Centre Armand-Frappier Santé Biotechnologie

RAB7 PALMITOYLATION BY ZDHHC11 AND PHOSPHORYLATION BY NEK7 AS MODULATORS OF ENDO-LYSOSOMAL TRAFFICKING IN BATTEN DISEASE

By

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RÉSUMÉ

La lipofuscinose céroïde neuronale (NCL) est un groupe de maladies neurodégénératives héréditaires récessives chez l'enfant produite par une mutation des gènes NCL tels que CLN5. Notre laboratoire a montré que CLN5 participe à la modulation du trafic du réseau Golgi (TGN) endosome-trans en régulant les interactions entre Rab7 et Retromer. Rab7 module la récupération endosome vers TGN des récepteurs de tri lysosomal, du récepteur du mannose 6-phosphate (CI-MPR) et de la Sortilin via le recrutement de rétromères. La régulation de ces interactions effectrices est facilitée par des modifications post-traductionnelles (PTM), telles que la phosphorylation et la palmitoylation médiées par les palmitoyl transférases (PAT) et la phosphorylation médiée par les kinases.

Le cellulestoylation de Rab7 et le recrutement de retromer sont réduits dans les cellules CLN5KO Hela. En fait, les protéines NCL CLN3 et CLN5 sont également nécessaires au recrutement et au fonctionnement des rétromères. Nous proposons que CLN5 joue un rôle crucial dans le processus de palmitoylation de Rab7 en facilitant l'interaction Rab7/PAT. Pour tester cela, nous avons utilisé la complémentation de fluorescence biomoléculaire (BiFC) pour dépister les modifications des interactions Rab7-PAT. Parmi les 20 PAT testés, nous avons constaté que les interactions de ZDHHC11 étaient diminuées dans CLN5KO. Pour caractériser davantage ces PAT, nous avons utilisé siRNA et CRISPR-CAS9 pour épuiser DHHC8 et ZDHHC11 et avons trouvé significativement moins de Rab7 palmitoylé dans ces cellules par rapport au type sauvage. L'épuisement de ZDHHC11 a également entraîné una bimiution rétromère moins lié à la membrane. Nous avons également constaté que la phosphorylation de Rab7 par NEK7 est nécessaire pour une palmitoylation ultérieure et pour le recrutement de rétromères.

Mots-clés : Tri endolysosomal, Rab7, Maladie de Batten, Palmitoylation, Phosphorylation, Recrutement de Rétromères.

ABSTRACT

Neuronal Ceroid Lipofuscinosis (NCL) is a group of childhood recessive inherited neurodegenerative diseases produced by a mutation in the NCL genes such as CLN5. Our laboratory has shown that CLN5 modulates endosome-to-trans Golgi network (TGN) trafficking by regulating interactions between Rab7 and Retromer. Rab7 modulates endosomes to TGN retrieval of the lysosomal sorting receptors mannose 6-phosphate receptor (CI-MPR) and Sortilin through retromer recruitment. The regulation of these effector interactions is facilitated by post-translational modifications (PTM), such as phosphorylation and palmitoylation mediated by palmitoyl transferases (PATs) and Phosphorylation mediated by Kinases.

Rab7 Palmitoylation and the recruitment of Retromer are reduced in CLN5KO Hela cells. The NCL proteins CLN3 and CLN5 are likewise necessary for retromer recruitment and function; we propose that CLN5 plays a crucial function in the palmitoylation process of Rab7 by facilitating the Rab7/PAT interaction. To test this, we used biomolecular fluorescence complementation (BiFC) to screen changes in Rab7-PAT interactions. Among the 20 PATs tested, we found that the interactions of ZDHHC11 were diminished in $CLN5^{KO}$. To further characterize these PATs, we used siRNA and CRISPR-CAS9 to deplete DHHC8 and ZDHHC11 and found significantly less palmitoylated Rab7 in these cells compared to the wild type. Depletion of ZDHHC11 also resulted in a less membrane-bound retromer. We also discovered that Phosphorylation of Rab7 by NEK7 is required for posterior Palmitoylation and retromer recruitment.

Keywords: Endo-lysosomal Sorting, Rab7, Batten disease, Palmitoylation, Phosphorylation, Retromer recruitment.

SOMMAIRE RÉCAPITULATIF

INTRODUCTION

L'activité catalytique des endosomes et des lysosomes dépend de l'apport de protéines hydrolytiques solubles. Diriger ces protéines vers les lysosomes repose sur un mécanisme dépendant du signal dans lequel les hydrolases subissent des modifications avec différents motifs reconnus par le tri des protéines, comme c'est le cas avec le mannose 6-phosphate (Ludwig et al., 1993 ; Coutinho et al., 2012) reconnus par les récepteurs M6P (CMPR) au sein de l'appareil de Golgi, pour un transport ultérieur vers les endosomes (Yang & Wang, 2021). Cependant, toutes les enzymes solubles et protéines non enzymatiques n'adhèrent pas à cette voie ; certains suivent une trajectoire indépendante de M6P (Bonifacino & Traub, 2003 ; Braulke & Bonifacino, 2009). Des récepteurs alternatifs, comme LIMP-2 ou sortilin, guident le transport unique des enzymes hydrolytiques (par exemple, les cathepsines D et H), façonnant ainsi un mécanisme cellulaire distinctif. (Canuel et coll., 2008). Il a été démontré que la sortiline facilite le transport de nombreuses enzymes lysosomales résidentes vers le lysosome (Lefrancois et al., 2003 ; Morales, 2006) et joue un rôle dans le trafic de diverses autres protéines destinées à la dégradation lysosomale (Evans et al., 2011 ; Nielsen et al., 1999 ; Li, 2017).

Comme M6PR, les récepteurs sortilin au niveau des endosomes tardifs sont recyclés en TGN par trafic rétrograde pour un cycle de tri supplémentaire après que la cargaison soit déposée au niveau de l'endosome tardif (Cullen & Steinberg, 2018). Le trafic moléculaire dans les endosomes précoces, les endosomes tardifs et les lysosomes est modulé par la famille Rab des GTPases, qui sont des commutateurs moléculaires qui alternent d'un état inactif entre lié au GTP (état actif) et lié au GDP (états inactifs) pour activer ou désactiver fonctions biologiques (Villarroel-Campos et al., 2014 ; Rojas et al., 2008).

Rab7 contrôle la maturation des endosomes, la biogenèse des lysosomes, l'autophagie et la récupération du CI-MPR et de la Sortilin au niveau des endosomes tardifs (Rojas et al., 2008 ; Seaman et al., 2007). Rab7 nécessite différents effecteurs pour démarrer différentes fonctions (Modica & Lefrancois, 2020). Les interactions avec le complexe HOP facilitent la fusion endosome-lysosome (Nickerson et al., 2009 ; Peralta et al., 2010). L'interaction avec la protéine lysosomale interagissant avec Rab7 (RILP) module la morphologie des endo-lysosomes et leur mouvement à travers les microtubules (Jordens et al., 2001), et son interaction avec le rétromère régule le trafic endo-lysosomal (Rojas et al., 2008).

La régulation entre Rab7 et son effecteur est régulée par des modifications post-traductionnelles (PTM), telles que la phosphorylation et la palmitoylation (Modica & Lefrancois, 2020). La palmitoylation est une PTM réversible catalysée par un groupe d'enzymes appelées palmitoyl transférases (PAT) (Jin et al., 2021). Ces protéines ajoutent une modification lipidique, une chaîne d'acide gras attachée aux protéines à un résidu cystéine par une liaison thioester (Jiang et al., 2018 ; Stix et al., 2020) ; cette modification est inversée par les thioestérases (Zeidman et al., 2009). En d'autres termes, la palmitoylation ajoute un motif hydrophobe qui transfère la protéine entre l'espace cytoplasmique et les membranes biologiques (Jiang et al., 2008). La palmitoylation peut également moduler la localisation, la stabilité, les interactions protéine-protéine et l'activité enzymatique des protéines (Blaskovic, 2014).

