INRS Centre Armand-Frappier Santé Biotechnologie

Caractérisation du rôle du *facteur de fragmentation de l'ADN 40* dans l'acquisition d'éléments clés de la tumorigenèse.

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LES ISSUES OBTENUES À LA SUITE DES TRAVAUX DE CETTE THÈSE

Les travaux de cette thèse ont mené à la publication de 4 articles scientifiques à titre de première auteure, publiés ou soumis dans de périodiques de qualités d'envergures, tels *Toxicology* (facteur d'impact de 4.2), *Biochemical Pharmacology* (facteur d'impact de 5.8), *Molecular and Cellular Biochemistry* (facteur d'impact de 3.8) et *Cell Biology and Toxicology* (article soumis ; facteur d'impact de 6.7).

De plus, deux revues de littératures scientifiques ont été rédigées. La première revue de littérature a relevé le rôle du DFF40 dans la stabilité génomique et a été publié dans le périodique *Apoptosis* (facteur d'impact de 4.7). La deuxième revue de littérature portait sur les mécanismes de résistance à l'apoptose et a été publié dans le périodique *Journal of Cellular Biochemistry* (facteur d'impact de 4.5).

Au cours de ma thèse de doctorat, les travaux de recherches ont été présentés sous forme de communication orale à deux congrès provinciaux. Les travaux de recherche ont aussi été disséminés dans un congrès international américain, un congrès national, six congrès provinciaux et trois journées scientifiques institutionnelles. Au total, deux prix de finalistes, un second prix et un premier prix pour la meilleure présentation par affiches ont été obtenus.

RÉSUMÉ

Le facteur de fragmentation de l'ADN 40 (DFF40) est l'endonucléase régissant l'étape ultime de l'apoptose et est nécessaire au maintien de l'homéostasie cellulaire et tissulaire. Des altérations dans son expression ont été démontrée dans de multiples tumeurs et associées avec un mauvais pronostic. De nombreuses études ont suggéré un rôle du DFF40 dans la stabilité génomique et dans le développement de la tumorigenèse. Dans ce projet de thèse, nous avons généré une lignée cellulaire stable déficiente en l'expression du DFF40 (DFF40 KO) par CRISPR-cas9, en utilisant les cellules T Jurkat comme modèle d'étude. Nous avons par la suite caractérisé la réponse cellulaire des cellules DFF40 KO à divers agents inducteurs d'apoptose et étudié les mécanismes moléculaires impliqués. Premièrement, nous avons démontré que les cellules déficientes en DFF40 ont une augmentation de l'expression basale de Mcl-1. Deuxièmement, une déficience en DFF40 altère le métabolisme énergétique basal. Les cellules DFF40 KO ont une augmentation de la production de ROS, une modification du potentiel de membrane mitochondriale, une augmentation de la quantité d'ADN mitochondriale et une production d'énergie dépendante de la glycolyse. L'exposition à des agents inducteurs d'apoptose a démontré que les cellules Jurkat DFF40 KO sont résistantes à la mort cellulaire induite par le tributylétain (TBT), la staurosporine (STS) et les antimétabolites, dont le méthotrexate (MTX), 6-mercaptopurine (6-MP) et cytarabine (Ara-C). De manière intéressante, les cellules déficientes en DFF40 ont une plus grande sensibilité aux inhibiteurs de la topoisomérase II (TOP2), dont l'etoposide (ETO). Nous avons démontré que la résistance au TBT est due à un retard d'activation de l'apoptose, caractérisé par un retard de clivage de la procaspase-3 et PARP, ainsi qu'un retard d'activation de la caspase-6. De plus, les cellules DFF40 KO ont une augmentation de la phosphorylation de Bcl-2 suivant l'induction de l'apoptose au TBT. L'abolition de l'expression du DFF40 altère les mécanismes de réparation de l'ADN. Les cellules DFF40 KO ont une absence de phosphorylation de l'histone H2AX. L'analyse de l'activation des voies de réparation de l'ADN démontre une phosphorylation accrue de CHK1 et une diminution significative de la phosphorylation de CHK2. Nos résultats supportent l'hypothèse que le DFF40 est impliqué dans l'instabilité génomique au sein des cellules cancéreuses et dans la résistance aux agents de chimiothérapie.

Mots-clés : Apoptose, résistance, DFF40, instabilité génomique, métabolisme énergétique, voies de réparation de l'ADN.

ABSTRACT

The DNA fragmentation factor (DFF40) is the endonuclease governing the final step of apoptosis and is necessary for the maintenance of cellular and tissue homeostasis. Alterations in its expression have been demonstrated in multiple tumors and associated with poor prognosis. Numerous studies have suggested a role of DFF40 in genomic stability and in the development of tumorigenesis. In this thesis project, we generated a stable cell line deficient in DFF40 expression (DFF40 KO) by CRISPR-cas9, using Jurkat T cells as a study model. We then characterized the cellular response of DFF40 KO cells to various apoptosis-inducing agents and studied the molecular mechanisms involved. First, we demonstrated that DFF40-deficient cells have increased Mcl-1 expression at a basal state and impaired energy metabolism. DFF40 KO cells have increased ROS production, altered mitochondrial membrane potential, increased amount of mitochondrial DNA, and a glycolysis-dependent energy production in non-apoptotic conditions. Exposure to apoptosis-inducing agents demonstrated that Jurkat DFF40 KO cells are resistant to cell death induced by tributyltin (TBT), staurosporine (STS), and antimetabolites, namely methotrexate (MTX), 6-mercaptopurine (6-MP) and cytarabine (Ara-C). Interestingly, cells deficient in DFF40 have a greater sensitivity to topoisomerase II (TOP2) inhibitors, such as etoposide (ETO). We demonstrated that resistance to TBT is due to delayed activation of apoptosis, characterized by delayed cleavage of procaspase-3 and PARP, as well as delayed activation of caspase-6. In addition, DFF40 KO cells have increased Bcl-2 phosphorylation following TBT induction of apoptosis. Abolition of DFF40 expression alters DNA repair mechanisms. DFF40 KO cells have an absence of histone H2AX phosphorylation. Analysis of the activation of DNA repair pathways demonstrates an increased phosphorylation of CHK1 (*i.e.* activation of ATR) and a significant decrease in phosphorylation of CHK2 (*i.e.* inhibition ATM). Our results support the hypothesis that DFF40 is involved in genomic instability within cancer cells and in resistance to chemotherapy agents.

Keywords : Apoptosis, resistance, DFF40, genomic instability, cell metabolism, DNA repair pathways

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6-MP	6-mercaptopurine
ADNmt	ADN mitochondriale
Apaf-1	Protéine *apoptotic protease activating factor 1*
Ara-C	Cytarabine
ASK1	*Apoptosis signal-regulating kinase* 1
ATF4	Facteur de transcription activateur 4
ATF6	Facteur de transcription activateur 6
ATM	Ataxia telangiectasia mutated
ATR	*Ataxia telangiectasia and Rad3-related protein*
Bad	*Bcl-2-associated death promoter*
Bak	*Bcl-2 homologous antagonist killer*
Bax	Protéine *Bcl-2-associated X* protein
Bcl-2	*B-cell lymphoma 2*
Bcl-w	*Bcl-2-like protein 2*
Bcl-xL	*B-cell lymphoma extra large*
BDB	Bris double brins
Bid	*BH3 interacting-domain death agonist*
Bim	*Bcl-2-like protein 11*
Bip/GRP78	Protéine chaperonne *immunoglulin-binding protein*
Bok	*Bcl-2 related ovarian killer*
BSB	Bris simple brins
c-FLIP	Protéine inhibitrice FLICE
c-FLIP L	Isoforme long c-FLIP
c-FLIP S	Isoforme court c-FLIP
CD95/Fas	Récepteur CD95
CDKs	Cyclines-dépendantes kinases
CHK-1	*Checkpoint kinase 1*
CHK-2	*Checkpoint kinase 2*
CHOP	*C/EBP homologous protein*
CICD	Voie caspase-indépendante
CIDE	*Cell death-inducing DFF45-like effector*

CRD	Domaines riches en cystéines
CrmA	*Cytokine response modifier A*
DAMPs	*Damage-associated molecular patterns*
DcR	Récepteur leurre
DD	Domaine de mort
DDR	*DNA damage response*
DED	Domaine effecteur de mort
DFF	Facteur de fragmentation de l'ADN
DFF45-L	Isoforme long du DFF45
DFF45-S/DFF35	Isoforme court du DFF45
DFS	Taux de survie sans maladie
DNA-PK	*DNA-dependant protein kinase*
DXR	Doxorubicine
EGF	Facteur de croissance épidermique
ETO	Etoposide
FADD	Protéine adaptatrice associée au Fas
GST	Glutathione S-transférase
HIF-1	*Hypoxia-inducing factor 1*
HMGB1	*High-mobility group box 1*
HSP	Protéine de choc thermique
IAPs	Protéines inhibitrices de l'apoptose
IRE1a	*Inositol-requiring protein 1*
JNK	*c-Jun N-terminal kinase*
Mcl-1	*Myeloid leukemia cell differentiation protein*
MDC1	Protéine *mediator of DNA damage checkpoint 1*
mTOR	La cible de la rapamycine chez les mammifères
MTS	Séquence de localisation mitochondriale
MTX	Méthotrexate
MX	Mitoxanthrone
NFkB	*Nuclear-factor k-light-chain-enhancer of activated B cell*
NLS	Signal de localisation nucléaire
NOXA	*Phorbol-12-myristate-13-acetate-induced protein 1*
OS	Taux de survie globale
PERK	Protéine kinase *RNA-like ER kinase*

PKC	Protéine kinase C
PLAD	Domaine d'assemblage pré-ligand-liaison
pRb	Protéine du rétinoblastome
PUMA	*p53 upregulated modulator of apoptosis*
RAGE	*Receptor for advanced glycation end products*
RE	Réticulum endoplasmique
RIP	Protéine d'interaction avec les récepteurs
s-CD95L	Forme soluble du CD95L
Smac/Diablo	Protéine second mitochondria-derived activator of caspases
sTNF	TNFa soluble
STS	Staurosporine
TBT	Tributylétain
TNFa	Facteur de nécrose tumorale alpha
TNFR	Récepteur de facteurs de nécrose tumorale
TNFRSF	Superfamille des récepteurs du facteur de nécrose
TOP2	Topoisomerase II
TRADD	Protéine adaptatrice associée au TNFR
TRAF2	Facteur 2 associé au TNFR
TRAIL	*Tumor necrosis factor related apoptosis inducing ligand*
TRAIL-R	Récepteur tumor necrosis factor related apoptosis inducing ligand
UPR	*Unfolded protein response*
XIAP	X-linked IAP
$\Delta\Psi m$	Potentiel de membrane mitochondriale

1 CHAPITRE 1 : INTRODUCTION

L'être humain est un organisme multicellulaire composé de cellules somatiques et de cellules germinales. Les cellules germinales sont impliquées dans la reproduction sexuée et leurs mutations génétiques sont transmises à leur descendance. Bien qu'elles soient à l'origine de certaines pathologies, c'est l'accumulation de mutations de l'ADN dans les cellules somatiques qui est fortement impliquée dans le développement de cancer (Olafsson *et al.*, 2021). Les cellules somatiques sont propres à chaque individu et accumulent au cours de leur vie de nombreuses mutations. Le type de mutation, ainsi que le degré de l'atteinte, permet de prédire la progression tumorale. Dans un contexte clinique oncologique, la caractérisation de mutations permet de dresser le portrait génotypique d'une cellule cancéreuse et d'adapter la thérapie à celle-ci.

Les cellules tumorales sont très complexes ; elles contiennent de nombreuses mutations et le cumul de celles-ci détermine la stabilité génomique, un concept qui sera discuté ultérieurement. Les mutations sont induites par des dommages à l'ADN (Loeb *et al.*, 2000). Les principales mutations regroupent les gènes qui sont impliqués dans le contrôle du cycle cellulaire et l'apoptose. Parmi celles-ci, il a été démontré que les mutations qui impliquent les gènes codant pour les protéines c-myc, p53, la protéine du rétinoblastome (pRb), les cyclines-dépendantes kinases (CDKs) et les cyclines régulent la progression tumorale (Pucci *et al.*, 2000). De plus, près de 50% des cellules tumorales ont une perte de fonction de p53, une protéine transcrite par les gènes suppresseurs de tumeurs (Pucci *et al.*, 2000).

Au fil des dernières années, de nombreuses protéines impliquées dans le cycle cellulaire et l'apoptose ont été démontrées comme étant mutées dans certains types de cancer. L'étude de ces mécanismes est laborieuse, car l'impact d'une simple mutation peut avoir des répercussions à plusieurs niveaux dans les voies de signalisation. Récemment, des études ont suggéré un rôle du facteur de fragmentation de l'ADN (DFF), une endonucléase codée par *DFFB*, dans l'acquisition d'un phénotype malin de certaines tumeurs (Banas *et al.*, 2017, Banas *et al.*, 2018a, Sánchez-Osuna *et al.*, 2016).

Dans les prochaines sections, nous passerons en revue les concepts reliés à l'acquisition d'une mutation du DFF dans la progression tumorale. Nous expliquerons en détail la voie de signalisation de l'apoptose, tout en nous concentrant sur le DFF, les mécanismes moléculaires impliqués dans l'instabilité génomique et les caractéristiques phénotypiques des cellules cancéreuses qui permettent de prédire la malignité tumorale.

1.1 Les divers types de mort cellulaire

Les connaissances scientifiques entourant la mort cellulaire dans le maintien de l'homéostasie de la cellule ont grandement évolué au courant des dernières décennies. Les premières avancées suggéraient que les cellules étaient principalement éliminées par nécrose ou par apoptose. Or, au fil des années, de nombreux phénomènes de mort cellulaire ont été caractérisés, dont l'autophagie, la mitophagie, la ferroptose et la nécroptose.

Le sujet de cette présente thèse se concentre sur les processus d'apoptose. Le Tableau 1 résume les principaux changements physiologiques lors des divers types de mort cellulaire.

1.1.1 Différences clés entre l'apoptose, la nécrose, la nécroptose, la ferroptose, l'autophagie et la mitophagie

Premièrement, la nécrose est un mécanisme de mort cellulaire non programmée et non spécifique (Festjens *et al.*, 2006). Elle est induite par des insultes physicochimiques, dont le choc osmotique, stress mécanique ou par acidification extracellulaire (Festjens *et al.*, 2006). Du point de vue morphologique, les cellules nécrotiques ont une perte de l'intégrité membranaire, ce qui mène à un gonflement cellulaire. Ultimement, la cellule se dégrade par lyse cellulaire avec libération du contenu cytoplasmique dans le micro-environnement (Festjens *et al.*, 2006). La libération du contenu intracellulaire permet aux cellules environnantes d'induire une cascade inflammatoire par la reconnaissance de motifs moléculaires, connus sous le nom de *damage-associated molecular patterns* (DAMPs) (Yang *et al.*, 2015). Par exemple, la protéine *high-mobility group box 1* (HMGB1), relâchée par les cellules nécrotiques, induit l'inflammation cellulaire via la stimulation de ses récepteurs cibles, le *receptor for advanced glycation end products* (RAGE) (Yang *et al.*, 2015).

Deuxièmement, contrairement à la nécrose, l'apoptose est un mécanisme de mort cellulaire programmée et très spécifique. De plus, les processus inflammatoires sont généralement absents. Du point de vue microscopique, la cellule apoptotique va subir une diminution de sa taille cellulaire et une condensation de sa chromatine (connu sous le nom de *pyknosis*) (Elmore, 2007). L'ADN sera ensuite fragmenté (*karyorrhexis*), pour finalement observer une dissolution complète de la chromatine (*karyolyse*) (Elmore, 2007). Des corps apoptotiques seront produits et ceux-ci seront éliminés à l'aide des cellules phagocytaires. Les voies de signalisation de l'apoptose seront expliquées en détail dans les chapitres subséquents.

Au fil des années, il a été démontré que l'apoptose n'est pas le seul mécanisme de mort cellulaire ayant une régulation. Des études ont permis de découvrir que la nécrose peut être régulée et

dans ce cas-ci, elle se nomme la « nécroptose » (Berghe *et al.*, 2014). La nécroptose est induite par des stimuli externes, tels l'hypoxie ou l'inflammation (Berghe *et al.*, 2014), qui vont mener à l'activation du récepteur de facteurs de nécrose tumorale (TNFR). Cette voie de signalisation est indépendante de l'activation des caspases et mène à la production de nombreux médiateurs pro-inflammatoires, dont des cytokines, par l'entremise de la voie de signalisation *nuclear-factor κ-light-chain-enhancer of activated B cell* (NFκB) (Berghe *et al.*, 2014). La cascade inflammatoire a pour effet d'inactiver de manière rapide et irréversible la cellule, où celle-ci va subir de nombreux changements phénotypiques. Du point de vue moléculaire et morphologique, la cellule en nécroptose subit les mêmes changements que la cellule nécrotique décrit ci-dessus.

L'autophagie décrit un phénomène d'autodestruction, qui permet d'éliminer les organelles non fonctionnelles ou qui ont subi un dommage. Ce processus fut découvert par le chercheur de Duve avec l'utilisation de la microscopie électronique : il décrit le processus d'autophagie comme un processus de dégradation du contenu intracellulaire par le lysosome (De Duve C Fau - Pressman *et al.*, 1995). Elle se définit donc comme un processus de recyclage et de renouvellement cellulaire. L'autophagie peut être divisée en trois types, soit la macroautophagie, la microautophagie et l'autophagie médiée par protéine chaperonne (Ohsumi, 2014). La macroautophagie implique la fusion des autophagosomes avec les lysosomes (Ohsumi, 2014). En effet, les composants cellulaires, dont les macro-protéines et les organelles, seront séquestrés dans des compartiments vésiculaires, soit les autophagosomes (Ohsumi, 2014). Ensuite, il y aura une fusion des autophagosomes avec les lysosomes, où les protéases dégraderont le contenu de la vésicule (Ohsumi, 2014). D'autre part, la microautophagie implique la capture directe de matériaux cellulaires par le lysosome par pinocytose (Das *et al.*, 2012). Ceci implique la dégradation intracellulaire d'un plus petit compartiment que la macroautophagie (Ohsumi, 2014). Enfin, le troisième type d'autophagie implique le transport direct de protéines dans le lysosome par une interaction avec des molécules chaperonnes (Das *et al.*, 2012). Du point de vue tumoral, l'autophagie peut exercer un effet positif ou négatif sur la survie cellulaire. À des niveaux basaux d'autophagie, ce processus est considéré comme un mécanisme de suppression tumorale (Yun *et al.*, 2018). Par contre, lorsque l'autophagie est sous-régulée ou possède une altération dans son mécanisme de signalisation, celle-ci favorise le développement tumoral par une accumulation d'organelles altérées (Yun *et al.*, 2018).

La mitophagie repose sur le même principe que l'autophagie, où le compartiment vésiculaire dans ce cas-ci est la mitochondrie (D'Arcy, 2019). Ce processus permet d'éliminer les mitochondries dont le métabolisme énergétique est altéré. Dans les cellules cancéreuses, il a été reporté qu'il

existe divers niveaux de mitophagie et son rôle est distinct selon le stade de développement tumoral (Yigang Wang *et al.*, 2020). L'inhibition de la mitophagie favoriserait initialement la tumorigenèse, tandis que dans les stades de progression tumorale, une activité mitophagique est nécessaire pour la survie des cellules cancéreuses (Yigang Wang *et al.*, 2020).

La dernière voie de mort cellulaire programmée décrite dans la littérature est celle de la ferroptose. La ferroptose est impliquée dans le développement de nombreuses maladies, dont le cancer (Stockwell *et al.*, 2017). Elle fut décrite pour la première fois en 2012 par l'équipe du chercheur Stockwell comme un processus de mort cellulaire dépendante de l'accumulation de fer et de la peroxydation des lipides (Dixon *et al.*, 2012). Elle peut être activée par de nombreux agents, qui vont principalement agir sur 3 phénomènes biocellulaires, soit le métabolisme lipidique, le métabolisme en fer ou le métabolisme oxydatif (Jie Li *et al.*, 2020a). Du point de vue cellulaire, aucun changement au sein de la membrane cellulaire et du noyau n'est observé (Stockwell *et al.*, 2017). La ferroptose est principalement caractérisée par une diminution de la taille des mitochondries, avec une augmentation concomitante de la densité membranaire (Stockwell *et al.*, 2017). Or, de nombreux éléments restent à être étudiés, dont l'identification vigoureuse de marqueurs clés de la ferroptose, en complément à ceux déjà identifiés dans la littérature (Stockwell *et al.*, 2017).

En somme, il existe une relation étroite entre les divers processus de mort cellulaire dans les cellules cancéreuses. Selon le stress de leur micro-environnement, il y aura des adaptations cellulaires de survie ou de mort cellulaire, dans l'objectif commun de favoriser la croissance et progression tumorale.

Tableau 1. Comparaison des changements cellulaires impliqués dans l'apoptose, la nécrose, l'autophagie et la mitophagie.

	Apoptose	Nécrose	Autophagie/Mitophagie	Ferroptose
Membrane cellulaire	Exposition PS[a]	Gonflement cellulaire	-	-
	« Blebbing » au stade tardif	Rupture au stade tardif		
Cytoplasme	Formation de corps apoptotiques	Lyse cellulaire	Formation d'autophagosomes	Diminution de la masse mitochondriale
	Pas de libération du contenu dans le ME[a]	Libération du contenu dans le ME[a]	Pas de libération du contenu dans le ME[a]	Rupture de la membrane externe mitochondriale
	Désintégration du cytosquelette			
Noyau cellulaire	Clivage spécifique	Clivage non-spécifique	-	-
	Pyknosis			
	Karyorrhexis			
	Karyolyse			

[a.] Abréviations : PS, phosphatidylsérine ; ME, micro-environnement.

1.2 L'apoptose

Le terme « *apoptosis* » a été attribué pour la première fois en 1972, à la lumière des recherches de John Kerr, Alastair Currie et Andrew Wyllie (Kerr *et al.*, 1972). Grâce à leurs observations en microscopie de diverses coupes tissulaires, ils ont rapporté les changements structuraux et cellulaires qui se produisent lors du renouvellement cellulaire, et y ont attribué le terme « *apoptosis* » à la suite des recommandations du Professeur James Cormack (Kerr *et al.*, 1972). Ce terme vient du mot «*apoptōsis* » en grec, qui signifie « *falling off from* », telle la chute de pétales de roses d'une fleur (Kerr *et al.*, 1972). Or, il est important de mentionner que les premières observations remontent au 19^{e} siècle. À travers les années 1800, de nombreux chercheurs ont documenté la présence de mort cellulaire dans divers types cellulaires, en débutant avec Carl Vogt en 1842 (Clarke *et al.*, 1996).

Les trouvailles relatives aux mécanismes de régulation génomiques de l'apoptose ont d'écoulées des premières découvertes obtenues chez le nématode *C. elegans* (Ellis Hm Fau - Horvitz *et al.*, 1986, Metzstein *et al.*, 1998, Reddien *et al.*, 2001). Les Pr Sydney Brenner, John Sulston et Robert Horvitz ont contribué de manière significative à l'identification de gènes impliqués dans la régulation de la mort cellulaire programmée (Brenner *et al.*, 2002). Les études qui ont suivi au courant des années subséquentes ont permis de caractériser les étapes moléculaires de l'apoptose.

L'apoptose se décrit comme un mécanisme de mort cellulaire programmée, qui est activé subséquemment à un signal suicidaire. Ce signal peut être de diverses étiologies, qui seront discutées dans les sous-sections suivantes. Son rôle est primordial dans l'élimination non inflammatoire des cellules altérées, afin de préserver l'homéostasie et le fonctionnement du système immunitaire (Elmore, 2007). De multiples altérations dans la voie de signalisation de l'apoptose et sa régulation ont été démontrées comme étant impliquées dans le développement de pathologies, dont l'Alzheimer (Crews *et al.*, 2011, Stanga *et al.*, 2010), l'insuffisance cardiaque (Fujita *et al.*, 2011, Whelan *et al.*, 2010), les maladies auto-immunes (Eguchi, 2001, Nagata, 2007) et les néoplasies (Dunkle *et al.*, 2011, Tamiya *et al.*, 1998).

Les voies de signalisation de l'apoptose peuvent être subdivisées en deux grandes catégories : (1) les voies dépendantes des caspases et (2) les voies indépendantes des caspases (CICD). Cette dernière se produit lorsqu'un signal activateur de la voie de signalisation de l'apoptose ne mène pas à l'activation de caspases. Les phénomènes cellulaires diffèrent entre ces deux voies, particulièrement en ce qui concerne l'activation des protéases et les changements nucléaires (Tait *et al.*, 2008). Dans la voie CICD, la cinétique de signalisation est relativement réduite. La

perte du potentiel de membrane mitochondriale (ΔΨm) se produit de manière graduelle et un grand nombre d'autophagosomes cytoplasmique est présent (Tait *et al.*, 2008). Enfin, il y a une condensation partielle de la chromatine avec une absence de fragmentation de l'ADN (Tait *et al.*, 2008). Les principales différences entre les voies caspases-dépendantes et CICD sont décrites dans le Tableau 2.

Tableau 2. Principales caractéristiques de l'apoptose et de la voie indépendante des caspases[a].

Adaptations	Voie dépendante des caspases (apoptose)	Voie indépendante des caspases (CICD)
Nucléaire	Condensation de la chromatine	Condensation partielle de la chromatine
	Fragmentation nucléaire	Absence de fragmentation
	Clivage des substrats de caspases	Marginalisation de la chromatine au pourtour de la membrane nucléaire
Mitochondriaux	Perméabilisation de la membrane mitochondriale	Perméabilisation de la membrane mitochondriale
	Perte rapide du ΔΨm	Perte graduelle du ΔΨm
Au cytosol	Augmentation du Ca^{2+}	Autophagosomes abondants
	Formation d'adduits de protéines	Agrégation de ribosomes
	Condensation	Vacuolisation
	Clivage des substrats de caspases	
Membranaires	Formation de bulles membranaires	Membrane déchiquetée
	Perte de la symétrie de phospholipides	
	Clivage des substrats de caspases	
Cellulaires	Perte de la prolifération	Perte de la prolifération
	Détachement de la matrice	Attaché à la matrice
	Phagocytose des débris	Clairance des débris par des mécanismes inconnus

[a]Tableau adapté de : (Tait *et al.*, 2008).

1.2.1 La voie dépendante des caspases de l'apoptose

Les voies dépendantes des caspases sont subdivisées en trois catégories : (1) la voie extrinsèque, (2) la voie intrinsèque et (3) la voie du stress du réticulum endoplasmique (RE). Ces trois voies ont en commun l'activation de caspases effectrices. Dans cette section, nous verrons en détail les signaux activateurs et régulateurs de chaque voie de signalisation caspase-dépendantes, ainsi que les mécanismes cellulaires impliqués dans celles-ci. La Figure 1 est une illustration schématique de ces trois voies dépendantes des caspases.

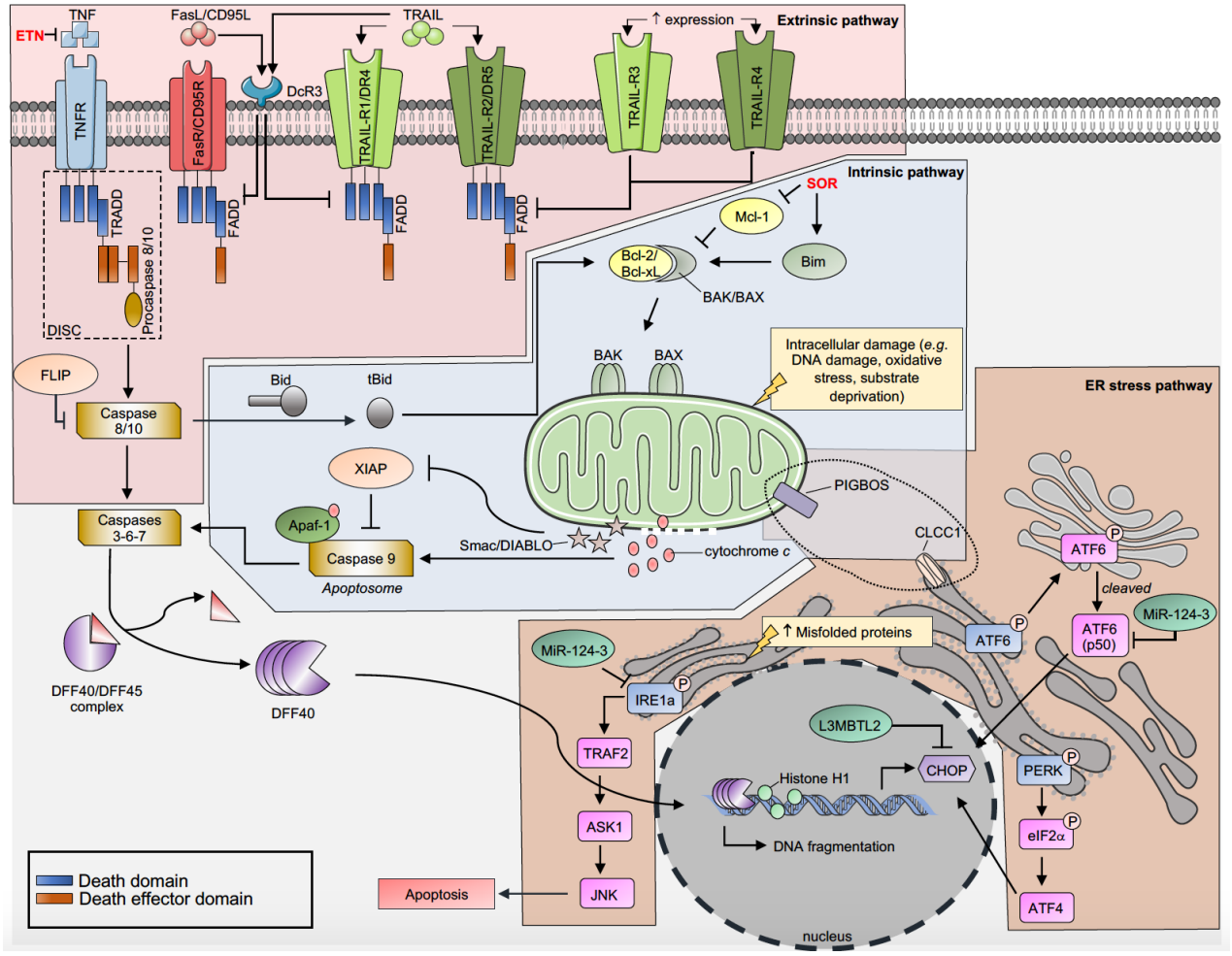


Figure 1. Illustration schématique des voies caspases-dépendantes de l'apoptose.

Image tirée de (M. Kulbay *et al.*, 2021c).

1.2.2 La voie extrinsèque

La voie extrinsèque de l'apoptose a rigoureusement été étudiée dans la littérature scientifique. Au cours des dernières décennies, de nombreuses recherches ont permis d'identifier les ligands et récepteurs respectifs, appelés récepteurs de mort cellulaire, ainsi que les molécules inhibitrices, impliqués dans l'activation de la voie extrinsèque de l'apoptose.

La voie extrinsèque se distingue par la nécessité d'un complexe ligand-récepteur pour la transduction du signal cellulaire. Elle révèle une grande complexité par la nature très variée des récepteurs de mort cellulaire. Dans cette section, nous discuterons des principaux récepteurs de morts cellulaires, ainsi que des mécanismes de signalisation et régulation cellulaires.

1.2.2.1 Les récepteurs de mort cellulaire

La première étape dans l'activation de la voie extrinsèque de l'apoptose repose sur la liaison d'un ligand exogène à son récepteur de mort cellulaire respectif. Ces récepteurs font partie de la superfamille des récepteurs du facteur de nécrose tumorale (TNFRSF). Les membres de cette famille les mieux étudiées sont les TNFR, le récepteur CD95 (CD95/Fas) et le *tumor necrosis factor related apoptosis inducing ligand* (TRAIL) *receptor* (TRAIL-R) (Locksley *et al.*, 2001). Or, il est important de mentionner que divers autres récepteurs de mort cellulaire ont été identifiés dans la littérature.

Les TNFR sont les récepteurs transmembranaires les mieux caractérisés et étudiés. Les deux principaux récepteurs sont le TNFR1 et TNFR2. L'expression de ceux-ci varie selon le type cellulaire. TNFR1 est exprimé au sein de toutes les cellules, tandis que le TNFR2 est retrouvé seulement à la surface des cellules du système immunitaire, des cellules souches mésenchymateuses et les cellules neuronales (Naserian *et al.*, 2020). La structure de ces récepteurs peut être divisée en trois parties distinctes, soit les domaines extracellulaires, intracellulaires et transmembranaires. Les domaines extracellulaires sont constitués de multiples domaines riches en cystéines (*cysteine-rich domains*; CRD) (Harald Wajant *et al.*, 2019). En ce qui concerne le domaine intracellulaire, il existe des subtilités selon le sous-type de TNFR. Le TNFR1 possède un domaine de mort (DD) de 70 à 80 acides aminés en projection du cytosol (Elmore, 2007, Nair *et al.*, 2014), qui a pour fonction la transduction du signal apoptotique. Or, le TNFR2 ne possède pas de DD, mais contient plutôt un court motif d'acides aminés près du domaine C-terminal, qui permet le recrutement d'une protéine adaptatrice, le facteur 2 associé au TNFR (TRAF2) *(Harald Wajant et al., 2019)*. Étant donné l'absence de DD chez le TNFR2, la

transduction du signal apoptotique est régie par l'activation de la voie signalétique de NFκB et de multiples kinases (H. Wajant *et al.*, 2003).

Le ligand agoniste de ces récepteurs est le facteur de nécrose tumorale α (TNFα). Le TNFα est exprimé sous une forme transmembranaire ou soluble (sTNF), issu du clivage protéolytique de la forme transmembranaire (Harald Wajant *et al.*, 2019). Le sTNF existe sous la forme d'un trimère, mais peut se retrouver en équilibre sous la forme d'un monomère ou dimère également (Sedger *et al.*, 2014). L'affinité de liaison de sTNF a été démontrée comme étant supérieure pour le TNFR1 en comparaison au TNFR2 (Kemanetzoglou *et al.*, 2017). De nombreux agents qui peuvent induire l'apoptose médient leurs actions par la régulation de la concentration de TNF. Il est intéressant de noter que certains agents toxiques, tels que le bisphénol A et certaines nanoparticules, peuvent induire une augmentation de la relâche de TNF, ce qui permet l'activation de l'apoptose (Urriola-Muñoz *et al.*, 2018). De l'autre côté, les récepteurs solubles et les anticorps monoclonaux permettent de neutraliser le TNF et ainsi inhiber l'activation de la voie extrinsèque par les TNFR (Chadwick *et al.*, 2018, Jing Liu *et al.*, 2016).

Le récepteur CD95, aussi connu sous le nom de Fas, est un récepteur transmembranaire trimérique qui, contrairement au TNFR, contient 3 CRD (Peter *et al.*, 2003, Siegel Richard *et al.*, 2000). Les CRD sont situés au niveau N-terminal et sont cruciaux dans l'induction de l'oligomérisation du récepteur en trimère (Papoff *et al.*, 1999, Siegel Richard *et al.*, 2000). Le CRD1 contient le domaine d'autoassociation (*c.-à-d.* le domaine d'assemblage de liaison préligand (PLAD)), un domaine partagé entre les divers membres de la famille des TNFR (Siegel Richard *et al.*, 2000). La liaison du ligand FasL, qui est sous une forme trimérique, permet le rapprochement des trimères et la transmission d'un signal intracellulaire (Fu *et al.*, 2016). La Figure 2 est une représentation schématique de la structure du récepteur CD95/Fas.

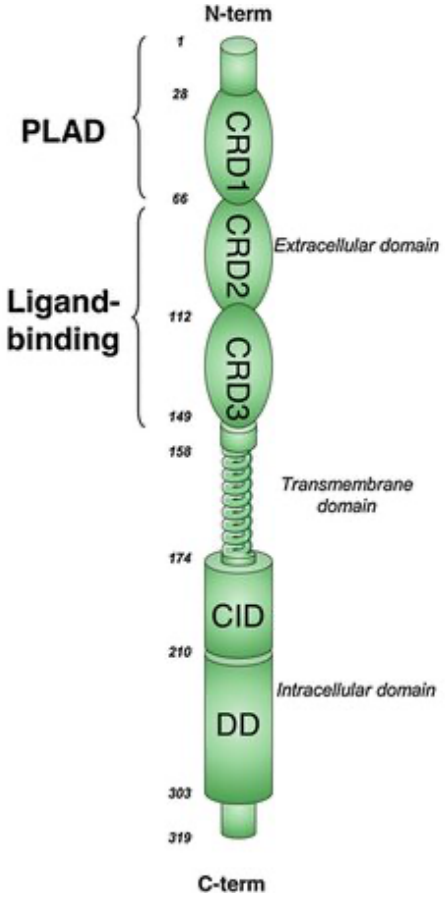


Figure 2. Représentation schématique de la structure du récepteur CD95/Fas.

Le domaine intracellulaire (résidu 174 à 319) est composé d'un domaine de mort (DD) et du domaine d'induction du calcium (CID). Le domaine transmembranaire est composé des acides aminés 158 à 174. Le domaine extracellulaire (résidus 1 à 149) est composé des domaines riches en cystéines (CRD). Abréviations : PLAD, domaine d'assemblage préligand. Image adaptée de : (Guégan *et al.*, 2020).

L'agoniste du récepteur CD95 est le CD95L/FasL. La liaison du ligand au récepteur induit un changement de conformation qui permet la transduction du signal apoptotique dans le cytosol (Qadir *et al.*, 2020, Sharma *et al.*, 2019). Le CD95L est une protéine transmembranaire contenant 4 régions distinctes : un domaine cytoplasmique, un domaine transmembranaire, une région homologue au TNF permettant l'homotrimérisation, et un domaine C-terminal impliqué dans la liaison avec le CD95 (Levoin *et al.*, 2020). Les domaines transmembranaires du CD95L sont impliqués dans l'agrégation et l'induction de l'apoptose (Levoin *et al.*, 2020). Le domaine transmembranaire du CD95L peut être clivé par des métalloprotéinases, libérant ainsi la forme soluble du CD95L (s-CD95L) (Levoin *et al.*, 2020). Le s-CD95L est présent sous forme d'homotrimères et n'est pas un inducteur de l'apoptose (Levoin *et al.*, 2020). Il fut démontré qu'une injection de CD95L dans des souris n'induit pas l'activation de l'apoptose, car elle est sujette à des modifications post-traductionnelles par les métalloprotéinases (Al-Saeedi *et al.*, 2018, D. C. Huang *et al.*, 1999, Tanaka *et al.*, 1998).

Finalement, les récepteurs TRAIL-R sont des récepteurs membranaires trimériques qui peuvent être catégorisés selon la présence ou non de DD. Les récepteurs qui contiennent un DD sont le TRAIL-R1 (DR4) (Schneider *et al.*, 1997) et TRAIL-R2 (DR5) (Vunnam *et al.*, 2017) et exercent une fonction proapoptotique. Les récepteurs ne contenant pas de DD, soit TRAIL-R3 et TRAIL-4, n'induisent pas l'apoptose (von Karstedt *et al.*, 2020, Yoshimura *et al.*, 2019). TRAIL-R1 et TRAIL-R2 possèdent dans leur domaine extracellulaire 2 à 4 CRD (Shulin Wang *et al.*, 2003). Leur domaine intracellulaire est composé d'environ 70 acides aminés (Yuan *et al.*, 2018). Les ligands agonistes sont des protéines membres de la TNFSF et consistent en un total de 282 acides aminés (Almasan *et al.*, 2003).

1.2.2.2 Activation et régulation de la voie dépendante des caspases

L'activation et la transduction du signal apoptotique sont régies par la liaison du ligand agoniste à son récepteur, ce qui va permettre l'oligomérisation de celui-ci et induire une cascade de signalisation complexe. Il est important de mentionner que cette étape d'activation de l'apoptose peut être régulée à plusieurs niveaux. L'expression des récepteurs de mort cellulaire possède une variabilité cellulaire et tissulaire. De plus, des variations de l'expression des divers types de récepteurs furent reportées au sein du même type cellulaire (Spierings *et al.*, 2004), suggérant une spécificité à certains ligands. Ensuite, il existe des récepteurs leurres (de l'anglais *Decoy*) qui ont pour fonction d'inhiber l'apoptose par l'entremise d'une régulation des récepteurs de mort cellulaire. Deux exemples de récepteurs leurres sont les TRAIL-R3 et TRAIL-R4 (Pan *et*

al., 1997). Ils exercent leur fonction en inhibant le transfert du signal apoptotique au niveau intracellulaire (Neumann *et al.*, 2014). Un autre récepteur leurre (DcR) bien caractérisé dans la littérature est le DcR3 membre de la TNFRSF (Takahashi *et al.*, 2011). Le DcR3 se lie de manière compétitive à FasL de par une similarité dans sa structure avec le récepteur CD95 (Liang *et al.*, 2017).

La transduction du signal est dépendante du ligand : chaque ligand induit le recrutement de protéines adaptatrices spécifiques au récepteur de mort cellulaire à la membrane cellulaire dans le cytosol. Les protéines adaptatrices permettent d'effectuer le pont entre la transmission du signal du récepteur vers le compartiment intracellulaire. L'interaction CD95L-CD95 va permettre le recrutement de la protéine adaptatrice associée au Fas (FADD), tandis que l'activation du TNFR induit le recrutement de la protéine adaptatrice associée au TNFR (TRADD) en complément au FADD (Cavalcante *et al.*, 2019). Ces protéines adaptatrices sont localisées à la hauteur des DD du récepteur. La dimérisation du complexe va ensuite engendrer le recrutement de la procaspase-8, formant ainsi le complexe signalétique inducteur de la mort cellulaire, nommé le DISC (Seo *et al.*, 2018). Une fois le DISC formé, il y a un clivage autolytique de la procaspase-8 en caspase effectrice 8.

Le rôle de la caspase-8 est crucial, car c'est cette protéine qui permet la transmission du signal apoptotique aux caspases effectrices subséquentes. Cette caspase contient un domaine effecteur de mort (DED) (Shen *et al.*, 2018). Son activation nécessite absolument la formation de structures oligomériques, appelées filaments DED (Ivanisenko *et al.*, 2019). Ces structures sont formées suivant l'interaction des DED de la protéine adaptatrice avec celles de la procaspase-8 et procaspase-10 (Hillert *et al.*, 2020). Dans le cas des TNFR (*e.g.* TNFR1), l'induction de la voie extrinsèque de l'apoptose mène au recrutement de TRADD, qui à son tour mène au recrutement de FADD. Contrairement au TRADD, la protéine adaptatrice FADD possède un DED, nécessaire à l'interaction avec la caspase-8 via son DED respectif. À la suite de ce processus, une activation en cascade de caspases effectrices s'ensuit, comme démontré à la Figure 1, pour aboutir à l'ultime activation de la caspase-3 (Masuda *et al.*, 2020). L'activation de la caspase-3 est le point de non-retour de la voie extrinsèque de l'apoptose. Cette caspase effectrice est en effet responsable de l'activation du facteur de fragmentation de l'ADN 40 (DFF40), par le clivage de la sous unité inhibitrice le DFF45 (Bagheri *et al.*, 2015b). Le DFF40 activé est responsable de la fragmentation de l'ADN.

À travers la voie de signalisation de l'apoptose, l'activation des caspases peut être régulée à l'aide d'inhibiteurs physiologiques, telles les protéines p35, la *cytokine response modifier A*

(CrmA) et les protéines inhibitrices de l'apoptose (IAPs) (Ekert *et al.*, 1999). La protéine p35 inhibe l'apoptose en se liant aux sites cibles des protéases (Qiao Zhou *et al.*, 1998), tandis que la protéine CrmA inhibe de manière directe l'activation des caspases en se liant à leur site catalytique (Callus *et al.*, 2007). Il existe huit IAPs, dont la X-linked IAP (XIAP) (Kewei Wang *et al.*, 2013). Celle-ci est la seule parmi les diverses IAPs à avoir la capacité à inhiber de manière directe l'activation des caspases, particulièrement les caspases-9 (Feltham *et al.*, 2012). Enfin, l'activation de la caspase-8 peut être inhibée par la protéine inhibitrice FLICE (c-FLIP), via une régulation des filaments DED (Meng *et al.*, 2020). Il existe deux isoformes majeures de c-FLIP : l'isoforme long (c-FLIP L) et l'isoforme court (c-FLIP S) (Meng *et al.*, 2020). L'isoforme c-FLIP S est un inhibiteur de la caspase-8 et empêche l'activation DISC-dépendante de la procaspase-8 en interférant avec l'oligomérisation protéique (Tummers *et al.*, 2017). En effet, c-FLIP S est la forme tronquée de la procaspase-8 n'ayant que deux DED (Tummers *et al.*, 2017). D'autre part, la fonction de l'isoforme c-FLIP L est complexe. Elle a une fonction proapoptotique jusqu'à des concentrations intermédiaires, tandis qu'à des concentrations élevées, elle exerce une fonction antiapoptotique (Hillert *et al.*, 2020, Schleich *et al.*, 2016). Cela dit, l'inhibition de l'activation de la voie extrinsèque de l'apoptose par c-FLIP permet de faire le pont vers la nécroptose via l'intermédiaire de la protéine d'interaction avec les récepteurs (RIP). Dans le cas des TNFR, le recrutement de la kinase RIPK1, une sérine/thréonine kinase, au DD de la protéine adaptatrice TRADD permet l'activation des voies de signalisation pro-inflammatoire par ubiquitination, dont celle de NFκB (Tummers *et al.*, 2017). L'état d'ubiquitination de la protéine RIPK1 détermine l'induction ou non de la voie de signalisation de l'apoptose (Tummers *et al.*, 2017).

1.2.3 La voie intrinsèque

La voie intrinsèque de l'apoptose fut très bien caractérisée dans la littérature au fil des ans. Un des chercheurs avec une renommée internationale pour ses contributions dans la caractérisation des mécanismes moléculaires de cette voie de signalisation est le Pr Douglas R. Green. Dans cette section, nous verrons les diverses étapes d'activation et de signalisation de la voie intrinsèque de l'apoptose, tout en relatant la contribution des divers experts du domaine.

De multiples stimulus endogènes et exogènes sont reconnus pour activer la voie intrinsèque de l'apoptose, dont les dommages à l'ADN, le stress oxydatif par la production d'espèces réactives de l'oxygène (ROS) et une augmentation en concentration intracellulaire de calcium (Elmore, 2007, Loreto *et al.*, 2014, Solano-Gálvez *et al.*, 2018). Il est à noter que ce sont les ROS induits par la réaction de Fenton qui sont à l'origine de l'induction de la voie intrinsèque de l'apoptose, où celles induites par la peroxydation des lipides enclenchent la ferroptose, comme discuté précédemment. La mitochondrie est l'organelle clé où les processus physiologiques apoptotiques sont initiés. La perte du $\Delta\Psi m$ est l'étape initiatrice de la cascade de signalisation.

La signalisation induite par la voie intrinsèque de l'apoptose est médiée par la modulation de l'activité des protéines de la famille de *B-cell lymphoma 2* (Bcl-2). Les protéines de la famille de Bcl-2 peuvent être divisées en trois catégories distinctes : (1) les protéines antiapoptotiques, (2) les protéines proapoptotiques impliquées dans la formation de pores et (3) les protéines avec un domaine unique BH3 (de l'anglais BH3-only) (Green, 2022). Nous survolerons en premier les membres de chaque catégorie, puis nous détaillerons le rôle de chacune des protéines dans la voie intrinsèque de l'apoptose.

La première catégorie regroupe les protéines antiapoptotiques, dont la protéine Bcl-2, la protéine *B-cell lymphoma extra large* (Bcl-xL), la protéine *Bcl-2-like protein 2* (Bcl-w) et la protéine *myeloid leukemia cell differentiation protein* (Mcl-1). Bcl-xL, Bcl-2 et Bcl-w possèdent quatre domaines BH et un domaine hydrophobe en C-terminal (Tzifi *et al.*, 2012), tandis que la protéine Mcl-1 possède trois domaines BH (L. W. Thomas *et al.*, 2010). Les domaines BH1 à BH3 sont impliqués dans les interactions avec les protéines BH3-only, tandis que le domaine BH4 est impliqué dans la stabilisation de la structure tridimensionnelle (Tzifi *et al.*, 2012).

La deuxième catégorie regroupe les protéines proapoptotiques. Il existe trois protéines proapoptotiques impliquées dans la formation de pores mitochondriaux, soit la protéine *Bcl-2-associated X* protein (Bax), la protéine *Bcl-2 homologous antagonist killer* (Bak) et la protéine *Bcl-*

2 related ovarian killer (Bok). Comme les protéines antiapoptotiques, ils possèdent aussi quatre domaines BH (Westphal *et al.*, 2011).

La troisième catégorie, soit les protéines proapoptotiques BH3-only, englobe entre autres les protéines *BH3 interacting-domain death agonist* (Bid), *Bcl-2-associated death promoter* (Bad), *Bcl-2-like protein 11* (Bim), *p53 upregulated modulator of apoptosis* (PUMA) et *Phorbol-12-myristate-13-acetate-induced protein 1* (NOXA). Celles-ci possèdent seulement le domaine BH3 de 100 à 200 acides aminés (Hardwick *et al.*, 2013). La classification ainsi que les rôles respectifs de chaque protéine sont résumés dans le Tableau 3 (Dai *et al.*, 2014, Kale *et al.*, 2018). Les rôles physiologiques seront expliqués en détail dans le paragraphe suivant.

Tableau 3. Classification des protéines de la famille de Bcl-2 et leurs rôles respectifs.

Classification	Protéines	Rôles – Mécanismes d'action	Références
Protéines antiapoptotiques	Bcl-2	Bloque la formation de pores mitochondriaux – liaison inhibitrice avec les protéines Bax et Bak Inhibition de la formation de l'apoptosome – Bloque le relâche du cytochome C Modulation de l'activité des caspases – régulation de l'activité de la caspase-2	(Hardwick *et al.*, 2013, Tzifi *et al.*, 2012)
	Bcl-xL	Bloque la formation de pores mitochondriaux – liaison inhibitrice avec les protéines Bax et Bak Inhibe l'activation de Bax – Translocation continue de Bax de la membrane mitochondriale au cytoplasme Empêche la polymérisation de Bax et Bak – Formation d'hétérodimères avec les protéines BH3-only	(Edlich *et al.*, 2011, Hardwick *et al.*, 2013, Tzifi *et al.*, 2012)
	Bcl-w	Bloque la formation de pores mitochondriaux – liaison inhibitrice avec les protéines Bax et Bak Empêche la polymérisation de Bax et Bak – Formation d'hétérodimères avec les protéines BH3-only	(Hartman *et al.*, 2020, Ku *et al.*, 2011, Tzifi *et al.*, 2012)

	Mcl-1	Empêche la polymérisation de Bax et Bak – Formation d'hétérodimères avec les protéines BH3-only Bloque la formation de pores mitochondriaux – Séquestration des protéines Bax et Bak par liaison directe	(L. W. Thomas *et al.*, 2010)
Protéines proapoptotiques	Bax Bak Bok	Perturbation du potentiel de membrane mitochondriale – Formation de pores au niveau de la membrane mitochondriale Favorisent le relâche du cytochrome *c* – Altération de l'intégrité mitochondriale	(Honglin Li *et al.*, 1998, Y. Yao *et al.*, 2009)
Protéines proapoptotiques BH3-only	tBid	Favorise l'oligomérisation et l'activation proapoptotique de Bax – Liaison directe avec Bax	(Honglin Li *et al.*, 1998, Y. Yao *et al.*, 2009)
	Bim	Favorise l'oligomérisation et l'activation proapoptotique de Bax – Liaison directe avec Bax	(Hardwick *et al.*, 2013)
	Bad	Favorise l'induction de l'activité proapoptotique de Bax – Dimérisation et séquestration des protéines Bcl-xL et Bcl-2	(Hardwick *et al.*, 2013)
	PUMA	Favorise l'oligomérisation et l'activation proapoptotique de Bax – Liaison directe avec Bax	(Hardwick *et al.*, 2013)
	NOXA	Inhibition de l'activité antiapoptotique de Mcl-1 – Liaison directe avec Mcl-1	(Hardwick *et al.*, 2013)

Avant de se pencher sur les divers mécanismes induits par l'apoptose dans la voie intrinsèque, il est important de bien comprendre la dynamique des protéines de la famille des Bcl-2 dans les cellules non apoptotiques (Figure 3, Tableau 4). Les protéines antiapoptotiques figurent au centre des régulations moléculaires. Un débalancement dans leur expression ou activité permet d'activer ou inhiber la voie de signalisation de l'apoptose.

Du point de vue physiologique, les protéines antiapoptotiques vont assurer la survie cellulaire via des interactions moléculaires avec les protéines proapoptotiques et les protéines BH3-only. Leur rôle primaire est de prévenir la perméabilisation de la membrane mitochondriale (Green, 2022). Cela dit, les protéines Bcl-2 et Mcl-1 figurent parmi les protéines antiapoptotiques les mieux étudiées. Mcl-1 se distingue de ses partenaires par sa structure et sa taille ; Mcl-1 possède un total de 350 résidus et ne possède que trois domaines BH (L. W. Thomas *et al.*, 2010). Mcl-1 a la capacité de former des hétérodimères avec les autres protéines de la famille de Bcl-2, particulièrement les protéines BH3-only (L. W. Thomas *et al.*, 2010). Cette interaction permet d'inhiber la polymérisation de Bax et Bak et donc, l'activation subséquente de la voie intrinsèque de l'apoptose. Il a été démontré que Mcl-1 possède une affinité de liaison supérieure pour les protéines BH3-only Bid, PUMA et NOXA, tandis que celle-ci est de faible intensité pour la protéine Bad (L. W. Thomas *et al.*, 2010). De plus, Mcl-1 empêche la progression de l'apoptose en liant de manière directe les protéines proapoptotiques Bak et Bax, induisant ainsi leur séquestration et inhibition (L. W. Thomas *et al.*, 2010). Il est important de noter que la liaison de Mcl-1 à Bax et Bak n'est pas un phénomène universel. L'affinité de liaison de la protéine Bak pour Mcl-1 est supérieure à celle de la protéine Bax lorsque celles-ci sont présentes de manière concomitante (Green, 2022). En effet, dans une cellule ayant une expression positive pour Bak et Bax, Mcl-1 va favoriser l'inhibition de la voie de signalisation de l'apoptose en se liant davantage à Bak (Green, 2022). Or, lorsque l'apoptose est médiée principalement par Bax, Mcl-1 inhibe tout de même l'activation de la voie intrinsèque de l'apoptose en se liant à cette dernière (Green, 2022). Il fut démontré dans les cellules de leucémies aiguës myéloïdes qu'une élévation de l'expression de Mcl-1 par des mécanismes intrinsèques à la cellule cancéreuse promeut la survie cellulaire en diminuant l'expression de Bak (Yakymiv *et al.*, 2021).

La protéine Bcl-2 médie ses effets antiapoptotiques en préservant l'intégrité mitochondriale : Bcl-2 est l'unique protéine antiapoptotique constitutionnellement retrouvée aux membranes (Tzifi *et al.*, 2012). Par exemple, Bcl-xL et Bcl-w iront s'ancrer à la membrane mitochondriale suivant un signal cytotoxique (Tzifi *et al.*, 2012). Ceci étant dit, la liaison de Bcl-2 à la membrane mitochondriale empêche l'oligomérisation de Bak et Bax par interaction directe (Green, 2022). En

comparaison à Mcl-1, Bcl-2 possède une meilleure affinité de liaison pour Bax que Bak (Green, 2022). Or, dans les cellules où l'apoptose est médiée par Bak, Bcl-2 peut tout de même se lier à celle-ci et inhiber l'activation de la voie intrinsèque de l'apoptose (Green, 2022). Bcl-2 possède aussi des actions physiologiques en aval : il a été démontré que Bcl-2 inhibe de manière directe le relâche du cytochrome C (Tzifi *et al.*, 2012), un phénomène biomoléculaire impliqué dans l'amplification du signal de mort cellulaire. De plus, Bcl-2 pourrait posséder une activité en amont par une régulation de l'activité des caspases, dont celle de la caspase-2 (Tzifi *et al.*, 2012).

Les protéines Bcl-xL et Bcl-w partagent une grande homologie de séquence (Hartman *et al.*, 2020). De manière similaire à Bcl-2, il a été démontré que Bcl-xL et Bcl-w interagit de manière directe avec Bax et Bak afin de prévenir la perméabilisation de la membrane mitochondriale aussi (Tzifi *et al.*, 2012). Cela dit, l'affinité de liaison de Bcl-w pour Bax est supérieure que pour celle de Bak (Ku *et al.*, 2011). Il fut d'ailleurs démontré que Bcl-xL exerce son rôle antiapoptotique en transloquant de manière continue Bax de la membrane mitochondriale au cytoplasme (Edlich *et al.*, 2011). Il existe une régulation de l'activité des protéines BH3-only par les protéines antiapoptotiques Bcl-w et Bcl-xL. Il a été démontré que Bcl-w lie les protéines Bad, tBid, Bim et PUMA, empêchant ainsi leur activité proapoptotique (Hartman *et al.*, 2020). De plus, il fut démontré que Bcl-xL empêche l'activation de Bax médiée par tBid (Hardwick *et al.*, 2013).

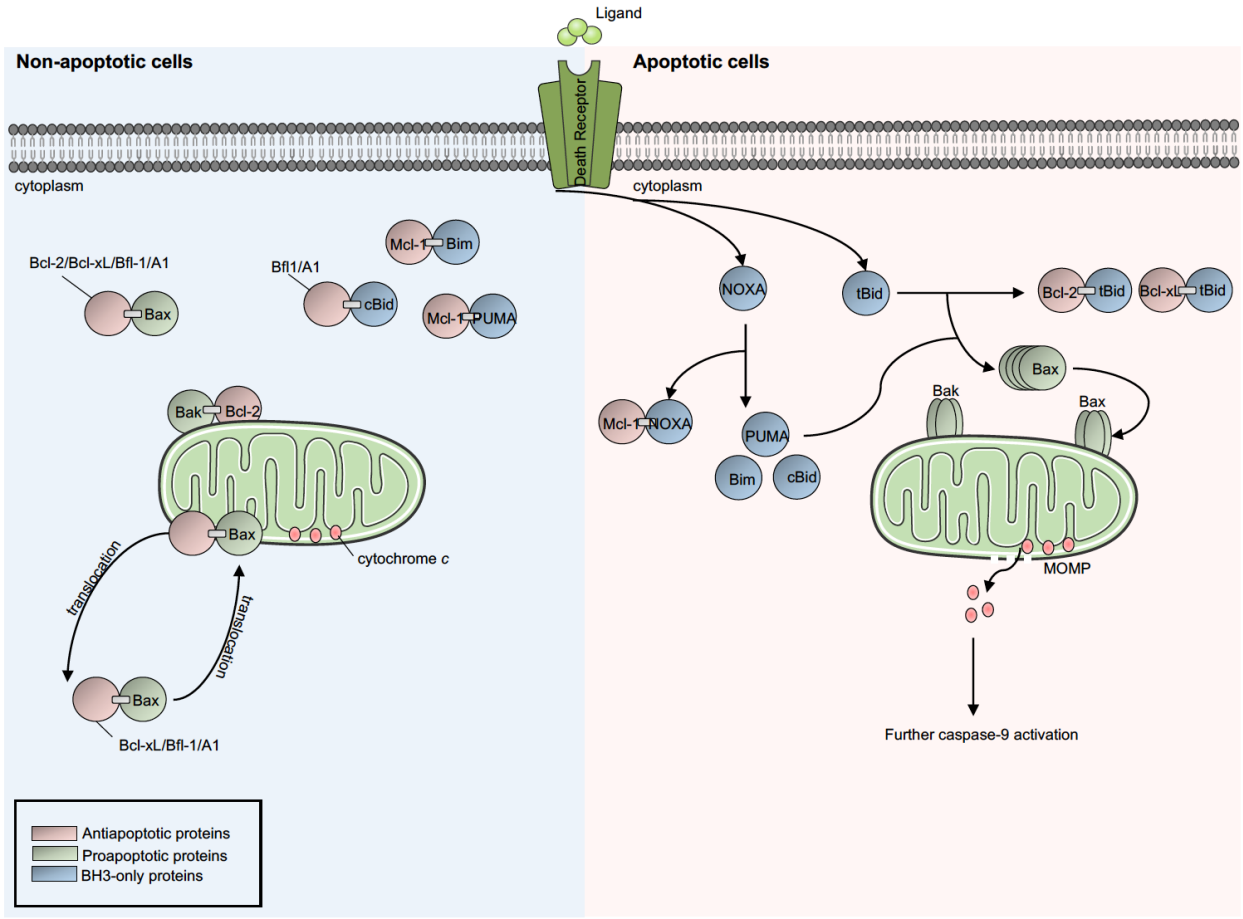


Figure 3. Régulation des protéines de la famille de Bcl-2.

Image tirée de : (M. Kulbay *et al.*, 2021c).

L'activation de la voie intrinsèque de l'apoptose peut avoir lieu par deux mécanismes, soit par activation directe par une atteinte mitochondriale ou une activation indirecte par la voie extrinsèque de l'apoptose. Les protéines BH3-only jouent un rôle crucial dans l'activation des protéines proapoptotiques. En effet, la protéine tBid médie la communication entre les deux voies de l'apoptose. Suivant l'induction de la voie extrinsèque de l'apoptose, l'activation de la caspase-8 va mener au clivage de Bid en la forme active, tBid, où celle-ci va transloquer du cytoplasme à la membrane mitochondriale (Honglin Li *et al.*, 1998). tBid possède une grande affinité pour Bcl-xL (Honglin Li *et al.*, 1998), ainsi que Bax (Y. Yao *et al.*, 2009). D'un côté, la liaison à Bcl-xL permet d'inhiber l'activation de l'apoptose, tandis que de l'autre côté, la liaison de tBid à Bax favorise la formation de pores au niveau de la membrane mitochondriale (Y. Yao *et al.*, 2009). Les protéines Bim et PUMA médient aussi leur action proapoptotique en liant Bax, tel tBid

(Hardwick *et al.*, 2013). Enfin, sous sa forme non phosphorylée, Bad dimérise avec les protéines Bcl-xL et Bcl-2, permettant ainsi le relâche de Bax et l'induction de son activité proapoptotique (Hardwick *et al.*, 2013).

Une fois activés, Bax et Bak vont former des pores à la membrane mitochondriale. La différence primordiale entre Bak et Bax est leur localisation cellulaire. Bax est retrouvée dans le cytosol, tandis que Bak a une localisation mitochondriale (Green, 2022). L'altération de l'intégrité de la membrane mitochondriale a pour effet de perturber le ΔΨm. Ceci va mener au relâchement du cytochrome c au niveau cytoplasmique, ainsi que de la protéine *second mitochondria-derived activator of caspases* (Smac/DIABLO) (Honglin Li *et al.*, 1998). Dans un premier temps, le cytochrome *c* va induire la formation de l'apoptosome en formant un complexe avec la protéine *apoptotic protease activating factor 1* (Apaf1) et la procaspase-9 (Dorstyn *et al.*, 2018). L'apoptosome est une structure heptaédrique, où les molécules d'Apaf1 forment une interaction directe avec trois à quatre molécules de procaspase-9 (Dorstyn *et al.*, 2018). Cette structure tridimensionnelle induit le clivage de la procaspase-9 en sa forme active, la caspase-9, ce qui a pour but de mener à l'activation des caspases -3 et -7 (Dorstyn *et al.*, 2018). Dans un deuxième temps, Smac est responsable de l'inhibition des protéines IAP, dont particulièrement XIAP (Adrain *et al.*, 2001). Une fois l'apoptosome activée, les étapes subséquentes de la voie intrinsèque sont similaires à celles de la voie extrinsèque : les deux voies ont convergé.

1.2.4 La voie du stress du réticulum endoplasmique

La voie du stress du RE est principalement induite par l'accumulation de protéines mal repliées dans le RE (Solano-Gálvez *et al.*, 2018). Le RE est impliqué dans de nombreuses fonctions physiologiques, dont le repliement des protéines. Les autres fonctions du RE impliquent la synthèse de lipides et stérols, ainsi que le stockage du calcium libre. À la suite d'un stress pathologique cellulaire, les niveaux de protéines mal repliées augmentent de manière significative. Ceci perturbe l'équilibre physiologique entre la capacité du RE à replier des protéines et la demande en repliement de protéines (Marchi *et al.*, 2012). Ce phénomène est à la base du signal de stress au sein du RE, qui est détectée et signalée par la voie de réponse de protéines non repliées (de l'anglais *unfolded protein response* (UPR) (Marchi *et al.*, 2012).

La voie UPR peut être subdivisée en trois voies de signalisation selon l'initiateur (Figure 1) : (1) la voie de la protéine kinase *RNA-like ER kinase* (PERK), (2) la voie de l'*inositol-requiring protein 1* (IRE1a) et enfin, (2) la voie facteur de transcription activateur 6 (ATF6) (Solano-Gálvez *et al.*,

2018). À l'état physiologique, les initiateurs PERK, IRE1a et ATF6 sont présents sous forme d'un complexe hétérodimérique avec la protéine chaperonne *immunoglulin-binding protein* (BiP/GRP78) (Kadowaki *et al.*, 2013). Les niveaux de BiP peuvent être modulés par la formation d'oligomères, de modifications post-traductionnelles et par l'induction de la voie UPR (Adams *et al.*, 2019). Lorsque le niveau de stress au sein du RE augmente, BiP se dissocie de ses partenaires, menant à l'activation des voies de signalisation (Kadowaki *et al.*, 2013). Chacune des molécules initiatrices induit donc son propre mécanisme de signalisation, avec pour objectif commun d'induire la transcription de facteurs proapoptotiques (*e.g. C/EBP homologous protein* (CHOP)) (Kadowaki *et al.*, 2013). De nombreuses protéines proapoptotiques sont sous la régulation du facteur de transcription CHOP, dont l'expression du DR5 et les protéines Bim et PUMA (Kadowaki *et al.*, 2013). Dans les paragraphes suivants, nous survolerons les mécanismes moléculaires impliqués dans la voie de signalisation des trois initiateurs de la voie UPR.

PERK est une molécule transmembranaire de type I et possède une activité kinase dans son domaine intracellulaire (Kadowaki *et al.*, 2013). La voie de signalisation de PERK, connue sous le nom regroupé de la voie PERK-eIF2α-ATF4, est strictement sensible à l'augmentation de protéines mal repliées dans le cytosol (Kadowaki *et al.*, 2013). La voie de signalisation de PERK peut soit favoriser la survie cellulaire ou l'apoptose, les deux processus ayant les mêmes partenaires de signalisation. Suivant la dissociation de PERK de la molécule chaperonne BiP, celle-ci va s'oligomériser puis induire une autophosphorylation activatrice (Kadowaki *et al.*, 2013). La forme activée de PERK induit subséquemment une phosphorylation de eIF2α au résidu sérine 51 (Ser51) (Kadowaki *et al.*, 2013). eIF2α est responsable de contrôler la quantité d'ARNm qui subit une traduction, afin de minimiser le taux de stress infligés au RE en cas de dysfonctionnement (Kadowaki *et al.*, 2013). De plus, lorsque activé, eIF2α induit la traduction de ATF4, qui a pour fonction ultime de réguler le métabolisme des acides aminés et du stress oxydatif (Kadowaki *et al.*, 2013). Lorsque la survie cellulaire n'est plus possible, ATF4 va induire l'activation de CHOP.

La voie IRE1a est similaire à celle de PERK, où l'autophosphorylation de IRE1a induit l'activation subséquente d'une cascade de phosphorylation. En plus d'une activité de kinase telle la protéine PERK, IRE1a possède aussi une activité d'endoribonucléase dans son domaine C-terminal (Kadowaki *et al.*, 2013). Les mécanismes menant à l'action de IRE1a sont identiques à celles de PERK. Lorsque la cellule est soumise à un stress du RE prolongé, il va y avoir une interaction entre IRE1 et TRAF2. Cette interaction va favoriser le recrutement de l'*apoptosis signal-regulating kinase* 1 (ASK1) et la formation du complexe IRE1-TRAF2-ASK1, qui aura pour fonction d'activer

la voie de la *c-Jun N-terminal kinase* (JNK) (Kadowaki *et al.*, 2013). La voie JNK induit l'apoptose via l'augmentation de la transcription des ARNm de Bax/Bak (Kadowaki *et al.*, 2013).

Enfin, la voie de ATF6 possède des particularités qui lui sont propres. À l'état physiologique, ATF6 est localisé au RE. Suivant l'activation de la voie du stress du RE, il y a une phosphorylation de ATF6 qui induit sa translocation à l'appareil de Golgi (Kadowaki *et al.*, 2013). À cet endroit, ATF6 subit des modifications ; il y a un clivage du domaine C-terminal et transmembranaire (Kadowaki *et al.*, 2013), ce qui permet la libération d'un fragment du domaine N-terminal (ATF6(N)) (Kadowaki *et al.*, 2013). ATF6(N) est transporté au noyau, afin d'induire les gènes cibles de la voie UPR (Kadowaki *et al.*, 2013).

Un état de stress chronique du RE a été rapporté dans de multiples cancers (Corazzari *et al.*, 2017). L'activation constante de la voie UPR permet l'angiogenèse et la prolifération cellulaire (Corazzari *et al.*, 2017). Il a été démontré que l'activation de PERK permet l'expansion tumorale par une action dans le cycle cellulaire. En effet, l'abolition *in vitro* de l'expression de PERK induit un retard de transition dans le cycle cellulaire à la phase G2, secondaire à l'activation des voies de réparation de l'ADN (Bobrovnikova-Marjon *et al.*, 2010). De plus, de nombreuses mutations au niveau de la voie de signalisation de l'apoptose ont été démontrées comme étant des événements activateurs de la voie du stress du RE. La perte de fonction de p53 active la voie d'IRE1a (Namba *et al.*, 2015). Chez les cellules cancéreuses issues de cancer du sein triple négatif, l'activation d'IRE1a permet la survie cellulaire en condition hypoxique (Tingting Zhang *et al.*, 2020). De plus, l'induction du stress du RE confère aux cellules cancéreuses une résistance à l'apoptose induite par l'inhibiteur de Bcl-2, le ABT-263 (Bellini *et al.*, 2020). À la suite de ces observations, la question suivante se pose : Une nouvelle mutation dans la voie de signalisation de l'apoptose agira-t-elle de manière directe sur la régulation de la voie du stress du RE ?

1.3 Le facteur de fragmentation de l'ADN

Jusqu'à présent, les études à propos des voies de signalisation impliquées dans la résistance des cellules cancéreuses aux agents inducteurs de l'apoptose se concentraient davantage sur les partenaires moléculaires impliqués en amont de l'activation du DFF. De nombreuses recherches ont démontré l'importance des protéines de la famille de Bcl-2 (Yip *et al.*, 2008), des mutations impliquant les caspases (Olsson *et al.*, 2011) et des inhibiteurs de PARP (Alice Chen, 2011) dans la régulation de l'induction de l'apoptose. Depuis les dernières années, il y a un intérêt croissant envers le DFF40, où plusieurs études ont démontré une corrélation inversement proportionnelle entre l'expression du DFF40 et le taux de survie chez ces patients (Banas *et al.*, 2017, Banas *et al.*, 2018a, Sánchez-Osuna *et al.*, 2016). Or, peu d'études se sont penchées vers les mécanismes cellulaires pouvant justifier la résistance au traitement. Dans cette section, nous passerons en revue les propriétés biomoléculaires du DFF40, ainsi que les études qui supportent son importance dans la résistance à l'apoptose, faisant de lui une cible d'intérêt en oncologie et particulièrement dans cette thèse.

1.3.1 Structure moléculaire

En 1998, une première étude de séquençage, dans l'optique de mieux comprendre les mécanismes d'activation du DFF45, a été réalisée par Inohara et ses collègues (N. Inohara *et al.*, 1998, N. Inohara *et al.*, 1999b). À partir de la base de données scientifique GenBank, ils ont pu démontrer une homologie de séquence entre les gènes *DFFA* (codant pour la protéine DFF45), *CIDEA* (codant pour la protéine *cell death-inducing DFFA-like effector* a (CIDE-A)), *CIDEB* (codant pour la protéine CIDE-B) et *Drep1* (codant pour la protéine *DNA fragmentation factor-related protein 1* (Drep1)) (N. Inohara *et al.*, 1998). Ils ont démontré que les protéines codées par ces gènes ont une homologie de séquence dans la région N-terminal, soit un domaine conservé entre les espèces au cours de l'évolution.

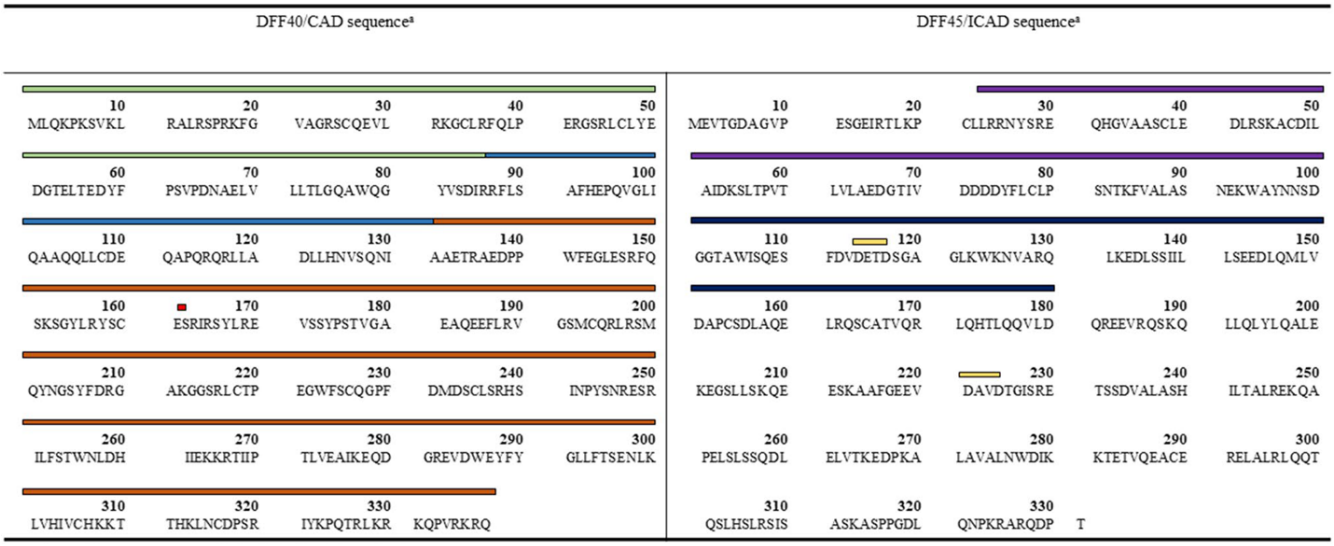
Les protéines de la famille CIDE sont un regroupement de trois protéines, CIDE-A, CIDE-B et CIDE-C (*c.-à-d.* FSP27). L'expression de chaque protéine varie en fonction de la localisation tissulaire (N. Inohara *et al.*, 1998). Elles ont été découvertes pour leur rôle proapoptotique (N. Inohara *et al.*, 1998, Slayton *et al.*, 2019). Il a été démontré que l'expression de la protéine CIDE-a ou CIDE-b dans les cellules 293T par transfection induit l'activation de l'apoptose par la présence de changements morphologiques précoces, tandis qu'une coexpression avec le DFF45 ou la protéine DREP-1 inhibe l'activation de l'apoptose et la mort cellulaire (N. Inohara *et al.*, 1998). Ainsi, les protéines CIDE sont considérées comme des effecteurs de la promotion de

l'apoptose et la fragmentation de l'ADN. De plus, il fut démontré que l'apoptose induite par CIDE-B requiert une localisation mitochondriale de la protéine (Zhengming Chen *et al.*, 2000). De nouveaux rôles pour les protéines CIDE ont ainsi été suggérés. Il a été démontré que la protéine CIDE-A localise à la mitochondrie et inhibe le thermogénine (*c.-à-d.* UCP1), une protéine découplant (Valousková *et al.*, 2008), tandis que la protéine CIDE-C régule l'expression des gènes impliqués dans la mitobiogenèse du tissu adipeux humain (Moreno-Navarrete *et al.*, 2014). De nombreuses études ont étudié et démontré le rôle physiologique des protéines CIDE, particulièrement en ce qui a trait à la régulation métabolique (Slayton *et al.*, 2019).

Ayant démontré une homologie de séquence entre la protéine DREP-1 et le DFF45, Inohara et Nuñez se sont par la suite intéressés aux potentielles protéines de la famille CIDE ayant une homologie de séquence avec le DFF40 (N. Inohara *et al.*, 1999b). En utilisant la séquence N-terminal des protéines CIDE et une base de données de séquençage, ils ont identifié trois nouvelles protéines reliées au DFF chez la drosophile : Drep-2, -3 et -4 (N. Inohara *et al.*, 1999b). Les protéines Drep-1, -2 et -3 ne possèdent pas d'activité de nucléase (N. Inohara *et al.*, 1999b). Drep-2 et Drep-3 pourraient interagir avec Drep-1 afin de moduler son activité (N. Inohara *et al.*, 1999b). L'homologue du DFF40 chez la drosophile est la protéine Drep-4 (N. Inohara *et al.*, 1999b). Ces résultats ont permis de reconfirmer la présence d'un domaine conservé en N-terminal chez les protéines de la famille CIDE ; les protéines de la famille CIDE auraient pour rôle de réguler l'activité du domaine effecteur. Il est important de souligner l'importance des protéines Drep dans les études de mutagenèse, car ce sont des modèles standards lorsque nous effectuons des études de génétiques. Plus de 75% des gènes humains ont un homologue chez la drosophile, qui ne présente pas d'obstacles significatifs dans la modification génétique (Lin *et al.*, 2014).

Suivant les multiples études de mutagenèse chez le modèle de la drosophile, la première étude de structure par cristallographie du DFF40 fut réalisée en 2004 par l'équipe de Byung-Ha Oh (Woo *et al.*, 2004). Cette étude a permis d'établir les acides aminés du DFF40 qui sont importants dans son activité catalytique, ainsi que de caractériser l'homologie de séquence dans le domaine N-terminal du DFF40 et DFF45. Les domaines structuraux, incluant les acides aminés fonctionnellement importants, sont représentés dans le Tableau 4. Dans les prochains paragraphes, nous discuterons donc en détail des trois domaines fonctionnels du DFF40 ainsi que des particularités de chacune.

Tableau 4. Comparaison des séquences d'acides aminés des molécules DFF40 et DFF45 chez l'humain



Les domaines CIDE du DFF40 sont représentés par les bandes de couleurs distinctes : le premier domaine (C1 ; bande verte), deuxième domaine (C2 ; bleu clair) et troisième domaine (C3 ; bande orange). La bande rouge représente le début du segment contenant l'activité catalytique du DFF40. La bande mauve représente le domaine d'interaction du DFF40 avec le DFF45. La bande bleue royale représente les sites de liaison de la sous-unité inhibitrice. Les sites de clivages du DFF45 par la caspase-3 sont représentés par les bandes jaunes. [a]Les séquences d'acides aminés ont été obtenues avec la base de données Uniprot. Tableau tiré de : (Merve Kulbay *et al.*, 2021a).

Le premier domaine du DFF40 (C1, Figure 4) est le domaine N-terminal, connu sous le nom du domaine CIDE-N. Ce domaine possède 80 acides aminés qui sont hautement conservés entre le DFF40 et DFF45 et est impliqué dans leur interaction (Woo *et al.*, 2004). De plus, le domaine CIDE-N est nécessaire à l'oligomérisation du DFF40 à la suite de son activation (Choi *et al.*, 2017). Il a été démontré que le domaine CIDE-N de la protéine Drep-4 possède des surfaces acides et basiques qui sont responsables de la formation de structures filamenteuses, par l'entremise d'une interaction dépendante des charges (Choi *et al.*, 2017, Lee *et al.*, 2013). Il a récemment été démontré que la mutation des résidus G92I, S96K, Y100A et S103K du gène *Drep4* inhibe la formation de structures filamenteuses (Ha *et al.*, 2022). De plus, cette interaction est dépendante de l'osmolarité extracellulaire ; des concentrations salines élevées inhibent la formation de complexes oligomériques de Drep-4 (Ha *et al.*, 2022). Chez son homologue humain le DFF40, le domaine CIDE-N est impliqué dans la modulation de l'activité catalytique de par sa conformation moléculaire. Une mutation du résidu G51I (correspondant au mutant G92I chez Drep-4) diminue significativement l'activité catalytique du DFF40, suggérant que la conformation filamenteuse est nécessaire à son activité catalytique. Il a été démontré que la boucle charnière et l'hélice de liaison du DFF40 sont localisées entre le domaine CIDE-N et le domaine catalytique (voir Figure 4), et que celle-ci est cruciale pour l'activité d'endonucléase (Ha *et al.*, 2022). En effet, le résidu W78 du domaine CIDE-N du DFF40 (correspondant au résidu I119 de Drep-4) est localisé à l'extrémité de celle-ci, près de la boucle charnière, et est impliqué dans des interactions hydrophobes (Ha *et al.*, 2022). Ceci permet à l'hélice de liaison d'effectuer une rotation de 32° et d'optimiser le positionnement du domaine catalytique du DFF40 sur le brin d'ADN (Ha *et al.*, 2022).

Le second domaine (C2, Figure 4) est composé des acides aminés 86 à 131 et de trois hélices alpha (Woo *et al.*, 2004). Ce domaine est principalement impliqué dans la dimérisation du DFF40 par le rapprochement des hélices alpha (Woo *et al.*, 2004). Il a été démontré par analyse cristallographique que le résidu H128 médie cette interaction dimère-dimère (Woo *et al.*, 2004).

Le troisième domaine (C3, Figure 4), composé des acides aminés 132 à 345, est celui qui possède l'activité catalytique du DFF40 (Woo *et al.*, 2004). Il est composé d'hélices alpha, de quatre feuillets beta et d'une boucle à son extrémité C-terminal (Woo *et al.*, 2004). La boucle contient le signal de localisation nucléaire (Woo *et al.*, 2004), qui permet la translocation du DFF40 du cytoplasme au noyau. Chez le modèle d'étude murin, il a été démontré par mutagenèse dirigée, à l'aide de plasmide contenant le glutathion S-transférase (GST), que quatre résidus d'histidines (H242, H263, H308, H313) étaient cruciaux à l'activité catalytique du DFF40 (Meiss

et al., 2001). Chez l'humain, les premières études ont démontré qu'une délétion des acides aminés histidines 162-345 ou 290-345 provoque une inhibition totale de la fragmentation de l'ADN dans des cellules épithéliales d'embryons de rein (Naohiro Inohara *et al.*, 1999a). Ensuite, des études de mutagenèse dirigée ont démontré qu'une substitution des résidus His263, His308, His310 ou His313 abolit la fragmentation de l'ADN, sans intervenir avec sa capacité de liaison à l'ADN (Korn *et al.*, 2002, Sakahira *et al.*, 2001).

À la lumière de ces résultats, nous pouvons donc suggérer qu'une absence de DFF40 dans les cellules cancéreuses pourrait être à l'origine d'une résistance à l'apoptose, expliquée par l'absence de fragmentation d'ADN lorsque nous modulons son domaine catalytique. De plus, étant donné qu'un rôle dans l'homéostasie lipidique et la régulation métabolique pour les protéines de la famille CIDE a été démontré, un potentiel rôle du DFF40 dans ces mécanismes n'est pas exclu. Jusqu'à ce jour, aucune étude n'a suggéré une implication du DFF40 dans la régulation métabolique. Cependant, il a été rapporté à de multiples reprises que les cellules cancéreuses ont une reprogrammation de leur métabolisme énergétique ; un concept qui sera revu en détail dans la section 1.7. De nombreuses questions hypothétiques restent à être prouvées, dont certaines seront discutées dans cette thèse.

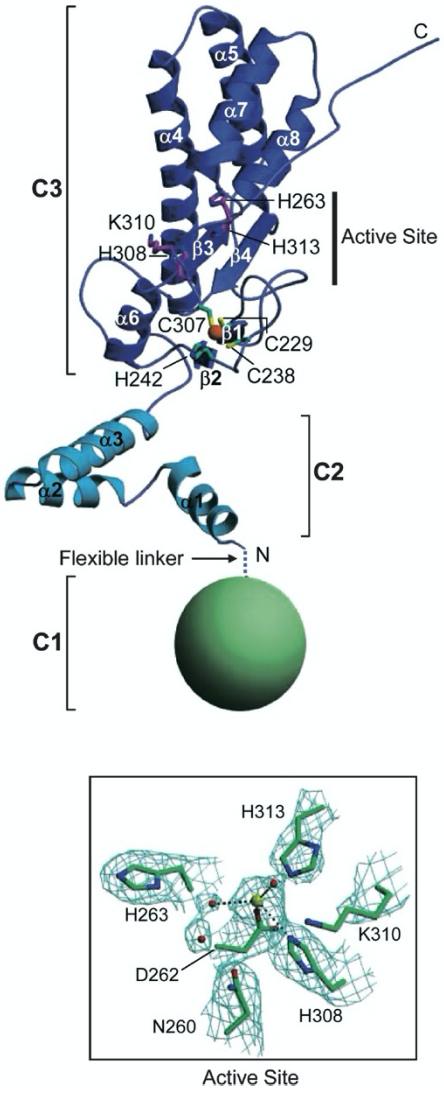


Figure 4. Aspects structuraux du DFF40.