La palmitoylation de Rab7 est requise pour le recrutement des rétromères (Modica et al., 2017). Retromer est un complexe protéique trimérique qui comprend Vps35, Vps29 et Vps26 ; cette association protéique joue un rôle dans la récupération endosome-Golgi des récepteurs CI-MPR et sortilin (Braulke et Bonifacino, 2009 ; Staudt et al., 2016). Rab7 recrute le rétromère sur la membrane lysosomale par interaction directe (Rojas et al., 2008 ; Seaman, 2007) ; une fois que le complexe se trouve sur la membrane tardive de l'endosome, le rétromère identifie et recycle le récepteur vers Golgi, et la cargaison est livrée au site luminal de l'endosome (Seaman, 2021). Le dysfonctionnement de Rab7 et de rétromère a été associé à plusieurs maladies neurodégénératives (ND), notamment PS, AD et NCL. Par conséquent, Rab7 et rétromère sont devenus des cibles thérapeutiques potentielles (Kiral et al., 2018).

La phosphorylation est une addition d'un groupe phosphate à la sérine ou à la tyrosine résiduelle d'une protéine qui peut activer ou désactiver les fonctions d'une protéine ; les kinases ajoutent ce PTM et pourraient être inversées par les phosphatases (Linder & Deschenes, 2007). Les interactions de phosphorylation et de palmitoylation sont associées au trafic cellulaire (Shetty et al., 2023), et l'activité et la fonction des enzymes palmitoylantes ou dépalmitoylantes sont régulées par la modification de la phosphorylation (Jin et al., 2021 ; Jansen & Beaumelle, 2022). L'interaction entre la palmitoylation et la phosphorylation a été rapportée dans la dynamique des neurones de la protéine Synapsin1, dans laquelle la phosphorylation module négativement la palmitoylation (Yan et al., 2022).

Rab7 peut être phosphorylé sur les sites sérine72 (S72) et tyrosine 183 (Y183) et peut être palmitoylé dans deux résidus cystéine, C83 et C84 (Modica & Lefrancois, 2020) ; ces modifications ne sont pas nécessaires pour la délimitation membranaire de Rab7 puisque les mutants sans table palmitoyle et sans table phosphoryle n'affectent pas la distribution

membranaire dans les endosomes (Modica et al., 2017 ; Modica et al., non publié). Cependant, la palmitoylation de Rab7 par Rab7 est nécessaire pour recruter efficacement Retromer (Modica et al., 2017). Nous avons constaté que chez le mutant s72 Rab7 non phosphorylable, les niveaux de palmitoylation diminuaient (Modica et al., non publié) et, par conséquent, le recrutement de rétromères était moindre.

Nous émettons l'hypothèse qu'une Kinase doit phosphoryler Rab7 en S72 pour permettre la palmitoylation et recruter un rétromère. La phosphorylation de Rab7,module différentes fonctions indépendamment du site phosphorylé et de la Kinase qui ajoute la PTM (Modica & Lefrancois, 2017). Par exemple, la phosphorylation de la sérine 72 par la kinase 1 répétée riche en leucine (LRRK1) facilite les interactions entre Rab7 et RILP, permettant le transport de l'EGFR vers la région périnucléaire, ce qui signifie que la phosphorylation de S72 par LRRK1 module le mouvement spécifique de la cargaison des endosomes. (Hanafusa et al., 2019). Au niveau du résidu tyrosine183, la kinase Src phosphoryle Rab7 a un effet opposé à la phosphorylation par LRRK1. La phosphorylation par Src induit l'EGFR et altère l'interaction avec RILP (Lin et al., 2017). TBK1 est une autre kinase responsable de la phosphorylation de Rab7 en S72, une modification cruciale pour l'initiation de la mitophagie. Cette kinase joue un rôle régulateur négatif en perturbant l'association avec l'inhibiteur de dissociation du RAB GDP tout en améliorant l'interaction avec l'hétérodimère FLCN-FNIP1 contenant le domaine DENN. En cas de dommages aux mitochondries, FLCN-FNIP1 est recruté et les cellules exprimant la table non-phosphoryle RAB7 présentent une mitophagie inefficace, altérant le recrutement de vésicules ATG9A-positives dans les mitochondries endommagées (Heo et al., 2018). Néanmoins, ni LRRK1 ni TBK1 ne sont impliqués dans le recrutement des rétromères par phosphorylation de s72 Rab7 (données non publiées).

Non seulement Rab7 est nécessaire au recrutement des rétromères, mais CLN5 et CLN3 sont également nécessaires au recrutement et à la fonction des rétromères. En effet, CLN3 interagit avec Rab7, et CLN5 module cette interaction (Yasa et al., 2020 ; Yasa et al., 2021). CLN5 et CLN3 font partie des 13 gènes mutés pour conduire au céroïde neuronal, une maladie cérébrale mortelle chez l'enfant (Butz et al., 2020). CLN3 est une protéine transmembranaire endo-lysosomale glycosylée avec six domaines transmembranaires avec des terminaisons N et C faisant face au cytosol (Oetien & Hermey, 2016 ; Mirza et al., 2019). CLN3 est requis pour l'interaction Rab7- rétromère et au sein de rétromère -Sortilin (Yasa et al., 2020) dans un complexe avec CLN5 pour moduler les fonctions de Rab7 ; dans les cellules CLN5ko, Rab7 n'est

pas capable de recruter le rétromère, dans les cellules CLN3KO le rétromère est recruté mais non activé (Yasa et al., 2021)

CLN5 est une protéine résidente endo-lysosomale dont la fonction reste insaisissable. Les premières études suggéraient que CLN5 était une protéine intégrale avec au moins un domaine transmembranaire qui est ensuite transformé en une protéine soluble avec plusieurs sites glycosylés (Basak et al., 2021). CLN5 a proposé des rôles dans le maintien d'un environnement acide approprié dans les lysosomes (Holopainen, 2001; Best et al., 2017), la respiration mitochondriale, la mitophagie (Best et al., 2017) et le métabolisme des lipides (Schmiedt et al., 2012). . Dans le trafic endosome vers TGN, CLN5 régule le processus en contrôlant la localisation du rétromère sur la membrane de l'endosome (Mamo et al., 2012 ; Basak et al., 2021). L'absence de CLN5 entraîne un phénotype semblable à celui observé dans les cellules présentant des complexes rétromères appauvris ou dépourvues de palmitoylation médiée par Rab7 ; la fraction de rétromère liée à la membrane est réduite (Modica et al., 2017).

Nous montrons que la phosphorylation de la sérine 72 de Rab7 est nécessaire à son interaction avec le rétromère. De plus, nous avons identifié NEK7 comme la kinase responsable de la phosphorylation de RAB7 dans ce processus. Nous avons également constaté que la palmitoylation de RAB7 est réduite dans les cellules $NEK7^{KO}$, ce qui suggère une interaction entre la phosphorylation et la palmitoylation dans le recrutement du rétromère ; nous identifions ZDHHC11 comme l'une des palmitoyl transférases qui palmitoylent Rab7 pour recruter un rétromère dans la membrane. Puisque CLN5 est requis pour la phosphorylation de Rab7 (Yasa et al., 2021), nous nous sommes demandés si CLN5 pourrait également influencer la phosphorylation de Rab7 s72 ; nous n'avons trouvé aucun changement dans les cellules $CLN5^{KO}$ lors de la phosphorylation de s72 Rab7 suggérant la modulation directe de la palmitoylation après la phosphorylation. De plus, nous avons identifié les Palmitoyl transférases interagissant avec CLN3 comme premier indice du PAT possible pour la palmitoylation de CLN3

MÉTHODES

- **Culture cellulaire et transfections**

Les cellules Hela et HEK ont été cultivées dans du milieu Eagle modifié par Dulbecco (DMEM) additionné de 10 % de FBS (Wisent et al.) à 37 °C dans une chambre humidifiée à 95 % d'air et 5 % de CO2. Les cellules ont été placées dans des plaques à 12 puits avec une densité d'ensemencement de 2 × 10 ^ 5 cellules par puits et dans des plaques à 6 puits avec une densité d'ensemencement de 5 × 10 ^ 5 cellules par puits 24 heures avant la procédure de transfection. Les transfections ont été réalisées avec de la polyéthylèneimine (PEI). La solution 1 a été préparée en diluant le plasmide dans Opti-MEM (ThermoFisher Scientific). La solution 2 a été préparée en diluant du PEI (1 µg/µl) dans Opti-MEM dans un rapport de 1:4 avec l'ADN à transfecter. Après une incubation de 5 minutes, les deux solutions ont été mélangées, vortexées pendant 3 secondes, incubées à température ambiante (TA) pendant 15 minutes et ajoutées aux cellules. Des expériences ultérieures ont été réalisées 48 heures après la transfection.