Représentation d'un monomère du DFF40. Panneau du haut : les résidus d'histidine et de lysines impliqués dans l'activité catalytique du DFF40 sont représentés par des lignes. Le site de liaison du zinc est démontré par une sphère orange. Panneau du bas : Schématisation du noyau du DFF40 permettant son activité catalytique. Le Mg2+ est représenté par une sphère jaune. Image adaptée de (Woo et al., 2004).

1.3.2 Propriétés biomoléculaires du DFF40

Afin que le DFF40 puisse exercer son rôle d'endonucléase au maximum, certaines conditions physiologiques doivent être respectées. L'activité catalytique du DFF40 est maximale à un pH de 7.5 et requiert la présence d'ions de magnésium (Piotr Widlak *et al.*, 2000). Il a été démontré par cristallographie que les résidus impliqués dans l'activité catalytique du DFF40 sont organisés avec une configuration spatiale bien particulière, où elles forment un cercle avec le résidu Asp262 en position centrale, comme démontré à la Figure 4 (Woo *et al.*, 2004). La substitution du résidu Asp262 par une alanine diminue de manière très significative l'activité catalytique du DFF40 (Scholz *et al.*, 2003); le résidu Asp262 est un chélateur de Mg^{2+}.

Des concentrations optimales en zinc doivent être présentes pour la formation de dimères de DFF40 ; une concentration élevée de Zn^{2+} inhibe l'activité du DFF40 (Piotr Widlak *et al.*, 2000). La liaison des molécules de Zn^{2+} au DFF40 nécessite des chaines latérales invariantes possédant les résidus Cys229, Cys238, His242 et Cys207 (Figure 5) (Woo *et al.*, 2004). Il a été démontré qu'une substitution du résidu His242 par une alanine inhibe la liaison du zinc au DFF40 (Woo *et al.*, 2004). Ceci inhibe subséquemment la fonction proapoptotique du DFF40 due à une précipitation de la protéine (Woo *et al.*, 2004). Les ions de Zn^{2+} sont donc importants pour la stabilité structurale du DFF40.

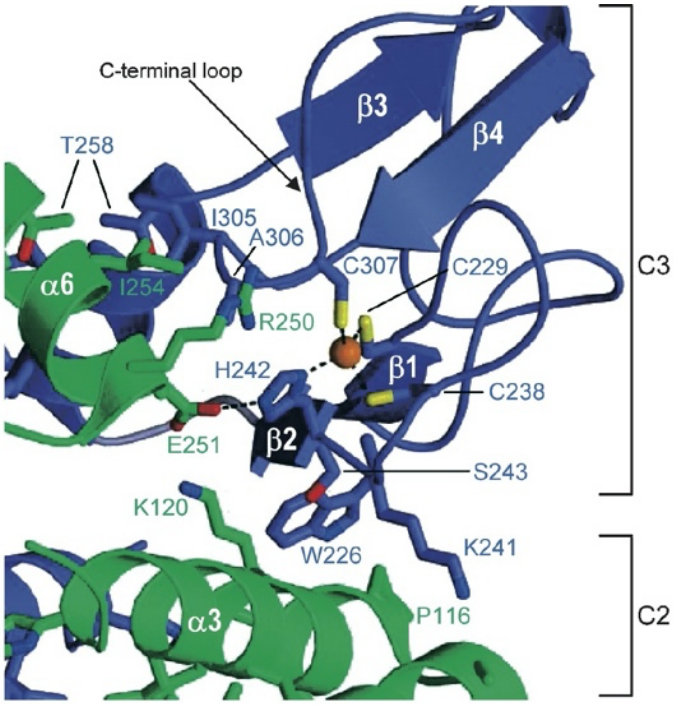


Figure 5. Représentation des acides aminés fonctionnels du domaine catalytique du DFF40.

Le site de liaison du zinc est représenté par une sphère orange. L'unique pont d'hydrogène se situe entre les résidus His242 et Glu251. Image adaptée de (Woo *et al.*, 2004).

1.3.3 Le complexe DFF40/DFF45 et les partenaires d'interaction

Dans les cellules non apoptotiques, le DFF40 est présent sous forme d'hétérodimère, connu sous le nom du complexe du DFF. Ce dernier contient le DFF40 qui est lié au DFF45, soit la sous-unité inhibitrice. Non seulement le DFF45 empêche l'activation du DFF40 et donc la fragmentation de l'ADN, mais il permet aussi le repliement du DFF40 à la suite de la traduction (Samejima *et al.*, 1998).

Le DFF45 peut être retrouvé sous forme de deux isoformes suivant l'épissage alternatif de son ARN messager (ARNm), à savoir l'isoforme long (DFF45-L) ou court (DFF45-S/DFF35) (Kawane *et al.*, 1999). L'affinité de liaison de l'isoforme DFF45-S est plus grande pour le DFF40 (Gu *et al.*, 1999). Le ratio de DFF45 et DFF35 au sein des cellules est d'une importance primordiale afin d'assurer une fragmentation de l'ADN optimale (Xialu Li *et al.*, 2005). Il a été démontré que lorsque les niveaux de DFF35 sont diminués précocement dans l'apoptose, le transport nucléaire du complexe du DFF est favorisé (Sunatani *et al.*, 2018). Les deux isoformes exercent une activité inhibitrice sur le DFF40, or seulement le DFF45-L permet son repliement et la translocation du complexe dans le compartiment nucléaire grâce à son signal de localisation nucléaire (NLS) (Sakahira *et al.*, 1999a, Samejima *et al.*, 2000). Il est à noter que la localisation du DFF40 est sujette à débat depuis de nombreuses années. Les premières études ont démontré que suivant sa traduction dans le cytosol, le complexe du DFF est directement transporté au noyau par la voie de l'importine α/β (Neimanis *et al.*, 2007, Widłak, 2000, Woo *et al.*, 2004). Une abolition de l'exterminé C-terminale contenant le NLS inhibe le transport du DFF40 au noyau (Neimanis *et al.*, 2007). D'autres études stipulent que le complexe du DFF est cytoplasmique dans les cellules non apoptotiques (Enari *et al.*, 1998, Sabol *et al.*, 1998).

L'interaction entre le DFF40 et le DFF45 est régie par les domaines CIDE-N, qui implique les domaines fonctionnels du DFF45 (D1, D2, D3) et les domaines activateurs et catalytiques du DFF40 (McCarty *et al.*, 1999). Deux interactions peuvent être décrites dans la littérature. La première interaction implique la région basique du DFF40, qui inclut les acides aminés Lys9, Lys18, Lys32 et Arg36, et le noyau acide du DFF45, qui inclut les acides aminés Asp66, Asp71, Asp72 et Asp74 (P. Zhou *et al.*, 2001). La seconde interaction de plus grande puissance implique quant à elle le domaine CIDE-N du DFF40 et le domaine juxtaposé au site de clivage du DFF45 par la caspase-3 (voir Tableau 1) (Kutscher *et al.*, 2012). Il a été démontré que les acides aminés 101 à 180 du DFF45 sont impliqués dans son interaction avec le DFF40, tandis que les acides aminés 23 à 100 sont responsables de son activité inhibitrice (Gu *et al.*, 1999).

Le DFF45 peut être clivé à deux sites, soit les régions DEPD (acides aminés 114 à 117) et DAVD (acides aminés 221 à 224) (P. Zhou *et al.*, 2001). Ces clivages permettent de libérer le DFF40 et induire son homo-oligomérisation. Une fois activé, le DFF40 existe sous forme de tétramère avec une configuration spatiale très spécifique (Figure 6). Des études de cristallographie ont démontré que suivant son activation, les domaines CIDE-N du DFF40 forment des structures semblables à des filaments (*filament-like structures*) qui permettent l'oligomérisation du DFF40 sous forme d'une hélice (Choi *et al.*, 2017, Ha *et al.*, 2018). Des études de mutagenèse dirigée ont permis de mettre en évidence que cette oligomérisation se produit de manière crânio-caudale. En effet, la mutation des résidus E57, D58, F60 et W81 abolit les formations filamenteuses du DFF40 à la suite de son activation et nuit au clivage de l'ADN (Choi *et al.*, 2017). En somme, la conformation spatiale du DFF40 permet de retrouver l'activité catalytique en distal avec au centre du noyau les domaines CIDE, telle une paire de ciseaux.

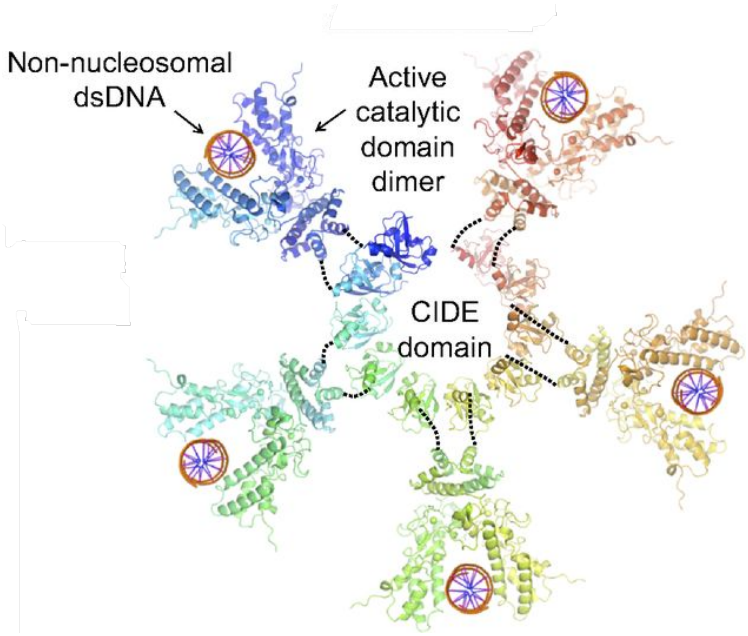


Figure 6. Configuration spatiale 3D du DFF40.

Les domaines CIDE des monomères du DFF40 se retrouvent au centre, tandis que les domaines catalytiques sont orientés vers l'extérieur. L'image a été reproduite en utilisant la structure moléculaire de CAD chez la souris. Image adaptée de (Choi *et al.*, 2017).

Une fois activé, le DFF40 va générer des bris doubles brin de l'ADN. Des études complémentaires ont démontré que le DFF40 peut aussi être impliqué dans la génération de bris au brin unique de l'ADN (Iglesias-Guimarais *et al.*, 2013, P. Widlak *et al.*, 2001). Il a été démontré qu'une abolition de l'expression du DFF40 par un siRNA dans les cellules humaines épithéliales dérivées de neuroblastes (SK-N-AS) abolit la presque totalité des bris simples brins induient par la staurosporine (Iglesias-Guimarais *et al.*, 2013). L'utilisation du même siRNA dans les cellules épithéliales HeLa a aussi permis de démontrer une inhibition de l'induction de la fragmentation de l'ADN induite par la roténone, un inhibiteur du complexe mitochondrial I (Tsuruta *et al.*, 2007).

De nombreuses protéines ont été identifiées comme étant des régulateurs de l'activité catalytique du DFF40 (Tableau 5). La protéine de choc thermique (HSP) 70 stimule et stabilise le DFF40 activé (Hanus *et al.*, 2010). L'interaction du DFF40 avec l'histone H1 permet à l'endonucléase de se lier à l'ADN au niveau du bris et de stimuler son activité catalytique (Xuesong Liu *et al.*, 1999). Il existe 6 isoformes de l'histone H1 et elles possèdent toutes le même potentiel de manière équivalente (Piotr Widlak *et al.*, 2005). Certaines protéines comme les HMGB 1 et 2 stimulent le clivage de l'ADN sans interaction directe avec le DFF40, ni par modulation de son activité (Kalinowska-Herok *et al.*, 2008). En effet, elles induisent des distorsions locales de l'ADN suivant leur liaison, afin de rendre les brins accessibles au clivage par l'endonucléase (Kalinowska-Herok *et al.*, 2008). De premières études vers le 21^{e} siècle ont suggéré une interaction de la topoisomérase II (TOP2) avec le DFF40, plus particulièrement la TOP2α qui est connue pour son rôle dans la ségrégation de chromosomes et la réplication de l'ADN (Grue *et al.*, 1998). Initialement, les études à ce sujet étaient controversées. Une première étude avait démontré qu'une inhibition sélective de l'activité de TOP2 avec ICRF-193 n'était pas nécessaire pour la fragmentation de l'ADN (Beere *et al.*, 1996). Par contre, l'induction de l'apoptose par le peroxyde d'hydrogène (H_2O_2) chez les monocytes humains U937 induisait l'excision de boucles de fragments d'ADN via la TOP2, où une mutation dans l'expression de celle-ci diminuait de manière significative les niveaux de fragments d'ADN (T. K. Li *et al.*, 1999). Dans une seconde étude, il a été démontré que l'inhibition de la TOP2 avec 3 inhibiteurs distincts chez les cellules HeLa abolit la condensation de la chromatine (Durrieu *et al.*, 2000). L'ajout de protéines purifiées de TOP2 dans des extraits de noyaux permet de rétablir les phénomènes apoptotiques, dont la condensation de la chromatine (Durrieu *et al.*, 2000). À la suite des nombreuses études qui en sont d'écoulés, une revue de littérature exhaustive supporte le rôle de la TOP2 dans les voies de dommages à l'ADN (Calderwood, 2016). La TOP2 serait responsable d'induire des bris doubles brins (BDB) à l'ADN. Ceci permettrait le recrutement de protéines impliquées dans les voies de réparation de l'ADN, dont la DNA-PK (*c.-à-d.* la protéine kinase dépendante de l'ADN)

(Calderwood, 2016). Récemment, il a été démontré que les cellules cancéreuses de la prostate résistantes aux taxanes ont une expression élevée de TOP2α, où cette corrélation est associée avec un mauvais pronostic (Hongo *et al.*, 2021). Or, il est possible de renverser la sensibilité des cellules résistantes aux taxanes avec des inhibiteurs de la TOP2 (Hongo *et al.*, 2021). Dans un contexte où l'expression du DFF40 est abolie, nous nous posons donc la question suivante : est-ce qu'une diminution de l'expression du DFF40 dans les cellules cancéreuses pourrait être compensée par une augmentation de l'expression de la TOP2α ?

Tableau 5. Identification des protéines partenaires du DFF40 et caractérisation de leurs rôles

Protéines d'interaction du DFF40	Rôle physiologique
Histone H1	Permet la capacité de liaison à l'ADN au DFF40 et stimule son activité d'endonucléase
Histone H2AX	Recrutement nécessaire à la fragmentation de l'ADN par le DFF40
HMGB 1/2	Stimule indirectement le clivage de l'ADN par distorsions locales
Topoisomérase IIα	Participe au processus de condensation de la chromatine Induit le recrutement de protéines de la voie de réparation de l'ADN suivant des dommages doubles brins à l'ADN
HSP70	Stimule et stabilise le DFF40 activé

Tableau adapté de : (Merve Kulbay *et al.*, 2021a)

1.3.4 Relations entre l'expression du DFF40 et DFF45 dans la malignité tumorale

Jusqu'à présent, nous avons démontré l'importance du DFF40 lors l'induction *in vitro* de l'apoptose dans des études fondamentales. Cependant, qu'en est-il de l'importance clinique d'une mutation fonctionnelle du DFF40 ? De nombreuses études ont mis en évidence une corrélation entre l'expression du DFF40 et/ou du DFF45 et l'agressivité de diverses tumeurs, que nous survolerons dans cette section.

Les premières études se sont penchées sur la relation entre l'expression du DFF45 et le pronostic cancéreux. Une analyse quantitative des ARNm du DFF45 dans des tumeurs épidermoïdes du cancer de l'œsophage a démontré qu'une diminution du taux d'ARNm conférait aux patients un mauvais pronostic (Konishi *et al.*, 2002). Chez les patients avec un stade avancé, une atteinte de plusieurs ganglions et ayant une métastase au réseau lymphatique, l'expression des ARNm du DFF45 était significativement diminuée (Konishi *et al.*, 2002). La même tendance fut observée chez les cellules épithéliales du cancer du côlon (Youssef Errami *et al.*, 2013b) et de l'adénocarcinome rénal (Rajandram *et al.*, 2016). De manière intéressante, quelques études ont démontré une tendance opposée. En effet, dans les cancers de l'ovaire de type séreux (Brustmann, 2006) et l'hyperplasie typique et atypique de l'endomètre (Brustmann, 2007), une augmentation de l'expression du DFF45 est liée à une résistance à l'apoptose.

En ce qui concerne la relation entre le DFF40 et la tumorigénicité, les observations sont similaires aux premières corrélations rapportées avec le DFF45. Chez les patientes atteintes de cancer de l'endomètre non endométrioïdes et endométrioïdes de haut grade avec une diminution de l'expression du DFF40 au sein des tumeurs, il fut démontré que leur taux de survie sans maladie (DFS) et leur taux de survie globale (OS) étaient significativement diminués en comparaison aux patientes avec un cancer de bas grade (Banas *et al.*, 2018a). La même observation fut observée chez les patientes atteintes de léiomyosarcomes utérins (Banas *et al.*, 2017). En effet, il a été démontré que le DFS chez les patients atteints de léiomyosarcomes utérins n'exprimant pas le DFF40 est réduit d'environ 100 mois, tandis que l'OS est réduit de 80 mois, en comparaison aux patients ayant une tumeur positive pour l'expression du DFF40 (Banas *et al.*, 2017). Parmi les tumeurs les plus agressives, près de 80% des glioblastomes sont malins et ils représentent à eux seuls près de 30% des tumeurs du système nerveux central (Ostrom *et al.*, 2014). Une étude réalisée par l'équipe de Yuste en 2016 a démontré que les glioblastomes ont un trait commun, soit une déficience intrinsèque en DFF40 qui est impliquée dans un défaut de fragmentation de l'ADN (Sánchez-Osuna *et al.*, 2016). De plus, les patients atteints d'un glioblastome avec une

inhibition de l'expression du DFF40 ont une diminution du taux d'OS, qu'ils aient reçu préalablement ou non un traitement néoadjuvant combiné à la radiothérapie (Hanus *et al.*, 2010). Il fut aussi démontré que tous les astrocytomes, peu importe leur grade, ont une diminution de l'expression du DFF40 (Sánchez-Osuna *et al.*, 2016). Or, aucune corrélation ne fut établie avec le résultat clinique. Dans l'ensemble, ces études suggèrent qu'une expression négative du DFF40 dans les cellules tumorales est liée à un mauvais pronostic.

Certaines études se sont penchées sur l'impact d'une modulation de l'expression du DFF40 sur la sensibilité des cellules cancéreuses aux agents inducteurs d'apoptose. Il a été démontré que la surexpression du DFF40 dans les cellules cancéreuses du sein T-47D augmente la sensibilité à la doxorubicine (Bagheri *et al.*, 2015b), ainsi qu'à l'acétazolamide et la sulfabenzamide (Bagheri *et al.*, 2014). Une récente étude a d'ailleurs démontré que l'induction de l'expression génique du DFF40 par un promoteur couplé à la survivine dans les cellules cancéreuses de mélanome augmente l'apoptose induite par la dacarbazine, un agent de chimiothérapie (Minaiyan *et al.*, 2021). De plus, il est possible d'activer l'agglomération de DFF40 nucléaire diminuée dans les glioblastomes à l'aide du gossypol et induire l'apoptose de manière dépendante des caspases (Martínez-Escardó *et al.*, 2021).

À la lumière de ces résultats, nous pouvons comprendre qu'un réel enjeu existe dans la prise en charge de patients ayant des tumeurs avec un génotype déficient en DFF. Plusieurs études ont démontré l'impact à long terme d'une tumeur déficiente en DFF40 sur la survie des patients. Or, jusqu'à ce jour, aucune n'a élucidé les voies de signalisation impliquées dans cette modulation. L'avancement des connaissances dans la médecine personnalisée requiert une meilleure compréhension des processus moléculaires impliqués, afin de permettre le développement de meilleurs outils thérapeutiques.

1.4 Anomalies de l'apoptose sans fragmentation de l'ADN

Il est important de noter que nous pouvons observer une activation de l'apoptose avec absence de fragmentation de l'ADN. En effet, au courant des dernières décennies, de nombreuses études ont démontré l'activation de la voie signalétique de l'apoptose, ainsi que des changements nucléaires reliés à l'apoptose, mais sans la présence de fragmentation d'ADN.

Des études de structure ont permis de démontrer qu'il était possible d'induire une activation de l'apoptose sans fragmentation de l'ADN. La première étude menée en 1999 par l'équipe de Sakahira portait sur l'impact d'une mutation dans l'expression du DFF45 chez les cellules lymphocytaires Jurkat dans l'apoptose induite par la staurosporine (STS) (Sakahira *et al.*, 1999b). Ils ont démontré qu'une substitution des acides aminés Asp117 et Asp224 par l'acide glutamique induisait une condensation de la chromatine en périphérie du noyau (Sakahira *et al.*, 1999b). Or, aucune fragmentation de l'ADN n'était identifiable. Les résidus Asp117 et Asp224 sont les deux sites de clivage du DFF45 par la caspase-3 active, comme expliqué ci-haut (Sakahira *et al.*, 1999b). Ainsi, en l'absence de clivage du DFF45, le DFF40 inactif ne peut induire la fragmentation de l'ADN. Cette découverte a mené à la réalisation d'études similaires, où une équipe différente a pu aussi démontrer qu'une mutation dans la séquence du DFF45-L au site de clivage de la caspase-3 chez les cellules Jurkat et TF-1 induisait le même phénotype (McIlroy *et al.*, 1999). Des expériences de mutagenèse dirigée et de substitution des acides aminés ayant un rôle majeur dans l'activité catalytique du DFF40, comme énumérées à la section 1.3, ont aussi démontré l'absence de fragmentation de l'ADN en présence d'induction d'apoptose.

Dans les modèles murins, il a été démontré qu'une déficience en DFF45 induit non seulement une résistance à la fragmentation de l'ADN dans les splénocytes et thymocytes, mais ainsi qu'une résistance à la condensation de la chromatine (J. Zhang *et al.*, 1998). Or, dans le contexte d'une insulte secondaire à un impact cortical chez les souris déficientes en DFF45, il a été démontré qu'il est possible d'observer une fragmentation de l'ADN par d'autres nucléases que le DFF40 (Yakovlev *et al.*, 2001).

Du côté des modèles cellulaires humains, il a été démontré qu'une déficience intrinsèque dans l'expression du DFF40 dans les glioblastomes perturbe l'hydrolyse de l'ADN, sans nuire au processus d'activation de la caspase-3 et du clivage subséquent du DFF45 (Sánchez-Osuna *et al.*, 2016). Une absence en DFF dans les cellules de carcinome rénal RCC-91 permet l'activation de l'apoptose en l'absence de fragmentation de l'ADN (Yamaguchi *et al.*, 2004). Les cellules neuronales SK-N-AS exposées à la STS ont une absence totale de fragmentation de l'ADN, en comparaison aux cellules neuronales de la lignée SH-SY5Y, malgré des niveaux similaires de

DFF40 (Iglesias-Guimarais *et al.*, 2012). En effet, il a été démontré que seule une expression de DFF40 n'est pas suffisante : l'expression du DFF40 au cytosol est cruciale pour la fragmentation nucléaire (Iglesias-Guimarais *et al.*, 2012). De plus, notre laboratoire a démontré qu'une déficience dans l'expression du récepteur CD45 dans des cellules lymphocytaires inhibait la fragmentation de l'ADN malgré une activation appropriée de la caspase-3 et un clivage du DFF45 (Desharnais *et al.*, 2008).

Toutes les anomalies de clivage du DFF40 rapportées dans la littérature soulèvent la question suivante : les accumulations de mutations génomiques causent-elles une instabilité génomique ? Lorsque la cellule humaine possède une mutation initiatrice ne pouvant être réparée, celle-ci risque de se transmettre de manière exponentielle lors des divisions mitotiques subséquentes. Il a été démontré à l'aide de cellules d'embryons de souris déficiente en DFF que celles-ci étaient plus susceptibles à la carcinogenèse induite par la radiation (Yan *et al.*, 2006a). Le concept d'instabilité génomique sera discuté de manière plus approfondie dans la section 1.6.

1.5 Les agents cytotoxiques et inducteurs d'apoptose

Plusieurs agents cytotoxiques et inducteurs d'apoptose peuvent être utilisés lors des études de mécanismes moléculaires. Ils sont d'une très grande variété et possèdent leurs propres caractéristiques selon leur mode de fonctionnement. Le choix d'un bon agent inducteur devient donc très complexe et est principalement régie par l'objectif de l'expérience. Dans cette section, nous reverrons les principales caractéristiques de la STS, du tributylétain (TBT) et de certains médicaments de chimiothérapies, à savoir ceux de la classe des antimétabolites et les inhibiteurs de la TOP2.

1.5.1 La staurosporine

La STS fut découverte en 1977 et isolée pour la première fois par Omura et ses collègues à partir de la bactérie *Streptomyces staurosporeus* (Omura S Fau - Iwai *et al.*, 1977). L'intérêt envers cet agent s'est accru dans les années 90; il a été démontré que la STS est un inhibiteur de la protéine kinase C (PKC), une enzyme essentielle à la prolifération cellulaire (Ward *et al.*, 1992). Depuis, de nombreuses études se sont penchées sur les mécanismes d'action de la STS, en particulier sur sa puissance d'induction de l'apoptose.

1.5.1.1 Les mécanismes d'action

Les premières études de cinétique ont permis d'établir que la STS médie son effet antiprolifératif par sa liaison à un complexe de la PKC (*c.-à-d.* MgATP et l'histone III-S) au domaine catalytique (Ward *et al.*, 1992). Les premiers effets moléculaires observés sont l'accumulation de substrats, en particulier l'inositol phosphate (King *et al.*, 1989). Il a été démontré dans les cellules d'adénocarcinome du côlon que l'inhibition de la prolifération est ultimement secondaire à un arrêt de transition de la phase G2 à M, résultant en une apoptose des cellules (Qiao *et al.*, 1996).

Au courant des dernières décennies, de nombreuses études se sont penchées sur les changements biomoléculaires induits par la STS dans la voie de signalisation de l'apoptose. Dans la voie intrinsèque de l'apoptose, il a été démontré qu'une induction de l'apoptose est initialement marquée par une hyperpolarisation précoce du potentiel de membrane mitochondriale, permettant ainsi la relâche du cytochrome C (Scarlett *et al.*, 2000). Une régulation des protéines de la famille de Bcl-2 a aussi été démontrée. L'effet proapoptotique de la STS serait dû à une sous-régulation de la protéine Bcl-2 et à une augmentation de la régulation de la protéine Bcl-XL

(Giuliano *et al.*, 2004), ainsi qu'à une activation de la caspase-9 (Malsy *et al.*, 2019). Ensuite, il a été démontré que la STS induit l'activation de la voie extrinsèque de l'apoptose par induction de la caspase-8 et clivage de Bid dans les cellules MCF7 (Tang *et al.*, 2000). La STS exerce son effet de manière dépendante des caspases majoritairement; il a été démontré que l'apoptose induite par la STS est médiée par l'activation de multiples caspases, dont les caspases-3, -2 et -9 (Caballero-Benítez *et al.*, 2003).

1.5.2 Le tributylétain

Le TBT est un puissant perturbateur endocrinien reconnu pour ses propriétés antibactériennes et fongicides (Suzdalev *et al.*, 2015). Pendant près de 60 ans, avant son abolition en 2008, il fut utilisé dans l'industrie marine en tant qu'agent antisalissure (Batista-Andrade *et al.*, 2018). Or, son utilisation ne se limite pas seulement au milieu marin ; le TBT peut aussi être retrouvé dans les plastiques (*c.-à-d.* PVC), dans les désinfectants et dans les pesticides (Taewoo Kim *et al.*, 2017).

Le TBT, comme la majorité des perturbateurs endocriniens, fut d'intérêt important avant son abolition dans ces domaines étant donné ses propriétés physico-chimiques. En effet, c'est un agent liposoluble, ayant un haut taux d'absorption dans la terre (Meador, 2000). Malgré l'interdiction d'usage du TBT depuis maintenant plus d'une décennie, la concentration de TBT peut atteindre plus de 1 μg/L dans l'eau dans les régions fortement atteintes de l'Asie, et la concentration sanguine de TBT chez l'être humain varie entre 50 à 400 nM (Martínez *et al.*, 2017, Yamada *et al.*, 2014). L'exposition systémique humaine est donc non négligeable.

1.5.2.1 Les mécanismes d'action

Le groupe de recherche du Pr Denizeau de Montréal a caractérisé les effets induits à la suite d'une exposition *in vitro* au TBT, à l'aide de différents modèles cellulaires. Le TBT induit l'apoptose en activant les voies intrinsèques et extrinsèques de l'apoptose (Jurkiewicz *et al.*, 2004). Il mène au clivage de la protéine proapoptotique Bid, tout en amplifiant la cascade d'activation des caspases (Jurkiewicz *et al.*, 2004). Le TBT peut agir au niveau des divers marqueurs de l'apoptose, tel le clivage des procaspases dont la procaspase-3, la perturbation de la perméabilité et potentiel membranaire mitochondrial, ainsi que la surexpression des protéines proapoptotique.

En complément à ces résultats, notre laboratoire a démontré dans le passé que le TBT induisait une mobilisation du calcium intracellulaire et une activation des canaux à chlore, responsable de l'acidification du milieu intracellulaire (Dupéré-Minier *et al.*, 2009). Une perturbation des concentrations de calcium permet de réguler de nombreux mécanismes cellulaires, dont l'apoptose et la prolifération cellulaire. Ces effets découleraient d'une altération du potentiel mitochondrial (Desharnais *et al.*, 2008).

1.5.3 Les agents de chimiothérapies : un survol

Actuellement, il existe une variété d'agents de chimiothérapies utilisées en clinique pour le traitement des cancers. Il a été recensé dans la littérature que la majorité des patients diagnostiqués d'un cancer vont opter pour la chimiothérapie selon les recommandations des médecins (Goffin *et al.*, 2010). Ces agents peuvent être distingués en différentes catégories en fonction de leur action pharmacologique.

Plusieurs algorithmes existent concernant le choix de la thérapie pharmacologique. Les patients nouvellement diagnostiqués subissent un traitement divisé en trois étapes, consistant principalement en l'utilisation de médicaments de chimiothérapies conventionnels, seuls ou en combinaisons (Jayachandran *et al.*, 2014). Ces médicaments comprennent essentiellement les médicaments inhibiteurs de la TOP2, qui empêchent le dépliement de l'ADN double brin lors de sa synthèse, et les antimétabolites, à savoir les agents qui inhibent la synthèse de l'ADN. La Figure 7 représente de manière schématique le mécanisme d'action de ces classes de médicaments.

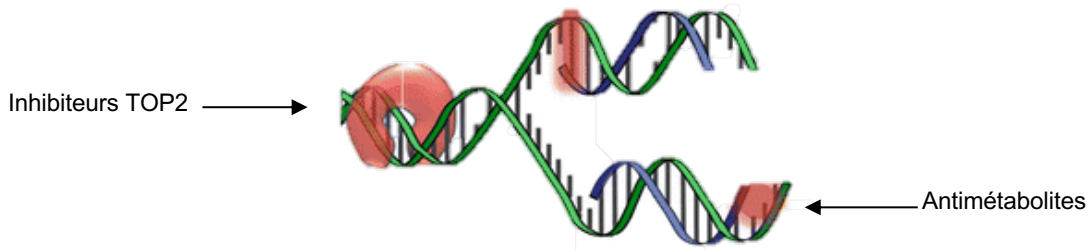


Figure 7. Représentation schématique du mécanisme d'action des antimétabolites et des inhibiteurs de la topoisomérase II.

Abréviations : TOP2, topoisomérase II. Image adaptée de : (Margaret L. Thomas *et al.*, 2015).

1.5.4 Les antimétabolites

La première ligne de traitement consiste généralement en l'administration d'une combinaison de médicaments de type antimétabolites (Jayachandran *et al.*, 2014). Ces composés exercent une compétition avec les ligands endogènes naturels pour les sites actifs des récepteurs impliqués dans la synthèse protéique (J. *et al.*, 2000). Ils présentent une meilleure spécificité pour ces récepteurs et une meilleure efficacité lorsque la cellule est dans la phase de synthèse d'ADN (S-phase) du cycle cellulaire (J. *et al.*, 2000). Dans cette catégorie, nous pouvons retrouver plusieurs composés, dont le méthotrexate (MTX), le 6-mercaptopurine (6-MP) et la cytarabine (Ara-C).

Le MTX agit en bloquant la synthèse de thymidine monophosphate catalysée par la thymidylate synthase, et celle du tétrahydrofolate (J. *et al.*, 2000). La 6-MP, un analogue des purines, est une prodrogue qui nécessite une activation afin d'exercer son effet (J. *et al.*, 2000). Elle a pour fonction d'inhiber la synthèse de nucléotides en s'incorporant dans le brin d'ADN (J. *et al.*, 2000). Enfin, l'Ara-C, un analogue des pyrimidines, a pour fonction d'inhiber l'ADN polymérase qui est responsable de la synthèse du nouveau brin d'ADN lors de la réplication (J. *et al.*, 2000).

1.5.5 Les inhibiteurs de la topoisomérase II

La seconde classe d'agents de chimiothérapies est celle des inhibiteurs de TOP2. Ceux-ci constituent la deuxième ligne de traitement, lorsque les antimétabolites s'avèrent inefficaces (Jayachandran *et al.*, 2014). Ces molécules stabilisent le complexe ADN et TOP2, puis induisent un bris dans la chaîne d'ADN, ce qui inhibe la réplication d'ADN (Shaaban *et al.*, 2014). Dans cette catégorie, nous pouvons retrouver plusieurs composés, dont la doxorubicine (DXR), le mitoxanthrone (MX) qui est un analogue avec un potentiel toxique inférieur à celle de la DXR et l'étoposide (ETO) (Shaaban *et al.*, 2014).

De manière générale, la sélection d'un agent de chimiothérapie est fortement dépendante du type de cancer. La diversité dans les traitements actuellement disponibles permet au clinicien de choisir parmi une gamme d'options thérapeutiques. Or, il est nécessaire de considérer que selon le type d'agent employé, une certaine forme de résistance peut être observée, découlant de mécanismes cellulaires inconnus jusqu'à présent (Shaaban *et al.*, 2014).

1.6 L'instabilité génomique

L'instabilité génomique fait référence à l'accumulation de mutations somatiques dans l'ADN, menant ultimement à l'initiation de la tumorigenèse. Deux mécanismes principaux pour la génération d'une instabilité génomique sont soulevés dans la littérature : (1) l'instabilité des microsatellites ou (2) les aberrations de chromosomes (Loeb *et al.*, 2000). Le premier mécanisme implique des mutations génomiques dans les régulateurs de la réparation des mésappariements de l'ADN, tandis que le deuxième cible les gènes requis pour la ségrégation des chromosomes (Loeb *et al.*, 2000).

Dans cette section, nous reverrons en détail les mécanismes de régulation du cycle cellulaire et les mécanismes de réparation de l'ADN. Une altération de ces voies de signalisation peut être à l'origine d'une instabilité génomique.

1.6.1 Le cycle cellulaire

Les instabilités génomiques peuvent être le résultat d'une dérégulation dans l'activité des CDK dans le cycle cellulaire (Malumbres *et al.*, 2009). Le rôle du cycle cellulaire est de déceler des anomalies de séquence dans l'ADN en induisant un arrêt de division cellulaire et une réparation subséquente si possible. Les anomalies peuvent être décelées à deux points de contrôle : (1) le point de contrôle des phases G1/S et (2) le point de contrôle des phases G2/M. Une altération des points de contrôle G1/S ou G2/M peuvent donc être à l'origine de développement tumoral.

Le cycle cellulaire est divisé en quatre phases : (1) la phase G1 où le contenu cellulaire est dupliqué, (2) la phase S où les chromosomes sont dupliqués, (3) la phase G2 où les erreurs de réplication du matériel génétique sont réparées et enfin (4) la phase M où la division cellulaire se complète par la mitose (Figure 8).

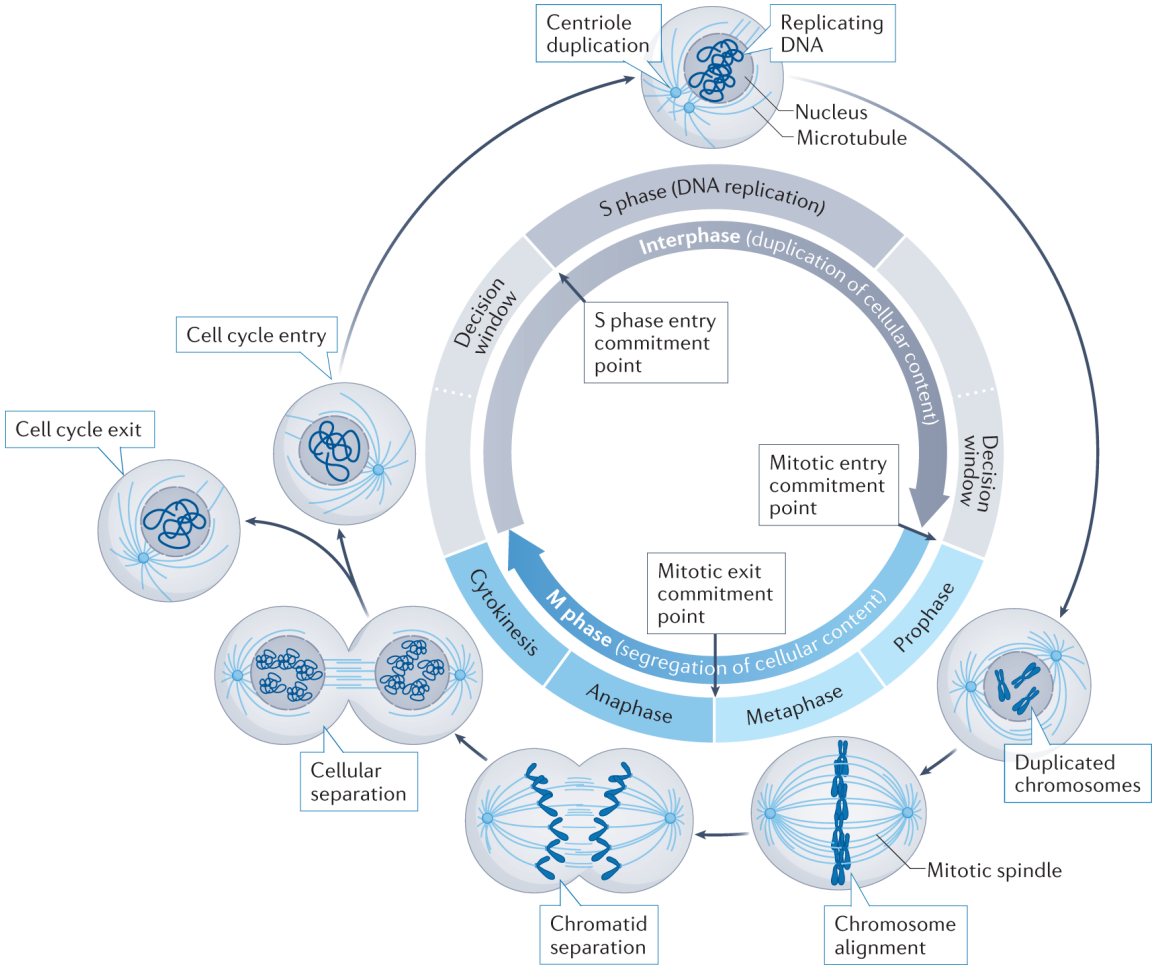


Figure 8. Représentation schématique des étapes du cycle cellulaire.

Image tirée de : (Matthews *et al.*, 2022).

Le point de contrôle G1/S permet la transition entre la phase G1 et S. Elle est dépendante de l'activité de la protéine p53 et de l'expression du gène suppresseur de tumeur du pRb (Vermeulen *et al.*, 2003). À l'état physiologique dans les cellules quiescentes, la protéine Rb est hypophosphorylée et bloque l'activité transcriptionnelle du facteur de transcription E2F-1 (Brehm *et al.*, 1998, Buchkovich *et al.*, 1989). Ceci permet de réprimer l'expression des gènes qui sont impliqués dans la progression du cycle cellulaire. Lorsque la cellule est stimulée par des facteurs de croissance, la CDK2 induit la phosphorylation de la protéine Rb et un changement dans sa conformation (Hinds *et al.*, 1992). Ceci a pour effet de libérer le facteur E2F-1 et d'activer la transcription de gènes nécessaires à la réplication de l'ADN. Si des dommages irréversibles à l'ADN sont détectés au point de contrôle G1/S, l'expression de la protéine p53 est stimulée, ce qui induit de manière irréversible l'apoptose (Vermeulen *et al.*, 2003).

Le point de contrôle G2/M permet la transition entre la phase G2 et M, où son objectif est de déceler les dommages à l'ADN avant la mitose pour éviter le transfert horizontal de mutations somatiques (DiPaola, 2002). Ce point de contrôle est initié par la cycline B1 et la CDK1 (DiPaola, 2002). Lorsqu'un dommage à l'ADN est détecté, le *checkpoint kinase 1* (CHK-1) phosphoryle Cdc25C, inhibant ainsi la possibilité d'activer cdc2 par une activité de phosphatase (DiPaola, 2002, Kousholt *et al.*, 2012). Le rôle de Cdc25c et cdc2 est donc crucial au point de contrôle G2/M. Il existe de nombreux autres modulateurs du point de contrôle G2/M, dont les protéines des voies de réparations ataxia telangiectasia mutated (ATM) et *ataxia telangiectasia and Rad3-related protein* (ATR), ainsi que la DNA-PK et la protéine p53 (Kousholt *et al.*, 2012).

Il est important de noter que la protéine kinase B (PKB), connue sous le nom d'AKT, possède un rôle crucial dans la régulation du cycle cellulaire (Xu *et al.*, 2012). AKT va permettre la répression ou l'activation d'une multitude de protéines en fonction des signaux de croissance cellulaire, qui sont représentés à la Figure. 9.

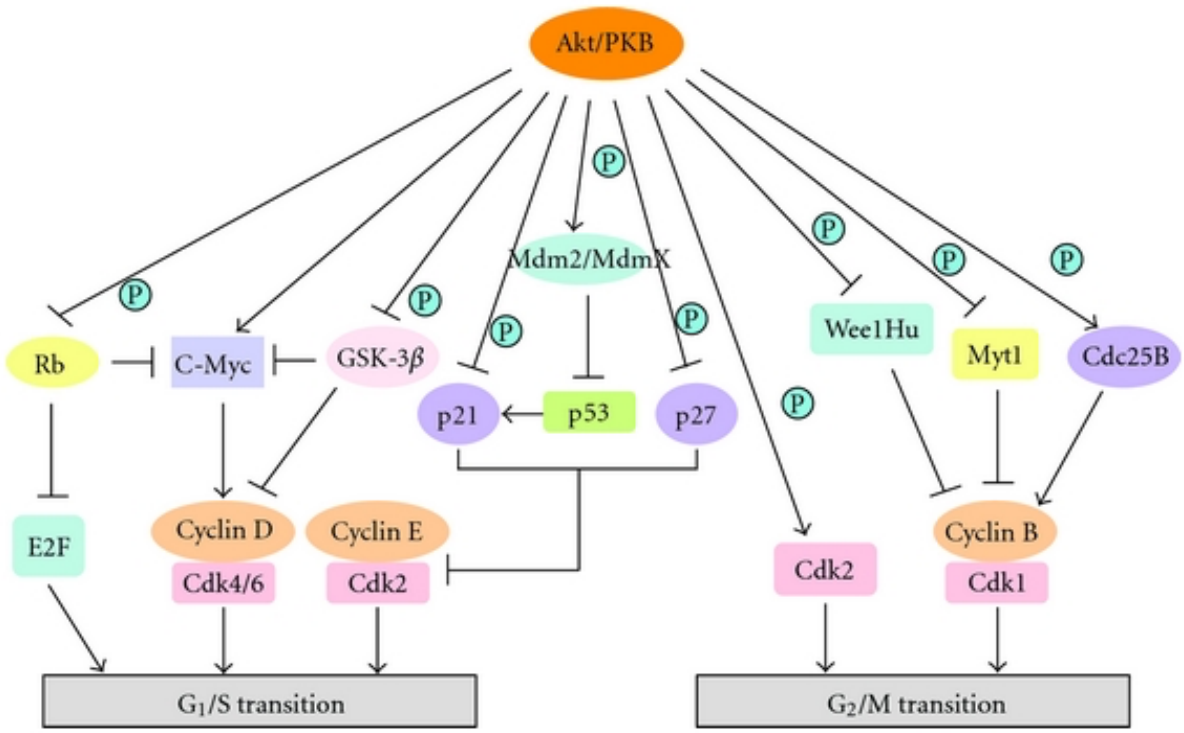


Figure 9. Schématisation des protéines des phases du cycle cellulaire modulées par AKT.

Image tirée de (Xu *et al.*, 2012)

1.6.2 Les voies de réparation de l'ADN

Les voies de réparation de l'ADN peuvent être divisées en deux voies principales, soit la voie ATM et la voie ATR. Ces voies sont activées en réponse à des dommages à l'ADN (de l'anglais *DNA damage response* ; DDR). Les bris simples brins (BSB) de l'ADN activent la voie ATR, tandis que les BDB de l'ADN activent la voie ATM. Dans cette section, nous survolerons les principaux partenaires de signalisation de chaque voie de réparation de l'ADN.

1.6.2.1 Voie ATM

La voie ATM est activée en réponse à des BDB de l'ADN. L'histone H2AX est le marqueur clé des dommages à l'ADN. Suivant l'induction de dommages, l'histone H2AX est phosphorylée

(γH2AX) au résidu sérine 139 par ATM ou la DNA-PK et induit de manière rapide la signalisation reliée aux DDR (Rogakou *et al.*, 1998). Certaines études ont souligné l'importance de l'induction de BDB pour la formation de foci de γH2AX. En effet, il a été démontré que l'inhibition de l'activité des caspases par l'inhibiteur zLEHD-fmk compromet la formation de γH2AX (Hain *et al.*, 2016b), et que la formation de γH2AX est nécessaire pour la fragmentation de l'ADN par le DFF40 (Chengrong Lu *et al.*, 2006).

Une réaction de nucléation se produit lorsque l'histone H2AX est phosphorylée. La protéine *mediator of DNA damage checkpoint 1* (MDC1) est recrutée au site de phosphorylation, suivi du recrutement du complexe MRN (Kinner *et al.*, 2008). Le complexe MRN, composé des protéines NBS1, MRE11 et RAD50, induit une rétroaction positive sur la formation de γH2AX (Kinner *et al.*, 2008). La régulation à la hausse de l'expression de γH2AX permet l'activation de la protéine kinase *checkpoint 2 (*CHK-2), où cette dernière régule l'expression de la protéine p53 (Podhorecka *et al.*, 2010). De plus, γH2AX facilite l'interaction des extrémités libres de l'ADN (Reina-San-Martin *et al.*, 2003). À la lumière de ces mécanismes de signalisation moléculaire, l'objectif cellulaire est d'induire la réparation de l'ADN par recombinaison non homologue ou homologue.

1.6.2.2 Voie ATR

De son côté, la voie ATR est induite suivant la détection de BSB. Les BSB se produisent généralement aux fourchettes de réplication de l'ADN, suivant une erreur de réplication par l'ADN polymérase ou par l'induction directe de dommages à l'ADN (Shiotani *et al.*, 2009). Lorsqu'un BSB est produit, il y a un recrutement rapide du complexe *replication protein A* (RPA) et de la protéine *ATR-interacting* (ATRIP) (Flynn *et al.*, 2011). La formation de ce complexe initiateur de la voie ATR permet d'activer et d'induire le recrutement du complexe surnommé 9-1-1, composé des protéines Rad9-Hus1-Rad1 (Flynn *et al.*, 2011). L'interaction du complexe ATR-ATRIP au site de lésion induit la réparation de l'ADN.

1.6.3 Évidences d'un rôle potentiel du DFF40 dans l'instabilité génomique

Nous avons précédemment abordé l'importance du DFF dans l'induction de l'apoptose, où son absence peut être à l'origine d'anomalies de fragmentation de l'ADN (voir section 1.4). Il est reconnu que les causes majeures reliées à l'instabilité génomique impliquent des dysfonctions dans la réplication de l'ADN ou dans les voies de dommages à l'ADN (Aguilera *et al.*, 2013). Les

mutations induisant une modulation sur ces voies ont un impact sur la carcinogenèse. Il a été rapporté que les lymphoblastes, issus de la rate (*c.-à-d.* les cellules TK6), déficients en DFF40 ont une absence d'accumulation de mutations suivant une induction à l'apoptose avec le TRAIL : la mutagenèse induite par le TRAIL dépend de l'activation de caspases et du DFF40 (Miles *et al.*, 2017). Il a été démontré que les dommages à l'ADN induits par le TRAIL dépendent de l'activation de la caspase-8 (Lovric *et al.*, 2010). L'activation de la caspase-3 effectrice, suivie du clivage du DFF45, est nécessaire à l'induction subséquente de la mutagenèse (Lovric *et al.*, 2010).

En 2004, Fernandez-Capetillo et ses collègues ont proposé que les cellules cancéreuses ayant une expression diminuée ou abolie du DFF40 aient une diminution de la formation de γH2AX, pierre angulaire de l'activation du DDR (Fernandez-Capetillo *et al.*, 2004). En complément à ces études, en 2016, l'équipe de Hain a démontré que lors d'un arrêt mitotique, l'utilisation d'inhibiteur de caspases réduit non seulement la formation de γH2AX, mais augmente les niveaux de TRF2 aux télomères (Hain *et al.*, 2016b). Ces observations supportent l'hypothèse que les dommages à l'ADN induits par les voies de signalisation dépendantes des caspases produisent des BDB et activent la DNA-PK (Bernal *et al.*, 2018). La DNA-PK aurait pour rôle d'augmenter les focis de γH2AX et induire une déplétion de TRF2 aux télomères. De nouvelles études émergentes supportent l'idée que les extrémités des télomères seraient dans une configuration en boucle, connue sous le nom de *T-loop (de Lange, 2004, Van Ly et al., 2018)*. Les *T-loop* aux extrémités des télomères permettent aux cellules d'échapper aux voies de réparation de l'ADN (de Lange, 2004). En effet, la suppression de la voie de signalisation d'ATM est nécessaire au maintien de cette configuration (Van Ly *et al.*, 2018), ainsi que la présence de complexes contenant le TRF2 ou TFR1 (de Lange, 2004). Dans les cellules apoptotiques, lors de l'activation des DDR, les extrémités télomériques optent pour une configuration linéaire, permettant ainsi l'activation de la voie d'ATM (Van Ly *et al.*, 2018). Une étude réalisée à l'aide de cellules déficientes en DFF45 a suggéré que la perte de TRF2 au sein de celles-ci pourrait être secondaire à un rôle physiologique du DFF40 (Podhorecka *et al.*, 2010). Jusqu'à ce jour, aucune étude n'a réellement étudié le rôle du DFF40 dans l'intégrité des télomères. Cependant, les observations cliniques sont en faveur d'un rôle potentiel du DFF40 dans l'intégrité des télomères. En effet, comme rapporté précédemment, de nombreuses études ont démontré la résistance à l'apoptose dans les cellules cancéreuses déficientes en DFF40 et l'accumulation subséquente de mutations génomiques. Ceci pourrait partiellement être expliqué par la configuration T-loop : les cellules déficientes en DFF40 ne pouvant induire un changement de conformation des extrémités télomériques échapperaient aux voies de réparation de l'ADN. En somme, le DFF40 pourrait être comparé à

l'activité biomoléculaire de la DNA-PK. L'absence d'activité du DFF40 pourrait théoriquement inhiber la coupure de l'ADN, l'activation de la PK-DNA, l'augmentation de foyer de γH2AX et la perte de TRF2 des télomères (Hain *et al.*, 2016b), soit la machinerie cellulaire impliquée dans la réparation des dommages à l'ADN. Ces hypothèses sont schématisées à la Figure 10.

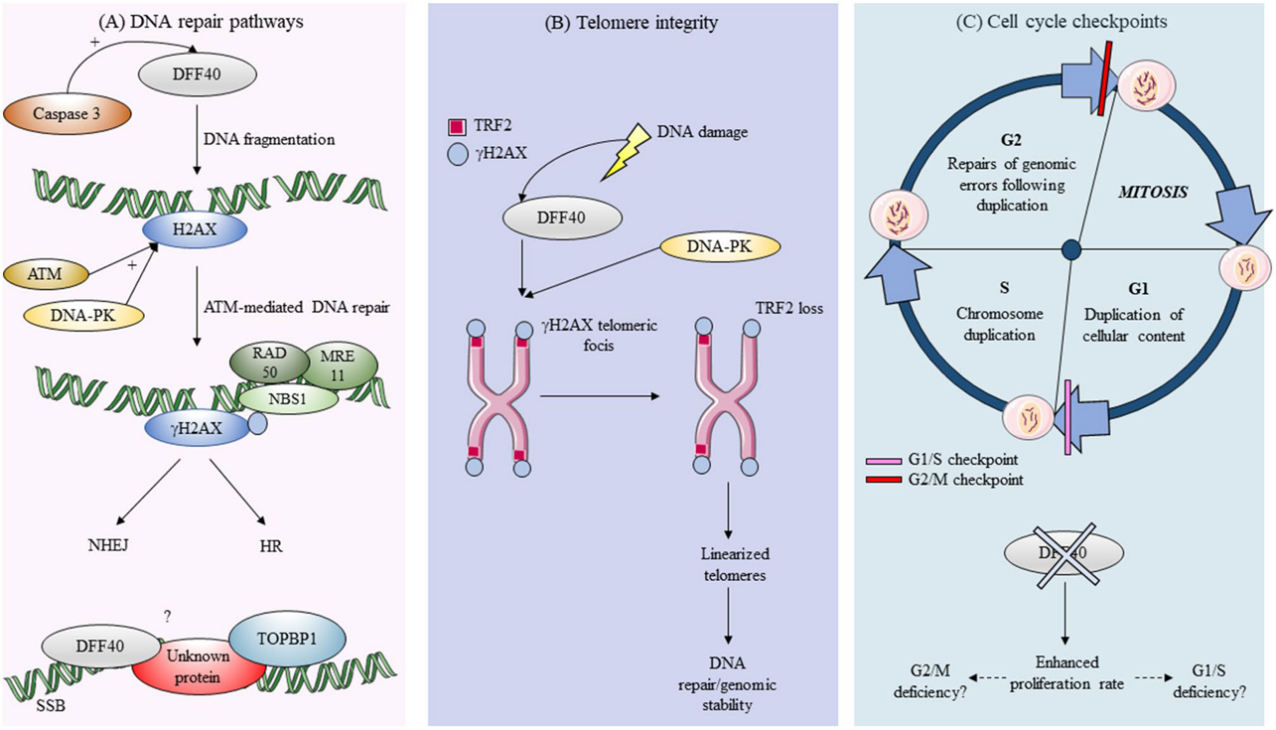


Figure 10. Représentation schématique des rôles hypothétiques du DFF40 dans la stabilité génomique.

Image tirée de (Merve Kulbay *et al.*, 2021a)

Des études complémentaires ont démontré que les cellules cancéreuses du cancer du côlon déficientes en DFF45 ont une instabilité génomique importante, caractérisée par des amplifications et délétions de séquences du génome (Youssef Errami *et al.*, 2013b). De plus, les souris déficientes en DFF40 ($CAD^{-/-}$) ont une résistance à l'apoptose et démontrent une accumulation significative d'aberrations chromosomiques (Yan *et al.*, 2006a). L'incidence de papillomes est augmentée dans les souris $CAD^{-/-}$, où la croissance de ces tumeurs bénignes est accélérée de manière significative (Yan *et al.*, 2009). De nombreux modulateurs de l'ADN sont connus comme étant des modulateurs du cycle cellulaire, dont la DNA-PK qui agit au point de contrôle G2/M du cycle cellulaire. Précédemment, nous avons proposé un rôle similaire du DFF40 à la DNA-PK, particulièrement dans la formation de γH2AX. Or, qu'en est-il de la régulation du cycle cellulaire? De nombreuses études que nous avons soulevées à travers cette revue de littérature supportent une augmentation de la prolifération cellulaire dans les cellules cancéreuses déficientes en DFF40. Or, aucune étude n'a rapporté un rôle direct du DFF40 dans la régulation du métabolisme cellulaire. Étant donné le phénotype des cellules cancéreuses déficientes en DFF40, nous croyons que le DFF40 pourrait être un modulateur clé dans le cycle cellulaire (voir Figure 10).

.

1.7 Le cancer

En 2022, au Canada, il fut déterminé par la Société canadienne du cancer que le cancer est la première cause de décès et il est responsable de près de 30% des décès canadiens (SCC, 2022). La survie nette à 5 ans prédite pour tous les types de cancers combinés est de 64% (SCC, 2022). Cliniquement, il devient donc très important de développer de nouvelles interventions afin de diminuer le taux de mortalité associé à cette maladie.

Les cellules cancéreuses au sein d'une tumeur sont très diversifiées, ayant cumulé un grand nombre de mutations et étant grandement influencées par le micro-environnement. Au fil des années, plusieurs caractéristiques ont été identifiées, celles-ci se regroupant principalement dans 10 catégories distinctes (Figure 11). Dans cette étude, nous nous sommes essentiellement intéressés aux phénomènes de résistances à la mort cellulaire, à l'instabilité génomique et aux modifications du métabolisme énergétique. Les deux premières ayant été approfondies dans les sections ci-dessus, ici, nous reverrons les principales caractéristiques de l'énergie métabolique chez les cellules cancéreuses.

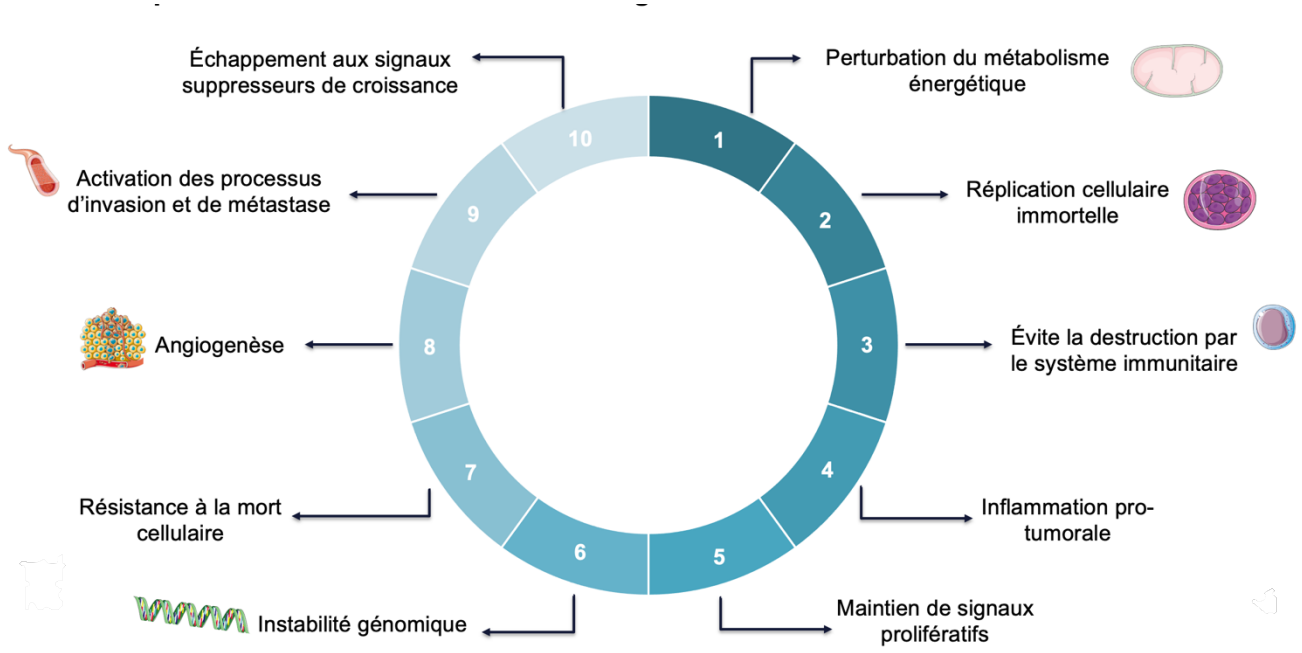


Figure 11. Événements clés des cellules cancéreuses

Image adaptée de : (Hanahan *et al.*, 2011).

1.7.1 Métabolisme énergétique des cellules cancéreuses

À l'état physiologique, l'énergie requise au fonctionnement cellulaire est obtenue par la production d'adénosine triphosphate (ATP). Il existe deux principaux mécanismes de production d'ATP dans les cellules, soit la glycolyse ou la phosphorylation oxydative (OXPHOS). Bien que chacune des deux voies ait leur propre particularité, les deux travaillent vers un objectif commun de production d'ATP. La voie glycolytique génère de l'ATP à une vitesse supérieure que la voie de l'OXPHOS, mais les quantités produites sont significativement moindres. Ainsi, en fonction de l'homéostasie cellulaire, la cellule va privilégier une voie ou l'autre. Dans les cellules normales, nous observons généralement de faibles taux de glycolyse avec des niveaux d'OXPHOS significative (Xue-Bing Li *et al.*, 2015)

Dans cette section, nous reverrons les principaux mécanismes moléculaires impliqués dans ces voies, ainsi que les adaptations cellulaires engendrées dans les cellules cancéreuses.

1.7.1.1 La glycolyse

La voie de la glycolyse repose sur la conversion du glucose en pyruvate dans le cytosol, afin de générer de l'ATP. La voie de signalisation repose sur des réactions chimiques en 10 étapes, nécessitant l'implication de 10 enzymes spécifiques, dont le pyruvate kinase, qui est responsable de la transformation de la molécule de phosphoenolpyruvate en pyruvate (Xue-Bing Li *et al.*, 2015). En conditions d'hypoxie, dans les cellules normales et cancéreuses, le pyruvate est réduit en acide lactique, soit le lactate, par l'enzyme lactate déshydrogénase (Xue-Bing Li *et al.*, 2015), ce que nous appelons la glycolyse.

De nombreux signaux moléculaires sont responsables de la régulation de la voie de la glycolyse. Le facteur de transcription *hypoxia-inducing factor 1* (HIF1) favorise le « switch » de la glycose à l'OXPHOS (Papandreou *et al.*, 2006). Ceci est médié par une régulation à la hausse de l'expression des transporteurs à glucose, dont le transporteur GLUT1, à la membrane cellulaire et une diminution de la mitobiogenèse (Denko, 2008, Xue-Bing Li *et al.*, 2015). De plus, une délétion du gène *TP53* privilégierait des taux de glycolyse supérieure (Xue-Bing Li *et al.*, 2015).

1.7.1.2 La phosphorylation oxydative

Lorsque le micro-environnement cellulaire devient riche en molécules d'oxygène (O_2), donc un milieu en aérobie, les cellules privilégient l'OXPHOS pour la production d'ATP. Au lieu de transformer le pyruvate en lactate dans la voie de la glycolyse, celui-ci va plutôt cheminer vers le cycle de Krebs à la mitochondrie (Xue-Bing Li *et al.*, 2015).

Les réactions enzymatiques du cycle de Krebs ont lieu à la chaine de transport des électrons dans la mitochondrie. Cette chaîne est composée de quatre complexes d'OXPHOS (I à IV), ainsi que d'une ATP synthase (complexe V). Le premier complexe (I) contient l'enzyme NADH déhydrogénase (Chaban *et al.*, 2014). Le complexe II, ou le succinate déhydrogénase, est responsable de l'oxydation du succinate et du subséquent transfert d'électrons à l'ubiquinone (Chaban *et al.*, 2014). Le complexe III, ou le cytochrome *c* oxy-réductase, oxyde l'ubiquinone en ubiquinol, induisant le relâche de deux protons dans l'espace intermembranaire (Chaban *et al.*, 2014). Enfin, le complexe IV, soit le cytochrome c oxydase, permet la catalyse d'une molécule d'oxygène en molécules d'eau, aboutissant en la relâche subséquente de quatre protons dans l'espace intermembranaire (Chaban *et al.*, 2014). Suivant la création d'un gradient électrochimique au sein de l'espace intermembranaire par les super-complexes, l'ATP synthase catalyse la transformation de l'ADP en ATP, permettant ainsi à la cellule de réguler sa fonction biologique (Chaban *et al.*, 2014).

L'OXPHOS peut être régulée à plusieurs niveaux, particulièrement par la modification de l'activité des complexes de la chaîne de transport des électrons. Il a été démontré que dans les cellules cancéreuses, des mutations dans l'ADN mitochondrial (ADNmt) ou génomique, régulant l'expression des complexes de la chaîne de transport d'électrons, augmente de manière significative la production de ROS, favorisant ainsi la prolifération cellulaire (Brandon *et al.*, 2006). La surproduction de ROS contribuerait d'ailleurs à la chimiorésistance dans les cellules souches cancéreuses (Diehn *et al.*, 2009).

1.7.1.3 L'Effet Warburg

Le terme « Effet Warburg » fut introduit pour la première fois en 1956 (Warburg, 1956). Le chercheur Otto Heinrich Warburg a découvert que les besoins énergétiques des cellules cancéreuses étaient plus grands que ceux des cellules normales (Xue-Bing Li *et al.*, 2015). Les cellules cancéreuses privilégient la voie de la glycolyse, et ce, même lorsque l'environnement est riche en molécules d'O_2 (concept de la glycolyse aérobie).

Plusieurs études se sont penchées sur les mécanismes cellulaires impliqués dans l'effet Warburg. Les taux de glycolyse augmentés dans les cellules cancéreuses seraient secondaires à une régulation à la hausse du récepteur de glucose GLUT1 (Chang Liu *et al.*, 2021). En effet, plusieurs tumeurs peu différenciées sont associées avec une surexpression du récepteur GLUT1 et un mauvais pronostic (Min Yu *et al.*, 2017). L'altération des voies de signalisation ayant pour substrat l'activation de NFκB et la cible de la rapamycine chez les mammifères (mTOR), dont celles de PI3K/AKT, Ras et Myc, permettent la surexpression de GLUT1 (Chang Liu *et al.*, 2021). Globalement, la transduction des signaux intracellulaires induits par l'activation du récepteur GLUT1 permettra l'expression génique et l'activation de protéines cellulaires favorisant la prolifération cellulaire, les métastases et la résistance à l'apoptose induite par les drogues de chimiothérapie (Chang Liu *et al.*, 2021). La Figure 12 représente les voies de signalisations et protéines impliquées dans la glycolyse.

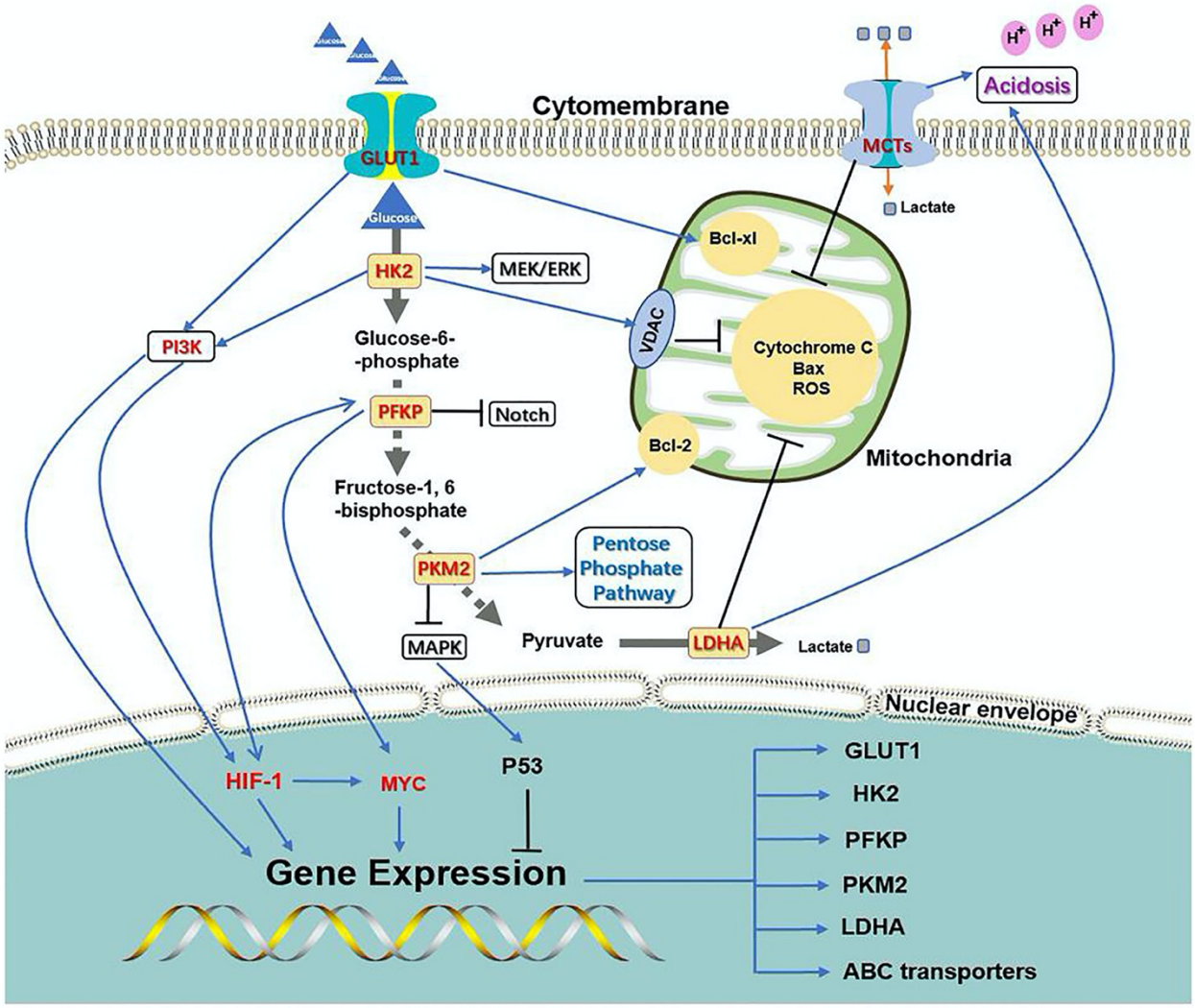


Figure 12. Représentation des voies de signalisation et partenaires d'interaction impliquées dans la glycolyse.

Image tirée de : (Chang Liu *et al.*, 2021).

1.8 Hypothèses

Il a été démontré que les tumeurs ayant une altération à la baisse de l'expression du DFF40 sont associées avec un mauvais pronostic (Banas *et al.*, 2017, Banas *et al.*, 2018a, Sánchez-Osuna *et al.*, 2016). Récemment, Hain et ses collègues ont démontré que l'inhibition de l'expression de la DNA-PK induit une absence de formation de foci de γH2AX et prévient la perte de TRF2 des extrémités télomériques (Hain *et al.*, 2016b). Ces altérations moléculaires sont associées avec une survie cellulaire augmentée. Les premières études concernant le DFF40 au sein de la tumorigenèse avaient permis de stipuler que celui-ci serait impliqué dans la stabilité génomique : l'absence d'activation du DFF40 induit une hausse de la fréquence de mutations induites par la radiation, menant à l'accumulation d'aberrations chromosomiques (Yan *et al.*, 2006a). Globalement, ces études suggèrent que l'absence du DFF40 dans les cellules cancéreuses pourrait médier la résistance aux traitements de chimiothérapie par des mécanismes moléculaires altérant la stabilité génomique. Jusqu'à ce jour, aucune étude n'a caractérisé les altérations des voies de signalisation induite par une absence de DFF40.

Notre hypothèse de recherche est que le DFF40 possède d'autres rôles physiologiques et son absence dans les cellules cancéreuses pourrait permettre d'induire une résistance à l'apoptose par l'altération des voies de signalisation impliquées dans la prolifération cellulaire et la stabilité génomique.

1.9 Objectifs

L'objectif général du projet de recherche est de caractériser de nouveaux rôles physiologiques pour le DFF40, qui permettront d'expliquer les mécanismes de résistance cellulaire. Les objectifs spécifiques sont :

1) Établir des lignées cellulaires cancéreuses n'exprimant pas le DFF40
2) Déterminer la susceptibilité des cellules déficientes en DFF40 à des agents inducteurs d'apoptose et de chimiothérapies, dont les antimétabolites et les inhibiteurs de la topoisomérase II
3) Étudier l'impact d'une déficience en DFF40 sur les voies de signalisation de i) l'apoptose, ii) du contrôle du cycle cellulaire et iii) du métabolisme énergétique à la suite d'une exposition aux xénobiotiques mentionnés ci-haut.
4) Étudier le lien entre le DFF et la stabilité génomique

INTRODUCTION AUX PUBLICATIONS

Les chapitres subséquents (chapitres 2 à 5 inclusivement) se concentrent les quatre articles scientifiques issus de cette thèse. Ici, nous survolerons rapidement les conclusions tirées de chaque étude.

Le chapitre 2 porte sur l'étude d'une déficience en DFF40 chez les cellules Jurkat dans l'apoptose induite par le TBT. L'étude démontre que les cellules Jurkat DFF40 KO sont résistantes à l'apoptose induite par le TBT par un retard d'activation des caspases-3 et caspases-6 et un retard de clivage de PARP. La sensibilité au TBT est partiellement restaurée lorsqu'on exprime à nouveau le DFF40 dans les cellules déficientes.

Le chapitre 3 porte sur l'impact d'une déficience en DFF40 chez les cellules Jurkat dans la sensibilité aux médicaments de chimiothérapies. Nous avons démontré que les cellules Jurkat DFF40 KO sont résistantes à l'apoptose induite par les antimétabolites, mais sensibles aux inhibiteurs de la TOP2, dont l'ETO. Les effets peuvent partiellement être expliqués par une modulation de la voie de signalisation de l'apoptose.

Le chapitre 4 étudie l'impact d'une déficience en DFF40 sur le métabolisme énergétique des cellules Jurkat. Nous avons démontré que les cellules Jurkat DFF40 KO reposent sur l'effet Warburg pour leur production d'énergie. Ceci pourrait partiellement être expliqué par un potentiel rôle mitochondrial du DFF40 : le DFF40 subit une translocation à la mitochondrie lors de l'apoptose.

Le chapitre 5 démontre le rôle du DFF40 dans la stabilité génomique. Une déficience en DFF40 chez les cellules Jurkat perturbe l'activation des voies de réparation de l'ADN suivant des dommages induits par le TBT.

2 CHAPITRE 2: DNA FRAGMENTATION FACTOR 40 EXPRESSION IN T CELLS CONFERS SENSIBILITY TO TRIBUTYLTIN-INDUCED APOPTOSIS

Titre de l'article : *L'expression du facteur de fragmentation de l'ADN 40 dans les cellules T confère une sensibilité à l'apoptose induite par le tributylétain*

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Contribution des auteurs :

MK : Conception de la méthodologie, exécution des expériences, analyses statistiques, rédaction et révision du manuscrit.

BJ : Exécution des expériences – génération des lignées cellulaires par CRISPR-cas9, analyses statistiques et révision du manuscrit.

JB : Conception du projet et de la méthodologie, analyses statistiques, supervision, rédaction et révision du manuscrit.

2.1 Résumé en français

Le facteur de fragmentation de l'ADN 40 (DFF40) est une endonucléase qui intervient dans l'étape finale et irréversible de l'apoptose, où elle médie la fragmentation oligonucléosomal de l'ADN. De nouvelles études ont proposé un rôle du DFF40 dans la stabilité génomique. Il a été démontré que la surexpression du DFF40 dans les cellules tumorales augmente leur sensibilité aux médicaments de chimiothérapie. Dans cette étude, nous avons voulu déterminer si l'expression du DFF40 influence la toxicité du tributylétain (TBT), un composé cytotoxique et inducteur d'apoptose bien connu. La stratégie utilisée était de générer une lignée cellulaire Jurkat déficiente en l'expression du DFF40 par la méthode du CRISPR-cas9, puis d'évaluer la toxicité du TBT. Les cellules DFF40 KO Jurkat montrent une diminution significative de la mortalité cellulaire suivant une exposition au TBT pendant 24 h ($p < 0,05$). Les cellules Jurkat DFF40 KO sont résistantes à l'apoptose induite par le TBT, comme démontré par l'Annexine V/PI ($p < 0,05$). Ensuite, le niveau basal de ROS est significativement supérieur dans les cellules Jurkat DFF40 KO comparativement aux cellules DFF40 WT. Les cellules T Jurkat déficientes en DFF40 n'ont pas une modulation des niveaux de ROS suivant une exposition au TBT. Les niveaux de mortalité cellulaire diminuée suivant une exposition au TBT dans les cellules Jurkat DFF40 KO peuvent être expliqués par un retard d'activation de l'apoptose : le clivage de la procaspase-3, de PARP et l'activation de la caspase-6 sont significativement retardés chez les cellules DFF40 KO, comparativement aux cellules DFF40 WT. De plus, les cellules déficientes en DFF40 ont une absence totale de phosphorylation de l'histone H2AX, alors que les cellules DFF40 WT ont une phosphorylation significative suivant une exposition au TBT ($p < 0,001$). La réexpression du DFF40 dans les cellules KO restaure les effets cytotoxiques du TBT. Globalement, ces données suggèrent un rôle du DFF40 dans la sensibilité des cellules T Jurkat au TBT et possiblement dans la stabilité génomique.

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DNA fragmentation factor 40 expression in T cells confers sensibility to tributyltin-induced apoptosis

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ABSTRACT

DNA fragmentation factor 40 (DFF40), an endonuclease, mediates the final and irreversible step of apoptosis by conducting oligonucleosomal DNA fragmentation. New emerging studies have proposed a role of DFF40 in genomic stability, besides its nuclease activity. Overexpression of DFF40 in tumoral cells increases their sensitivity to chemotherapeutic drugs. In this study, we sought to determine if DFF40 expression influences the toxicity of tributyltin (TBT), a well-known immunotoxic and apoptosis-inducing compound. The strategy used was to knockout DFF40 expression by CRISPR-cas9 method in Jurkat T cells and to determine the toxicity of TBT in DFF40 KO cells and DFF40 WT Jurkat cells. DFF40 KO Jurkat cells show an increase of cell viability following a 24-h TBT exposure ($p < 0.05$). There is a resistance to TBT-induced apoptosis determined by annexin V/PI am labeling ($p < 0.05$). Interestingly, the basal level of ROS rises in DFF40 KO Jurkat cells, but ROS production levels after TBT exposure remains at the same basal level. Other apoptosis or DNA damage makers (procaspase-3, caspase-6, and PARP cleavage) are significantly delayed and decreased. DFF40 deficient cells do not present histone H2AX phosphorylation, whereas wild-type cells present a phosphorylation following a 6-h exposure to TBT ($p < 0.001$). The re-expression of DFF40 in DFF40 KO cells restores the cytotoxic effects of TBT. Overall, these data suggest a role of DFF40 in cells sensitivity to TBT and possibly in DNA stability.

1. Introduction

Programmed cell death, also known as apoptosis, is one of the most common cell death pathways required for homeostasis maintain and organism development through the deletion of non-necessary cells. Perturbations in its pathway have been liked multiple times to severe diseases such as cancer (Dunkle et al., 2011; Tamiya et al., 1998), neurological disorders (Crews et al., 2011; El Hindy et al., 2011) and autoimmune disorders (Eguchi, 2001). Apoptosis is characterized by specific biochemical hallmarks such as cell shrinkage and cellular fragmentation called apoptotic bodies. For many years, the main nuclear hallmark of apoptosis has been the internucleosomal fragmentation of genomic DNA (Saraste and Pulkki, 2000). This step is mediated by the activation of the DNA fragmentation factor (DFF) and its subsequent endonuclease activity. In non-apoptotic cells, the DFF is present in cells as a heterodimeric protein consisting of two subunits: the DFF40 (DFF40/CAD) and the DFF45 (DFF45/ICAD) (Widlak, 2000). The DFF45/ICAD protein acts as a chaperone molecule that allows the proper folding of the DFF40 following its cytoplasmic translation, and is as well the inhibitory subunit of the DFF40/CAD (Enari et al., 1998; Halenbeck et al., 1998). The location of the inactive complex following its synthesis has been up to debate for a while over the past years. The DFF40/DFF45 complex was reported to be located in both the nucleus (Lechardeur et al., 2000; Liu et al., 1998; Samejima and Earnshaw, 1998) or the cytosol (Enari et al., 1998; Iglesias-Guimarais et al., 2012; Sabol et al., 1998), a cell-specific localization. Following apoptosis-inducing signals, the activation of the DFF occurs by the cleavage of specific regions of the DFF45/ICAD by caspase -3 or -6 (Zhou et al., 2001). This leads to the release of the DFF40 subunit into the nucleus and subsequent internucleosomal DNA fragmentation, producing multiple DNA fragments of 180–200 bp (Di Filippo and Bernardi, 2009; Yan et al., 2006). Based on RNA sequencing of DFF40, it was revealed that the lymph node is amongst the tissues highly expressing DFF40, besides the spleen, duodenum, small intestines, and the ovaries (Fagerberg et al., 2014). Also, studies have shown that DFF40 expression was significantly downregulated in different cancer cell lines (Banas et al., 2017, 2018; Luciano et al., 2002).

The DNase activity of the DFF40 is enhanced by DNA binding proteins and their respective interactions, e.g., histone H1 (Liu et al., 1999), HMG-1/2 (Kalinowska-Herok and Widlak, 2008) and topoisomerase IIα (Durrieu et al., 2000; Li et al., 1999). Furthermore, histone H2AX phosphorylation (γH2AX) on Ser 139 was shown to be the early

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maker for internucleosomal DNA fragmentation in apoptotic cells (Chen et al., 2000; Hanus et al., 2010). Phosphorylation of γH2AX is a widely used biomarker to detect DNA double-stranded breaks (DSB) (Sharma et al., 2012). The expression of γH2AX is significantly enhanced by the ataxia-telangiectasia mutated (ATM) kinase, which subsequently allows the activation of DNA repair pathways (Kinner et al., 2008) or cell death if the damage is irreversible.

Toxic effects of environmental pollutants on human health is a tremendous concern in toxicology studies. Many environmental mutagens, found in the soil and potentially in the human blood through the consumption of contaminated food and water, are being suspected to be linked to cancer incidence (Sato and Aoki, 2002). Amongst those pollutants, tributyltin (TBT) is a widely studied organotin compound due to its bioaccumulative and persistent properties in the environment (Suzdalev et al., 2015). Organotin compounds are broadly used in many applications, such as stabilizers in plastics and as biocides in antifouling paints (Appel, 2004). Even though TBT use as an antifouling agent on ships has been banned since the late 90′s-early 20′s, studies have shown that TBT concentrations can reach up to 1.35 μg/L in freshwater near South America (Martinez et al., 2017), and 31 ng/ml in one human subject blood (Whalen et al., 1999), which corresponds to approximately 107 nM. TBT metabolites, dibutyltin (DBT) and monobutyltin (MBT), were also found in human liver samples at 28.3 ng/g and 4.7 ng/g respectively in one subject (Nielsen and Strand, 2002). Furthermore, it was shown that exposure risk was age related; younger men subjects (40 years old) have higher TBT metabolite concentration in their liver, probably associated with a higher risk of exposure or lower detoxifying capacity (Nielsen and Strand, 2002). Furthermore, oral dosing of rat dams with TBT chloride revealed that TBT and DBT distributes in most tissues, whereas neonates of rat dams exposed during gestation contain TBT and DBT the most in the brain and liver tissues (Cooke et al., 2008). Although hepatotoxicity and neurotoxicity of TBT have been widely shown, immunotoxicity of TBT metabolites remains the most obvious effect (Appel, 2004) due to its interaction with hematological cells. Immune organs mostly sensitive to organotin compounds are the spleen and thymus where lymphocyte depletion was noted. After exposure to TBT, induction of oxidative damage, apoptosis, and necrosis were present in each organ (Aw et al., 1990; Gennari et al., 2002, 1997).

Oxidative damage induced by TBT comes from mitochondrial perturbation, which leads to apoptosis (Dupere-Minier et al., 2004; Nishikimi et al., 2001). Its alterations are associated with the loss of mitochondrial membrane potential (MMP) and subsequent cytochrome c and apoptosis-inducing factor (AIF) release (Ly et al., 2003). In the cytoplasm, the cytochrome c, in association with Apaf-1 and caspase-9, forms the apoptosome and leads to the activation of caspase-3 and subsequent DFF activation (Hill et al., 2004). The toxicity associated with TBT involves many other cellular mechanisms. Toxicogenomic analysis has shown that exposure to low levels of TBT upregulates the expression of genes implicated in apoptotic processes (Zhou et al., 2016). The major altered pathways are the tumor necrosis factor (TNF), mitogen-activated protein kinase (MAPK), p53 protein, and toll receptor signaling pathways (Zhou et al., 2016). TBT is also known to disturb calcium homeostasis (Desharnais et al., 2008; Nakatsu et al., 2007; Reader et al., 1994) and increase procaspase cleavage (Nakatsu et al., 2007). A recent study performed on isolated ventricular myocytes showed that TBT induces mitochondrial reactive oxygen species (ROS) production by reducing MMP, which can be the cause of dysfunction in calcium homeostasis (Pereira et al., 2019). Also, it was shown in primary hypothalamic neurons that TBT-induced apoptosis occurs following the phosphorylation of jun N-terminal kinase (JNK) when exposed to 150 μg/L of TBT (Cong et al., 2019). Furthermore, in pancreatic β-cells, exposure to TBT for up to 24 h induces poly (ADP-ribose) polymerase (PARP) cleavage and phosphorylation of extracellular-signal-regulated-kinase (ERK) and JNK1/2 (Huang et al., 2018). Our laboratory has shown in a previous study that TBT induces internucleosomal DNA fragmentation in Jurkat and HPB-ALL cells (Desharnais et al., 2008; Dupere-Minier et al., 2004), and intracellular acidification and chloride channel phosphorylation (Dupere-Minier et al., 2004).

Exposure to environmental pollutants has been linked to inducing multiple mutations in the genome (Sato and Aoki, 2002), mainly in DNA repair pathways (Nikolaev and Yang, 2017) or the apoptosis signaling pathway (Plati et al., 2008). The capacity of TBT to induce DNA damage in rat peripheral blood has been previously reported (Liu et al., 2006). TBT-initiated oxidative stress leads to ROS production, and in its turn, can lead to DNA breakage. Different teams, including our own, have demonstrated a link between the expression and/or activity of DFF40 and the sensitivity of cells to pharmacological agents or environmental toxins (Bagheri et al., 2014, 2015; Dupere-Minier et al., 2010, 2004). We have previously demonstrated the importance of CD45 phosphatase expression in T cells to control DFF40 activity (Dupere-Minier et al., 2010, 2004). The inhibition of DFF activity confers T cells a resistance towards nuclear apoptosis induced by TBT. Recently, it has been shown that DFF40 plays a crucial role in caspase-dependent DNA damage responses (DDR) at telomeres and may predict cell survival following a mitotic arrest (Hain et al., 2016). These different studies suggest that the expression and activity of DFF40 can potentially influence the sensitivity of cells to toxic agents and comprise its genomic stability. Since oxidative stress induced by TBT causes DNA breakage followed by the activation of the DNA repair system (Li et al., 2015), absence of DFF40 can increase genomic instability, which can result in tumoral transformation and/or cells surviving after exposure to such xenobiotic. It is therefore important to determine the role of DFF40 upon toxic effect after TBT exposure, knowing that immune organs are the most susceptible to its adverse effects.

The current study aims to examine the role of DFF40 in TBT-induced apoptosis in Jurkat cells. To address the importance of DFF40 directly, we performed CRISPR/Cas9-based knockout of DFF40 gene in Jurkat T cells. Our results show that the absence of DFF40 protein expression is associated with apoptosis cell resistance to TBT, but not to staurosporine (STS), an inhibitor of kinases. DFF40 expression was associated with reduced cell mortality and the absence of DNA DSB after TBT exposure and an increase in ROS production at basal state. Overall, our results suggest a link between DFF40 expression, ROS production, and TBT toxicity.

2. Materials and methods

2.1. Materials

RPMI 1640 (11875-093), penicillin/streptomycin (15140-122), HEPES (15630-080) and propidium iodide (PI, P3566) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). TBT chloride (96% purity, T50202), hydrogen peroxide (H_2O_2; 516813) and fetal bovine serum (FBS, F1051) were from Sigma-Aldrich Corporation (Milwaukee, WI, USA).Anti-caspase-3 (9668S) was from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-DFF45 antibody (1141) was from Prosci Inc. (Poway, CA, USA) and the anti-DFF40 antibody (ab71083) and anti-caspase 6 (ab185648) were from Abcam (Toronto, ON, CA). The anti-tubulin (625902), anti-PARP (614302) and anti-phospho-H2AX (613402) antibodies were from BioLegend (San Diego, CA, USA). Anti-mouse IgM (sc-2064) and bovine serum albumin (BSA; sc-2323) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and anti-rabbit IgG was from New England Biolabs Ltd. (Whitby, ON, Canada). The H2DCFDA (D399) and caspase 3/7 (R37111) probes were obtained from Invitrogen Corporation (Carlsbad, CA, USA) and the DHR 123 probe (85,100) was from Cayman Chemical Company (Ann Arbor, MI, USA). The annexin V-FITC Apoptosis Detection Kit (B32115) was purchased from Cedarlane Laboratories (Burlington, ON, Canada). Clarity Western ECL substrate (170–5060) was obtained from Bio-Rad Laboratories, Ltd. (Mississauga, ON, Canada). The XL10-Gold®

Ultracompetent cells were purchased from Stratagene (San Diego, CA, USA). The QIAGEN Plasmid midi kit was purchased from Qiagen (Toronto, ON, Canada). The sgRNA/cas9-all-in-one expression plasmids were obtained from GeneCopoeia (Rockville, MD, USA). A FACScan® from Becton Dickinson (Oakville, ON, Canada) was used for flow cytometer analysis. EtBr was visualized using a Fluor S-Multilmager by UV transillumination from Bio-Rad Laboratories, Ltd. (Mississauga, ON, Canada). Protein bands were visualized by chemiluminescence with the ChemiDoc Gel Imaging System – Universal Hood III from Bio-Rad Laboratories, Ltd. (Mississauga, ON, Canada).

2.2. Cell culture

The Jurkat cells (subclone J77.6.8) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg streptomycin and 10 mM HEPES.

2.3. Jurkat CRISPR-cas9 induced knockout of DFF40 and gene transfection

Gene deletions were performed with the CRISPR-cas9 method. Jurkat cells were transfected with 3x sgRNA/cas9-all-in-one expression plasmids targeting DFF40 gene by electroporation. Briefly, 10^7 cells were washed in phosphate-buffered saline (PBS) and suspended in 1 ml RPMI medium supplemented with 10% FBS. Cells were electroporated 3 times at 250 V (pulse length: 10 ms) and immediately transferred in complete culture medium supplemented with 10% FBS. Positive cells were selected with geneticin (G418) for 7 days post-transfection. On the 8th day post-transfection, cells were suspended in complete RPMI medium without G418, and populations were selected by two rounds of serial dilutions for the loss of DFF40 (KO Jurkat, Jurkat-BG11). DFF40 expression was assessed by Western Blot. After the first serial dilutions, Jurkat cells which have conserved DFF40 expression were used as a negative control (WT Jurkat, Jurkat-AD10). No differences between Jurkat AD10 and parental Jurkat cells were observed in the different assays. To confirm the role of DFF40, re-expression of DFF40 was performed in KO cells with Lipofectamine LTX using pcDNA3.4-TOPO plasmids containing the ORF of full-length DFF40. Briefly, 10^7 cells were washed in cold-serum free RPMI and plated in 6-well plates in OPTI-MEM culture medium. For the transfection, 250 µl of complexes consisting of 2.5 µg plasmid DNA, 2.5 µl PLUS reagent and 7 µl Lipofectamine LTX, in Opti-MEM, was added to the cells and incubated overnight. Cell selection was performed as described above.

2.4. Cell treatments

Cells were treated as previously described with some modifications (Desharnais et al., 2008). Briefly, cells were washed twice with PBS and seeded in 24-well plates at 1×10^6 cells/ml in complete RPMI 1640 medium supplemented with 10% FBS. Cells were exposed to increasing doses of TBT (0.2–0.6 µM) or STS (0.1–10 µM), both diluted in ethanol (EtOH), for 24 h, or to a single dose of TBT [0.4 µM] or STS [1 µM] for different time points (4–24 hrs). Short time exposure to TBT (0–6 hrs) was done in RPMI with 0.1% FBS. Control cells were exposed to 0.1% EtOH.

2.5. Cell viability assay

Following treatments, cells were washed twice with cold PBS and suspended in a solution containing propidium iodide (PI; 1 µg/ml). Samples were kept on ice. Cells were then immediately analyzed by flow cytometer on a FACScan® at 488 (FL-2 channel). A total of 10 000 events were analyzed.

2.6. Apoptosis assessment

The internucleosomal DNA fragmentation was assessed by cell cycle analysis using the G1/G0 subdiploid peak quantification as previously described but with some modifications (Desharnais et al., 2008; Johnson et al., 2014). Briefly, following treatments, cells were washed twice with PBS and suspended in Krishan buffer (0.1% sodium citrate, 0.3% NP-40, 20 µg/ml RNase and 50 µg/ml PI). Samples were incubated at 37 °C for 30 min and analyzed by flow cytometer on a FACScan® at 488 (FL-2 channel). The analysis was performed using live gates to discriminate between doublets and cells exhibiting reduced DNA content corresponding to the sub G1/G0 diploid peak. These were defined as the apoptotic cells. A total of 5 000 events were analyzed. The results were confirmed by the visualization of internucleosomal DNA fragmentation by standard agarose gel electrophoresis as previously described with some modifications (Dupere-Minier et al., 2004; Gong et al., 1994). Briefly, following treatments, cells were washed twice with PBS and resuspended in 20 µl of the apoptotic cell lysis buffer (10 mM Tris – HCl pH 7.4, 0.5% SDS, 10 mM EDTA) containing proteinase K (0.1 mg/ml) and incubated at 50 °C overnight in a water bath. RNAse was then added to the samples at a final concentration of 0.5 mg/ml and further incubated another 30 min at 50 °C. Samples were then diluted in 20% (v/v) loading buffer (10 mM EDTA, pH 8.0, 1.0% (w/v) low-gelling T^0 agarose, 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose) and incubated at 70 °C for 10 min. Samples were loaded onto a 2% (w/v) agarose gel and electrophoresed at 100 V for 3 h in TBE 0.5 X (10% (v/v) TBE 5X (0.45 M Tris base, 0.45 M boric acid, 2% (v/v) EDTA 0.5 M pH 8.0) at pH 8.3). The DNA ladder was stained with 0.5 µM ethidium bromide (EtBr) at room temperature for 30 min and visualized by UV transillumination. Finally, the percentage of cells in apoptosis was quantified by the analysis of phosphatidylserine (PS) translocation to the extracellular membrane with the Annexin V-FITC apoptosis detection kit. Briefly, following treatments, cells were washed twice with PBS and incubated with annexin-V (5% (v/v)) and PI (5% (v/v)) at room temperature for 15 min. Cells were then analyzed by flow cytometer on a FACScan®. Compensation was done with the FL-2 channel. A total of 10 000 events were analyzed.

2.7. Oxidative stress levels assay

Intracellular ROS were quantified using the 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and dihydrorhodamine 123 (DHR 123) probes. First, untreated cells were incubated with the H2DCFDA [5 µM] or DHR 123 [25 µM] probes for 1 h at 37 °C. Cells were then washed once with PBS and treated or not with TBT [0.4 µM], H_2O_2 [50 µM] or arsenic trioxide (As_2O_3; 100 µM) in serum-free medium for up to 1 h. Control cells were exposed to 0.1% EtOH. Following treatments, cells were washed once with PBS and analyzed by flow cytometer. Background from cells, the probe, and ROS-inducing agents, diluted in the assay medium, were used as the blank value, to deduce self-fluorescence of compounds. ROS results were normalized using basal data of DFF40 WT cells. Nitric oxide levels were quantified using the Griess method as previously described by Wu and Yotnda (Wu and Yotnda, 2011) with some modifications. Briefly, for this assay, supernatants of treated samples were used. Samples were incubated with the Griess reagent A (1% (w/v) sulfanilamide diluted in 5% phosphoric acid) for 10 min in the dark and then with the Griess reagent B (0.1% (w/v) naphthyl ethylenediamine dihydrochloride diluted in water) for 30 min in the dark, at ratio of 1/1/1. A standard curve was made using sodium nitrite (1.56–200 µM), diluted with sample medium. The absorbance of samples was measured using the SpectraMax® M5 Microplate Reader with a filter of 538 nm.

2.8. Western blotting

Following treatments, cells were washed twice with PBS and suspended in laemmli buffer (12.5% (v/v) 0.5 M Tris – HCl pH 6.8, 10% (v/v) glycerol, 20% (v/v) SDS 10%, 5% (v/v) β-mercaptoethanol, 5% (v/v) bromophenol blue 0.5%). Samples were then incubated at 95 °C

for 5 min and stored at −20 °C for further analysis. Equal amounts of protein were loaded on SDS-polyacrylamide gels (SDS-PAGE) ranging from 7.5% to 12% and transferred separately onto nitrocellulose membranes. The membranes were then blocked in TBS-0.1% Tween 20 (TBST 1X) with 5% skimmed milk overnight. Membranes were washed 3 times with TBST 1X and then incubated with the following human antibodies (ab): whole anti-DFF40 ab, anti-DFF45 ab, anti-caspase-3 ab, anti-phospho-H2AX ab or anti-tubulin ab for 2 h at room temperature, or with anti-PARP ab or anti-caspase-6 ab overnight at 4 °C. Primary ab were diluted (1: 2 000) in TBST 1X containing 5% bovine serum albumin (BSA). Membranes were then washed 3 times with TBST 1X and incubated with horseradish peroxidase-conjugated goat anti-mouse IgM secondary Ab (1: 20 000) or with the horseradish peroxidase-conjugated sheep anti-rabbit IgG secondary Ab (1: 20 000) for 1 h at room temperature. Visualization of the bands was done by chemiluminescence with the Clarity Western ECL substrate. The quantification of the bands was performed using Image Lab.

2.9. Caspase-3/7 activity detection assay

Caspase-3/7 activity was assessed using the Caspase-3/7 Green detection reagent according to the manufacturer's instructions, with some modifications. Briefly, cells were washed once with PBS and suspended in RPMI 1640 medium supplemented with 0.1% FBS, at a density of 1×10^6 cells/ml, in sterile round-bottom polypropylene tubes. Caspase-3/7 Green detection reagent was then added to the samples (2 drops/ml), and cells were treated or no with TBT [0.4 μM]. At desired time points (2, 4 and 6 h), cells were analyzed by flow cytometer. During the kinetic assay, cells were incubated at 37 °C.

2.10. Statistical analysis

Each experiment was performed independently three times or more. Datas are presented as mean ± SEM of three independent experiments in triplicates. Differences between the two cell lines, treated or not, were tested by two-way ANOVA followed by Tukey's post hoc correction. Multiple *t*-test comparisons were performed to compare statistical differences within the same cell line when treated or not, or between two groups. A value of $p < 0.05$ was considered significant.

3. Results

3.1. DFF40 is essential for TBT-induced cellular mortality

Overexpression of DFF40 in T-47D cells was associated with an increased sensitization to different drugs (Bagheri et al., 2014, 2015). To investigate the role of DFF40 in TBT-induced apoptosis, we deleted its expression in Jurkat T cells (DFF40 WT) by using CRISPR/Cas9-mediated gene editing. As shown in Fig. 1A, the DFF40 gene was successfully deleted from Jurkat T cells (DFF40 KO), without altering the expressing of DFF45. Next, we exposed DFF40 KO and DFF40 WT Jurkat cells to TBT (0.2–0.4 μM) for up to 24 h, and the viability was assessed. Following TBT exposure, DFF40 KO cells present significantly lower levels of cellular mortality (51.09% ± 7.93%) than DFF40 WT cells (74.67% ± 3.61%) at 0.4 μM (Fig. 1B). This effect is enhanced at the concentration of 0.6 μM, where all DFF40 WT cells are dead. Kinetic of exposure to 0.4 μM of TBT for 24 h shows no significant changes at 4, 8, 16 h of exposure (Fig. 1C and D). To assess if this effect was only specific to TBT-induced apoptosis, cells were exposed to STS (0.1 to 10 μM) for up to 24 h or to a single dose of STS [1 μM] for 4 to 24 h. No differences in both cell lines are detected following STS-induced apoptosis (Fig. 1E and F). These results demonstrate that, in contrast to DFF40 WT Jurkat cells, DFF40 KO Jurkat cells are less sensitive to cytotoxicity induced by TBT exposure.

3.2. DFF40 KO T cells are resistant to TBT-induced apoptosis

Next, apoptotic cell death induced by TBT was assessed by the analysis of the cell cycle, using the G1/G0 subdiploid peak quantification, an indicator of DNA fragmentation (Fig. 2A). The results show that after a 24h exposition to TBT [0.4 μM], DFF40 WT cells present significantly higher levels of DNA fragmentation, at almost 40% following PI labeling, in comparison to DFF40 KO Jurkat cells, who do not have any DNA fragmentation (less than 1%). The absence of DNA fragmentation was confirmed on an agarose gel (Fig. 2B). As shown in Fig. 2B, TBT-exposed DFF40 WT cells exhibit a time-related DNA fragmentation, although DFF40 KO cells show a total absence of DNA bands. Since apoptosis can occur in the absence of DNA fragmentation, we next determined PS translocation to the extracellular cell membrane, an early marker of apoptosis (Wlodkowic et al., 2009). DFF40 WT Jurkat cells show significantly more cells in early apoptosis (annexin V^+- PI^-, 23.35% ± 3.85%), in comparison to DFF40 KO cells (6.53% ± 1.10%) (Fig. 2C). When early apoptotic (annexin V^+ PI^-) and late apoptotic/necrotic cells (annexin V^+ PI^+) are taken into account, DFF40 WT Jurkat cells have approximately 50.30% (± 6.87%) of the population stained positive versus 18.33% (± 2.49%) for DFF40 KO Jurkat cells. Together these results demonstrate that DFF40 KO Jurkat cells are resistant to TBT-induced apoptosis, by the lack of nuclear apoptosis.

3.3. DFF40 KO Jurkat T cells have a decrease of ROS production following TBT exposure

TBT-induced oxidative stress resulting in cell death by apoptosis has been previously demonstrated (Coutinho et al., 2016; Liu et al., 2006; Nakatsu et al., 2007). Both cell lines were pre-incubated with H2DCFDA, a ROS probe, and treated or not with TBT [0.4 μM], H_2O_2 [50 μM] or As_2O_3 [100 μM] for 1 h. Interestingly, as shown in Fig. 3A, untreated DFF40 KO cells have significantly higher levels of basal ROS production (GeoMFI 33.20 ± 5.82), approximately 3 folds higher than DFF40 WT cells (GeoMFI 11.50 ± 1.57). When T cells are treated with TBT, no significant ROS production is observed in DFF40 KO cells in comparison to DFF40 WT cells, who have an increase of about 3 folds (Fig. 3B, C). These later results were repeated with another mitochondrial perturbing agent, H_2O_2. Results were obtained with a concentration of 50 μM of H_2O_2 after a 1 h incubation, with an average of 4 folds increase of ROS production in DFF40 WT (Fig. 3B) cells as compared to no significant increase in DFF40 KO cells (Fig. 3C). Since H_2O_2 can in some condition directly activate DCFDA fluorescence, cells were exposed to As_2O_3, a well-characterized ROS inductor (Gupta et al., 2003). Exposure to As_2O_3 causes a significant increase in ROS production in DFF40 WT cells but not in DFF40 KO cells. In parallel, NO production levels were assessed by the Greiss method, but no significant effects are detected in both cell lines (Fig. 3D, E). Cells were also labeled with DHR 123, a molecular probe that detects peroxynitrite ($ONOO^-$) production levels. Both cell lines show no change in the production of ONOO- when treated or not with TBT [0.4 μM] for 6 h. An increase of about 2 folds is detected in DFF40 WT cells when treated with H_2O_2 as compared to a lower level in DFF40 KO cells (Fig. 3F, G). Overall, these results suggest that DFF40 deficiency influences the antioxidant response.

3.4. Reduced cleavage of procaspase-3, caspase-6 activation and γH2AX formation after TBT exposure in Jurkat DFF40 KO T cells

It has been reported in the literature that DDR plays an essential role in TBT genotoxicity (Liu et al., 2006; Morales et al., 2013). Like many endocrine disruptors, TBT causes DNA adducts and induces a genomic instability (Attaluri et al., 2010; Liu et al., 2006). Knowing that cells undergo apoptosis when their integrity is compromised, we next assessed procaspase-3 cleavage, caspase-3/7 activity, caspase-3 substrate cleavages such as PARP, caspase-6 activation, and DSB identified by γH2AX formation. Both cell lines were treated with TBT [0.4 μM] for up

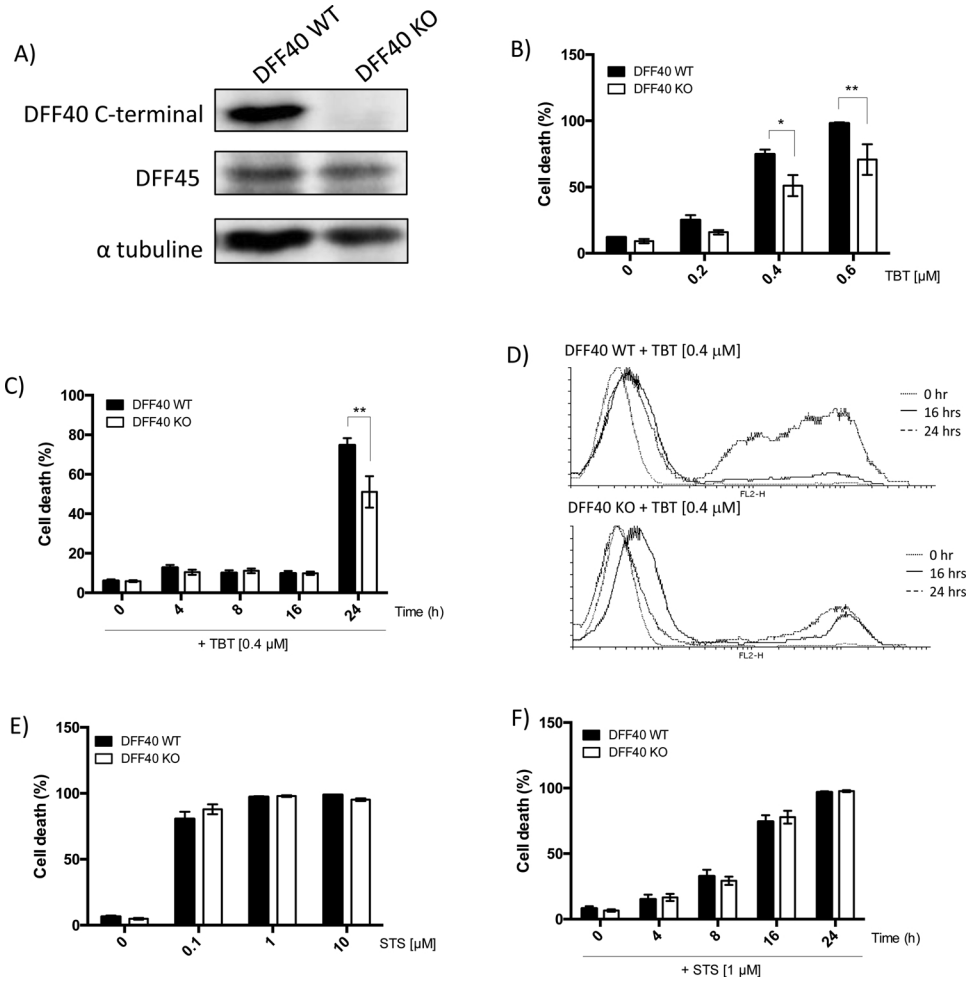

Fig. 1. DFF40-deficient Jurkat cells present lower cellular mortality levels following exposure to tributyltin. A: Jurkat DFF40 KO cells were obtained by the CRISPR cas-9 method. Proteins were extracted from the cells, then loaded onto 10% SDS-PAGE gels and (A) immunoblotted with anti-C-terminal-DFF40 or anti-DFF45 antibodies. B: Jurkat DFF40 WT and DFF40 KO cells were treated or not for 24 h with TBT (0–0.6 μM) in RPMI 1640 with 10% FBS. C: Cells were treated or not with TBT [0.4 μM] as described in B for 4 to 24 h. D: Flow cytometry plots of selected exposure times in C are presented. E: Cells were treated or not with STS (0–10 μM) for 24 h as described in B. F: Cells were treated with STS [1 μM] as described in B for 4 to 24 h. Control cells were incubated with 0.1% (v/v) EtOH. Cells were analyzed by flow cytometer with propidium iodide (1 μg/ml). All data are representative of at least 3 independent experiments. Data are shown as (mean ± SEM). Two-way ANOVA with Tukey's posthoc test was performed, followed by multiple *t*-test comparisons (*p < 0.05, **p < 0.001).

to 6 h, and procaspase-3, caspase-6, cleaved PARP and γH2AX levels were determined. As shown in Fig. 4A, DFF40 KO T cells treated with TBT have a higher expression of procaspase-3, corresponding with its delayed cleavage and consequently of its activation. The quantification of caspase-3/7 activity confirms that DFF40 KO cells have a delayed activation of caspase-3/7 following TBT exposure (Fig. 4C). Next, we confirmed the decrease of caspase-3 activation in Jurkat KO cells by the determination of two substrates, namely procaspase-6 and PARP (Fig. 4A, 4D). For both substrates, their cleavage follows the same pattern of caspase-3 activation in Jurkat KO and Jurkat WT cells. Caspase-dependent and independent apoptosis were associated with γH2AX formation, which corresponds with the presence of DSB. Following TBT treatment, the analysis shows no phosphorylation of the histone H2AX in DFF40 KO cells. The phosphorylation in DFF40 WT cells is approximately 6 folds higher (Fig. 4A, 4D). To confirm the absence of any DNA cleavage, γH2AX was assessed after a 24h exposure to TBT (Fig. 4B, 4D). As expected, there is no significant γH2AX formation in DFF40 KO cells. These results taken together demonstrate a delayed and reduced caspase-3/6 activation, PARP cleavage, and γH2AX formation in DFF40 Jurkat KO cells exposed to TBT.

3.5. DFF40 expression in DFF40 KO cells restores TBT-induced apoptosis

To further confirm and validate the role of DFF40 in TBT-induced resistance to apoptosis, DFF40 KO cells were transfected with the plasmid encoding the DFF40 gene (DFF40 KO transfected cells). As shown in Fig. 5A, the re-expression of DFF40 in DFF40 KO Jurkat cells is re-established, but at a lower level of expression. Treatment with TBT [0.4 μM] for 24 h significantly enhances cell mortality in DFF40 KO transfected cells (55.13% ± 8.47%) at a level comparable to DFF40 WT cells (52.97% ± 3.39%) (Fig. 5B). DNA fragmentation occurs in DFF40 KO transfected cells after exposure to TBT (Fig. 5C and 5D). Basal ROS levels and their production after H_2O_2 treatment is significantly increased in DFF40 KO transfected cells (Fig. 5E). Finally, DFF40 KO transfected cells re-established caspase-3 activation and γH2AX formation, but at a later time as compared to DFF40 WT cells (Fig. 5F). These results provide evidence for the requirement of DFF40 for the induction of nuclear apoptosis, leading to more massive cellular mortality levels.

4. Discussion

Among the apoptosis-inducing agents, the environment pollutant TBT is known to induce apoptosis in many cell types (Chow et al., 1992; Marinovich et al., 1996; Reader et al., 1999). Our study demonstrates that a 24h exposure to TBT of DFF40 WT Jurkat T cells in optimal culture (10% FBS) condition significantly reduces cell viability, in a dose-dependent manner (Fig. 1B). No significant effect is observed preceding this time point (Fig. 1C and 1D). Accordingly, TBT-induced apoptosis depends on the concentration and exposure time (Nakatsu et al., 2007; Sroka et al., 2008). Consistent with our results, where approximately 75% cell mortality was detected in DFF40 WT cells following a 24h TBT exposure, it was demonstrated that a 24h exposure to 500 nM TBT in PC12 cells significantly enhances cytotoxicity for up to 80% (Nakatsu et al., 2007). In Jurkat cells, some studies have demonstrated that exposure to 2 μM TBT for up to 100 min was sufficient to induce apoptosis, as well as 1 μM for a 4h exposure in Jurkat A3 T-cells (Krug, 2012; Stridh et al., 1999). The differences in cell toxicity to

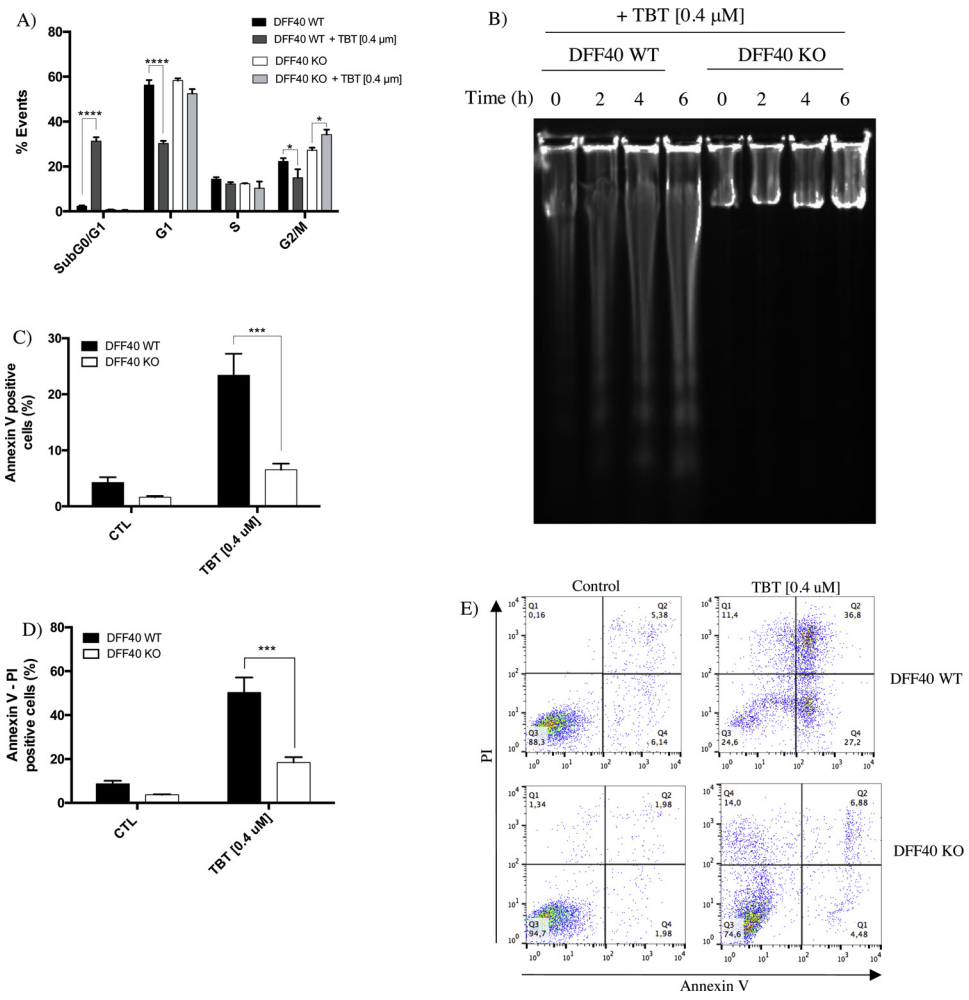

Fig. 2. DFF40 deficiency in Jurkat cells inhibits DNA fragmentation and delays tributyltin-induced apoptosis. Jurkat DFF40 WT and DFF40 KO cells were treated or not with TBT [0.4 µM] for 24 hs in complete medium. A: Apoptosis was determined by sub-G0 cell cycle analysis after staining with Krishan buffer. B: DNA fragmentation was visualized by a 2% agarose gel stained with ethidium bromide C–E: Apoptosis was determined by phosphatidylserine/annexin V based assay following a 6 h exposure to TBT [0.4 µM] in RPMI plus 01% FBS. C: Annexin V^+-PI^- cells, D: Annexin V^+-$PI^{+,}$ and E: Representative flow cytometry analysis. Control cells were incubated with 0.1% (v/v) EtOH. All data are representative of a biological triplicate. Data are shown as (mean ± SEM). Two-way ANOVA with Tukey's posthoc test was performed, followed by multiple *t*-test comparisons (*$p < 0.05$, ***$p < 0.0001$).

TBT obtained in these various studies may be explained by the composition of the medium and the physicochemical properties of the pollutant (Pagliarani et al., 2013; Stridh et al., 1999). The majority of the biological effects of organotin compounds are attributable to their amphiphilic nature, where the tin core can establish strong covalent bonds with atoms of biomolecules, such as cysteine residues of small proteins (Pagliarani et al., 2013). The variability in the percentage of serum during cell treatment may explain the interactions of TBT with these residues, which can further diminish the availability of TBT to induce toxicity. This could explain why no significant cellular mortality is induced following a 16h exposure to TBT in complete medium with 10% FBS. *in vivo*, the cytotoxicity of TBT is highly influenced by plasmatic albumin levels, that predicts its bioavailability, and thus its cytotoxicity. Sun and colleagues demonstrated that TBT has a binding coefficient (Log K_b) of approximately 5.68 with human serum albumin and binds through noncovalent interactions (Sun et al., 2012). Covalent interactions between TBT and BSA was also demonstrated (Zhang et al., 2007). Also, our *in vitro* study was performed using 400 nM TBT, a concentration that is 4 times higher than what was previously detected in one human blood sample (Whalen et al., 1999).

On the other hand, DFF40 KO Jurkat cells presented a less toxicity pattern than DFF40 WT cells (Fig. 1). The abolition of the DFF40 gene expression rescued cell death of only 23.5% in comparison to DFF40 WT Jurkat cells (Fig. 1C and 1D). TBT-induced apoptosis mechanism depends on TBT concentration, as mentioned above (Nakatsu et al., 2007). At low doses, relevant to those in the environment, it was shown that TBT acts on cholesterol homeostasis in primary theca cells by perturbating RXR pathway (Pu et al., 2019). Moreover, TBT can induce both apoptosis and necrosis, where apoptosis is more prevalent at lower doses (below 250 nM) in porcine aortic endothelial cells (Botelho et al., 2015). Using high doses of TBT in the present study and a prolonged time of exposure, a percentage of cells underwent necrosis rather than apoptosis, as shown in Fig. 2E. In this case, deletion of the DFF40 gene had a lower impact on total cell viability. This observed effect of DFF40 expression abolition on cellular mortality was specific to TBT-induced apoptosis. In fact, no effect between both cell lines is present in the STS-induced apoptosis (Fig. 1E and 1 F), a protein kinase inhibitor widely used for its apoptosis-inducing properties. This result could be explained by the fact that STS and TBT have distinct mechanisms of action which could activate different effectors. The present study is the first to generate DFF40 KO cells with the CRISPR-cas9 method and assess the toxicity of pollutants. Although, an intrinsic deficiency of DFF40, especially in cancerous cells, has shown to impair oligonucleosomal DNA hydrolysis and predict an overall better survival rate (Banas et al., 2018; Sanchez-Osuna et al., 2016).

The involvement of DFF40 in internucleosomal DNA fragmentation and chromatin condensation is widely known (Liu et al., 1998). As shown by our results, cells lacking the expression of DFF40 and exposed to TBT presented no DNA fragmentation after analysis of cell cycle and agarose gel migration (Fig. 2A-B). Our lab has previously reported that HPB-ALL and Jurkat T cells natural revertant CD45-deficient were resistant to TBT- or H_2O_2-induced nuclear apoptosis (Desharnais et al., 2008). The last resistance was associated with a defect of T cell signaling, as noted for Raji B lymphoma cells, with hypothetically a structural or functional defect in DFF40 (Kawabata et al., 1999). It is suggested that a defective apoptotic signal transduction downstream

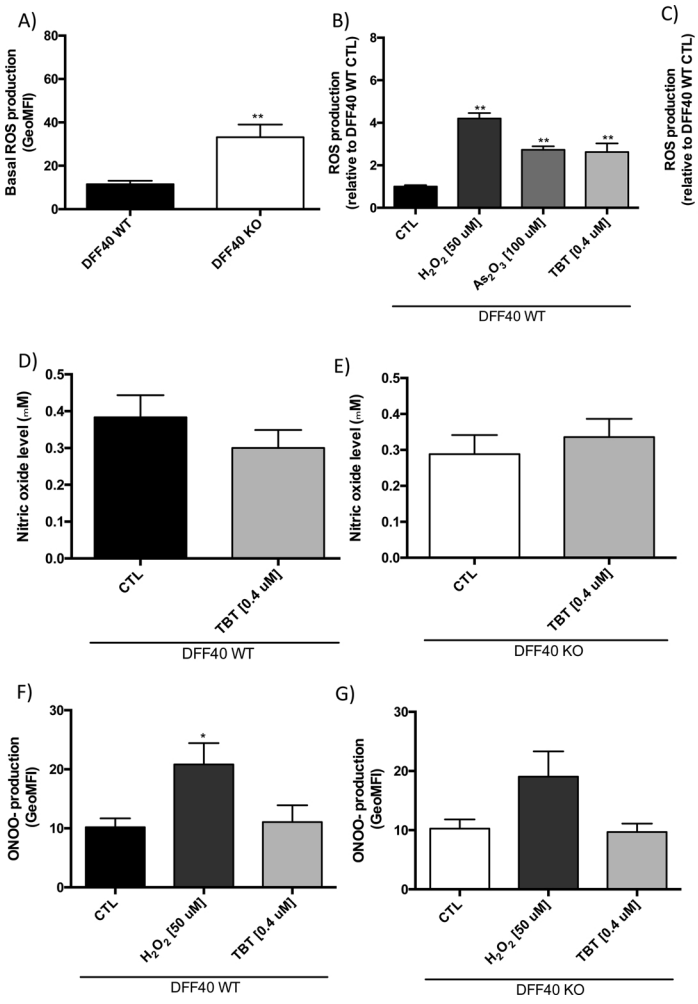

Fig. 3. Exposure to tributyltin in Jurkat DFF40 KO cells inhibits ROS production levels in comparison to wild-type cells. A: Jurkat DFF40 WT and DFF40 KO cells were pre-incubated for 1 h with H2DCFDA and basal level of ROS was determined. B: Same as in A, but Jurkat DFF40 WT were then treated or not with H_2O_2 [50 µM], As_2O_3 [100 µM] or TBT [0.4 µM] for 1 h at 37 °C, in RPMI plus 0.1% FBS. Control cells were incubated with 0.1% (v/v) EtOH. Cells were analyzed by flow cytometer. C: Same as in B but the assay was performed with Jurkat DFF40 KO cells. D: NO production was assessed with the Griess method following treatment of Jurkat DFF40 WT cells with TBT [0.4 µM] for 6 h. E: Same as in D but instead Jurkat DFF40 KO cells were used. F: $ONOO^-$ production was determined using DHR123 probe following H_2O_2 [50 µM] or TBT [0.4 µM] exposure of Jurkat DFF40 WT cells for 1 h. G: Same as in F but instead Jurkat DFF40 KO cells were used. All data are representative of at least 3 independent experiments. Data are shown as (mean ± SEM) of the geomean fluorescence intensity (GeoMFI) or relative to DFF40 CTL values. Multiples *t*-test comparisons were performed (*$p < 0.05$, **$p < 0.001$).

caspase-3 activation may be responsible for nuclear apoptosis resistances (Kawabata et al., 1999). Interestingly, the absence of apoptosis was confirmed with the Annexin-V staining assay, an early marker of apoptosis associated with PS exposure on the outer membrane (Lee et al., 2013) (Fig. 2C-E). At high doses and prolonged exposure time, TBT is known to induce the necrotic pathway (Nakatsu et al., 2007; Stridh et al., 1999). As shown, DFF40 KO cells do not bind to Annexin-V, on the contrary to DFF40 WT cells, which also stains positively to PI (Fig. 2). Our results are in concordance with those in the literature, whereas TBT at 100 nM has shown to induce PS exposure in rat thymocytes (Nakata et al., 1999). It is proposed that concentrations greater than 300 nM are likely to cause necrosis in some cells (Nakata et al., 1999).

Among the early onsets of the apoptotic pathway, we can find ROS generation in many cases as the consequence of cellular disruption during cell death (Perrone et al., 2008). TBT at low doses induces oxidative stress in many cell types, such as pancreatic β-cells (Huang et al., 2018), Jurkat T cells (Katika et al., 2011) and PC12 cells from rat adrenal medulla (Nakatsu et al., 2007). This molecular effect is the consequence of intracellular Ca^+ increase (Nakatsu et al., 2007). Furthermore, it has been reported that TBT induces interferon-gamma (IFNγ) and TNFα synthesis (Lawrence et al., 2018). Pro-inflammatory cytokines are highly involved in mitochondrial ROS generation (Yang et al., 2007). These results are similar to the present study, where exposure to TBT enhanced ROS generation in DFF40 WT cells (Fig. 3B). TBT did not induce ROS generation in DFF40 KO cells in comparison to DFF40 WT cells (Fig. 3C). The same pattern is observed following H_2O_2 exposure, but with a much greater ROS production in DFF40 WT cells. The limitations regarding the use of the DCFDA probe for H_2O_2-induced ROS detection has been debated for a while (Kalyanaraman et al., 2012). Knowing that H_2O_2 and the molecular probe DCFDA can interact together and cause biased mean fluorescence, another well-known ROS inducer was used – As_2O_3. It was shown in HeLa cells that As_2O_3 induces apoptosis through a ROS-dependant mechanism by perturbating MMP (Woo et al., 2002). In Jurkat cells, As_2O_3 [2.5 µM] has shown its ability to significantly increase ROS production (Lu et al., 2012). In comparison to those results, As_2O_3 did significantly induce ROS production in our Jurkat DFF40 WT cells. In the DFF40 knock-out cells, a higher level of ROS at a basal state was noted (Fig. 3A) suggesting a redox imbalance. No significant differences were seen in ONOO- levels and NO production in both cell lines. The primary source of ROS in cells is the mitochondria. A perturbation of the respiratory chain or in the number of mitochondria can be associated with an increase of ROS (Fleury et al., 2002). A link between AIF in the regulation of complex I of the mitochondrial respiratory chain and an increase in mitochondrial content in B and T cells was demonstrated (Milasta et al., 2016), suggesting a tight interaction between apoptotic machinery and this organelle. Current analysis in our laboratory indicates that Jurkat DFF40 KO cells have a significant increase in mitochondrial mass and a higher MMP (manuscript in preparation). Increase in the basal level of ROS can be explained by the relationship between those two later factors, where the mitochondrial mass and MMP positively influence ROS levels in the cell (Suski et al., 2012).

Caspase-3 is a serine protease activated during apoptosis, having for the primary function to cleave the DFF45 to induce internucleosomal DNA fragmentation in the apoptotic pathway (Kawabata et al., 1999). It

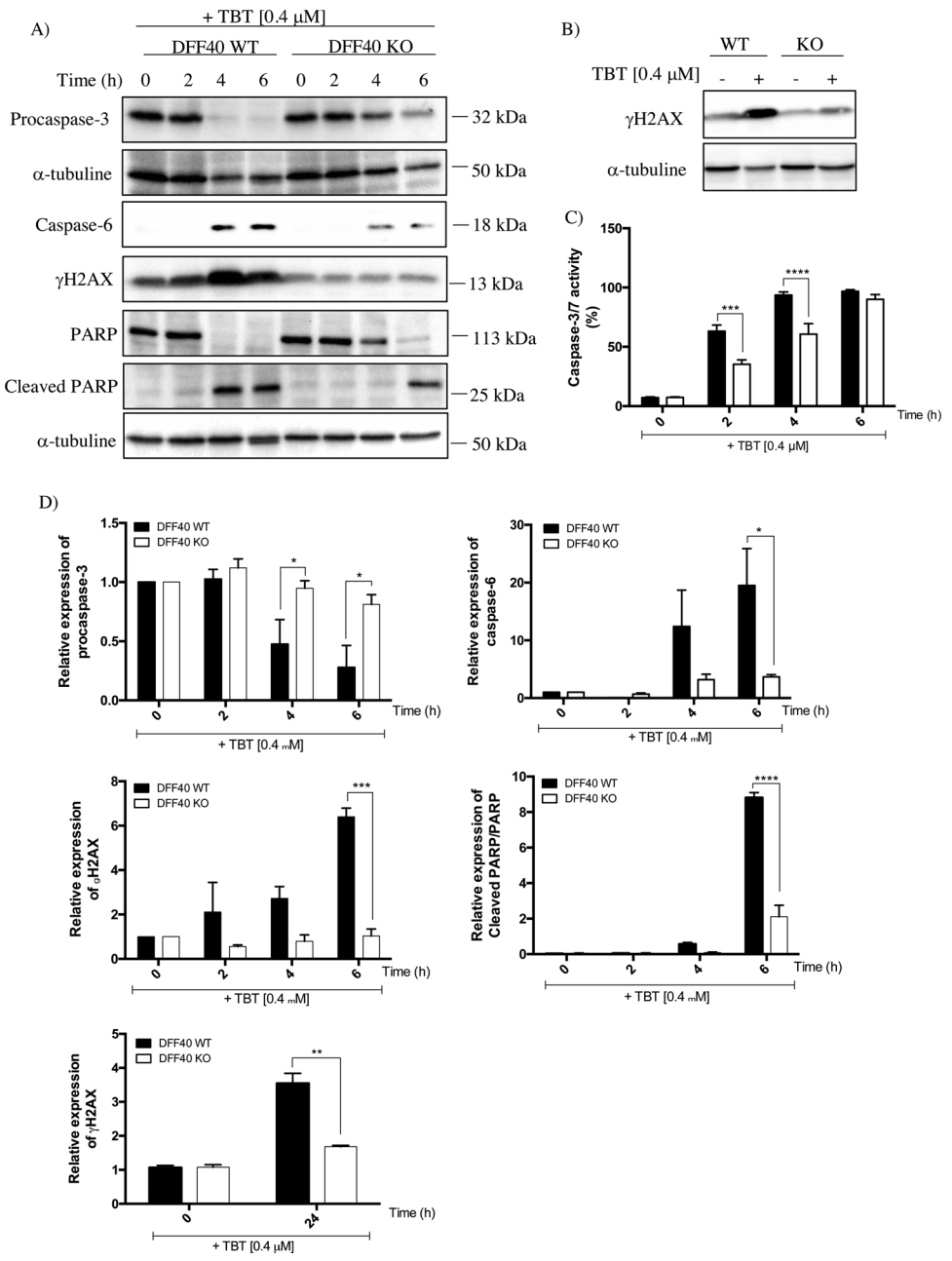

Fig. 4. Caspase-3/7 activity, PARP cleavage and histone H2AX phosphorylation is significantly reduced and inhibited in DFF40 KO cells following tributyltin-induced apoptosis. A: Jurkat DFF40 WT and DFF40 KO cells were treated or not with TBT [0.4 μM] for 2, 4 and 6 h and then immunoblotted with anti-caspase-3, anti-phospho-H2AX, anti-caspase-6 or anti-PARP antibodies following SDS-PAGE gels (ranging from 7.5%–12%). B: Cells were treated as described in A but for 24 h. Cells were lysed, and anti-phospho-H2AX determined. C: Jurkat DFF40 WT and DFF40 cells were treated as in A and caspase-3/7 Green detection reagent was added to each sample (2 drops/ml). Caspase-3/7 activity was assessed by flow cytometer. D: Densitometric analysis of each blot is shown. Analysis was performed with the Image Lab program. All data are representative of at least 3 independent experiments. Data are shown as (mean ± SEM). One-way Anova with Dunnett's post-hoc test was performed. (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$, ****$p < 0.00001$).

acts as a convergence point between the intrinsic and extrinsic apoptosis pathway, making it a target of interest in apoptosis studies (Snigdha et al., 2012). The absence of significant activation of effector caspase-3/6 in DFF40 KO cells confirms the lack of caspase-dependent apoptosis as observed in DFF40 WT Jurkat cells. A deficient DNA repair system is known to active cell death by nucleases such as the DFF40, which induces specifically DNA DSB (Hanus et al., 2008). This molecular change is always followed by γH2AX formation, a widely used biomarker for DNA damage (Kuo and Yang, 2008). The use of caspase-3 inhibitors in Jurkat cells have shown to inhibit TBT-induced apoptosis, suggesting a pivotal role for caspase-3 in the cytotoxic effects of TBT (Krug, 2012). The cytotoxic effect of TBT methoxide on HepG2 cell line has shown to decrease γH2AX formation (Khoury et al., 2016). These results are in concordance with our results, where TBT exposure induced procaspase-3 cleavage, caspase-3/7 activity enhancement followed by cleavage of their substrates caspase-6, PARP, and DFF45 in WT Jurkat cells (Fig. 4). These effects were reduced and delayed in DFF40 KO cells. Surprisingly, TBT-mediated apoptosis induced γH2AX in DFF40 WT cells, in contrary to DFF40 KO cells where no change was observed. γH2AX is considered to be critical for DNA ladder formation, where DFF40 binds to γH2AX to mediate its effects (Lu et al., 2006). It is proposed that the lack of γH2AX in p53 deficient background cells, as our Jurkat DFF40 KO cells, might be responsible for apoptosis resistance (Lu et al., 2006). These differences in γH2AX may explain the variabilities in the cellular changes following TBT exposure. The absence of γH2AX could be linked to cell cycle malfunctions, abnormal cell survival, and compromised DNA integrity. The intended function of γH2AX as a docking site for DNA damage and repair proteins could explain the resistance to apoptosis and the genomic instability in DFF40 KO cells (Podhorecka et al., 2010). Also, γH2AX seems to play a role in cell cycle checkpoints, where its phosphorylation contributes to the recruitment of ATM kinase to DSB sites and activates cell cycle checkpoints (Podhorecka et al., 2010). These results strongly suggest a role of DFF40 in genomic stability, where its deficiency can impact DNA integrity mediating apoptotic processes.

To confirm the importance of DFF40 in nuclear apoptosis, Jurkat

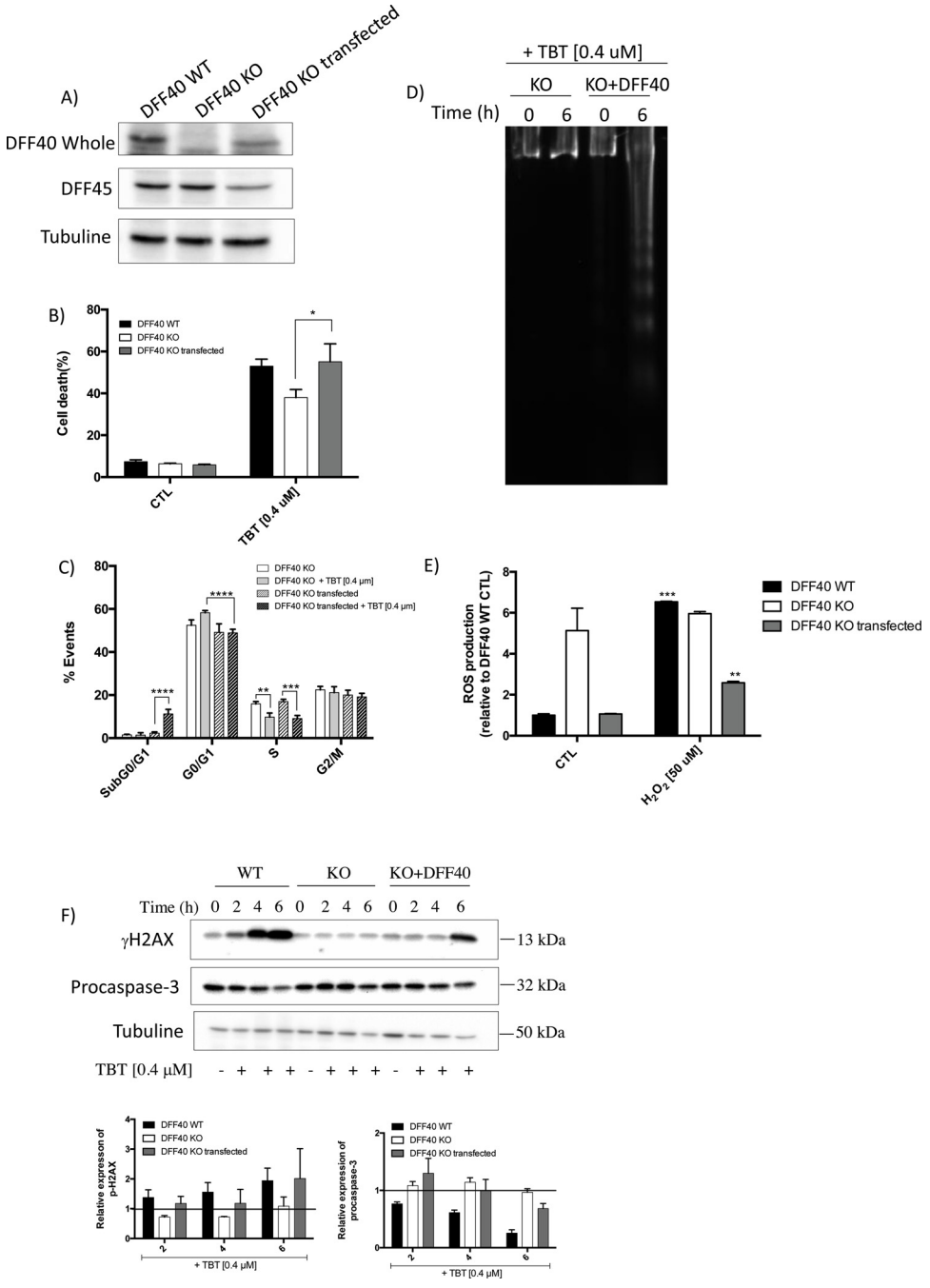

Fig. 5. Transfection of DFF40 in DFF40 KO cells restore the apoptosis induced by tributyltin. Jurkat DFF40 WT, DFF40 KO and DFF40 KO transfected cells were treated or not with TBT [0.4 µM] for (B, C) 24 hs in RPMI plus 10% FBS or for up to 6 hs in RPMI plus 0.1% FBS. Control cells were incubated with 0.1% (v/v) EtOH. A: DFF40 expression after transfection. B: Cell dead analysis after TBT exposure. C: Sub-G0 and cell cycle analysis after TBT exposure. D: DNA fragmentation visualized by a 2% agarose gel stained with ethidium bromide. E: Jurkat DFF40 WT, DFF40 KO and DFF40 KO transfected cells were pre-incubated for 1 h with H2DCFDA and were then exposed or not to H_2O_2 [50 µM] for 1 h. ROS production levels were determined by flow cytometer. F: Caspase 3 cleavage and γH2AX phosphorylation after TBT exposure. Densitometric analysis was showed at the right of panel F. All data are representative of a biological triplicate. Data are shown as (mean ± SEM). One-way Anova with Dunnett's posthoc test was performed, followed by multiple *t*-test (*p < 0.05, **p < 0.001 *** p < 0.0001,****p < 0.00001).

DFF40 KO cells were transfected with DFF40, and the same assays as above were performed (Fig. 5). Studies have shown that overexpressing DFF40 in T-47D breast cancer cells enhanced apoptosis, compromised cell viability, and enhanced DFF45 and DFF40 expression (Bagheri et al., 2014, 2015). Our results are similar to those in which the transfection of DFF40 diminished cellular viability (Fig. 5B) and re-established DNA laddering following TBT exposure (Fig. 5D). Following cell cycle analysis (Fig. 5C), the transfection of DFF40 in KO Jurkat cells restored DNA fragmentation. There were no differences in the percentage of cells in the G0/G1 phase for transfected cells, before or after TBT exposure. Two main reasons could explain this observation. On the first hand, transfected cells did not exhibit a total re-expression of DFF40 as compared to DFF40 WT cells, which could explain the variability in the susceptibility to TBT between those two cell lines. On the other hand, similar to DFF40 KO cells, treated DFF40 KO transfected Jurkat T cells had a significant diminution in cells in the S-phase. The DNA fragmentation that occurs during the S-phase causes cells to accumulate in the G0/G1 phase and thus, not change the G0/G1 proportions before and after treatment. Furthermore, γH2AX formation occurred after a more extended time of exposure to TBT (Fig. 5F). As shown in our results, re-expression of DFF40 did not establish the basal expression levels of DFF40, compared to DFF40 WT cells (Fig. 5A). This could explain a lower ROS generation in DFF40 KO transfected cells (Fig. 5E) and delayed γH2AX formation (Fig. 5F). Nevertheless, DFF40 transfection in KO cells confirm the potential role of DFF40 in genomic stability and propose distinct roles than DNA laddering exclusively.

Several new emerging studies have been proposing a unique role for DFF40 in genomic stability, where its low expression compromises DNA laddering (Sanchez-Osuna et al., 2016; Yan et al., 2006). Following exposure to antimitotic drugs, a study has shown that DFF45 cleavage and DFF40 activation was necessary for a mitotic arrest and the occurrence of DNA damage (Orth et al., 2012). These new findings

suggest that DFF40 may play a more crucial role in cellular mechanisms. In the perspective to find new molecular partners to DFF40, immunoprecipitations of the DFF45/DFF40 complex were performed, and potential protein interactions were identified. Preliminary results point out that the metabolic, proliferative, DNA repair and protein synthesis markers were amongst the identified potential partners (data not shown). Several new studies have been demonstrating the impact of TBT on cholesterol homeostasis via protein synthesis (Pu et al., 2019), on DNA damage through human breast carcinoma cells (Hunakova et al., 2019) and on the metabolism and proliferation of cells implicated in spermatogenesis (Cardoso et al., 2018; Wu et al., 2017). On the other hand, a study demonstrated that the loss of DFF40 in apolipoprotein E-deficient mice conferred protection against atherosclerosis development (Chao et al., 2016). This suggests an additional role of DFF40 in other mechanisms than DNA fragmentation, such as MAPK signaling pathway (Chao et al., 2016), that is important in cell proliferation, differentiation, migration, and apoptosis (Sun et al., 2015). Knowing that DFF40 may play a role in those effects through its hypothesized interactions by immunoprecipitations, we could hypothesize that DFF40 deficiency may regulate other distinct effects of TBT, and thus compromise cell integrity and function following environmental exposure. Our preliminary analysis revealed that the most significant interactions were with DNA repair and protein synthesis markers, determined by a higher number of Mascot Server score. Nucleophosmin/B23 was highly detected in every analyzed sample, a DNA repair marker. B23 is known to interact with DFF40 directly and inhibit its activity (Ye, 2005). Other interactions are not known up to this date in the literature. Further studies are necessary to assess the importance of those interactions following apoptosis-inducing agents and to identify new targets that mediate TBT-induced cytotoxicity.

In conclusion, the results in the present study show not only the importance of DFF40 in TBT-induced toxic response but propose a new role of DFF40 in toxin-induced DNA damage. DFF40 deficiency confers Jurkat T cells resistance to TBT-induced apoptosis, by interacting with genomic markers as the γH2AX. There is an increase in γH2AX formation in DFF40 WT cells and no change in phosphorylation levels in DFF40 KO cells. These results prove no implication of another nuclease such as AIF and endo G. It could be hypothesized that the DFF40 endonuclease activity can be involved not only in the final apoptosis phase but also in the early checkpoint of the cell cycle.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3 CHAPITRE 3: DFF40 DEFICIENCY IN CANCEROUS T CELLS IS IMPLICATED IN CHEMOTHERAPY DRUG SENSITIVITY AND RESISTANCE THROUGH THE REGULATION OF THE APOPTOTIC PATHWAY

Titre de l'article : *Une déficience en DFF40 dans les cellules cancéreuses lymphocytaires est impliquée dans la sensibilité et la résistance à la chimiothérapie via la régulation de la voie signalétique de l'apoptose*

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MK : Conception de la méthodologie, exécution des expériences, analyses statistiques, rédaction et révision du manuscrit.

BJ : Exécution des expériences – génération des lignées cellulaires par CRISPR-cas9, analyses statistiques et révision du manuscrit.

SF : Exécution des expériences – analyse bio-informatiques à partir de la base de données TCGA, analyses statistiques, révision du manuscrit.

RJD : Supervision de SF, analyses statistiques, révision du manuscrit.

JB : Conception du projet et de la méthodologie, analyses statistiques, supervision, rédaction et révision du manuscrit.

3.1 Lien entre l'article précédant et les suivants :

Dans l'article précédent, nous avons démontré que les cellules déficientes en DFF40 étaient résistantes à l'apoptose induite par le TBT. Ceci était marqué par un retard d'activation de l'apoptose ; les cellules DFF40 KO ont un retard de clivage de la procaspase-3 et de PARP, ainsi qu'une diminution de l'activation de la caspase-6. De plus, nos résultats de travaux de recherche ont permis de complémenter la littérature en regard du rôle du DFF40 dans la stabilité génomique : les cellules déficientes en DFF40 ont une absence totale de formation de foci de γH2AX suivant un stimulus cytotoxique, pouvant témoigner d'une altération des voies de réparation de l'ADN. Or, est-ce que les mêmes phénomènes peuvent être observés dans l'apoptose induite par les médicaments de chimiothérapies ? Dans cet article, nous avons voulu étudier la réponse cellulaire et les régulations moléculaires suivant une exposition aux agents de chimiothérapie chez les cellules déficientes en DFF40. Les résultats de cet article nous ont permis de caractériser l'effet des médicaments de chimiothérapie chez les cellules T Jurkat DFF40 KO, afin de mieux pouvoir orienter nos travaux au sujet de la régulation de la stabilité génomique (Article 4).

3.2 Résumé en français :

La régulation de la voie de signalisation de l'apoptose est l'un des mécanismes les plus étudiés en ce qui concerne la résistance des cellules cancéreuses. De nombreuses mutations ont été démontrées comme étant impliquées dans la résistance aux médicaments de chimiothérapie. Durant la dernière décennie, le facteur de fragmentation de l'ADN 40 (DFF40), l'endonucléase régissant la fragmentation de l'ADN dans la voie de l'apoptose, a suscité de l'intérêt dans l'étude de la résistance des cellules cancéreuses à la chimiothérapie. Il a été démontré que les tumeurs de glioblastomes et de léiomyosarcomes utérins ont une sous-régulation de l'expression du DFF40 et ceci est impliqué dans le mauvais pronostic des patients. En concordance avec ces observations, dans cette étude, nous avons démontré que l'expression de l'ARNm transcrit par le gène codant pour la protéine DFF40 est sous-exprimée dans les cancers du sein, endocervicaux, ovariens, pulmonaires, pancréatiques et du cerveau. Dans cette étude, nous avons voulu déterminer si un déficit en DFF40 dans les lymphocytes T Jurkat pourrait avoir un impact sur la sensibilité aux médicaments de chimiothérapie conventionnels. La lignée de cellules T Jurkat déficiente en DFF40 (DFF40 KO) a été générée par CRISPR-cas9, puis les cellules DFF40 WT et DFF40 KO ont été traitées avec différents antimétabolites et inhibiteurs de la topoisomérase II (TOP2). Nos résultats démontrent que les cellules DFF40 KO ont une chimiorésistance aux antimétabolites (*c.-à-d.* le méthotrexate (MTX), la 6-mercaptopurine (6-MP) et la cytarabine (Ara-C)) et de manière surprenante, ils sont plus sensibles aux inhibiteurs de TOP2 (*c.-à-d.* étoposide (ETO) et téniposide (VM-26)). Ensuite, nous avons démontré que les cellules déficientes en DFF40 traitées avec l'Ara-C ont des niveaux de translocation de la phosphatidylsérine à la membrane externe significativement diminués en comparaison avec les cellules DFF40 WT. L'exposition des cellules DFF40 KO à l'ETO induit des niveaux de mortalité significativement plus élevés et une régulation significativement négative de l'expression de Bcl-xL, en comparaison aux cellules DFF40 WT. Enfin, l'abolition de l'expression de DFF40 dans les cellules Jurkat altère significativement les niveaux de phosphorylation de l'histone H2AX suivant un traitement à l'ETO et à l'Ara-C. Globalement, nos résultats suggèrent que le DFF40 est une nouvelle cible clé dans la résistance des cellules cancéreuses à la chimiothérapie et celle-ci régule potentiellement la stabilité génomique.

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DFF40 deficiency in cancerous T cells is implicated in chemotherapy drug sensitivity and resistance through the regulation of the apoptotic pathway

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ABSTRACT

The regulation of the apoptotic pathway is one of the most studied mechanisms regarding cancer cell resistance. Many mutations have been linked to drug resistance. The DNA fragmentation factor 40 (DFF40) has been gaining interest regarding cancer cell response to chemotherapy and patient outcomes. Glioblastomas and uterine leiomyosarcomas have been shown to have a downregulation of DFF40 expression, conferring a poor patient prognosis. In concordance with these observations, in this study, we showed that DFF40 gene is also downregulated in breast, endocervical, ovarian, lung, pancreas and glioblastomas. DFF40 is the endonuclease responsible of DNA fragmentation during apoptosis. In this study, we sought to determine if a DFF40 deficiency in Jurkat T cells could impact the sensitivity to conventional chemotherapy drugs. CRISPR-cas9 generated DFF40 knockout (DFF40 KO) stable Jurkat cells and wild-type (DFF40 WT) cells were treated with different antimetabolites and topoisomerase II (TOP2) inhibitors, and cell viability was subsequently assessed. DFF40 deficient cells show chemoresistance to antimetabolites (*e.g.* methotrexate, 6-mercaptopurine and cytarabine) and surprisingly, they are more sensitive to TOP2 inhibitors (*e.g.* etoposide and teniposide). DFF40 deficient cells exposed to cytarabine present lower phosphatidylserine translocation levels to the outer cell membrane layer. Etoposide exposure in DFF40 deficient cells induces higher mortality levels and downregulation of Bcl-xL cells compared to DFF40 expressing T cells. The abolition of DFF40 expression in Jurkat cells significantly impairs histone H2AX phosphorylation following etoposide and cytarabine treatments. Our findings suggest that DFF40 is a novel key target in cancer cell resistance that potentially regulates genomic stability.

1. Introduction

Apoptosis is a highly regulated programmed cell-death mechanism that preserves cell and tissue homeostasis. It allows the elimination of mutated or senescent cells and is important to avoid the horizontal transfer of mutated genes to phagocytes [1]. The latter step of apoptosis is regulated by the DNA fragmentation factor (DFF). The physiological role of the DFF is to maintain cell homeostasis by regulating chromatin condensation and DNA fragmentation following apoptosis-inducing triggers. The DFF is an heteromeric complex, consisting of the endonuclease DFF40/caspase-activated DNase (CAD) and its inhibitory subunit DFF45/inhibitor of CAD (ICAD). The DFF45 subunit also acts as a chaperone molecule. Under normal circumstances, the chaperone molecule binds with the DFF40 subunit following its translation, allowing its proper folding, as well as blocking its homo-oligomerization [2]. Following apoptosis induction, several caspases are activated (*e.g.* caspase-8, −6, −7, −9) from the extrinsic and intrinsic apoptotic pathways [3], ultimately leading to the activation of the caspase-3 [4]. This last effector cleaves the DFF45 subunit that further allows the release of the DFF40 and its subsequent homo-oligomerization in the cytoplasm [4]. The active oligomer is then translocated into the nucleus and leads the internucleosomal DNA fragmentation process, generating DNA fragments of 180–200 bp [4,5]. It is to be noted that two models for the localization of the inactive DFF40/DFF45 complex have been proposed, studies revealing a cytoplasmic or nuclear localization [3].

Over the past decades, multiple mutations in the apoptotic genes or proteins have been identified to have a role in cancer development and chemoresistance [6–8]. Among these mutations, alterations in the

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expression of DFF40 or mutations of its gene have been linked to tumorigenesis and cancer malignancy [3,9,10]. Evidence has shown that DFF-deficient mice are more susceptible to develop cancer [11,12]. Grade IV astrocytoma, also known as glioblastoma, is an extremely aggressive brain tumor that is characterized by an absence of oligonucleosomal DNA fragmentation during apoptosis and a reduction in the pool of cytoplasmic DFF40 [10]. Cell lines over-expressing caspase-resistant DFF45 display increased levels of chromosomal aberrations and aneuploidy following irradiation [13]. TK6 DFF40-deficient cells don't accumulate mutations following TRAIL treatment [14]. In addition, a group of scientists have shown that a deregulation in DFF40 and DFF45 ratio is one of the reasons of TRAIL-induced apoptosis resistance in lymphoma's [15]. Human lymphoma cells with function disorders downstream of caspase-3 do not demonstrate DNA fragmentation to chemical-induced apoptosis [16]. Furthermore, DFF40 activity and expression may also affect the elimination of cancer cells by chemotherapy. Our laboratory has previously shown that Jurkat T cells deficient in DFF40 are more resistant to tributyltin (TBT)-induced apoptosis [17]. Mammary gland epithelial cells overexpressing DFF40 are less resistant to doxorubicin (DXR) [18], which could be a novel approach in personalized treatments.

Clinically, numerous malignancies have shown a deregulation in the expression of DFF40 and DFF45 [3]. The expression of DFF40 was shown to be downregulated in non-endometrial and high-grade endometrioid endometrial cancers [19], in uterine leiomyosarcomas [9] and in glioblastomas [10], and associated with poor clinical outcomes. Even though multiple studies demonstrate the link between DFF40 expression and cancer cell sensitivity to chemotherapeutic drugs, very few studies have sought to determine the underlying mechanisms. Our laboratory has shown a delay in caspase-3 activation, poly (ADP-ribose) polymerase (PARP) cleavage and a total absence of histone H2AX phosphorylation in DFF40 knocked out cells treated with TBT [17]. No data is available regarding the regulation of the apoptotic pathway in DFF40 deficient cancer cells following an exposure to chemotherapeutic drugs. Furthermore, no evidence proves cancer cell response (*e.g.* sensibility) to chemotherapeutic drugs *in vitro* in DFF40 deficient cells. It is important to consider this aspect due to the low contribution of chemotherapy on overall survival rates [20,21]. A better understanding of the role of DFF40 in cancer therapy would allow a better patient management and clinical outcomes.

The aim of this study was to compare the relation between DFF40 expression abolition with cancer cell resistance to conventional chemotherapy drugs. Antimetabolite drugs (*e.g.* methotrexate (MTX), cytarabine (Ara-C), 6-mercaptopurine (6-MP)), as well as topoisomerase II (TOP2) inhibitors (*e.g.* etoposide (ETO), teniposide (VM-26), mitoxantrone (MIT), DXR) have been compared to assess cell mortality rates. Results suggest an important role of DFF40 in the resistance of cancer cells to antimetabolites drugs. Jurkat DFF40-deficient cells did not display oligonucleosomal DNA fragmentation and were more viable following exposure to MTX, Ara-C and 6-MP. Surprisingly, DFF40-deficient cells were more sensitive to TOP2 inhibitors. These results may propose a new algorithm for cancer treatment depending on biological markers of tumors.

2. Materials and methods

2.1. Cell culture

The Jurkat (subclone J77.6.8 from ATCC TIB-152) cells were grown in RPMI 1640 (Gibco, Thermo Fisher Scientific, MA, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Wisent, Thermo Fisher Scientific, MA, USA), 100 U/ml penicillin (Wisent, Thermo Fisher Scientific, MA,USA), 100 µg streptomycin (Wisent, Thermo Fisher Scientific, MA,USA) and 10 mM HEPES (Wisent, Thermo Fisher Scientific, MA,USA). Jurkat cells were kept in culture at a cell density of 0.3 to $1x10^6$ cells per ml. Cells in culture were replaced by freshly thawed Jurkat cells every 45 days. Mycoplasma tests were regularly conducted on cells.

2.2. Jurkat CRISPR-cas9 induced knockout of DFF40

Gene deletions in Jurkat were performed with the CRISPR-cas9 method as previously described [17]. To confirm knockout cell lines, protein extracts were blotted for the expression of DFF40.

2.3. Cell treatments

Prior to treatments, Jurkat DFF40 WT and DFF40 KO cells were washed twice with phosphate-buffered saline solution (PBS 1X). Jurkat DFF40 WT and DFF40 KO cells were seeded in 24-well plates at a density of $2x10^5$ cells/ml in complete RPMI 1640 medium, supplemented with 10% FBS. Cells were exposed i) to increasing doses (0.01 to 10 µM) of MTX (Cayman Chemical, MI, USA, Cat# 13960), 6-MP (Abcam, ON, CA, Cat# ab142389), Ara-C (Cayman Chemical, MI, USA, Cat# 16069), ETO (Cayman Chemical, MI, USA, Cat# 12092), MIT (Cayman Chemical, MI, USA, Cat# 14842), DXR (Cayman Chemical, MI, USA, Cat# 15007) or VM-26 (Cayman Chemical, MI, USA, Cat# 14425) diluted in DMSO (Sigma-Aldrich, WI, USA) for 24 h, or ii) to a fixe dose of these drugs (1 or 10 µM) for different time points (4 to 24 h).

2.4. Cell viability assay

Cell viability assays were performed as previously described [17], without any modifications.

2.5. Apoptosis assessment

Changes in the nuclear morphological events, following exposure to conventional chemotherapy drugs, were assessed by the visualization of internucleosomal DNA fragmentation and cell cycle analysis as previously described [17,22,23]. First, evaluation of DNA fragmentation was performed by standard agarose gel electrophoresis [17]. Following treatments of 16 h and 24 h with ETO (10 µM) or Ara-C (10 µM), $1x10^6$ Jurkat cells cells were washed twice with cold PBS and suspended in the apoptotic cell lysis buffer. The subsequent steps were performed as previously described [17]. Cell cycle analyses were performed using the G1/G0 subdiploid pek quantification as previously described without any modifications [17]. Finally, phosphatidylserine (PS) translocation to the extracellular membrane was assessed with the Annexin-V FITC apoptosis detection kit (Thermo Fisher Scientific, MA, USA Cat# BMS500FI/100, RRID: AB_2575598), according to the manufacturer's instructions but with some modifications. Briefly, treated cells ($2x10^5$ cells/ml) were washed twice with PBS 1X in Eppendorf tubes and incubated in 150 µl of Annexin V-FITC solution in binding buffer 1X for 10 min at room temperature in the dark. Cells were then washed with 300 µl of binding buffer 1X and resuspended in 200 µl of a PI solution at a final concentration of 20 µg/ml. A total of 10 000 cells were analyzed with the FACSCalibur from BD (BD FACSCalibur Flow Cytometry System, RRID:SCR_000401). Compensation was done with only Annexin V positive or PI positive cells.

2.6. Mitochondrial membrane potential assessment

Mitochondrial membrane potential (MMP) was assessed with the TMRE-Mitochondrial membrane potential assay kit (Abcam, ON, CA, Cat#ab113852) according to the manufacturer's instructions with some modifications. Briefly, cells were treated with ETO (10 µM) or Ara-C (10 µM) for 8 h in complete RPMI medium and then cells were washed once with PBS 1X. Cell pellets were suspended in 200 µl of PBS 1X containing TMRE at a final concentration of 300 nM. Samples were incubated for 20 min at 37°C with 5% CO_2. Cells were then transferred on ice and analyzed by flow cytometer.

2.7. Cell proliferation assays

Prior to treatment, Jurkat cells were washed once with PBS 1X and seeded in 24-well plates at a density of $1x10^6$ cells/ml, in complete RPMI 1640 media supplemented with 10% FBS. Cells were treated with MTX (10 μM), 6-MP (10 μM), Ara-C (10 μM), ETO (10 μM), MIT (1 μM) or VM-26 (1 μM) and incubated for 16 h at 37 °C with 5% CO_2. Control cells were treated with 0.13% DMSO. Cells were then washed once with PBS 1X and suspended in complete RPMI 1640 media with 10% FBS, supplemented with Embryomax® nucleosides 100X (Millipore, MA, USA, Cat# ES-008-D) diluted at a final concentration of 10X, in 96-well plates at a density of $2x10^5$ cells/well. Plates were incubated for 5 h at 37 °C with 5% CO_2. Alamar Blue (Thermo Fisher Scientific, MA, USA, Cat# DAL1100), diluted at 10%, was then added to each well and plates were incubated for an additional 3 h. Dye fluorescence intensity (FI) was assessed with the SpectraMax® M5 Microplate Reader at an excitation wavelength of 570 nm and emission wavelength of 585 nm.

2.8. Western-Blot

Jurkat DFF40 WT and DFF40 KO cells ($1x10^6$ cells/ml) were exposed to Ara-C (10 μM) or ETO (10 μM) for 8, 16 or 24 h. Following treatments, cells were washed twice and lysed with laemmli 1X (12.5% (v/v) 0.5 M Tris-HCl pH 6.8, 10% (v/v) glycerol, 20% (v/v) SDS 10%, 5% (v/v) β-mercaptoethanol, 5% (v/v) bromophenol blue 0.5%) at 95 °C for 10 min. Protein lysates were loaded onto 12% SDS-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membranes. Transfers onto nitrocellulose membranes were made using the Trans-Blot® Turbo Transfer Pack (Bio-Rad, ON, CA, Cat# 1704159). Proteins were then blocked with TBS-0.1% Tween 20 (TBST 1X) with 5% skimmed milk for 2 h at room temperature. Membranes were then washed 3 times with TBST 1X for 10 min and then incubated with the following antibodies (ab): human anti-DFF40 C-terminal ab (Abcam, ON, CA, Cat# ab71083), anti-DFF45 ab (Millipore, MA, USA, Cat# AB16961, RRID:AB_90977), anti-phospho-H2AX ab (BioLegend, CA, USA, Cat# 613402, RRID: AB_315795) or anti-tubulin ab (BioLegend, CA, USA, Cat# 625902, RRID:AB_493416) for 2 h at room temperature, or with anti-procaspase-3 ab (Cell Signaling Technology, MA, USA, Cat# 9668, RRID: AB_2069870), anti-procaspase-6 ab (Abcam, ON, CA, Cat#ab185648), anti-XIAP ab (Cell Signaling Technology, MA, USA Cat# 2045, RRID: AB_2214866), the bcl-2 family ab kit (Cell Signaling Technology, MA, USA Cat# 17229 T), anti-PARP ab (BioLegend, CA, USA Cat# 614302, RRID:AB_2299318) or anti-HMGB1 ab (Abcam, ON, CA, Cat# ab18256, RRID:AB_444360) overnight at 4 °C. Primary ab's were diluted (1: 2 000) in TBST 1X containing 5% BSA. Following incubations, membranes were washed 3 times with TBST 1X and incubated with the secondary ab for 1 h at room temperature. Horseradish peroxidase-conjugated goat anti-mouse IgM (Santa Cruz Biotechnology, CA, USA, Cat# sc-2064) or sheep anti-rabbit IgG (New England Biolabs Ltd., ON, CA, Cat# 7074) were used at a dilution of 1: 20 000 in TBST 1X. Selectivity of each antibody was assessed before use. Bands were visualized by chemiluminescence with the Clarity Western ECL substrate (Bio-Rad, ON, CA, Cat# 170–5060). Density quantification was done using the Image Lab software (Image Lab Software, RRID:SCR_014210).

2.9. Caspase-3/7 activity detection

Caspase-3/7 activity was assessed by flow cytometer using the Caspase-3/7 Green detection reagent (Thermo Fisher Scientific, MA, USA, Cat# R37111) according to the manufacturer's instructions, but with some modifications. Briefly, following cell treatments, samples were washed twice with PBS 1X and suspended in 200 μl of Caspase-3/7 Green Reagent (prepared in PBS 1X using 3 drops in 2.5 ml). Cells were then incubated at room temperature in the dark for 30 min. Samples were transferred on ice and analyzed using the FACSCalibur from Becton Dickinson (BD FACSCalibur Flow Cytometry System, RRID: SCR_000401). A total of 10 000 events were analyzed and Caspase-3/7 activity was assessed by gating viable cells.

2.10. Statistical analysis

Data's are presented as means ± SEM of three independent experiments in triplicates. Differences between the two cell lines, treated or not, were tested by two-way ANOVA followed by Tukey's post-hoc correction using the GraphPad Prism software version 6. A value of $p < 0.05$ was considered significant.

2.11. TCGA analyses

TCGA analyses were performed using the RNA-seq data's for TCGA projects and normal expression values for the corresponding tissue from the GTEx project [24]. GTEx project was downloaded from the Recount2 project using package TCGABiolinks in R [25–27]. For the TCGA datasets, only samples who were in both the GDC and Recount2 datasets were kept. Samples with a tumour purity of over 60% were kept for analyses. Recurrent tumour samples from the TCGA datasets were excluded from analyses. Normalization of the expression data was performed using the software edgeR (version 4.0.3).

3. Results

3.1. DFFB gene expression is downregulated in breast, endocervical, ovarian, lung, pancreas and brain tumors.

Few studies have reported in the past alterations in DFF40′s expression in human cancers, mainly glioblastomas, uterine leiomyosarcomas and endometrial cancers [9,10,19]. This abnormality was linked to higher malignancy of tumors. Knowing that many other known cancers are aggressive, we first sought to determine DFF40 gene expression in common cancers, such as breast, cervical, ovarian, lung, pancreas, colorectal and brain cancers (Fig. 1). We performed an analyses of *DFFB* gene expression using the RNA-seq data's in the cancer genome atlas (TCGA projects), and normal expression values for the corresponding tissue were obtained from the genotype-tissue expression (GTEx portal). For these purpose, we compared the *DFFB* expression in normal breast tissue versus i) different subtypes of breast cancer based on hormone receptor expression or ii) different subtypes of breast cancer classifications; normal cervix tissue versus cervical squamous cell carcinoma (SCC) and endocervical adenocarcinomas combined; normal ovarian tissue versus ovarian cancer subtypes; normal lung tissue versus adenocarcinoma of the lung or versus squamous cell carcinoma (SCC) of the lung; normal pancreatic tissue versus pancreatic adenocarcinoma; normal colorectal tissue versus colon adenocarcinoma or rectum adenocarcinoma; and finally, normal brain tissue versus glioblastoma multiforme or low grade glioma. Our analysis reveals overall a decrease of *DFFB* expression in all tumor samples, with an exception for colorectal cancer.

3.2. DFF40 deficiency in cancer cells confers resistance to antimetabolite chemotherapy drugs

Conventional antimetabolite chemotherapeutic agents are the first generation of targeted drugs and are widely used in cancer treatments [28]. They are the most commonly used therapies in first lines treatments for neoplastic diseases [29]. The emergence of cancer cell resistance notion, based on the molecular features of the tumor, has put a tremendous interest in the study of the cellular mechanisms laying behind those effects [29,30]. DFF40 deficiency was shown to impair caspase-dependant cell death in human glioblastoma cells using STS [10]. We thus sought to determine if DFF40 deficiency could possibly alter the cell-death response in cancer cells following an exposure to antimetabolite drugs. We first generated DFF40 deficient Jurkat and

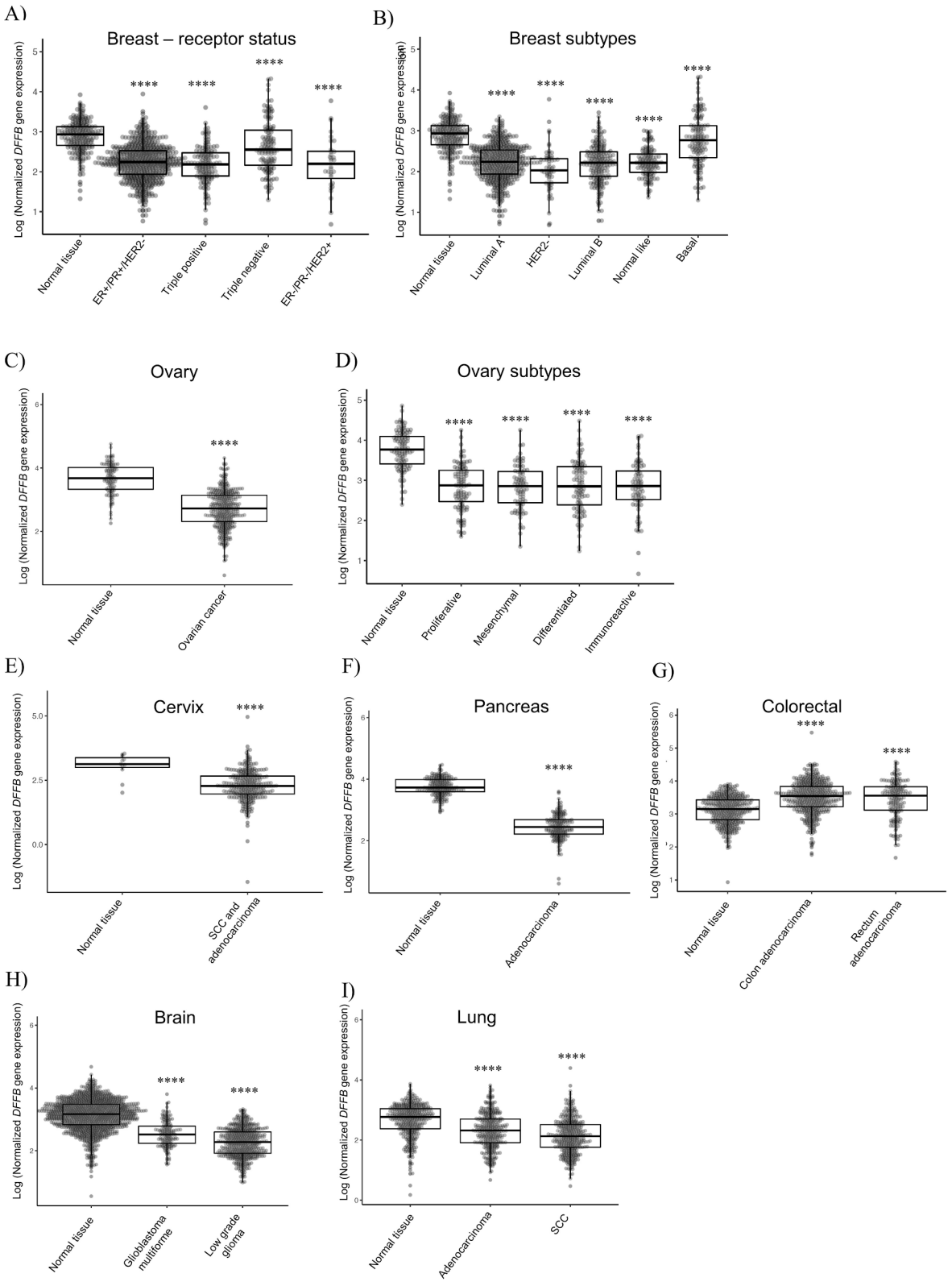

Fig. 1. *DFFB* gene expression is downregulated in breast, endocervical, ovarian, lung, pancreas and brain tumors. TCGA analyses were performed using the RNA-seq data's for TCGA projects and normal expression values for the corresponding tissue from the GTEx project. A: n = 218 for normal breast tissue, n = 601 for ER+/PR+/HER2-, n = 141 for triple positive, n = 131 for triple negative and n = 32 for ER-/PR-/HER2 + . B: n = 218 for normal breast tissue, n = 506 for luminal A, n = 59 for HER-, n = 196 for luminal B, n = 124 for normal like and n = 139 for basal. C: n = 108 for normal ovarian tissue and n = 370 for ovarian cancer. D: n-108 for normal ovarian tissue, n = 102 for proliferative subtype, n = 85 for mesenchymal subtype, n = 99 for differentiated subtype, n = 77 for immunoreactive subtype. E: n = 11 for normal cervix tissue and n = 289 for cervical squamous cell carcinoma (SCC) and endometrial adenocarcinomas. F: n = 197 for normal pancreatic tissue and n = 177 for pancreatic adenocarcinoma. G: n = 376 for normal colorectal tissue, n = 450 for colon adenocarcinoma and n = 164 for rectum adenocarcinoma. H: n = 1146 for normal brain tissue, n = 147 for glioblastoma multiforme and n = 506 for low grade glioma. I: : n = 374 for normal lung tissue, n = 322 for adenocarcinoma of the lung and n = 367 for squamous cell carcinoma (SCC) of the lung. ****$p < 0.0001$.