- **CRISPR/CAS9**

Nous avons utilisé la technologie CRISPR/Cas9 pour concevoir des cellules HEK ou HeLa knock-out. Les lignes déjà générées incluent les cellules NEK7 knock-out ($NEK7^{KO}$), $DHHC8^{KO}$ et $ZDHHC11^{KO}$. En bref, les cellules ont été transfectées avec un plasmide tout-en-un contenant Cas9, l'ARN guide et un marqueur de section fluorescent. Quarante-huit heures après la transfection, les cellules ont été triées en fonction de l'expression de la fluorescence (une cellule par puits) et laissées se développer. L'efficacité du KO a été testée par Western blot et confirmée par séquençage. $CLN5^{KO}$ et $Rab7^{KO}$ ont déjà été décrits (Yasa et al., 2021 ; Yasa et al., 2020).

- **Génération de lignées cellulaires stables**

Pour les expériences de sauvetage, les cellules HEK293 $NEK7^{KO}$ ont été transfectées avec 500 ng de plasmide HA-NEK7 et de plasmide HA-$NEK7^{K64M}$ en utilisant du PEI à une concentration de 1:4. Quarante-huit heures après la transfection ; les cellules ont été exposées à de la gentamicine (G418) à une concentration de 500 ng/ml dans du DMEM 10 % SBF. Les cellules non transfectées ont été utilisées comme point de référence pour mesurer la dose mortelle. Après la mort des cellules traitées qui n'ont pas été transfectées positivement, la cellule a été maintenue dans la culture à une concentration égale à la moitié de la dose létale (250 ng/ml). Ces lignées cellulaires stables ont été utilisées dans les tests sur la cathepsine L et sur la membrane de séparation, car un pourcentage élevé de cellules transfectées était nécessaire pour sauver les phénotypes.

- **Complémentation bimoléculaire de fluorescence (BiFC).**

Les cellules HEK ont été ensemencées dans des plaques à 12 puits et co-transfectées avec les plasmides VN-Rab7 et CC-DHHC et, de manière alternative, CC-Rab7 avec les VN-DHHC. Quarante-huit heures après la transfection, les cellules ont été lavées dans du PBS, détachées avec de l'EDTA 5 mM dans du PBS et collectées dans 500 ml de PBS. La quantité de cellules a été ajustée à l'aide d'un spectrophotomètre ; les cellules ont été transférées sur des plaques noires à 96 puits en triple et la fluorescence totale a d'abord été mesurée avec le lecteur de plaques Tecan Infinite M1000 Pro avec l'excitation et l'émission réglées à 510 nm et 530 nm, respectivement. Des cellules transfectées avec du sperme de saumon ont été utilisées pour contrôler le fond de fluorescence. La fluorescence finale a été calculée en soustrayant le fond cellulaire de la fluorescence initiale.

- **BRET**

Les cellules HEK ont été ensemencées dans des plaques à 12 puits et co-transfectées avec les plasmides Rluc-Rab7, VPS26-Venus et Rluc-Rab7 avec Sortilin-YFP. Quarante-huit heures après la transfection, les cellules ont été lavées dans du PBS, détachées avec de l'EDTA 5 mM dans du PBS et collectées dans 500 ml de PBS. Les cellules ont été transférées sur des plaques blanches à 96 puits en triple. La fluorescence totale a d'abord été mesurée avec le lecteur de plaques Tecan Infinite M1000 Pro avec l'excitation et l'émission réglées à 500 et 530 nm pour BRET1. Le substrat h-coelentérazine a été ajouté à tous les puits (concentration finale de 5 mM) et les signaux de luminescence ont été mesurés 2 minutes plus tard. Les signaux BRET ont été calculés comme le rapport de la lumière émise à 525 ± 15 nm sur la lumière émise à 410 ± 40 nm. Les signaux BRETnet ont été calculés comme la différence entre le signal BRET dans les cellules exprimant à la fois les constructions de fluorescence et de luminescence et le signal BRET des cellules où seule la construction fusionnée par luminescence était exprimée, comme expliqué dans Yasa et al. (2021).

- **Western blot**

Pour l'analyse par Western blot, les cellules ont d'abord été détachées à l'aide d'une solution contenant 5 mM d'EDTA dans du PBS, suivie d'une augmentation de PBS 1 × et d'une collecte ultérieure par centrifugation. Pour lyser les cellules, un tampon TNE (composé de NaCl 150 mM, Tris 50 mM à pH 7, 5, EDTA 2 mM, Triton X-100 à 0, 5% et un cocktail d'inhibiteurs de protéase) a été utilisé, avec incubation sur glace pendant 30 minutes. Ensuite, les lysats ont subi une

centrifugation à grande vitesse pendant 10 minutes et les surnageants résultants (comprenant le lysat de cellules entières) ont été préparés pour une analyse par Western blot.

Les échantillons préparés ont été combinés avec un tampon d'échantillon 3 × pour atteindre une concentration finale de 1 × (contenant 62, 5 mM de Tris – HCl à pH 6, 5, 2, 5% de SDS, 10% de glycérol et 0, 01% de bleu de bromophénol). Ces échantillons ont ensuite été chauffés à 95 ° C pendant 5 minutes et soumis à une séparation par SDS – PAGE, suivie d'un transfert humide sur des membranes de nitrocellulose. La détection ultérieure a été réalisée par immunoblot en utilisant les anticorps spécifiés. La quantification a été réalisée à l'aide d'ImageJ.

- **Essai de séparation membranaire**

Après une transfection de 24 heures dans 6 plaques saines, les cellules ont été récoltées en utilisant une solution contenant 5 mM d'EDTA dans du PBS. Ces cellules ont été rapidement congelées par immersion dans de l'azote liquide et décongelées à température ambiante pendant 5 minutes. Les cellules ont été remises en suspension dans le tampon 1 (Mes-NaOH 0,1 M à pH 6,5, MgAc 1 mM, EGTA 0,5 mM, orthovanadate de sodium 200 µM et saccharose 0,2 M), suivies d'une centrifugation à 10 000 g pendant 5 minutes à 4 ° C. Le surnageant résultant contenant les protéines cytosoliques (S, fraction soluble) a été collecté.

Le culot restant a été remis en suspension dans le tampon 2 (Tris 50 mM, NaCl 150 mM, EDTA 1 mM, 0,1 % de SDS et 1 % de Triton X-100) et centrifugé à 10 000 g pendant 5 minutes à 4°C. Des volumes égaux d'échantillons ont été chargés dans des gels SDS – PAGE. L'intensité des bandes a été quantifiée à l'aide du logiciel ImageJ. Pour déterminer la distribution des protéines, l'intensité de chaque fraction a été calculée et divisée par l'intensité totale.

- **Activité lysosomale, cathepsine B et L :**

Pour évaluer l'activité des cathepsines D et L, les cellules ont été cultivées dans 12 plaques saines puis collectées à une densité de 3 x 106 cellules/ml. Les cellules ont ensuite été transférées en triple sur une plaque noire à 96 puits. Le réactif Magic Red a été ajouté au PBS pendant 60 min à 37 ° C et protégé de la lumière. L'intensité de la fluorescence a été mesurée avec un lecteur de plaques Tecan Infinite M1000 Pro (Tecan et al., Suisse) avec l'excitation et l'émission réglées à 592 nm et 628 nm, respectivement.