HeLa cells by CRISPR-cas9. The knockout cell lines do not express any DFF40 protein expression and exhibit a total absence of DNA fragmentation following apoptosis induction with STS (data not shown and [17]). To determine the cellular response of malignant cancerous cells to antimetabolites, DFF40 WT and DFF40 KO Jurkat cells were exposed to MTX, 6-MP and Ara-C, and dose–response and time-dependent kinetic curves were generated (Fig. 2). Cell death was assessed with PI incorporation. DFF40 KO Jurkat cells are significantly resistant to a 24 h exposure of MTX (10 μM), 6-MP (10 μM, and Ara-C (1 and 10 μM). MTX induces in average 21.80 ± 2.35% cell death in DFF40 WT cells vs 13.08 ± 1.50% cell death in DFF40 KO cells. As for Ara-C (10 μM), we obtained 45.36 ± 1.06% cell death in DFF40 WT cells vs 35.51 ± 3.23 % in DFF40 KO cells. Finally, 6-MP induced 30.27 ± 0.75% cell death in Jurkat DFF40 WT cells in comparison to 16.27 ± 1.98% cell death in DFF40 KO cells. HeLa cells exposed to the same drugs showed a significantly higher resistance phenotype (data not shown). In fact, it required a 48 h exposure time to MTX and Ara-C to induce approximately 25% of cell death in DFF40 WT HeLa cells, whereas only about 10% of cell death was quantified in DFF40 KO HeLa cells for both drugs. Exposure of DFF40 WT and DFF40 KO HeLa cells to 6-MP (up to 10 μM) did not induce cell death in both cell lines (data not shown).

3.3. Sensibility to inhibitors of topoisomerase II is increased in DFF40 deficient cancer cells

Another widely used category of chemotherapeutic agents in the management of hematological malignancies and solid tumors are the TOP2 inhibitors [31,32]. Depending on the drug class, these components have diverse indications in the treatment of various cancers and may be used as first-line treatments as well, in combination with other components [33]. The resistance to these compounds has been documented in the literature [31,34]. We thus sought to determine if the observed cell-death resistance of DFF40 deficient cancer cells to antimetabolite drugs was also present with TOP2 inhibitors. First, Jurkat DFF40 WT and DFF40 KO cells were treated with 4 distinct TOP2 inhibitors (ETO, VM-26, DXR, MIT) and cell death was assessed with PI incorporation (Fig. 3). Interestingly, DFF40 KO Jurkat cells are more sensitive to ETO (10 μM), VM-26 (1 and 10 μM) and MIT (0.1 and 1 μM) following a 24 h exposure. Expression of DFF40 in Jurkat cells does not have an impact on DXR-induced cell death. In order to perform time-dependent kinetics, the highest significant dose from dose-dependent curves was used. Significant differences regarding cell death in DFF40 WT and DFF40 KO Jurkat cells is observed as early as a 16 h exposure time to ETO (10 μM), VM-26 (1 μM) and MIT (1 μM), where DFF40 deficient cells have a higher proportion of dead cells. Cell death values are as follow (DFF40 WT values : DFF40 KO values): ETO (10 μM) induces a (26.67 ± 2.80)% : (37.73 ± 3.70)% cell death ratio, VM-26 (1 μM) induces a (26.81 ± 3.63)% : (43.68 ± 4.41)% cell death ratio and MIT (1 μM) induces a (20.12 ± 2.03)% : (39.38 ± 4.49)% cell death ratio, following 16 h treatments. The gap between the cell death values of both cell lines diminishes when the exposure time is prolonged up to 24 h for MIT and VM-26. Higher cell death levels are obtained in DFF40 KO Jurkat cells (67.36 ± 5.43%) when treated with ETO (10 μM) for 24 h, in comparison to DFF40 WT Jurkat cells (46.40 ± 4.64%). As for HeLa DFF40 WT and DFFKO cells, no significant differences in cell death is observed with VM-26 and MIT according to DFF40 expression (data not shown). ETO induces greater cell death in DFF40 KO HeLa cells in comparison to DFF40 WT HeLa cells, but at a lower scale compared to Jurkat cells (data not shown).

Following the dose–response and kinetics curves, we could see that HeLa cells were overall much more resistant to the employed drugs, and cell death values of at least 30% were difficult to obtain with further exposure times (data not shown). Thus, the following experiments were conducted with only Jurkat DFF40 WT and DFF40 KO cells. In order to establish the lethal concentrations where 50% of the cell population is dead (LC_{50}), we performed multiple dose–response curves with both Jurkat cell lines with a 24 h exposure time. It was difficult to obtain cell death values greater than 60% in some conditions in order to perform sigmoidal curves (data not shown) and obtain LC_{50} values. Thus, we represent in Table 1 the delta value (mean differences) for cell death values in both Jurkat cell lines. The largest mean difference in cell death values for antimetabolites is observed with cells exposed to 6-MP,

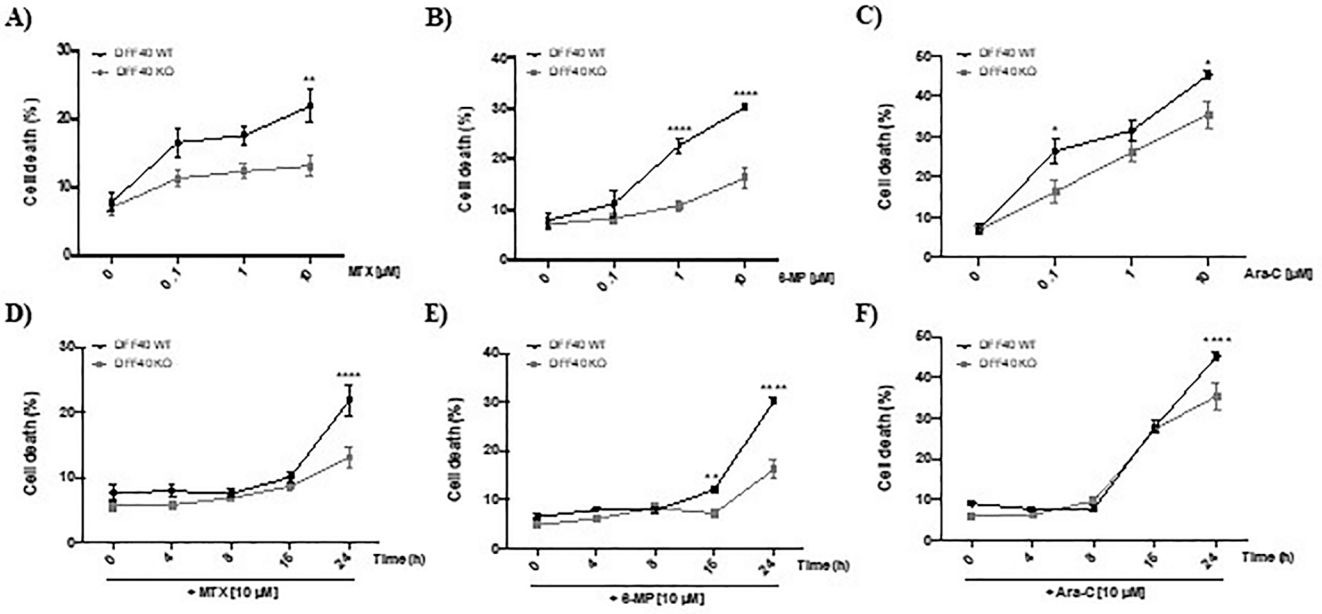

Fig. 2. DFF40 deficient Jurkat T cells are less sensitive to antimetabolite chemotherapeutic drugs. A to F: DFF40 WT and DFF40 KO Jurkat cells ($2x10^5$ cells/ml) were treated or not with MTX (0.1 to 10 μM), 6-MP (0.1 to 10 μM) or Ara-C (0.1 to 10 μM) for 24 h, or with a fixe dose [10 μM] of the drug for up to 24 h. Control cells were incubated with 0.1% (v/v) DMSO. Cells were analyzed by flow cytometer with propidium iodide (1 μg/ml). All data are representative of at least 3 independent experiments. Two-way Anova with Tukey's post-hoc test was performed (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$). Abbreviations: methotrexate (MTX), 6-mercaptopurine (6-MP), cytarabine (Ara-C).

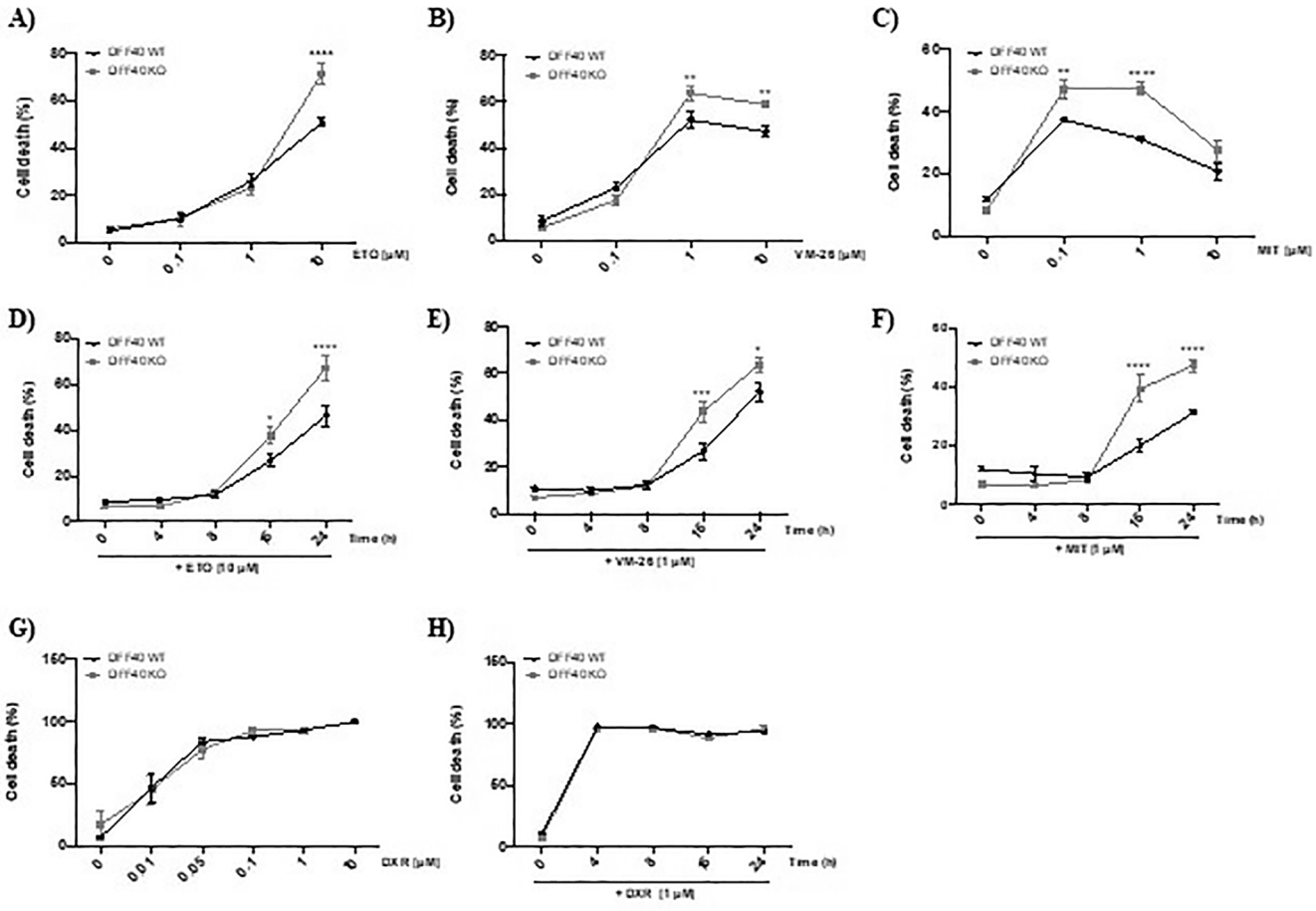

Fig. 3. DFF40 deficient Jurkat cells are more sensitive to topoisomerase II inhibitors. A to C, G: DFF40 WT and DFF40 KO Jurkat cells ($2x10^5$ cells/ml) were treated or not with ETO (0.1 to 10 µM), VM-26 (0.1 to 10 µM), MIT (0.1 to 10 µM) or DXR (0.01 to 10 µM) for 24 h. D to F, H: Cells in A were treated or not with ETO (10 µM), VM-26 (1 µM), MIT (1 µM), DXR (1 µM) for 4, 8, 16 and 24 h. Control cells were incubated with 0.1% (v/v) DMSO. All cells were analyzed by flow cytometer with propidium iodide (1 µg/ml). Data's are representative of at least 3 independent experiments. Two-way Anova with Tukey's post-hoc test was performed (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Abbreviations: etoposide (ETO), teniposide (VM-26), mitoxantrone (MIT), doxorubicin (DXR).

followed by Ara-C and then MTX. Although, higher values are obtained with Ara-C, whereas 6-MP induces close to little effect on DFF40 deficient cells. As for TOP2 inhibitors, the largest mean difference in cell death values and the higher mortality values are obtained following an exposure to ETO. According to this results, we thus decided to pursue the remaining experiments with Ara-C and ETO.

3.4. Cell cycle phase arrests are related to DFF40 expression in Jurkat cells

To understand how DFF40 KO Jurkat cells are protected from antimetabolite-induced cell death, their cell cycle was first analyzed (Fig. 4). Jurkat DFF40 WT and DFF40 KO cells were treated to 10 µM of 6-MP, MTX or Ara-C for 24 h and then every cell cycle phase proportion were analyzed by flow cytometer. Every drug induces an increase of the SubG0/G1 phase, synonymous with DNA fragmentation. As expected, DFF40 KO cells showed no DNA fragmentation in the absence of DFF40 for all drugs. 6-MP treatment induces a shift of the cell population from the G0/G1 phase to the S phase, independently of DFF40 expression (Fig. 4A). A similar shift is observed in cells treated with MTX and Ara-C, but the percentage of cells in the S phase is significantly higher ($p < 0.0001$) in DFF40 KO cells (Fig. 4B and 4C). Next, we investigated the impact of TOP2 inhibitors on cell cycle analysis in DFF40 deficient cells. TOP2 inhibitors induces a decrease in every cell cycle phase for DFF40 WT cells. ETO didn't induce any changes in the cell cycle phases in DFF40 deficient cells (Fig. 4D). MIT induces a higher accumulation of cells in the G2/M phase, whereas VM-26 induces a higher accumulation in the S phase in DFF40 deficient cells. Finally, the importance of nucleotides in cell proliferation is widely known, due to their utility in DNA synthesis. Intracellular nucleotide depletion can be a factor for altered proliferative states. We thus sought to determine if the addition of extracellular nucleosides could facilitate cell cycle transition and ultimately increase cell proliferation rates following chemotherapeutic drug exposure. DFF40 WT and DFF40 KO Jurkat cells were exposed to the respective drugs and Embryomax® nucleosides were added in the media for 8 h before cell proliferation measurement by Alamar Blue (Fig. 5). The addition of extracellular nucleosides on Jurkat cells exposed to TOP2 inhibitors doesn't impact cell proliferation rates in both DFF40 WT and DFF40 KO Jurkat cell lines (Fig. 5B). Extracellular nucleosides increase's cell proliferation rates of DFF40 deficient Jurkat cells exposed to Ara-C (10 µM) or 6-MP (10 µM).

3.5. DFF40 deficient Jurkat cells do not exhibit DNA fragmentation following etoposide and cytarabine exposure but show altered phosphatidylserine translocation dynamics and mitochondrial membrane potential regulation

Before diving into the investigation of the apoptotic pathway, we wanted to confirm the presence of apoptosis or necrosis in both cell lines, as well as the activation of early apoptotic markers such as PS translocation to the outer cell membrane and MMP alteration. It is to note that further experiments are all conducted with either ETO or Ara-

Table 1
Cell death values in Jurkat cells according to DFF40 expression following chemotherapeutic drug exposure. Jurkat DFF40 WT and DFF40 KO cells were treated or not with the methotrexate (MTX; 10 μM), 6-mercaptopurine (6-MP; 1 or 10 μM), cytarabine (Ara-C; 0.1 or 10 μM), etoposide (ETO; 10 μM), teniposide (VM-26; 1 or 10 μM) or mitoxantrone (MIT; 0.1 or 1 μM) for 24 h in complete RPMI media. Cell viability was assessed by propidium iodide by flow cytometer. Mean cell death differences between DFF40 WT and DFF40 KO were calculated by subtracting corresponding values and represented as the delta value and respective p-values were calculated.

	Mortality values (%)[a]			
Drug [μM]	Jurkat DFF40 WT	Jurkat DFF40 KO	Δ[b]	P value[c]
Antimetabolites				
MTX				
10	21.80	13.08	8.72	0.0201
6-MP				
1	22.66	10.72	11.92	0.0006
10	30.27	16.27	14.00	0.0006
Ara-C				
0.1	26.37	16.36	10.01	0.0413
10	45.36	35.51	9.85	0.0273
Topoisomerase II inhibitors				
ETO				
10	46.40	67.36	−20.96	0.0149
VM-26				
1	52.16	63.84	−11.68	0.0446
10	47.15	58.15	−11	0.0036
MIT				
0.1	37.44	47.18	−9.74	0.0134
1	31.23	47.27	−16.04	<0.0001

[a] Cell death values are represented as the mean value in percentage (%) of at least three independent experiments for a 24 h exposure time.
[b] Delta value (Δ) was calculated by subtracting the cell death mean values obtained in DFF40 WT Jurkat cells by the cell death values obtained in DFF40 KO Jurkat cells.
[c] t-test's were performed between both cell lines exposed to the same drug to obtain the respective p-values.

C, the representative chemotherapeutic drugs for each category (see Table 1 for details). First, exposure to ETO (10 μM) for 24 h induces 58.47 ± 7.21% DNA fragmentation, whereas fragmentation levels are way lower in DFF40 WT Jurkat cells exposed to Ara-C (10 μM) for 24 h (29.96 ± 5.61%) (Fig. 6A). As expected, no DNA fragments were quantified by flow cytometer in DFF40 deficient Jurkat cells nor observed by agarose gel (Fig. 6B). MMP is a key phenomenon in apoptosis that further leads to the activation of caspases [35]. Permeabilization of the mitochondrial outer membrane leads to the disruption of the electron chain transport and further loss of mitochondrial transmembrane potential [35]. We thus sought to determine if ETO or Ara-C disrupts MMP in Jurkat DFF40 KO cells (Fig. 6C). Exposure to ETO doesn't alter MMP in Jurkat DFF40 WT cells, whereas MMP in DFF40 KO Jurkat cells is significantly diminished by 0.60-fold. Exposure to Ara-C enhanced MMP in DFF40 WT Jurkat cells by approximately 1.32 fold, but did not affect DFF40 KO Jurkat cell's MMP. Sensibility to ETO in DFF40 KO cells could be explained by MMP alteration. Next, we wanted to quantify caspase 3/7 activity in both cell lines (Fig. 6D), knowing that MMP loss is linked to caspase activation. Treatment with Ara-C enhances caspase 3/7 activity in both DFF40 WT and DFF40 KO Jurkat cells with no significant differences. As for ETO, DFF40 KO Jurkat cells exhibit significantly lower caspase 3/7 activity following apoptosis induction. PS translocation to the outer cell membrane layer is a key step in apoptosis and underlying cell pathway activation [36]. We thus sought to determine if this early step could be significantly altered in chemo -sensitive or -resistant Jurkat DFF40 KO cells (Fig. 6E to 6G). DFF40 KO Jurkat cells have a lower proportion of cells who exhibit PS translocation following a 24 h Ara-C (10 μM) treatment (21.36 ± 3.42%) in comparison to DFF40 WT Jurkat cells (33.16 ± 3.81%). In concordance with our viability results, DFF40 deficient Jurkat cells have a higher proportion of Annexin V and PI positive cells (25.88 ± 3.30%) vs DFF40 WT Jurkat cells (16.98 ± 2.79%) when treated with ETO. These results suggest that chemosensitivity to ETO or chemoresistance to Ara-C in DFF40 KO cells could be mediated by apoptosis.

3.6. Chemoresistance to cytarabine and chemosensitivity to etoposide in DFF40 deficient Jurkat cells is not mediated through the regulation of caspases, XIAP and anti-apoptotic protein Mcl-1

To better understand the sensitivity or resistance of DFF40 deficient cells to chemotherapeutic drugs, we investigated the regulation of the apoptosis pathway by gel electrophoresis. Briefly, DFF40 WT and DFF40 KO Jurkat cells were exposed to ETO (10 μM) or Ara-C (10 μM) for 8, 16, and 24 h and protein expression was assessed by western blotting. (Fig. 7A and 7B). One of the major regulators of caspase activation in the apoptotic pathway are inhibitor of apoptosis proteins (IAPs). The most well defined and potent IAP protein is the X-linked IAP (XIAP), which mediates its action by inhibiting and neutralizing caspases −9, −3 and −7 [37]. XIAP expression regulation can thus ben implicated in cancer cell chemoresistance. Exposure to ETO significantly diminishes XIAP expression in both Jurkat DFF40 WT and DFF40 KO cells. No significant differences in the expression of XIAP in each time point between both cell lines is observed, following ETO exposure. Surprisingly, XIAP is significantly downregulated at a later exposure time in DFF40 deficient cells (16 h) in comparison to DFF40 WT cells, who exhibit a down-regulation as early as 8 h. Ara-C doesn't have any impact on XIAP's protein expression in both Jurkat cell lines. Having no differences in XIAP's expression regulation, we next sought to determine if caspase expression was regulated according to DFF40 expression following ETO and Ara-C exposure. Procaspases −3 (data not shown) and −6 are cleaved in DFF40 WT and DFF40 KO Jurkat cells as early as an 8 h exposure to the respective chemotherapeutic drugs. No differences in caspase expression is observed for both cell lines following ETO and Ara-C treatments. Furthermore, there isn't any significative difference in PARP1 cleavage between DFF40 WT and DFF40 KO Jurkat cells treated or not with ETO (Fig. 7B) or Ara-C (data not shown). Although, DFF40 deficient Jurkat cells are less sensitive to DNA damages. Jurkat DFF40 KO cells have significantly less histone H2AX phosphorylation following ETO and Ara-C exposure, in comparison to DFF40 WT cells. We next sought to determine if anti-apoptotic proteins could be regulated and thus explain the chemoresistance or chemosensitivity observed in Figs. 2 and 3. First, Mcl-1 expression is significantly downregulated following ETO and Ara-C treatment in both DFF40 WT and DFF40 KO Jurkat cells, without any statistical differences between both cells lines (data not shown). As for Bcl-xL, its expression is not altered in Ara-C-treated Jurkat DFF40 WT and DFF40 KO cells. In ETO-treated DFF40 WT cells, Bcl-xL's expression is significantly up regulated at 16 h, before its level rises back down, in comparison to DFF40 KO cells. In fact, DFF40 KO Jurkat cells express significantly lower levels of Bcl-xL levels following a 16 h exposure to ETO in comparison to DFF40 WT cells. Finally, we assessed the expression of HMGB1 in our cells exposed to ETO or Ara-C in order to assess necrosis levels. HMGB1 expression is significantly enhanced in DFF40 KO Jurkat cells exposed to ETO in comparison to DFF40 WT cells (Fig. 7). No differences in HMGB1 expression between DFF40 WT and DFF40 KO Jurkat cells exposed to Ara-C was noted (data not shown).

4. Discussion

Cancer cell resistance to chemotherapeutic treatments is a multifactorial phenomenon, that involves the mutation of multiple genes or proteins mainly implicated in cell growth, proliferation and survival. Apoptosis pathway mutations involved in chemoresistance are the most characterized. Multiples studies have reported mutations in death receptors [38], caspases [7], proapoptotic proteins [39–41] and anti-apoptotic proteins [42,43] to be involved in cell resistance. In the last

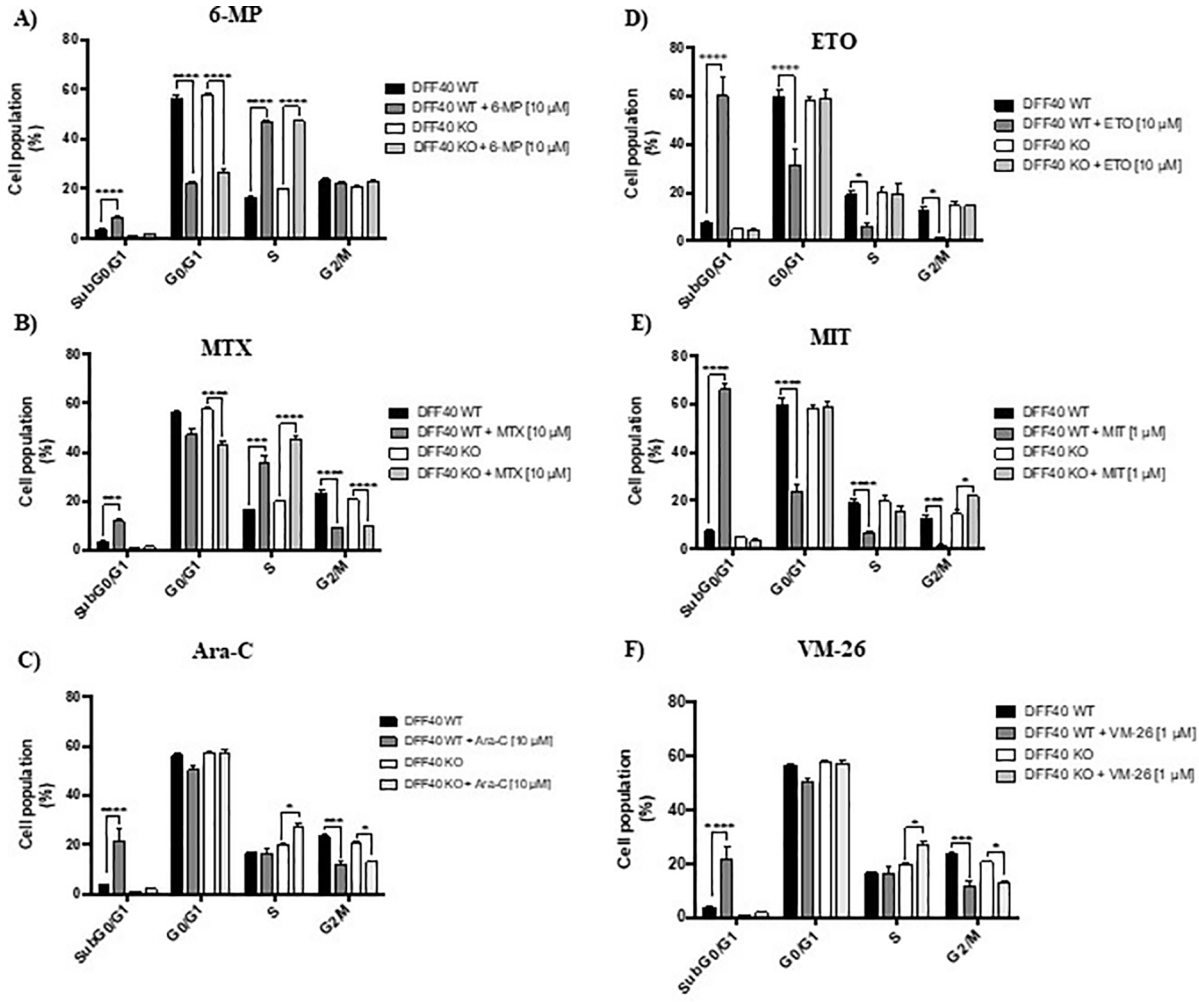

Fig. 4. DFF40 deficiency in Jurkat cells induces a larger cell cycle arrest in S-phase following exposure to cytarabine and methotrexate. Jurkat DFF40 WT and DFF40 KO cells ($2x10^5$ cells/ml) were treated for 24 h with (A) 6-MP (10 µM), (B) MTX (10 µM), (C) Ara-C (10 µM), (D) ETO (10 µM), (E) MIT (1 µM) or (F) VM-26 (1 µM). Control cells were incubated with 0.1% (v/v) DMSO. Cells were incubated for 1 h at 37 °C with RNAse/PI and then analyzed by flow cytometer. Data's are representative of at least 3 independent experiments. Two-way Anova with Tukey's post-hoc test was performed (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Abbreviations: methotrexate (MTX), 6-mercaptopurine (6-MP), cytarabine (Ara-C), etoposide (ETO), teniposide (VM-26), mitoxantrone (MIT).

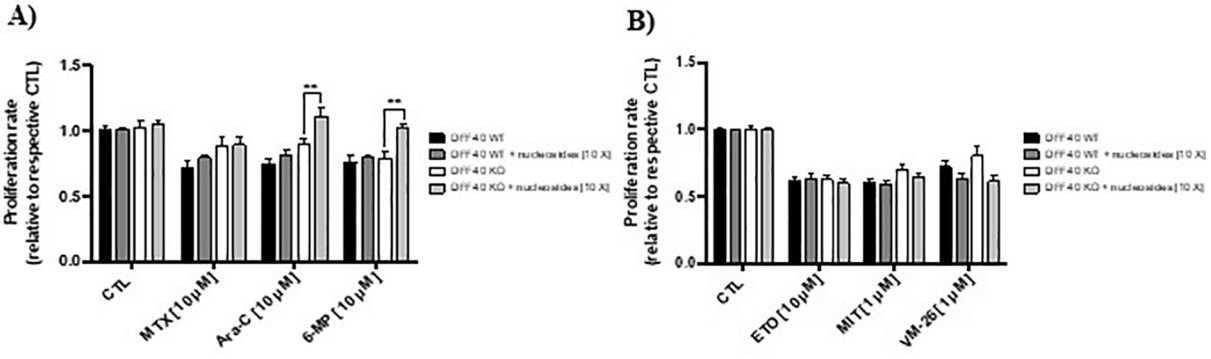

Fig. 5. The addition of extracellular nucleosides enhances cell proliferation of DFF40 deficient cells treated to cytarabine and 6-mercaptopurine. Jurkat DFF40 WT and DFF40 KO cells ($1x10^6$ cells/ml) were treated for 16 h with (A) antimetabolites (MTX, Ara-C, 6-MP) or (B) TOP2 inhibitors. Embryomax® nucleosides were then added at a final concentration of 10 X and incubated for 8 h. Cell fluorescence was assessed with alamar blue with the SpectraMax® M5 Microplate Reader at an excitation wavelength of 570 nm and emission wavelength of 585 nm. Data's are representative of at least 3 independent experiments. Two-way Anova with Tukey's post-hoc test was performed (**$p < 0.01$). Abbreviations: methotrexate (MTX), 6-mercaptopurine (6-MP), cytarabine (Ara-C), etoposide (ETO), teniposide (VM-26), mitoxantrone (MIT).

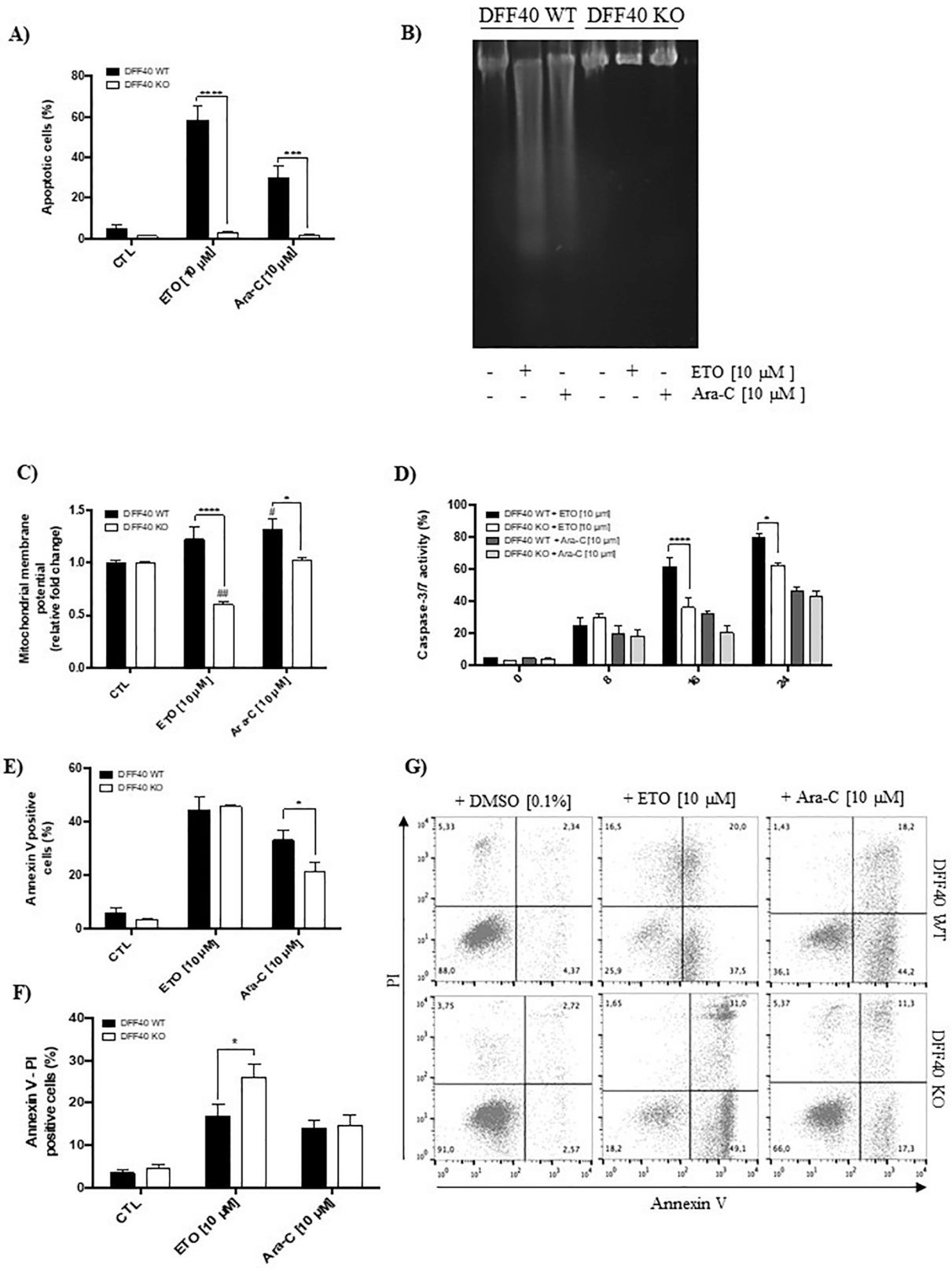

Fig. 6. DFF40 deficiency impacts phosphatidylserine translocation following cytarabine and etoposide exposure. A and B: Jurkat DFF40 WT and DFF40 KO were treated for 24 h with ETO or Ara-C and apoptosis was assessed by cell cycle analysis or by agarose gel. C: Same as in A and B, but treatment time was of 8 h. MMP was quantified by flow cytometer with the TMRE-Mitochondrial Membrane potential assay kit, according to the manufacturer's instructions. D: Same as in A, but treatments were performed over a 24 h time-course. Caspase 3/7 activity was assessed by flow cytometer. E to G: Same as in A, but Annexin V and PI staining was performed following treatments. Data's are representative of at least 3 independent experiments. Two-way Anova with Tukey's post-hoc test was (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$). Abbreviations: cytarabine (Ara-C), etoposide (ETO).

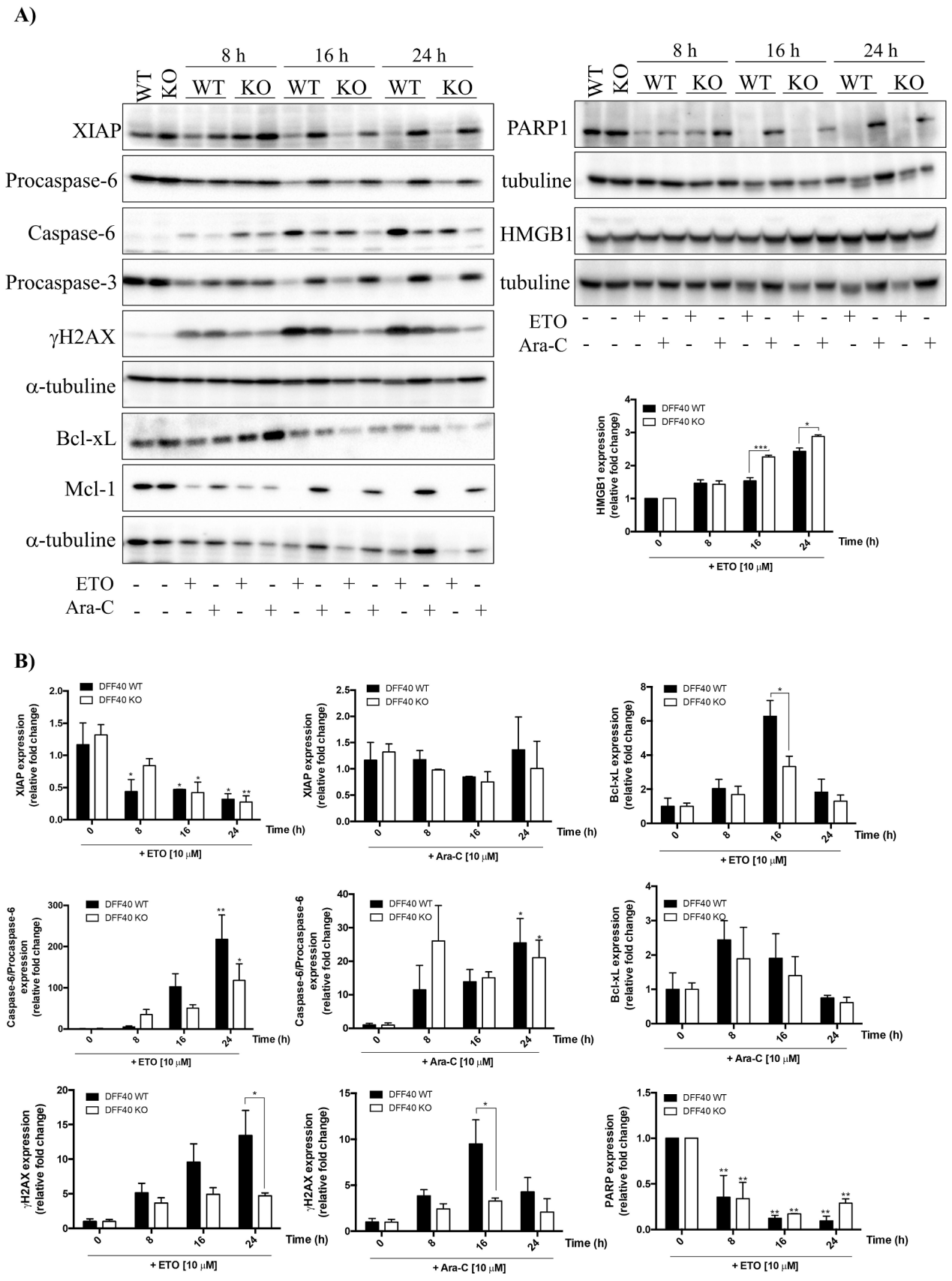

Fig. 7. DFF40 deficient cells have lower Bcl-xL levels following etoposide treatment. A: Jurkat DFF40 WT and DFF40 KO cells ($1x10^6$ cells/ml) were treated for 8 h, 16 h or 24 h with ETO (10 μM) or Ara-C (10 μM) and apoptosis pathway protein expression was assessed by gel electrophoresis. B: Represents densitometric analysis of protein expression following gel electrophoresis in A. Data's are representative of at least 3 independent experiments. Two-way Anova with Tukey's post-hoc test was (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Abbreviations: cytarabine (Ara-C), etoposide (ETO).

years, there has been an increasing interest towards the DFF40 protein and its role in cancer cell resistance to apoptosis inducing agents. The DFF40 protein is an endonuclease responsible of the fragmentation of DNA during apoptosis. Following caspase-3 activation, the inhibitory subunit (DFF45) of DFF40 is cleaved which allows its release, oligomerization and translocation to the nucleus, where it cleaves DNA. For many years, the role of DFF40 was thought to be limited to DNA fragmentation, but new studies are proposing novel roles, mainly in genomic stability [3,44]. Our laboratory has previously shown that DFF40 deficient cells lack histone H2AX phosphorylation following TBT-induced apoptosis [17], a crucial step in DNA damage pathway signalization.

Knowing that cancer cells present a large amount of genomic instabilities, multiple studies have wondered the expression of DFF40 protein in primary tumors. Downregulation of DFF40 expression was noted in uterine leiomyosarcomas [9], in non-endometrioid and high-grade endometrioid endometrial cancers [19], in glioblastomas [10] and in astrocytoma's [10]. In order to complement the literature on DFF40′s expression in tumor samples, we performed a biostatistical analysis with the RNA-seq data's available through the TCGA database. Our results revealed a significant downregulation of *DFF40* gene expression in many primary tumors in comparison to normal tissue (Fig. 1). *DFF40* gene is downregulated in breast (all subtypes), endocervical, ovarian (all subtypes), lung, pancreas and brain tumors. The importance of DFF40 expression regulation has been reported in the past. DFF40 under expression in uterine leiomyosarcoma's was shown to be associated with a poorer disease-free survival (DFS) and overall survival (OS) rates [9], due to inefficacy of treatment. The same trend was observed in glioblastoma's, where DFF40 mRNA expression was shown to negatively correlate with OS rate [10], and in non-endometrioid and high grade endometrioid endometrial cancers, where negative DFF40 expression was associated with lower OS and DFS rates [19]. On the other hand, DFF40 was shown to be over expressed in uterine leiomyoma's [9], suggesting that malignancy may be acquired through a loss of DFF40 expression. In fact, over expression of DFF40 in breast cancer cells [18] and in glioblastoma cells [10] increased the sensitivity of cells to DXR, a TOP2 inhibitor, and to staurosporine (STS) respectively, enhancing cell death by apoptosis. In concordance with these results, our laboratory has previously shown that DFF40 deficient T cells were resistant to apoptosis, but we could restore their sensitivity to TBT-induced apoptosis by DFF40 gene transfection [17]. Furthermore, it was shown that overexpression of DFF40 gene with the surviving promotor, a tumor specific promotor, sensitizes melanoma cancer cells to drug-induced apoptosis [45]. Overall, these results are strong evidences of the importance of DFF40 in cancer cell resistance, mainly in its therapeutic potential in malignant tumors.

Studies surrounding the role of DFF40 in chemoresistance are thus becoming of crucial interest. A better understanding of DFF40 deletion on chemosensitivity would help adapt and personalize current treatments. Antimetabolites, developed more than 50 years ago, are amongst the most common anticancer drugs used in clinical oncology [46]. They interfere with DNA synthesis by competing with natural intracellular nucleotides, therefore leading to cell suicide [46]. Antimetabolite drugs are classified according to their structural analogues [46]. The main drugs are either folate antagonists (*e.g.* MTX, Ralitrexed), purine antogonists (*e.g.* 6-MP, thioguanine (6-TG)) or pyrimidine antagonists (*e.g.* Ara-C, 5-Fluorouracil (5-FU)) [47]. Due to their unselective activity, antimetabolites induce cell death in all proliferative cells [47]. We thus wondered if DFF40 deficient cells would be equally affected by antimetabolites (Fig. 2). DFF40 deficient cells were much more resistant to MTX, Ara-c and 6-MP, in comparison to DFF40 expressing Jurkat cells. Greater differences were observed with 6-MP, followed by Ara-C and then MTX (Table 1). It is important to mention that although differences in cell death are slight, the clinical significance is important. Malignant tumors are known for their high proliferative rate, which renders them genetically unstable [48]. The survival of one unstable cell is sufficient to produce a malignant tumor and subsequent metastasis. Thus, it is important to develop new therapies that enhance sensitization of cancer cells to chemotherapy. Resistance to antimetabolites have been thoroughly discussed in the literature, where underlying mechanisms involve gene mutations, modifications in drug transport and efflux, alterations in cell cycle pathways and DNA damage pathways [49]. MTX-linked resistance was shown in breast cancer cells via diminished formation of MTX polyglutamates [50] and in osteosarcoma cell lines through a much more complex pathway [51]. Deletions in DNA mismatch repair (MMR) pathways (*e.g.* deletion in *MSH6* gene) have also been linked to thiopurine resistance [52]. MSH6 deletion represses the activation of checkpoint regulators (CHK1, γH2AX and p53) following 6-MP treatments [52]. Nuclear factor erythroid 2-related factor (Nrf2) deficiency has also been linked to altered MMR pathways through MutS Homolog 2 (MSH2) protein inhibition, following Ara-C-induced apoptosis [53]. Studies have shown a role for DFF40 in DNA repair pathways though the regulation of γH2AX formation [17,54]. It is hypothesized that DFF40 deficiency could impair DNA damage responses (DDR) via DNA-PK and ATM activity inhibition [3]. The impact of DFF40 deficiency on DNA repair pathways remains to be elicited in order to better understand chemoresistance.

TOP2 inhibitors are also important in the therapy of many neoplasms such as breast cancer, lung cancer and lymphomas [55]. In normal cells, TOP2 forms double strand breaks (DSB) and allows the relaxation of coiled DNA [55]. They are essential for DNA transcription and replication [56]. The main TOP2 inhibitors approved by the FDA are ETO, VM-26, DXR, idarubicin, epirucibin and MIT [55]. They have been widely used in the treatment of many cancers through their ability to induce DNA damage. Many *in vitro* studies have reported a mechanism of resistance to TOP2 inhibitors [34]. In 2 Epstein-Barr virus (EBV)-associated Burkitt cell lines, ETO-induced apoptosis resistance was shown to be linked to an impairment in the activation of pathways upstream caspase activation [57]. A laboratory generated neuroblastoma etoposide-resistant cell line has shown to evade apoptosis by altering the Bax/Bcl2 ratio, upregulate BMI-1 oncoprotein and decrease p16 tumor suppressor expression [58]. In breast cancer cell lines, resistance to DXR was in part due to extracellular matrix proteins that leaded to an upregulation of pro-survival proteins [59]. Having shown that DFF40 deficient cells were resistant to antimetabolites, the DFF40 deficient cells are more sensitive to ETO, VM-26 and MIT (Fig. 3). The sensitivity is higher for ETO, followed by MIT and then VM-26 (Table 1). A recent study from Hernandez and his colleagues has demonstrated that genome editing by CRISPR-cas9 of TOP2α, in order to enhance intron 19 removal, overcomes etoposide resistance in leukemia cell [60]. Human TOP2α introduces temporary DSB during mitosis for chromosomal segregation [32,61], making it a crucial factor for cell proliferation and survival. TOP2 inhibitors are known to stabilize those DNA-enzyme complexes by occupying the break sites and therefore inducing permanent DSBs which lead to cell death due to impaired DNA repair [62]. Hypothesis regarding DFF40′s interaction with TOP2α were recently presented from our laboratory [3]. DFF40 is known to bind to TOP2α and enhance its activity [63]. It was shown that chromatin condensation is not specific to DFF40 expression cells. In fact, cells where DFF40′s activity is inhibited also present chromatin condensation mediated by TOP2α [63]. TOP2 inhibitor sensitivity in DFF40 deficient cells could thus be linked to enhanced TOP2α activity during apoptotic execution, knowing that decreased TOP2α is clinically associated with chemoresistance [60]. Overall, our cell viability assays suggest that DFF40 could play a role in response to DNA damage induced by antimetabolites and TOP2 inhibitors. DFF40 has been linked to DNA damage responses in the past [64–66]. Antimetabolites leads to an increasing number of DNA errors, which will generate single and double strand DNA breaks once the capacity of the DNA repair pathways are overwhelmed, leading to cell death [67]. We also suggest a potential role for DFF40 in the repair of DSBs induced by the DNA-TOP2 complex, which could explain the reason behind the chemosensitivity of DFF40 KO cells to TOP2 inhibitors.

Having characterized the cell response of DFF40 deficient Jurkat cells to antimetabolites and TOP2 inhibitors, we next sought to determine the impact of these drugs on cell cycle analyses (Fig. 4). DNA damage induced by chemotherapy agents is known to lead to cell cycle arrest, when the damage exceeds the capacity of the cell to repair strand breaks. A delayed progression of cells in the S phase was observed (Fig. 4B and C), following treatment with antimetabolites due to the inhibition of DNA replication. Similar results to ours was observed by other groups, following treatment with MTX, 6-MP and Ara-C [68–70]. Antimetabolite's toxicity is known to be time dependent and their cytotoxic mainly takes part in the S-phase of cell cycle [47]. Interestingly, the absence of DFF40 induced a greater number of cells to arrest in the S-phase, following treatment with antimetabolites. It is possible that DFF40 is implicated in the DNA damage response to antimetabolites and that its absence leads to a slower resolution of the cell toward repair or apoptosis [71]. PARP1, one of the key protein in the DNA damage response, is a known regulator of DFF40 [72,73]. Our laboratory has previously shown that a deficiency in DFF40 expression in Jurkat cells leads to a delayed activation of PARP1 following TBT exposure [17]. On the other hand, exposure to TOP2 inhibitors reduced the proportion of cells in each phase of the cell cycle in DFF40 WT cells (Fig. 4D, E and F). MIT and VM-26 induced a largest cell cycle arrest in the G2/M phase and S-phase respectively (Fig. 4E and F).

Progression through the cell cycle is highly dependent on the availability of intracellular nucleotides, where their *de novo* synthesis is regulated according to the proliferative state [74]. Studies investigating the impact of extracellular nucleotides on cell proliferation have shown to have opposite effects depending on cell type. The addition of extracellular nucleotides in human progenitor endothelial cells [75] and human esophageal cancer cells [76] have shown to inhibit cell proliferation. On the other hand, their addition in MCF-7 breast cancer cells [77] and human glioma cell lines [78] have shown to significantly enhance cell proliferation. Agonists of P2Y nucleotide receptors, as well as low concentrations of adenosine, enhance cell proliferation in combination with growth factors [79]. The regulation of purinoceptors is also involved in the promotion of the tumor's proliferation and progression [77,78]. Based on these studies, we thus wondered if extracellular nucleotides could have an impact on cell cycle phase transition and consequently on cell proliferation following cell cycle arrest induced by the chemotherapeutic drugs (Fig. 5). In concordance with the literature, an excess of extracellular nucleosides enhances cell proliferation in DFF40 deficient Jurkat cells exposed to 6-MP and Ara-C, but not to TOP2 inhibitors or in DFF40 WT Jurkat cells. TOP2 inhibitors do not directly impact DNA synthesis steps (*e.g.* incorporation of purines and pyrimidines), but mainly act upstream by inducing nonreversible DSBs [80]. Extracellular nucleosides thus have no effect on TOP2 inhibitors. 6-MP and Ara-C are purine and pyrimidine antagonists respectively, whereas MTX is a folate antagonist [47]. 6-MP and Ara-C directly block purine (*e.g.* adenine, guanine) and pyrimidine (*e.g.* cytosine, thymine) uptake for DNA synthesis, whereas MTX inhibits the dihydrofolate reductase enzyme involved in pyrimidine synthesis [47]. The dihydrofolate reductase increases reduced folate levels that are further used by the cell to synthesize thymine [47]. The mechanism of action of MTX is indirect, whereas 6-MP and Ara-C directly act on DNA synthesis. This pathway could explain the positive impact of extracellular nucleosides on cell proliferation in DFF40 deficient Jurkat cells exposed to 6-MP and Ara-C. Extracellular nucleosides could replace antimetabolites from the DNA binding site and allow proper DNA synthesis and cell proliferation. But why do DFF40 WT cells do not exhibit an augmented cell proliferation following nucleoside addition? As mentioned above, we suspect a role for DFF40 in DNA repair pathways and cell cycle regulation. DFF40 could be a key regulator in cell cycle phase transition, hence its absence could facilitate mitosis in cancer cells. These hypotheses have not yet been demonstrated and require further investigation.

DNA fragmentation is not required for apoptosis [81–83], due to the cleavage of a plethora of other caspase targets. However, it remains unknown whether apoptosis is a faster or more efficient process when DNA fragmentation occurs. Recovery is possible for cells that underwent mitochondrial outer membrane permeabilization or caspase activation [84–86]. However, excessive DNA fragmentation represents a no-return step for the dying cell [87]. The importance of DFF40 in ETO- and Ara-C-induced apoptosis was investigated in Figs. 6 and 7. ETO significantly induced DNA fragmentation in DFF40 WT Jurkat cells, at a much greater scale then Ara-C in comparison to DFF40 KO cells, who totally lack DNA fragmentation (Fig. 6A and 6B). Similarly to these results, we have previously shown that DFF40 deficient cells do not show DNA fragmentation following TBT exposure [17]. Human glioblastomas deficient in DFF40 also show an impairment in DNA hydrolysis [10]. Overall, the lack of DNA fragmentation in apoptosis anomalies has been previously reported multiple times [3]. The exposure of PS on the outer cell membrane layer and loss of MMP are one of the early steps of apoptosis activation [36]. We thus investigated at first PS dynamics in our study model (Fig. 6). In parallel to our viability results, DFF40 deficient cells exposed to ETO present a significantly higher proportion of double positive cells (Annexin V and PI positive), but no significant differences were observed in the amount of only Annexin V positive cells. Double positive cells with the Annexin V staining protocol are known to be cells in necrosis (post-apoptotic necrosis or late apoptosis), whereas Annexin V positive cells alone are cells in early apoptosis. Surprisingly, caspase activity analysis (Fig. 6D) revealed significantly lower caspase 3/7 activity in DFF40 KO Jurkat cells exposed to ETO, in comparison to DFF40 WT cells. It is important to understand that cells can exhibit a rearrangement of PS at the outer layer of cell membrane without exhibiting DNA fragmentation. It was shown that activation of mixed lineage kinase-like (MLKL) leaded to PS exposure, but independently of caspase or receptor-interacting protein kinase-3 (RIPK3) activity [88]. Also, PS exposure independently to caspase-activation is not necessarily associated with cell death [89]. These results suggest that ETO treatment in DFF40 KO Jurkat cells could induce higher necrosis levels rather than apoptosis in comparison to DFF40 WT cells. This hypothesis was confirmed with HMGB1 expression, where DFF40 deficient Jurkat cells have a significantly higher regulation of the necrosis marker following ETO exposure (Fig. 7). Extracellular release of HMGB1 in necrotic Jurkat cells exposed to heat was shown to be enhanced [90]. As for Ara-C, it induces less apoptosis activation in DFF40 deficient cells, which have a significantly lower proportion of Annexin V positive cells, but no significant changes in caspase 3/7 activity. Dysfunction in mitochondria has been reported to participate in apoptosis induction [91]. ETO-treated DFF40 KO Jurkat cells have a significantly loss in MMP in comparison to DFF40 WT Jurkat cells (Fig. 6C). In concordance with our results, it was shown that mitochondrial membrane depolarization enhanced cell death induced by TRAIL in human granulose tumor cells [92].

Apoptosis pathway key proteins expressions were then analyzed (Fig. 7), knowing that PS translocation requires the activity of caspases [93]. Surprisingly, no differences in protein expression is observed between both cell lines following treatment with ETO or Ara-C for 16 h and 24 h (Fig. 7A and E). Chemotherapy treatments equally activates caspases −3 (data not shown), −6 and PARP1 or inhibits XIAP and Mcl-1 expression. Interestingly, we found an upregulation of Bcl-xL expression in both cell lines following a 16 h exposure to ETO, but levels were significantly lower in DFF40 deficient Jurkat cells. Bcl-xL is an anti-apoptotic protein where its function is to inhibit Bax and Bak activation [94]. Downregulation of Bcl-xL expression was shown to enhance chemosensitivity in hepatoblastoma cells [95], prostate cancer cells [96], mesothelioma cells [97] and glioblastoma cells [98]. The difference in Bcl-xL expression between DFF40 WT and DFF40 KO cells could explain the chemosensitivity to ETO observed in Fig. 3. As for the chemoresistance observed with Ara-C in DFF40 deficient, we didn't find any significant regulations in the apoptotic pathway that could explain this observation. Although, we did observe that γH2AX formation is significantly lower in DFF40 deficient Jurkat cells treated with ETO and Ara-

C. γH2AX formation is an important DNA damage marker, where its phosphorylation is a required step to active DNA repair pathways [3]. We have previously shown that DFF40 deficient cells lacked total histone H2AX phosphorylation following TBT treated for up to 24 h [17]. These results support the previously stipulated hypothesis by our laboratory [3], that DFF40 might have an important role in genomic stability regulation.

Taken together, our study is the first to concretely demonstrate the impact of DFF40 gene regulation in chemotherapy response. DFF40 protein expression can potentially impact patient outcome depending on the first line chemotherapeutic drugs used. DFF40 deficient cancer cells are less sensitive to antimetabolites, whereas the use of TOP2 inhibitors enhances chemosensitivity of those cells. Bcl-xL protein expression regulation and MMP loss can explain the chemosensitivity of DFF40 deficient cancer cells to ETO, a TOP2 inhibitor, whereas the underlying mechanism to cytarabine chemoresistance seems to not be related to the apoptotic pathway regulation. In concordance with the emerging new studies, we support with our data the role of DFF40 in genomic stability. It is important to note that tumor growth is highly dependent of its microenvironment and these results would have to be confirmed *in vivo*. Finally, further investigations regarding its role in DNA repair pathways are currently being conducted in our laboratory to better understand the response of DFF40 deficient cancer cells to chemotherapeutic drugs.

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CRediT authorship contribution statement

Merve Kulbay: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Bruno Johnson:** Investigation, Methodology, Writing – original draft. **Sophie Fiola:** Methodology, Formal analysis, Investigation. **Roberto J. Diaz:** Methodology, Formal analysis, Investigation. **Jacques Bernier:** Conceptualization, Funding acquisition, Supervision, Validation, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4 CHAPITRE 4: ENERGETIC METABOLIC REPROGRAMMING IN JURKAT DFF40-DEFICIENT CANCER CELLS

Titre de l'article : *Reprogrammation métabolique chez les cellules cancéreuses Jurkat déficientes en DFF40*

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GR : Exécution des expériences – Expériences de RT-qPCR, analyses statistiques, révision du manuscrit.

MNSG : Exécution des expériences – Expériences d'immunofluorescence, analyses statistiques, révision du manuscrit.

JB : Conception du projet et de la méthodologie, analyses statistiques, supervision, rédaction et révision du manuscrit.

4.1 Lien entre les articles précédents et le suivant :

Dans nos deux articles précédents, nous avons démontré la réponse cellulaire des cellules déficientes en DFF40 aux agents inducteurs d'apoptose, à savoir le TBT et les médicaments de chimiothérapie. Les cellules T Jurkat sont résistantes à l'apoptose induite par le TBT et les antimétabolites, mais sensibles à l'apoptose induite par les inhibiteurs de TOP2. Par la suite, nous avons caractérisé les adaptations moléculaires dans la voie de signalisation de l'apoptose impliquée dans ces phénotypes. Ici, nous avons étudié l'impact d'une déficience en DFF40 sur les mécanismes d'adaptations des cellules cancéreuses en conditions non apoptotiques. En effet, plusieurs études supportent l'hypothèse qu'une mutation dans un locus génomique peut affecter l'expression d'autres protéines ou marqueurs moléculaires impliqués dans les voies de signalisation complémentaire. De ce fait, nous avons étudié l'expression de diverses protéines de l'apoptose dans les cellules cancéreuses. De plus, de nombreuses études supportent l'hypothèse que les tumeurs déficientes en DFF40 ont un profil plutôt malin : la sous-régulation de l'expression du DFF40 dans plusieurs tumeurs est liée à un mauvais pronostic. Donc, nous avons voulu étudier le métabolisme cellulaire des cellules T déficientes en DFF40, sachant que les tumeurs malignes dépendent de l'effet Warburg. Les résultats de cet article nous permettront de confirmer l'impact du DFF40 dans la régulation de la stabilité génomique, puis d'étudier par la suite les adaptations moléculaires en détail.

4.2 Résumé en français :

Le facteur de fragmentation de l'ADN 40 (DFF40), ou DNase activée par la caspase (CAD), est une endonucléase spécifique pour les bris doubles brins de l'ADN. Des altérations de sa fonction et de son expression ont été liées à la résistance à l'apoptose, un mécanisme d'adaptation utilisé par de nombreuses cellules cancéreuses. Cependant, les modulations impliquées dans la voie de résistance à l'apoptose liée au DFF40 restent incertaines. Ici, nous avons voulu déterminer si l'expression du DFF40 pourrait être liée au métabolisme cellulaire par la régulation de l'intégrité et la fonction mitochondriale. Nous avons démontré que les cellules déficientes en DFF40 sont plus résistantes à l'apoptose induite par la staurosporine (STS) et le tributylétain (TBT), et expriment des niveaux basaux de Mcl-1 plus élevés. Le traitement au TBT induit une augmentation de la transcription de l'ARNm de Bcl-2 et de la caspase-9 dans les cellules DFF40 KO Jurkat, ainsi qu'une phosphorylation augmentée de Bcl-2. Une perte d'expression du DFF40 induit une augmentation de la masse mitochondriale, du nombre de copies d'ADNmt, du potentiel de membrane mitochondriale au repos et du taux de glycolyse. Les cellules déficientes en DFF40 présentent le phénotype de l'effet Warburg, où elles dépendent beaucoup plus de la glycolyse que de la phosphorylation oxydative, et ont un état prolifératif plus élevé, démontré par une expression plus élevée du facteur de transcription Ki-67 et de l'augmentation de la phosphorylation d'AKT. Enfin, nous avons démontré avec le fractionnement cellulaire que le DFF40 peut subir une translocation vers la mitochondrie suivant l'induction de l'apoptose. Notre étude révèle que le DFF40 peut agir comme un régulateur mitochondrial lors de l'apoptose et sa perte d'expression pourrait compromettre l'intégrité mitochondriale et entraîner une reprogrammation énergétique dans des pathologies, telles que le cancer.

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Energetic metabolic reprogramming in Jurkat DFF40-deficient cancer cells

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Abstract
DNA fragmentation factor 40 (DFF40), or the caspase-activated DNase (CAD), is an endonuclease specific for double-stranded DNA. Alterations in its function and expression have been linked to apoptosis resistance, a mechanism likely used by cancer cells. However, how the DFF40-related apoptosis resistance pathway occurs remains unclear. Here, we sought to determine if DFF40 expression could be linked to cell metabolism through the regulation of mitochondrial integrity and function. We demonstrated that DFF40-deficient cells are more resistant to staurosporine and tributyltin (TBT)-induced apoptosis, and express higher levels of Mcl-1 at basal state. Treatment with TBT induces higher Bcl-2 and caspase-9 mRNA transcripts in DFF40 KO Jurkat cells, as well as enhanced Bcl-2 phosphorylation. A loss of DFF40 expression induces a higher mitochondrial mass, mtDNA copy number, mitochondrial membrane potential, and glycolysis rates in resting T cells. DFF40-deficient cells exhibit the Warburg effect phenotype, where they rely significantly more on glycolysis than oxidative phosphorylation and have a higher proliferative state, demonstrated by a higher Ki-67 transcription factor expression and AKT phosphorylation. Finally, we demonstrated with cell fractioning that DFF40 can translocate to the mitochondria following apoptosis induction. Our study reveals that DFF40 may act as a regulator of mitochondria during cell death and its loss could compromise mitochondrial integrity and cause an energetic reprogramming in pathologies such as cancer.

Keywords Apoptosis · DFF40 · Mitochondria · Energetic metabolism · Cell proliferation · DNA · Reprogramming · Warburg effect · Cancer

Introduction

Cancer cell resistance mechanisms involve complex molecular signaling pathways that continuously evolve. Among those, mutations in the apoptotic pathway have been shown to be crucial in resistance acquisition to cell death [1, 2]. Apoptosis is a programmed cell death mechanism highly important in mammalian development and tissue homeostasis. The DNA fragmentation factor 40 (DFF40) is the primary endonuclease responsible for the fragmentation of DNA into nucleosomal fragments of about 180 to 200 bp and subsequent chromatin condensation during apoptosis [3]. Following apoptosis-triggering signals, the extrinsic and intrinsic apoptotic pathways are activated. These pathways lead to the activation of effector caspases, mainly the caspase-3, which cleaves the inhibitory subunit of DFF40, the DNA fragmentation factor 45 (DFF45) [3]. DFF40 is then released and homo-oligomers are formed, representing the active form of the protein [4, 5]. Multiple studies have reported the importance of DFF40 in cancer cell resistance, by linking DFF40-deficient tumors with poor prognosis. Under expression of DFF40 in uterine leiomyosarcomas [6], non-endometrioid, and high-grade endometrioid cancers [7], glioblastomas and astrocytomas [8] were shown to be associated with poor prognosis in patients. Recently, our laboratory has shown that knocking out the DFF40 gene in Jurkat cells by CRISPR-cas9 conferred cell resistance to tributyltin (TBT)-induced apoptosis through a delayed activation of the extrinsic apoptotic pathway [9], raising a new hypothesis for its potential novel role in genomic stability [10].

Mitochondria of cancer cells are known to be one of the most crucial bio cellular features in malignant tumor progression, by the regulation of mitochondrial DNA (mtDNA)

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and cell metabolism [11]. Mitochondrial perturbations have been shown to impair tumorigenesis [12]. DFF40, DFF45, and proteins of the cell death-inducing DFF45-like effector (CIDE) family share great sequence similarities in their N-terminal CIDE-N domain [13, 14]. CIDE proteins also contain a conserved CIDE-C domain, located in the C-terminal domain (CTD) [15]. Various localizations of CIDE proteins have been listed in the literature. CIDE-A, CIDE-B and CIDE-C/FSP27 proteins were shown to be mainly found in lipid droplets but also found in other compartments [16]. CIDE-A is located in mitochondria and can inhibit the mitochondrial ion coupler thermogenin (UCP1) [17]. The dimerization and localization of CIDE-B in mitochondria are necessary for its proapoptotic function [18]. CIDE-C/FSP27 was shown to regulate the expression of mitobiogenesis-related genes in human adipose tissue [19]. In addition to these results, CIDE domains of DFF-related proteins of drosophila (Drep2 and Drep4) were also found to be critical for DNA fragmentation and for lipid droplet fusion [16]. Throughout the literature, we underlay the importance of CIDE domains in regulating mitochondria-related biological functions. It is known that disruption of CIDE domains can cause metabolic disorders (*e.g.*, diabetes, obesity, cardiovascular diseases) [16] and the alteration of their expression by gene deletion can be related to cancer development [6, 8]. Our laboratory recently demonstrated that DFF40 deficiency in Jurkat T cells induced a higher redox state at basal state [9]. Recently, it was shown that elevated ROS production in cancer cells was associated with higher mtDNA copy number (mtDNAcn) [20]. These results brought our laboratory to suggest a role of DFF40 in preserving mitochondria homeostasis and function. Our laboratory has found an interest in investigating the possibility of DFF40, a CIDE domain-containing protein, to potentially localize outside the nuclei and regulate mitochondria integrity.

The aims of this study were to investigate the impact of a DFF40 deficiency on mtDNA and mitochondrial functions. First, we demonstrated that DFF40-deficient cells are resistant to staurosporine (STS)-induced apoptosis and TBT-induced apoptosis. DFF40-deficient cells show enhanced Bcl-2 phosphorylation following TBT-induced apoptosis and present higher Mcl-1 protein expression in non-apoptotic cells. The abolition of DFF40 expression by CRISPR-cas9 in Jurkat cells induces higher glycolysis rates, mitochondrial membrane potential (MMP) levels, mitochondrial mass, and mtDNAcn. TBT treatment does not alter ATP production and mitochondrial membrane integrity in DFF40 KO Jurkat cells. Subsequently to these results, in silico analysis of DFF40's amino acid sequence was realized to determine if mitochondria were a potent target because members of the CIDE family proteins have been found in this organelle previously [18]. Apoptotic stimuli induced an increase in the mitochondrial localization of the protein.

Materials and methods

Cell culture

The Jurkat (subclone J77.6.8 from ATCC TIB-152) and HeLa (ATCC CCL-2) cells were cultivated in RPMI 1640 (Gibco) and DMEM/F12 (Gibco) mediums, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Wisent), 100 U/ml penicillin (Wisent), 100 µg/ml streptomycin (Wisent), and 10 mM HEPES (Wisent). Jurkat cells were kept in culture at a range of 0.3 to 1 M cells per ml. Fresh vials were thawed every 90 days. HeLa cells were kept at a confluency of 80% before passages and were used until the 20th passage.

Jurkat and HeLa CRISPR/Cas-9 induced knockouts of DFF40

The CRISPR-cas9 system was used to perform gene deletions. Jurkat DFF40-*knockout* cells were generated as previously described by electroporation [9]. For HeLa cells, DFF40 KO cells were generated by Lipofectamine LTX (Thermo Fisher Scientific Cat# 15,338,100) transfection using 3 × sgRNA/cas9-all-in-one expression plasmids-targeting DFF40 gene (GeneCopoeia Cat# HCP256543-CG01-3). Briefly, HeLa cells were seeded in 6-well plates (Sarstedt) in complete DMEM/F12 medium supplemented with 10% FBS in order to reach an 80% confluency state overnight. The next morning, the culture medium was replaced with Opti-MEM medium (Gibco), and 250 µl of Lipofectamine LTX complex (2.5 µg plasmid DNA, 2.5 µl PLUS reagent and 7 µl Lipofectamine LTX) was added to the cells and left overnight. Transfection media were replaced with complete DMEM/F12 medium supplemented with 10% FBS the following day and 48 h post-transfection, geneticin (G418) at 1 µg/ml was added for a 5 day selection. Cells were passaged in complete DMEM/F12 medium without G418 on the 8th day, and single colonies were expanded twice to obtain monoclonal populations. After the first expansion of a single colony, HeLa cells, which have conserved a positive expression of DFF40, were used as a negative control for further assays (HeLa DFF40 WT).

Cell viability assays

Jurkat DFF40 WT and DFF40 KO cells were treated with STS [1 µM] or TBT [0.4 µM] for up to 6 h in RPMI medium supplemented with 0.1% FBS. Following treatments, cell viability was assessed as previously described [9].

Apoptosis assessment

DNA fragmentation was evaluated by cell-cycle analysis using the G1/G0 subdiploid peak quantification as described previously with some modifications [9]. Briefly, 1×10^6 DFF40 WT and DFF40 KO Jurkat cells were treated with STS [1 µM] or TBT [0.4 µM] for up to 6 h in RPMI medium supplemented with 0.1% FBS. Treated cells were then washed once in PBS 1X and resuspended in a propidium iodide (PI)/RNase solution (0.1% sodium citrate, 0.3% NP-40, 20 µg/ml RNase, and 50 µg/ml propidium iodide). Samples were incubated for 1 h at 37 °C and then immediately analyzed by flow cytometry with a FACSCalibur from Becton Dickinson (BD FACSCalibur Flow Cytometry System, RRID:SCR_000401). Analyses were performed using live gates to discriminate between doublets and cells exhibiting reduced DNA content corresponding to the sub G1/G0 diploid peak; the latter cells were defined as apoptotic cells. A total of 10 000 events per sample were analyzed. Early apoptosis was assessed with Annexin-V staining with the Annexin-V FITC apoptosis detection kit (Thermo Fisher Scientific Cat# BMS500FI/100, RRID: AB_2575598) according to the manufacturer's instruction without any modifications. Finally, caspase-3/7 activity was assessed by flow cytometer with the Caspase-3/7 Green detection reagent (Thermo Fisher Scientific, Cat#R37111) according to the manufacturer's instructions with some modifications. Briefly, 1×10^6 Jurkat cells were treated or not with STS [1 µM] for up to 2, 4, and 6 h in RPMI medium supplemented with 0.1% FBS. Cells were then washed twice with PBS 1X and suspended in PBS 1X containing the caspase-3/7 probe (3 drops in 4 ml). A total of 10 000 events were analyzed by flow cytometer.

Western blotting

Following treatments and mitochondria isolations, protein dosage was performed using the Pierce BCA protein assay kit (Thermo Fisher Scientific Cat#A53225), as per the manufacturer's recommendations. Proteins were then diluted in laemmli buffer 1X (12.5% (v/v) 0.5 M Tris–HCl pH 6.8, 10% (v/v) glycerol, 20% (v/v) SDS 10%, 5% (v/v) β-mercaptoethanol, 5% (v/v) bromophenol blue 0.5%) at a final concentration of 1 µg/µl and incubated at 95 °C for 10 min before electrophoresis on SDS–polyacrylamide gels. Proteins were transferred on nitrocellulose membranes using the Trans-Blot Turbo Transfer System of Bio-Rad, as per the manufacturer's instructions. Membranes were blocked in TBS-0.1% Tween 20 (TBST 1X) solution containing 5% (m/v) skimmed milk overnight. Primary human antibodies anti-N-terminal DFF40 (Abcam Cat# ab8408, [RRID]:AB_2092919), anti-interior DFF40 (Abcam Cat# ab8406, [RRID]:AB_2092921), anti-C-terminal DFF40 (Abcam Cat# ab69438, [RRID]:AB_2040661), anti-tubulin (Covance Cat# MMS-410P-250, RRID:AB_10063407), anti-TOMM20 (Santa Cruz Biotechnology Cat# sc-17764, RRID:AB_628381), anti-actine (Sigma-Aldrich Cat# A5441, RRID:AB_476744), and anti-phospho-H2AX (BioLegend Cat# 613,402, RRID:AB_315795) were incubated for 2 h at room temperature. Primary antibodies anti-COXIV (Abcam Cat# ab33985, RRID:AB_879754), anti-pro-survival Bcl-2 family antibodies (Cell Signaling Technology Cat# 9941, RRID:AB_2274727), anti-Bak (Cell Signaling Technology Cat# 12,105, RRID:AB_2716685), anti-caspase-3 (Cell Signaling Technology Cat# 9668, RRID:AB_2069870), anti-caspase-9 (Cell Signaling Technology Cat# 9508, RRID:AB_2068620), anti-caspase-6 (Abcam Cat# ab185648), anti-caspase-7 (Abcam Cat#69,540), anti-XIAP (Cell Signaling Technology Cat# 2042, RRID:AB_2214870), anti-phosho-AKT (Cell Signaling Technology Cat# 9271, RRID:AB_329825), and anti-phospho-p44/42 (Cell Signaling Technology Cat# 9101, RRID:AB_331646) were incubated overnight at 4 °C. Primary antibodies were all diluted at a final concentration of 1: 2 000 in TBST 1X solution containing 5% (m/v) BSA. Respective secondary HRP-conjugated antibodies were incubated for 1 h at room temperature at a final concentration of 1:20 000. Bands were visualized by chemiluminescence with the SuperSignal West Femto substrate (Thermo Fisher Scientific Cat#34,095). Densitometric analyses were performed using Image Lab software (Image Lab Software, RRID:SCR_014210).

RNA extraction and RT-qPCR assays

Jurkat DFF40 WT and DFF40 KO cells were plated in 6-well plates at a density of 5×10^6 cells/well in RPMI 1640 medium supplemented with 0.1% FBS. Cells were then treated or not with TBT [0.4 µM] for 2, 4, or 6 h. Cells were washed once with PBS 1X and RNA was extracted using the conventional phenol–chloroform extraction using the TRIzol reagent by Sigma. The integrity of ribosomal subunits was assessed with the Experion RNA StsSens and HighSens analysis kit (Bio-rad Cat#100-7103) as recommended by the manufacturer's instructions. RNA concentrations following the extractions were assessed by spectrophotometer using the NanoDrop 1000 by Thermo Fisher. RT-PCRs were performed using 50 ng of sample ARN per reaction with the SsoAdvanced Universal SYBR Green Supermix kit (Bio-rad Cat#1,725,271), as recommended by the manufacturer's instructions. For qPCR assays, the iScript gDNA Clear cDNA Synthesis kit (Bio-rad Cat#1,725,035) was used to prepare sample mixes and the following cycling program was used: 10 min denaturation at 95 °C and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Forward and reverse primers (see table below; Thermo Fisher Scientific) were used at a

concentration of 0.01 nmol per reaction per primer. Results were analyzed with the CFX Maestro™ Software (Bio-rad Cat# 12,004,110) (Table 1).

Mitochondrial membrane potential quantification

MMP was assessed with the TMRE-Mitochondrial membrane potential assay kit (Abcam Cat#ab113852), according to the manufacturer's instructions with some modifications. Cells were washed once with PBS 1X and suspended in 200 µl of PBS 1X containing 0.2% (m/v) BSA. TMRE was then added to cells at a final concentration of 300 nM and samples were incubated 20 min at 37 ºC before analyses by flow cytometer.

Glucose uptake assays

Glucose uptake was assessed using the 2-NDBG glucose analog (Thermo Fisher Scientific Cat#N13195). Briefly, treatments described above were performed in RPMI medium supplemented with 0.1% FBS without glucose. 2-NDBG was then added to samples at a dilution of 1: 500, and cells were incubated another 45 min at 37 °C. Cells were washed once and suspended in PBS 1X for further analyses by flow cytometer.

Seahorse assay

Extracellular acidification rates (ECAR) and oxygen consumption rates (OCR) were assessed using the Seahorse XF Cell Mito Stress Test Kit (Agilent Cat#103,015), according to the manufacturer's instructions with some modifications. Briefly, Jurkat cells were seeded in Seahose XFe96 cell culture microplates at a density of 0.2×10^6 cells/well in Agilent Seahorse XF RPMI medium, supplemented with sodium pyruvate (1 mM), glutamine (2 mM), and glucose (10 mM) or galactose (10 mM). Cells grown in galactose media were first starved for 2 h in EBSS (Thermo Fisher Scientific Cat#14,155,063) at a density of 1×10^6 cells/ml. Cells were then seeded at a density of 0.2×10^6 cells/well and incubated for 1 h at 37 °C with 5% CO_2 before treatments. Cells were treated or not with TBT [0.4 µM] or STS [1 µM] for 60 min. Plates were then centrifuged at 1200 RPM for 7 min and placed in a CO_2-free incubator for 60 min prior loading of the injection ports of the sensor cartridge. Oligomycin, FCCP, and Rotenone/Antimycin were loaded in the injection ports as instructed by the manufacturer. OCR and ECAR were measured using the Seahorse XFe96 analyzer by Agilent. Results were normalized with total protein in each well, determined by a Bradford assay following the experiment. Results were analyzed with the Seahorse Wave software (Seahorse Wave, RRID:SCR_014526).

Cell proliferation

Cell proliferation was first quantified with Alamar Blue by spectrophotometer. Briefly, 2×10^5 Jurkat DFF40 WT and DFF40 KO cells were plated in 96-well plates and Alamar Blue (Thermo Fisher Scientific, Cat# DAL1100), diluted at 10%, was added to each well. Plates were incubated for 3 h at 37 °C with 5% CO_2. Dye fluorescence intensity was assessed with the SpectraMax® M5 Microplate Reader at an excitation wavelength of 570 nm and emission wavelength of 585 nm. Increase of cells density was confirmed by microscopy with trypan blue dye (data not shown). Finally, Ki-67 (Cell Signaling Technology Cat# 9129, RRID:AB_2687446) staining was performed according to the manufacturer's instruction but with some modifications. Briefly, 5×10^6 cells were starved with EBSS (Gibco) for 4 h at a density of 1×10^6 cells/ml. Cells were then washed once with PBS 1X, plated in 6-well plates in RPMI medium supplemented with 10% FBS at a density of 1×10^6 cells/ml and incubated for

Table 1 Primer sequences for RT-qPCR

Gene	Sequences	
	Forward (5′-3′)	Reverse (3′-5′)
Bax	TCAGGATGCGTCCACCAAGAAG	TGTGTCCACGGCGGCAATCATC
Bak	TTACCGCCATCAGCAGGAACAG	GGAACTCTGAGTCATAGCGTCG
BIM	CAAGAGTTGCGGCGTATTGGAG	ACACCAGGCGGACAATGTAACG
Bcl-2	ATCGCCCTGTGGATGACTGAGT	GCCAGGAGAAATCAAACAGAGGC
Bcl-xL	GCCACTTACCTGAATGACCACC	AACCAGCGGTTGAAGCGTTCCT
Bfl-1/A1	GGATAAGGCAAAACGGAGGCTG	CAGTATTGCTTCAGGAGAGATAGC
Mcl-1	CCAAGAAAGCTGCATCGAACCAT	CAGCACATTCCTGATGCCACCT
Caspase-3	GGCCTGCCGTGGTACA	AGCATGGCACAAAGCGACT
Caspase-9	GTTTGAGGACCTTCGACCAGCT	CAACGTACCAGGAGCCACTCTT
NRF2	CACATCCAGTCAGAAACCAGTGG	GGAATGTCTGCGCCAAAAGCTG
SOD1	CATCTGTTATCCTGCTAGCTG	GGCAAAATACAGGTCATTGA
SOD2	TTGGCCAAGGGAGATGTTACA	CAACAGATGCAGCCGTCAG

3 h. Cells were then washed twice with PBS 1X and fixed with 4% paraformaldehyde (PFA) at room temperature for 15 min. Cells were then washed thrice and permeabilized with PBS 1X/Triton X-100 0.2% for 15 min at room temperature. Cells were then washed twice with PBS 1X and the antibody dilution buffer (0.5% BSA in PBS 1X). Samples were stained with the primary antibody Ki-67 (1:400) for 1 h at room temperature and then washed twice with the antibody dilution buffer before staining with the secondary antibody (1:1000) conjugated to Alexa Fluor 488 (Cell Signaling Technology Cat# 4412, RRID:AB_1904025) for 30 min at room temperature in the dark. Samples were then washed once with PBS 1X and analyzed by flow cytometer.

Mitochondrial mass and mitochondrial DNA analyses

For the analysis of mitochondrial mass, Jurkat or HeLa DFF40 WT and DFF40 KO cells were synchronized by serum starvation for 24 h. The next day, 100 nM Mitotracker Deep Red FM (Thermo Fisher Scientific Cat#M22426) was added for 30 min and cells were washed twice in PBS 1X, before analysis by flow cytometer. A total of 10 000 events were analyzed. Relative mtDNA copy number in Jurkat and HeLa DFF40 WT and DFF40 KO cells was determined by qPCR. The NovaQUANT™ Human Mitochondrial to Nuclear DNA Ratio Kit (Sigma-Aldrich Cat#72,620) was used to compare the levels of nuclear to mtDNA in DNA samples. Genomic DNA of Jurkat and HeLa cells was purified with the GenElute Miniprep kit from Sigma-Aldrich, as per the manufacturer's recommendation. 1 ng of DNA was added in a NovaQuant 96-well plate, and samples were treated as per the manufacturer's protocol. Toyobo Thunderbird™ SYBR® qPCR Mix was used to prepare sample mixes, and the following cycling program was used: 10 min denaturation at 95 °C and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Finally, mitochondrial biogenesis capacity was assessed using the commercial MitoBiogenesis flow cytometry kit (Abcam Cat#ab168540), according to the manufacturer's instructions with some modifications. Briefly, Jurkat DFF40 WT and DFF40 KO cells were washed once with PBS 1X and suspended in complete RPMI medium and fixed with 4% (v/v) PFA for 15 min at room temperature. Samples were then vortexed 5 min at 400×*g*, and pellets were suspended in PBS 1X. Following steps were done according to the instructions provided by the manufacturer.

Immunofluorescence

Briefly, DFF40 WT or DFF40 KO Jurkat cells were seeded in 24-well plates at a density of 1×10^6 cells/ml for 2 h in complete RPMI media supplemented with 10% FBS. Cells were then washed once with PBS 1X and incubated with 500 nM of Mitotracker Deep Red FM for 45 min at 37 °C with 5% CO_2. Following incubations, cells were washed once in PBS 1X, and 0.3×10^6 cells per sample were layered on microscope slides by cytospin with a speed of 800 RPM for 3 min. Microscope slides were pretreated with collagen type I for 1 h at room temperature and then washed with sterile H_2O. Following cytospin, cells were fixed with PFA 4% and permeabilized with Triton 0.02%. Cells were then stained with DAPI (dilution of 1:15 000) for 10 min at room temperature and washed 3 times with PBS 1X. Slides were covered with coverslips using fluoromount-G mounting medium and left overnight at room temperature. The next morning, slides were stored at 4 °C until imaging. Immunofluorescence images were obtained with a Nikon A1R + confocal microscopic laser equipped with a spectral detector. The NIS Elements advanced research software (version 4.20.00) was used for imaging.

Mitochondria purification and pellet treatments

Mitochondria were purified from 1×10^7 Jurkat DFF40 WT cells using a Mitochondria isolation kit for cultured cells as per the manufacturer's instructions (Thermo Fisher Scientific Cat# 89,874) with some modifications. Briefly, cells were treated or not with STS [1 µM] (Sigma-Aldrich Cat# S5921) and/or with Z-VAD-FMK [25 µM] (Abcam Cat#ab120382) for 2 h. Cells were then washed twice with PBS 1X in 15 ml Falcon tubes, and 400 µl of cold buffer A containing proteinase inhibitors (1X) was added. Cells were vortexed at medium speed for 5 s. After 2 min, 5 µl of buffer B was added, and cells were vortexed thoroughly for 9 s. Samples were vortexed 5 s per min for 5 min, and 400 µl of cold buffer C was added. Samples were mixed 5 times by inversion and centrifuged for 7 min at 800×*g*. Cytoplasm-containing supernatant was transferred to a new falcon tube and was centrifuged again to remove nuclei and unbroken cells contamination. Supernatant was transferred to microtubes and was centrifuged 15 min at 7000×*g*. Mitochondria-containing pellets were washed once with cold buffer C, and mitochondria pellets were used immediately or saved at − 80 °C. Proteinase K protection assay was done on freshly isolated mitochondria. Mitochondria pellets were treated with 100 µg/ml proteinase K in 30 µl of buffer for 20 min at room temperature for different time points (0, 10 or 20 min). SDS sample buffer was then added, and the samples were immediately boiled to stop the reaction.

In silico and statistical analysis

In silico prediction of human DFF40 translocation to mitochondria was determined with MitoProt II software (v.1.101). Experimental data are expressed as mean ± SEM. Student's t test was performed when two groups were

compared, or Two-way ANOVA with Tukey's post-hoc test was performed when 3 groups or more were compared. Statistical analysis was performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Differences were considered statistically significant when $p < 0.05$.

Results

DFF40-deficient Jurkat cells are more resistant to staurosporine and tributyltin-induced apoptosis

DFF40 deficiency has been linked multiple times to apoptosis resistance [6, 8, 9]. Recently, our laboratory has shown that DFF40-knockout Jurkat cells were much more resistant to apoptosis induced by TBT, following a 24 h exposure time [9]. Mitochondrial alterations are known to occur early in the apoptotic pathway, ahead of cell death. We, thus, sought to determine if resistance to TBT-induced apoptosis could be observed with a narrower exposition window, providing us with lower cell death levels, in order to study mitochondrial pathways (Fig. 1). As hypothesized, DFF40 KO Jurkat cells have significantly lower levels of cell death (14.66% ± 0.77%) in comparison to DFF40 WT Jurkat cells (22.55% ± 2.32%) following a 6 h TBT treatment in RPMI medium supplemented with 0.1% FBS (Fig. 1a and b). In addition to this agent, we sought to investigate the resistance of our cell line to STS, a protein kinase inhibitor. STS is known to induce cell death via the intrinsic pathway through changes in mitochondrial function [21, 22], making it an agent of choice for this study. We, thus, investigated if DFF40-deficient cells were resistant to this agent using the same experimental design. Previously, we had shown that DFF40 KO Jurkat cells did not have a different sensitivity to STS following a 24 h exposure time [9]. Interestingly, in this experimental design, DFF40 KO cells are significantly more resistant to STS-induced apoptosis, as early as a 4 h exposure time (Fig. 1c and d). DFF40 KO Jurkat cells exhibit significantly lower Annexin-V-positive cells following STS exposure, as well as lower caspase-3/7 activity, demonstrated by flow cytometer (Fig. S1b and S1c). No differences in caspase-3 expression is observed between DFF40 WT and DFF40 KO cells (Fig. S1D). In addition, DFF40 KO cells lack histone H2AX phosphorylation, whereas this phenomenon is observed at its peak after a 2 h exposure in DFF40 WT cells (Fig. S1). As expected, no DNA fragmentation was observed following TBT and STS treatments in DFF40 KO cells, following cell-cycle analysis with PI/RNase staining (Fig. 1e and f). These results confirmed our choices of apoptosis-inducing agents in order to study mitochondrial alterations.

DFF40 deficiency regulates Bcl-2 family genes transcription based on cell death

During apoptosis, mitochondria play a key role, mainly in the intrinsic pathway, by increasing the oxidative stress levels, releasing caspase activators, and regulating the expression of Bcl-2 family proteins [23]. Previously, our laboratory had shown that a DFF40 deficiency altered the redox balance in healthy cells and that the apoptosis pathway activation was delayed following TBT treatments [9]. In an attempt to better understand the importance of DFF40 in TBT-induced apoptosis resistance, we assessed mRNA expressions of apoptotic genes in DFF40 WT and DFF40 KO Jurkat cells (Fig. 2a). In total, 3 proapoptotic (Bak, Bax, BIM), 4 antiapoptotic (Bcl-2, Bcl-xL, A1/Bfl-1, Mcl-2), and 2 caspases (caspase-3,-9) mRNA transcript levels were assessed in resting cells. No significant differences in all mRNA transcript levels were observed between DFF40 WT and DFF40 KO Jurkat cells (Fig. 2a). We next wondered if DFF40 deficiency could alter protein expression although gene expression was normal. Thus, we assessed protein expression levels of the Bcl-2 family protein members by western blot in resting cells (Fig. 2b and c). Interestingly, DFF40-deficient Jurkat cells display approximately 3 times higher levels of Mcl-1 (fold increase of 3.19 ± 1.78) in comparison to DFF40 WT cells. No differences in the proapoptotic protein levels (e.g., procaspase-3-9-6-7) and in the expression of XIAP were noted between DFF40 WT and DFF40 KO Jurkat cells (Fig. 2b and c). Apoptosis resistance is not only regulated by basal protein and gene expressions. Following initiation of apoptosis, gene and protein expression regulations can impact the sensitivity of cells to the agent. We thus sought to determine if exposure to TBT could impact mRNA levels of apoptosis-regulating genes and their subsequent protein expressions. Following TBT exposure, there was a significant increase in most of mRNA transcript levels of apoptosis regulation genes in both cell lines (Fig. 2d and e). Bcl-2 mRNA transcripts were significantly upregulated in DFF40 KO Jurkat cells (fold increase of 7.02 ± 0.21) in comparison to DFF40 WT Jurkat cells (fold increase of 4.31 ± 0.45) following a 6 h treatment with TBT (Fig. 2S). Interestingly, we found that caspase-9 mRNA transcripts were as well significantly upregulated in DFF40-deficient cells (fold increase at 6 h of 9.06 ± 0.93) in comparison to DFF40 WT cells (fold increase at 6 h of 4.46 ± 0.29) following a 4 h and 6 h exposure to TBT (Fig. 2S). No differences in both cell lines regarding Mcl-1, Bak, BIM, Bcl-xL, caspase-3, and A1/Bfl-1 mRNA transcript levels were observed following TBT treatment. Our protein expression analyses of antiapoptotic proteins reveals a significant upregulation of Bcl-2 phosphorylation in DFF40 KO Jurkat cells in comparison to DFF40 WT cells following a 4 h TBT exposure (Fig. 2f and g). We do not observe any significant modulations in

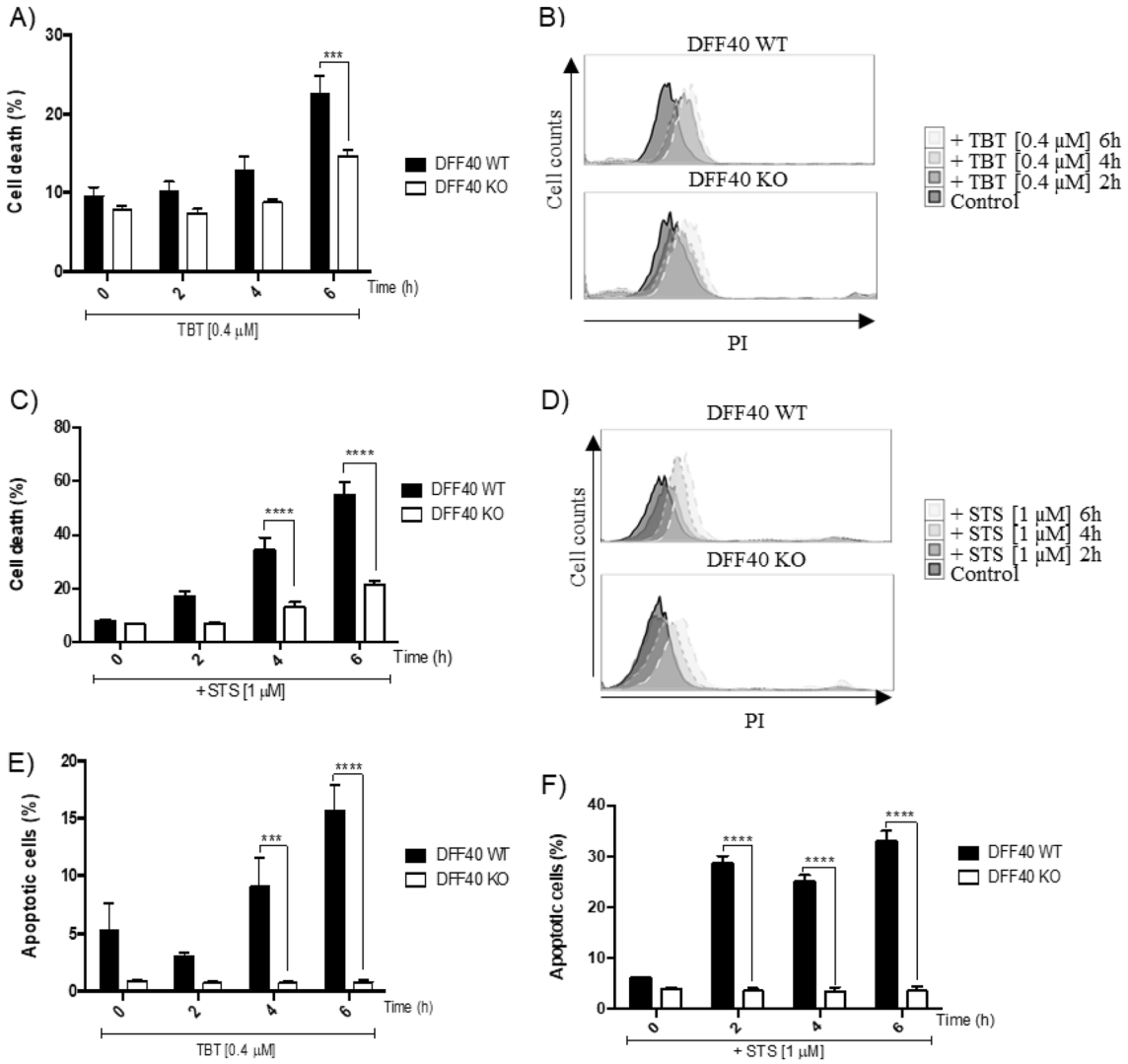


Fig. 1 DFF40-knockout Jurkat cells are more resistant to staurosporine and tributyltin-induced apoptosis. **A** and **B**: Jurkat DFF40 WT and DFF40 KO cells (1×10^6 cells) were treated or not with TBT [0.4 µM] for 2, 4, and 6 h in RPMI medium supplemented with 0.1% FBS. **C** and **D**: Cells in (**A**) were treated or not with STS [1 µM] for 2, 4, and 6 h in RPMI medium supplemented with 0.1% FBS. Cell viability was assessed with propidium iodide (PI; 1 µg/ml) by flow cytometer. **E** and **F**: Same as in **A** and **B**, respectively, but cells were suspended in a PI/RNase solution and cell fragmentation was assessed by flow cytometer. Apoptosis percentages were determined by subG0/G1 cell-cycle phase analysis. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed (***$p < 0.001$, ****$p < 0.0001$)

Bcl-2, Mcl-1 and Bcl-xL protein expressions when comparing Jurkat DFF40 WT and DFF40 KO cells treated with TBT. On the other end, STS did not induce proapoptotic mRNA transcription (e.g., BAK, caspase-3, and caspase-9) (Fig. 2S). Surprisingly, Bcl-2 mRNA was significantly more upregulated in DFF40 WT Jurkat cells in comparison to DFF40 KO cells (Fig. 2S). DFF40 KO Jurkat cells had lower caspase-3/7 activity when treated with STS (Fig. 1S) and lower Annexin-V + positive cells (Fig. 1S). Overall, our results suggest that TBT-induced apoptosis resistance could be partially explained by higher levels of p-Bcl-2 in DFF40 KO Jurkat cells.

DFF40 deficiency upregulates mitochondrial DNA, mitochondrial mass and mitochondrial-potential membrane

Mitochondria are complex organelles with a diverse aggregation of proteins within their membranes that allow the precise regulation of cell metabolism. Endonucleases, such as the nuclear-encoded endonuclease G, have been found to localize to mitochondria and regulate mtDNA metabolism [24]. Our laboratory has previously shown that DFF40-deficient Jurkat cells had higher ROS production at basal state [9]. Recently, it was shown that Caco-2 cancer cells

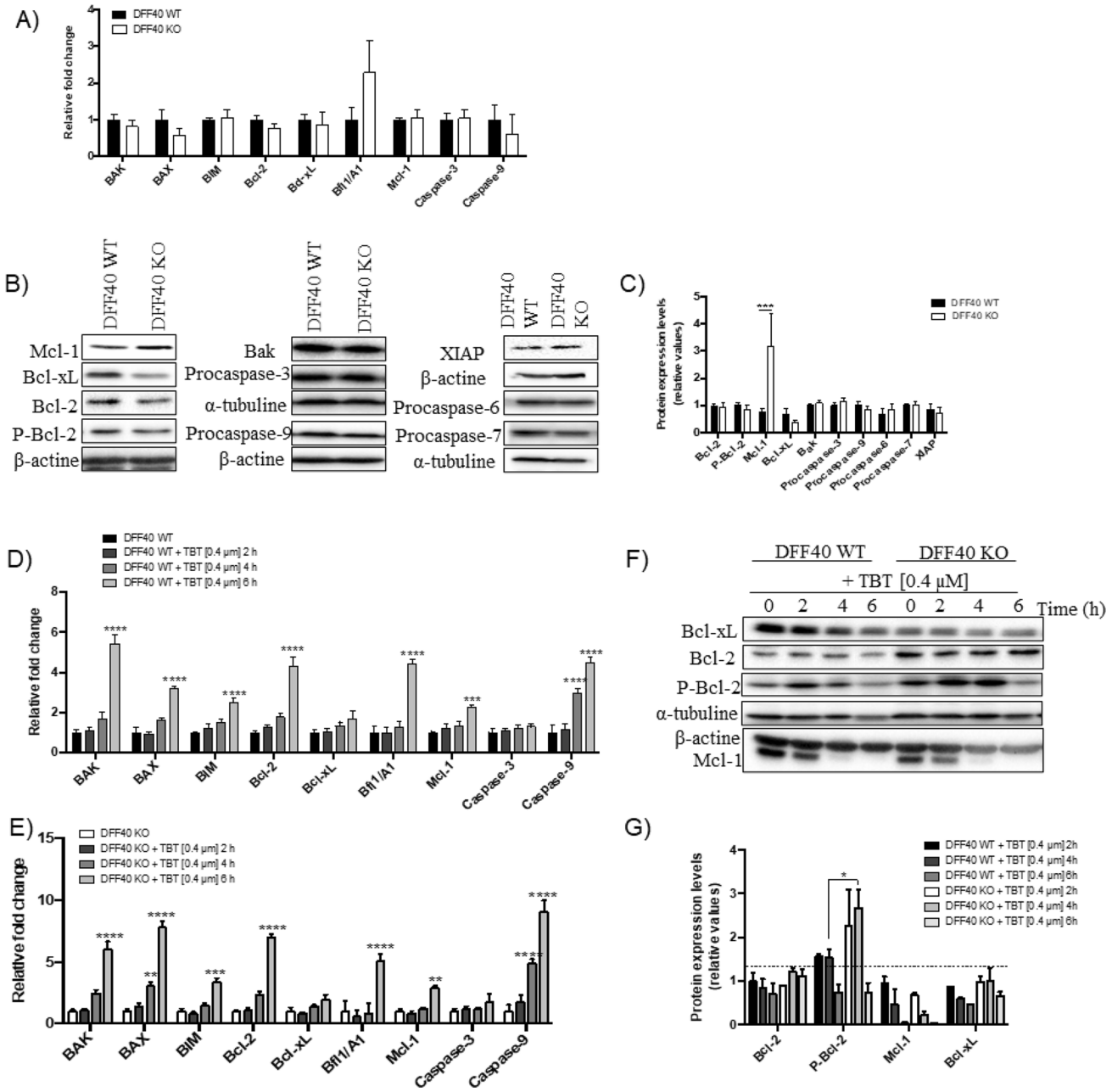


Fig. 2 DFF40-deficient Jurkat cells have enhanced phosphorylation of Bcl-2 protein following tributyltin-induced apoptosis. **A**: RNA of Jurkat DFF40 WT and DFF40 KO cells (5×10^6 cells) was extracted using the TRIzol reagent. RT-PCRs were performed on RNA samples (50 ng RNA) followed by a qPCR. Results were analyzed with the CFX Maestro software. Results are expressed as a relative fold change value in comparison to DFF40 WT cells. **B** and **C**: Jurkat DFF40 WT and DFF40 KO cells were grown for 24 h in RPMI media supplemented with 10% FBS at a density of 1×10^6 cells/ml. Protein extracts of Jurkat DFF40 WT and DFF40 KO cells were immunoblotted for Bcl-2, p-Bcl-2, Mcl-1, Bcl-xL, Bak, procaspase-3-9-6-7, and XIAP. Results are expressed as a relative fold change in protein expression according to DFF40 WT cells. **D** and **E**: Jurkat DFF40 WT and DFF40 KO cells were seeded in 6-well plates and treated or not with TBT [0.4 µM] for up to 6 h in RPMI media supplemented with 0.1% FBS. RT-qPCRs were performed as described in A. Results are expressed as a relative fold change value in comparison to their respective untreated control cell. **F** and **G**: Cells were treated as described in (**D**) and immunoblotted for the same antibodies as in B. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$)

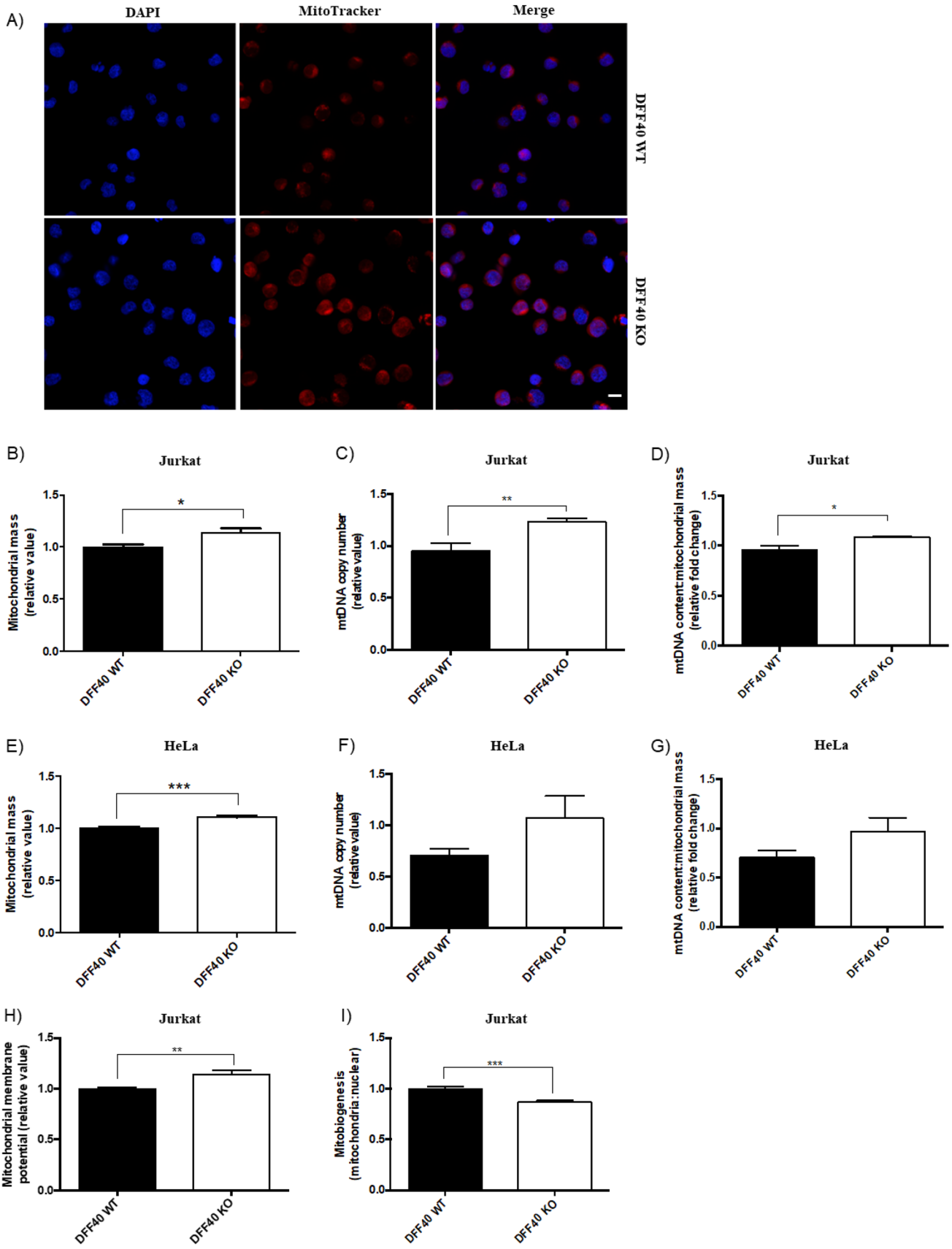
exhibiting higher ROS production at basal state had higher mtDNAcn [20]. We thus sought to determine the impact of DFF40 deficiency in Jurkat and HeLa cells on mtDNAcn and mitochondrial mass (Fig. 3). DFF40 KO Jurkat and HeLa cells exhibit a greater mitochondrial mass than DFF40 WT cell lines (Fig. 3b and e). Mitochondrial mass is 1.14-folds higher in DFF40 KO Jurkat cells and 1.11-folds higher in DFF40 KO HeLa cells in comparison to their respective control. Qualitative observation also demonstrates higher MitoTracker Deep Red fluorescence in DFF40 KO cells (Fig. 3a). In concordance with these results, the number of mtDNAcn was greater in DFF40 KO Jurkat cells in comparison to DFF40 WT Jurkat cells (Fig. 3c). DFF40 KO Jurkat cells have 1.23 times more mtDNAcn in comparison to DFF40 WT Jurkat cells. In HeLa cells, a similar tendency was observed, but no significant differences were noted in DFF40 KO and DFF40 WT cells (Fig. 3f). Since mtDNAcn is strongly affected by mitochondrial mass, we next calculated the ratio between mtDNAcn and MitoTracker fluorescence in both cell lines (Fig. 3d and g). Following normalization, DFF40 KO Jurkat cells express significantly higher mtDNAcn per mitochondria per cell (1.08 ± 0.02) in comparison to DFF40 WT Jurkat cells (0.95 ± 0.11) (Fig. 3d). No differences in HeLa cells is reported (Fig. 3g). We next investigated the impact of DFF40 deficiency on MMP. MMP is 1.14-folds significantly higher at resting state in DFF40 KO Jurkat cells (Fig. 3h). Finally, mitobiogenesis was measured by flow cytometry as a ratio in protein expression of mtDNA-encoded complex IV subunit 1 (COX-I) on nDNA-encoded 70 kDa subunit of complex II (SDH-A). At a basal state, DFF40 KO Jurkat cells exhibit a significantly lower mitobiogenesis ratio when normalized in comparison to DFF40 WT Jurkat cells (Fig. 3i).

DFF40 deficiency induces an energetic reprogramming in Jurkat cells

Changes in mtDNAcn can significantly impact cell metabolism and further oxygen consumption [25]. It has been known for over a few decades that cancer cells present a glycolytic phenotype in an aerobic environment, a process described as the Warburg effect [26]. Until today, no studies have shown a link between DFF40 expression in cancer cells and cell metabolism. We thus sought to determine the impact of DFF40 gene knockout on essential mitochondrial respiratory functions (e.g., OCR, glycolysis, glucose uptake and oxidative phosphorylation (OXPHOS)). Therefore, we first assessed the OCR and extracellular acidification rates (ECAR) in our study model, before and after apoptosis induction (Fig. 4). OCR and ECAR in DFF40 WT and DFF40 KO Jurkat cells were measured with the Seahorse XFe96 analyzer from Agilent following exposure to TBT [0.4 µM] for 1 h (Fig. 4a). DFF40-deficient cells had significantly higher OCR rates in comparison to DFF40 WT Jurkat cells (Fig. 4c and d). No changes in OCR between DFF40 WT and DFF40 are observed under OXPHOS conditions (Fig. 4c and S4). Under apoptotic conditions, DFF40-deficient cells also exhibit significantly higher OCR (Fig. 4e). The relation between OCR and ECAR was used as an indicator of glycolysis. Jurkat DFF40 KO cells have a higher glycolysis rate at basal state, demonstrated by a reduced OCR/ECAR ratio (Fig. 4f). The OCR/ECAR ratio of Jurkat DFF40 WT cells is of 5.44 ± 0.64, in comparison to DFF40 KO cells, which has a ratio of 3.74 ± 0.27 (Fig. 4f). Following STS exposure, the glycolysis ratio is equally enhanced in both cell lines (Fig. S3). TBT significantly reduces the OCR/ECAR ratio in both cell lines (Fig. 4g), with significantly higher glycolysis in DFF40 WT Jurkat cells (OCR/ECAR: 0.46 ± 0.01) in comparison to DFF40 KO Jurkat cells (OCR/ECAR: 0.70 ± 0.01). In the glycolysis pathway, glucose molecules go through different transformations to produce ATP and pyruvate. We next assessed the glucose uptake profile in our DFF40 KO cell line by quantifying 2-NDBG uptake by flow cytometer (Fig. 4h and i). No differences are noted at the basal state in both cell lines for 2-NDBG uptake (Fig. 4h). We observe an increase in 2-NDBG uptake in both cell lines following TBT-induced apoptosis (Fig. 4i). The uptake is significantly much greater in DFF40 KO cells ($55.83\% \pm 2.74\%$) than DFF40 WT cells ($27.43\% \pm 1.95\%$) following TBT exposure. Glycolysis generates fewer ATP molecules than OXPHOS, but at a much faster rate. DFF40 WT and DFF40 cells produce similar levels of ATP at a basal state (Fig. 4b). Following TBT exposure, basal respiration and ATP production levels were significantly reduced in DFF40 WT cells, whereas proton leak was increased by 2 folds (Fig. 4b). Surprisingly, no significant changes in the mitochondrial respiratory chain were observed in DFF40 KO Jurkat cells treated with TBT (Fig. 4b). In an OXPHOS prone environment (Fig. 4S), DFF40 KO Jurkat cells produce lower ATP levels. Following TBT-induced apoptosis, ATP production levels are significantly decreased in DFF40 WT and DFF40 KO cells (Fig. S4). Overall, these results suggest that DFF40 deficiency is involved in energetic reprogramming in cancer cells, favoring a glycolysis phenotype.

DFF40 deficiency induces a higher proliferative state in Jurkat cells through the upregulation of the Ki-67 antigen and AKT pathway

Having shown that DFF40-deficient cells rely more on glycolysis and have higher OCR, we next sought to determine their proliferative state with the Alamar Blue assay, Ki-67 expression and the activation of MAPKs (Fig. 5). First, alamar blue fluorescence was assessed by spectrophotometer. DFF40-deficient Jurkat cells proliferate 1.13 times more



◂**Fig. 3 DFF40 expression modulates mitochondrial mass, mitochondrial DNA, and mitochondrial membrane potential. A**: Jurkat DFF40 WT and DFF40 KO cells were stained for mitochondria (MitoTracker Deep Red) and nucleus (DAPI). Cell imaging was performed using a confocal microscope. Bar = 1.15 µm. **B** and **E**: Jurkat and HeLa DFF40 WT and DFF40 KO cells were stained for mitochondria (MitoTracker Deep Red), and mitochondrial mass was determined with the BD FACSCalibur flow cytometer using the mean fluorescence. Values are expressed as the relative fold change according to DFF40 WT cells. **C** and **F**: Relative mtDNA copy number in Jurkat and HeLa DFF40 WT and DFF40 KO cells was determined by qPCR. Data represent the relative value of mtDNA per 5 g of DNA in comparison with the DFF40 WT Jurkat cells. **D** and **G**: Mathematical transformation of mtDNAcn divided by mitochondrial mass. **H**: The mitochondrial membrane potential of Jurkat DFF40 WT and DFF40 KO cells (10^6 cells/cell line) at basal state was quantified by TMRE staining of active mitochondria with the BD FACSCalibur flow cytometer **I**: The capacity of mitochondrial biogenesis at basal state of Jurkat DFF40 WT and DFF40 KO cells was assessed with the BD FACSCalibur flow cytometer according to the manufacturer's instructions, as a ratio of the expression of mitochondrial-encoded COX-1 gene on nuclear-encoded SDH-A gene. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). *t* tests were performed (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

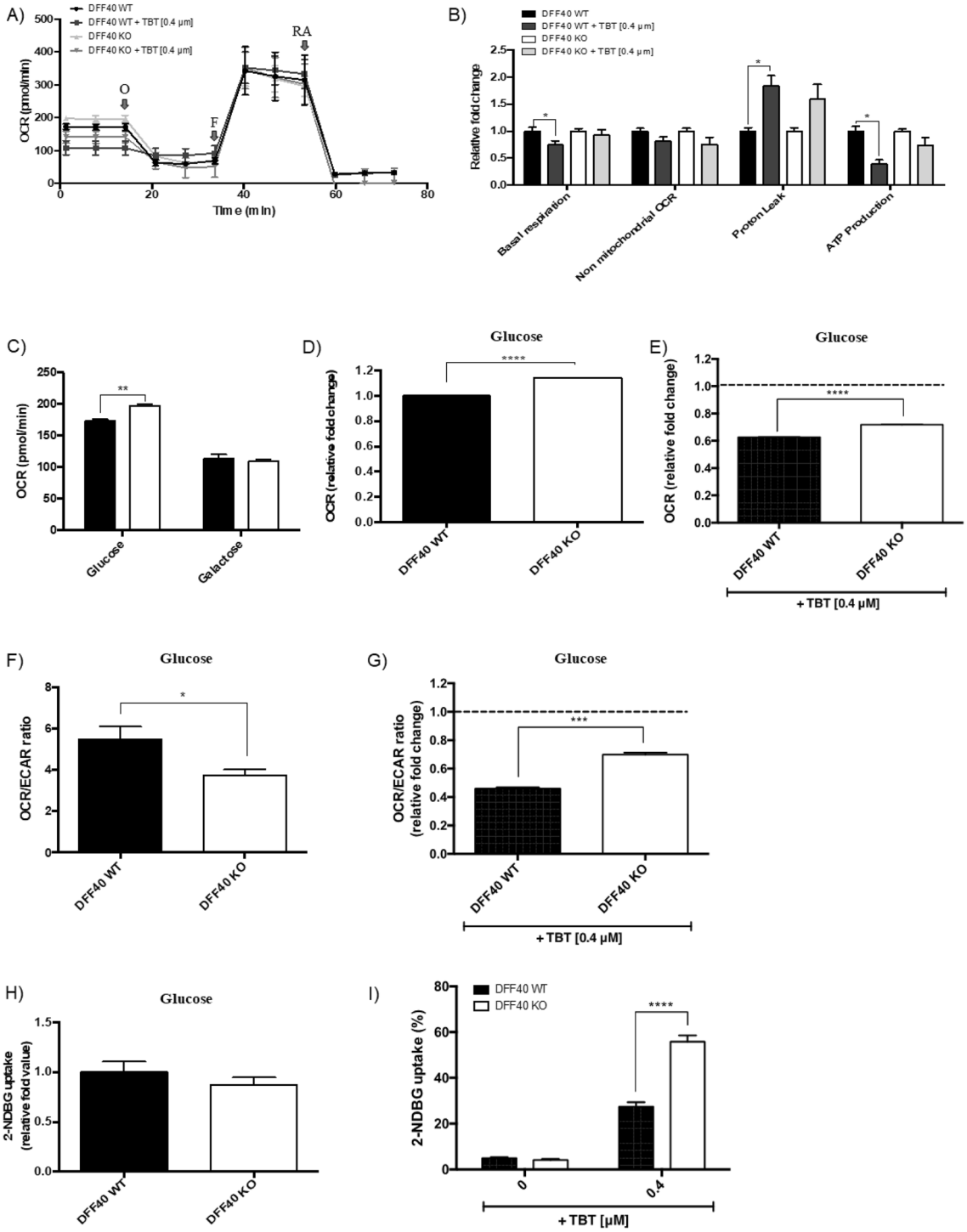
than DFF40 WT Jurkat cells (Fig. 5a). The higher proliferative state correlates with higher Ki-67 expression. In fact, Jurkat DFF40 KO Jurkat cells express approximately 18% more the Ki-67 marker in comparison to DFF40 WT Jurkat cells (Fig. 5b and c). Ki-67 was shown to have a role in cell cycle, where the PI3K/AKT pathway plays a center role in cell-cycle progression [27, 28]. We thus wondered if the higher proliferative state could be linked to a higher phosphorylation level of AKT. DFF40-deficient cells had a significantly higher phosphorylation of AKT following FBS addition to the media (Fig. 5d and e). Surprisingly, starved DFF40-deficient cells also express a higher phosphorylated state of ERK1/2 (Fig. 5d). The phosphorylation of ERK1/2 is totally abolished in both cell lines following FBS addition to the media. Overall, these results suggest that DFF40 deficiency plays a key role in cell-cycle progression through the upregulation of Ki-67 and AKT phosphorylation.

Evidence of mitochondrial localization of the DFF40 endonuclease in Jurkat cells

The localization of the DFF complex following its synthesis has been up to debate for many years, studies reporting its presence in both the nucleus or the cytosol [29, 30]. No studies until today have reported an extra-nuclear or cytoplasmic localization of DFF. Thus, we investigated if the DFF40 could be present in mitochondria. On the first hand, we performed an *in silico* analysis of the DFF40 amino acid sequence intending to identify a possible site of localization of DFF40 outside the nucleus. As seen in Fig. 6a, the presence of a potential N-terminal mitochondrial localization peptide was predicted using MitoProt II (v.1.101). The computed probability of export to mitochondria of DFF40 is 0.9389, and the predicted cleaved peptide would potentially be of 26 amino acid residues (cleaved sequence). To demonstrate the presence of DFF40 in mitochondria, we isolated mitochondria of Jurkat cells treated or not with STS [1 µM] for 2 h. After cell fraction isolation, the purity of cytoplasmic and mitochondrial fraction was determined with α-tubulin and COX-4 markers, respectively. The presence of DFF40 was determined with antibodies specific to N-terminal domain and the interior domain of DFF40 (Fig. 6b). In non-apoptotic DFF40 WT cells, we observed a major band in cytoplasmic fraction and several weak bands in the mitochondrial fraction with lower molecular weight. Using specific peptide to DFF40, we established in the mitochondrial fraction that the lower molecular weight band is non-specific (identified with an arrow, data not shown). Upon apoptosis induction, we detected two bands corresponding to DFF40 in the mitochondrial fraction. Interestingly, using the DFF40 N-terminal antibody, we detected only a high molecular band suggesting a N-terminal cleavage in mitochondrion. To confirm the DFF40 migration to mitochondria upon apoptosis, we repeated the experiment in the presence of a caspase-3 inhibitor, Z-VAD-FMK, to reduce DFF40 activation. Simultaneous treatment of cells with the caspase inhibitor reduced the translocation of DFF40 to mitochondria (Fig. 6c). To further determine in which mitochondrial subcompartment resides DFF40, we performed the proteinase K protection assay. Purified mitochondria from Jurkat cells, pretreated or not with STS [1 µM], were exposed to proteinase K [100 µg/ml] for up to 20 min (Fig. 6d). The DFF40 band that appears during apoptosis was completely removed like TOMM20, a protein localized at the outer mitochondrial membrane. Since proteinase K cannot penetrate to the outer and inner mitochondrial membranes, COX-4 does not disappear. Overall, our results suggest that DFF40 can translocate to the outer mitochondrial membrane following apoptosis induction.

Discussion

Cancer resistance mechanisms are widely studied throughout the literature. One of the main targets of study is the regulation of the apoptotic pathway in cancer cells. In eukaryotic cells, the DFF40 endonuclease is primordial for DNA fragmentation following the induction of apoptosis to preserve genomic stability. Over the past years, emerging new studies have been proposing additional roles for DFF40, such as in genomic stability [9, 10, 31]. At this day, the most accepted role for DFF40 is DNA fragmentation. Following the activation of the intrinsic or extrinsic apoptosis pathway, the DFF40 forms active oligomers in



the cytoplasm, subsequently to the cleavage of DFF45 by the caspase-3, and translocates to the nucleus to cleave the DNA into 180 to 200 bp fragments [31]. On a biomolecular scale, the DFF40 is composed of CIDE domains, where its CIDE-N domain is involved in complex formation by mediating CIDE-N/CIDE-N interactions [16]. A great sequence similarity has been shown between DFF40 and DFF45 CIDE-N domain and CIDE family proteins, which have a role in mitochondria homeostasis [32].

Many tumors have shown to have a downregulation of DFF40's expression, positively correlating with the degree of malignancy. Uterine leiomyosarcoma, high-grade endometrioid cancers and glioblastoma tumors all have shown to under express DFF40 and have poor clinical outcome [6–8]. In addition, we have recently demonstrated by

◂**Fig. 4 DFF40 deficiency in T cells alters the mitochondrial respiratory chain. A**: Oxygen consumption rates (OCR) of untreated and treated Jurkat DFF40 WT and DFF40 KO cells were measured with the Seahorse XFe96 analyzer by Agilent according to the manufacturer's instructions. Cells (2×10^5 cells/cell line) were treated or not with TBT [0.4 µM] for 1 h in RPMI media supplemented with glucose (10 mM). Arrows represent the injection times. (O: Oligomycin; F: FCCP, RA: Rotenone/antimycin A). **B**: Cell were treated as in (**A**). Mitochondrial respiratory function parameters were calculated using the Seahorse XF Cell Mito Stress Test Report Generator by Agilent. Values were compared to DFF40 WT cells and relative fold changes were calculated. **C**: OCR results of (**A**) and Fig. S4 presented in a histogram. **D** and **E**: Transformed OCR results of (**A**) presented in a histogram. Dashed line represents the baseline (normalized control values). **F and G**: Same as in (**A**) but OCR/ECAR ratios were calculated for each cell line and condition. Dashed line represents the baseline (normalized control values). **H**: Jurkat DFF40 WT and DFF40 KO cells (10^6 cells/well) were incubated with 2-NDBG (1: 500) for 45 min in RPMI medium without glucose and analyzed with the BD FACSCalibur flow cytometer for glucose uptake. **I**: Same as in (**H**), but cells were first treated or not TBT [0.4 µM] for 2 h. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$)

bioinformatical analyses that mRNA levels of *DFF40* gene are downregulated in breast, ovarian, cervical, pancreas, brain, and lung cancers [33]. Very few studies have investigated the impact of DFF40 expression alterations on apoptosis resistance. It was shown in T-47D breast cancer cell line that overexpression of DFF40 enhances cell sensitivity to doxorubicin-induced apoptosis [34]. DFF40 expression abolition in Jurkat cells induces a resistance to TBT-induced apoptosis [9], as well as to antimetabolites (methotrexate, 6-mercaptopurine, and cytarabine) [33]. We have previously shown that a resistance to TBT exposure (6 h) was mediated by delayed procaspase-3 and PARP cleavage, and lower caspase-6 activation [9]. It is important to note that mitochondrial alterations are known as the hallmarks of early apoptosis [35]. In order to better quantify mitochondrial alterations, lower cell death levels are thus needed. We demonstrated in this study that the delayed activation of apoptosis following a 6 h exposure to TBT in DFF40 KO Jurkat cells was linked to significantly lower cell death levels in DFF40 KO Jurkat cells. TBT activates apoptosis through multiples pathways, mainly by disturbing calcium homeostasis [36, 37], MMP [38, 39], and mitogen-activated protein kinase (MAPK) pathways [40], and directly enhancing caspase activation [41]. Another widely used apoptosis-inducing agent is STS, a protein kinase inhibitor. Multiple studies have demonstrated the importance of STS in the activation of the intrinsic apoptotic pathway [22, 42–45], making it an agent of choice for the study of mitochondrial alterations. In this study, we have shown that Jurkat DFF40 KO cells are also resistant to STS-induced apoptosis (Fig. 1), showing lesser positivity to Annexin-V staining (Fig. S1). STS induces apoptosis in Jurkat cells through procaspase-8 and Bid cleavage [46] and subsequent mitochondrial activation [47]. Surprisingly, no differences in procaspase-3 cleavage is observed between DFF40 WT and DFF40 KO Jurkat cells (Fig. S1). Protein expression does not necessarily correlate with its activity [48]. In fact, we showed that although the expression of procaspase-3 is not significantly altered in DFF40 KO Jurkat cells following STS exposure, there is a decrease in caspase-3/7 activity, which can partially explain the resistance towards STS-induced apoptosis. We have previously shown that DFF40 KO cells have a different sensibility to apoptosis-inducing agents and the observed phenotype can be described by distinct mechanisms [33]. The kinetics to STS and TBT-induced resistance to apoptosis slightly differ in DFF40 KO cells, as shown in this study. These results taken together, we decided to pursue our apoptosis pathway studies with TBT only and use STS solely for mitochondrial localization of DFF40 following apoptosis induction, since TBT is a protein cross-linker, rendering himself not suitable for protein localization assays and dynamic studies.

Our laboratory has previously shown the impact of DFF40 gene deletion on the regulation of the extrinsic apoptotic pathway [9]. However, more remains to be elucidated regarding the intrinsic apoptotic pathway and mitochondrial alterations. To better understand why DFF40 deficiency impairs cell death, we first assessed the genomic and protein expression of pro- and anti-apoptotic genes in resting DFF40 WT and DFF40 KO Jurkat T cells (Fig. 2a to c). We found that DFF40-deficient T cells exhibit significantly higher levels of Mcl-1 when grown in optimal conditions (RPMI media supplemented with 10% FBS) (Fig. 2b and c). Mcl-1 overexpression in DFF40 KO cells is lost when grown in a serum-deprived media (Fig. 2f and g). Many studies have reported a modification in protein expression in serum-depleted conditions [49, 50]. Following apoptosis induction by TBT (Fig. 2d to g), we observed an enhancement in the transcriptional activity of the genes Bak, Bax, Bim, Bcl-2, Bcl-xL, Bfl-1/A1, Mcl-1, caspase-3, and caspase-9 in both cell lines. We discovered that the fold change in Bcl-2 and caspase-9 RNA transcripts is significantly ($p < 0.0001$) enhanced in DFF40 KO cells in comparison to DFF40 WT Jurkat cells when treated with TBT [0.4 µM] for 6 h, and 4 and 6 h, respectively (Fig. 2S). Phosphorylation of Bcl-2 is also significantly more enhanced in DFF40 KO cells in comparison to DFF40 WT cells (Fig. 2g). The BCL2 gene codes for the Bcl-2 protein, an antiapoptotic protein that is permanently found in the mitochondrial outer membrane (MOM), but as well as on the nuclear envelope and the ER [51]. Bcl-2 prevents the oligomerization of BAX/BAK, which indirectly inhibits MOMP [51]. An increased expression of BCL2 transcripts in DFF40 KO cells could explain the resistance to TBT-induced apoptosis. In fact, it was shown in the literature that overexpression of Bcl-2 inhibits cell death in human mammary epithelial cells [52] and

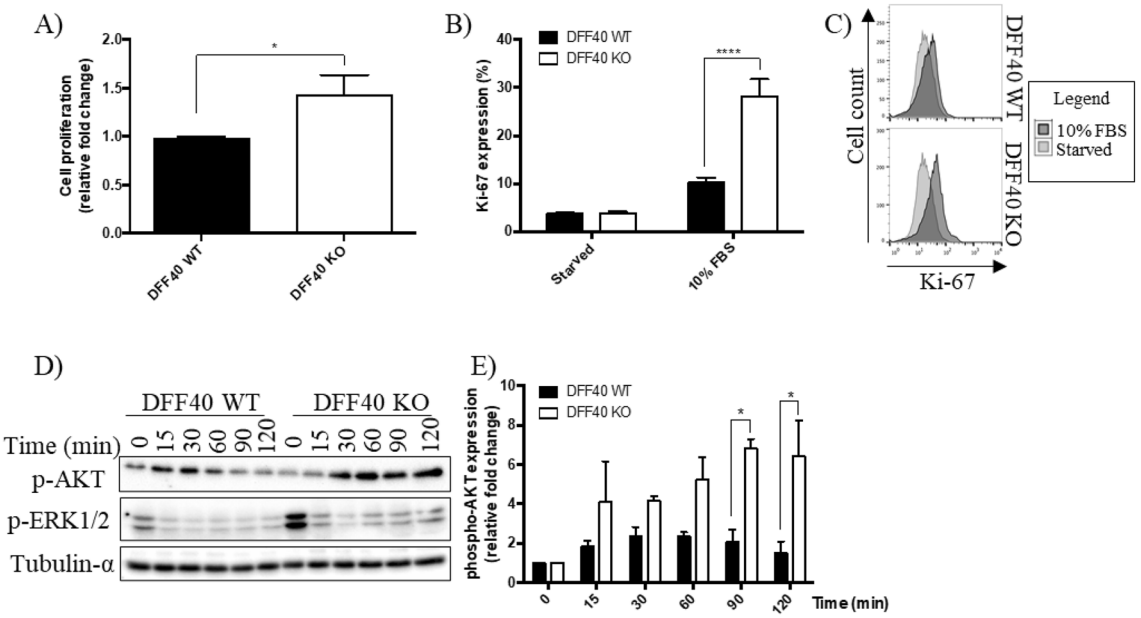


Fig. 5 DFF40-deficient Jurkat cells have higher Ki-67 expression, as well as AKT and ERK 1/2 phosphorylation. **A**: Jurkat DFF40 WT and DFF40 KO cells were seaded in a 96-well plate at a density of 1×10^6 cells/ml and incubated for 3 h with Alamar Blue (10%). Dye fluorescence was measured with the Spectra M5 Microplate reader at an excitation wavelength of 570 nm and emission wavelength of 585 nm. **B** and **C**: Jurkat DFF40 WT and DFF40 Jurkat cells were starved for 4 h in EBSS and incubated in RPMI medium supplemented with 10% FBS for 3 h. Cells were stained with the Ki-67 primary antibody (1:400) and Alexa-488 secondary antibody (1:1000). Cells were analyzed by flow cytometer. **D and E**: Jurkat DFF40 WT and DFF40 KO cells were starved for 4 h in EBSS and then incubated for up to 120 min in RPMI medium supplemented with 10% FBS. SDS-page nitrocellulose membranes were blotted for respective antibodies. Band densitometry was calculated using the Image Lab software. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed (*$p<0.05$, ****$p<0.0001$)

in lymphoid cells of transgenic zebrafish [53]. Pro-survival Bcl-2 family members, such as the Bcl-2 protein, have been shown to regulate invasion-related pathways via ROS production [54]. In non-apoptotic cells, overexpression of Bcl-2 enhances ROS production, which regulates the expression of invasion-related proteins such as mitogen-activated protein kinases (MAPK) [55–57]. In non-small cell lung carcinoma (NSCLC) cell lines, Mcl-1, a member of the Bcl-2 family proteins, was shown to promote cell migration through ROS generation [58]. The overexpression of Mcl-1 in non-apoptotic DFF40 KO cells could, thus, explain the higher redox state previously shown [9]. On the other hand, the inhibition of ROS induction inhibits Bcl-2 family members and their capacity to promote cell invasion [59]. All these results taken together point out that DFF40-deficient cells seem to present a more malignant profile of T cell leukemia. T cell leukemia are known for their metastatic profile and migratory patterns [60]. Thus, their capacity of metastasis could be enhanced, and the higher Bcl-2 gene transcription and Mcl-1 protein levels that are observed could be linked to a migratory pattern.

One of the key factors in cancer malignancy is the ability of cancer cells to extensively proliferate through metabolic reprogramming [61]. The Warburg effect is one of the most important metabolic alterations in cancer cells, where cancer cells have a preference for glycolysis in the presence of oxygen [62]. The elevated metabolic rate in cancer cells enhances ROS production, which is crucial for malignancy transformation [63, 64]. Our laboratory has previously shown that DFF40-deficient cells have a higher redox state in non-apoptotic conditions [9]. Numerous studies have reported the importance of ROS production on mitochondrial function and integrity through the regulation of mtDNA. Exposing isolated yeast mitochondria to hydrogen peroxide significantly enhance mtDNAcn [65]. In human mesangial cells, enhancing ROS levels through high glucose exposure increases mtDNAcn [66]. We thus sought to determine the impact of DFF40 deletion on mtDNA. In these following assays, we used two different cell models (e.g. Jurkat and HeLa cells) to provide confirmation that the observations are not cell specific but mostly gene related. We first assessed mitochondrial mass with the MitoTracker Deep Red probe. DFF40-deficient Jurkat and HeLa cells presented a significantly higher mitochondrial mass, in comparison to DFF40 WT cells (Fig. 3b and e). Qualitative measurement also showed a higher mitochondrial mass

A)

Parameters	**Computed values**
Net charge of query sequence	+13
Analysed region	27
Number of basic residues in targeting sequence	8
Number of acidic residues in targeting sequence	0
Cleavage site	27
Cleaved sequence	MLQKPKSVKLRALRSPRKFGVAGRSC
Probability of export to mitochondria	0.9389

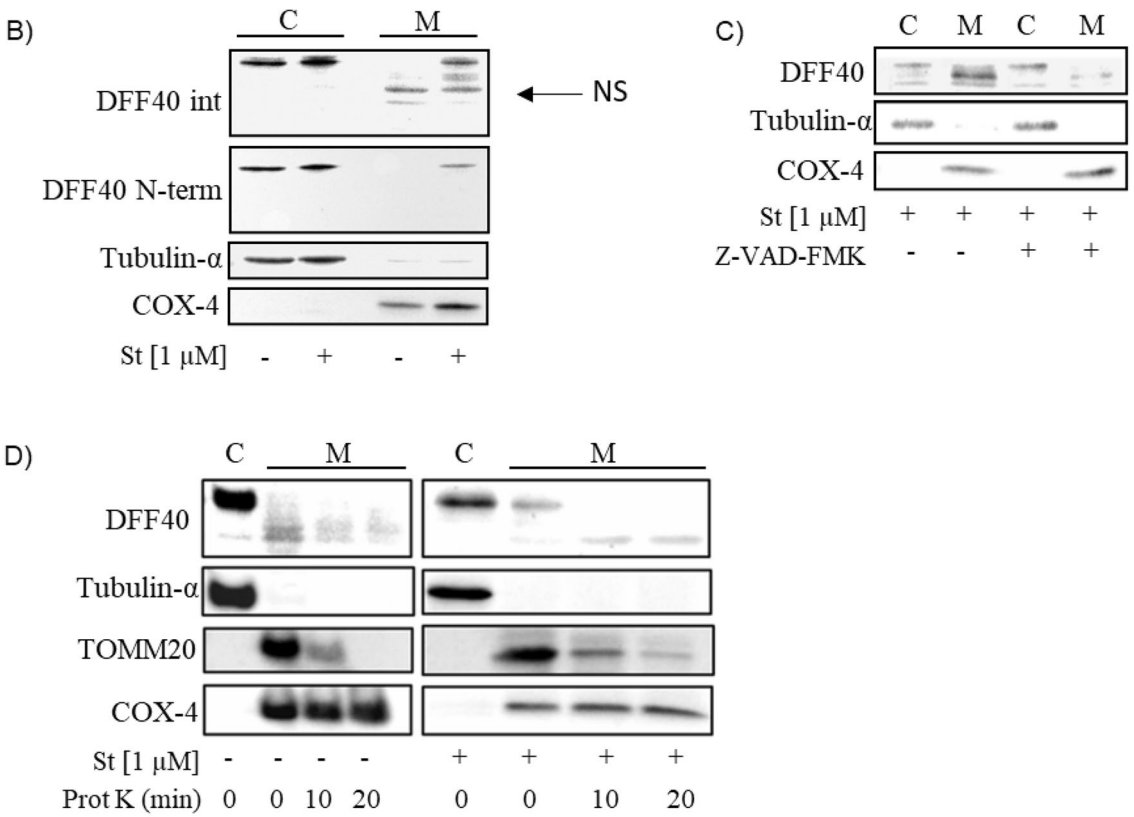


Fig. 6 Evidence of DFF40 in mitochondria of apoptotic Jurkat cells. A: Prediction of human DFF40 translocation to the mitochondria is of 0.9380; probability determined by in silico analysis using Mitoprot II (v.1.101) software. **B**: The presence of cytoplasmic (**C**) or mitochondrial (M) DFF40 was assessed by western blotting in Jurkat DFF40 WT cells after apoptosis induction with 1 μM STS for 2 h. Note that DFF40, identified with antibody specific to internal sequence (DFF int) or N-terminal sequence (DFF40 N-term) is highly expressed in the cytoplasmic fraction. NS: non-specific band. Tubulin-α and COX-4 were used as cytoplasm and mitochondrial markers, respectively. **C**: Same as in (**B**), except cells were also exposed or not to a caspase-3 inhibitor, Z-VAD-FMK [25 μM]. **D**: Proteinase K protection assay. Following cell treatments as in (**B**), mitochondria were isolated and pellets were exposed for 0, 10, or 20 min to proteinase K [100 μg/ml]. Correct digestion of mitochondrial proteins was assessed by immunoblotting with TOMM20. All experiments were performed at least 3 independent times. Images are representative of all data

through enhanced MitoTracker fluorescence in DFF40 KO cells (Fig. 3a). MitoTracker Deep Red has been shown to be a mitochondrial-potential-dependent dye [67]. We thus suspected that the higher mitochondrial mass could be linked to higher ROS production at basal state. In order to confirm that the higher mitochondrial mass was truly linked to higher mtDNA content, we quantified mtDNAcn by RT-qPCR (Fig. 3c and f). mtDNAcn is significantly enhanced in DFF40-deficient Jurkat cells and a similar non-significant pattern was observed in DFF40 KO HeLa cells. mtDNAcn is highly dependent on the number of mitochondria per cell. Once normalized per mitochondria per cell, DFF40 KO Jurkat cells still exhibit a significantly higher production of mtDNAcn (Fig. 3d). Having a higher mtDNAcn in stem-like cancer cells was shown to confer resistance to chemotherapy [68]. It was also shown in hair and HEI-OC1 cells that

having increased mtDNAcn confers a protection to drug-induced apoptosis [69]. DFF40-related apoptosis resistance could, thus, be partially explained by the upregulation of mtDNAcn. The relationship between ROS, mtDNA, and MMP has been thoroughly discussed in the literature [70]. Higher MMPs were found in lung cancer cell lines [71], as well as glioblastoma and breast cancer cell lines [72]. mtDNA deficiency in the T-47D breast cancer cell line was shown to induce lower MMP levels and impair cell proliferation and tumorigenicity [73]. We thus suspected that DFF40-deficient cells might present a higher MMP due to an increase in their mtDNAcn. MMP was assessed using the TMRE fluorescent probe (Fig. 3h). DFF40-deficient Jurkat cells have a significantly higher MMP at basal state. The depolarization of the outer mitochondrial membrane triggers the activation/recruitment of different signaling partners, such as PINK1 and PARKIN, which ultimately lead to mitophagy [11, 74]. Having a higher MMP in DFF40-deficient T cells, the required threshold to observe a depolarization in these cells would have to be higher in order to have the mitophagy process; a lower MMP induces the accumulation of PINK1 leading to the removal of damaged mitochondria [75]. We, therefore, suggest that these alterations in the MMP participate in the cell resistance mechanisms. Mitobiogenesis is a highly regulated process that allows the growth, division, and expansion of mitochondria [76]. In this study, we have shown that DFF40-deficient Jurkat cells have a higher mitobiogenesis response (Fig. 3i). Multiple studies have demonstrated the regulation of SDH-A (nuclear encoded) and COX-1 (mitochondrial encoded) protein levels as mitochondrial biogenesis markers [77–79], as they both localize and mediate their function in the mitochondria. It was shown that mitobiogenesis promotes tumor growth through autophagy resistance in epithelial cancer cell overexpressing PGC-1α [80]. Our results are in concordance with those in the literature, where we have demonstrated in this study that the higher mitobiogenesis response in DFF40-deficient Jurkat cells is mainly due to the higher mtDNA content, which can in its turn explain the previously demonstrated higher redox state in DFF40-deficient Jurkat cells [9]. Overall, these results suggest that DFF40 deficiency-mediated apoptosis resistance is linked to higher mtDNAcn and MMP, phenomenon of the metabolic reprogramming observed in tumorigenesis [81].

As stated above, malignant cancer cells rely on the Warburg effect to produce their energy, one of the key points to be addressed in metabolic reprogramming [11]. We thus sought to determine the energetic profile of DFF40-deficient cells. To assess the bioenergetic profile of our cell lines, we used the Seahorse XF technology by Agilent (Fig. 4). First, DFF40-deficient cells have a higher OCR at basal state and following apoptosis induction (Fig. 4c to e). The OCR/ECAR ratio is also higher in DFF40 KO cells and is less affected by TBT-induced apoptosis (Fig. 4f and g). Consistent with these results, TBT-induced apoptosis generated a larger intake of the glucose analog 2-NDBG in DFF40 KO Jurkat cells (Fig. 4i). A recent study performed on colon cancer cells has shown that a deficiency in BAX/BAK conferred a selective resistance to FDA-approved drugs [82]. This resistance to apoptosis came with an upregulated glycolysis pathway [82]. Cancer cells are known for their increased energetic metabolism through increased OCR [83]. Bioenergetic profile analyses of multiple cancer cell lines have shown that proliferation rates positively correlate with glycolysis ratios and are opposite to ROS generation [83]. We have previously demonstrated a more malignant profile of our DFF40 KO Jurkat cells throughout their resistance to TBT-induced apoptosis [9] and to STS-induced apoptosis in this study (Fig. 1). In addition to the higher OCR, many cancer cells are known to have an enhanced lactate production [84]. Lactate production is linked to the acidification of the extracellular compartment. The analysis of the OCR/ECAR ratio is a marker of glycolysis; a diminished ratio value suggests a higher glycolysis rate proven by an increased ECAR due to lactate production. Our results show that DFF40 KO cells have indeed a higher lactate production and depend largely on glycolysis for their metabolic energy in comparison to DFF40 WT cells. In concordance with the literature, our results show that TBT exposure does not alter basal respiration and OCR in DFF40 KO cells, in comparison to DFF40 WT cells who show a decrease in basal respiration and ATP production (Fig. 4b). When grown in an OXPHOS prone environment (Fig. 4c and S4), DFF40-deficient Jurkat cells exhibited lower ATP production levels, with no significant differences in OCR between both cell lines (DFF40 WT and DFF40 KO cells), suggesting non-altered levels of OXPHOS. It was shown in a meta-analysis that the contribution of OXPHOS to ATP production is highly variable according to cell type [85]. Furthermore, OXPHOS up- or downregulation may be related to mtDNA mutations or mtDNAcn [86]. Having shown in this study that mtDNAcn is upregulated, it was expected to observe normal or enhanced OXPHOS levels in DFF40-deficient cells. Recent studies support the idea that highly metastatic cancer cells depend on OXPHOS regardless of their mtDNAcn, and having higher mtDNAcn is not an absolute factor to enhanced OXPHOS levels [87]. Overall, this new role of DFF40 in the regulation of mitochondria indicates that apoptosis and responses to stress could be altered in the mitochondria of DFF40 KO cells, a phenotype found in malignant brain tumors [8].

In the literature, it is suggested that OCR regulates the growth of tumors, thereby providing a more malignant profile [88]. No studies until today have reported a link between DFF40 expression and cell proliferation. We thus sought to determine the impact of DFF40 deficiency on

cell proliferation. We first quantified cell proliferation with Alamar Blue, a fluorescent probe that is widely used as an indicator of metabolic function [89]. DFF40-deficient Jurkat cells have a significantly higher proliferation rate (Fig. 5a). We next wanted to confirm the higher cell proliferation rate with a more specific marker. One of the most widely used proliferation markers is the expression of Ki-67, which its expression is strictly associated to cell proliferation [90]. We found that DFF40-deficient cells express 30% more the Ki-67 marker (Fig. 5b and c). Recently, roles for Ki-67 in cell-cycle regulation have been proposed [28]. One of the main pathways that is involved in cell-cycle progression and neoplastic transformation is the PI3K/AKT pathway [27]. Many studies have reported the importance of AKT phosphorylation in tumor cell lines and human malignancies [91]. Overactivation of AKT is frequently detected in ovarian cancers and its inhibition by LY294002 promotes apoptosis [92]. It was shown in human colorectal carcinoma that phosphorylation of AKT was required for tumor progression and suppression of cell apoptosis [93]. Overactivation of AKT is also linked to phosphorylation of mTOR, a serine/threonine protein kinase of the PI3K-related kinase family that promotes cell growth [92, 94]. Our results are in concordance with those in the literature, where we have demonstrated that apoptosis-resistant DFF40-deficient Jurkat cells have higher phosphorylation levels of AKT in a time-dependent manner (Fig. 5d and e). DFF40 WT Jurkat cells displayed an early phosphorylation of AKT, but this process was rapidly inhibited by dephosphorylation of AKT. The change in the state of phosphorylated AKT between both cell lines could thus explain the higher proliferative state in DFF40-deficient cells. A close association between AKT phosphorylation and Ki-67 expression was also demonstrated in human colorectal carcinoma, supporting our data from this study [93]. Many studies have also reported that an aberration in AKT pathway is commonly linked with alterations in the ERK pathway [95]. In this study, we have also demonstrated that starved DFF40-deficient Jurkat cells present higher phosphorylated ERK1/2 levels (Fig. 5d). It was shown that high ERK protein expression influences tumorigenicity; patients with triple-negative breast cancer tumors that have a higher expression of ERK1/2 have a lower overall survival rate [96]. On the other hand, the addition of growth factors to the media abolishes ERK phosphorylation (Fig. 5d). The ERK pathway has many functions such as in cell growth, migration, and differentiation [97]. In restrictive conditions (e.g. withdrawal of survival factors), the ERK signaling pathways allow cell protection towards apoptosis [98]. In this context, the addition of FBS rescues cell from potential cell death by apoptosis, explaining the inhibition of ERK pathway activation. Overall, our results suggest that DFF40 deficiency could be an important key element in tumorigenicity. Resistance to apoptosis in DFF40-deficient cells could be explained by an upregulation of AKT and ERK 1/2 phosphorylation, as well as Ki-67 upregulation that allows a faster transition through the cell cycle.

Having shown that DFF40 deficiency alters mitochondrial function and integrity, we next wondered if DFF40 could localize to mitochondria. Intending to verify the hypothesis of a potential localization of DFF40 in the mitochondria, we performed an *in silico* analysis with the Mitoprot II biotool. The analysis confirmed that a pool of the protein might be localized in the mitochondria (probability of export to mitochondria = 0.9389) and the predicted sequence would consist of 26 amino acids cleaved in the NTD. The expression of DFF40 in the mitochondria was confirmed with western blot analysis on mitochondria pellets, showing an expression of the protein in the cytoplasm as expected (Fig. 6b). We did not observe any mitochondrial DFF40 in non-apoptotic cells. To assess how DFF40 is translocated to the mitochondria, the importance of apoptosis in this process was next studied. Cell fractionation (Fig. 6b) confirmed an increase in the mitochondrial localization of the endonuclease following an exposure to STS [1 µM]. To confirm if this translocation is dependent on apoptosis signals, we used an inhibitor of caspase-3 and DFF40 expression was evaluated in mitochondria pellets following treatment with STS (Fig. 6c). The Z-VAD-FMK inhibitor reduced the translocation of DFF40 in STS-treated Jurkat cells, indicating that the mitochondrial accumulation of the enzyme is an apoptotic process. Mitochondrial proteins are mostly imported and go through multiple proteolytic processing and refolding [99]. Heavy membranes from the endoplasmic reticulum and lysosomes are two major potential contaminants of purified mitochondrial fractions. Topologies regarding protein anchoring at the MOM exist. They can be anchored by respecting 7 different topologies, depending on whether the *N*- and/or *C*- terminal regions are in the intermembrane space, or completely inserted in the MOM [100]. Proteins of the mitochondria are majorly located in the intracellular compartment, whereas only 8 to 10% of total proteins can be found on the MOM [100]. These proteins are encoded in the nucleus and synthesized as precursors in the cytosol before being transported to the mitochondria using different pathways [100]. Intending to assess if DFF40 is a MOM protein, we performed a proteinase K protection assay, following STS exposure (Fig. 6d). Results demonstrated that DFF40 localizes exclusively to the outer mitochondrial membrane. Protection to proteinase K resulting to outer and inner mitochondrial membranes localization point out that DFF40 does not enter mitochondria in its native form. These results need more investigation to understand the reason for DFF40

localization. Nevertheless, it is known that multiple apoptosis-regulating proteins translocate to the mitochondria during cell death, such as p53, Akt, and Bcl-2 family members [101–103]. The mechanism in which DFF40 expression abolition induces resistance to apoptosis being still unclear, further investigation on the involvement of mitochondrial proteins have been needed.

In conclusion, the results in the present study suggest the importance of DFF40 in regulating mitochondrial functions and integrity following exposure to apoptosis-inducing agents such as STS and TBT. DFF40 KO Jurkat cells are resistant to STS and TBT-induced apoptosis and present higher Mcl-1 protein levels at basal state. Following TBT treatment, DFF40 KO Jurkat cells have an enhanced Bcl-2 phosphorylation. A deficiency in DFF40 confers Jurkat cells an increased mitochondrial mass, mtDNAcn, MMP and glycolysis rates in a non-apoptotic environment that could explain their malignancy. The induction of apoptosis in those cells mimics the Warburg effect, where cancer T cells have an increase of glucose uptake and higher OCR, without any change in OXPHOS levels. Our team is the first one to provide evidences that following STS-induced apoptosis, DFF40 translocates to the mitochondria. It could be hypothesized that a deficiency in DFF40 could be responsible of chemotherapy resistance by regulating the metabolism of tumor cells through mitochondrial and cell-cycle alterations.

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Data availability Data and raw material will be available upon request.

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Declarations

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5 CHAPITRE 5: DFF40/CAD DEFICIENCY IN LEUKEMIA T-CELLS IMPAIRS GENOMIC STABILITY THROUGH AKT-MEDIATED P38 INHIBITION AND INDUCES ALTERATIONS IN DNA REPAIR PATHWAYS

Titre de l'article : *La déficience en DFF40/CAD dans les cellules T de leucémies altère la stabilité génomique par l'inhibition de la voie p38 MAPK dépendante de AKT et induit des altérations dans les voies de réparation de l'ADN.*

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CV : Analyses statistiques et révision du manuscrit.

JB : Conception du projet et de la méthodologie, analyses statistiques, supervision, rédaction et révision du manuscrit.

5.1 Résumé en français

Une altération de la voie de signalisation de l'apoptose a été rapportée dans de nombreuses cellules cancéreuses et elle serait liée à la tumorigenèse. Le facteur de fragmentation de l'ADN 40 (DFF40) est l'endonucléase clé impliquée dans la clairance du matériel génomique par la génération de cassures d'ADN double brin. Une altération de son expression et/ou de son activité a été rapportée dans de nombreux sous-types de cancer et liée à un mauvais pronostic clinique. Des études récentes ont souligné son importance dans l'instabilité génomique, où sa sous-expression s'est avérée induire l'accumulation de gènes mutés. Cependant, l'impact du déficit en DFF40 sur les voies de réponse aux dommages à l'ADN reste encore incertain. Dans cette étude, nous avons cherché à déterminer l'impact du déficit en DFF40 sur les voies de réparation de l'ADN et ses voies de régulation. Nous avons démontré que les cellules Jurkat déficientes en DFF40 ont une réponse au stress oxydatif plus élevée après l'apoptose induite par le TBT, marquée par des niveaux de ROS plus élevés et une régulation significativement retardée des transcrits d'ARNm de NRF2 et SOD1. En outre, la résistance à l'apoptose induite par le TBT chez les cellules déficientes en DFF40 induit l'inhibition de la voie p38 MAPK médiée par AKT. L'abolition de l'expression du DFF40 dans les cellules Jurkat inhibe la formation de foyers de γH2AX induite par le TBT, tandis que la formation de γH2AX dans les cellules DFF40 WT n'est pas colocalisée aux extrémités télomériques. Nous avons ensuite démontré que les cellules Jurkat déficientes en DFF40 ont des niveaux d'activation significativement plus faibles de la voie de réparation ATM par rapport aux cellules DFF40 WT, marquées par des niveaux inférieurs de CHK2 phosphorylé. Nous avons démontré que l'apoptose induite par le TBT augmentait significativement la phosphorylation de CHK1 dans les cellules KO DFF40, suggérant une activation de la voie de réparation d'ATR. Dans l'ensemble, notre étude révèle qu'une déficience en DFF40 dans les cellules cancéreuses confère une résistance à l'apoptose par la régulation positive d'AKT et l'inhibition ultérieure de la voie p38 MAPK. Le sauvetage de la survie cellulaire est assuré par l'activation d'ATR et la désacétylation des histones, conférant ainsi une instabilité génomique.

5.2 Abstract

Impairment of the apoptotic pathway has been reported in many cancer cells and been linked to tumorigenesis. The DNA fragmentation factor 40 (DFF40) is the key endonuclease involved in genomic material clearance through the generation of double-strand DNA breaks. An alteration in its expression and/or activity has been reported in many cancer subtypes and linked to a poor clinical outcome. Recent studies have been underlying its importance in genomic instability, where its under-expression has shown to induce the accumulation of mutated genes. However, the impact of DFF40 deficiency on DNA damage response pathways still remains unclear. In this study, we sought to determine the impact of DFF40 deficiency on DNA repair pathways and its regulatory pathways. Here, we demonstrated that DFF40-deficient Jurkat cells have a higher oxidative stress response following TBT-induced apoptosis, marked by higher ROS levels and a delayed upregulation of NRF2 and SOD1 mRNA transcripts. Furthermore, TBT-induced apoptosis resistance in DFF40 deficient induced AKT-mediated p38 MAPK pathway inhibition. DFF40 expression abolition in Jurkat cells inhibited TBT-induced γH2AX foci formation, moreover γH2AX formation in DFF40 WT cells did not colocalize at telomeric ends. We then demonstrated that DFF40 deficient Jurkat cells have significantly lower activation levels of the ATM repair pathway in comparison to DFF40 WT cells, marked by lower levels of phosphorylated CHK2. We showed that TBT-induced apoptosis significantly increased CHK1 phosphorylation in DFF40 KO cells, suggesting an activation of the ATR repair pathway. Overall, our study reveals that DFF40 deficiency in cancer cells confers resistance to apoptosis through AKT upregulation and subsequent p38 pathway inhibition. Cell survival rescuing is ensured by ATR activation and deacetylation of histones, thus conferring genomic instability.

5.3 Introduction

Apoptosis is a programmed cell death mechanism that is highly sophisticated and involves a series of caspase-dependent molecular events ultimately leading to DNA fragmentation (M. Kulbay *et al.*, 2021c). The end goal of apoptosis is to destroy and eliminate abnormal cells in order to ensure cell and tissue homeostasis. An impairment in the apoptotic pathway can lead to genomic instability, therefore allowing tumorigenesis (Zhivotovsky *et al.*, 2004). An important player of the apoptotic pathway is the DNA fragmentation factor 40 (DFF40) endonuclease, whose role is to induce the internucleosomal fragmentation of genomic DNA (Merve Kulbay *et al.*, 2021a). Following the activation of the extrinsic and intrinsic apoptotic pathways, the caspase-3 effector cleaves the inhibitory DFF45 subunit, allowing the release and activation of the catalytic activity of the DFF40 (M. Kulbay *et al.*, 2021c). Over the past years, a role for DFF40 in genomic instability has been proposed (Y. Errami *et al.*, 2013a, Hain *et al.*, 2016a, Merve Kulbay *et al.*, 2021a, Yan *et al.*, 2006b, Yan *et al.*, 2009). Primary evidence has supported the hypothesis of a malignant phenotype in cancer cells with low DFF40/DFF45 expression. A homozygous deletion in the 1p36.2-p36.3 genomic locus of neuroblastoma cancer cell lines was reported and shown to be linked to an unfavorable prognosis (Ohira *et al.*, 2000). Poor clinical outcomes were also reported in esophageal carcinomas (Konishi *et al.*, 2002), human hepatoma cancer cell lines (Hsieh *et al.*, 2003a), uterine leiomyosarcomas (Banas *et al.*, 2017), non-endometrioid and high-grade endometrioid endometrial cancers (Banas *et al.*, 2018b) and glioblastomas (Sánchez-Osuna *et al.*, 2016) with altered DFF40 expression. More recently, it was shown that DFF40 expression rescue in T47-D breast cancer cells (Bagheri *et al.*, 2015a, Bagheri *et al.*, 2014) and in melanoma cancer cells with a survivin promoter (Minaiyan *et al.*, 2021) established the sensitivity of these cells to apoptosis-inducing agents. Yan and colleagues were amongst the first groups to demonstrate a direct link between DFF40 and genomic stability *in vitro*: In DFF45/ICAD mutant cancer cell lines, which lack DFF activation and subsequent DNA fragmentation, an increase in the frequency of mutated genes following radiation exposure was observed (Yan *et al.*, 2006b). Furthermore, they demonstrated an increase in chromosol aberrations (*i.e.* chromosomal terminal deletion, ring chromosome, dicentric chromosome and chromatid break) in bone marrow cells collected from $CAD^{-/-}$ mice following radiation exposure (Yan *et al.*, 2006b). These findings were associated with an increased survival (Yan *et al.*, 2006b). Overall, the impairment of the DFF40 or DFF45 gene expressions favours a pro-oncogenic environment, allowing the expansion of these tumoral cells possibly through altered DNA damage response (DDR) pathways, thus establishing genomic instability.

The harmonious interaction between the regulation of apoptosis and genomic instability is ensured by DNA-damage repair proteins, mainly involved in the ATM (*i.e.* ataxia telangiectasia mutated) and ATR (*i.e.* ATM and rad3-related) pathways (Menolfi *et al.*, 2020). The DDR pathways are initiated through the phosphorylation of the histone H2AX (γH2AX) (Mah *et al.*, 2010). Following double-strand DNA breaks (DSBs), rapid γH2AX foci formation creates an epigenetic signal that initiates DSBs signalling by increasing chromatin accessibility and enhancing the recruitment and accumulation of DDR proteins at these phosphorylated sites (Kinner *et al.*, 2008, Melillo, 2004). In first hand, ATM pathway activation relies on the recruitment of a number of molecular partners at damaged sites following DSBs exclusively (Nam *et al.*, 2011). The MRN complex (MRE11-RAD50-NBS1) is one of the crucial components involved in ATM activation (Shiloh *et al.*, 2013). Once activated, ATM induces the phosphorylation and modulation of several protein kinases (Shiloh *et al.*, 2013). The main substrate and the best documented kinase is the checkpoint kinase 2 (CHK2). The main substrates of the CHK2 are proteins involved in DNA repair, cell cycle regulation and apoptosis (Zannini *et al.*, 2014). On the other hand, regarding the ATR pathway, it is activated following the recruitment of ATRIP (*i.e.* ATR interacting protein), a checkpoint clamp (*i.e.* RAD9-HUS1-RAD1) and TOPBP1 (*i.e.* topoisomerase binding protein 1) to damaged sites (Nam *et al.*, 2011). DNA damages that lead to an ATR activation are more diverse, including DSBs, protein adducts and crosslinks (Cimprich *et al.*, 2008). The ATR pathway ultimately leads to the phosphorylation and activation of the checkpoint kinase 1 (CHK1), which is involved in the regulation of cell cycle (Nam *et al.*, 2011).

A recent study by Hain and colleagues demonstrated that in the absence of DFF40/CAD-mediated DNA damage, TRF2 loss from telomeres is compromised (Hain *et al.*, 2016a). The DNA-dependant protein kinase (DNA-PK) was shown to induce γH2AX foci formation, enhance TRF2 loss from telomeres and activate DNA repair pathways (Hain *et al.*, 2016a). Previously, our team has shown that DFF40 deficient Jurkat cells were resistant to tributyltin (TBT)-induced apoptosis, through apoptotic pathway modulations and lacked γH2AX foci formation (Merve Kulbay *et al.*, 2019). Furthermore, DFF40-deficiency conferred sensibility to topoisomerase II (TOP2) inhibitors, such as etoposide (ETO), but a resistance to antimetabolite chemotherapeutic drugs such as cytarabine (Ara-C) was observed (Merve Kulbay *et al.*, 2021b). Hypothesizing that DFF40 might act as the DNA-PK (Merve Kulbay *et al.*, 2021a), the aim of this study was to demonstrate the impact of DFF40 deficiency on DNA repair pathways following insult-specific DNA damages.

5.4 Methods

5.4.1 Cell culture

The Jurkat DFF40 WT and DFF40 KO cell populations were previously generated by our lab using the CRISPR-cas9 technology (Merve Kulbay *et al.*, 2019). Jurkat DFF40 WT and DFF40 KO cells were kept in culture in RPMI 1640 (Gibco, ON, CA) media, supplemented with 10% heat-inactivated foetal bovine serum (FBS; Wisent, QC, CA), 100 U/ml penicillin (Wisent, QC, CA), 100 µg/ml streptomycin (Wisent, QC, CA) and 10 mM HEPES (Wisent, QC, CA). Cell density was kept between a range of 0.3 to 1 M cells per ml and cells in culture were replaced every 90 days.

5.4.2 Reactive oxygen species quantification

Intracellular ROS was quantified using the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe (Invitrogen, Cat# D399). Briefly, Jurkat DFF40 WT and DFF40 KO cells ($1x10^6$ cells/ml) were pre-incubated with the H_2DCFDA probe (10 µM) for 45 min at 37 °C with 5% CO_2. Cells were then washed once with PBS 1X and resuspended in complete RPMI 1640 media supplemented with 0.1% FBS. Cells were treated or not with TBT (0.4 µM) and ROS production was assessed continuously at different time points by flow cytometer with a FACSCalibur from Becton Dickinson (BD FACSCalibur Flow Cytometry System, RRID:SCR_000401). A total of 10,000 events per condition was acquired.

5.4.3 Mitochondrial membrane potential measurement

Mitochondrial membrane potential (MMP) was assessed using the TMRE-Mitochondrial membrane potential assay kit (Abcam Cat#ab113852), as previously described (Merve Kulbay *et al.*, 2022). Briefly, Jurkat DFF40 WT and DFF40 KO cells ($1x10^6$ cells/ml) were treated or not with TBT (0.4 µM) or FCCP (20 µM) in complete RPMI media supplemented with 0.1% FBS for 2 h or 15 min respectively. Cells were then washed twice with PBS 1X and stained with TMRE (300 nM) for 20 min at 37 °C. A total of 10,000 cells were analyzed by flow cytometer.

5.4.4 RNA extractions and RT-qPCR assays

RNA extractions and RT-qPCRs were performed exactly as previously described (Merve Kulbay *et al.*, 2022). Prior to RNA extractions, Jurkat DFF40 WT and DFF40 KO cells were treated or not with TBT [0.4 µM] for 2, 4 or 6 h in RPMI media supplemented with 0.1% FBS. Forward and reverse primers are listed in Table 1. RT-qPCRs were performed using the SsoAdvanced

Universal SYBR Green Supermix kit (Bio-Rad, Cat#1,725,271) and the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad, Cat#1,725,035). Results were analyzed with the CFX Maestro™ Software (Bio-Rad, Cat# 12,004,110).

Table 1. Primer sequences for RT-qPCR

	Sequences	
Gene	Forward (5' – 3')	Reverse (3' – 5')
NRF2	CACATCCAGTCAGAAACCAGTGG	GGAATGTCTGCGCCAAAAGCTG
SOD1	TGCTGGTTTGCGTCGTAGTCT	CATGCAGGCCTTCAGTCAGTC
SOD2	GTTGGCTTGGTTTCAATAAGG	GAAGGTAGTAAGCGTGCTCC

5.4.5 Western blotting

SDS-PAGE was performed as previously described, but with some modifications (Merve Kulbay *et al.*, 2022). Briefly, following treatments, Jurkat DFF40 WT and DFF40 KO cells were washed twice with PBS 1X and suspended in 100 µl of laemmli buffer 1X. Samples were then incubated at 95 °C for 10 min before electrophoresis. Following protein transfers on nitrocellulose membranes using the Trans-Blot Turbo Transfer System of Bio-Rad, membranes were blocked in 5% (m/v) skimmed milk for 2 h at room temperature. Following primary antibodies were incubated overnight at a final dilution of 1: 2 000 at 4 °C: anti-SOD2, anti-phosphoSer473-AKT (Cell Signaling Technology Cat# 9271, RRID:AB_329825), anti-phosphoThr180/Tyr182-p38 (Cell Signaling Technology Cat# 9211, RRID:AB_331641), anti-acetyl histone H3 (Cell Signaling Technology Cat# 9677, RRID:AB_1147653), anti-acetyl histone H4 (MBL International Cat# JM-3656-100, RRID:AB_591894), anti-CHK2 (Cell Signaling Technology Cat# 2662, RRID:AB_2080793), anti-CHK1 (Cell Signaling Technology Cat# 2360, RRID:AB_2080320), anti-phosphoThr68-CHK2 (Cell Signaling Technology Cat# 2661, RRID:AB_331479), anti-phosphoSer345-CHK1 (Cell Signaling Technology Cat# 2348, RRID:AB_331212), anti-HMGB1 and anti-GRP78 (Cell Signaling Technology Cat# 3177, RRID:AB_2119845). Respective

secondary HRP conjugated antibodies were incubated for 1 h at room temperature (diluted 1: 20 000). Analyses was performed according to previously described method.

5.4.6 Immunofluorescence assays

Immunofluorescence staining was performed as previously described, with some modifications (Freppel *et al.*, 2018). Briefly, Jurkat DFF40 WT and DFF40 KO cells were treated or not with TBT (0.4 μM) in complete RPMI media supplemented with 0.1% FBS for 1 h. Following treatments, cells were washed twice with PBS 1X. Microscope slides were coated with collagen type I during 1 h at room temperature, then washed 3 times with sterile ddH_2O and dried. Cells were layered on microscope slides by cytospin (3 min, 800 RPM). Cells were fixed for 20 min at room temperature with 4% PFA, then washed 3 times with PBS 1X. Cells were permeabilized with PBS/Triton X-100 solution (0.2%) for 15 min at room temperature. Cells were then washed thrice with PBS 1X and incubated for 1 h at room temperature in the blocking solution (5% (m/v) BSA, 0.05% (m/v) sodium azide and 10% (v/v) goat serum in PBS). Slides were then incubated with primary antibodies anti-H2AX (Cell Signaling Technology Cat# 9718, RRID:AB_2118009) and anti-TRF2 (Abcam Cat# ab13579, RRID:AB_300474), diluted at 1:600 and 1:200 respectively in the blocking solution without goat serum, and incubated for 2 h at room temperature. Respective secondary antibodies (diluted at 1:1000) were incubated for 1 h at room temperature in the dark, followed by DAPI staining (diluted 1: 15 000) for 10 min at room temperature in the dark. Slides were washed thrice in between each step with PBS 1X, followed by a last wash with sterile H_2O. Coverslips were mounted on slides using fluoromount G and left at room temperature overnight in the dark. The following day, slides were stored at 4 °C until imaging. Immunofluorescence images were acquired using a LSM780 confocal microscope (Carl Zeiss Microimaging) at the Confocal Microscopy Core Facility of the INRS-Centre Armand-Frappier Santé Biotechnologie.

5.4.7 Statistical analyses

All experimental data are expressed as mean ± SEM. Student's t-test were performed when two groups were compared, or Two-way ANOVA with Tukey's posthoc test was performed when 3 groups or more were compared. Statistical analysis was performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Differences were considered statistically significant when $p < 0.05$.

5.5 Results

5.5.1 DFF40 deficient cells exhibit an upregulation of their oxidative stress metabolism after apoptosis induction through delayed downregulation of NRF2 and SOD1

Cancer cells are known to adapt to their microenvironment by regulating their redox balance. Levels of ROS production can either have a tumor-promoting or tumor-inhibiting function (Liou *et al.*, 2010). We thus sought to determine the impact on oxidative stress response of DFF40 deficient Jurkat cells, by measuring ROS production, mitochondrial mass and MMP following TBT-induced apoptosis (Fig. 1). Both cell lines display a similar kinetic curve form for ROS production, its peak being reached at 90 min following TBT exposure (Fig.1a and 1b). DFF40 deficient Jurkat cells exhibit a greater response to oxidative stress, with higher levels of ROS production (47.67% ± 4.93%) at the 90 min time point, in comparison to DFF40 WT cells (24.35% ± 2.89%). The maximal production of ROS is maintained until 120 min in both cell lines, followed by a progressive decrease. One of the early hallmarks of apoptosis is the depolarization of the inner MMP, mainly due to ROS production (M. Kulbay *et al.*, 2021c, Park *et al.*, 2011). MMP was shown to be linked to cancer malignancy, where cancer cells exhibit elevated levels of MMP (Porporato *et al.*, 2018, Bei-bei Zhang *et al.*, 2015). We have previously shown that DFF40 deficient Jurkat cells have a higher MMP level at basal state (Merve Kulbay *et al.*, 2022). We thus sought to determine the impact of apoptosis induction on MMP levels (Fig. 1c and 1d). Following TBT-induced apoptosis, both DFF40 WT and DFF40 KO Jurkat cells have a decrease in their MMP. The decrease is significantly lower in DFF40 KO Jurkat cells; DFF40 KO Jurkat cells have a 0.68-fold decrease in MMP, whereas the decrease in DFF40 WT Jurkat cells is of 0.54-fold following TBT-induced apoptosis. Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), an uncoupling agent, almost completely abolishes MMP in both cell lines.