- **Acyl-rac (capture assistée par résine)**

Les cellules ont été lysées avec un tampon RIPA pendant 30 minutes à quatre ° C additionné de N-éthylamine (NEM) 50 nM pour bloquer les groupes thiol libres. Les échantillons ont ensuite été

centrifugés pendant 10 minutes à 13 000 tr/min à 4°C. Les surnageants ont été incubés pendant deux heures à température ambiante sur une roue rotative. Les échantillons ont été précipités pendant une nuit avec deux volumes d'acétone froide à -20 °C. Le lendemain, les échantillons ont été lavés à l'acétone froide et centrifugés. Le culot a été séché à l'air et remis en suspension dans un tampon de liaison (BB) (HEPES 100 mM, EDTA 1 mM, SDS 1%) avec 250 mM d'hydroxylamine (Sigma – Aldrich) (NH2OH) pH 7,5 pour couper la liaison thioester entre le groupe palmitoyle. et les résidus de cystéine modifiés. Les échantillons témoins ont été remis en suspension dans du BB contenant 250 mM de NaCl mais pas d'hydroxylamine et incubés pendant 2 heures, en rotation à température ambiante jusqu'à ce que les pastilles soient complètement diluées. 50 µl ont été enregistrés en entrée. Des billes de thiopropylsépharose ont été ajoutées et les échantillons ont été incubés pendant deux heures à température ambiante sur une roue rotative. Les billes ont été lavées quatre fois avec BB et les protéines capturées ont été éluées avec un tampon d'échantillon contenant 100 mM de DTT

RÉSULTATS

Des travaux antérieurs dans notre laboratoire suggèrent que la phosphorylation de Serine72 est nécessaire pour le recrutement du rétromère Rab7 ; l'implication des kinases en interaction précédemment connues au niveau de S72 LRRK1 et TBK1 a été testée par des inhibiteurs chimiques, siARN et CRISPR-cas9 sans aucun effet sur la distribution membranaire des rétromères (données non publiées Modica et al., 2024). Nous émettons l'hypothèse que cette voie est contrôlée par une kinase qui n'a pas été décrite auparavant comme une protéine interagissant avec RAB7. Dans cette étude, nous évaluons NEK7, une kinase mitotiquement active, en tant que candidat potentiel responsable de la phosphorylation de Rab7 et de la facilitation du recrutement ultérieur de protéines rétromères. De plus, des recherches antérieures ont fait allusion à l'implication de la kinase mitotique CDK1 dans la phosphorylation de Rab, illustrée par les premières découvertes de la phosphorylation de Rab1 (Bailly et al., 1991).

NEK7 est une protéine sérine/thréonine kinase membre de la famille des kinases liées au NIMA (jamais en mitose) caractérisée par un manque de domaine C-terminal (NEK) (Fry et al., 2012). La structure protéique est limitée à un domaine kinase avec un bref N-terminal, ce qui pourrait avoir une importance dans la reconnaissance du substrat, comme dans la structure protéique NEK6, qui est similaire à 87 pour cent. Néanmoins, les deux protéines ne partagent pas le même Interactiveome (Vaz et al., 2010 ; De Souza et al., 2014). NEK7 implique différentes fonctions cellulaires en fonction de son activité protéique en interaction et dépendante ou indépendante de la kinase. La plupart de la littérature sur NEK7 tourne autour du fait que cette protéine est impliquée dans l'assemblage et l'activation de l'inflammasome NLRP3 lors d'événements mitotiques. NLRP3 fonctionne comme un capteur d'inflammasome qui interagit directement avec NEK7 pour déclencher une réponse inflammatoire à des stimuli tels que les ROS, l'efflux de potassium, la déstabilisation lysosomale, la signalisation NF-κB, l'agrégation incorrecte des protéines et les substances toxiques (Xu et al., 2016 ; Sharif et al. ., 2019 ; Anderson et al., 2023), cette voie ne nécessite pas d'activité catalytique NEK7. Cependant, la régulation de l'instabilité dynamique des microtubules et la facilitation de l'assemblage du fuseau nécessitent l'activité catalytique de NEK7, notamment en interphase (He et al., 2016). NEK7, dans le rôle mitotique, interagit avec NEK9, en compétition et en régulant les événements de mitose et d'inflammation ; lorsque NEK7 interagit avec NLRP3, il ne peut plus interagir avec NEK9 pour la mitose (Shi et al., 2016 ; Sharif et al., 2019).

Zhang et coll. (2023) ont révélé le lien complexe entre le trafic endocytaire impliquant l'interacteur NEK7 NLRP3. Leur étude a démontré que la déstabilisation des sites de liaison entre le réticulum

endoplasmique et la membrane de l'endosome entraîne une accumulation de phosphatidylinositol 4-phosphate (PI4P) dans les endosomes. Cette accumulation conduit ensuite à la rétention du transport rétrograde du réseau trans-Golgi (TGN) CI-MPR et de la cargaison TGN46, la majeure partie de la protéine étant située au niveau des endosomes, facilitant finalement le recrutement de NLRP3 dans les endosomes. Cependant, dans cette étude, on ne sait pas clairement comment NLRP3 perturbe le trafic, et NEK7 n'a aucun lien avec le processus.

En général, il a été démontré que les kinases liées à NIMA pourraient jouer un rôle essentiel dans le contrôle du trafic intracellulaire, l'inactivation de l'orthologue NEKl-3 C. Les protéines élégantes de NEK7 génèrent des défauts d'endocytose que NEK7 et NEK6 peuvent sauver chez les vers mutants NIMA, et ces fonctions sont conservées entre espèces puisque la protéine humaine est capable de sauver le phénotype (Joseph et al., 2020). Une étude récente montre une colocalisation de plus de 50 pour cent entre NELKL-3, Rab7 et Rab5, ce qui conduit à l'association du NEK7 humain avec des structures endosomales. D'autres protéines NEKL homologues à NEK8 ont une colocalisation principalement au niveau des endosomes précoces, et sa déplétion génère une augmentation de la taille des endosomes précoces mais pas des endosomes tardifs, ce qui suggère que différentes protéines NEK pourraient être impliquées dans différentes étapes du trafic endocytaire. De plus, il n'y a pas de colocalisation entre NEKL-2 et NEKL-3, ce qui suggère des fonctions différentes, contrairement à l'épuisement de NEK-2, l'épuisement de NEKL-3 a conduit à des défauts substantiels au niveau des endosomes précoces et tardifs (Joseph et al., 2023).

Joseph et al., 2023 ont découvert que les changements dans la voie rétrograde des endosomes vers le réseau Trans-Golgi semblent être affectés par la destruction de NEK6 et NEK7, entraînant un mauvais tri et une dégradation du CI-MPR des endosomes, ce qui peut être attribué à un problème lié au rétromère (Arighi et al., 2004) mais la relation de NEK7 avec le rétromère n'a pas été prouvée dans la littérature. L'épuisement de NEK7 augmente également la taille et le nombre d'endosomes, affectant éventuellement les événements de fusion/fission. Cependant, cela n'a pas été testé directement (Joseph et al., 2023).

Compte tenu de ces informations, nous avons décidé de générer des lignées cellulaires NEK7KO à l'aide de la technologie CRISPR-Cas9, de tester si NEK7 phosphoryle Rab7 à S72 et d'évaluer son rôle dans le recrutement des rétromères. Quarante-deux cellules monoclones ont été évaluées à l'aide d'un anticorps spécifique de NEK7 (Figure 5.1A).

1. NEK7 phosphoryle Rab7 en S72.

Nous avons testé si NEK7 était nécessaire à la phosphorylation de Rab7 en utilisant un anticorps produit pour détecter Rab7 phosphorylé au niveau de la sérine 72. En utilisant cet anticorps, nous avons constaté une diminution de Rab7 phosphorylé dans les cellules NEK7^{KO} (Figure 5.2), qui a été sauvée en exprimant NEK7 de type sauvage (HA-NEK7), mais pas en exprimant le mutant kinase mort (HA-NEK7^{K64M}). Le signal de Rab7 phosphorylé ne disparaît pas entièrement, probablement à cause des autres kinases qui modulent Rab7 au niveau de la sérine72. Rab7 en tant que cible pour la phosphorylation de NEK7 n'a pas été signalé auparavant.