Oxidative stress levels are regulated by many signaling pathways, involving mediators of the mitogen-activated protein kinases (MAPKs) pathways and antioxidant gene expression levels (Liou *et al.*, 2010). We thus sought to determine if ROS-promoting signaling pathways were regulated upon apoptosis induction in DFF40 deficient Jurkat cells (Fig. 2). First, following apoptosis induction by TBT, DFF40 deficient Jurkat cells have significantly lower levels of upregulation of antioxidant genes, mainly NFR2 and SOD1 (Fig. 2a and 2b), in comparison to DFF40 WT Jurkat cells. NRF2 RNA expression is significantly increased by 1.68-fold in DFF40 WT cells while it remained unchanged in DFF40 KO Jurkat cells, 2 h following TBT exposure. On the other hand, SOD1 RNA levels are 1.12-fold higher in DFF40 WT Jurkat cells, following a 4 h

TBT exposure time. No significant changes are observed in SOD2 RNA levels between both cell lines following TBT-induced apoptosis (Fig. 2c). Knowing that protein expression might be regulated downstream of RNA levels through post-transcriptional modifications, we investigated the protein expression of SOD2 in Jurkat DFF40 WT and DFF40 KO cells (Fig. 2d and 2e). No significant differences are observed in SOD2 protein expression regulation between DFF40 WT and DFF40 KO Jurkat cells following apoptosis induction. Overall, enhanced ROS production in DFF40 KO Jurkat cells following TBT-induced apoptosis could partially be explained by higher MMP and a delayed upregulation of NRF2 and SOD1 mRNA.

5.5.2 DFF40 deficient Jurkat cells exhibit AKT mediated downregulation of p38 signaling pathway following TBT-induced apoptosis.

Redox homeostasis and pro-tumorigenic signaling in cancer cells has been shown to be linked to PI3K/AKT hyperactivation (N. Koundouros *et al.*, 2018b). Furthermore, studies have demonstrated the importance of ROS production through the MAPK pathway activation, a key molecular signaling pathway for cell proliferation and differentiation (Son *et al.*, 2011). We thus sought to determine whether DFF40 deficiency altered MAPK and AKT activation pathways following TBT-induced apoptosis (Fig. 3). First, DFF40 KO Jurkat cells grown in serum deprived media (*i.e.* control cells) exhibit approximately 3.5 times higher phosphorylation levels of AKT (Fig. 3a and b). Following apoptosis induction by TBT, AKT activation is significantly higher in DFF40 deficient Jurkat cells up until 60 min in comparison to DFF40 WT cells, which do not have a significant change in phospho-AKT levels. On the other hand, the expanded role of p38 MAPK has been thoroughly described in the literature (Gräb *et al.*, 2019). Sustained activation thresholds of p38 was shown to be linked to cell senescence and cell death, whereas low activation levels in cancer cells promotes cell survival (Martínez-Limón *et al.*, 2020). We thus wondered the impact of DFF40 deficiency on p38 activation following an apoptotic signal. Following TBT-induced apoptosis, DFF40 KO Jurkat cells have significantly lower levels of p38 activation in comparison to DFF40 WT cells (Fig. 3a and c). At its highest peak of activation (*i.e.* at 60 min), we observe a 5.7 fold increase in phospho-p38 levels in DFF40 WT cells, in comparison to a 1.7 fold increase in DFF40 KO cells (Fig. 3c). Overall, these results suggest that TBT-induced ROS production in DFF40 deficient Jurkat cells mediates the activation of the AKT pathway, a key component involved in cell proliferation, which in its turns allow cell survival through downregulation of the p38 signaling pathway.

5.5.3 H2AX foci formation following TBT-induced apoptosis is abolished in DFF40-deficient cells

One of the major roles of ROS production is the mediation of DNA damages through double strand breaks (DSBs) (Srinivas *et al.*, 2019). The phosphorylation of the H2AX histone (γH2AX) is required for the activation of the DNA damage response (DDR) pathways (Srinivas *et al.*, 2019). DNA damages can occur at telomeres (Longhese, 2008) and it was shown that γH2AX foci formation is more likely to occur at telomeric regions (Olive, 2009). Previously, we have shown that DFF40 deficient Jurkat cells do not exhibit γH2AX formation following apoptosis-induction by Western Blot (Merve Kulbay *et al.*, 2019). Here, we sought to quantify the exact number of γH2AX foci per cell and determine the localization of these modifications (*i.e.* telomeric colocalization) (Fig. 4). As expected, TBT-treated DFF40 WT cells have a significant induction of γH2AX foci formation (Fig. 4a). Approximately eleven γH2AX foci are detected per cell. Although γH2AX focis in DFF40 WT Jurkat cells are localized in the nucleus, we did not find any colocalization of γH2AX with TRF2, a protein present at telomeres. As expected, DFF40 KO Jurkat cells do not exhibit histone H2AX phosphorylation following TBT-induced apoptosis (Fig. 4).

5.5.4 DFF40 deficient cells have an altered ATM and ATR pathway signalization through downregulated CHK2 and upregulated CHK1 phosphorylation respectively

DNA damages are sensed by two main DDR pathways, known as the ATM or ATR pathway. The relationship between ATM and ATR activation in cancer cells is highly complex: cancer cells can survive with mutations in the ATM pathway, whereas ATR inhibition can enhance tumor cell death (Smith *et al.*, 2010). As mentioned above, DNA repair pathways are activated following H2AX phosphorylation. Having shown that DFF40 KO Jurkat cells are impaired in H2AX phosphorylation following TBT-induced apoptosis, we thus sought to determine the impact of DFF40 deficiency on ATM and ATR activation. First, we assessed the levels of HMGB1 protein in DFF40 WT and DFF40 KO cells, in order to confirm the absence of necrosis, a physiological cell death mechanism that lacks DNA damages. Following TBT-exposure, DFF40 WT and DFF40 KO do not have any changes in HMGB1 expression (Fig. 5b and 5f). Next, we demonstrated that DFF40 deficient Jurkat cells have lower phosphorylation levels of CHK2 at residue Thr68 (1.2-fold increase) in comparison to DFF40 WT cells (4.3-fold increase) following TBT-induced apoptosis at 4 h (Fig. 5a and 5c). Furthermore, exposure to TBT for 2 h significantly enhances CHK1 phosphorylation at residue Ser345 in DFF40 KO Jurkat cells by a 2.9-fold change, whereas no changes are observed in DFF40 WT cells (Fig. 5a and 5d).

Chromatin structure remodeling is highly dependent on histone acetylation and deacetylation, a process that is crucial for the maintenance of genomic stability (Bhaskara *et al.*, 2010). Histones are required to be deacetylated to ensure proper chromatin structure following the repair of DSBs (Bhaskara *et al.*, 2010). We thus wondered if the altered signaling in DNA repair pathways in DFF40 deficient cells could be explained by histone modulations. We have shown that DFF40 KO Jurkat cells show a delayed modulation in acetyl-histone H3 expression (1.03-fold increase) in comparison to DFF40 WT cells, which exhibit a 1.7-fold increase following a 4 h exposure to TBT (Fig. 5b and 5e, left panel). No changes in histone H4 acetylation is observed between DFF40 WT and DFF40 KO Jurkat cells following TBT-induced apoptosis (Fig. 5b and 5e, right panel). We have previously shown that DFF40 deficiency conferred sensitivity to ETO, a TOP2 inhibitor, whereas a resistance to Ara-C (an antimetabolite) was observed (Merve Kulbay *et al.*, 2021b). We thus investigated the impact of DFF40 deficiency on DNA repair pathway activation (Fig. 2S). No significant differences were observed between DFF40 WT and DFF40 KO cells in CHK2 and AKT phosphorylation following ETO or Ara-C treatments. Overall, our results suggest that DFF40 deficiency favors ATR activation in cancer cells and a downregulation in ATM pathway activation following DNA damages, through histone H3 acetylation.

5.6 Discussion

Genomic stability maintenance is required for cellular integrity in order to prevent DNA replication errors (Y. Yao *et al.*, 2014). Establishment of a genomic instability marks the basic characteristics of most cancer cells. Roles for the DFF40/CAD protein have been proposed in genomic stability (Y. Errami *et al.*, 2013a, Hain *et al.*, 2016a, Yan *et al.*, 2006b, Yan *et al.*, 2009). DFF40 is an endonuclease activated by the cleavage of DFF45 by caspase-3, and is involved in DNA fragmentation in the later steps of apoptosis (M. Kulbay *et al.*, 2021c). Studies have demonstrated that a lack in DFF40 expression confers genomic instability to cancer cells. Spleen lymphoblasts TK6 cells that are deficient in DFF40 do not accumulate mutations following TRAIL-induced apoptosis (Miles *et al.*, 2017). An alteration in the ratio of DFF40 and DFF45 expression partially explains the resistance to TRAIL-induced apoptosis in lymphoma cells (Luciano *et al.*, 2002). The lack of DFF expression in renal carcinoma cells impairs oligonucleosomal DNA fragmentation, thus causing a threat in mutated gene transfer (Yamaguchi *et al.*, 2004). Furthermore, *in vivo* models have also demonstrated that $CAD^{-/-}$ mice have a higher incidence of papilloma, which display an increased growth rate (Yan *et al.*, 2009). Taken together, these results highly suggest a potential role of DFF40 in genomic stability.

Cancer cells drive their tumorigenesis by the accumulation of mutations in genes that are involved in cellular growth, differentiation and proliferation. It was suggested that in myeloid malignancies, treatment-induced DNA damages, mainly DSBs, are an important hallmark in the development of tumors (Sallmyr *et al.*, 2008). The endogenous generation of ROS allows the formation of DSBs and their repair subsequently leads to genomic instability. Studies have demonstrated that multiple cancer cells have high levels of ROS. Overexpression of oncogenic H-RasG12V in 3T3 fibroblasts was shown to increase ROS production (Irani *et al.*, 1997). Tumoral cells which had enhanced levels of ROS were shown to indirectly promote epithelial-mesenchymal transition (EMT) (Okon *et al.*, 2015). Previously, we have shown that DFF40 deficient Jurkat cells were resistant to TBT-induced apoptosis (Merve Kulbay *et al.*, 2019) and had an altered mitochondrial function in nonapoptotic cells (Merve Kulbay *et al.*, 2022). Following apoptosis induction, DFF40 deficient cells have shown to have a shift of their metabolism towards aerobic glycolysis, known as the Warburg effect (Merve Kulbay *et al.*, 2022). We thus sought to determine if cancer cell resistance could be explained by an altered redox state following apoptosis induction (Fig. 1). In this study, we have shown that DFF40 deficient Jurkat cells have enhanced ROS production levels following TBT-induced apoptosis (Fig. 1a and b) and higher MMP (Fig. 1c and d). Higher ROS levels in DFF40 KO Jurkat cells following TBT treatment is consistent with lower NRF2 and

SOD1 mRNA expressions (Fig. 2). Several studies have shown that a high glycolytic metabolism is linked to enhanced ROS production (Talior *et al.*, 2003, Jie Zhou *et al.*, 2005). Interestingly, it was further brought to light that the shift towards a glycolysis-dependent metabolism in cancer cells could be an adaptation to promote drug resistance (Marcucci *et al.*, 2021). The higher levels of ROS produced through glycolysis in DFF40 deficient cells could promote cancer survival by modulating cell proliferation and genomic instability.

One of the most dysregulated pathways in cancer cells is the PI3K/AKT pathway, an oncogenic signaling pathway (Nikos Koundouros *et al.*, 2018a). Aberrant AKT pathway activation is known to induce mitochondrial ROS production through the activation of NADPH oxidases (Nikos Koundouros *et al.*, 2018a), as well as mediate multidrug resistance of cancer cells (Rui Liu *et al.*, 2020). In this study, we have shown that DFF40 deficient cancer cells have a prolonged AKT phosphorylation activity following TBT exposure in comparison to DFF40 expressing cells (Fig. 3). It was shown in colon carcinoma cells that AKT phosphorylation enables cell proliferation and apoptosis resistance (Itoh *et al.*, 2002). In HeLa cells, expression of constitutively active AKT inhibits ASK1-induced apoptosis (A. H. Kim *et al.*, 2001). Furthermore, in hybrid motor neuron 1 (HMN1) cell line, phosphorylation of AKT inhibits apoptosis by preventing caspase-3 and -9 activation (H. Zhou *et al.*, 2000). The MAPK pathways are a complex signaling network involving the ERK 1/2, JNK/SAPK and p38 pathways (Wei Zhang *et al.*, 2002). Over the last two decades, multiple studies have demonstrated the relationship and cross-talk between the PI3K/AKT and MAPK pathways, mainly in the regulation of cell proliferation (Wei Zhang *et al.*, 2002). Moscat and colleagues were amongst the first groups to demonstrate that p38 mediated apoptosis can be counter-blocked by PI3K/AKT activation (Berra *et al.*, 1998). Furthermore, p38 pathway inhibition with the inhibitor SB203580 showed to enhance cell survival through colony formation (Deschesnes *et al.*, 2001). Similar studies have demonstrated the regulation of the AKT pathway in human cells for cell survival. In endothelial cells, AKT mediated p38 downregulation showed to confer cytoprotection towards growth factor deprivation-induced apoptosis (Jean-Philippe Gratton *et al.*, 2001a). Pharmacological inhibition of PI3K/AKT induced p38 activation, following stimulation of endothelial cells with vascular endothelial growth factor (VEGF), leading to cell apoptosis (Jean-Philippe Gratton *et al.*, 2001a). In human colorectal adenocarcinoma cells, it was shown that cowanin, a xanthone extracted from the *G. cowa* tropical plant, -induced apoptosis was the result of a downregulation in the AKT pathway and activation of the p38 MAPK signaling network (Chowchaikong *et al.*, 2018). Our findings are in line with previous reports. Indeed, we show in this study that DFF40 deficient cells show an upregulation in AKT activation following apoptosis induction, which subsequently inhibits p38 activation (Fig. 3) and confers resistance to

cell death. DFF40 deficient cells can thus exhibit a more malignant profile, through an upregulation in cell proliferation and resistance to genotoxic signals.

Besides its role in tumor progression through apoptosis inhibition, AKT has gained notable interest for its role in genomic instability (Gan *et al.*, 2015). In response to DNA damages, AKT can be activated by multiple mediators, such as ATM, ATR or DNA-PK (Gan *et al.*, 2015). Previously, we have shown that DFF40 deficient Jurkat cells lacked total H2AX phosphorylation (Merve Kulbay *et al.*, 2019), a key DNA damage marker (Mah *et al.*, 2010). Knowing that DFF40 KO cells show an altered response to DNA damages and enhanced AKT phosphorylation, we thus sought to determine the impact of DFF40 deletion in cancer cells on ATM and ATR repair pathways. First, we showed that foci of H2AX are formed following apoptosis induction in DFF40 WT Jurkat cells but do not obviously localize to telomeric ends (Fig. 4). In line with our previous results, we do not observe H2AX formation in DFF40 deficient Jurkat cells (Fig. 4). Surprisingly, DFF40 deficient cells exhibit opposite activation of the ATM and ATR repair pathways; DFF40 deficient Jurkat cells were shown to have lower activation levels of CHK2, whereas enhanced phosphorylation of CHK1 was observed following TBT-induced apoptosis (Fig. 5). Lower levels of H2AX phosphorylation in cancerous cells have been reported in the past. It was shown that radioresistant human squamous cell carcinoma's display lower H2AX foci formation in comparison to radiosensitive cancer cells (Taneja *et al.*, 2004). In parallel, CHK2 depletion in prostate cancer was shown to increase cancer cell survival through resolution of DSBs (Ta *et al.*, 2020). Furthermore, breast cancer cells can be sensitize to cisplatin-induced cell death through activation of the ATM-CHK2 pathway (Shi *et al.*, 2012). On the other hand, emerging new studies have been supporting the role of the ATR-CHK1 pathway in tumorigenesis (Y. Zhang *et al.*, 2014). CHK1 is a crucial partner in controlling G2/M transition. Once activated, CHK1 prevents G2/M transition until DNA repair is achieved (Y. Zhang *et al.*, 2014). In fact, we have previously show that DFF40 deficient Jurkat cells accumulated in the G2/M phase following TBT-induced apoptosis (Merve Kulbay *et al.*, 2019). Thus, we hypothesize that rapid activation/inactivation of CHK1 in DFF40 deficient cells might be implicated in cell survival through DNA repair. It noteworthy that DNA repair is possible only after chromatin remodeling through histone expression (Bhaskara *et al.*, 2010). In this study, we have shown that DFF40 deficient Jurkat cells do not have a significant increase in acetylated histone H3 (Fig. 5). It was shown that uncoordinated acetylation of histones is responsible for DNA repair dysfunction and altered cancer progression (Shiqin Li *et al.*, 2020b). Histone deacetylases have a primordial role in breast and ovarian tumorigenesis to maintain the survival of cancer steam cells (Caslini *et al.*, 2019).

In opposition to TBT, a powerful cytotoxic agent, chemotherapeutic drugs mediate their pro-death action mainly through DNA synthesis/replication halt. We have previously shown that DFF40 deficient Jurkat cells were resistant to Ara-C, an antimetabolite drug, whereas they displayed higher sensitivity to ETO, an inhibitor of TOP2 (Merve Kulbay *et al.*, 2021b). In this study, we did not observe any significant changes in AKT or ATM pathway activations in DFF40 deficient following ETO or Ara-C treatments (Fig. 2S). These results suggest an ATM/ATR-independent response to chemotherapeutic drugs in DFF40 deficient cells. Our team was the first to demonstrate the impact of DFF40-deficiency on cell cycle checkpoint modulations: TBT-induced toxicity conferred an accumulation in the G2-phase (Merve Kulbay *et al.*, 2019), whereas DNA targeting agents such as Ara-C induced S-phase accumulation (Merve Kulbay *et al.*, 2021b). The addition of extracellular nucleotides rescued cell survival following Ara-C-induced cell cycle arrest in DFF40 deficient cancer cells (Merve Kulbay *et al.*, 2021b). Another group showed that a genotoxic stress by radiation induced DFF40/CAD-mediated single strand DNA breaks (SSBs) with subsequent G2-phase checkpoint halt, allowing DNA repair pathway activation and cancer cell survival (Larsen *et al.*, 2022). DFF40-deficient cells have shown to have an altered mitochondrial metabolism, exhibiting higher mitochondrial DNA (mtDNA) and relying on aerobic glycolysis for their energy production (Merve Kulbay *et al.*, 2022). In apoptotic cells, DFF40 was shown to translocate to the mitochondria (Merve Kulbay *et al.*, 2022). Overall, this suggests that depending on the type of DNA insult (*i.e.* direct or indirect DNA damage inducing agents), DFF40 deficient cells have the ability to rescue apoptotic cells either by mitochondrial alterations or direct DNA repair pathway activations.

In regard to our previous (Merve Kulbay *et al.*, 2021b, Merve Kulbay *et al.*, 2022) and current data, we propose that the absence of DFF40 in genomic instability appears to be linked to damages in the mitochondrial function, which induce a pro-oxidant environment via increased production of ROS. We have shown that DFF40 deficient cancer cells favor an activation of the ATR and AKT pathways and downregulation of the ATM and p38 pathways to promote their survival, particularly during genotoxic insults such as TBT. TBT-induced G2-phase arrest, as shown previously (Merve Kulbay *et al.*, 2019), could induce ATR activation and subsequent cell rescue from apoptosis. It is important to note that many apoptosis-inducing agents have a different mechanism of action on DDR pathways. Adaptations in cancer cells towards these insults are multifactorial and further investigations are required to better understand insult-specific DFF40-related modulations. This along with other factors, appears to significantly contribute to genomic instability.

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5.8 References

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5.9 Figures

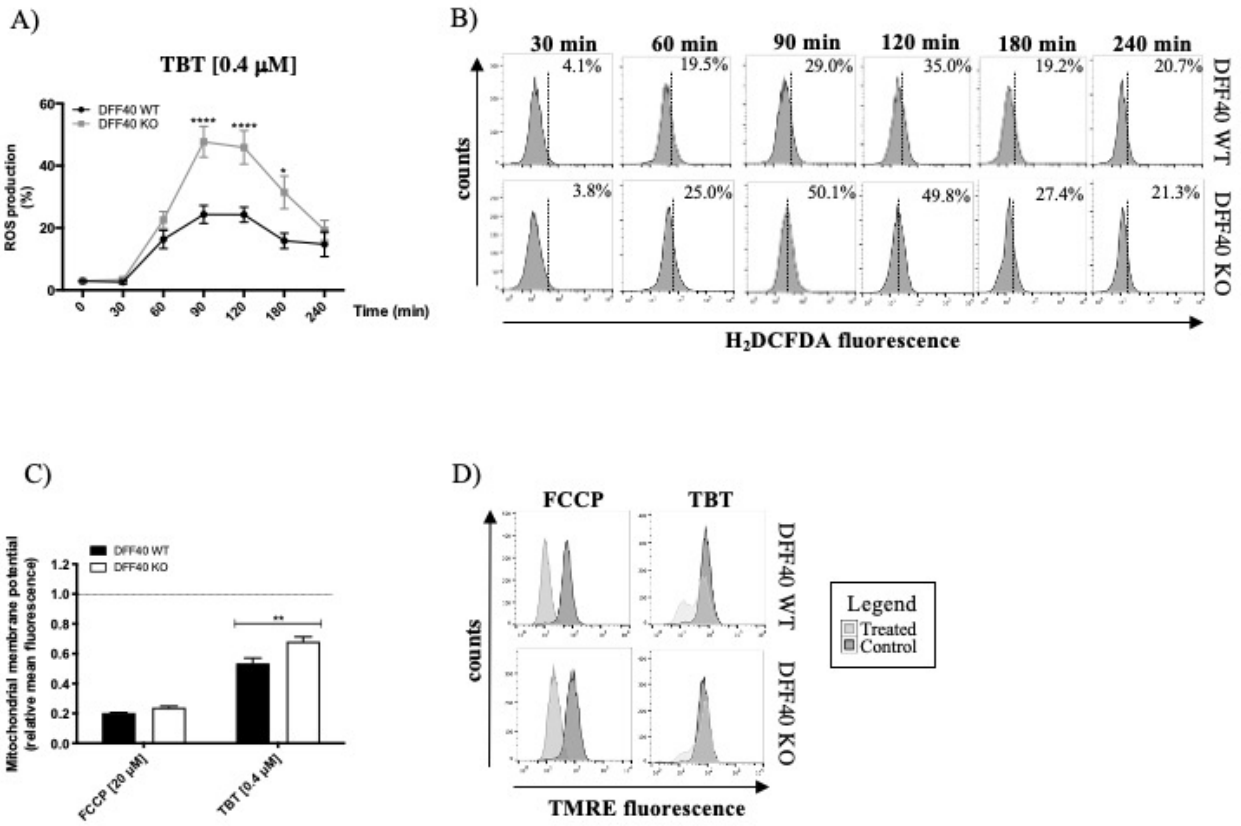


Figure 1. DFF40-deficient Jurkat cells exhibit significantly higher levels of reactive oxygen species production and delayed mitochondrial membrane potential loss following tributyltin-induced apoptosis resistance. A and B: Jurkat DFF40 WT and DFF40 KO cells (1 x 10^6 cells) were pre-incubated with the molecular probe H_2DCFDA [10 μM] for 45 min and then treated or not with TBT [0.4 μM] for different time points (0, 30, 60, 90, 120, 180 and 240 min) in RPMI media supplemented with 0.1% FBS. Cells were analyzed by flow cytometer at each time point. **C and D:** Jurkat DFF40 WT and DFF40 KO cells (1 x 10^6 cells) were treated or not with TBT [0.4 μM] for 2 h or FCCP [20 μM] for 15 min and then stained with the TMRE probe for 20 min before analysis by flow cytometer. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

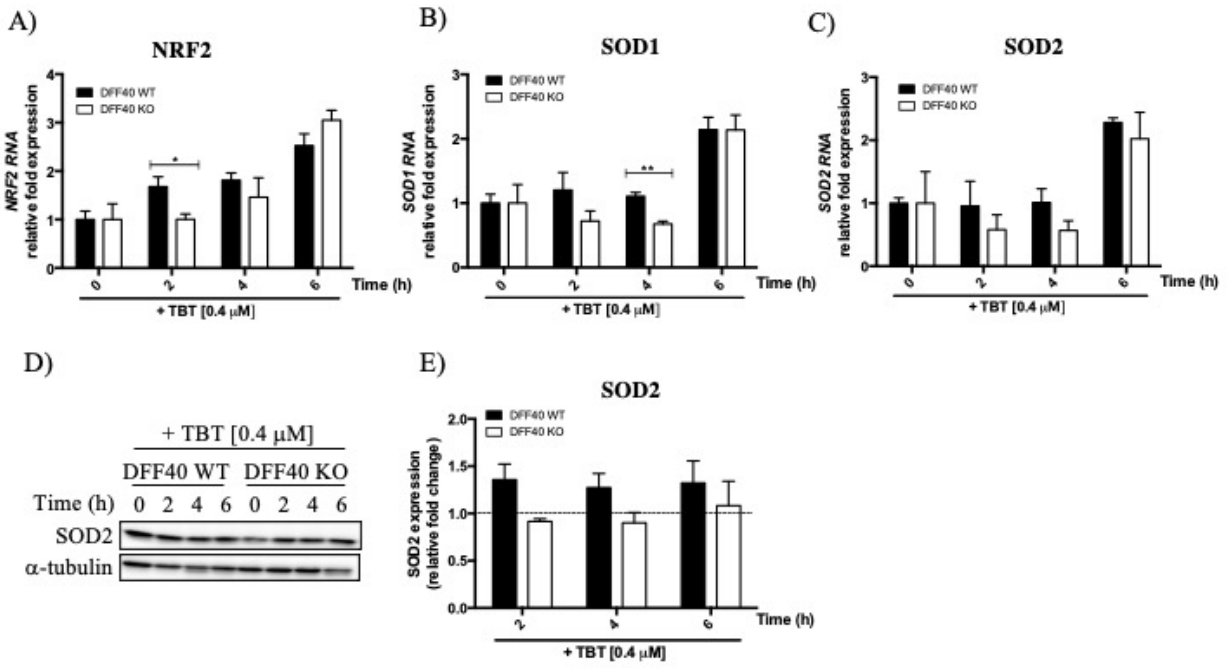


Figure 2. DFF40-deficient Jurkat cells have a delayed upregulation in NRF2 and SOD1 mRNA transcripts following TBT-induced apoptosis resistance. A, B and C: Jurkat DFF40 WT and DFF40 KO cells (5 x 10^6 cells) were treated or not with TBT [0.4 μM] for 2, 4 or 6 h in RPMI media supplemented with 0.1% FBS. RNA extractions and RT-qPCRs were then performed. **D:** Same treatments as in A, B and C, but following treatments proteins were extracted and immunoblotted for SOD2. **E:** Densitometric analysis of immunoblots in D. Results are express as a relative fold change in protein expression according to DFF40 WT cells. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed (* $p < 0.05$).

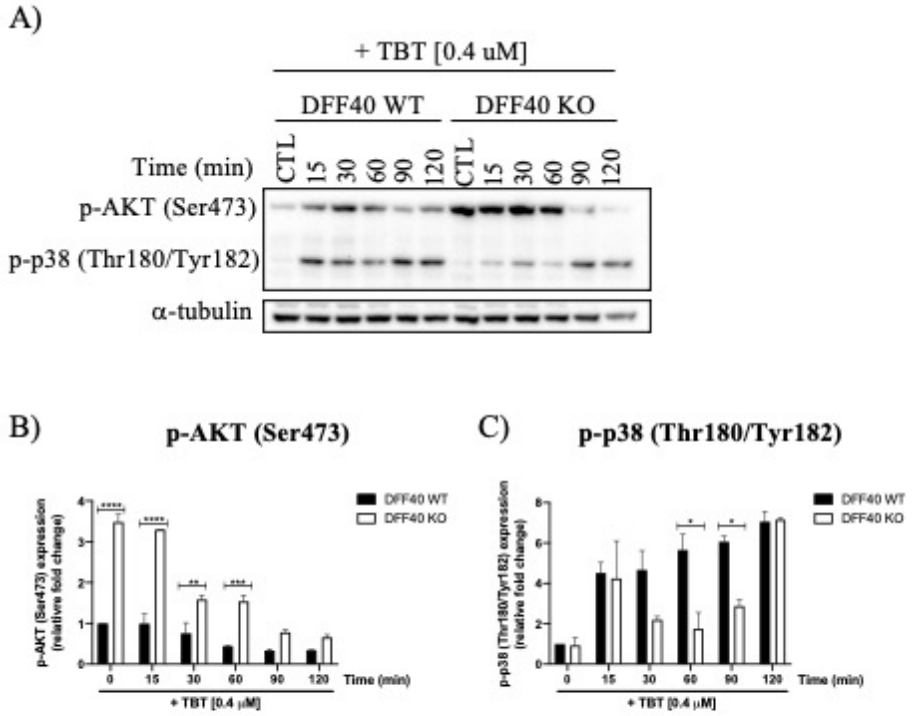
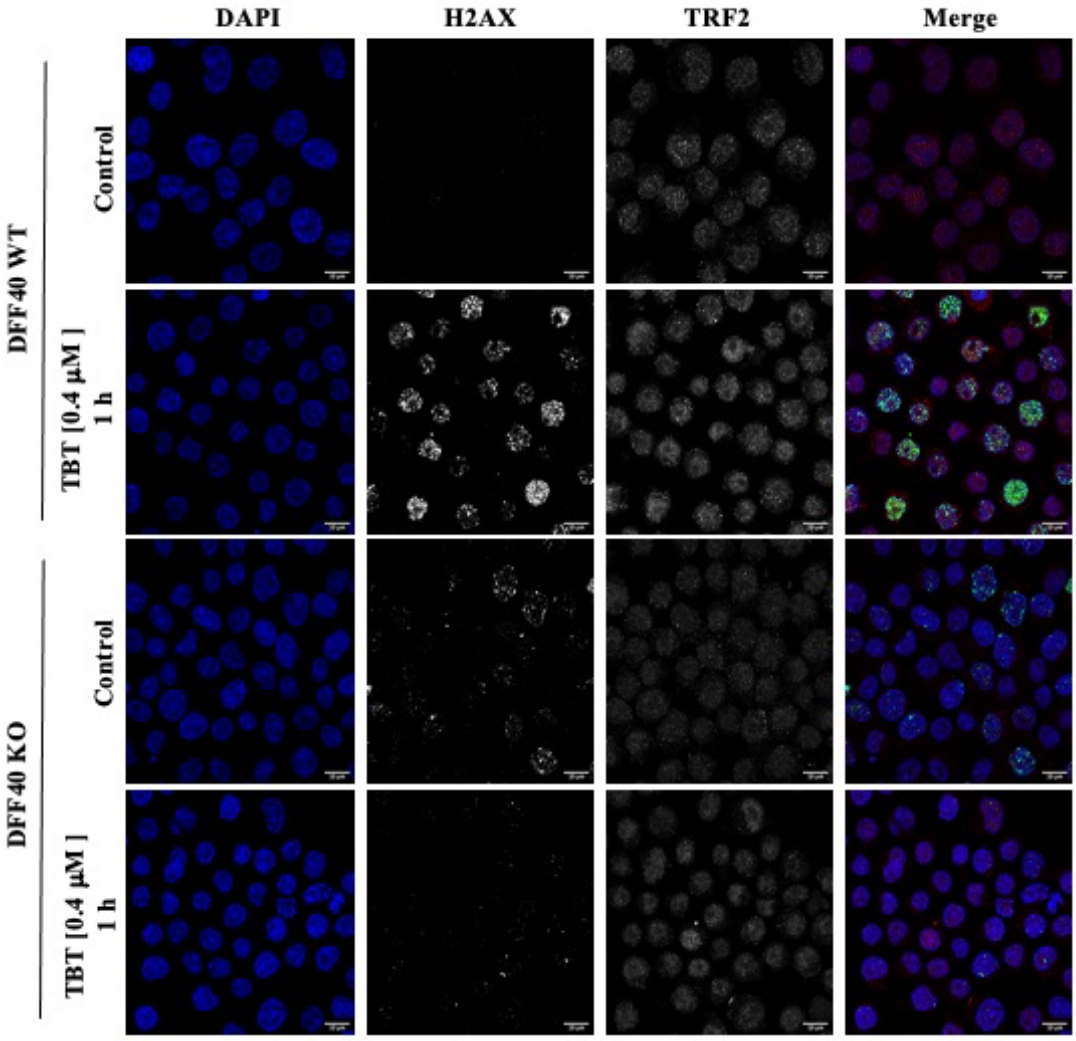


Figure 3. DFF40-deficient Jurkat cells show AKT-mediated p-38 pathway inhibition following TBT-induced apoptosis resistance. A: Jurkat DFF40 WT and DFF40 KO cells (1 x 10^6 cells) were treated or not with TBT [0.4 μM] for different time points (0, 15, 30, 60, 90, 120 min) in RPMI media supplemented with 0.1% FBS. Following treatments, proteins were extracted and immunoblotted for phospho-AKT, phospho-p-38 and α-tubulin. **B and C:** Densitometric analysis of immunoblots in A. Results are express as a relative fold change in protein expression according to DFF40 WT cells. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

A)


DAPI
H2AX
TRF2
Merge
DFF40 WT
Control
TBT [0.4 µM]
1 h
DFF40 KO
Control
TBT [0.4 µM]
1 h

B)

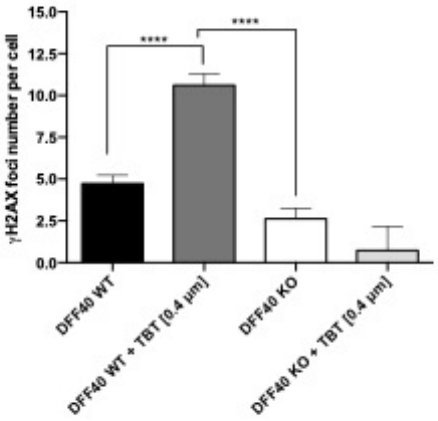

15.0
12.5
10.0
7.5
5.0
2.5
0.0
γH2AX foci number per cell
****
****
DFF40 WT
DFF40 WT + TBT [0.4 µm]
DFF40 KO
DFF40 KO + TBT [0.4 µm]

Figure 4. H2AX phosphorylation in DFF40-positive Jurkat cells does not form at telomeric ends. A and B: Jurkat DFF40 WT and DFF40 KO cells (1 x 10^6 cells) were treated or not with TBT [0.4 μM] for 1 h. Cells were then cyto-spinned and fixed on microscopic slides and blotted with phospho-H2AX and TRF2 for 2 h. Respective conjugated secondary antibodies were blotted for 1 h. Immunofluorescence images were acquired using a LSM780 confocal microscope (Carl Zeiss Microimaging). The number of γH2AX foci in both cell lines were manually counted. A total of 100 cells per condition was assessed. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed (**** $p < 0.0001$).

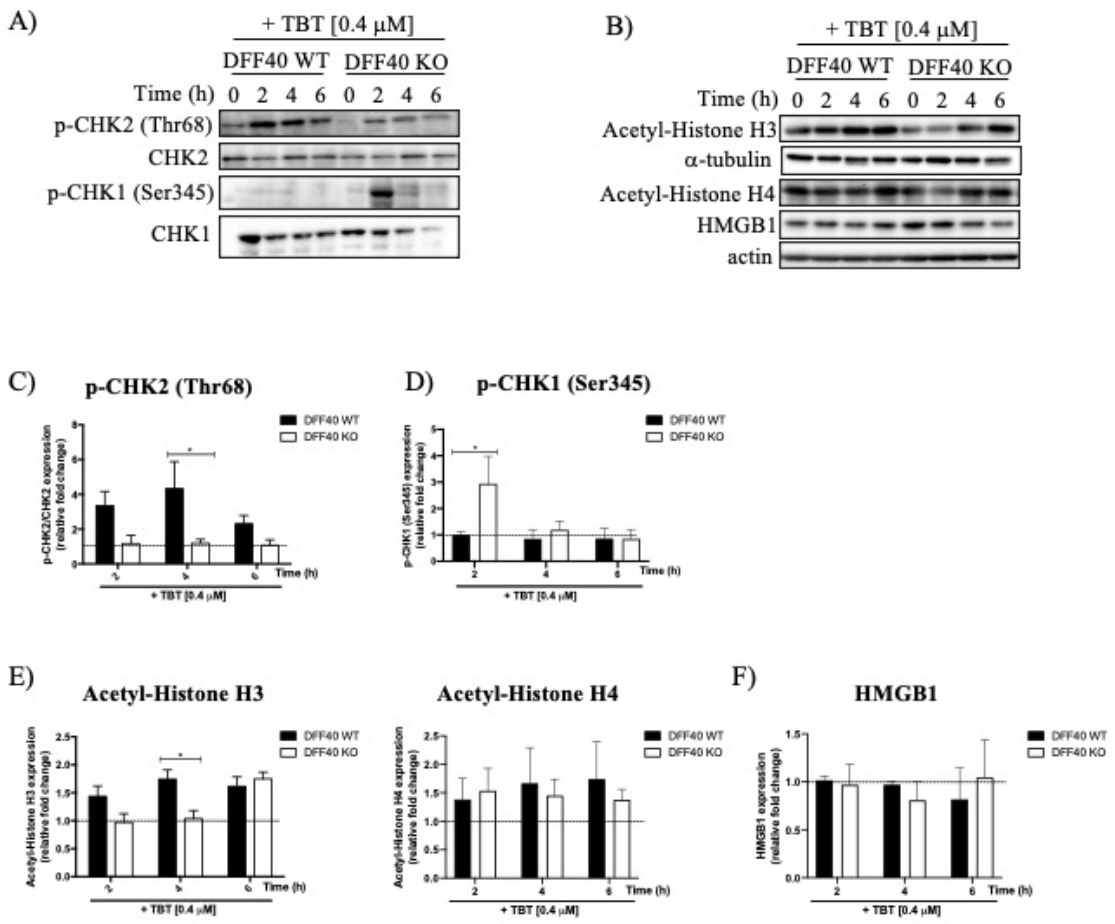


Figure 5. ATM pathway is inhibited in DFF40-deficient Jurkat cells, whereas ATR pathway is induced following tributyltin-induced apoptosis. A and B: Jurkat DFF40 WT and DFF40 KO cells (1 x 10^6 cells) were treated or not with TBT [0.4 μM] for 2, 4 or 6 h in RPMI media supplemented with 0.1% FBS. Following treatments, proteins were extracted and immunoblotted for phospho-CHK2, phospho-CHK1, CHK2, CHK1, acetyl-histone H3, acetyl-histone H4, HMGB1, α-tubulin and actine. **C to F:** Densitometric analysis of immunoblots in A and B. Results are express as a relative fold change in protein expression according to DFF40 WT cells. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed (* $p < 0.05$).

6 CHAPITRE 6 : DISCUSSION GÉNÉRALE

Les cellules cancéreuses possèdent de nombreuses mutations génomiques impliquant la voie de signalisation de l'apoptose, qui ont été démontrées comme jouant un rôle pivot dans l'acquisition d'un phénotype malin (Fernald *et al.*, 2013, M. Kulbay *et al.*, 2021c). Depuis les dernières années, de nombreuses études se sont intéressées au rôle de l'endonucléase DFF40 dans la progression tumorale. L'étude de Ohira et ses collègues fut une des premières à démontrer la présence d'une délétion homozygote de la région 1p36.2-p36.2, soit le locus du gène du DFF40/DFF45, dans les cellules de neuroblastomes – une tumeur avec un mauvais pronostic (Ohira *et al.*, 2000). Dans cette étude, nous avons pu démontrer de nombreuses évidences scientifiques. Nous avons démontré une corrélation inversement proportionnelle de l'expression du DFF40 dans les cellules tumorales avec le pronostic clinique ; l'altération de l'expression du DFF40 dans les cellules tumorales du cancer de l'œsophage (Konishi *et al.*, 2002), hépatique (Hsieh *et al.*, 2003b), de l'endomètre (Banas *et al.*, 2018a) et du cerveau (Sánchez-Osuna *et al.*, 2016) est liée avec un mauvais pronostic. La sensibilité aux agents inducteurs d'apoptose dans les cellules cancéreuses avec une altération de l'expression du DFF40 pourrait être récupérée à l'aide de modulations transcriptionnelles en induisant à la hausse l'expression du DFF40 (Bagheri *et al.*, 2015b, Minaiyan *et al.*, 2021). Étant donné que la résistance cellulaire à l'apoptose est le siège de l'instabilité génomique et que peu d'études ont caractérisé l'implication du DFF40 dans ces phénomènes moléculaires, nous nous sommes donc intéressés à celle-ci. Notre objectif primaire était d'établir de nouveaux rôles pour le DFF4, puis d'étudier les modulations moléculaires et cellulaires liées à ces rôles.

6.1 Génération d'une lignée cellulaire déficiente en DFF40

La première étape de mon projet de doctorat était d'établir au moins une lignée cellulaire humaine stable ayant une délétion génique du gène du DFF40. En effet, jusqu'en 2016, aucune étude n'avait évalué les modulations de la voie de signalisation de l'apoptose en utilisant un modèle *in vitro* stable, dépourvu du gène du DFF40 directement. Les premières études *in vitro* ont démontré l'implication du DFF40 dans la stabilité génomique en utilisant des techniques de mutagenèse dirigée (X. Liu *et al.*, 1997). Des mutants humains géniques stables pour le DFF45, où l'activation du DFF et la fragmentation subséquente de l'ADN sont abolies, furent générés par transfection d'un vecteur rétroviral pLPCX contenant le gène muté créé par PCR dans les cellules HeLa (Yan *et al.*, 2006a). Les mutations ciblaient les sites de clivage du DFF45 par la caspase-3

(X. Liu *et al.*, 1997, Yan *et al.*, 2006a). Bien que cette étude fut une des premières à créer de manière stable une lignée cellulaire exempte d'activité du DFF40, ce dernier était tout de même exprimé dans les cellules HeLa. Des études ont aussi rapporté l'utilisation de cellules issues de souris déficientes en DFF40/CAD ($CAD^{-/-}$) (Yan *et al.*, 2006a, Yan *et al.*, 2009). L'extrapolation des résultats issus d'une autre espèce possède ses propres enjeux : l'expression des partenaires de signalisation moléculaires de la voie de signalisation de l'apoptose diffère légèrement chez la souris. Une analyse comparative a démontré que l'expression de certaines caspases, protéines reliées à la famille de Bcl-2 et protéines en amont de l'activation des caspases sont altérées chez le modèle murin (Reed *et al.*, 2003). Ainsi, l'objectif primaire était d'établir une lignée cellulaire humaine déficiente en DFF40.

Durant la dernière décennie, l'émergence du système du CRISPR-cas9 a révolutionné le domaine de la biologie moléculaire en regard des technologies d'édition du génome. Contrairement aux autres technologies d'édition du génome récentes, le CRISPR-cas9 (Figure 13) est composé d'une nucléase (*c.-à.-d.* cas9), guidé par de petits ARN avec une région PAM (*c.-à-d. protospacer adjacent motif*), responsable de l'induction de BDB de l'ADN (Ran *et al.*, 2013). Cette région permet de cibler de manière très spécifique la région adjacente à la séquence d'ADN à cliver (Ran *et al.*, 2013). Ce système possède de nombreux avantages en comparaison aux autres technologies d'édition du génome (Ran *et al.*, 2013). En effet, le système CRISPR-cas9 ne nécessite pas la construction manuelle d'ARN-guides, ceux-ci étant disponibles en grandes variétés auprès des fournisseurs (Ran *et al.*, 2013). Il est donc très facile de personnaliser l'édition du génome. De plus, le clivage moléculaire de l'ADN est plus spécifique avec le système cas-9 : la nucléase effectue une coupure franche entre les 17^{e} et 18^{e} bases de la séquence cible du PAM (Ran *et al.*, 2013). Ces caractéristiques font que de nombreux locus génomiques peuvent être ciblés simultanément, augmentant ainsi l'efficacité de l'édition génomique.

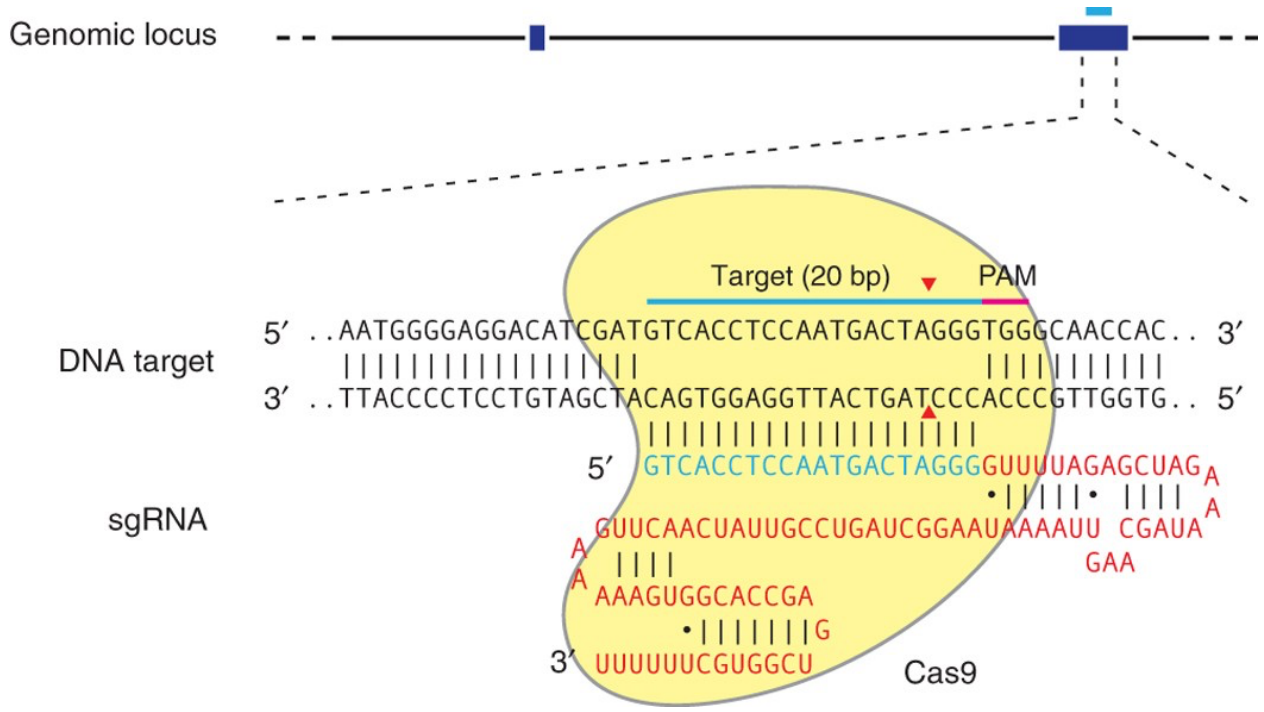


Figure 13. Représentation du système d'édition du génome CRISPR-cas9.

La nucléase Cas9 est représentée par l'encadré jaune. Le Cas9 est ciblé à l'ADN génomique d'intérêt (DNA target) par un ARN guide (sgRNA) constitué de 20 nucléotides (targe) et d'une séquence de localisation et d'initiation du clivage (PAM). Le sgRNA se lie à la séquence cible en amont de la séquence PAM, puis le clivage par la cas9 effectue un clivage à une distance d'environ 3 paires de bases de la séquence PAM. Image tirée de : (Ran *et al.*, 2013).

Pour ces raisons, nous avons eu recours à la technologie du CRISPR-cas9 pour générer notre lignée cellulaire déficiente en DFF40. Nous avons généré une lignée cellulaire stable déficiente en DFF40 par électroporation, en utilisant comme modèle les cellules T immortalisées Jurkat, où l'expression protéique du DFF40 a été abolie (Article 1, Fig. 1). Il est important de mentionner que notre laboratoire est le premier à générer une lignée cellulaire non-adhérente déficiente dans l'expression du DFF40. Plusieurs modèles cellulaires ont couramment été utilisés dans la littérature scientifique lors d'études fondamentales sur la carcinogenèse. Peu importe le modèle cellulaire utilisé, plusieurs facteurs peuvent influencer l'extrapolation des résultats *in vitro* aux modèles *ex vivo* et *in vivo* (Gillet *et al.*, 2013). Le micro-environnement tumoral, l'interaction cellule-cellule, les conditions de cultures *in vitro* figurent parmi les facteurs influençant significativement les résultats scientifiques (Gillet *et al.*, 2013). Dans mon projet de doctorat, nous avons utilisé la lignée cellulaire Jurkat pour de nombreuses raisons. Les mutations génomiques dans les cellules Jurkat ont été répertoriées de manière vigoureuse (Gioia *et al.*, 2018). Les principales mutations impliquées dans la régulation de la stabilité génomique regroupent celles touchant les gènes *TP53*, *BAX* et *MSH2* (Gioia *et al.*, 2018). Il fut démontré que le gène TP53 dans les cellules Jurkat possède 4 mutations hétérozygotes impliquant les codons 196, 256, 259 et 260 (Cheng *et al.*, 1990). La mutation touchant le codon 196 serait due à la substitution d'un unique nucléotide ayant pour effet un arrêt de lecture du cadre par un codon-stop (Cheng *et al.*, 1990). Ainsi, la protéine p53 n'est pas exprimée chez les cellules Jurkat. Ensuite, deux mutations hétérozygotes au codon 41 ont été rapportées dans le gène *BAX*, qui serait responsables d'un décalage du cadre de lecture et d'une abolition de l'expression protéique de son substrat, Bax (Meijerink *et al.*, 1998). Enfin, la protéine MSH2, qui est impliquée dans les voies de réparation de l'ADN, n'est pas exprimée dans les cellules Jurkat dues à une mutation ponctuelle dans l'exon 13 (Brimmell *et al.*, 1998). Les autres raisons qui nous ont motivés à choisir les cellules Jurkat comme modèle sont que de nombreuses études ont caractérisé de manière extensive les phénomènes moléculaires engendrés suite à l'activation de la voie de signalisation de l'apoptose (Gottlieb *et al.*, 1996, Ben Chang Guo *et al.*, 2001, Karpinich *et al.*, 2006, Ruiz-Ruiz *et al.*, 1999).

6.2 Caractérisation des cellules T Jurkat déficientes en DFF40

Ayant émis l'hypothèse que les cellules déficientes en DFF40 ont un phénotype malin, la seconde étape de mon projet de doctorat était de caractériser les atteintes moléculaires du modèle d'étude généré. Des études ont démontré que la modification génique par la délétion d'un gène engendre essentiellement une perte de l'équilibre génomique, et des mutations supplémentaires des gènes complémentaires peuvent se produire (Teng *et al.*, 2013). De ce fait,

nous nous sommes questionnés à savoir si la perte du gène du DFF40 pourrait causer des adaptations moléculaires au sein des cellules cancéreuses.

6.2.1 L'absence de DFF40 régule à la hausse l'expression de Mcl-1

Nous avons étudié l'impact d'une délétion du gène du DFF40 par CRISPR-cas9 sur l'expression protéique de d'autres partenaires moléculaires de la voie de signalisation de l'apoptose. Nous avons démontré que l'absence de DFF40 dans les cellules cancéreuses Jurkat n'a pas d'impact sur l'expression protéique du DFF45 (Article 1, Fig. 1), de Bcl-2, Bcl-xL, Bak, XIAP et des procaspases-3, -6, -7 et -9 (Article 3, Fig.2). Cependant, une absence de DFF40 dans les cellules cancéreuses Jurkat augmente l'expression protéique de Mcl-1 (Article 3, Fig.2). Comme expliqué précédemment, la protéine Mcl-1 a une fonction antiapoptotique via la modulation de l'activité des protéines BH3-only et des protéines Bak et Bax (L. W. Thomas *et al.*, 2010). L'épissage alternatif de l'ARNm de Mcl-1 permet d'induire l'expression de deux isoformes, soit l'isoforme long (Mcl-1L) ou court (Mcl-1S) (Morciano *et al.*, 2016). Il a été démontré que les cellules cancéreuses HeLa ont des niveaux d'ARNm de Mcl-1L supérieur, comparativement au niveau de Mcl-1S, malgré une expression protéique similaire (Morciano *et al.*, 2016). Le traitement des cellules HeLa avec des oligonucléotides antisens à commutation d'épissage (sc-ASO) induit une élévation préférentielle de l'isoforme Mcl-1S qui à son tour, diminue de manière significative les niveaux de Mcl-1 (Morciano *et al.*, 2016). Il a donc été conclu que l'isoforme court est la variante proapoptotique. Une déficience en DFF40 pourrait être impliquée dans la régulation des modifications post-traductionnelles de l'ARNm de Mcl-1, favorisant ainsi l'expression de Mcl-1L dans les cellules cancéreuses.

Le rôle de Mcl-1 ne se limite pas exclusivement à la régulation de l'apoptose. Il a été démontré que Mcl-1 est impliqué dans la différenciation et développement cellulaire des lymphocytes T et B (Opferman *et al.*, 2003), du système nerveux central (Arbour *et al.*, 2008) et des cellules souches hématopoïétiques (Opferman Joseph *et al.*, 2005). De plus, Mcl-1 module la progression du cycle cellulaire via une interaction avec la CDK-1 (Jamil *et al.*, 2005) et la protéine CHK-1 (Jamil *et al.*, 2008). Il a été démontré qu'une surexpression de Mcl-1 induit une phosphorylation de la protéine CHK-1 au résidu 345 en l'absence de dommages à l'ADN (Jamil *et al.*, 2008). Lorsque l'expression de Mcl-1 est déplétée, une augmentation de l'instabilité génomique et une diminution de la survie cellulaire est observée suivant une irradiation aux rayons ultraviolets (UV) ; la radiosensibilité des cellules déficientes en Mcl-1 est due à la perte de signal télomérique, de formation de chromosomes dicentriques ou multicentriques et l'aneuploïdie (Mattoo *et al.*, 2017).

Dans les cellules positives pour l'expression de Mcl-1, il a été démontré que Mcl-1 transloque au noyau suivant l'induction de l'apoptose par des mécanismes causant des dommages à l'ADN, tels les rayons UV (Mattoo *et al.*, 2017, L. W. Thomas *et al.*, 2010). La présence de Mcl-1 nucléaire permet d'induire la réparation de l'ADN par recombinaison homologue, où une absence de Mcl-1 interfère avec ce processus par l'augmentation d'arrêts à la fourche de réplication de l'ADN (Mattoo *et al.*, 2017). Ces résultats suggèrent une première évidence du rôle du DFF40 dans la régulation de la stabilité génomique. L'augmentation de l'expression de Mcl-1 dans les cellules Jurkat déficientes en DFF40 pourrait potentiellement induire un dérèglement des voies de signalisation DDR et ainsi, permettre une survie cellulaire augmentée, voir une promotion de la tumorigenèse, par l'altération du cycle cellulaire.

6.2.2 Les cellules T cancéreuses déficientes en DFF40 ont un taux d'ERK 1/2 phosphorylé augmenté en condition de stress secondaire à une privation en sérum

Les cellules cancéreuses possèdent de nombreux mécanismes d'adaptations cellulaires favorisant la survie et la prolifération cellulaire (Fodale *et al.*, 2011). Il est important de noter que le micro-environnement tumoral est très complexe ; l'interaction de la cellule cancéreuse dans son micro-environnement est un processus ultra-dynamique et complexe, regroupant de nombreux médiateurs (Baghban *et al.*, 2020). Au centre de cette dynamique, les fibroblastes associés aux cancers sont responsables de la création d'un micro-environnement riche en stress oxydatif, privilégiant la prolifération tumorale (Baghban *et al.*, 2020). À travers ce réseau biocellulaire et biomoléculaire complexe, les cellules cancéreuses vont développer des caractéristiques protumorales, tels l'indépendance aux signaux de croissance, l'échappement à la mort cellulaire et l'accumulation de mutations génomiques. Sachant que l'absence de DFF40 dans les cellules cancéreuses est liée à un mauvais pronostic, nous nous sommes donc penchés à savoir si une déficience en DFF40 altère le phénotype des cellules cancéreuses, en privilégiant les caractéristiques phénotypiques des cellules malignes.

Nous avons démontré que les cellules déficientes en DFF40 ont une phosphorylation accrue d'ERK1/2 dans un environnement de stress (*c.-à.d.* secondaire à une privation en sérum) (Article 3, Fig. 5). Des études similaires ont démontré l'induction de la phosphorylation d'ERK en condition de privation de sérum (De Iuliis *et al.*, 2018, Yan-Jun Guo *et al.*, 2020). Dans les cellules de mélanome (M13) la privation en sérum induit la phosphorylation d'ERK, la relâche du facteur de croissance épidermique (EGF) et la formation d'autophagosomes (De Iuliis *et al.*, 2018), suggérant l'activation de l'autophagie dans ces cellules cancéreuses. Le rôle de l'autophagie

dans les cellules cancéreuses semble être mitigé : l'autophagie peut agir à titre de processus favorisant la tumorigenèse ou de suppresseur de tumeur (Yun *et al.*, 2018). Jusqu'à présent, aucune étude n'a démontré le rôle du DFF40 dans le processus d'autophagie. Des études supplémentaires sont donc nécessaires afin d'élucider ces mécanismes cellulaires pouvant potentiellement être impliqués dans la malignité des cellules cancéreuses.

6.3 Modulations de la voie de signalisation de l'apoptose

6.3.1 Caractérisation de la sensibilité des cellules déficientes en DFF40 aux agents inducteurs d'apoptose

Les mutations au sein des cellules cancéreuses sont généralement utilisées comme des marqueurs de sensibilité ou résistance aux agents inducteurs d'apoptose (Levatić *et al.*, 2022). Du point de vue clinique, l'expression de nombreux marqueurs tumoraux est utilisée afin d'orienter la thérapie. Par exemple, des inhibiteurs de Bcl-2 sont utilisés dans les cancers ayant une surexpression de cette protéine, dont les leucémies lymphoblastiques chroniques et les leucémies myéloïdes aiguës (Roberts, 2020). Ainsi, nous avons voulu caractériser la réponse cellulaire des cellules Jurkat déficientes en DFF40 à une gamme d'agents inducteurs d'apoptose, dont I) le TBT, II) la STS et III) les agents de chimiothérapie. Il est à noter que jusqu'à présent, aucune étude n'avait démontré la réponse cellulaire de cellules avec une altération dans l'expression du DFF40 sur de nombreux agents inducteurs d'apoptose.

À travers nos trois premières publications, nous avons démontré que les cellules Jurkat déficientes en DFF40 sont résistantes à l'apoptose induite par le TBT (Article 1, Fig. 1), la STS (Article 3, Fig. SX) et les antimétabolites, dont le MTX, le 6-MP et l'Ara-C (Article 2, Fig. 2). Or, les cellules Jurkat déficientes en DFF40 ont démontré une sensibilité accrue aux inhibiteurs de TOP2, dont l'ETO, le VM-26 et le MX (Article 2, Fig. 3). La réponse cellulaire observée peut être en partie expliquée par les mécanismes d'action des divers agents inducteurs d'apoptose (voir Chapitre Introduction). Nous croyons que la sensibilité aux inhibiteurs de TOP2 observée chez les cellules déficientes en DFF40 peut partiellement être expliquée par son mécanisme d'action : les inhibiteurs de TOP2 interfèrent directement avec l'intégrité structurelle de l'ADN, contrairement aux antimétabolites qui vont agir au niveau du brin naissant d'ADN.

Les médicaments de chimiothérapie utilisés dans mon projet de recherche font partie des agents de chimiothérapie conventionnels. Il est important de mentionner que cliniquement, le choix de l'agent de chimiothérapie va fortement être influencé par le type de néoplasie. Dans les leucémies, les agents les plus couramment utilisés sont la vincristine, l'Ara-C, le DXR, le 6-MP et

le MTX, pour n'en nommer que quelques-uns (ACS, 2018). Le choix de nos agents pour notre projet de recherche concorde donc avec la réalité clinique. Or, dans les tumeurs non hématologiques, les traitements pharmacologiques ont grandement évolué. Le capecitabine, une prodrogue du fluorouracile, est un antimétabolite le plus utilisé dans de nombreux cancers, dont dans le traitement du cancer du sein (Varshavsky-Yanovsky *et al.*, 2020), cancer colorectal (Auber *et al.*, 2021) et cancer du poumon (C. Y. Huang *et al.*, 2017). Cependant, l'évolution de la recherche a permis de développer de nouveaux traitements personnalisés, dont l'immunothérapie, qui occupe aujourd'hui une place importante dans la prise en charge du cancer (Falzone *et al.*, 2018).

Un des enjeux de mon projet de recherche concerne l'extrapolation des résultats aux tumeurs solides. Il a été démontré que les médicaments d'une même classe pharmacologique peuvent avoir des effets distincts, en fonction des paramètres pharmacocinétiques et pharmacodynamiques (Sweeney, 1983). Afin de valider nos résultats, nous pourrions donc utiliser la capecitabine et évaluer la viabilité cellulaire.

6.3.1 Le DFF40 est impliqué dans la résistance à l'apoptose induite par le tributylétain via un retard d'activation de l'apoptose et l'accumulation de cellules en phase G2/M

Dans notre premier article, nous avons démontré l'impact d'une délétion du DFF40 sur l'activation de la voie de signalisation de l'apoptose par le TBT. La résistance aux effets cytotoxiques du TBT chez les cellules déficientes en DFF40 peut en partie être expliquée par un retard d'activation de l'apoptose, marquée par une diminution de la translocation de phosphatidylsérine à la membrane extracellulaire, diminution de l'expression protéique de caspase-6, un délai de clivage de la procaspase-3 et PARP, diminution de l'activité de la caspase-3/7 et une absence de fragmentation de l'ADN dans les cellules déficientes en DFF40 (Article 1, Fig. 2 et 4). De plus, nous avons démontré que les cellules Jurkat déficientes en DFF40 ont une augmentation de la phosphorylation de Bcl-2 après une exposition au TBT (Article 3, Fig. 2). Aucune différence dans l'expression des ARNm de Bak, Bax, Bim, Bcl-2, Bcl-xL, Bfl1/A1, Mcl-1, caspase-3 et caspase-9 n'a été observé entre les cellules Jurkat DFF40 WT et DFF40 KO (Article 3, Fig. 2). Plusieurs études ont démontré l'importance de la surexpression de Bcl-2 dans la résistance à l'apoptose. Il a été démontré dans les cellules cancéreuses humaines de prostate (LNCaP) (Raffo *et al.*, 1995), de thyroïde (FRO) (Mitsiades *et al.*, 2007) et de larynx (Sai Li *et al.*, 2017) qu'une augmentation de l'expression de Bcl-2 par transfection induit une résistance à l'apoptose. Depuis les deux dernières décennies, un rôle dans la régulation de la stabilité génomique pour Bcl-2 a été

proposé. Il a été démontré qu'une surexpression de Bcl-2 dans des lignées cellulaires de mammifères contenant la séquence en tandem pour les substrats de la recombinaison (*e.g.* CHO-DRA10, pJS3-10) inhibe la recombinaison homologue suivant des dommages à l'ADN induits par les rayons UV (Saintigny *et al.*, 2001). La voie de réparation de l'ADN impliquant RAD51 pour les BDB est altérée, induisant l'accumulation de mutagenèse (Saintigny *et al.*, 2001). Une seconde étude a démontré que la surexpression de Bcl-2 dans les cellules Jurkat augmente la formation de micronoyaux suivant une exposition à 50 μM de peroxyde d'hydrogène (Cox *et al.*, 2007). Ayant démontré dans notre projet de recherche qu'une délétion en DFF40 dans les cellules Jurkat augmente l'expression de Bcl-2 suivant un stimulus apoptotique, de nombreuses questions sont soulevées par rapport à son implication dans la stabilité génomique. L'absence de DFF40 dans les cellules cancéreuses pourrait être un mécanisme d'adaptation aux insultes physico-chimiques dans l'optique de promouvoir la tumorigenèse.

Le TBT induit un arrêt des cellules déficientes en DFF40 dans la phase G2/M du cycle cellulaire (Article 1, Fig. 2). Les arrêts mitotiques induits dans la phase G2/M sont les conséquences de dommages à l'ADN d'ampleur plus importante. Dans les cellules cancéreuses, ceux-ci peuvent être surmontés grâce à l'activation de la voie de signalisation d'ATR. Il fut démontré à l'aide de modèles de levures que lors de dommages génomiques importants induits par les rayons UV, l'activation de la kinase wee1 permet d'induire un arrêt mitotique à la phase G2/M, induisant ainsi l'activation de la voie d'ATR et la réparation subséquente des dommages (O'Connell *et al.*, 1997). À la lumière de ces résultats, un rôle hypothétique est donc suggéré pour le DFF40 dans la régulation du point de contrôle G2/M également : le type de dommages à l'ADN médie le contrôle du cycle cellulaire par le DFF40.

6.3.2 Une déficience en DFF40 induit une altération du point de contrôle G1/S du cycle cellulaire lors de l'apoptose induite par les antimétabolites

Dans l'optique de valider l'hypothèse que le DFF40 est impliqué dans la régulation du cycle cellulaire, nous avons quantifié la proportion de cellules dans les phases du cycle cellulaire suivant une exposition aux antimétabolites ou inhibiteurs de TOP2. Nous avons démontré que les cellules déficientes en DFF40 ont un arrêt mitotique plus significatif à la phase S du cycle cellulaire, suivant une exposition à l'Ara-C et le MTX, ainsi qu'une résistance à l'apoptose et une augmentation de la prolifération cellulaire (Article 2, Fig. 2, 4-5). La phase S du cycle cellulaire est responsable de la synthèse d'ADN via l'activation des voies de réparation de l'ADN (*c.-à-d.* la voie de signalisation d'ATM et d'ATR). Quelques études ont démontré l'importance des arrêts mitotiques dans l'acquisition d'une résistance aux médicaments de chimiothérapie chez les

cellules cancéreuses. Une première étude réalisée par l'équipe du professeur Hong Ma a étudié l'impact d'une délétion de la protéine de liaison au p53-1 (53BP1) sur la résistance à l'apoptose induite par les médicaments de chimiothérapie dans les cellules cancéreuses colorectales HCT116 et HT-29 (Jing Yao *et al.*, 2017). Ils ont démontré qu'une abolition de l'expression de 53BP1 à l'aide de shARN dans les cellules HCT116 et HT29 induit une accumulation de cellules cancéreuses dans la phase S du cycle cellulaire suivant une exposition au 5-fluorouracil (5-FU), un agent de chimiothérapie de type antimétabolite (Jing Yao *et al.*, 2017). L'arrêt mitotique dans la phase S induit conséquemment une résistance à l'apoptose induite par le 5-FU via une diminution de l'expression des protéines effectrices de la voie de signalisation d'ATM, de la caspase-3, caspase-9 et par une augmentation de l'expression de Bcl-2 (Jing Yao *et al.*, 2017). De plus, il a été démontré qu'une accumulation de cellules cancéreuses ovariennes A2780 dans la phase S du cycle cellulaire est impliquée dans la résistance de celles-ci à la toxicité du cisplatine (Kielbik *et al.*, 2018).

La résistance cellulaire pourrait potentiellement être expliquée par une phosphorylation accrue de la protéine ERK1/2 dans des conditions de stress, telle une privation en sérum (Article 3, Fig. 5) : l'utilisation d'un inhibiteur de l'activation d'ERK1/2 augmente la sensibilité à la cisplatine dans la lignée cellulaire A2780 (Kielbik *et al.*, 2018). Parallèlement, les mêmes observations ont été rapportées dans les cellules cancéreuses de sein MCF7, où une exposition à l'hydroxyurée induit la phosphorylation d'ERK1/2 et un arrêt mitotique dans la phase S du cycle cellulaire (Wu *et al.*, 2006). Le rôle d'ERK1/2 dans la résistance à la chimiothérapie suivant un arrêt mitotique peut être expliqué par la régulation à la hausse de la voie de DDR. Il a été démontré que l'activation d'ERK1/2 mène à la formation de focis d'ATR au niveau nucléaire (Wu *et al.*, 2006). De nombreuses cellules cancéreuses sont dépendantes de la voie de signalisation d'ATR dans la réparation des dommages à l'ADN (Barnieh *et al.*, 2021). L'activation d'ATR permet aux cellules cancéreuses de surmonter le stress de réplication induit lors des arrêts mitotiques à la phase S du cycle cellulaire (Barnieh *et al.*, 2021). Le stress de réplication peut causer des BSB de l'ADN et induire la mort cellulaire de manière rapide. Dans les cellules cancéreuses, l'activation d'ATR par la phosphorylation d'ERK1/2 permettrait donc à celles-ci d'avoir une réparation plus rapide de leur matériel génomique et favoriser la prolifération cellulaire (Barnieh *et al.*, 2021). Nos résultats de recherche supportent fortement le rôle du DFF40 dans la régulation de la stabilité génomique via le contrôle du cycle cellulaire dans la chimiorésistance. Le DFF40 pourrait agir à titre de partenaire de contrôle dans la transition de la phase S à la phase G2/M. À l'état physiologique, le DFF40 pourrait prévenir l'activation inappropriée des voies de DDR, ainsi promouvoir l'apoptose des cellules mutées. Dans un contexte tumoral, l'abolition de l'expression

du DFF40 enlèverait les signaux inhibiteurs sur l'activation de la voie de signalisation d'ATR, permettant la prolifération des cellules cancéreuses.

6.3.3 La sensibilité accrue aux inhibiteurs de la topoisomérase II dans un fond DFF40-déficient est partiellement attribuable à une augmentation du taux de nécrose

Nous avons démontré que la sensibilité à la toxicité engendrée par l'ETO peut être en partie attribuable à des taux de nécrose supérieurs (Article 2, Fig. 6), comme démontré par une expression augmentée de la protéine HMGB1 (Article 2, Fig. 7). La différence dans la sensibilité aux inhibiteurs de TOP2 comparativement aux antimétabolites peut être attribuable à leurs mécanismes d'action distincts. D'un côté, les antimétabolites agissent en s'incorporant dans les brins d'ADN en réplication et sont plus susceptibles d'induire des arrêts mitotiques. De l'autre côté, les inhibiteurs de TOP2 interfèrent avec les mécanismes de changement de conformation de l'ADN et sont responsables de la production de BSB ou BDB de l'ADN. Dans ce contexte, le DFF40 pourrait agir à titre de sauveteur de la mort cellulaire lorsque présent dans les cellules apoptotiques par la régulation du cycle cellulaire au point de contrôle G2/M (Merve Kulbay *et al.*, 2021a). Nos résultats supportent cette hypothèse : en l'absence de DFF40, les cellules cancéreuses ont une sensibilité accrue aux inhibiteurs de TOP2 pouvant être expliqués par leurs incapacités à induire les voies de réparation de dommages à l'ADN. Une nouvelle étude est venue appuyer notre hypothèse. Il a été démontré que l'absence de DFF40 dans les cellules cancéreuses U2OS d'ostéosarcome diminue de manière significative la croissance tumorale suivant une irradiation due à une instabilité génomique accrue et arrêt mitotique à la phase G2 (Larsen Brian *et al.*, 2022). Lors de dommages à l'ADN, le groupe de chercheurs ont démontré que le DFF40 et DFF45 sont recrutés au niveau de la chromatine et sont impliqués dans la production de BSB d'ADN de manière séquence-spécifique (Larsen Brian *et al.*, 2022). L'activité d'ATM et ATR sont nécessaires au recrutement du DFF40 suivant des dommages à l'ADN induits par la radiation, afin de maintenir une prolifération et survie cellulaire optimale (Larsen Brian *et al.*, 2022). Les conclusions tirées de cette étude sont extrapolables à notre projet de recherche. En l'absence de DFF40, les dommages à l'ADN induits par les inhibiteurs de TOP2 ne peuvent être réparés due à une absence de mécanisme de régulation intrinsèque des DDR.

6.4 Reprogrammation énergétique dans les cellules déficientes en DFF40

6.4.1 Une déficience en DFF40 induit une modification du métabolisme énergétique des cellules cancéreuses vers l'effet Warburg

Parmi les caractéristiques des tumeurs malignes mentionnées ci-dessus, les changements dans la régulation du métabolisme énergétique sont parmi les mieux répertoriés (Xue-Bing Li *et al.*, 2015). En effet, les cellules cancéreuses dépendent de la glycolyse aérobie pour leur production d'ATP (Xue-Bing Li *et al.*, 2015). De nombreuses mutations impliquées dans l'activation des oncogènes et inactivation des gènes suppresseurs de tumeurs ont un impact sur l'activité enzymatique des kinases responsables de la glycolyse aérobie (Jang *et al.*, 2013). Parmi celles-ci, les mutations de la voie de signalisation de PI3K, responsable de l'activation en aval d'AKT, sont les plus fréquentes et cruciales dans la prolifération et survie des cellules cancéreuses humaines (Jang *et al.*, 2013). De nombreuses fonctions physiologiques sont d'ailleurs régulées par AKT, dont la dynamique mitochondriale, le métabolisme énergétique, l'état redox, l'apoptose, l'autophagie et la mitophagie (Xie *et al.*, 2022). Sachant que les tumeurs déficientes en DFF40 ont un profil malin plus élevé, comme démontré dans la littérature (Banas *et al.*, 2018a, Sánchez-Osuna *et al.*, 2016), nous nous sommes donc intéressés à l'impact d'une abolition de l'expression du DFF40 sur la sphère énergétique. Nous avons démontré que les cellules Jurkat DFF40 KO ont une augmentation significative de leur masse mitochondriale, ainsi que de leur quantité d'ADNmt (Article 3, Fig. 3). Du point de vue métabolique, les cellules DFF40 KO produisent plus de ROS (Article 1, Fig. 3), ils ont un $\Delta\Psi m$ perturbé à la hausse, une augmentation du taux de consommation d'oxygène et de glycolyse (Article 3, Fig. 4), ainsi qu'une augmentation de la prolifération cellulaire marquée par une augmentation de l'expression d'AKT et du facteur de transcription Ki-67 (Article 3, Fig. 5). Le rôle de la mitophagie dans le développement de cancer est encore à ce jour un processus non élucidé à part entière. Les hypothèses actuelles suggèrent que selon le stade de tumorigenèse, la mitophagie peut être induite ou inhibée, dans le but ultime de favoriser la survie tumorale (Yigang Wang *et al.*, 2020). Lors de la carcinogenèse, il a été démontré qu'une perturbation de la voie de signalisation de PINK1/Parkin par une sous-régulation de l'expression de Parkin inhibe la mitophagie et promeut le développement tumoral (Yigang Wang *et al.*, 2020). De plus, il a été démontré que le recrutement et l'activation de Parkin et PINK1 à la mitochondrie sont reliés à la dépolarisation de la membrane mitochondriale externe (Lazarou *et al.*, 2015, Zong *et al.*, 2016). Qu'advient-il dans les cellules cancéreuses avec un potentiel de membrane de repos augmenté, comme démontré dans nos résultats? De manière hypothétique, il se pourrait que le DFF40 soit un partenaire

d'interaction de la mitochondrie pour le maintien de la stabilité mitochondriale. En l'absence de DFF40, une hausse du ΔΨm induirait une dépolarisation moindre suivant un stimulus physico-chimique, inhibant ainsi le recrutement de PINK1/Parkin et favorisant la carcinogenèse. De nombreuses études ont démontré l'importance d'une régulation du ΔΨm dans les cellules tumorales. Les lignées cellulaires de cancer de poumon (Ye *et al.*, 2011) et de glioblastomes (Bonnet *et al.*, 2007) ont un ΔΨm augmenté et ceux-ci figurent parmi les tumeurs avec la plus grande agressivité. De plus, il a été démontré qu'une déficience en ADNmt dans les cellules du cancer du sein T-47D diminue de manière significative le ΔΨm et est responsable d'une altération de la prolifération cellulaire (Man Yu *et al.*, 2007). Le rôle protecteur d'un ΔΨm élevé dans les cellules cancéreuses pourrait aussi être attribué à un état oxydatif basal augmenté dans ce contexte. En effet, nous avons démontré que non seulement les cellules Jurkat DFF40 KO ont une augmentation du ΔΨm basal et du nombre de copies d'ADNmt (Article 3, Fig. 3), mais aussi une élévation de la production de ROS (Article 1, Fig. 3). Le rôle des ROS dans les cellules cancéreuses a longuement été étudié ; des taux optimaux de ROS sont nécessaires pour la tumorigenèse. Ceux-ci sont augmentés dans de nombreux cancers et sont impliqués dans la prolifération, différenciation et survie cellulaire, ainsi que dans le métabolisme du glucose (Liou *et al.*, 2010). La production de ROS active la voie de signalisation de PI3K/AKT. En accord avec la littérature, nous avons démontré que dans des conditions non apoptotiques, les cellules déficientes en DFF40 ont une phosphorylation augmentée d'AKT, concordant avec une augmentation de la prolifération cellulaire et une augmentation de l'expression du facteur de transcription Ki-67 (Article 3, Fig. 5). Tous ces changements biologiques sont responsables du phénomène de Warburg, où les cellules cancéreuses optent pour la glycolyse aérobie pour leur production d'énergie. Les changements métaboliques observés dans les cellules cancéreuses déficientes en DFF40 pourraient être attribués à l'expression augmentée de Mcl-1. Il a été démontré que la bioénergétique mitochondriale requiert la présence de Mcl-1 à la matrice extracellulaire ; Mcl-1 subit une translocation à la membrane externe mitochondriale à l'aide d'une séquence de localisation mitochondriale (MTS) (Perciavalle *et al.*, 2012). Il a été démontré qu'une délétion de Mcl-1 diminue le ΔΨm, tandis qu'une surexpression de celle-ci permet d'améliorer le ΔΨm (Perciavalle *et al.*, 2012). Il fut également démontré qu'une délétion de Mcl-1 réduis la prolifération cellulaire et les niveaux de production d'ATP (Perciavalle *et al.*, 2012).

Le rôle de la protéine kinase AKT est versatile ; elle est impliquée dans la régulation de la prolifération et de la survie cellulaire, le métabolisme énergétique et la migration tissulaire (Xu *et al.*, 2012). Du point de vue du cycle cellulaire, AKT permet la transition de la phase G1 à S, ainsi que de la phase G2 à M, en induisant ou inhibant de nombreux effecteurs qui vont à leur tour

réguler l'expression des cyclines (Xu *et al.*, 2012). Depuis les dernières décennies, de nombreuses études se sont intéressées au rôle d'AKT dans la régulation de la stabilité génomique. Un des signaux activateurs de la voie de signalisation d'AKT est le dommage à l'ADN. Il a été démontré que l'activation d'AKT permet de prévenir les arrêts mitotiques de la phase G2, en favorisant la réparation de l'ADN, indépendamment de l'expression de p53 (Kandel *et al.*, 2002). Dans les cellules de glioblastomes, la prévention de l'arrêt mitotique en phase G2 diminue le recrutement de la protéine CHK2 aux sites de dommages à l'ADN (Hirose *et al.*, 2005). Ces phénomènes permettent une prolifération accrue des cellules cancéreuses et l'instauration d'une instabilité génomique qui favorise la tumorigenèse. Ayant démontré que les cellules déficientes en DFF40 ont une phosphorylation accrue d'AKT, est-ce que le DFF40 pourrait non seulement être un partenaire des points de contrôle, mais aussi un inhibiteur et un régulateur de la voie de signalisation d'AKT? L'acquisition d'un génotype déficient en DFF40 dans les cellules cancéreuses pourrait permettre de contrer les signaux inhibiteurs de la prolifération cellulaire et d'opter pour un phénotype caractéristique de l'effet Warburg.

6.4.2 Le DFF40 transloque à la mitochondrie lors de l'apoptose

La majorité des protéines mitochondriales sont encodées dans le compartiment nucléaire, puis elles sont traduites dans le cytosol et transportées à la membrane mitochondriale par l'entremise de nombreux processus physiologiques (Betz *et al.*, 2013). L'ADNmt est responsable de l'encodage de 13 protéines mitochondriales seulement. Dans notre projet de recherche, nous nous sommes penchés à savoir si le DFF40 pourrait jouer un rôle dans la mitochondrie lors de l'apoptose. En effet, la structure du DFF40 a une grande homologie avec les protéines de la famille CIDE en son domaine N-terminal, constitué de 80 acides aminés hautement conservés (Woo *et al.*, 2004). De nombreuses localisations et fonctions mitochondriales ont été rapportées pour les protéines de la famille CIDE (Moreno-Navarrete *et al.*, 2014, Slayton *et al.*, 2019, Valousková *et al.*, 2008). Nous avons démontré la présence d'une MTS à l'aide d'une analyse in silico : la probabilité que la séquence de 27 acides aminés soit une MTS était de 0.9389 (Article 3, Fig. 6). Les MTS varient en taille de 15 à 70 résidus habituellement et sont composés de résidus basiques chargés positivement (Mengxia Li *et al.*, 2010). Notre séquence correspondant à ces critères, nous avons donc étudié la présence de DFF40 mitochondriale à l'aide d'essais de précipitation protéiques. Nous avons pour la première fois démontré que lors de l'apoptose, le DFF40 subit une translocation à la membrane externe de la mitochondrie (Article 3, Fig. 6). L'absence de DFF40 dans les cellules cancéreuses pourrait induire des anomalies d'activation de la voie de signalisation intrinsèque de l'apoptose.

Il est à noter que dans notre expérience de localisation mitochondriale, le DFF40 a été précipité sous forme d'un complexe avec le DFF45 : nous n'étions pas en mesure de précipiter le DFF40 seul, ce dernier formant des précipités insolubles dans les solvants. Des essais d'immunoprécipitation en conditions non dénaturantes nous ont permis d'isoler de manière hypothétique la protéine d'intérêt. Or, il était impossible de le confirmer dû à la présence d'une multitude de bandes non spécifiques.

En date d'aujourd'hui, aucune étude n'a réussi à faire des essais d'immunoprécipitation avec le DFF40 humain. Une seule étude a rapporté avec succès l'immunoprécipitation du DFF40 dans les cellules hépatiques de la souris en utilisant des billes d'agarose/sepharose conjuguées à l'anticorps du DFF40 (Xiang *et al.*, 2006). Cependant, la protéine d'intérêt fut détectée à un poids de 52 kDa et de 20 kDa lorsque clivée par la caspase-3 (Xiang *et al.*, 2006). Les difficultés d'immunoprécipitation du DFF40 peuvent être expliquées par sa structure moléculaire ; la structure moléculaire du DFF40 est composée de régions complémentaires chargées positivement ou négativement qui sont impliquées dans les interactions d'homooligomères (Ha *et al.*, 2022). Il a été démontré que la protéine Drep-4, l'homologue du DFF40 chez la drosophile, forme des interactions homotypiques, lorsqu'elle est activée (voir Figure 14), où 10 molécules de Drep-4 vont interagir pour former la structure d'hélice (Ha *et al.*, 2022). Cette configuration spatiale est similaire à celle opté par le DFF40, où celui-ci forme des oligomères à l'aide de quatre molécules de DFF40 (Choi *et al.*, 2017). De plus, le DFF40 possède en sa région CIDE-N un domaine hydrophobe qui est impliqué dans les interactions avec les protéines de la famille CIDE (Ha *et al.*, 2022). Afin de pallier cet enjeu, nous pourrions générer par mutagenèse dirigée une lignée stable dont le DFF40 a été muté aux sites d'interactions homotypiques. Ceci pourrait permettre d'inhiber la formation d'oligomères lorsque le DFF40 est dissocié de sa sous-unité inhibitrice. La surface basique du DFF40 sera maintenue dans le but de permettre son interaction avec le DFF45.

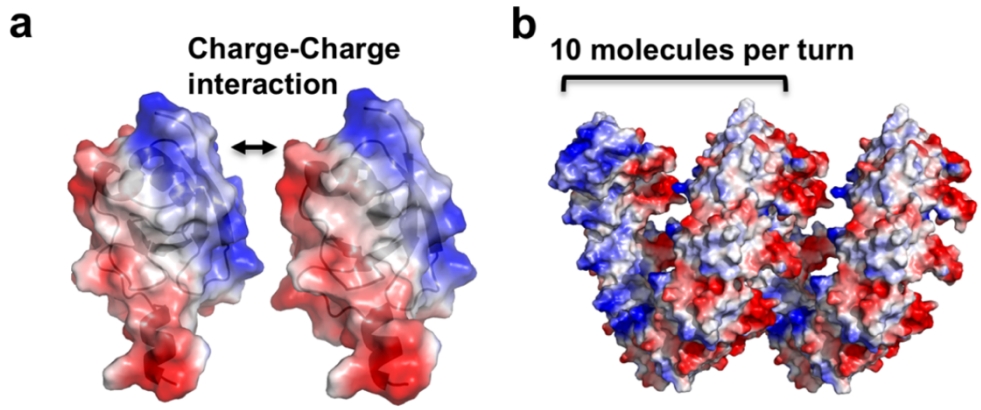


Figure 14. Représentation des propriétés dynamiques de la protéine DREP4 de la drosophile.

Panneau A : Représentation de la distribution électrostatique de surface de la protéine Drep-4. Panneau B : Représentation d'un oligomère de Drep-4; 10 molécules de Drep-4 sont nécessaires pour une structure d'hélice. Image adaptée de : (Ha *et al.*, 2022).

6.4.3 Le DFF40 pourrait-il être impliqué dans certaines fonctions mitochondriales?

Un des rôles importants de la mitochondrie dans la régulation du métabolisme cellulaire est la synthèse d'acides aminés (Spinelli *et al.*, 2018). Ayant démontré que le DFF40 localise à la mitochondrie lors de l'apoptose et que les cellules déficientes en DFF40 ont un métabolisme cellulaire orienté vers l'effet Warburg, nous croyons que le DFF40 pourrait être un régulateur de la synthèse d'acides aminés. Les antimétabolites sont des agents de chimiothérapie qui interfèrent avec la synthèse de l'ADN en s'incorporant dans les brins naissants par une compétition avec les nucléotides. Nous avons démontré que les cellules déficientes en DFF40 étaient résistantes à l'apoptose induite par ces agents de chimiothérapie (Article 2, Fig. 2), et qu'un ajout de nucléotide extracellulaire permet d'augmenter la prolifération cellulaire des cellules déficientes en DFF40 traités avec l'Ara-C et la 6-MP (Article 2, Fig. 5). La localisation mitochondriale pourrait être un facteur protecteur contre la croissance tumorale, où son absence permettrait la prolifération de cellules cancéreuses, comme démontré dans notre projet de recherche. Jusqu'à ce jour, aucune étude ne s'est penchée sur le rôle métabolique du DFF40 et de nombreuses recherches restent à être effectuées.