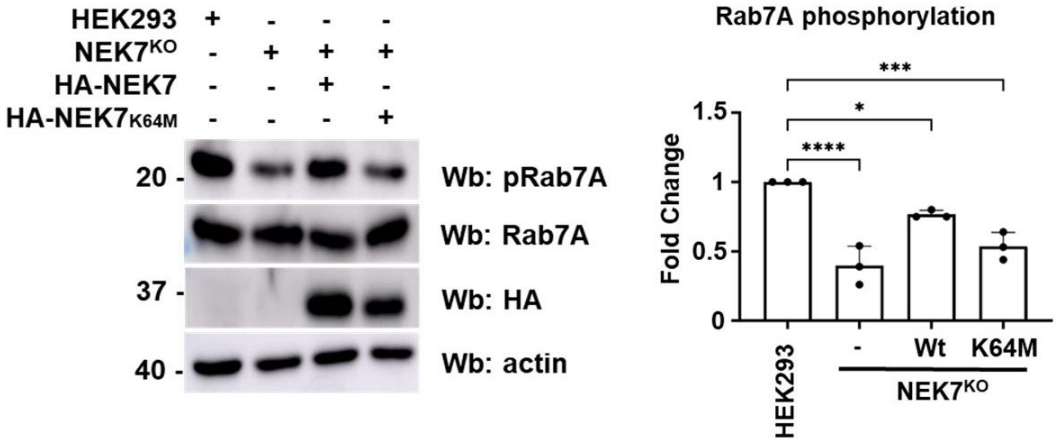

Figure 5.2. Niveaux de phosphorylation de Rab7 à Serine72 : Figure 5.2 Niveaux de phosphorylation de Rab7 à Serine72

Les lysats cellulaires dérivés de cellules HEK293, NEK7^{KO} et NEK7^{KO} exprimant soit HA-NEK7 de type sauvage, soit le mutant HA-NEK7^{K64M} mort par kinase ont été analysés par Western blot (Wb) en utilisant des anticorps spécifiques contre Rab7A phosphorylé au niveau de la sérine 72 (pRab7A), total Rab7A, HA-tag (pour détecter les variantes HA-NEK7) et actine (utilisée comme contrôle de chargement). La phosphorylation de la sérine 72 de Rab7A a été quantifiée dans trois expériences indépendantes et est présentée sous forme de moyenne ± écart type (SD). La signification statistique a été déterminée à l'aide d'une ANOVA unidirectionnelle avec le test post-hoc de Tukey, où les niveaux de signification sont indiqués par * ($P < 0,05$), *** ($P < 0,001$) et **** ($P < 0,0001$). Niveaux de phosphorylation de Rab7 chez Serine72 avec une diminution de la phosphorylation dans NEK7ko par rapport à WT HEK, le phénotype a été sauvé par transfection transitoire de Ha-NEK7 dans des cellules NEKKO. En revanche, le mutant de mort de la kinase Ha-NEK7K64M ne sauve pas le phénotype.

2. Phosphorylation of s72 in Rab7 is required to recruit Retromer.

To confirm that NEK7 regulates endosome-to-TGN retrieval through retromer, we evaluated the membrane distribution of retromer in NEK7^{KO} cells. We found a 20 percent increase in the retromer subunit VPS26 in the cytosolic fraction in NEK7^{KO} cells compared to WT cells (Figure

5.3 A, B). This phenotype was in the stable cell line expressing HA-NEK7, while the K64M kinase death did not rescue the membrane distribution of retromer (Figure 5.3 C, D).

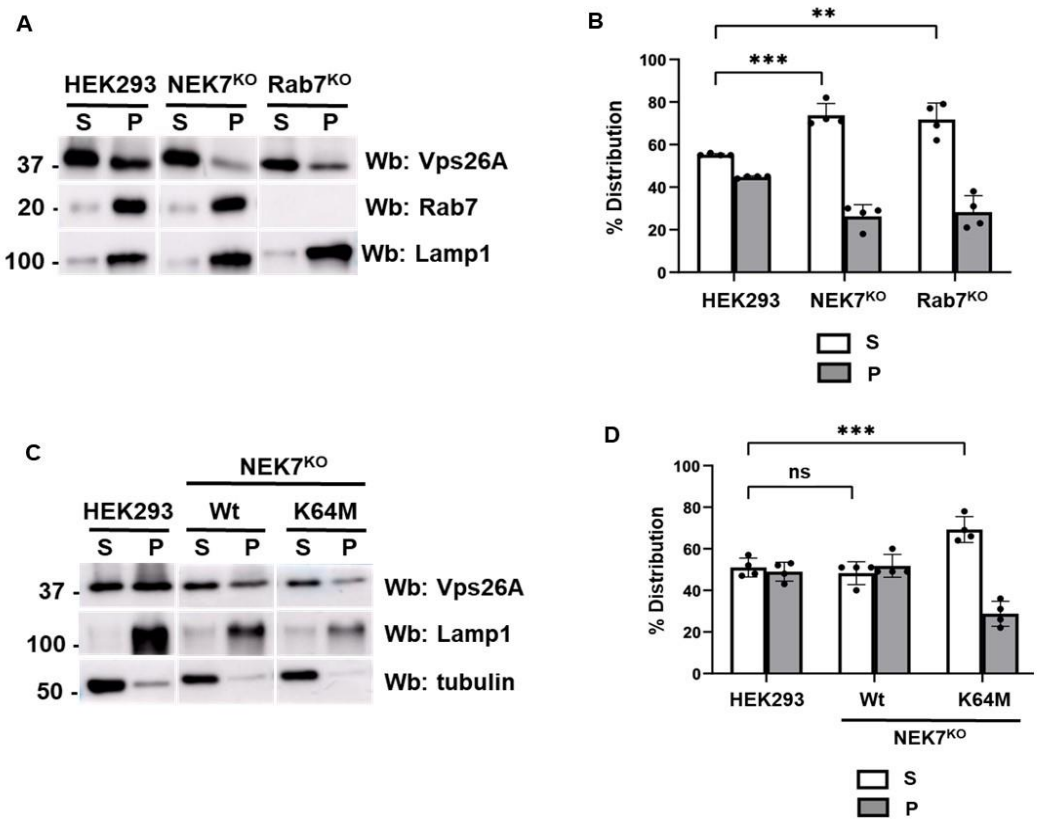

Figure 5.3. Distribution des sous-unités VPS26 Retromer au cytosol avec Rab7 dans les cellules NEK7ko et sauvetages avec des lignées cellulaires stables. Distribution des sous-unités VPS26 Retromer entre l'espace cytosolique et la protéine liée à la membrane, coloration pour le contrôle membranaire LAMP1 et tubuline pour la fraction cytosolique. (A, B) Une analyse quantitative basée sur trois expériences indépendantes montre que les cellules NEK7^{KO} HEK293 présentaient des niveaux réduits de rétromères liés à la membrane (26,25 % dans la fraction de pellets) par rapport aux cellules HEK293 de type sauvage (44,75 % dans la fraction de pellets). Les cellules NEK7^{KO} présentaient des niveaux comparables de rétromère lié à la membrane aux cellules Rab7^{KO} HEK293 (28,25 % dans la fraction des pellets), (C, D) Analyse quantitative basée sur trois expériences indépendantes. Les cellules NEK7^{KO} HEK293 exprimant HA-NEK7 ont présenté une distribution de rétromères comparable à celle des cellules HEK293 de type sauvage (51,64 % dans la fraction de culots). En revanche, les cellules NEK7^{KO} HEK293 exprimant HA-NEK7^{K64M} n'ont montré aucune amélioration significative de la distribution des rétromères (30,32 % dans la fraction de pellets). Analyse des données ANOVA à deux voies avec le test post-hoc de Dunnett, où les niveaux de signification sont indiqués par * (P < 0,05), *** (P < 0,001) et **** (P < 0,0001).

Nous avons également évalué les interactions entre Rab7 et VPS26 par BRET pour confirmer ces données. Cette technique nous permet d'évaluer les interactions protéine-protéine (IPP) dans les cellules vivantes sans détruire les membranes où se produisent certaines interactions, contrairement à l'immunoprécipitation et à d'autres méthodes pull-down où des lysats cellulaires

sont nécessaires. BRET, une méthode ancrée dans le transfert d'énergie par résonance (RET), implique l'interaction des spectres d'émission et d'excitation entre un chromophore donneur, comme la luciférase Renilla (Rluc), et un chromophore accepteur, généralement la GFP. Ce transfert d'énergie non radioactive résulte du chevauchement spectral, renforçant l'émission de l'accepteur (Sauvage et al., 2019). Les chromophores sont attachés à deux protéines exprimées dans des cellules vivantes et l'apparition de RET est interprétée comme une interaction directe entre les protéines. Nous avons trouvé un BRET50 inférieur dans les cellules NEK7^{KO} (Figure 5.4 A, B), ce qui signifie une interaction plus faible ; nous évaluons les interactions en aval entre VPS26 et Sortilin, montrant une interaction plus faible (Savageau et al., non publié). Ces données suggèrent que NEK7 module le recrutement des rétromères par la phosphorylation de Rab7 ; cependant, sur la base des résultats de Joseph et al. (2023), NEK7 peut moduler d'autres interactions Rab7 liées aux événements de fusion et de fission ; cette possibilité sera évaluée dans des expériences futures.

3. NEK7 est requis pour la palmitoylation de Rab7

Étant donné que la palmitoylation de Rab7 est requise pour le recrutement du rétromère (Modica et al., 2017), nous nous demandons si la palmitoylation de Rab7 pourrait être affectée par l'épuisement de NEK7 ; en utilisant acyl-rac, nous avons trouvé une diminution de la palmitoylation de Rab7 dans les cellules NEK7KO, un phénotype qui a été sauvé par la transfection transitoire de HA-NEK7 mais pas par le mutant mortel de la kinase (Figure 5.5), ces données montrent une interaction entre la phosphorylation à Serine72 et Palmitoylation Rab7.

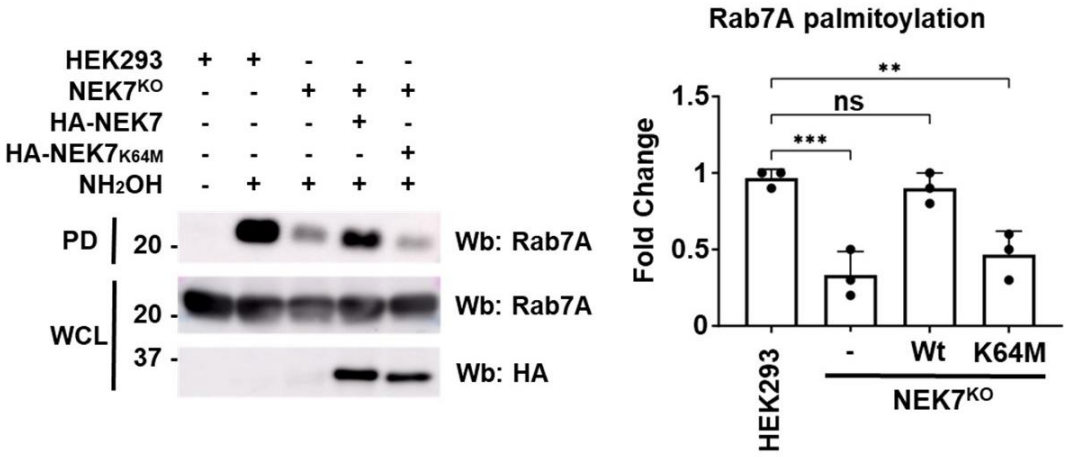

Figure 5.5. Palmitoylation Rab7 dans les cellules NEK7KO par rapport aux cellules HEK WT Les lysats de cellules HEK293, ainsi que les cellules NEK7^{KO} et NEK7^{KO} exprimant soit HA-NEK7 de type sauvage, soit

le mutant HA-NEK7^{K64M} mort par kinase, ont été soumis à Acyl-RAC pour évaluer les niveaux de palmitoylation de Rab7A. La procédure impliquait un traitement au NH2OH, une analyse pulldown (PD) et des lysats de cellules entières (WCL). Une analyse quantitative basée sur trois tests Acyl-RAC indépendants a été réalisée pour déterminer les niveaux de palmitoylation de Rab7A. Les données sont exprimées en moyenne ± écart type (SD). La signification statistique a été déterminée à l'aide d'une ANOVA unidirectionnelle avec le test post-hoc de Tukey, où les niveaux de signification sont notés NS (non significatif), ** ($P < 0,01$) et *** ($P < 0,001$).

4. ZDHHC11 Palmitoylates Rab7

Des recherches antérieures dans notre laboratoire ont montré que Rab7 doit être palmitoylé pour recruter un rétromère (Modica et al., 2017). Dans les cellules HeLa CLN5 knock-out (CLN5KO), Rab7 est moins palmitoylée que les cellules de type sauvage (Yasa et al., 2021). Ces résultats nous ont amenés à proposer que CLN5 joue un rôle crucial dans le processus de palmitoylation de Rab7 et médiatise l'interaction Rab7/PAT. En utilisant cela, nous avons expliqué que CLN5 pourrait jouer un rôle dans la palmitoylation de Rab7 en méditant l'interaction Rab7-PAT.

Pour comprendre la dynamique de la palmytoilation et de la phosphorylation de Rab7 et leurs implications dans la lipofuscinose céroïde neuronale, nous devons identifier le PAT qui palmitoyle Rab7. Notre première approche a évalué les interactions possibles entre les cellules WT et les cellules CLN5KO puisque la palmitoylation de Rab7 est réduite dans CLN5ko, entraînant une réduction du recrutement des rétromères dans la membrane de l'endosome (Yasa et al., 2021). Comme approche initiale pour tester quel PAT palmitoyle Rab7, nous avons utilisé la complémentation par fluorescence biomoléculaire (BiFC) pour dépister les modifications dans les interactions Rab7-PAT.

BiFC est une technique utilisée pour identifier les protéines qui interagissent dans les cellules vivantes. Elle consiste à compléter deux moitiés d'une protéine fluorescente attachées à deux protéines d'intérêt. Lorsque les deux protéines en interaction sont suffisamment proches pour interagir, les moitiés des protéines fluorescentes se réunissent et émettent un signal de fluorescence. Parmi les 20 PAT testés, nous avons observé de fortes interactions entre Rab7 et DHHC2, DHHC6, DHHC8 et ZDHHC11. Les interactions de ZDHHC11 ont été diminuées dans CLN5KO (Figure 5.8 A).

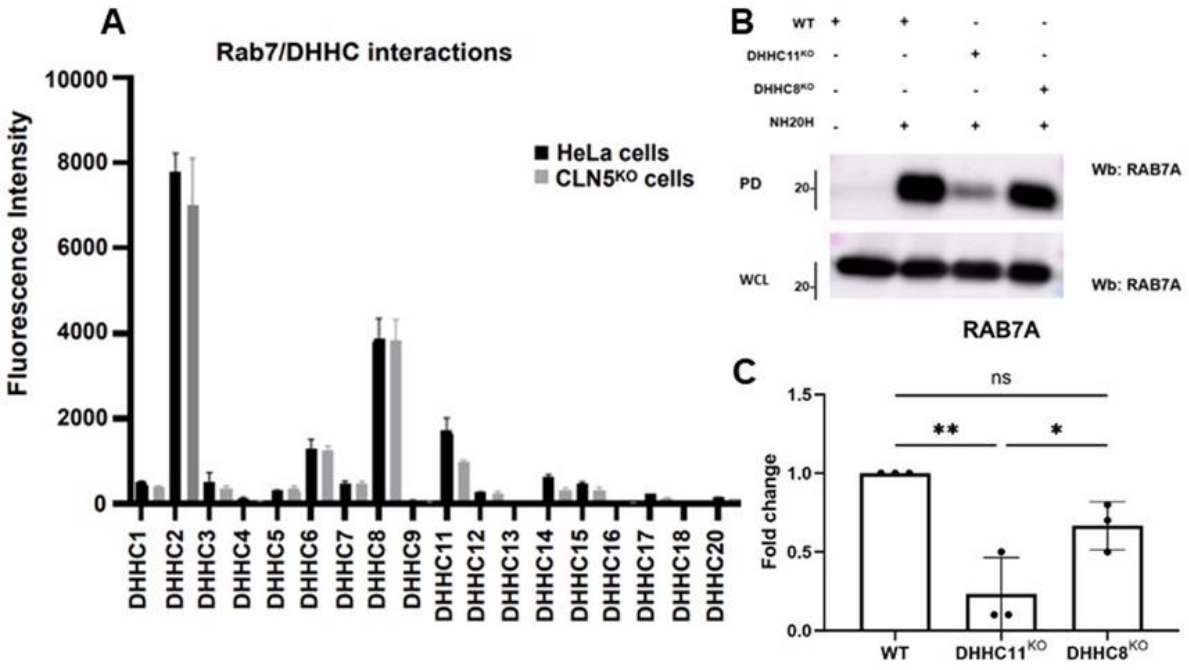

Figure 5.8 Interactions de Rab7 avec les protéines DHHC et Acyl Rac pour Rab7 dans les cellules DHHC11^{KO} (A) Criblage du signal de fluorescence des interactions entre Rab7 et différents PATS dans les cellules WT par rapport aux cellules CLN5KO, (B, C) Les cellules WT, ZDHHC11KO et ZDHHC8KO ont été soumises à Acyl-RAC pour évaluer les niveaux de palmitoylation de Rab7A. Les données de Rab7 palmitoylé tiré de trois expériences indépendantes sont exprimées en moyenne ± écart type (SD). La signification statistique a été déterminée à l'aide d'une ANOVA unidirectionnelle avec le test post-hoc de Tukey, où les niveaux de signification sont notés NS (non significatif), * ($P < 0,05$) et ** ($P < 0,01$).