6.5 Implication du DFF40 dans la stabilité génomique

6.5.1 Les cellules déficientes en DFF40 maintiennent leur stress oxydatif suivant l'induction de l'apoptose

Les premiers changements biomoléculaires de la voie intrinsèque de l'apoptose impliquent la production de ROS et l'altération du ΔΨm. Nous avons démontré que chez les cellules Jurkat déficientes en DFF40, lors de l'apoptose induite par le TBT, il y a une augmentation de production de ROS et un retard de perturbation du ΔΨm, pouvant être expliqué par un retard d'activation de NRF2 et SOD1 (Article 4, Fig. 1 et 2). La balance en ROS permet de maintenir la tumorigenèse chez les cellules cancéreuses, comme discuté dans notre Article 4. Le maintien tardif du ΔΨm dans les cellules cancéreuses déficientes en DFF40 pourrait permettre de favoriser la production de ROS et la survie cellulaire subséquente.

Il est à noter que nous avions précédemment démontré que suivant l'induction de l'apoptose par le TBT, il n'y avait pas de modulation significative dans les taux de production de ROS chez les cellules déficientes en DFF40 (Article 1, Fig. 3). Ceci peut être expliqué par le fait que les conditions de la méthode expérimentale n'étaient pas optimales. Dans notre premier article, nous avions utilisé une dose inférieure de sonde H_2DCFDA (*c.-à-d.* 5 μM), contrairement aux derniers résultats où nous avons doublé la dose (*c.-à.d.* 10 μM). Nous avons démontré qu'une dose sous optimale de sonde peut en effet sous-estimer la production de ROS (Article 4, Fig. S1). Il a été démontré que les niveaux de calcium et de magnésium corrèlent de manière positive avec les niveaux de ROS détectés (Jingru Zhou *et al.*, 2020). Dans l'article 1, les échantillons étaient analysés dans du PBS suivant deux cycles de lavage, tandis que dans l'article 4, les cellules ont été analysées dans le milieu de traitement, en présence de TBT. Il a été démontré que le TBT stimule la relâche de calcium dans le milieu extracellulaire (Kawanishi *et al.*, 2001). Les résultats ont donc pu être influencés par ceci. Nous croyons que le second design expérimental reflète mieux les conditions physiopathologiques : lors des traitements de chimiothérapie, l'agent inducteur d'apoptose se retrouve en circulation sanguine pendant plusieurs heures prolongées.

6.5.2 La résistance à l'apoptose est médiée partiellement par l'inhibition de la voie p38 par AKT

Les voies de réparation de l'ADN, ATM et ATR, peuvent être régulées par d'autres protéines kinase, telles qu'expliquées au chapitre 1. Dans cette thèse, nous nous sommes particulièrement intéressés à la modulation induite par AKT. Les résultats des travaux de cette thèse ont permis de démontrer que les cellules déficientes en DFF40 ont une inhibition de la voie p38 MAPK

médiée par AKT, suivant l'induction de l'apoptose par le TBT (Article 4, Fig. 3). Nos résultats concordent avec celles de la littérature : une activation de la voie d'AKT dans les cellules endothéliales confère une cytoprotection à l'apoptose induite par la privation de facteurs de croissance par l'entremise d'une inhibition de la voie de signalisation de p38 MAPK (J. P. Gratton *et al.*, 2001b). Or, qu'en est-il des autres voies de signalisation de la voie des MAPK ? Il a été démontré que la voie de signalisation de MEK/ERK est impliquée dans la transmission des signaux de mort cellulaire : l'inhibition de MEK/ERK prévient l'apoptose des cellules de la moelle osseuse de rats exposés à l'acide salvianolique (Biyan Lu *et al.*, 2010). Or, il a aussi été démontré que l'activation des voies de signalisation d'ERK et AKT par la toxine subtilase produite par *E. coli* confère une cytoprotection, particulièrement en modulant les niveaux d'activation de la voie du stress du RE (Tian *et al.*, 2011). ERK pourrait donc posséder un double rôle sur la stabilité génomique et varier son effet selon le type cellulaire.

L'implication des MAPK dans la régulation des voies de réparation de l'ADN est bien caractérisée. Il a été démontré que l'inhibition de la voie de signalisation de p38 permet de faciliter la réparation de l'ADN par recombinaison homologue (Golding *et al.*, 2007). L'utilisation d'inhibiteurs de la voie d'ATM inhibe la phosphorylation d'ERK et subséquemment la réparation de l'ADN (Golding *et al.*, 2007). L'activation d'AKT par ATM, ATR ou la DNA-PK permet d'instaurer une boucle de rétroaction positive : AKT médie la transition au niveau des points de contrôles G1/2 et G2/M, ainsi que la réparation de l'ADN (Xu *et al.*, 2012). Il a été démontré que les souris déficientes pour l'activation d'AKT ont un phénotype similaire aux souris déficientes en DNA-PK (Bozulic *et al.*, 2008). AKT co-localise à la chromatine au site des dommages avec la DNA-PK pour médier les réparations à l'ADN et la survie cellulaire (Bozulic *et al.*, 2008). Globalement, nos résultats, en accord avec celles de la littérature, supportent donc l'hypothèse que le DFF40 est impliqué directement dans la stabilité génomique via une modulation des voies de signalisation impliquées dans la prolifération et la réparation de l'ADN.

Cela dit, dans cette étude (Article 4), nous avons seulement étudié l'impact d'une délétion du DFF40 sur l'activation des voies de signalisation d'AKT et p38. Cependant, comme démontré dans la littérature, la modulation des autres voies de signalisation des MAPK est aussi impliquée dans la stabilité génomique. Nous croyons qu'une modulation d'ERK et JNK sera présente chez les cellules déficientes en DFF40, ainsi qu'au niveau de l'activation de la voie du stress du RE.

6.5.3 La déficience en DFF40 altère les voies de réparation de l'ADN suivant l'induction de l'apoptose

À travers notre projet de recherche, nous avons soulevé à plusieurs reprises le rôle potentiel du DFF40 dans la stabilité génomique. Dans les dernières sections, nous avons démontré des preuves essentielles supportant cette hypothèse. Le dernier objectif de notre projet de recherche visait donc à élucider l'impact d'une déficience en DFF40 sur les voies de réparation de l'ADN. De multiples études supportent le rôle des ROS dans l'instauration d'une instabilité génomique. Nous avons démontré que les cellules cancéreuses déficientes en DFF40 produisent plus de ROS à l'état basal (Article 1, Fig. 3), mais également dans l'apoptose (Article 4, Fig. 1). De plus, nous avons démontré que les cellules déficientes en DFF40 médient la résistance à l'apoptose par un retard d'activation de la voie de signalisation (Article 1), et par l'inhibition de la p38 MAPK par AKT (Article 4, Fig. 3).

Nos derniers résultats de travaux de recherche ont démontré que les cellules déficientes en DFF40 ont une activation supérieure de la voie d'ATR et une diminution significative de l'activation d'ATM, en comparaison aux cellules DFF40 WT (Article 4, Fig. 5). Nous croyons que la régulation de l'expression de Mcl-1 pourrait être impliquée dans la perturbation de la stabilité génomique. Il a été démontré qu'une surexpression de Mcl-1S induit une accumulation de dommages à l'ADN via une perturbation du cycle cellulaire (Streletskaia *et al.*, 2020). De plus, la transfection transitoire de lignées cellulaires cancéreuses avec Mcl-1 augmente la phosphorylation de CHK1 et l'accumulation de cellules en phase G2/M (Jamil *et al.*, 2008). Ceci est en lien avec une résistance supérieure aux effets cytotoxiques engendrés par l'ETO (Jamil *et al.*, 2008). En accord avec ces résultats, nous avons démontré que le TBT induit une accumulation de cellules en phase G2/M dans notre lignée cellulaire déficiente en DFF40 (Article 1). Nous émettons donc l'hypothèse que l'altération de la voie de signalisation d'ATR pourrait être médiée par une modulation de l'isoforme de Mcl-1. Afin de démontrer cette hypothèse, nous pourrions effectuer des essais à l'aide d'inhibiteurs pharmacologiques de Mcl-1 et caractériser la réponse cellulaire et moléculaire des cellules T déficientes en DFF40. De plus, dans le but de valider un débalancement dans les isoformes de Mcl-1 (*c.-à-d.* Mcl-1L et Mcl-1S), nous pourrions effectuer des essais d'immunoprécipitation de Mcl-1 dans nos cellules DFF40 WT et DFF40 KO et par la suite, séquencer les isoformes de Mcl-1.

En somme, de nombreux mécanismes moléculaires sont impliqués dans la stabilité génomique des cellules cancéreuses. Les modifications de la chromatine figurent parmi les mécanismes avec le plus grand niveau d'intérêt. Nous avons démontré que les cellules déficientes en DFF40 KO

ont une diminution de l'acétylation de l'histone H3 (Article 4, Fig. 5). Or, un des mécanismes régulateurs les plus importants dans la transcription génomique est la méthylation de l'ADN. Les cellules cancéreuses peuvent soit avoir une hypométhylation sur la totalité de leur génome ou des zones d'hyperméthylation à des sites spécifiques (*c.-à-d.* aux sites CpG) dans le promoteur de gènes (Wajed *et al.*, 2001). À notre connaissance, jusqu'à ce jour, aucune étude n'a démontré le lien direct entre la méthylation de l'ADN et la résistance à l'apoptose chez les cellules cancéreuses déficientes en DFF40. Cependant, des études ont démontré le rôle de la méthylation auprès des protéines de la famille CIDE. Dans les cellules ayant une sous-expression de la protéine CIDE-A, une hyperméthylation aux sites CpG des régions du promoteur est présente (D. Li *et al.*, 2008). Sachant que le DFF40 fait partie des protéines de la famille CIDE, nous croyons que des modifications dans la méthylation de l'ADN seraient impliquées dans la résistance des cellules cancéreuses aux médicaments de chimiothérapies. Des études supplémentaires sont nécessaires afin de valider cette hypothèse.

7 CONCLUSION

À la lumière des résultats des travaux de cette thèse, nous avons pu fournir une contribution significative à l'avancement des connaissances, particulièrement en ce qui a trait à la compréhension des mécanismes moléculaires impliqués dans la résistance des cellules cancéreuses aux traitements inducteurs d'apoptose.

L'utilisation de la technique CRISPR-cas9 nous a permis de créer pour la première fois dans la littérature une lignée cellulaire non-adhérente stable, déficiente en DFF40. Nos résultats ont démontré que les cellules déficientes en DFF40 possèdent une régulation physiologique de l'expression de Mcl-1 à la hausse et, du point de vue phénotypique, présentent des altérations suggestives d'une adaptation de l'effet Warburg. Les essais métaboliques de Seahorse ont révélé que les cellules T déficientes en DFF40 ont une augmentation de la consommation d'oxygène en conditions non apoptotiques et présentent des taux de glycolyse supérieurs. Ces trouvailles phénotypiques peuvent être expliquées au niveau moléculaire par une induction de l'expression du facteur de transcription Ki-67 et de la voie de signalisation de PI3K/AKT. Globalement, ces adaptations moléculaires sont d'un grand intérêt, car à l'aide d'analyses statistiques bio-informatiques, nous avons pu démontrer qu'un grand nombre de cellules tumorales malignes possèdent une régulation négative de l'expression du DFF40.

L'utilisation de divers agents inducteurs d'apoptose, dont le TBT, STS et les médicaments de chimiothérapie, ont permis d'élucider la réponse biocellulaire et biomoléculaire des cellules T déficientes en DFF40 à celles-ci. Nous avons démontré qu'une déficience en DFF40 confère une résistance à l'apoptose induite par de nombreux agents, dont le TBT, la STS et les antimétabolites. En revanche, nous avons démontré une sensibilité supérieure des cellules T déficientes en DFF40 aux inhibiteurs de TOP2. La résistance peut en partie être expliquée par un délai d'activation de la voie de signalisation de l'apoptose et une inhibition de la voie de signalisation p38 MAPK dépendante de l'activation de la voie d'AKT. Des études supplémentaires sont cependant nécessaires, afin de mieux caractériser les phénomènes moléculaires impliqués dans la sensibilité aux inhibiteurs de TOP2.

Finalement, en complément aux résultats de la littérature, nous avons permis de démontrer que le DFF40 possède un rôle dans la stabilité génomique. Une déficience en DFF40 dans les cellules T cancéreuses perturbe les voies de réparation de l'ADN suivant l'induction de l'apoptose, en favorisant une activation de la voie ATR et une inhibition de la voie d'ATM. De plus, nous avons fourni des évidences concernant un rôle potentiel du DFF40 dans la régulation du cycle cellulaire.

Les cellules T déficientes en DFF40 ont un arrêt de transition à la phase S du cycle cellulaire, lorsque exposé aux antimétabolites, tandis que l'apoptose induite par le TBT induit un arrêt à la phase G2/M. Des études complémentaires sont toutefois nécessaires afin d'élucider le rôle du DFF40 dans le cycle cellulaire.

8 PERSPECTIVES

Dans ma thèse de doctorat, nous avons utilisé comme modèle d'étude les cellules T Jurkat, pour les avantages énumérés ci-dessus (section Discussion). Or, la question suivante se pose : est-ce que le modèle Jurkat est un bon modèle d'étude afin d'atteindre une bonne validation externe ? Cliniquement, à notre connaissance, aucune étude n'a démontré jusqu'à ce jour une régulation de l'expression du DFF40 dans les divers types de leucémies. Dans nos travaux de recherche, nous nous étions penchées sur cette question. Cependant, nous n'avions pas pu établir une corrélation entre l'expression génique du DFF40 avec les différents types de leucémies aiguës ou chroniques ; la banque de données TCGA et TARGET n'ont pas d'échantillons contrôles pour ces types de cancer. Cela dit, nous avons tout de même pu observer une expression similaire du DFF40 entre les types de leucémies (résultats non démontrés dans la thèse). Or, nous n'avons pas pu confirmer si cette expression avait une régulation à la baisse comparativement à l'expression basale dans les cellules T et B physiologiques. Afin d'accroître la signifiance clinique des résultats de mon projet de doctorat, il serait intéressant de générer des lignées cellulaires supplémentaires déficientes en DFF40. Nous avons démontré que plusieurs tumeurs associées avec de mauvais pronostics possèdent une sous-régulation de l'expression du DFF40 (Article 2, Fig. 1). Nous pourrions donc générer au moins une lignée supplémentaire stable par CRISPR-cas9 pour valider les résultats de cette thèse. Une deuxième méthode d'édition serait d'utiliser de petits RNA interférents (siRNA). Plusieurs études ont rapporté l'utilisation de siRNA pour la régulation du DFF40 (Iglesias-Guimarais *et al.*, 2013, Martínez-Escardó *et al.*, 2021). Contrairement à la méthode de CRISPR-cas9 qui requiert énormément de temps, l'utilisation de siRNA permettrait de valider de manière rapide et efficace nos résultats.

Un second modèle d'étude pour caractériser le rôle du DFF40 dans le cycle cellulaire et la stabilité génomique serait d'utiliser le modèle du poisson-zèbre. Le séquençage du gène du DFF40 chez le poisson-zèbre a permis de démontrer que celui-ci avait une homologie de 100% avec son homologue humain. Plusieurs avantages existent à utiliser le poisson-zèbre comme modèle d'étude ; ce modèle possède un cycle de reproduction et de développement très rapide comparativement aux modèles murins (D'Costa *et al.*, 2009), qui ont déjà été utilisés dans les études impliquant le DFF40. En effet, les poissons-zèbres atteignent la maturité, c'est-à-dire l'âge adulte, dès le 90^{e} jour post-fertilisation (D'Costa *et al.*, 2009). De plus, le développement du système nerveux est possible dès le 2^{e} jour post-fertilisation grâce à la transparence des embryons (D'Costa *et al.*, 2009). Des études ont démontré l'importance du DFF40 dans les

fonctions cognitives : les souris mutantes pour l'expression du DFF45 possèdent une meilleure mémoire d'apprentissage spatiale et une rétention mnésique augmentée dans les tâches de reconnaissances d'objets (McQuade *et al.*, 2002, Slane *et al.*, 2000). Chez la drosophile, il a été démontré que Drep-2, un analogue du DFF40 et du DFF45, est une protéine synaptique impliquée dans l'apprentissage et les adaptations comportementales (Andlauer *et al.*, 2014).Ces résultats suggèrent donc un rôle potentiel du DFF40 dans le développement neuronal. L'approche expérimentale serait de créer des générations de poissons-zèbres hétérozygotes pour l'expression du DFF40 par CRISPR-cas9. Le croisement de deux espèces hétérozygotes permettrait l'obtention d'une espèce déficiente en DFF40 (génération F3). À partir des embryons déficients en DFF40, plusieurs approches expérimentales sont possibles. Nous pourrions séquencer le génome des poissons-zèbres déficients en DFF40 afin de caractériser les modulations géniques secondaires à notre mutation. Ensuite, les marqueurs de régulation du cycle cellulaire, de l'apoptose et de la stabilité génomique peuvent être analysés par immunofluorescence.

Ensuite, dans les travaux de cette thèse, nous avons démontré des modulations au niveau protéique des partenaires de la voie de signalisation de l'apoptose par SDS-PAGE, dont Mcl-1. Nous pourrions approfondir l'étude des partenaires d'interaction potentielle du DFF40 à l'aide d'une méthode d'analyse avec une meilleure rentabilité. En effet, lorsque les cibles d'interaction ne sont pas connues, le SDS-PAGE n'est pas une approche idéale : plusieurs essais aléatoires doivent être effectués pour retrouver une régulation d'expression de protéines. Pour surmonter cet obstacle, nous pourrions utiliser la technique de profilage par « antibody array » (Haab *et al.*, 2013). Cette technique nous permettra de faire un premier triage concernant les cibles potentielles, puis les expressions protéiques pourront par la suite être validées par SDS-PAGE. Une fois les protéines d'intérêts identifiées, des études de fonctions peuvent être réalisées. Pour complémenter nos résultats, nous pourrions utiliser des inhibiteurs de Mcl-1. Nous croyons que Mcl-1 serait impliquée dans l'acquisition du phénotype malin chez les cellules déficientes en DFF40 et possèderait un rôle dans le cycle cellulaire et la réparation de l'ADN. Un exemple de design expérimental serait donc de traiter préalablement les cellules déficientes en DFF40 avec les inhibiteurs de Mcl-1, puis les exposer aux agents inducteurs d'apoptose. Nous pourrions par la suite mesurer les taux de mortalité, quantifier les taux de prolifération et vérifier les modulations dans les voies de réparation de l'ADN et du cycle cellulaire. Parallèlement, nous pourrions utiliser des inhibiteurs du cycle cellulaire, dont de la phase G2/M et de la phase S afin de valider le rôle du DFF40 à ces points de contrôle.

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10 ANNEXE I : FIGURES SUPPLÉMENTAIRES – ADDENDUM CHAPITRE 4

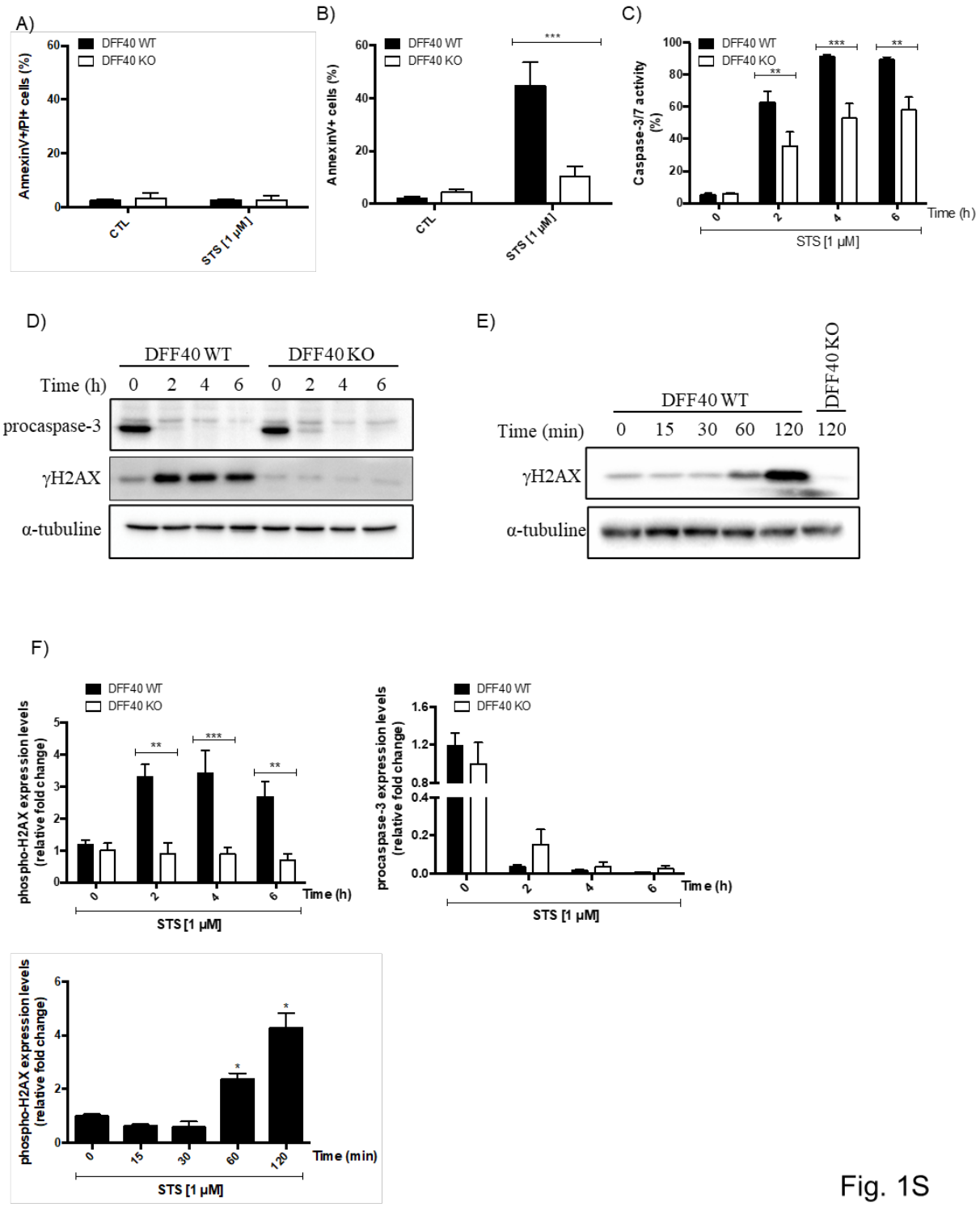


Fig. 1S

Figure 1S. DFF40 deficient Jurkat cells are resistant to STS-induced apoptosis. A and B: Jurkat DFF40 WT and DFF0 KO Jurkat cells ($1x10^6$ cells/ml) were treated or not with STS [1 μM] for 6 h in RPMI medium supplemented with 0.1% FBS and stained with a Annexin V/PI solution. Cell were analyzed by flow cytometer. C: Jurkat DFF40 WT and DFF0 KO Jurkat cells ($1x10^6$ cells/ml) were treated or not with STS [1 μM] for 2 to 6 h in RPMI medium supplemented with 0.1% FBS and stained with the Caspase-3/7 Green detection reagent. Cells were analyzed by flow cytometer. D: Same cell treatments as in C, but protein extracts were then run on a SDS-PAGE and immunoblotted for respective antibodies. Blots are representative of all data E: DFF40 WT Jurkat cells were treated or not with STS [1 μM] for 0 to 120 min, then handled as described as in D. F: Densitometric analysis of blots from panels D and E. Data's represent the mean of at least 3 independent experiments. Data's are presented as (mean ± SEM). Two-way ANOVA with Tukey's posthoc test was performed (**$p < 0.01$, ***$p<0.001$) or multiple t-test were performed for the last graph of panel F (* $p < 0.05$).

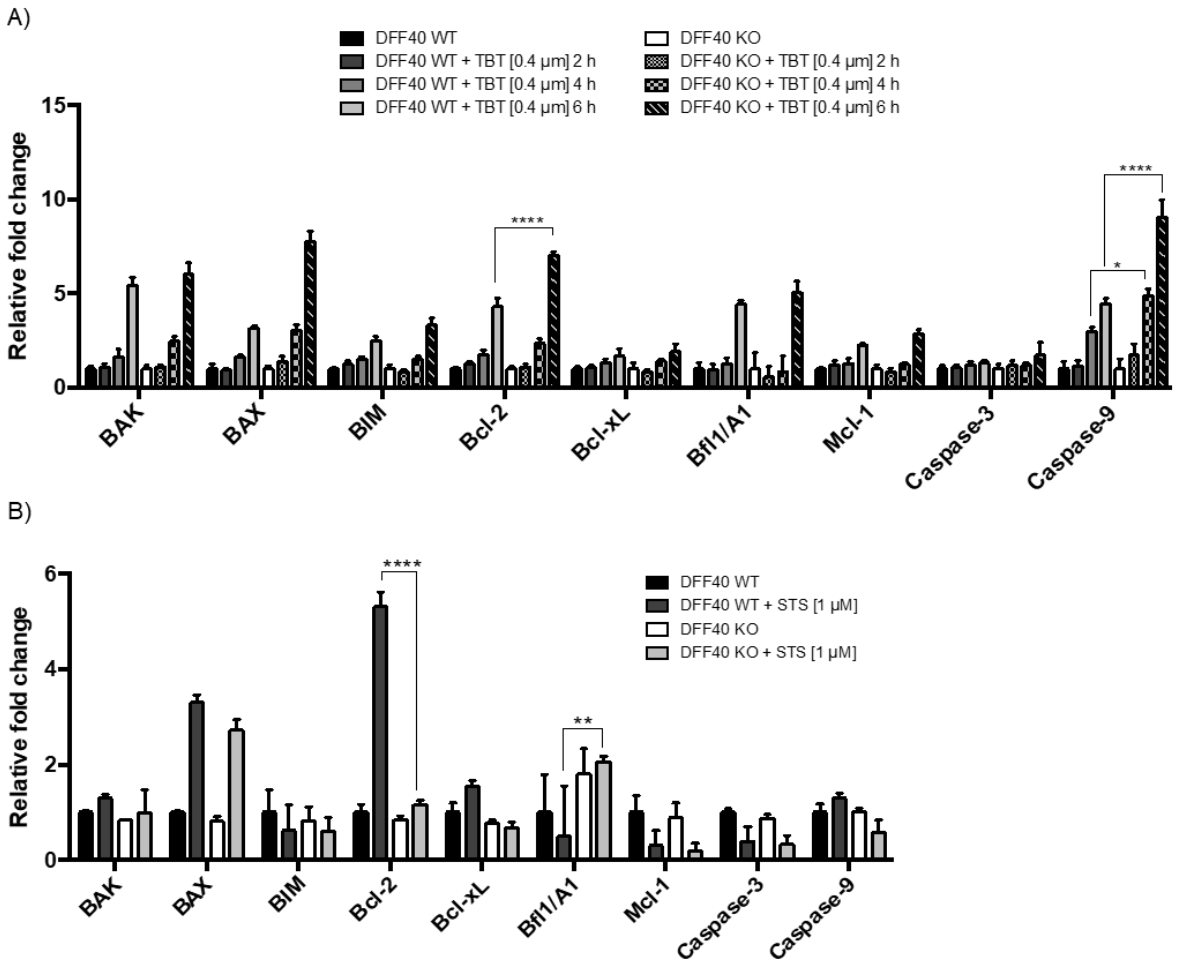
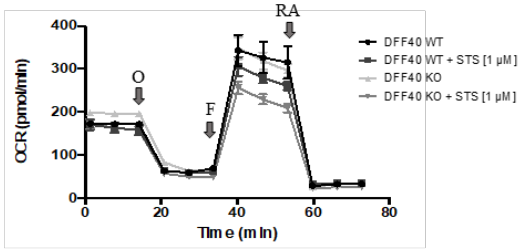

A)
DFF40 WT
DFF40 WT + TBT [0.4 µm] 2 h
DFF40 WT + TBT [0.4 µm] 4 h
DFF40 WT + TBT [0.4 µm] 6 h
DFF40 KO
DFF40 KO + TBT [0.4 µm] 2 h
DFF40 KO + TBT [0.4 µm] 4 h
DFF40 KO + TBT [0.4 µm] 6 h
Relative fold change
15
10
5
0
****
****
*
BAK
BAX
BIM
Bcl-2
Bcl-xL
Bfl1/A1
Mcl-1
Caspase-3
Caspase-9
B)
DFF40 WT
DFF40 WT + STS [1 µM]
DFF40 KO
DFF40 KO + STS [1 µM]
Relative fold change
6
4
2
0
****
**
BAK
BAX
BIM
Bcl-2
Bcl-xL
Bfl1/A1
Mcl-1
Caspase-3
Caspase-9

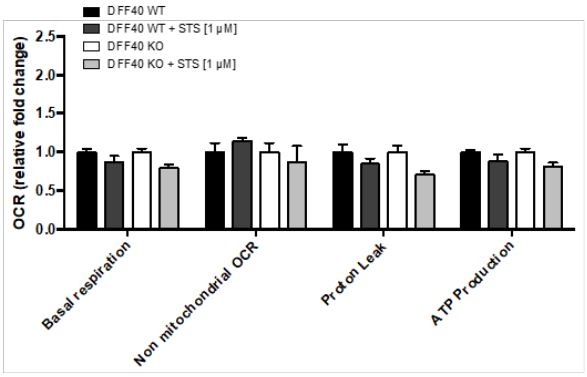
Fig. 2S

Figure 2S. Bcl-2 and caspase-9 RNA transcripts are significantly enhanced in DFF40 deficient T cells following tributyltin-induced apoptosis. A: RNA of Jurkat DFF40 WT and DFF40 KO cells ($5x10^6$ cells) treated with TBT [0.4 µM] for up to 6 h was extracted using the TRIzol reagent. RT-PCR's were performed on 50 ng RNA samples (50 ng RNA) followed by a qPCR. Results were analyzed with the CFX Maestro software. B: Same as in A but cells were treated with STS [1 µM] instead. Results are expressed as a relative fold change value in comparison to DFF40 WT cells. Data's represent the mean of at least 3 independent experiments. Data's are presented as (mean ± SEM). Two-way ANOVA with Tukey's posthoc test was performed (* $p < 0.05$, ** $p < 0.01$, **** $p<0.0001$).

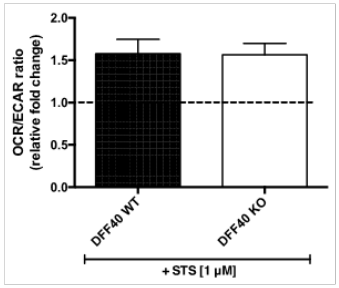
A)


RA
O
F
OCR(pmol/min)
Time (min)
DFF40 WT
DFF40 WT + STS [1 µM]
DFF40 KO
DFF40 KO + STS [1 µM]

B)


DFF40 WT
DFF40 WT + STS [1 µM]
DFF40 KO
DFF40 KO + STS [1 µM]
OCR (relative fold change)
Basal respiration
Non mitochondrial OCR
Proton Leak
ATP Production

C)


OCR/ECAR ratio
(relative fold change)
DFF40 WT
DFF40 KO
+ STS [1 µM]

D)

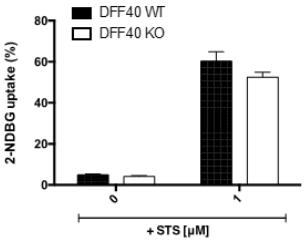
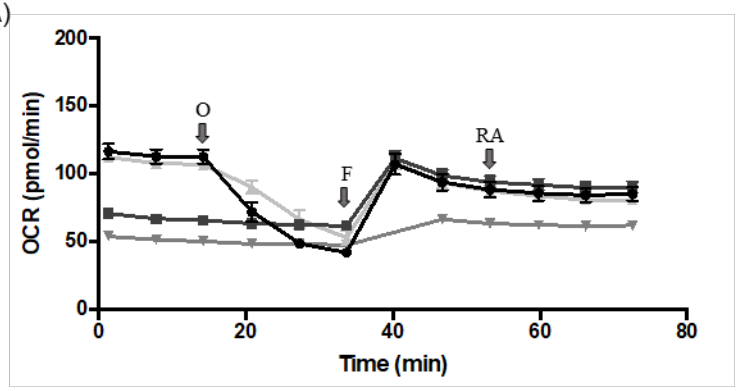

DFF40 WT
DFF40 KO
2-NDBG uptake (%)
+ STS [µM]

Fig. 3S

Figure 3S. STS-induced apoptosis does not alter cell metabolism in DFF40 deficient Jurkat cells. A: Jurkat DFF40 WT and DFF0 KO Jurkat cells ($2x10^5$ cells/ml) were treated or not with STS [1 μM] for 1 h in RPMI media supplemented with glucose (10 mM) and oxygen consumption rates (OCR) were measured with the Seahorse XFe96 technology by Agilent. Arrows represent the injection times. (O: Oligomycin; F: FCCP, RA: Rotenone/antimycin A). B: Same as in A. Mitochondrial respiratory function parameters were calculated using the Seahorse XF Cell Mito Stress Test Report Generator by Agilent. C: Same as in A but OCR/ECAR ratios were calculated for each cell line and condition. Dashed line represents the baseline (normalized control values). D: Jurkat DFF40 WT and DFF40 KO cells (10^6 cells/well) were treated or not with STS [1 μM] for 2 h, then incubated with 2-NDBG (1: 500) for 45 min in RPMI medium without glucose and analyzed with the BD FACSCalibur flow cytometer for glucose uptake. Data's represent the mean of at least 3 independent experiments. Data's are presented as (mean ± SEM). Two-way ANOVA with Tukey's posthoc test was performed.

A)


OCR (pmol/min)
200
150
100
50
0
O
F
RA
0
20
40
60
80
Time (min)

B)

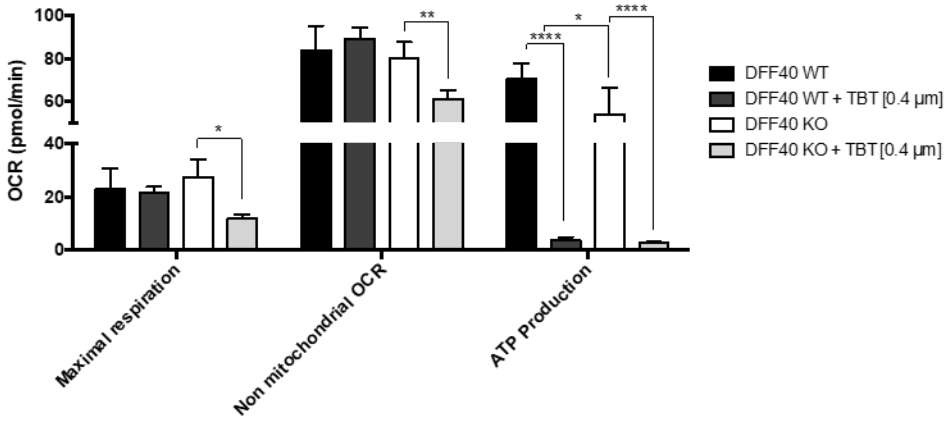

OCR (pmol/min)
100
80
60
40
20
0
*
**
****
*
****
Maximal respiration
Non mitochondrial OCR
ATP Production
DFF40 WT
DFF40 WT + TBT [0.4 µm]
DFF40 KO
DFF40 KO + TBT [0.4 µm]

Figure 4S

Figure 4S. DFF40 deficient cells do not entirely rely on oxidative phosphorylation for ATP production. A: Jurkat DFF40 WT and DFF0 KO Jurkat cells ($2x10^5$ cells/ml) were treated or not with TBT [0.4 μM] for 1 h in RPMI media supplemented with galactose (10 mM) and oxygen consumption rates (OCR) were measured with the Seahorse XFe96 technology by Agilent. Arrows represent the injection times. (O: Oligomycin; F: FCCP, RA: Rotenone/antimycin A). B: Same as in A. Mitochondrial respiratory function parameters were calculated using the Seahorse XF Cell Mito Stress Test Report Generator by Agilent. Data's represent the mean of at least 3 independent experiments. Data's are presented as (mean ± SEM). Two-way ANOVA with Tukey's posthoc test was performed.

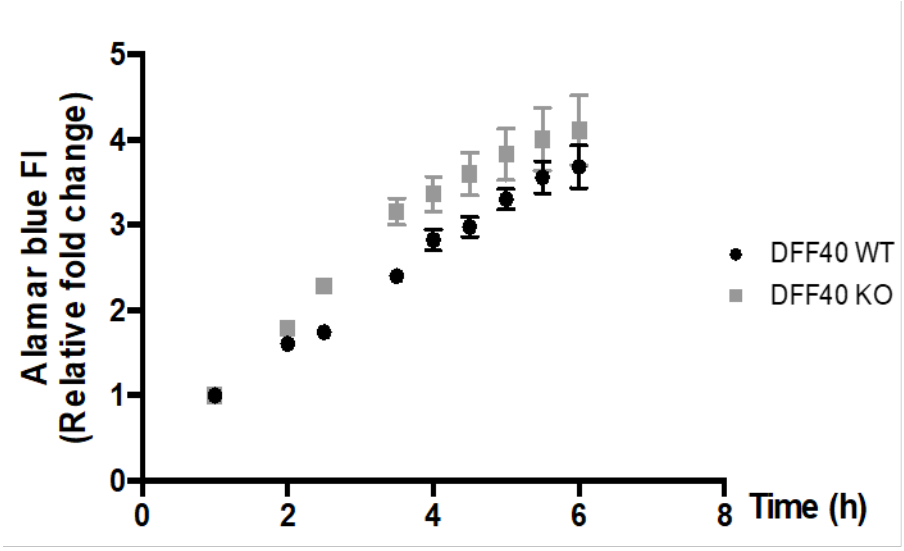

Alamar blue FI
(Relative fold change)
5
4
3
2
1
0
0
2
4
6
8
Time (h)
DFF40 WT
DFF40 KO

Figure 5S

Figure 5S. Cell proliferation kinetic curves of DFF40 WT and DFF40 KO Jurkat cells. Jurkat DFF40 WT and DFF40 KO cells ($1x10^6$ cells/ml) were incubated with Alamar Blue (10%) for up to 6 h. Cell proliferation at different time points was measured by dye fluorescence with the Spectra M5 Microplate reader at an excitation wavelength of 570 nm and emission wavelength of 585 nm. Results were normalized according to their respective control. Results are presented as the relative fold change in comparison to the control cells (incubation time = 0).

11 ANNEXE II : FIGURES SUPPLÉMENTAIRES – ADDENDUM CHAPITRE 5

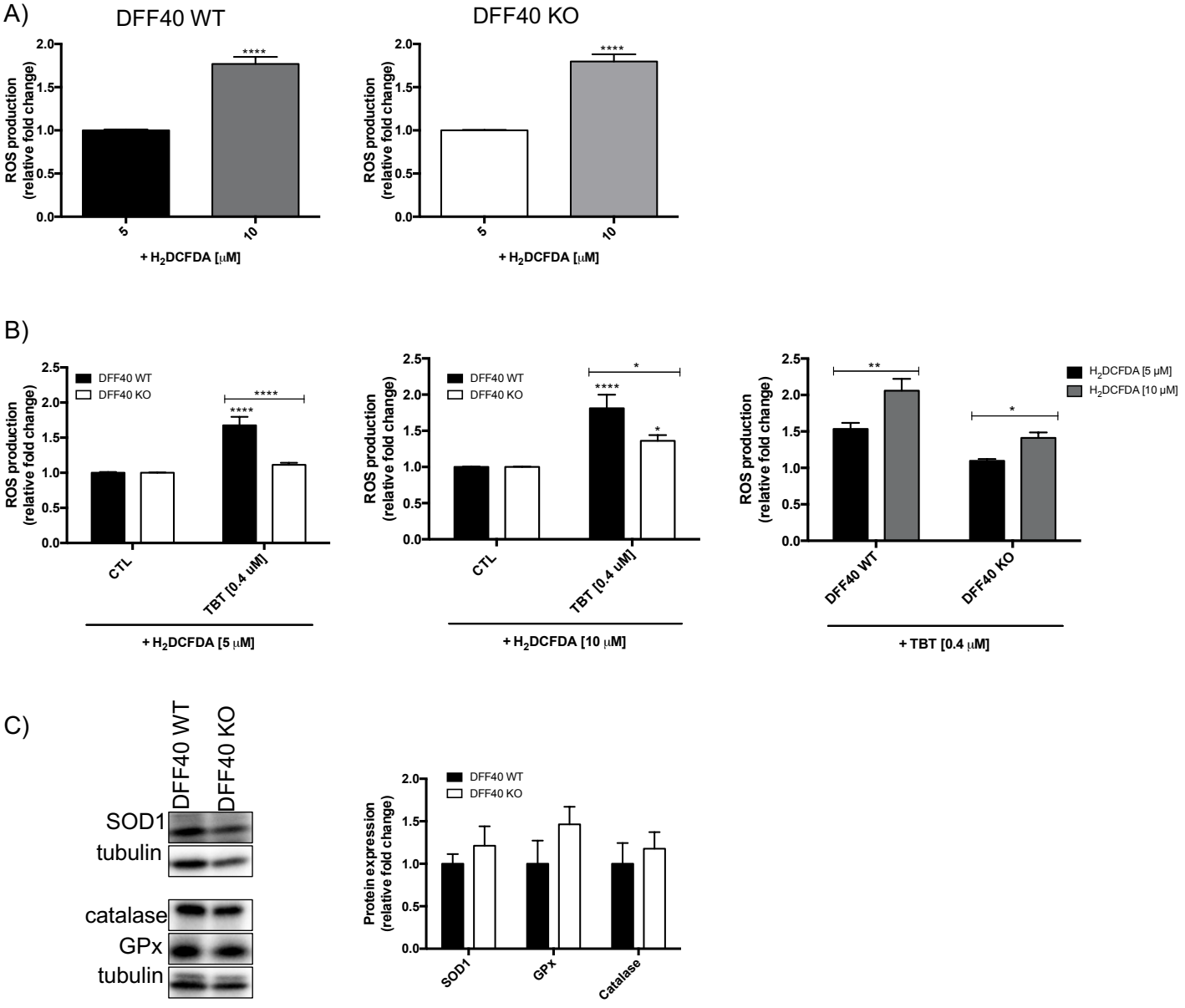


Figure 1S

Figure 1S. ROS production variations according to the H2DCFDA molecular probe concentrations. A: Jurkat DFF40 WT and DFF40 KO cells (1 x 10^6 cells) were incubated with the molecular probe H_2DCFDA [5 or 10 μM] for 45 min and then cells were analyzed by flow cytometer. Results are expressed as a relative fold change in geometric mean according to the lower concentration of H_2DCFDA. **B:** Jurkat DFF40 WT and DFF40 KO cells (1 x 10^6 cells) were treated or not with TBT [0.4 μM] for 2 h and then incubated with the H_2DCFDA probe [5 or 10 μM] for 45 min. Cells were analyzed by flow cytometer. Results are expressed as a relative fold change in geometric mean according to their respective control. **C:** Protein extracts from nonapoptotic Jurkat DFF40 WT and DFF40 KO cells were immunoblotted for SOD1, catalase, GPx and α-tubulin. Densitometric analysis are expressed as a relative fold change in protein expression according to DFF40 WT cells. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$).

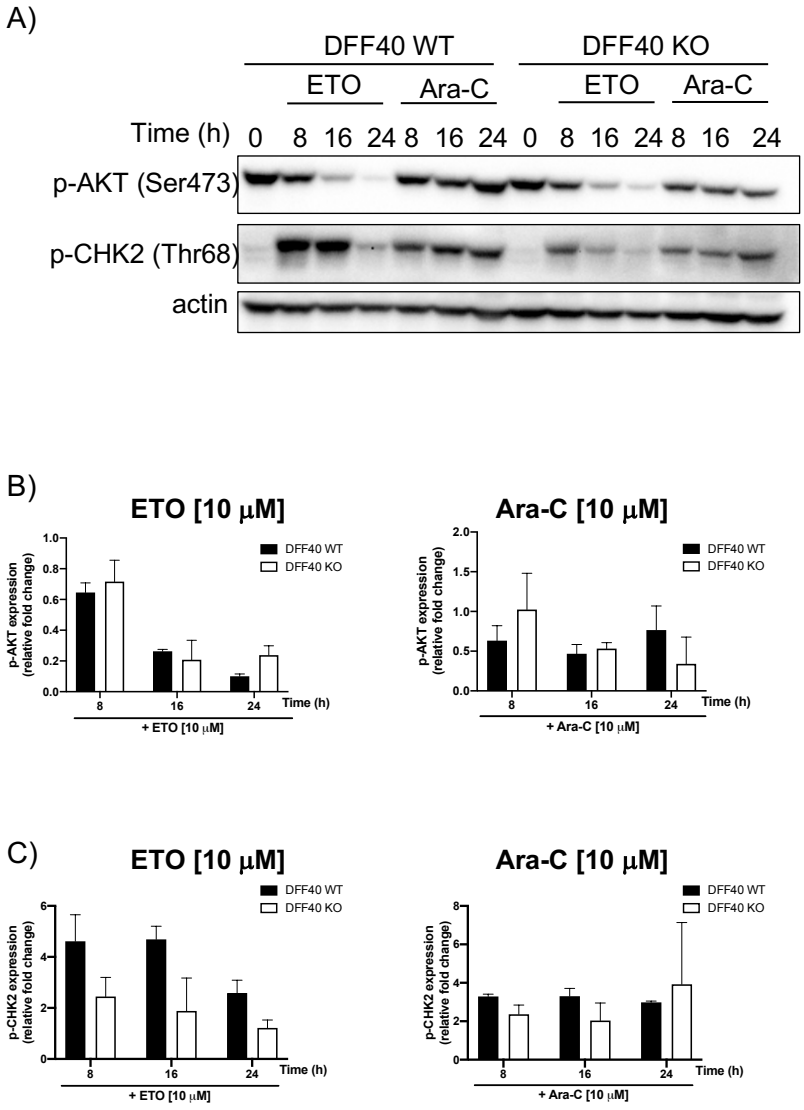

A)
DFF40 WT
DFF40 KO
ETO
Ara-C
ETO
Ara-C
Time (h) 0 8 16 24 8 16 24 0 8 16 24 8 16 24
p-AKT (Ser473)
p-CHK2 (Thr68)
actin
B)
ETO [10 μM]
Ara-C [10 μM]
DFF40 WT
DFF40 KO
p-AKT expression (relative fold change)
Time (h)
+ ETO [10 μM]
+ Ara-C [10 μM]
C)
ETO [10 μM]
Ara-C [10 μM]
p-CHK2 expression (relative fold change)

Figure 2S

Figure 2S. DFF40 deficiency doesn't alter ATM and ATR activation following etoposide- or cytarabine- induced apoptosis. A: Jurkat DFF40 WT and DFF40 KO cells (1 x 10^6 cells) were treated or not with ETO [10 μM] or Ara-C [10 μM] for 8, 16 or 24 h in RPMI media supplemented with 10% FBS. Following treatments, protein extracts were immunoblotted for phospho-CHK2, phospho-AKT and actine. **B and C:** Densitometric analysis of blots from A. Densitometric analysis are expressed as a relative fold change in protein expression according to respective control. Data represent the mean of at least 3 independent experiments. Data are presented as (mean ± SEM). Two-way ANOVA with Tukey's post-hoc test was performed.

12 ANNEXE III : REVUES DE LITTÉRATURE SCIENTIFIQUE

Apoptosis
https://doi.org/10.1007/s10495-020-01649-7

REVIEW

The role of the DFF40/CAD endonuclease in genomic stability

Merve Kulbay[1,2] · Nathan Bernier-Parker[3] · Jacques Bernier[1]

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Abstract
Maintenance of genomic stability in cells is primordial for cellular integrity and protection against tumor progression. Many factors such as ultraviolet light, oxidative stress, exposure to chemical reagents, particularly mutagens and radiation, can alter the integrity of the genome. Thus, human cells are equipped with many mechanisms that prevent these irreversible lesions in the genome, as DNA repair pathways, cell cycle checkpoints, and telomeric function. These mechanisms activate cellular apoptosis to maintain DNA stability. Emerging studies have proposed a new protein in the maintenance of genomic stability: the DNA fragmentation factor (DFF). The DFF40 is an endonuclease responsible of the oligonucleosomal fragmentation of the DNA during apoptosis. The lack of DFF in renal carcinoma cells induces apoptosis without oligonucleosomal fragmentation, which poses a threat to genetic information transfer between cancerous and healthy cells. In this review, we expose the link between the DFF and genomic instability as the source of disease development.

Keywords Apoptosis · DFF40 · Genomic stability · Cancer cell resistance

Apoptosis pathways and their regulation

Apoptosis is a programmed cell death mechanism that is necessary for the maintenance of cellular homeostasis. The term apoptosis was first used to identify a natural programmed cell death mechanism in adult tissues [1], and was later characterized with specific biochemical hallmarks of apoptosis leading to internucleosomal degradation of the genome [2, 3]. Over the past decades, two main pathways in the activation of apoptotic cell death have been deeply characterized: the intrinsic and extrinsic apoptotic pathways. These pathways are activated following cell suicide signals and are executed by specialized serine-protease enzymes named caspases.

The extrinsic pathway can be divided into 4 levels: (1) The activation of death receptors (DR), (2) subsequent serial pro-caspase cleavage leading to enhanced caspase-3 expression, (3) activation of the DNA fragmentation factor 40/caspase-activated DNAse (DFF40/CAD) and (4) internucleosomal DNA fragmentation (Fig. 1). DR are subsets of the tumor necrosis factor receptors (TNFR) superfamily [4]. One of the most studied TNFR is the Fas receptor (FasR or CD95), that directly signals apoptosis activation following the binding of Fas ligand (FasL or CD95L) [4]. The TNFR contains a 70–80 amino acid sequence in their cytoplasmic domain called the death domain [4, 5], which is crucial for intracellular signalization. Following the interaction of FasL with the FasR, an adapter death-domain protein, the Fas-associated death-domain (FADD) is recruited at the cell membrane. FADD associates immediately with the procaspase-8 via the dimerization of the death effector domain (DED) [5]. This results in the formation of a death-inducing signaling complex (DISC), which subsequently leads to the auto-catalytic activation of procaspase-8 into caspase-8 [6, 7]. Once activated, caspase-8 initiates the caspase cascade by directly cleaving procaspase-3, ultimately resulting in apoptotic cell death [6]. Activated caspase-3 is the primary necessary effector for the destruction of the genome to protect the neighboring cells of potential DNA instability. Caspase-3 induces the cleavage of DNA fragmentation factor 45/inhibitor of caspase-activated DNAse (DFF45/ICAD), which reveals the endonuclease activity of

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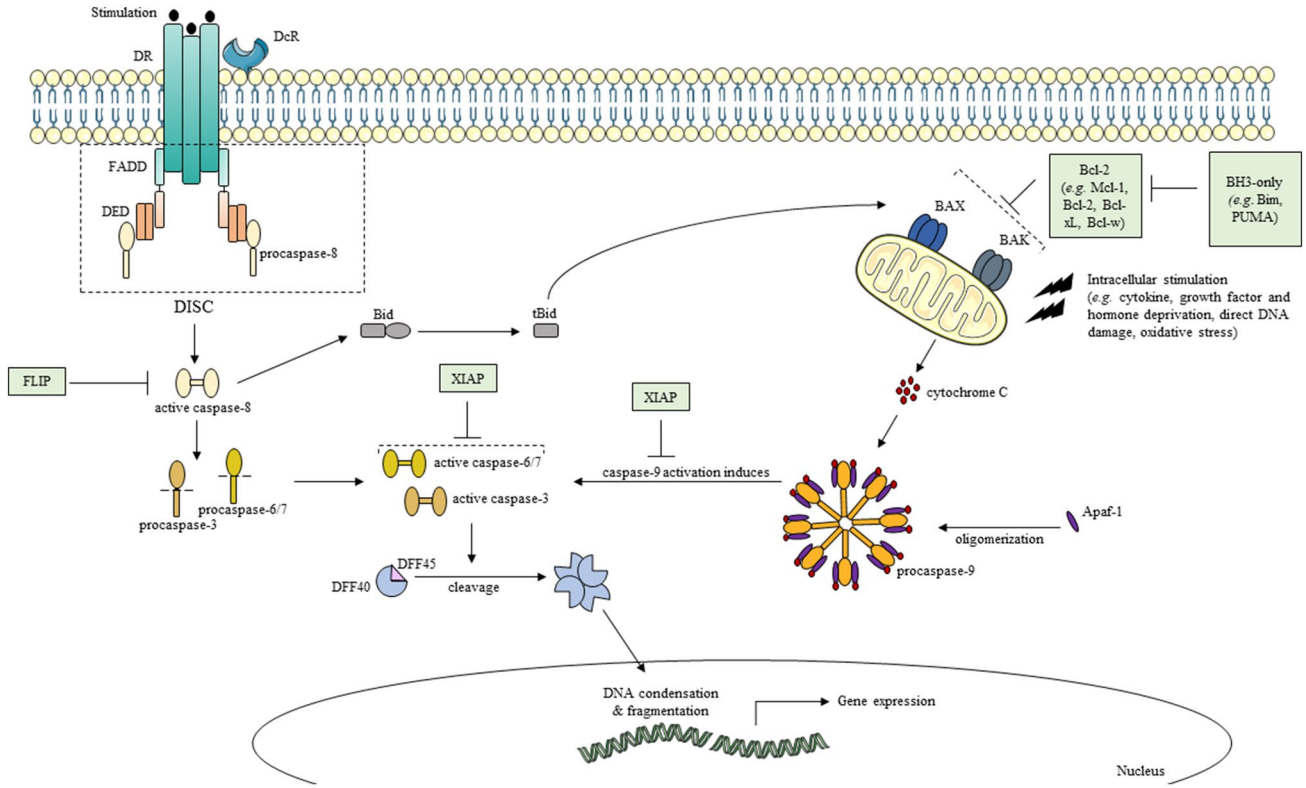


Fig. 1 Review of the main events in the extrinsic and intrinsic apoptosis pathway and their regulation

the DFF40 protein [8]. The DFF40 mediates the ultimate and irreversible step of apoptosis by inducing internucleosomal DNA fragmentation [8].

The extrinsic pathway is regulated upon many levels to limit unnecessary cell death. First, the expression of DR may vary from tissue and/or cell type to another, or may differ upon the same tissue and/or cell type depending on the DR [9, 10]. For example, the TNF-related apoptosis-inducing ligand receptor (TRAIL-R), a member of the TNFR superfamily, presents high variability in its expression depending on the human tissue site. TRAIL-R3 is not expressed in the human kidney but can be found in the liver and brain cells [10]. These distributions allow a ligand-specific apoptosis regulation process. Secondly, it was shown that apoptosis is regulated by death and decoy receptors (DcR) [11]. DcR function as inhibitors of signal transduction by binding to death ligands which cannot cause further activation of DR [11]. The most known DcR is the DcR3, a secreted and soluble protein [12], that binds FasL and allows cells to escape FasL-mediated apoptosis. DcR3 is highly expressed in many human cancers as a resistance mechanism to apoptosis [13]. Another regulatory mechanism involves the prevention of DISC formation and, consequently, the downregulation of caspase activation. The cellular FLICE-inhibitory protein (c-FLIP) polypeptide competes with procaspase-8 for FADD, inhibiting the activation of CD95 [14]. Finally, the X-linked inhibitor of apoptosis protein (XIAP) inhibits DNA fragmentation by inhibiting the activity of caspase-3 and caspase-7 [15].

The intrinsic apoptosis pathway is triggered by cellular insults, which leads to mitochondrial dysfunctions. The absence of specific growth factors, hormones, and cytokines [5], direct DNA damage [16] and oxidative stress [17] are known to induce the intrinsic apoptosis pathway (Fig. 1). There can also be a crosstalk between the extrinsic and intrinsic pathways via the pro-apoptotic protein Bid. The cleavage of Bid by caspase-8 into t-Bid leads to the permeabilization of the mitochondrial membrane by Bax, a pro-apoptotic protein [18]. Bax forms pores in the mitochondrial membrane [18], which results in the loss of mitochondrial membrane potential [19]. These cellular events lead to the release of pro-apoptotic proteins and cytochrome C into the cytoplasm [19, 20]. Once released, the cytochrome C binds to the Apaf-1 protein and induces the recruitment of the procaspase-9, where they form a molecular structure called the apoptosome [21]. This conformation induces the activation of the caspase-9, which in turn cleaves the procaspase-3 [5]. At this point, the intrinsic and extrinsic apoptosis pathways converge to cause the final step of apoptosis: the internucleosomal DNA fragmentation mediated by DFF40.

The intrinsic apoptosis pathway has its own regulatory mechanisms to inhibit apoptosis. The control and the

regulation of the pro-apoptotic proteins go through the members of the Bcl-2 family proteins. The anti-apoptotic proteins are the Bcl-2, Bcl-X_L, Bcl-W, A1/Bfl-1, and Mcl-1 proteins [22]. They mediate their action by binding to the pro-apoptotic protein members, Bax and Bak, and prevent mitochondrial damage [23]. Their inhibitory effect on apoptosis is controlled through their phosphorylation by different kinases [24]. Importantly, the mammalian BH3-only protein, a pro-apoptotic group of protein, plays a role of sensor and initiator of several cellular changes such as DNA damage and autophagy [25].

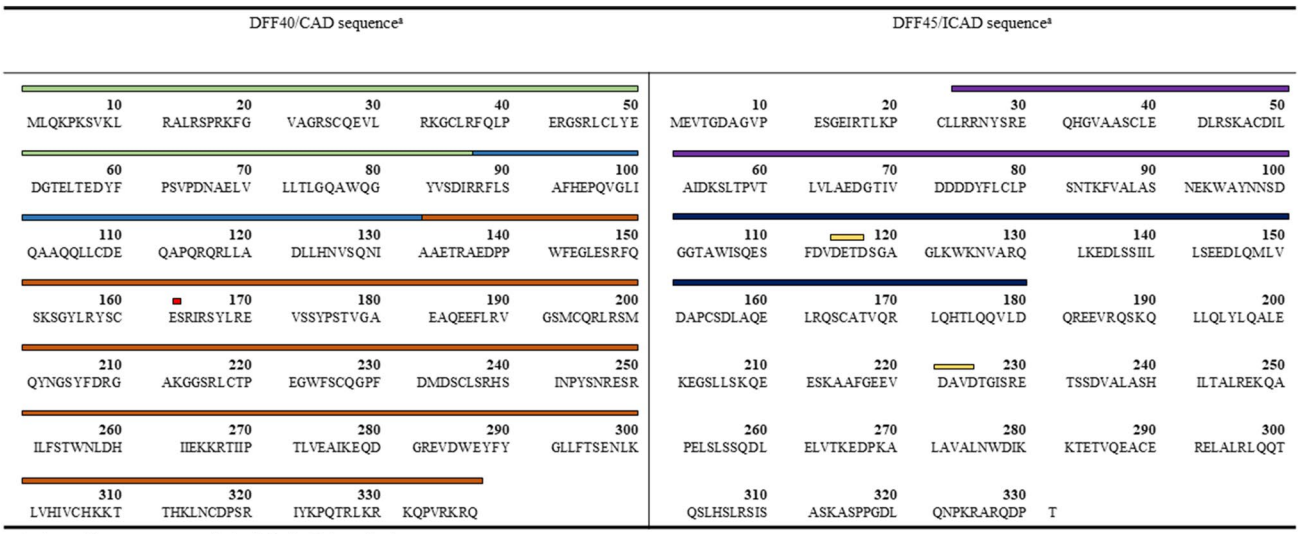
Biomolecular properties of the DFF40/CAD endonuclease

The biomolecular properties of the DFF40 are primordial to better understand its biological activity. The DFF40 has a molecular weight of 40 kDa, a maximal endonuclease activity at pH 7.5 and requires Mg^{2+} [26]. Its catalytic activity is inhibited by high concentrations of Zn^{2+} [26]. Its sequence contains a total of 345 amino acids and is subdivided into three main domains (see Table 1) [27]. The first domain, the CIDE-N domain, consists of 80 amino acids highly conserved between DFF45 and DFF40, and is important for the DFF40/DFF45 complex interactions as described later [28]. The second domain (C2) is the smallest of them all (amino acids 86–131) and contains 3 alpha-helices [28]. Finally, the third domain (amino acids 132–345) includes the catalytic activity of DFF40 and is formed with alpha-helices, 4 beta sheets and contains a loop on the C-terminal extremity. The C-terminal extremity being in an extra-molecular position holds the nuclear localization sequence (NLS) [28]. In mice, 4 histidine residues (residues 242, 263, 313, 308) were found to be crucial for the induction of DNA fragmentation by DFF40 [29]. In human embryonic kidney epithelium 293T cell line, the deletion of the residues 290–345 or 162–345 in the C-terminal domain of DFF40 results in a total loss of its catalytic activity, thus an abolition in DNA fragmentation [30]. The substitution of the residue 242 (His242) inhibits the binding of Zn^{2+} to DFF40, showing the importance of this divalent ion [28]. Zn^{2+} binding plays a role in DFF40 structural stability and information of functional dimer. Crystal structure showed that activated DFF40 is a homodimer forming a molecular scissor [28]. In murine models, substitution in the residues K155Q, K301Q, K310Q, and Y247F also inhibits its activity, but the binding of CAD to DNA isn't compromised [31]. Overall, the spatial configuration of DFF40 confers to the protein an essential catalytic activity, mediated by specific residues (in majority histidine residues) and co-modulator proteins, which is crucial to preserve genomic stability.

The DFF40/DFF45 complex

In the physiological state, the DFF complex contains the DFF40 endonuclease subunit and the DFF45 inhibitory subunit. As mentioned above, DFF40 acts as a pair of molecular scissors when activated to induce specific double-strand

Table 1 Amino acid sequence comparisons of the human DFF40/CAD and DFF45/ICAD molecules.



[a]Amino acid sequences were obtained via the Uniprot database

CIDE domains of the DFF40/CAD are represented with different color bars. The first domain is in green, the second domain in light blue and third domain in orange. The beginning of the segment containing the catalytic activity of DFF40/CAD starts with the red bar. Purple bars represent the binding sequence between DFF40/CAD and DFF45/ICAD, where as dark blue bars represent the inhibitory subunit binding sites. The yellow bars are the cleavage sequences of DFF45/ICAD by caspase-3.

breaks (DSB) in the DNA [32]. The DFF45 subunit inhibits the action of the endonuclease and also participates in the proper folding of DFF40 following its translation [33]. DFF45 exists in two isoforms obtained by alternative splicing of DFF45 pre-mRNA: DFF45-L (DFF45) and DFF45-S (DFF35) [34]. Both DFF45 isoforms can inhibit DFF40's activity, but only DFF45-L plays a role of chaperon and has a NLS participating in the targeting of DFF40 in the nucleus [35, 36]. The interaction between the DFF40/DFF45 complex is mediated by the CIDE-N domains. This interaction involves the functional domains (D1, D2, D3) of DFF45 and the activator and catalytic domains of DFF40 [37]. First, a basic region of DFF40, involving the Lys9, Lys18, Lys32, and Arg36 amino acids, surrounding a hydrophobic convex (phe19, Val21, Ala22), allows an interaction with the acidic core of DFF45 (Asp66, Asp71, Asp72, Asp74) that surrounds a concave hydrophobic core (Ile69, Val, Tyr75) [38]. Secondly, a more powerful interaction between the CIDE-N domain of DFF40 and the region surrounding the cleavage site by caspase-3 of the DFF45 solidifies this link. It inhibits the catalytic activity of DFF40 [39]. Gu et al. demonstrated that the amino acids 101–180 of DFF45 mediates its binding with DFF40, whereas the 23–100 residues are implicated in its inhibitory activity (see Table 1) [40]. Regarding the affinity of DFF45 isoforms for DFF40, the short form presents a higher affinity than the long-form [40]. The localization of the inactivated DFF complex has been subject to debate. First studies suggested that upon its translation in the cytoplasm, the inactive DFF40/DFF45 complex was directly transferred to the nucleus and awaited apoptosis-inducing triggers [27]. DFF40/DFF45 structure analysis showed the presence of a bipartite NLS in both C-terminal subunits that binds to the importin α/β, which mediates the translocation to the nucleus [28, 41]. DFF45-S seems to play a restricted role in the inhibition of DFF40 activity in the cytoplasmic part of cells, since the absence of C-terminal NLS inhibits the translocation of DFF40 to the nucleus [41]. The ratio in DFF45 and DFF35 is important to ensure a proper DNA fragmentation [42]. Decrease of DFF35 early after apoptosis induction increases favored nuclear import of DFF40/DFF45L, subsequent DFF40 activation and DNA fragmentation [43]. To obtain an active DFF40 oligomer, caspase-3 induces the cleavage of the DFF45 subunit on two specific sites, the DEPD (amino acids 114–117) and DAVD (amino acids 221–224) regions [38], which allows the release of the DFF40 subunit and its subsequent homo-oligomerization. The smallest units of oligomeric species consist of tetramers [44]. Crystal structure analysis showed that the CIDE-N domain of DFF40 forms filament-like structures [45], which favors helical homo-oligomerization assemblies of DFF40 molecules [46]. These higher-order assemblies involve a head-to-tail oligomerization of CIDE domains of DFF40 [46]. Importantly, the later conformation was not present in DFF complex since the surface charge distribution of DFF45 does not support this structure [46].

The cleavage of the DNA generates blunt ends or ends with 5′-overhangs with 5′-phosphate and 3′-hydroxyl groups [26]. The oligonucleosomal DNA fragmentation induced by DFF40 is considered to be implicated in stage II nuclear morphology; CAD deficient DT40 chicken cells do not exhibit stage II nuclear modifications after apoptosis-activating stimulus [47]. Surprisingly, aside from the specific DSB induced by DFF40, recent studies have shown the role of DFF40 in single-strand breaks (SSB) [48, 49]. The knockdown of DFF40 with siRNA in SK-N-AS human neuroblast-derived epithelial cells almost completely abolishes staurosporine-induced SSB [48]. Knockdown of DFF40 using siRNA in HeLa cells blocks DNA fragmentation induced by rotenone, an inhibitor of mitochondrial complex 1 [50]. In the literature, novel regulators of DFF40/CAD are identified and considered crucial to maintain a DFF40/CAD-mediated DNA fragmentation [51]. Heat-shock protein (HSP) 70 stimulates and stabilizes the activated DFF40, whereas the phosphorylated histone H2AX (γH2AX) is absolutely necessary for DNA fragmentation following apoptosis induction [51].

The nuclease activity of DFF40 oligomers is enhanced by different protein–protein interactions (see Table 2). First, the direct interaction of the DFF with the histone H1 (H1) confers the ability to the endonuclease to bind with the DNA and stimulate its activity [8]. All of the 6 isoforms of the H1 family have the same potential in an equivalent manner [52]. Secondly, the HMG-box-containing chromatin proteins (HMGB) 1 and 2 stimulate naked DNA cleavage by DFF40, without a direct interaction or neither by enhancing its activity [53]. Their activity is mediated by the HMG-boxes that confer local distortions to the DNA structure upon their binding [53]. These changes render the DNA more accessible to cleavage by DFF40. Recent

Table 2 Known molecular partners of the endonuclease DFF40/CAD

Protein partner	Role	References
Histone H1	Confers DNA binding ability to DFF40/CAD and stimulates its activity	[8, 36]
Histone H2AX	Necessary for DNA fragmentation	[47]
HMGB1/2	Indirectly stimulate naked DNA cleavage by local distortions	[37]
Topoisomerase IIα	Necessary for chromatin condensation	[38–41]
HSP 70	Stimulates and stabilizes activated DFF40/CAD	[47]

studies suggest an interaction of topoisomerase II (TOP2) with DFF40, particularly with TOP2α which is necessary for chromosome segregation and DNA replication in proliferating tissues [54]. Thus, the role of TOP2 activity in chromatin fragmentation during apoptosis has been controversial for a few years. Selective inhibition of its activity by the inhibitor ICRF-193 showed that TOP2 was not necessary for DNA cleavage into 50 kb fragments, but its proteolysis is a conserved event of apoptosis [55]. On the other hand, hydrogen peroxide-induced apoptosis in U937 human monocytes induces TOP2 mediated excision of loop-sized DNA fragments, where mutant cells for TOP2 expression have significantly lower levels of DNA fragments [56]. Using three different types of TOP2 inhibitors in HeLa cells abolishes chromatin condensation, but this apoptotic event is rescued when highly purified human TOP2 is added in nuclei extracts [57]. Overall, these co-modulators of DFF40 allows the endonuclease to induce preferential histone linker DNA cleavage, which produces DNA fragments of 180–200 bp with a specific pattern.

Evidence of apoptosis anomalies compromising DNA fragmentation

Although the typical apoptotic pathways lead to DNA fragmentation, there are multiple studies that have reported apoptotic nuclear morphological changes or an activation of apoptosis in human cells without the presence of this later step. In 1999, Sakahira et al. demonstrated that a mutation in the expression of ICAD in Jurkat cells, that replaces the residues Asp117 and Asp224 by glutamic acid, induces chromatin condensation near the nuclear periphery, whereas no clear DNA fragmentation is observed in staurosporine-exposed cells [58]. The absence of DNA fragmentation following apoptosis induction is also obtained in Jurkat and TF-1 cells expressing the mutated form of ICAD-L at the caspase-3 recognition site [59]. In vivo, DFF45 deficiency in mice induces not only a resistance to DNA fragmentation in splenocytes and thymocytes, but as well as a resistance to chromatin condensation [60]. Our laboratory has shown that a mutation downstream of DFF45 also alters oligonucleosomal DNA fragmentation following apoptosis induction. DFF40-deficient Jurkat T cells do not exhibit DNA fragmentation following apoptosis induction by tributyltin (TBT), although activation of caspase-3 is noted in these cells [61]. The same pattern was also previously reported in glioblastomas. A DFF40 deficiency in human glioblastomas impairs DNA hydrolysis in cells that show a correct activation of the caspase-3/ICAD apoptosis cascade [62]. Mutations in the apoptotic pathway upstream of caspase-3 have also been linked to DNA fragmentation abnormalities in cell death. CD45, a transmembrane molecule involved in cell signaling through TCR and cytokine modulations, regulates apoptosis in T cells [63]. An intrinsic deficiency of CD45 in T cells impairs DNA fragmentation, but not caspase-3 activation and DFF45 cleavage [64]. Overall, alterations in the apoptotic pathway that impairs DNA fragmentation can alter the maintenance of genomic stability.

Correlations between DFF40/DFF45 expression and malignancies

Malignant tumors are known to possess multiple mutations and protein expression alterations that contribute to their tumorigenesis. DFF40 and DFF45 expression have been linked multiple times to a malignant tumor profile. First studies have demonstrated a correlation between the expression of DFF45/ICAD and tumorigenesis. A decrease in DFF45 mRNA levels in patients with esophageal squamous cell carcinoma is associated with a poor prognosis [65]. In fact, DFF45 mRNA levels are decreased in patients with a higher pathological stage and tumor status, with lymph node metastasis and in patients who have extensive lymphatic invasions [65]. It was also shown that in human colon cancer epithelial cells, DFF45 expression is severely compromised [66]. In vivo and in vitro studies using mice colon epithelial cells show that DFF45/ICAD deficiency confers cells death resistance to the colon carcinogen dimethylhydrazine [66]. A tissue microarray analysis of human renal cell carcinoma's showed that DFF45/ICAD expression is significantly decreased in these samples compared to normal renal cells [67]. Downregulation of DFF40/DFF45 was also noted in uterine leiomyosarcomas and was associated with a poor prognosis [68]. Although multiple studies are in favor of a downregulation of DFF45/ICAD expression in resistant cancers, few studies have reported the opposite observation. In serous ovarian cancer, upregulation of DFF45/ICAD was shown to be associated with poor prognosis [69]. In non-atypical and atypical endometrial hyperplasia's, DFF45 expression is increased which may be involved in apoptosis resistance [70]. Studies regarding DFF40/CAD expression have reported the same general pattern of observations in different malignancies. DFF40 is underexpressed in non-endometrioid and high-grade endometrioid endometrial cancers [71], as well as in uterine leiomyosarcoma [68] and glioblastomas [62], and is associated with a poor prognosis. Overexpression of DFF40 in the T47-D breast cancer cell line was shown to sensitize cells to drug-induced apoptosis [72, 73]. These results suggest a crucial role for DFF40 in tumorigenesis development.

DFF anomalies in neuronal cells have shown its importance in genomic stability

Neuronal apoptosis is a highly conserved process between species. It is essential in the development of the nervous system, and it is also an underlying element in neurodegenerative disease. The importance of the relation between DFF and the brain was pointed out in DFF45 mutant mice, where they exhibit an enhanced spatial learning memory and longer memory retention in novel object recognition tasks [74, 75]. The absence of DFF45 expression in mice causes an increase in granule cell density and total granule number in the dentate gyrus region [74]. Non-functional DFF in the brain is associated with attenuated excitotoxic neuronal degeneration in the CA3 subfield of the hippocampus, as demonstrated with kainic acid-induced seizures [76]. The expression of DFF was associated with neuronal resistance to excessive activity-induced toxicity. Interestingly, it was reported in *Drosophila* brain that the CIDE-N family protein Drep-2, an analog of human DFF40 and DFF45, is a novel synaptic protein in learning and behavioral adaptation [77].

Apoptosis plays an essential pathophysiological role in neuronal cell loss and is associated with neurologic deficits following traumatic brain injury (TBI). Several studies confirm the role of DFF in DNA hydrolysis [78–80]. In rat focal brain ischemia-induced apoptosis, different cell death distributions were noted between the ischemic zones, with apoptosis and DFF40 activation in the regions where server ATP depletion is absent [80]. In a controlled cortical impact in DFF45 deficient mice, in a short time, a delayed oligonucleosomal cleavage of DNA by other nucleases occurs [81]. In this pathological condition, no behavioral outcomes were noted.

DFF40 has been identified in genomic instability as a link to glioblastoma development. It was established that abnormal accumulation of DFF40 in the nucleoplasm compartment could result in cell survival after irradiation [62]. A decrease in their expression in astrocyte tumors was also observed. Analysis of several glioblastoma multiforme's confirms the reduced expression of DFF40 [62]. Using neuroblastoma cell lines, it was established that parental SH-SY5Y cells and derived SK-N-AS cells, which express a similar level of DFF40, respond differently to staurosporine-induced apoptosis [82]. The absence of DNA fragmentation in SK-N-AS cells can be explained by the necessity of the cytosolic pool of DFF40 in achieving nuclear apoptosis [82]. Interestingly, as we observe in lymphoma, the absence of DNA fragmentation was not associated with a defect upstream caspase activation or DFF45 processing [62, 83]. DFF45 expression was also shown to be affected in brain cancers. Masvoka has shown differential expression of the inhibitory subunit of DFF between different brain cells. The short isoform DFF35 is predominant in glioblastomas, while the long isoform DFF45 is predominant in medulloblastomas [84]. In several human neuroblastomas, a homozygous deletion at chromosome 1p36.2-p36.3, which includes the DFF45 gene or different coding alterations in the N-terminal region of DFF40 were identified [85–89]. These alterations were associated with an unfavorable outcome. For example, NB-C201, which bears a deletion on chromosome 1, is much resistant to genotoxic stresses such as cisplatin [89].

DFF40/CAD deficiency and cellular dysfunctions ultimately leading to genomic instability

Genomic instability refers to the alteration of the genome by the accumulation of mutations on essential genes, mainly those regulating cell division and tumor suppressor genes [90]. This phenomenon is considered as being at the basis of tumor initiation and progression, and the selection of a more aggressive subclone that accentuates tumorigenesis [91]. Defects in the apoptosis pathways or its regulation have been linked multiple times to many diseases such as Alzheimer's disease [92, 93], heart failure [94, 95], autoimmune diseases [96, 97], leukemia [98, 99] and central nervous system diseases [100, 101]. As mentioned before, among the vast mutations altering the apoptotic pathway, alterations of the DFF have shown to be important in tumor development, apoptosis resistance and genomic instability. In cancer cells, somatic mutations occur at a high rate and have been related to cancer malignancy and tumorigenesis over the past decades [102]. Many causes have been attributed to genomic instability, whereas dysfunctions in DNA replication and DNA damage responses (DDR) would be the major sources [103]. The lack of DFF in renal carcinoma cells induces apoptosis without oligonucleosomal DNA fragmentation, which poses a threat for genetic information transfer between cancerous and healthy cells [104]. Interestingly, it was also demonstrated that spleen lymphoblasts TK6 cells deficient in DFF40 do not accumulate mutations following TRAIL exposition [105]. Caspase and DFF40 activation are necessary for TRAIL or vincristine-induced mutagenesis [105]. TRAIL-induced DNA damage is a caspase-8 dependent mechanism and the mutagenesis occurs following DFF45 cleavage by the executioner capase-3 [106]. Imbalance in the ratio of DFF40 and DFF45 could be one of the reasons of resistance to TRAIL-induced cell death in lymphoma cells [107]. DFF45 deficiency in human colon cancer cells is associated with a massive genomic instability characterized primordially by multiple genomic amplifications and sizeable deletions [66]. It was shown that the DFF40/DFF45 complex is

under expressed in uterine leiomyosarcomas and predicts poor prognosis among those patients [68]. In vivo studies, involving CAD deficient mice, have shown that CAD loss renders the bone marrow resistant to apoptosis-induced DNA fragmentation and induces a significant increase in chromosomal aberrations (e.g. chromosome terminal deletion, ring chromosome, and dicentric chromosomes) [108]. Also, $CAD^{-/-}$ mice show significantly higher papilloma's and their incidence is enhanced [109]. The growth rate of those tumors is mainly accelerated [109]. These findings and observations on neurobiological changes in DFF45 deficient mice support the idea that DFF deficiency promotes the survival of a subclone fraction, where those cells accumulate DNA damage and raise the risk of cancer transformation.

DFF40 co-factors are crucial for specific DSB of the DNA by the endonuclease, as mentioned above. The TOP2 mediates the first stages of DNA cleavage into fragments of 50–100 kb under oxidative stress [56]. Studies have shown that DNA cleavage mediated by TOP2 can potentially trigger chromosomal translocations and lead to specific types of leukemia due to the instauration of genomic instability [110, 111]. In fact, when the induced DNA damage is insufficient for complete DNA fragmentation by DFF40 and its co-factor TOP2 to induce cell death, cell survival pathways can lead to stable chromosomal translocations formation and ultimately tumorigenesis [110, 112]. The use of recombinant proteins of the linker H1, a ubiquitous chromatin protein necessary for the action of DFF40 [113], shows that all the somatic isoforms of H1 are necessary for DFF40 activity [52]. A knockdown of each H1 variant in breast cancer cells shows an alteration in gene expression and cell proliferation [114]. Transient knockdowns of HMGB1 using short interfering RNAs in human lung epithelium A549 and renal cancer UO31 cell lines is associated with a decreased sensitivity to cytarabine-induced cytotoxicity [115]. These genomic alterations all have an impact on DDR mechanisms. In concordance with this stipulation, it was shown that cancer cells lacking the expression of DFF40 have an inhibition of γH2AX formation [61, 116], which can partially explain the apoptosis-induced DNA laddering resistance. This mechanism is described in further sections. On the other hand, knockout of the isoform 1 of HSP70 in mice induces significantly higher levels of DNA fragmentation, release of cytochrome c into the cytoplasm and activation of caspase-3 in a cerebral ischemia context [117]. Overall, these findings suggest that an alteration in the co-factors of DFF40 has a similar impact as a deletion of the endonuclease itself in general, proposing that the genomic stability is mediated through a large pool of DNA-modulating proteins.

Genomic stability regulation through DFF40 expression

It is primordial for human cells to maintain their genomic stability for cellular integrity and protection against tumor transformation. Many environmental factors are known to alter the integrity of the genome, such as ultraviolet (UV) light, oxidative stress, exposure to mutagen chemical reagents, and radiation [118]. To limit their impact on the genome, human cells have equipped themselves with many mechanisms that prevent these irreversible lesions such as DNA repair pathways, cell cycle checkpoints and telomeric function. An alteration in those regulatory pathways could potentially lead to the accumulation of chromosomal aberrations and a disturbance of genomic stability. In this section, we review the principal mechanisms' and proteins that can lead to genomic instability in DFF40 deficient cells in neoplasia (Fig. 2).

DNA repair pathways and DFF40/CAD expression

Following DNA damage in cancer cells, many molecular mechanisms can be activated to bypass cell death and instore a cytotoxic-induced resistance phenotype. DDR over-activate cell reparation pathways in damaged cells via the expression of the DNA-dependent protein kinase (DNA-PK) and the loss of TRF2 at the telomeres [119]. We can separate the DNA repair pathways into two subdivisions according to the nature of the damage: (1) ATM pathway DSB specific or (2) ATR pathway SSB specific. In this review, we discuss the ATM pathway because of the specificity of DFF40 to induce DSB.

The ATM pathway is activated following enhanced formation of γH2AX on DSB. The phosphorylation of H2AX occurs on the serine residue 139 and is a rapid and sensitive response to DNA damage [120]. Following its phosphorylation by ATM or DNA-PK, a nucleation reaction is put into place where the mediator of DNA damage checkpoint protein 1 (MDC1) is first recruited at the damage site, following with the recruitment of the MRN complex (NBS1, MRE11, RAD50 proteins) [121]. The MRN complex proteins induce a positive retroaction on the phosphorylation of H2AX [121]. These mechanisms ultimately lead to the non-homologous end-joining (NHEJ) repair or homologous repair (HR) pathways. The contribution of γH2AX to DNA repair is been a matter of investigation for years. The first study conducted on a yeast model suggested that a deletion containing the effective region leads to an impairment in the NHEJ repair pathway. In contrast, the HR pathway does not present any alterations [122]. New studies suggest that DNA repair is directly

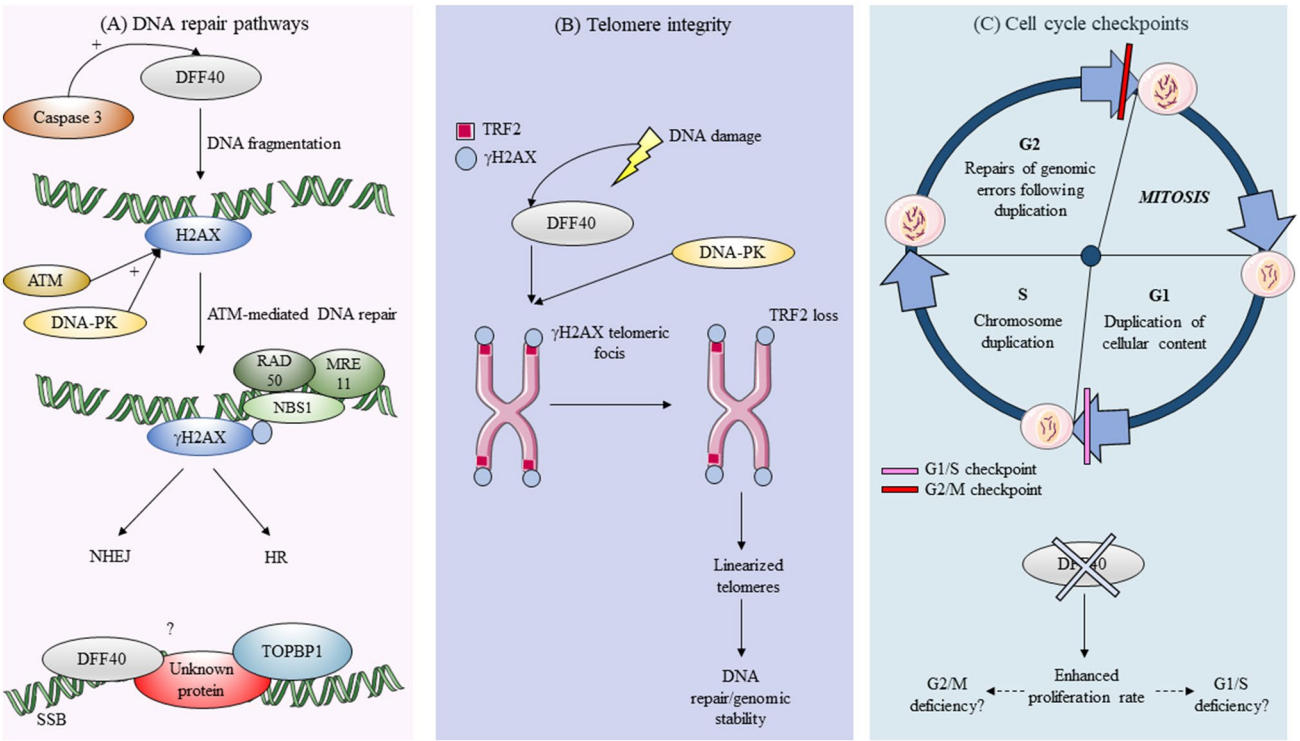


Fig. 2 Proposed mechanisms of the role of the DFF40/CAD endonuclease in genomic stability. Roles for DFF40/CAD in **a** DNA repair pathways, **b** telomeric integrity and **c** cell cycle checkpoint regulation are proposed

mediated by γH2AX formation by facilitating the interaction between the two loose DNA ends [123]. Enhanced γH2AX leads to the activation of the checkpoint kinase (CHK) 2, which activates at his turn, the p-53 tumor suppressor protein [124]. Upon its activation, γH2AX forms telomeric foci at the extremity of chromosomes and may predict genomic instability [125]. Overall, ATM pathway leads to the reparation of the DNA or cell suicide if the damage is too important to be repaired.