5. ZDHHC11 Palmitoylation est nécessaire pour recruter un rétromère.

Pour confirmer si ZDHHC11 était le PAT impliqué dans le recrutement des rétromères, nous avons séparé la fraction cellulaire membranaire de la fraction cytosolique cellulaire et quantifié la sous-unité rétromère dans les cellules ZDHHC11KO et DHHC8KO. Nous avons constaté une augmentation de 15 % de la fraction cytosolique dans la sous-unité VPS26, ce qui pourrait laisser entrevoir la possibilité de plus d'un recrutement de rétromères modulant la PAT par palmitoylation de Rab7 (Figure 5.10, A et B). Nous quantifions la distribution de Rab7 pour confirmer que les changements dans la distribution de VPS26 n'étaient pas causés par un effet sur le recrutement de Rab7 dans les membranes (Figure 5,10, C) ; les résultats ne montrent aucun changement entre les différentes lignées cellulaires, attribuant les changements à la capacité de Rab7 dans le recrutement de rétromères.

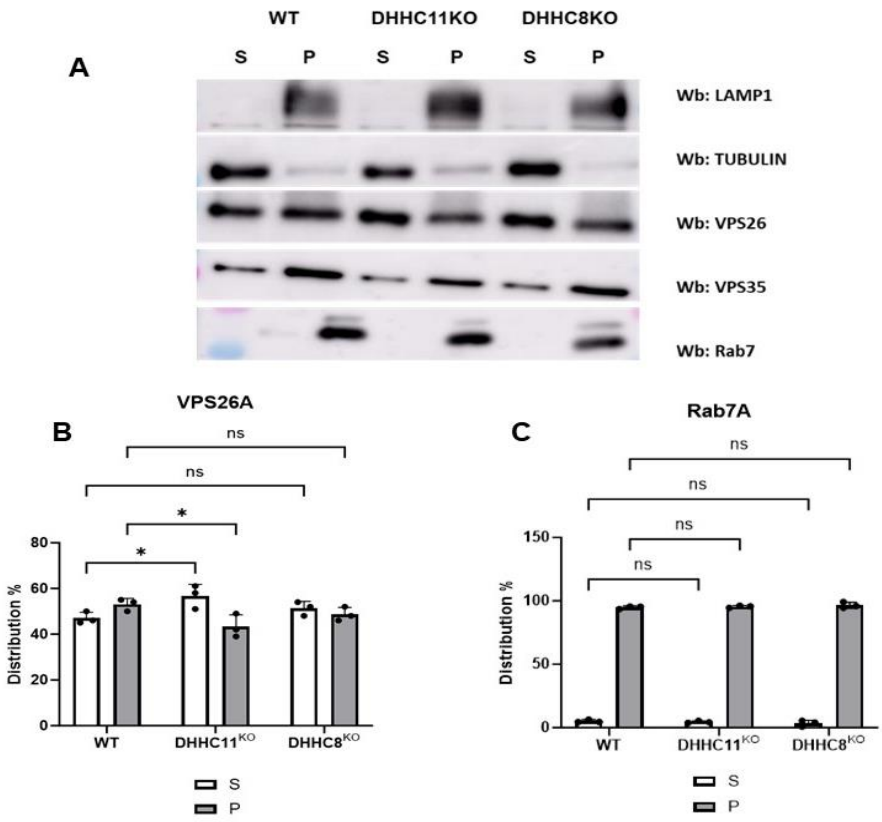

Figure 5.9 Distribution membranaire du rétromère BRET de VPS26 dans les cellules ZDHHCKO. (A) Criblage du signal de fluorescence des interactions entre Rab7 et différents PATS dans les cellules WT par rapport aux cellules CLN5KO, (B, C) Les cellules WT, ZDHHC11KO et ZDHHC8KO ont été soumises à Acyl-RAC pour évaluer les niveaux de palmitoylation de Rab7A. Les données de Rab7 palmitoylé tiré de trois expériences indépendantes sont exprimées en moyenne ± écart type (SD). La signification statistique a été déterminée à l'aide d'une ANOVA unidirectionnelle avec le test post-hoc de Tukey, où les niveaux de signification sont notés NS (non significatif), * ($P < 0,05$) et ** ($P < 0,01$).

CONCLUSIONS

Nous avons identifié la NEK7 kinase et la palmitoyl transférase ZDHHC11 comme régulateurs du recrutement de Retromer dans la membrane endosomale. La phosphorylation de Rab7 au niveau de la sérine 72 est cruciale pour sa palmitoylation et son recrutement membranaire ultérieur. Nous avons constaté que l'interaction de ZDHHC11 avec Rab7 pour la palmitoylation dépend de la phosphorylation de la sérine 72 par NEK7, suggérant un mécanisme potentiel de modulation du recrutement des rétromères. Des recherches plus approfondies sont nécessaires pour déterminer si cette modulation implique des changements conformationnels dans Rab7 qui facilitent l'accès de ZDHHC11 à ses motifs de cystéine. Il est intéressant de noter que le rôle de CLN5 dans la palmitoylation de Rab7 semble indépendant de la phosphorylation de NEK7, indiquant une modulation directe via la palmitoylation de Rab7.

L'épuisement de NEK7 entraîne une maturation altérée de la cathepsine D et une activité réduite de la cathepsine L en raison de l'incapacité du rétromère à recycler la sortiline. Cela souligne l'importance de NEK7 dans le maintien de la fonction lysosomale grâce à des mécanismes de tri médiés par les rétromères.

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AD
Alzheimer's disease.

ALS
Amyotrophic lateral sclerosis.

CD-MPR
Cation-dependent mannose 6-phosphate receptor.

CI-MPR
Cation-independent mannose 6 phosphate receptor.

CMPR
Cation mannose 6 phosphate receptor.

EGFR
Epidermal growth factor receptor.

EGRESS
ER-to-Golgi relaying of enzymes of the lysosomal system.

ER
Endoplasmic reticulum.

GGA
Golgi-localizing,γ-adaptin ear homology domain, ARF-binding.

GRIP1
Glutamate receptor interacting protein.

HOPs
Homotypic fusion and protein sorting.

IGF1R
Insulin-like growth factor.

IGF-II
insulin-like growth factor-II.

KO
Knockout.

LRRK1
leucine-rich repeat kinase 1.

M6P
mannose 6-phosphate.

NCL
Neuronal ceroid lipofuscinoses.

ND
Neurodegenerative diseases.

PAK1-3
Rac-p21-activated kinases 1 to 3.

PAT
Palmitoyl transferases.

PD
Parkinson's disease.

PI3P
phosphatidylinositol 3-phosphate.

PSD95
Postsynaptic density protein.

PTM
post-translational modifications.

RILP
Rab7-interacting lysosomal protein.

S72
Serine 72.

SNXs
sorting nexins.

TBK1
TANK-binding kinase 1.

TGN
Trans Golgi network.

Y183
Tyrosine 183

1. INTRODUCTION

The catalytic activity of endosomes and lysosomes depends on the delivery of soluble hydrolytic proteins. Directing those proteins to the lysosomes relies on a signal-dependent mechanism where the hydrolases undergo modification with different motifs that are recognized by sorting proteins, as is the case with mannose 6-phosphate (M6P) (Ludwig et al., 1993; Coutinho et al., 2012) recognized by M6P receptors (M6PR) within the Golgi apparatus, for subsequent transport to endosomes (Yang & Wang, 2021). However, not all soluble enzymes and non-enzymatic proteins adhere to this route as some follow an M6P-independent trajectory (Bonifacino & Traub, 2003; Braulke & Bonifacino, 2009). Alternative receptors, like LIMP-2 or sortilin, guide the unique transport of hydrolytic enzymes (e.g., cathepsin D and H), shaping a distinctive cellular mechanism. (Canuel et al., 2008). Sortilin has been demonstrated to facilitate the transportation of numerous resident lysosomal enzymes to the lysosome (Lefrancois et al., 2003; Morales, 2006) and plays a role in trafficking various other proteins earmarked for lysosomal degradation (Evans et al., 2011; Nielsen et al., 1999; Li, 2017).