On the other hand, the ATR pathway is initiated following the recognition of SSB. These damages are usually generated at DNA replication forks due to a miscoordination between the DNA polymerase and DNA helicase activity, or by SSB-inducing DNA damages [126]. The triggers that activate the ATR pathway are so versatile that it was suggested that its activation is mediated by DNA and/or protein structures commonly induced by replication problems [127]. Following SSB, the breaks are rapidly coated with the replication protein A (RPA) complex, which subsequently allows the recruitment of ATR-interacting protein (ATRIP) and the localization of the ATR-ATRIP complex to the damage site [127]. These crucial steps further recruit the Rad17-RFC complex following with the 9-1-1 (Rad9-Hus1-Rad1) complex [127], which is the limiting step of the ATR pathway. The phosphorylated Ser 387 residue of Rad9 binds with the domains of DNA topoisomerase 2-binding protein 1 (TOPBP1), whereas a distinct specific domain of TOPBP1 interacts with ATR-ATRIP [127]. This last interaction stimulates the ATR-ATRIP kinase complex at DNA damage sites to allow DNA repair [127].

In order to locate these DNA damages preceding DNA repair pathway, γH2AX is a widely used biomarker for this instance [128, 129]. It was shown that γH2AX formation is necessary for DNA laddering following DFF40 activation, but not for caspase-3 activation [116]. The inhibition of caspase activation via the caspase inhibitor zLEHD-fmk compromises the activation of the DNA damage marker [119] by directly inhibiting the endonuclease activity. These observations suggest that DSB are mandatory to observe γH2AX formation. On the other hand, a role for γH2AX formation in cell cycle checkpoint control was also proposed. It was shown that the absence of γH2AX alters G2/M checkpoint and leads to the accumulation of DNA damage [125]. Knowing that DFF40 deficiency impairs γH2AX foci formation [61, 116], it is wondered if this endonuclease could have a potential role in cell cycle control. This hypothesis is reviewed in the next section.

It is important to note that DNA damage occurs in the late phase after a prolonged mitotic arrest and involves DNA damage kinases, such as ATM and DNA-PK, as well as caspase activation [119]. It was shown in a study by Hain et al. that Bcl-2 family proteins, caspase-9, caspase-3/7 and

DFF40 regulate this pathway. The prolonged mitotic arrest induces a caspase-mediated TRF2 loss from telomeres and a DNA damage signaling [119]. According to these data, we hypothesize that DFF40 deficiency, in malignant tumors, could also impair DDR systems via an inhibition of DNA-PK and ATM activity, loss of γH2AX formation and by the maintenance of TRF2 at telomeres. These phenomena could impair the genomic stability of cancer cells and allow the tumors to progress by integrating mutations into their genome, due to the regulation of their telomeres and altered DNA repair systems. It is further hypothesized that TOPBP1 might interact with an unknown factor on damaged DNA [127]. If this factor involves a protein partner of DFF40, a deficiency of the endonuclease could as well compromise the ATR pathway. Overall, instabilities due to defective DNA repair systems can lead to chromosomal abnormalities.

Cell cycle checkpoints

Genomic instabilities are mainly the result of deregulation in the activity of cyclin-dependent kinases from the cell cycle [130]. There are two main checkpoints important in tumorigenesis that have been frequently identified as potential sites of mutation in cancer development: G1/S and G2/M checkpoints. These two checkpoints are crucial for the detection of chromatin damages that can potentially lead to genomic instability and cancer development [131].

The G1/S checkpoint is dependent on p53 activity and the expression of the product of retinoblastoma tumor suppressor gene (Rb). Following DNA damage detection, cells stimulate p53 expression that mediates cellular apoptosis, depending on the irreversibility of the lesion [131]. In non-dividing cells, Rb is hypophosphorylated and blocks the transcriptional activity of E2F-1, which is involved in gene expression related to G1 to S phase cell cycle progression, by recruiting repressing partners such as the histone deacetylase [132, 133]. Upon stimulation by growth factors, Rb is hyperphosphorylated by the cyclin-dependent kinase (CDK) 2-cyclin E [134]. The change in its conformation releases E2F and the transcriptional activation occurs [134]. This step allows the transcription of proteins necessary for DNA replication. It is important to note that different ectopic expression of human cyclins interferes in the G1/S checkpoint regulation. The substrates of CDK2-cyclin E are not limited to pRb. This cyclin also phosphorylates H1, in which this action may play a role for chromatin condensation during the replication [135].

On the other hand, the G2/M checkpoint is responsible for repairing DNA damages before entering mitosis, in order to preserve the genomic stability in somatic cells [136]. The role of the G2/M checkpoint seems to be more important; in G1/S checkpoint deficiencies, the G2/M checkpoint results in a longer arrest to repair DNA damages that may have bypassed the G1/S checkpoint [136]. This checkpoint is initiated by the cyclin B1 and the mitotic CDK1 (cdc2) [136]. When DNA damage is detected, the CHK1 phosphorylates Cdc25C, and therefore inactivates its ability to activate cdc2 by dephosphorylation [136, 137]. In order to pass through the checkpoint, the activity of Cdc25C and cdc2 is important. There are many regulators on many levels of the G2/M checkpoint. In fact, DNA-PK, ATM/ATR DNA repair pathways, and p-53 are amongst the regulators of G2/M transition [137]. Thus, any protein dysfunctions in the direct regulators could potentially lead to tumorigenesis.

The importance of DFF40 in chromatin condensation and nuclear collapse during programmed cell death is well known [27, 48, 62]. H1 seems to have a role during cell cycle by having its phosphorylation under the control of the CDK2-cyclin E complex for DNA replication. It is not to our surprise to note that DNA-modulating proteins are amongst the most predominant regulators of the cell cycle checkpoints. Knowing this, it is interesting to question the impact of DFF40 deficiency in the regulation of the G1/S transition. Overall, with these observations, we hypothesize that a lack of DFF40 expression may alter the efficiency of cell cycle checkpoints, thus, allow cells to undergo mitosis at a faster rate. This hypothesis is supported by studies that have shown that diminished expression of DFF40 is responsible for high proliferation and survival rates in cancer cells, despite many chromosol abnormalities [68].

May TRF2 loss from telomeres be regulated by DFF40/CAD?

Another mechanism used by cancer cells to significantly enhance their proliferation is the upregulation of the telomerase activity [90]. The enhanced cell division capacity of tumors predisposes cells to have higher levels of genomic instability due to chromosomal aberrations accumulated upon somatic cell division. To support this hypothesis, it is proposed that cancer cells, who present a lower or abolished expression of DFF40, have diminished levels of γH2AX that is crucial for DDR pathway activation [125]. Recently, Hain et al. investigated the biomolecular pathways involved in this response following a mitotic arrest [119]. The use of caspase inhibitors revealed a significant reduction in γH2AX levels and, surprisingly, an increased level of TRF2 at telomeres [119]. This observation supports the theory where caspase-dependent DNA damage at telomeres produces DSB and activates DNA-PK [138]. DNA-PK activation at its turn promotes γH2AX formation and TRF2 depletion. The mechanisms of DFF40 in telomere depletion aren't yet elicited and are subject to investigation.

Many upcoming new studies have been proposing a loop conformation for telomeres at the end of chromosomes to sequester chromatin segments [139–141]. The process of

T-loop formation consists of hiding the 3′ overhang into the loop, as well as a small portion of the 5′ overhang (< 100 nucleotides) [139]. In humans, this process is mediated by two different complexes: TRF2-containing complex and TRF1-containing complex [139]. The first complex has a role in protecting chromosome ends, whereas the TRF1-containing complex participates in the regulation of telomerase-mediated telomere maintenance [139]. The TRF2-containing complex participates in T-loop formation with the participation of the MRN complex at a low level [139]. It was recently shown that the TRFH domain of TRF2 regulates T-loop formation, and ATM pathway suppression at chromosome ends is required when the structure is held in this conformation [141]. This specific conformation of telomeres could be implicated in DNA repair bypass mechanisms by covering the detection of DNA damages by the DNA-damage sensors [139]. The ATM repair pathway is activated once the telomeres are linearized due to altered TRF2 or a mitotic arrest [141]. We can then consider that the loss of TRF2 at chromosomes ends is a necessary physiological process to maintain genomic stability via ATM. It was shown in U2OS cells, mutated in the expression of DFF45, that DFF40 could be implicated in the loss of TRF2 [124]. Oppositely, the use of the inhibitor NU7441, a DNA-PK inhibitor, prevents the loss of TRF2 during a mitotic arrest [124]. These data suggest a possible role of DFF40 in the regulation of chromosome ends, more precisely by mediating TRF2 expression at telomeres.

Alternative splicing of DFF45 can influence genomic stability

As mentioned before, DFF45 exists in two forms, DFF45 and DFF35. Alternative splicing of DFF45 pre-mRNA is regulated by serine- and arginine-rich splicing factor (SRSF1) which exist in short and long isoform [142]. This SR protein splicing factor is involved in the inhibition of R-loop formation, which can lead to DSB followed by DNA repair (NHEJ), genomic instability or apoptosis [42, 142]. SRSF1 depletion was associated with a decrease of DFF35 mRNA and protein levels [43]. During apoptosis, NHEJ repair pathway was inhibited following the cleavage of X-ray repair cross-complementing group 4 (XRCC4) protein by caspase-3/7, which liberates DNA ligase IV from XRCC4/ligaseIV/XLF [143]. XRCC4 cleavage results in an increase of oligonucleosomal DNA fragments by promoting the nuclear translocation of CAD. Inversely, mutation of the caspase-consensus sequence of XRCC4 decreases DNA fragmentation. Interestingly, inhibition of XRCC4 cleavage or of its expression was associated with a different isoform of SRSF1. Indeed, the ratio of SRSF1L/SRSF1S decreases in the absence of XRCC4 processing by caspases. On the other hand, SRSF1 activity was negatively regulated by SR protein kinase phosphorylation, leading to a decreased splicing of DFF45 pre-mRNA to DFF35. Consequently, DFF35 mRNA and protein decrease favored the formation of the DFF complex and their import to the nucleus. Later events cause an increase in DNA fragmentation following DFF45 cleavage by caspase-3. Thus, an alteration in either pre-mRNA splicing by inactivation of SRSF1 or in the absence of XRCC4 cleavage can induce G2 cell cycle arrest and the inhibition of internucleosomal DNA fragmentation leading to genomic instability.

Concluding remarks

This review on the DFF40 endonuclease proposes new potential roles in human cells. DFF40 was first characterized as an endonuclease mediating the ultimate step of apoptosis during cell death. Over the past years, emerging studies have been proposing a role for DFF40 in the maintenance of genomic stability. Could DFF40 work in concert with other intracellular proteins to maintain cell physiology and nuclear integrity? This hypothesis is being intensively studied. Our preliminary results propose a role of DFF40 in the regulation of histone H2AX phosphorylation [61]. As mentioned above, the ATM pathway is activated upon DSB, which are signaled through γH2AX formation. An abnormality in γH2AX formation could potentially lead to DNA disrepair and accumulation of signification gene mutations. Numerous malignant cell lines, such as glioblastomas as mentioned above, present a downregulation in DFF40 expression. These cell lines are known for their malignant profile. We therefore could suspect a potential role for DFF40 in cell metabolism regulation, such as the Warburg effect. It is crucial to better understand these proposed new roles for DFF40 in order to better understand biomolecular mechanisms underlying cancer cell resistance.

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Compliance with ethical standards

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REVIEW

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Review of cancer cell resistance mechanisms to apoptosis and actual targeted therapies

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Abstract

The apoptosis pathway is a programmed cell death mechanism that is crucial for cellular and tissue homeostasis and organ development. There are three major caspase-dependent pathways of apoptosis that ultimately lead to DNA fragmentation. Cancerous cells are known to highly regulate the apoptotic pathway and its role in cancer hallmark acquisition has been discussed over the past decades. Numerous mutations in cancer cell types have been reported to be implicated in chemoresistance and treatment outcome. In this review, we summarize the mutations of the caspase-dependant apoptotic pathways that are the source of cancer development and the targeted therapies currently available or in trial.

KEYWORDS
apoptosis, cell mutations, resistance, targeted therapy

1 | INTRODUCTION

Numerous cell death pathways have been identified throughout the literature. Cell signalization in apoptosis is the best described pathway until today, due to its implication in a large number of pathologies. This process was discovered by John KER, Wyllie and Currie in 1972.[1] Apoptosis is a programmed cell death mechanism known as a cellular suicide signal. This form of cell death describes a process by which cells trigger their self-destruction in response to a signal. Apoptosis plays several roles; it eliminates infected cells from the human body, contributes to the functioning of the immune system, and also the maintenance of homeostasis. There are two main apoptotic pathways that have been thoroughly described in the literature: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. On one hand, the intrinsic pathway is triggered by signals internal to the cell, such as damage to the DNA or infection of the cell by viral agents. On the other hand, the extrinsic pathway is triggered by signals that are external to the target cell that can be mediated by either natural killer (NK) lymphocytes or CD8 positive cytotoxic T cells (CTL/$CD8^+$ T cells). Overall, the apoptotic pathway lacks an inflammatory response, but some apoptotic signals were shown to induce one. Activation of CD95, a death receptor further discussed below, was shown to induce cell death and promote inflammation through pro-inflammatory cytokine production.[2] A pro-inflammatory role of human caspases related to caspase-1 was also reported in sepsis, through cleavage and activation of cytokines.[3] Although, it is fair to say that apoptosis does

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not lead to cell lysis unlike necrosis. Proapoptotic signals (e.g., DNA lesion, stress, viral infection, p53 status, chemicals) prompts the cell to make the decision to trigger a process of apoptosis.[4,5]

Cancerous cells are known to highly regulate the apoptotic pathway and its role in cancer hallmark acquisition has been discussed over the past decades.[6] Numerous mutations in cancer cell types have been reported to be implicated in chemoresistance and treatment outcome. In fact, cancer is among the leading death causes in the United States.[7] Current first-line therapies in oncology mainly involve chemotherapeutic drugs in combination with surgery and/or radiotherapy. Although, over the past years, the interest toward personalized therapies has increased to overcome deaths associated with cancer. Personalized medicine comes with a handful of challenges, mainly due to the highly diverse genetic background of each cancer cell type. Malignant cells can be highly diverse according to the type of cancer, but also within the same tumor.[8] Not only genetic heterogeneity is important in personalized medicine, but we also have to take into account the microenvironment in which the cancer cells divide and proliferate. These emerging discoveries in cancer genetic and patient management outcomes have led to clearly affirm that no patients have the same cancer cells within a subtype and thus, patient response to traditional first-line treatments may vary;[7,9] there's an increasing need for personalized medicine to alleviate cancer burden on society. It is clear that a better understanding of the mutations involved in cancer cell resistance to apoptosis will allow a better clinical management. Thus, in this review, we discuss the main pathways of apoptosis and their regulation in normal cells to better understand the adaptations in cancerous cells. The main mutations in malignancy acquisition are reviewed and the targeted therapies are presented.

2 | THE CASPASE-DEPENDANT APOPTOTIC PATHWAYS

2.1 | Apoptosis activating signals

The nature (e.g., extracellular or intracellular) of the apoptosis-inducing signal determines the pathways that are activated. In general, those signals induce the activation of effector caspases that mediate intracellular signaling, ultimately leading to DNA fragmentation. It is important to note that some signals do not lead to the activation of caspases (the caspase-independent apoptosis pathway).[10] Direct mitochondrial damage can induce the release of AIF and EndoG or HtrA2,[10] which are proteins that directly induce apoptosis through direct DNA damage or proteolytic breakdown of cytoskeleton.[11] In addition, non-caspase proteases, such as calpains, serine proteases, or lysosomal proteases, also mediate cell death by enhancing apoptosis signaling or inducing necrosis.[12] Caspase-independent PARP cleavage was also shown to regulate cell death.[13] Other non-apoptotic pathways involved in cancer cell clearance following DNA damage induction include autophagy,[14] mitophagy,[15] necrosis, and mitotic catastrophe (MC).[16] DNA damage-mediated autophagy is known to induce the DNA damage response pathway, which leads to subsequent cell cycle arrest and DNA repair pathway regulation.[17] In the case of extensive DNA damage that cannot be repaired, cell death is initiated. As for DNA damage-induced MC, cancer cells undergo cell cycle arrest which leads to different death-associated outcomes depending on the cell cycle phase arrest and the presence or not of DNA repair.[16] Although these non-apoptotic pathways are not covered in this review, it is important to acknowledge their existence and importance in cancer cell death. The caspase dependant pathway has three main sources for the activating signals: (1) extracellular signals that are detected and routed through ligand-specific transmembrane receptors to activate the extrinsic pathway, (2) intracellular stimuli that induce apoptosis through the mitochondria, and (3) intracellular stimuli that induce apoptosis through stress caused in the endoplasmic reticulum (ER).[10] The last two signals lead to the activation of the intrinsic pathway. In this section, we will thoroughly review the sources of these apoptosis-activating signals.

2.1.1 | Activation of the extrinsic pathway through ligand-specific transmembrane receptors

The binding of a ligand to a death receptor is the first step in the extrinsic apoptosis signaling cascade. The most studied death receptors are the receptors belonging to the tumor necrosis factor (TNF) receptor (TNFR) superfamily (TNFRSF) including the TNFR1, the CD95 receptor (CD95/Fas/APO-1/APT), and the two TNF-related apoptosis-inducing ligand (TRAIL) death receptors: TRAIL-R1 (DR4) and TRAIL-R2 (DR5).[18] Table 1 presents a summary of the major transmembrane receptors and their apoptosis-inducing ligands discussed in this review. Each of these death receptors has an importance within the apoptosis process and may even present similarities between them. Here, we discuss the characteristics of the most studied death receptors.

Amongst the TNFRSF, TNFR1 and TNFR2 are the most studied transmembrane receptors. TNFR1 is

TABLE 1 Common apoptosis-inducing death receptors and their ligands

TNFRSF	Ligands	References
TNFR1	*Activators* TNF-α *Inhibitors* Soluble receptors (e.g., etanercept) Monoclonal antibodies (e.g., adalimubab)	22,23 24,25,26
FasR	*Activators* FasL/CD95L *Inhibitors* Flavonoid resveratrol ONL1204 FAIM1, FAIM2, and FAIM3	29–33,37 44–48
TRAIL-R1/DR4	*Activators* TRAIL/Apo2L *Inhibitors* Sodium butyrate (NaBt)	49,50,54 61
TRAIL-R2/DR5	*Activators* TRAIL/Apo2L *Inhibitors* Sodium butyrate (NaBt) Nelfinavir	61,62 61,62

expressed in all cell types, whereas the expression of TNFR2 is limited to immune cells, mesenchymal stem cells, and neural cells (e.g., glial cells).[19] Both receptors contain multiple cysteine-rich domains (CRDs) in their extracellular domain.[20] TNFR1 contains a death domain (DD) of 70–80 amino acids[21] and is involved in apoptosis and chronic inflammation through the interaction with TNF-α.[22] On the other end, TNFR2 does not contain a DD, but has a short amino acid motif near its C-terminal domain that allows the recruitment of the adapter protein TRAF2 and its associated proteins as well.[20] Due to the lack of a DD, TNRF2 thus lacks an inducible intrinsic cell death activity. It mediates its activity through the NFκB signaling pathway and through the activation of other multiple kinases.[23] TNF-α is the ligand of both receptors and is expressed in two distinct forms (e.g., transmembrane protein or soluble variant (sTNF) issued from the proteolytic processing of the transmembrane form).[20] It was shown that sTNF has more affinity for TNFR1.[24] TNFR cell death signaling is thus activated by the binding of sTNF or membrane-bound TNF to the receptor. Antagonists of TNF-α such as soluble receptors (e.g., etanercept) or monoclonal antibodies (e.g., adalimumab) make it possible to neutralize TNF.[25,26] Bisphenol A (BPA) and nanoparticles (NPs) have been shown to induce the release of TNF and thus induce apoptosis.[27] The study by Crisafulli et al.[28] demonstrates that pharmacological, as well as genetic, inhibition of TNF binding to TNFR1 leads to a reduction in the degree of macrophage inflammation caused by LPS and IFN-γ stimulation.

CD95/Fas, DR4, and DR5 are also involved in the induction of apoptosis.[29] Their respective ligands, CD95L and TRAIL, are expressed as single-span type II trimeric membrane proteins, but can be cleaved in certain circumstances.[30] First, the CD95 receptors are pre-associated homotrimers expressed on the cell surface and contain three extracellular CRD.[31,32] It was shown that these domains are located on the NH2-terminal domain and are involved in the oligomerization of CD95.[32,33] The CRD1 contains the self-association domain (pre-ligand assembly domain [PLAD]), which is shared by the TNFRSF.[32] Cell surface CD95 proximity is linked to PLAD.[32] Furthermore, it was shown that CD95L is not necessary for apoptosis induction in cells overexpressing the cytoplasmic domain of CD95 or lacking the NH2-terminal domain (42 amino acids).[33] The binding of its ligand (FasL/CD95L) induces a conformational change allowing the formation of the DISC (further described below).[34,35] A study conducted by Liu et al.[36] using rats showed that in the reperfusion process of myocardial ischemia, FasL induces apoptosis of myocardial cells. Multiple studies have reported the importance of the anti-CD95 antibody (Jo2) in hepatocyte cell death.[37,38] Hepatocytes are type 2 cells that constitutively express CD95, rendering them sensitive to CD95L-mediated apoptosis. The injection of the agonistic Jo2 anti-CD95 antibody in mice was shown to significantly enhance hepatocyte cell death (e.g., liver toxicity), which is mediated by the activation of NK cells.[39,40] It is important to note that the injection of physiological soluble CD95L does not induce toxicity, due to posttranslational modifications of the ligand.[41–43] Many inhibitory molecules or modulators of CD95/Fas have also been reported in the literature. It was shown that resveratrol modulates FasL/Fas-mediated apoptosis.[44] ONL1204, a new derivate of Met12, was shown to be a small peptide inhibitor of Fas.[45] It was shown that Met12 inhibits caspase-8 activation following Fas-induced apoptosis in 661W cells.[46] Similarly, ONL1204 blocks membrane FasL-induced apoptosis and its subsequent signaling pathway.[45] Both molecules are actual derivates of Met, a receptor tyrosine kinase whose ligand is hepatocyte growth factor (HGF). Met was shown to directly bind to Fas in hepatocytes and induce its sequestration, therefore inhibiting FasL-induced apoptosis.[47] Derivates of Met are thus of interest due to their ability to inhibit Fas-induced apoptosis. The Fas inhibitor molecule (FAIM)1, FAIM2, and FAIM3 have also shown to inhibit Fas-induced cell death.[48] On

the other hand, TRAIL-R's are also expressed on cell surface, but they consist of five different receptor subtypes. The study by Staniek et al.[49] provided an analysis of the signaling capacity and induction of TRAIL-R-induced apoptosis in primary human B cells. TRAIL-R's that contain a DD, such as TRAIL-R1 (DR4)[50] and TRAIL-R2 (DR5) can induce apoptosis in cancer cells.[51] TRAIL-R's without a DD, such as TRAIL-R3, TRAIL-R4, and osteoprotegerin (OPG), do not induce apoptosis.[52,53] DR4 and DR5 have in their extracellular parts 2–4 similar CRDs and are type I membrane proteins.[54] Their DDs are composed of about 70 amino acids in the cytoplasmic part.[55] The trimeric bond will allow the intracellular DD structure of the DRs to be modified and thus the FADD bond will be formed.[56] The ligand of TRAIL-R (TRAIL) also belongs to the TNFSF and consists of 282 amino acids.[57] TRAIL is expressed in NK cells, B cells, monocytes, and dendritic cells,[58] and was shown to only activate extrinsic apoptosis in cancer cells.[59] Modulators of TRAIL-R include dulanermin, an apoptosis-inducing agent. It is an optimized form of the endogenous Apo2 ligand and can therefore bind to the DR4 and DR5 receptors.[60] Other modulators are Nabt and nelfinavir, which result in higher expression of membrane death receptors (DR4/5).[61,62]

2.1.2 | Activation of the intrinsic apoptosis pathway through the mitochondria

The intrinsic apoptosis pathway through mitochondrial damage is activated by a large number of endogenous or exogenous stimuli that can induce multiple bicellular damages such as DNA damage, oxidative stress, and a rise in intracellular Ca^{2+} concentrations.[1,10,63] The activation of this pathway involves the activation or inhibition of proteins from the B-cell lymphoma 2 (Bcl-2) family. This step will be discussed below. In oncology, multiple drugs are known to induce apoptosis of cancerous cells through this mechanism. Sorafenib, a small molecule *Raf* kinase inhibitor used in the treatment of solid tumors, induces the apoptosis of acute myeloid leukemias (AMLs) through the upregulation of BIM, an activator of the intrinsic pathway, and the downregulation of Mcl-1, an anti-apoptotic protein,[64] both members of the Bcl-2 protein superfamily. Synthetic silicon dioxide nanoparticles (SiNPs), used mainly in drug delivery and gene therapy, have shown to induce oxidative stress and mitochondrial damage.[65] Essential oils of *Cymbopogon citratus* induce the activation of the intrinsic pathway through the loss of the mitochondrial membrane potential ($\Delta\psi_m$).[66] In fact, an increase in reactive oxygen species (ROS) leads to the loss of $\Delta\psi_m$.[67] Every synthetic or natural molecule that can alter the redox balance of cells can thus induce the intrinsic apoptotic pathway through mitochondrial activation.

2.1.3 | Activation of the intrinsic apoptosis pathway through the endoplasmic reticulum stress

ER stress is mainly caused by misfolded proteins that accumulate in the ER compartment of normal cells.[10] The ER is the site for protein folding, lipid, and sterol synthesis as well as storage for free calcium. The highest levels of intracellular calcium are in fact found in the ER. Upon a pathological stress on the ER, the level of mutated proteins rises in the compartment, which results in an imbalance between the capacity of the ER to fold proteins and the demand for protein folding.[68] This phenomenon induces an ER stress that is sensed and transduced by the unfolded protein response (UPR). The protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1 (IRE1a), and the activating transcription factor 6 (ATF6) are activated when the levels of misfolded proteins reach a critical concentration.[10] Apoptosis is induced through the activation of Bax and Bak, two main proapoptotic proteins, by their association with IRE1.[10] These steps involved in the pathway activation will be discussed down below. In clinical oncology, many natural compounds are known to modulate cancer cell apoptosis through the ER stress pathway.[69] Thapsigargin (TG), extracted from plants and known as an ER stress inducer, has shown to induce apoptosis through caspase-3 activation in the myeloma cell line 12-PE.[70] DOT1L inhibition significantly suppresses ER stress pathway genes in sensitive multiple myeloma (MM) cells that undergo cell death.[71] The regulation of the UPR pathway could thus be a novel approach for cancer management by favoring the accumulation of unfolded proteins in cancer cells.[72,73]

2.2 | Cell signaling pathways

There are two main death signaling channels: the extrinsic and the intrinsic apoptotic pathways. The choice of the apoptotic pathway activated upon cell death-triggering signals depends on cell types and the nature of the stimuli. For example, type I cells are more likely to undergo the extrinsic apoptotic pathway, whereas type II cells require amplification to perform the intrinsic apoptotic pathway.[74] These specificities are due to biomolecular differences in both cell types. There is a high level of DISC and caspase-8 activation with Fas

stimulation in type I cells, which causes the activation of effector caspases-3 and -7 of the extrinsic pathway.[74] On the other hand, type II cells show low levels of DISC formation and their apoptotic signals are amplified by the release of cytochrome C from mitochondria through the intrinsic pathway.[74] The release of cytochrome C from the mitochondria leads to the formation of the apoptosome, resulting in the activation of the caspase-9.[75] In this section, we review the caspase-dependant apoptosis signaling pathways (Figure 1) in detail.

2.2.1 | The extrinsic apoptotic pathway

The extrinsic pathway is initiated by the binding of the DR's to their respective ligands. The binding of the receptor to the ligand leads to the oligomerization of the receptor and then attracts the adapter DD protein. Cell signaling in the extrinsic apoptotic pathway is ligand-specific. In fact, the recruited adapter DD proteins are specific to the ligand: each ligand induces the recruitment of an adapter DD protein associated with him. Thus, either the Fas-associated DD (FADD) or TNFR-associated DD (TRADD) proteins are recruited to the cell membrane.[76] Following the binding of FasL with Fas and the oligomerization of the receptor, FADD is recruited to the cell membrane at the DD sequence of the receptor.[21] FADD recruits the procaspase-8 (also known as FLICE) through its dimerization, leading to the formation of the death-inducing signaling complex (DISC).[77] Once the DISC is fully formed, the procaspase-8 is activated by its autolytic cleavage and forms the caspase-8, which initiates the apoptotic signaling pathway by directly cleaving the procaspase-3 into its active form.[78] Caspase-8 contains the death effector domain (DED).[79] The activation of caspase-8 requires the formation of specific

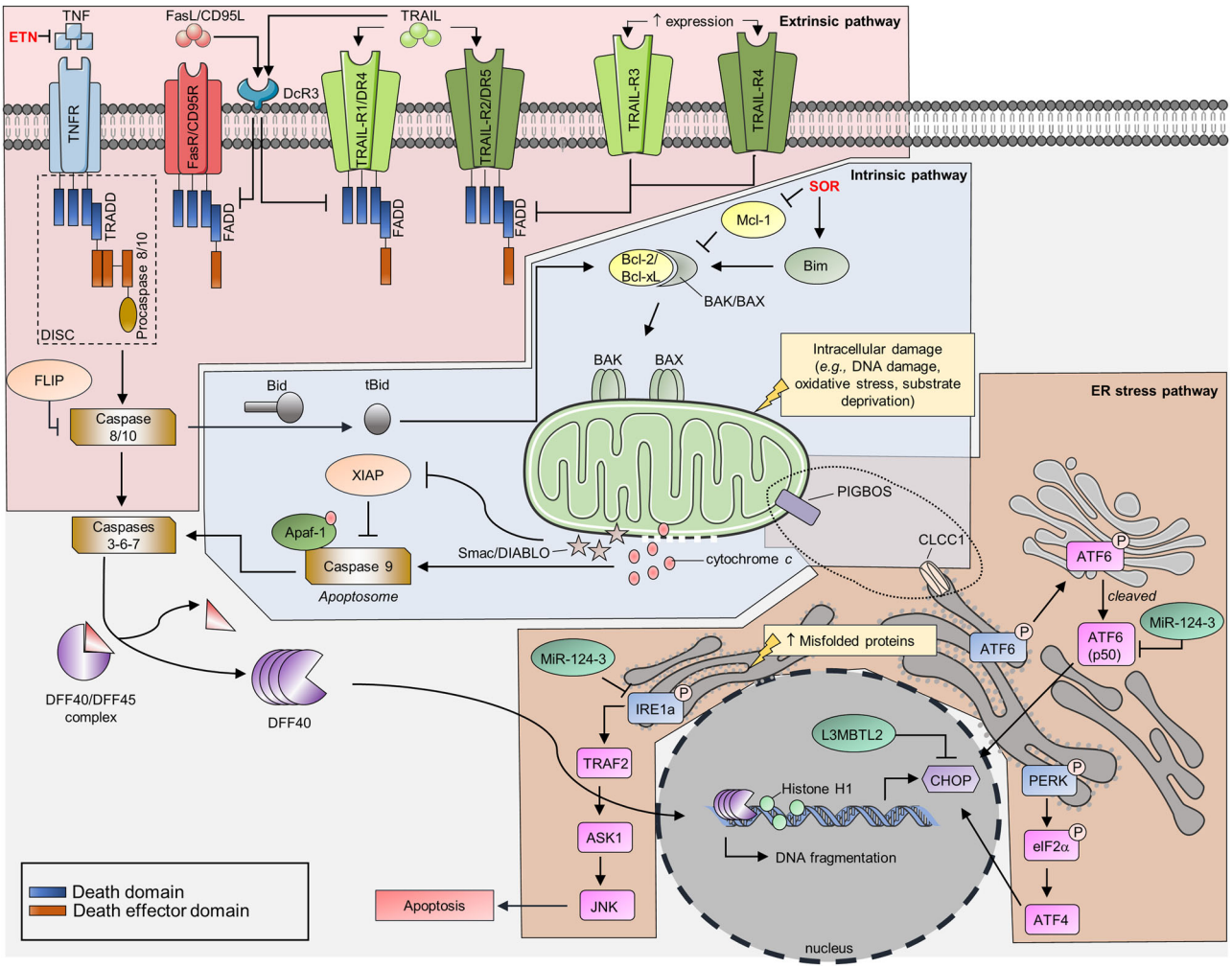

FIGURE 1 Illustration of the main caspase-dependent apoptotic pathways and their regulators. The extrinsic (pink box), intrinsic (blue box), and endoplasmic reticulum (ER) stress (orange box) pathways are represented. Mechanisms of actions of drugs are represented in red. ETN, etanercept; SOR, sorafenib

oligomeric structures, called DED filaments.[80] The formation of DED filaments is achieved through the interaction of FADD's DEDs with the DEDs of procaspase-8, procaspase-10, and cellular FLICE-inhibitory protein (c-FLIP).[81] The induction of the cascade of caspases is the result of the dimerization and activation of the procaspase-8 at the DED filament.[82] However, the activation of procaspase-8 would not be possible without the tandem DEDs (tDEDs), which allow several monomers of procaspase-8 to be brought together.[83] The direct activation of caspase-3 and caspase-7 by caspase-8 is the key step for the ultimate destruction of the genome.[21] Caspase-3 is responsible for the cleavage of the DNA fragmentation factor 45/inhibitor of caspase-activated DNAse (DFF45/ICAD), which switches on the endonuclease activity of the DNA fragmentation factor 40/caspase-activated DNAse (DFF40/CAD).[84] This final step leads to the internucleosomal fragmentation of the DNA. Overall, throughout the extrinsic apoptotic pathway, two groups of caspases participate in cell signalization: (1) apoptosis-initiating caspases, known as group II caspases (e.g., caspases-2 [Nedd2/ICH-1], -8 [FLICE/MACH/Mch5], -9 [ICE-LAP6/Mch6], and -10 [Mch4]) or (2) effector caspases of apoptosis, known as group III caspases (e.g., caspases-3 [Yama/CPP32/apopain], -6 [Mch2], and -7 [ICE-LAP3/Mch3/CMH-1]).[85] Group III caspases do not have DED or caspase recruitment domains (CARDs). On the contrary, group II caspases have one of the two domains according to their precise roles.[86]

2.2.2 | The intrinsic apoptotic pathway

The intrinsic apoptotic pathway can be activated by signals that directly regulate the Bcl-2 family proteins or through a crosstalk with the extrinsic apoptotic pathway.[21,87] The key member of the Bcl-2 family involved in the crosstalk between those two pathways is Bid, a BH3-only interacting protein.[88,89] Following the activation of the FasL/Fas apoptotic pathway, caspase-8 induces the cleavage of Bid into t-Bid, which allows its translocation from the cytoplasm to the membrane compartments of mitochondria.[88] This is due to its higher affinity to Bcl-xL, a member of the Bcl-2 family protein.[88] The activation of t-Bid leads to the mitochondrial membrane permeabilization by Bax or Bak through pore formation.[21] The binding of the BH3- only protein to Bax induces conformational changes and its subsequent translocation to the mitochondria and oligomerization.[90] Active Bak and Bax proteins form pores in the mitochondrial membrane allowing the leak of its content. It was shown in Cos-7 cells that $\Delta\psi_m$ loss precedes Bax association with the mitochondria following staurosporine (STS)-induced apoptosis.[91] The activation of the intrinsic pathway through the regulation of Bcl-2 family proteins is discussed further below. Both pathways lead to the release of cytochrome C and second mitochondria-derived activator of caspases (Smac/DIABLO) from the mitochondria. Cytosolic cytochrome C binds to the Apaf-1 protein, which further recruits procaspase-9 to form the apoptosome.[21] The apoptosome is a heptameric structure where Apaf-1 directly interacts with three or four procaspase-9 molecules via CARD–CARD interactions.[92] Procaspase-9 molecules form homodimers or heterodimers in the apoptosome.[92] This specific tridimensional conformation induces the cleavage of procaspase-9 to its active form, the caspase-9, which in its turn activates caspase-3/7 by procaspase-3/7 cleavage.[21] On the other hand, Smac was shown to inhibit inhibitor of apoptosis proteins (IAPs), mainly XIAP, allowing further caspase activation.[93] Overall, caspase-9 and t-Bid are the two main molecules that allow a link between the extrinsic and intrinsic apoptotic pathways. The subsequent steps following caspase-9 activation are similar to those in the extrinsic apoptotic pathway: both pathways have converged.

2.2.3 | The endoplasmic reticulum stress pathway

When the ER is subject to stress, the organelle cannot execute its proper function, leading to the accumulation of unfolded proteins in its intracellular compartment. The rise in the concentration of unfolded proteins is detected by specific membrane-associated sensors that activate the UPR. As mentioned above, three main sensors are involved in this pathway: PERK, IRE1, and ATF6. Here we will briefly have an overview of the three pathways, that were thoroughly reviewed by Kadowaki and Nishitoh.[94] Under physiological conditions, PERK, IRE1, and ATF6 are in a dimeric complex with the immunoglobulin-binding protein (BiP/GRP78).[94] Following the accumulation of misfolded proteins, BiP, the chaperone molecule, dissociates from the molecules leading to pathway activation. PERK, a type I transmembrane protein, possesses a serine/threonine kinase activity in its C-terminal cytosolic domain.[94] It senses the accumulation of unfolded proteins through its N-terminal domain.[94] Following the ER stress, the transcription of proapoptotic factors (e.g., C/EBP homologous protein [CHOP]) is activated by the PERK-eIF2α-ATF4 pathway.[94] A large number of proapoptotic proteins are under the regulation of CHOP, such as DR5, and two BH3-only proteins of Bcl-2 family, namely Bim

(binding to microtubule) and PUMA (p53 upregulated modulator of apoptosis).[94] The functions of these molecules are discussed below. The activation of these factors leads to apoptosis. The signaling pathway of IRE1 is pretty similar to that of PERK. The prolonged ER stress activates the IRE1-TRAF2-ASK1 pathway.[94] This pathway leads to the activation of the JNK/p38 pathway that's triggers apoptosis through Bak/Bax enhanced transcription.[94] Finally, the interaction of ATF6 with misfolded proteins induces its translocation from the ER to the Golgi, where it's processed and releases an N-terminal cytosolic domain.[94] The N-terminal domain is then translocated to the nucleus to induce UPR target genes (e.g., chaperones, X-box binding protein 1 [XBP1]).[94] Overall, the activation of the UPR pathways in the ER increases protein folding, upregulates proteolysis of unfolded proteins, and downregulates protein expression.[95] Overactivation of these pathways without a return to cell homeostasis will ultimately lead to cell apoptosis through an imbalance between pro- and anti-apoptotic Bcl-2 family proteins.[95,96]

3 | MECHANISMS OF REGULATION OF APOPTOSIS

3.1 | The extrinsic pathway is regulated upon many levels

3.1.1 | Cell death receptor regulations

Apoptosis can be regulated in several ways (see Table 2). The first level of regulation relies on tissue- and cell-specific expression of DRs. A variation in DR expression within the same cell has also been noted.[21,97] Second, decoy receptors (DcRs) have a huge role in apoptosis regulation. TRAIL-R3 and -4 are considered as decoy receptors and thus inhibit apoptosis.[98,99] The overexpression of TRAIL-R3 or TRAIL-R4 effectively inhibits apoptosis mediated by TRAIL-R1 or TRAIL-R2.[99] Since TRAIL3 and TRAIL4 are incorporated into signaling complexes, they can interfere by reducing or preventing signaling.[98] Many examples of DcRs exist in the literature. For example, one of the most studied DcR is the DcR3. DcR3, a member of the TNFRSF, is an antiapoptotic and pro-metastases factor.[100] It can competitively bind to FasL, TL1A (TNF-like cytokine 1A), and LIGHT, due to its similar sequence with OPG, TNF2, and Fas, and inhibit apoptosis and induce angiogenesis.[101] The DcR IL-1RII is a glycoprotein of 60 kDa and is expressed on monocytes, neutrophils, and B lymphocytes.[102] Its role is to inhibit signal transfer into the intracellular compartment.[102] Furthermore, glycoproteins play an important role in apoptosis modulation. In fact, the sensitivity to apoptosis induction of CD95 and TRAIL-R is due to their glycosylated structures, which modulates their sensitivity to apoptosis induction.[103] Galectins, by interacting with DRs and cell surface glycoproteins, can regulate apoptosis. N-glycosylation is required for the adjustment of CD95L/CD95L interactions, but is not required for the linkage from CD95L to CD95.[104]

3.1.2 | Regulation of the molecular signaling partners

The main regulation point of the extrinsic pathway is through caspases. First, caspase inhibitors such as p35, cytokine response modifier A (CrmA), and the IAPs play an important role in caspase activation.[105] The p35 protein prevents caspase activity by binding to protease target enzymes.[106] In the presence of active caspases, an intramolecular cleavage is performed at specific caspase sites by p35, which prevents its dissociation from caspases.[107] CrmA is the only member of the serine family that can inhibit the action of cysteine proteases, such as caspases, and serine proteases.[108] CrmA is the first identified caspase inhibitor and works by inhibiting caspase-1.[109] The inhibition of caspase-1 prevents the secretion of interleukin (IL)-1β and IL-18.[109] CrmA also inhibit caspase-8 and caspase-10.[109] The inhibition of caspases occurs by the binding of CrmA directly to the catalytic site of the protease.[109] Eight human IAPs are known to control caspases: X-linked IAPs (XIAP), cIAP1, cIAP2, ML-IAP, NAIP, ILP2, survivin, and Bruce.[110] However, only XIAP can directly inhibit caspases, whereas cIAP only prevents the formation of proapoptotic signaling complexes in the extrinsic apoptotic pathway.[111] XIAP contains three baculoviral IAP repeats (BIR) and contains an associated ubiquitin domain.[112] The BIR domain serves as a protein-protein interaction domain for the direct binding and inhibition of caspases-3, -7, and -9.[113] The BIR3 domain inhibits caspase-9 and the binding region between the BIR1 and BIR2 domains is responsible for the inhibition of caspase-3 and -7.[114] The ubiquitin-associated domain (UBA) allows the binding of poly-ubiquitin conjugates and the ring domain is necessary for the E3-ligase activity which promotes the degradation of proapoptotic proteins (SMAC and ARTS), on which the regulation of apoptosis essentially depends.[115,116] As mentioned above, there are two main cell types in Fas-induced apoptosis, which are type 1 (e.g., lymphocytes) and type 2 (e.g. hepatocytes, pancreatic β-cells) cells. It was shown that XIAP can discriminate between type I and type II Fas-induced apoptosis.[117] In thymocytes, Fas stimulation by its ligand rapidly abolishes XIAP levels, whereas in hepatocytes, treatment

TABLE 2 Regulators of the caspase-dependent apoptotic pathways

Regulators	Function(s)	References
The extrinsic pathway		
Soluble TNF-α receptors (e.g., etanercept)	Binds and neutralizes TNF-α, inhibiting the activation of TNFR.	25,26
Anti-TNF-α monoclonal antibodies (e.g., adalimubab)	Binds and neutralizes TNF-α, inhibiting the activation of TNFR.	25
TNF-α enhancing agents (e.g., Bisphenol A, nanoparticles)	Enhances the release of TNF-α induces apoptosis through TNFR signaling.	27
TRAIL-R3	Inhibits apoptosis mediated by TRAIL-R1/DR4.	99
TRAIL-R4	Inhibits apoptosis mediated by TRAIL-R2/DR5 by reducing or preventing signaling.	98,99
Decoy receptor 3	Competitively binds to FasL and TRAIL and inhibits apoptosis.	100,101
Decoy receptor IL-1RII	Inhibits apoptosis by impairing intracellular cell signaling.	102
p35 protein	Prevents caspase activity by binding to protease target enzymes.	105,106
CrmA	Inhibits the action of cysteine proteases (caspases) and serine proteases.	105,108
XIAP	Directly inhibits caspases-3, -7, and -9.	113,114
c-IAP	Prevents the formation of proapoptotic signaling complexes.	110,111
c-FLIP L	Antiapoptotic function at high concentrations. Inhibits procaspase-8 activation.	118,119
c-FLIP S	Inhibits procaspase-8 activation.	81,118
DFF45/ICAD	Inhibits DFF40/CAD activation, which is responsible for DNA fragmentation.	124
Histone H1	Stimulates DNA cleavage and activates DFF40/CAD through its C-terminal domain.	124
The intrinsic pathway		
Bcl-2	Inhibits the activation and oligomerization of Bax/Bak.	127
Bcl-xL	Directly interacts and inhibits Bax/Bak. Continuously retranslocates Bax from the mitochondria to the cytosol. Binds and inhibits Bax/Bak, with a preferential binding for Bax. Binds BH-3-only proteins and inhibits Bax/Bak activation.	127 130 132,133
Bfl-1/A1	Continuously retranslocates Bax from the mitochondria to the cytosol. Binds and inhibits cBid and Bax.	131
Mcl-1	Binds BH-3-only proteins and inhibits Bax/Bak activation.	134
Bim	Induces direct activation of Bax/Bak.	128,134
Noxa	Neutralizes Mcl-1, facilitating Bax/Bak activation.	128,134
PUMA	Induces direct activation of Bax/Bak.	128,134
Smac	Neutralizes IAPs (mainly XIAP), allowing caspase activation.	93
The ER stress pathway		
PIGBOS	Regulates the activation of the UPR pathways by interacting with the CLCC1 channel in the ER.	95
L3MBTL2	Directly suppresses CHOP transcription.	139
MiR-124-3	Suppresses CHOP transcription by neutralizing IRE1α, XBP1s, and active ATF6.	139

increases XIAP levels.[117] In BID/XIAP double-deficient hepatocytes of mice, FasL treatment induces cell death, which can be prevented by pretreatment with a caspase inhibitor.[117] Another major regulator of caspase activation is c-FLIP. The c-FLIP proteins regulate the activation of procaspase-8a/b.[118] This regulation is located on the DED filaments. There are 3 c-FLIP isoforms: c-FLIP L (Long), c-FLIP S (Short), and c-FLIP R (Raji).[118] c-FLIP L has a proapoptotic function at an intermediate concentration by forming catalytically active procaspase-8/c-FLIP L heterodimers and stabilizing the active center of procaspase-8.[119] Thus, caspase-8 will be active after sufficient heterodimer formation. However, c-FLIP L has an antiapoptotic function in high concentrations as do the short isoforms c-FLIP, c-FLIP S, and c-FLIP R.[81] Finally, regulations in the final step of apoptosis can also occur. The nuclease activity of DFF40/CAD can be activated by specific chromosomal proteins, such as the histone H1, the HMG-box-containing chromatin proteins (HMGB) 1 and 2, and topoisomerase II (TOP2). Histone H1, HMG-1/2, and the TOP2 enable the DFF to be activated by trapping DNA supercoils and cross-breeds, which induces DNA conformations that partially mimic nucleosomal DNA wrapping.[120] A study on histone H1 somatic cells showed that histone H1 stimulates DNA cleavage and that its C-terminal domain (CTD) is responsible for the activation of DFF40/CAD.[121] The binding of the HMGB 1/2 proteins makes the substrate more accessible to cleavage by the DFF40/CAD nuclease according to data shown in one study.[122] Finally, TOP2 allows the condensation and segregation of chromosomes.[123] DFF45/ICAD and DFF35/ICAD-S also have the ability to inhibit activated DFF40/CAD homo-oligomers. DFF45 also has a role of chaperone molecule where it allows the proper folding of newly synthesized DFF40.[124]

3.2 | Regulation of the intrinsic pathway through the Bcl-2 superfamily proteins

The intrinsic apoptosis pathway is regulated through the Bcl-2 superfamily proteins that mainly control mitochondrial outer membrane permeabilization (MOMP). The Bcl-2 family proteins can be divided into three groups: (1) the antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, Bcl-W, Mcl-1, and Bfl-1/A1), (2) the proapoptotic pore-forming proteins (e.g., Bak, Bax, and Bok), and finally the (3) proapoptotic BH3-only proteins, that can be divided into activator proteins (e.g., Bid, Bim, NOXA, and PUMA) or sensitizer proteins (e.g., Bad, NOXA).[125,126]

First, the interaction between the Bcl-2 family proteins is regulated through their BH3 domains. The antiapoptotic proteins perform their function through the mitochondrial membrane, the nuclear envelope, and the ER to prevent the activation of the proapoptotic proteins.[127] All members of the antiapoptotic subfamily (e.g., Bcl-2, Mcl-1, Bcl-xL, and Bcl-W) contain four BH domains and a C-terminal hydrophobic domain.[127] The BH1 to BH3 domains form the hydrophobic groove, known to interact with BH3-only proteins, whereas the BH4 domain is involved in structure stabilization.[127] BH3-only proteins consist of Bcl-2 family proteins that possess only the BH3 motif of 100–200 amino acids.[128] Their role is to either promote the direct activation of Bax and Bak (e.g., Bid, BIM, and PUMA) or inhibit the role of the antiapoptotic Bcl-2 proteins through their BH3-containing helix and the hydrophobic groove of the substrate.[128] Under nonapoptotic conditions, the Bcl-2 antiapoptotic subfamily is linked to BH3-only proteins through their BH1–BH3 groove, inhibiting the activation of proapoptotic proteins. The Bcl-2 protein inhibits the activation and oligomerization of Bax/Bak and is also known to directly bind and inhibit Bax.[127] It also directly stops the release of the cytochrome c in the cytoplasm, therefore inhibiting the formation of the apoptosome.[127] Like the Bcl-2 protein, Bcl-xL also directly interacts with Bax and Bak to prevent MOMP.[127] It is important to note that Bcl-xL is the antiapoptotic long isoform of Bcl-x.[129] It was shown that alternative splicing of Bcl-x also produces a short isoform, known as Bcl-xS, which has a proapoptotic function.[129] To prevent Bax activation, it was recently shown that Bcl-xL[130] and Bfl-1/A1[131] continuously retranslocates Bax from the mitochondria to the cytosol. Bfl-1/A1 has also the ability to form stable complexes with cBid and Bax, with a stronger binding affinity with cBid, and inhibit apoptosis.[131] On the other hand, Bcl-W binds with Bak and Bax, with a preferential binding for Bax.[132] It can also bind with numerous BH3-only proteins, like Mcl-1 and Bcl-2, to inhibit apoptosis.[133,134] Alternative splicing of Mcl-1 produces 3 distinct isoforms (Mcl-1L, Mcl-1S, Mcl-1ES), where the long isoform (Mcl-1L) is known for its antiapoptotic function and is commonly referred to as Mcl-1.[135] Mcl-1 can localize to cell membranes, especially to the mitochondrial outer membrane.[134] As for the two other isoforms, they mainly display proapoptotic functions. Mcl-1S can dimerize with Mcl-1L, thus inhibiting its antiapoptotic function, whereas Mcl-1ES is known to induce mitochondrial cell death, independently of Bak or Bax.[134] Bak and Bax are the proapoptotic proteins that also consist of four BH domains.[136] The main difference between Bak and Bax is their localization in normal cells. Prior to its activation, Bax is mainly located in the cytoplasm, whereas Bak is located in the mitochondrial membrane.[127] Phosphorylation of Bad inactivates its

tumor suppressor function.[137] Under apoptotic conditions (e.g., DNA damage, Bcl-2 inhibitors), the BH3-only proteins are released, subsequently activating the proapoptotic proteins through the hydrophobic groove interaction.[136] The molecular mechanisms involved in the activation of Bax and Bak have thoroughly been described by Westphal et al.[136] The hallmark of proapoptotic activation is the translocation of Bax to the MOM and its subsequent conformation change to allow homo-oligomerization. The relation between the different Bcl-2 family proteins under apoptotic and normal conditions is presented in Figure 2.

3.3 | Regulation of the endoplasmic reticulum stress pathway

As the other two caspase-dependent pathways, the ER stress pathway has its own regulatory mechanisms. First, levels of BiP are highly regulated by oligomer formation, Posttranslational modifications, and the induction of the UPR in the ER.[138] The PIGB opposite strand 1 (PIGBOS) microprotein, a mitochondrial outer membrane protein, was shown to interact with the chloride channel CLIC-like 1 (CLCC1) protein in the ER.[95] An abolition in the expression of PIGBOS leads to increase sensitivity to UPR activators such as TG and Brefeldin A (BFA).[95] In fact, there is an increase in the levels of target UPR genes, that lead to apoptosis through caspase-3 activation and PARP cleavage.[95] On the other hand, overexpression of PIGBOS in HEK293 cells lowers UPR activation levels.[95] A recent study, using the CRISPR-based loss of function genetic screening tool, discovered numerous suppressors of the ER stress pathway, such as a polycomb protein complex (lethal(3)malignant brain tumor-like 2 (L3MBTL2)) and a microRNA targeted toward IRE1 (MiR-124-3).[139] L3MBTL2 eases ER stress-induced apoptosis in HEK293T cells by suppressing CHOP expression.[139] MiR-124-3 also suppresses CHOP expression by directly

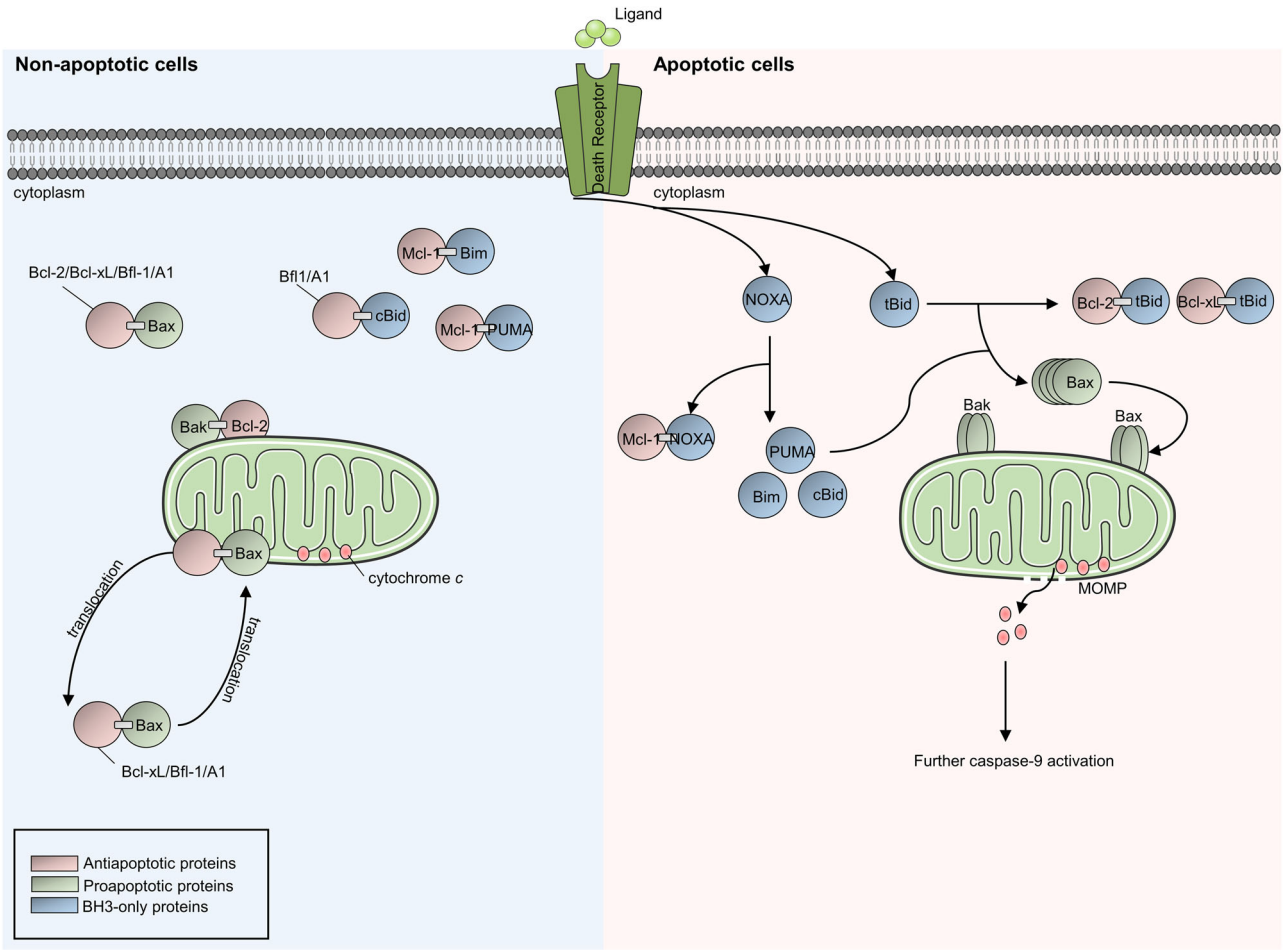

FIGURE 2 Representation of the cell signaling network between BH3-only proteins during apoptosis. Molecular interactions of Bcl-2 family proteins in non-apoptotic and apoptotic cells. Antiapoptotic proteins are represented in pink, proapoptotic proteins in green, and BH3-only proteins in blue

targeting IRE1, by diminishing the protein expression levels of IRE1α, spliced XBP1 (XBP1s), and active ATF6.[139]

4 | MUTATIONS IN THE CASPASE-DEPENDANT APOPTOSIS PATHWAYS THAT LEAD TO TUMORIGENESIS

Deregulation of apoptosis can lead to different types of diseases such as autoimmune diseases, neurodegenerative diseases, bacterial and viral diseases, heart disease, or cancer.[140] Many literature reviews have reported in the past mutations in the apoptosis pathway that lead to cancer and favors cell resistance to apoptosis.[141–143] Although, the mechanisms underlying cancer cell resistance to apoptosis are continuously investigated and new data found. It is important to know the main mutations and adaptations in cancer cells to provide a better clinical management and personalized treatment plan to patients. Here, we review the main mutations found in cancers and a large summary is reported in Table 3.

4.1 | Mutations in the extrinsic apoptotic pathway

4.1.1 | Mutations involved upstream caspase activation that lead to cancer

Mutations involving the DRs have been reported in cancers. In small cell lung carcinoma (SCLC) cell lines no surface DRs are detected.[144] Mutations and post-translational modulations in CD95-mediated cell death in cancer cells have been thoroughly described in the literature.[145] Numerous germinal and somatic mutations in the APT-1 gene, encoding for CD95, have been reported,[145] leading to the autoimmune lymphoproliferative syndrome (ALPS). ALPS often results from heterozygous dominant germline FAS or heterozygous somatic FAS mutations (10%–15% of ALPS-FAS cases).[146] Homozygous germline mutations are more rare.[146] ALPS caused by FAS/TNFRSF6 deficiency (ALPS-FAS) leads to a disruption of apoptotic signaling via the Fas DR pathway, which mainly compromises lymphocyte homeostasis.[147] Posttranslational modifications in the cytoplasmic portion of CD95, such as oxidations or covalent binding of palmitic acid, have also been linked to malignancy.[145] Furthermore, mutations on the human chromosome 11q13.3, which is the locus containing the FADD gene, are frequently observed in human malignancies.[148] This mutation prevents the transmission of apoptotic signals via FADD with DR.[149] For example, lymphoepithelioma-like hepatocellular carcinoma (LEL-HCC) is characterized by marked focal amplification of chromosome 11q13.3.[150] In vivo and in vitro studies conducted by Zhang et al. demonstrated that the amplification of miR-548k located in amplicon 11q13.3 promoted lymphangiogenesis and lymphatic metastasis.[151]

4.1.2 | Mutations in caspases and nucleases are largely involved in cancer therapy chemoresistance

Mutations involving caspases in cancer therapy chemoresistance have been widely reported in the past.[143] Caspase-8 mutations are mainly detected in gastric cancers.[152] The Q482H mutation of procaspase-8 abolishes apoptosis by causing dimerization attenuation of the protein monomers of procaspase-8, leading to resistance to chemotherapy.[153] Mutations in the caspase-8 gene mainly involve in-frame deletions, substitutions, and insertions of amino acids.[143] A deletion of the procaspase-8 protein was also noted in SCLC.[144] Although the majority of studies report a downregulation of caspase-8 in cancer cells, a few studies have reported the link between caspase-8 overexpression and oncogenesis. Caspase-8 overexpression was reported in glioblastomas.[154] It was shown that caspase-8 promotes tumor growth and angiogenesis through cytokine and growth factor secretion in glioblastomas.[155] Its overexpression was also shown to favor neoplastic transformation and upregulate cell proliferation.[156] Initially studied in neuroblastoma tissues with no evidence of somatic mutations, genomic DNA isolation by Soung et al. from normal and tumor tissues revealed silent mutations of caspase-9 splice sites in two colorectal carcinomas and one gastric carcinoma.[157,158] Park et al.[159] further looked into caspase-9 mutations in a case-control study in lung cancer patients and found that certain genotypes (−21263 GG) had significantly decreased risk of lung cancer. Soung et al.[160] also studied the caspase-3 somatic mutation in several cancers. Silent and missense mutations were found in stomach adenocarcinoma, lung cancer, colon cancer, hepatocellular carcinoma, and multiple myeloma. Chen et al. looked at caspase-3 mutations in squamous cell carcinoma of the head and neck (SCCHN), one of the most common cancers in the word, in a case-control study with 1863 patients (Chen et al.[161]). They found that the caspase-3 rs4647601:TT genotype was associated with an increased risk of SCCHN (Chen et al.[161]). Caspase-7 polymorphisms were seen in colon adenocarcinoma, oesophageal squamous cell carcinoma, and laryngeal squamous cell carcinoma.[162] The authors concluded that the alteration

TABLE 3 Known mutations of the caspase-dependent apoptotic pathways leading to cancer cell resistance

Gene	Mutations	Outcome	Cancers	References
The extrinsic pathway				
FasR/TNFRSF6	Deletion	Disruption of the Fas signaling death receptor pathway	Autoimmune lymphoproliferative syndrome	146,147
CD95	Somatic and geminal mutations; Posttranslational modifications			145
FADD	11q13.3 amplification	Inhibition of intracellular signal transmission	Lymphoepithelioma-like hepatocellular carcinoma	150,151
Procaspase-8	Q482H mutation	Inhibition of dimerization leading to diminished caspase-8 activation	Gastric cancers	144,152,153
	Deletion	Impaired activation of the extrinsic apoptotic pathways	Small cell lung carcinomas	
Caspase-8	Overexpression	Enhanced cell proliferation Angiogenesis	Glioblastomas	154–156
Procaspase-9	Silent mutations in splice sites	Impaired activity	Gastric cancers	157,158
	−21263 GG Genotype	Enhances cancer cell sensitivity to apoptosis	Lung cancer	159
Procaspase-3	Silent and missense mutations	Impaired activity	Stomach adenocarcinoma, lung cancer, colon cancer, hepatocellular carcinoma, and multiple myeloma	162
	rs4647601:TT genotype	Decreases cancer cell sensitivity to apoptosis	Squamous cell carcinoma of the head and neck	161
Procaspase-7	Nonsense mutation	Diminished caspase activity	Colon adenocarcinoma, esophageal squamous cell carcinoma, and laryngeal squamous cell carcinoma	162
DFF40	Hemizygous deletions of 1p36	Impaired DNA fragmentation	Human HCC cell lines	163
	Downregulation of protein expression	Impaired DNA fragmentation	Uterine leiomyosarcomas	165
c-FLIP	Deregulated protein expression	Enhanced inhibition of procaspase-8 activation	CCRF-HSB-2 leukemia cell line and the MCF-7 breast cancer cell line	170
XIAP	Deletion	Continuous activation of caspase-9	X-linked lymphoproliferative disease	171
The intrinsic pathway				
Bcl-2	Overexpression	Increased cell survival	Glioma, neuroblastoma, lung, colorectal, melanoma, and squamous carcinoma cancer cells	177–183
	Downregulation	Enhanced apoptosis	Uterine leiomyosarcomas	165,185
Bad	Active protein downregulation	Decreased activation of apoptosis	Grade II breast cancers	196

TABLE 3 (Continued)

Gene	Mutations	Outcome	Cancers	References
	Overexpression of inactive protein	Decreased activation of apoptosis	Triple-negative breast cancer, ovarian cancers	197,198
Bid	Frameshift and missense mutations	Inhibition of Bid activation	Gastric carcinomas	199,200
Bim	Deletions		Non-small-cell lung cancer	201
Bax	Downregulation	Decreased activation of apoptosis	Breast cancer cells, aggressive non-Hodgkin lymphoma, ovarian cancer, metastatic colorectal cancer, and pancreatic cancer	203–210
Bok	Downregulation	Decreased activation of apoptosis	Stage II and III colorectal cancers	211
The ER stress pathway				
GRP78/BiP	Overexpression	Adaptation to the ER pathway. Cancer cell survival.	Breast cancers, human pancreatic neuroendocrine tumors (PanNETs)	221,222
IRE1α	Somatic		Glioblastomas, lung carcinomas, and ovarian cancers	220,224
XBP1	Overexpression	Favors cell survival.	Human myelomas	228

of Arg-43 decreases the protease function of caspase-7 and the mutation leads to a failure to induce apoptosis.[162] Finally, defects in the expression of DFF are associated with several types of cancers.[84] Hemizygous deletions of chromosome 1p36 are often associated with some types of cancers.[163] DFF40 deficiency in Jurkat cells was shown to reduce cell sensibility to tributyltin-induced apoptosis.[164] DFF40 and DFF45 protein expressions were also shown to be downregulated in uterine leiomyosarcomas and linked with a poor prognosis.[165]

4.1.3 | Function deregulation of extrinsic apoptotic pathway regulators and cancer development

XAF1 plays an important role in cancer angiogenesis. High levels of XIAP to XAF1 expression in cancer cells may provide a survival advantage.[166] In addition, abnormally high expression of IAP proteins represents a frequent oncogenic event in human cancers.[167] Indeed, Jaiswal et al.[168] point out that survivin is strongly expressed in most cancers and is associated with poor clinical outcome. Deregulation of c-FLIP L expression was observed in tumors.[169] Increased expression of c-FLIP is implicated in resistance to CD95/Fas and TRAILR-induced apoptosis in various cancers.[170] A study was performed on 12 patients with the X-linked lymphoproliferative disease (XLP) a gene mutation encoding and leading to a BIRC4/XIAP deficiency.[171] Moreover, primary immunodeficiencies can be caused by XIAP deficiency due to the deletion of the BIRC4 gene.[172]

4.2 | Mutations in the intrinsic apoptotic pathway

Mutations in the Bcl-2 family proteins have been widely identified in numerous cancers. Amongst those proteins, the Bcl-2 protein is the most mutated protein in cancers. Bcl-2 is highly expressed in the majority of breast cancers as well as in other common tumors.[173] In vitro and in vivo studies have shown that overexpression of Bcl-2 in breast cancer cell lines increases metastasis to the lung in mice through the epithelial-to-mesenchymal transition,[174] promotes cancer cell growth by downregulating cell autophagy,[173] confers resistance to chemotherapy[175] and accelerates c-myc-induced tumorogenesis in transgenic mice.[176] Bcl-2 overexpression in other cancer cell types, such as glioma,[177,178] neuroblastoma,[179] lung,[180] colorectal,[181] melanoma,[182] and squamous carcinoma[183] cancer cells, has also been linked to cell migration, invasion, and metastasis.[184] Overexpression of Bcl-2 in

cancer cell types allows their survival, thus increases their malignancy. Surprisingly, Bcl-2 was shown to be downregulated in uterine leiomysarcomas[165,185] and negatively correlate with survival rates. Polymorphisms in the BCL2 gene may be associated with an increased risk for endometrial cancer.[186] Overexpression of the other antiapoptotic proteins (Mcl-1, BclxL, and Bcl-W) in cancer cell types, such as glioma, gastric, lung, and colorectal cancer cells, have also been shown to promote tumorigenesis and favor the cancer hallmarks.[181,187–194]

BH3-only proteins from the Bcl-2 family, primarily known as tumor suppressors, contribute to carcinogenesis through the regulation of their expression. First, in benign thyroid nodules, the gene expression level of Bad was significantly decreased, with a positive correlation according to nodule size.[195] Bad was shown to be underexpressed in the cytoplasm and nucleus of Grade II breast cancers.[196] Inactive phosphorylated Bad levels were shown to be increased in triple-negative breast cancer (TNBC) tumours[197] and in ovarian cancers,[198] which confers chemoresistance. Somatic mutations of Bad in colorectal cancers have also been reported and linked to cancer development.[199] On the other hand, a study performed on biopsies of cervix carcinoma from patients who received radiotherapy reveals that strong expression of Bid is associated with a poor outcome following treatment.[200] Moreover, analyses of 67 gastric carcinomas revealed a Bid mutation in 6% of cases, that consists of a frameshift and missense mutations.[199] Expression of the tumor-derived Bid mutants in HEK293T cells reveals a diminution in apoptosis frequency for the frameshift mutation.[199] Bim deletions have been detected in non-small-cell lung cancer positive for epidermal growth factor receptor (EGFR) and linked with shorter progression-free survival.[201]

Very few mutations in the mitochondrial proapoptotic proteins have been reported in the literature. A study performed by Kim et al.,[202] analyzing mutations in gastric ($n = 47$), colorectal ($n = 47$), breast ($n = 47$), lung ($n = 47$), and prostate ($n = 47$) carcinomas concluded that somatic mutations of BAX and BAK genes are rare in human cancers (besides those displaying a high microsatellite instability) and they would not be implicated in cancer tumorigenesis. Although, Bax has been shown to be underexpressed in breast cancer cells,[203–206] high-grade non-Hodgkin lymphoma,[207] ovarian cancer,[208] pancreatic cancer,[209] and metastatic colorectal cancer.[210] Bok protein levels have also been shown to be downregulated in stage II and III colorectal cancers.[211] As its fellow proapoptotic proteins, a downregulation in the expression of Bak in breast cancer tissues has been noted in the literature, and is linked with a reduced overall survival rate.[212]

4.3 | Expression modulation or mutations in DFF

The DFF has been linked multiple times to cancer malignancy throughout the past years. Here, we summarize the main mutations involved in cancer cell resistance to apoptosis. The decrease in expression of both subunits of DFF was shown to be associated with poor prognosis in patients. A decrease of DFF45 expression was associated with higher pathologic stage and metastasis.[213] Deletion of the 1p36.2-p36.3 locus in neuroblastoma causes a DFF45 mutation and correlates with unfavorable outcome.[214,215] NB-C201 cells bearing the 1p36.2 mutation are more resistant to cisplatin and DFF45 re-expression confers sensitivity to drug.[216] In melanoma cells resistant to etoposide and cisplatin, an absence of DNA fragmentation and PARP cleavage were associated with apoptosis deficiency.[217] Underexpression of DFF in uterine leiomyosarcomas (uLMS) influences disease-free survival.[165] Only the decrease of DFF40 and not DFF45 expression predicts disease-free and overall survival in non-endometroid and high-grade endometrioid endometrial cancers.[218] Later studies showed the role of DFF40 in tumor sensitivity to chemotherapeutic drugs. Interestingly, in uLMS, a decrease of DFF40 was associated with a lower expression of the antiapoptotic gene BCL-2. By targeting sequencing of chemoresistance genes, Hlavac identified DFFB genes modulation in neoadjuvant cytotoxic therapy.[219] Overall, direct modulation of DFF40 or indirect deregulation of functional DFF40 by DFF45 modulation affects cells' response in the presence of apoptosis-inducing drugs. Interestingly, results suggest a relation between the BCL-2 gene and the DFFB gene. In the study submitted, our lab has shown a direct connection between DFF40 expression and mitochondria, particularly some Bcl-2 members. Moreover, the loss of DFF40 correlated with particular sensitivity to some chemotherapeutic drugs.

4.4 | Mutations in the endoplasmic reticulum stress pathway

Cancer cells use intrinsic adaptive mechanisms such as the UPR pathway to adapt to an environment deprived of oxygen and nutrients, but also to meet increasing demands for the production of proteins and lipids necessary for rapid proliferation.[220] Their highly deprived microenvironment, and the presence of genomic instability, including multiple somatic mutations in protein secretion pathways, are sources of ER stress in cancer cells.[220] Several mutations of the ER stress pathway have been reported in the literature in cancer cells. Overexpression

of the glucose-regulated stress gene GRP78/BiP has been identified in solid tumor microenvironments in breast tissue cancers[221] and in human pancreatic neuroendocrine tumors (PanNETs).[222] These specific mutations could explain a more stable adaptation to the ER stress pathway, and allow the recurrence and survival of the cancer cells.[223] It should be noted that somatic mutations have rarely been found in IRE1α or PERK in cancers.[224] Mutations in the IRE1α gene have been reported in less than 1% of glioblastomas, lung carcinomas, and ovarian cancers.[224,225] However, Chen et al.[226] have shown that the homeostatic target XBP1 from IRE1α promotes tumor progression by controlling the HIF1α pathway in TNBC. Higher levels of cyclin A protein were observed by Thorpe and Schwarze[227] in cells with active IRE1α and were dependent on XBP1, enabling maintenance of cell division in cancer cells. Elevated levels of XBP1 were also found in 50% of human myelomas, suggesting a role of XBP1 in cell survival.[228] Ferrer et al. have demonstrated on breast cancer cells that O-GlcNAcylation regulates the metabolic reprogramming of cells as well as the signaling of survival stress via the regulation of HIF-1α.[229] Wang et al. revealed that XBP1s is an activator of the hexosamine biosynthesis pathway (BPH) and thus protects cells under stress.[230] Throughout these results, many have been wondering if the ER stress pathway promotes cancer cell survival. Oakes has addressed this matter in his review.[225] In vitro[231–234] and/or in vivo[235,236] studies have shown that all three pathways of the UPR are necessary for tumor growth and thus, cancer cell survival.

5 | ACTUAL TREATMENTS TO COUNTER APOPTOSIS RESISTANCE

In clinical oncology, the first-line treatments employ the use of chemotherapeutic drugs, often in combination with surgery and/or radiotherapy. As noted above, cancer cells are really well equipped with multiple mutations for chemoresistance. Morgan et al. have demonstrated in 2004 that the contribution of chemotherapy to 5-year survival rates was less than 5% in the United States.[7,237] There are a lot of factors to take into account when assessing the efficacy of a chemotherapeutic drug, such as factors related to the patient (e.g., age, medical history, and life habits) or to the cancer cell type (e.g., stage, grade, and location). These are among the reasons why there is an increasing interest in oncologists toward personalized medicine in patient management. In this section, we will review the main therapies that target the caspase-dependant apoptosis pathway. Although, it is important to note that there are many more targeted therapies involving different mutations that are not discussed in this review, such as cell cycle checkpoint[238] and immune system[239] regulators.

5.1 | Targeting the intrinsic apoptosis pathway

The treatment modalities involving the intrinsic caspase-dependant apoptosis pathway are made up of (i) dual Bcl-2 and Bcl-xL inhibitors, (ii) selective Bcl-2 inhibitors, (iii) Bcl-xL inhibitors, and (iv) Mcl-1 inhibitors, collectively referred to as BH3 mimetics, and (v) BAX agonists, some of which will be discussed in this review.[240] We will review major drugs approved by the FDA and their overall outcome on patients with chemoresistant tumors.

5.1.1 | Bcl-2 inhibitors

Bcl-2 inhibitors are one of the most effective treatments in cancer management. Having shown above that many tumors have a deregulation in the expression of Bcl-2, these inhibitors come in handy to treat those cancer cells, who will potentially be resistant to conventional chemotherapy. In 2016, the FDA approved the first-ever Bcl-2 inhibitor known as Venetoclax (ABT-199). ABT-199 is a BH3-mimetic and thus antagonizes the Bcl-2 anti-apoptotic protein.[241] It binds to the BH3 binding groove of the Bcl-2 protein, dislodging BH3-only proapoptotic proteins that are normally inactivated through the Bcl-2-BH3-groove interaction.[241] The released BH3-only proteins subsequently bind Bax and Bak and induce their activation/oligomerization which leads to MOMP. Although ABT-199 is known as a Bcl-2 inhibitor, it is important to mention that it also has an inhibitory action on Bcl-W.[241] In the preclinical trials, ABT-199 was shown to produce positive results when used to treat mouse xenografts injected with chronic lymphocytic leukemia (CLL) or non-Hodgkin lymphoma (NHL) cells.[242] A study conducted amongst patients with refractory CLL, treatment with ABT-199 induced a tumor response in 79% of patients and a complete response to treatment in 20% of patients.[243] These results brought scientists to recognize the importance of Bcl-2 inhibitors in the treatment of chemoresistant tumors. What if Bcl-2 inhibitors could be used as first-line treatments instead of treating only relapsed patients? Some tumors are known to be resistant to chemotherapy and have a higher relapse incidence rate (see Table 3 for tumors overexpressing Bcl-2). To respond to this major issue, recently, ABT-199, combined with an anti-CD20 antibody (Obinutuzumab), was approved for the frontline

treatment of patients with CLL, after it showed superior results compared to chemotherapy alternatives.[244] Bcl-2 inhibitors have also been effective in the treatment of myeloid malignancies, especially AML in elderly patients over 75 years old.[240] The FDA approved the treatment regimen, ABT-199, and 5-azacytidine or decitabine, for elderly patients with AML after Dinardo et al.255 showed in a phase I trial that a noteworthy complete response (CR) rate of 67.0% and a median overall survival (OS) of 17.5 months was achieved with this regiment.[245] Clinical oncology is evolving at a fast rate. Specialized treatments are becoming a lot more interesting, due to highly genetically diverse tumors and the ability to screen them.

5.1.2 | Dual Bcl-2 and Bcl-xL inhibitors

The ability to integrate the use of Bcl-2 inhibitors to treat hematological malignancies has led to further research and development of Bcl-2 selective and dual Bcl-2 and Bcl-xL inhibitors. Currently, there are experimental studies being conducted on dual inhibitors, but none have yet been approved by the FDA. One of the most studied dual inhibitors is Navitoclax (ABT-263). Its mechanism of action is similar to ABT-199, where it acts as a BH3-mimetic, allowing the release of BH3-only proteins and further MOMP through Bax/Bak activation.[241] In clinical trials, Robert and his colleagues examined the effect of ABT-263 in 118 patients with refractory CLL and recorded partial responses in 34.6% of patients in phase I trials.[246] Furthermore, Kipps et al.[247] combined ABT-263 with rituximab, a monoclonal antibody toward the protein CD-20 and a statistically significant overall response rate (ORR) of 70% were recorded in untreated patients with CLL compared to rituximab alone ($p = .003$).[247] Overall, these results are promising for the treatment of malignant tumors. Although, a major concern with this dual inhibitor is its side effect, where it induces thrombocytopenia in a dose-dependent manner, due to the role of Bcl-xL in platelet survival. There is also an additional concern regarding the development of cancer cell resistance to ABT-263, through the upregulation of Mcl-1 phosphorylation and further sequestration of Bim, a proapoptotic protein.[241] The clinical use of ABT-263 is thus limited and further studies are required before its approval.

5.1.3 | Bcl-xL, Mcl-1 inhibitors, and Bax agonists

Selective Bcl-xL inhibitors have shown antitumor activity in preclinical studies and are currently being assessed for their clinical use in phase I trials as monotherapy and combined therapy with taxanes in patients with refractory solid tumors and for patients with prostate cancer.[248] In vitro and in vivo studies have shown that selective Bcl-xL inhibitors, such as WEHI-539, mediate their proapoptotic actions by directly binding the hydrophobic groove of Bcl-xL,[249] thus allowing the release of BH3-only domain proapoptotic proteins and subsequent cancer cell death. Other studies have reported the importance of Bcl-xL regulators. Among those, there is tozasertib (VX-680), a selective small-molecular inhibitor of the Aurora kinases,[250] that has been undergoing clinical trials. VX-680 increases Bcl-xS fraction through Bcl-x splicing regulation by SRSF1 down-regulation, an antiapoptotic splice factor.[129,251] This regulation increases the sensitivity of cervical cancer cells to VX-680 induced apoptosis.

Another treatment modality that targets the intrinsic apoptosis pathway is Mcl-1 inhibitors, recently been approved for human trials, which could have clinical significance as Mcl-1 plays a role in the progression of several malignancies that have shown resistance to chemotherapy and dual Bcl-2/Bcl-xL inhibitors.[252] Mcl-1 inhibitors can briefly be classified into two distinct categories: (I) direct or (II) indirect Mcl-1 inhibitors. Some known indirect inhibitors of Mcl-1 are for example the cyclin-dependant kinase (CDK) inhibitors, mTOR inhibitors, and deubiquitinase inhibitors, which either block transcription, translation, or enhance degradation of Mcl-1 respectively.[135] Like other Bcl-2 protein inhibitors, direct and selective Mcl-1 inhibitors also mediate their action by binding the hydrophobic groove, thus allowing the BH3-only proteins to activate apoptosis.[135] Mcl-1 inhibitors are currently being tested in phase 1 or 2 clinical trials, and can either be used as a monotherapy or in combination with ABT-199.[135] A recent review from Wang et al.[135] lists the currently studied Mcl-1 inhibitors in clinical trials and the specific clinical application for each of them.

Finally, Bax agonist-induced apoptosis is another method of treatment via the intrinsic apoptosis pathway. Early studies looked at lung cancer in mice and observed apoptosis using BAX agonists, such as small molecule BAX agonist (SMBA)1-SMBA3.[253] SMBA's act by binding with high affinity the S184 pocket, where its phosphorylation enhances cell death by apoptosis.[253] Positive results with BAX agonists have led to further research and development of optimized compounds such as CYD-4-61 and GL0385, effective against breast cancer cell lines, and BAM-7 and BTSA1, effective in glioblastoma and AML cell lines.[240] Furthermore, Pirocanac et al.[254] used human telomerase reverse transcriptase (hTERT), an active enzyme in 85% of human cancers, to construct a

BAX gene expression inducer which led to apoptosis in human pancreatic cancer cells. Overall, currently, there are many ongoing experimental studies on specific inhibitors of the intrinsic apoptotic pathway. Developing such drugs can take several years. The major concern on developing specific inhibitors are the adverse effects. Every drug comes with adverse effects, but it is important to find a balance between the adverse effects and the clinical outcome to have the drug approved by the FDA.

5.2 | Targeting the extrinsic apoptosis pathway

The treatment modalities involving the extrinsic caspase-dependant apoptosis pathway are made up of death receptor agonists and activators, p53 pathway-related therapies, and other cell death mechanisms, such as activation of ATF4 and epigenetic mechanisms that favor intrinsic apoptosis.[240] The focus of treatment via death receptors have focused on DR4 and DR5 agonists as they have shown selective apoptosis of malignant cells without effects on surrounding nonmalignant cells unlike other agonists, such as CD95R/FasR, which was associated with severe liver toxicity, and TNFR, which was associated with inflammation.[255] Earlier studies extensively looked at dulanermin, a recombinant monoclonal antibody with anti DR4 and DR5 activity, as a monotherapy and as a combination therapy, but no clinical efficacy was noted, most likely secondary to its short half-life (30 min) and limited ability to induce receptor clustering.[256–261] To combat the short half-life and decreased receptor clustering ability of soluble agonists, DR4 and DR5 activating monoclonal antibodies (e.g., mapatumumab, lexatumumab, conatumumab, tigatuzumab, and drozitumab) were studied. Mapatumumab, as mono and combination therapy, was well tolerated but showed no clinical efficacy in both phase I and II trials in patients with non-small cell lung carcinoma (NSCLC), colorectal cancer, and other solid tumors, and was associated with grade 3–4 lymphopenia when compared to other arms in the study.[262–264] Lexatumumab, studied as mono and combination therapy, showed stable disease results in children with osteosarcoma and adults with sarcoma, but no statistically significant results were noted and dose-limited transaminitis limited the study.[265] Frequently inactivated in human cancers, the function of p53 has been the target of MDM2 and MDM4 inhibitors, which block the degradation of p53 without inducing DNA damage.[266] ATF4 activation, via ONC201, leading to CHOP-dependent DR5 upregulation and cell death, has shown to cause the death of tumor cells in both preclinical and clinical trials and remains under investigation as a therapeutic strategy.[267,268] Histone deacetylase (HDAC) or bromodomains and extra terminal domain (BET) protein inhibitors are epigenetic modulators that have shown anticancer activity via several mechanisms that lead to cell death.[240] BET inhibitors have shown positive activity in AML cell lines and patient-derived cutaneous T cell lymphoma cells via synergy when used in combination with Bcl-2 inhibitors, such as ABT-199.[269] HDAC inhibitors, which alter chromatin remodeling and induce apoptosis via downstream activation of the intrinsic pathway and subsequent cell death, have shown positive results in rhabdomyosarcoma cell lines when combined with ABT-199.[270–272] Positive results were also seen when HDAC inhibitors were used in combination with panobinostat and fimepinostat in diffuse large B cell lymphoma, multiple myeloma cell lines, and refractory lymphoma, respectfully.[273,274]

6 | CONCLUDING REMARKS

This review thoroughly describes the caspase-dependant apoptotic pathways and highlights the major mutations involved in cancer cell resistance to chemotherapy. Mutations in the apoptotic pathway are the main source of chemoresistance in cancer cells. The major advances in medicine led to the development of personalized medicine, that focuses on the treatment of each tumor according to its genetics. Experimental studies have thus led to the development of selective inhibitors of the Bcl-2 family of proteins, known to be the most abundant mutations in malignant tumors. But what about the other mutations? Every year, a significant number of researchers work on better understanding the impact of these mutations in cancer cell signaling, to develop potent therapies. It is important to have a solid knowledge of the fundamental molecular basics, to better understand the impact of these mutations. We thus gathered the main mutations identified in cancers until today, to open light on new studies.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Merve Kulbay and Adeline Paimboeuf performed the literature search and designed the figures. Merve Kulbay, Adeline Paimboeuf, and Derman Ozdemir wrote the

manuscript. Jacques Bernier guided during the writing process and revised the manuscript. All authors approved the final manuscript.

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