Like M6PR, Sortilin receptors at the late endosomes are retrieved to TGN by retrograde trafficking for additional rounds of sorting after the cargo is deposited at the late endosome (Cullen & Steinberg, 2018). Molecular trafficking in early endosomes, late endosomes, and lysosomes is modulated by the Rab family of GTPases, which are molecular switches that alternate from an inactive state between GTP-bound (active state) and GDP-bound (inactive states) to activate or deactivate biological functions (Villarroel-Campos et al., 2014; Rojas et al., 2008).

Rab7 controls endosome maturation, lysosome biogenesis, autophagy, and retrieval of M6PR and Sortilin at late endosomes (Rojas et al., 2008; Seaman et al., 2007). Rab7 requires different effectors to modulate different functions (Modica & Lefrancois, 2020). Interactions with the HOPs complex facilitate endosome-lysosome fusion (Nickerson et al., 2009; Peralta et al., 2010). The interaction with Rab7-interacting lysosomal protein (RILP) modulates the morphology of endolysosomes and their movement through microtubules (Jordens et al., 2001), and its interaction with the retromer regulates trafficking out of endolysosomes (Rojas et al., 2008).

Regulation between Rab7 and its effectors is regulated in part by post-translational modifications (PTM), such as phosphorylation and palmitoylation (Modica & Lefrancois, 2020). Palmitoylation is a reversible PTM catalyzed by a group of enzymes known as palmitoyltransferases (PATs) (Jin et al., 2021). Those proteins add a lipid modification, a fatty acid chain attached to the proteins to a cysteine residue by a thioester linkage (Jiang et al., 2018; Stix et al., 2020). This modification is

reversed by thioesterases (Zeidman et al., 2009). In other words, palmitoylation adds a hydrophobic motif that transfers the protein between cytoplasmic space and biological membranes (Jiang et al., 2008). Palmitoylation can also modulate protein localization, stability, protein-protein interactions, and enzymatic activity (Blaskovic, 2014).

Rab7 palmitoylation on cysteine 83 and 84 is required for retromer recruitment (Modica et al., 2017). Retromer is a trimeric protein complex comprised of Vps35, Vps29, and Vps26. Retromer performs a role in the endosome-to-Golgi retrieval of both M6PR and Sortilin receptors (Braulke & Bonifacino, 2009; Staudt et al., 2016). Rab7 recruits retromer to the endosomal membrane through direct interaction (Rojas et al., 2008; Seaman, 2007). Once the complex is on the late endosome membrane, the retromer identifies and recycles the receptor back to Golgi, after the cargo is delivered to the luminal site of the endosome (Seaman, 2021). Dysfunction in Rab7 and Retromer have been linked to several neurodegenerative diseases (ND), including PD, AD, and NCL. Consequently, Rab7 and Retromer have emerged as potential therapeutic targets (Kiral et al., 2018).

Phosphorylation is an addition of a phosphate group to a protein's serine or tyrosine residual that can activate or deactivate a protein's functions. Kinases add this PTM which can be reversed by phosphatases (Linder & Deschenes, 2007). Interplays of phosphorylation and palmitoylation are associated with cellular trafficking (Shetty et al., 2023), and the activity and function of PATs or thioesterases can be regulated by phosphorylation (Jin et al., 2021; Jansen & Beaumelle, 2022). The interplay between palmitoylation and phosphorylation has been reported in neuron dynamics in the Synapsin1 protein, in which phosphorylation negatively modulates palmitoylation (Yan et al., 2022).

Rab7 can be phosphorylated on serine 72 (S72) and tyrosine 183 (Y183). Rab7 phosphorylation, among other PTMs modulates different functions independently of the phosphorylated site and the Kinase that adds the PTM (Modica & Lefrancois, 2017). For example, phosphorylation at Serine 72 by leucine-rich repeat kinase 1 (LRRK1) facilitates the interactions between Rab7 and RILP, enabling EGFR transport to the perinuclear region, which means that phosphorylation on S72 by LRRK1 modulates the cargo-specific movement of endosomes (Hanafusa et al., 2019). At tyrosine183 residue, Src kinase phosphorylates Rab7 have an opposite effect to the phosphorylation by LRRK1. Phosphorylation by Src induces EGFR and impairs the interaction with RILP (Lin et al., 2017). TBK1 is another kinase responsible for phosphorylating Rab7 at S72, a crucial modification for the initiation of mitophagy. This kinase plays a negative regulatory role by disrupting the association with RAB GDP dissociation inhibitor while enhancing the interaction

with the DENN domain containing heterodimer FLCN-FNIP1. Upon damage to mitochondria, FLCN-FNIP1 is recruited, and cells expressing non-phosphorylatable RAB7 exhibit inefficient mitophagy, impairing the recruitment of ATG9A-positive vesicles to damaged mitochondria (Heo et al., 2018).

We found that the non-phosphorylatable mutant Rab7S72A, the palmitoylation levels of Rab7 are decreased (Modica et al., unpublished), and consequently, there is less retromer recruitment. LRRK1 nor TBK1 is involved in retromer recruitment by s72 Rab7 phosphorylation (unpublish data). We hypothesize that a different Kinase must phosphorylate Rab7 at S72 to allow palmitoylation and recruit a retromer.

Not only is Rab7 necessary to recruit retromer, but CLN5 and CLN3 are also necessary for retromer recruitment and function. Indeed, CLN3 interacts with Rab7, and CLN5 modulates this interaction (Yasa et al., 2020; Yasa et al., 2021). CLN5 and CLN3 are part of the 13 genes mutated to lead to Neuronal ceroid, a fatal childhood brain illness (Butz et al., 2020). CLN3 is a glycosylate Endo-lysosomal transmembrane protein with six transmembrane domains with both N- and C-terminals facing the cytosol (Oetien & Hermey, 2016; Mirza et al., 2019). CLN3 is required for Rab7-Retromer interaction and within Retromer-Sortilin (Yasa et al., 2020) in a complex with CLN5 to modulate Rab7 functions; in CLN5ko cells, Rab7 is not capable of recruiting retromer, in CLN3KO cells retromer is recruited but not activated (Yasa et al., 2021)

CLN5 is an endo-lysosomal resident protein whose function remains elusive. Early studies suggested CLN5 was an integral protein with at least one transmembrane domain that is later processed into a soluble protein with multiple glycosylated sites (Basak et al., 2021). CLN5 has proposed roles in maintaining a proper acidic environment in the lysosomes (Holopainen, 2001; Best et al., 2017), mitochondrial respiration, mitophagy (Best et al., 2017), and lipid metabolism (Schmiedt et al., 2012). In endosome-to-TGN trafficking, CLN5 regulates the process by controlling the localization of the retromer to the endosome membrane (Mamo et al., 2012; Basak et al., 2021). The lack of CLN5 results in a phenotype akin to that observed in cells with depleted retromer complexes or those lacking Rab7-mediated palmitoylation; the membrane-bound fraction of retromer is reduced (Modica et al., 2017).

We show that phosphorylation of Rab7 serine 72 is required for its interaction with the retromer. Furthermore, we identified NEK7 as the kinase responsible for RAB7 phosphorylation in this process. We also found that palmitoylation of RAB7 is reduced in NEK7KO cells, which suggests an interplay between phosphorylation and palmitoylation in recruiting retromer; we identify ZDHHC11 as one of the palmitoyl transferases that palmitoylates Rab7 for recruiting retromer to

the membrane. Since CLN5 is required for Rab7 phosphorylation (Yasa et al., 2021), we wondered if CLN5 could also influence Rab7 s72 phosphorylation; we found no changes in CLN5KO cells at s72 Rab7 phosphorylation that suggest the direct modulation of palmitoylation after phosphorylation. Additionally, we identified the palmitoyltransferases (PAT) interacting with CLN3 as the first clue of the possible PAT for CLN3 palmitoylation.

2. OBJECTIVES

- Identify the Kinase that phosphorylates at serine72 of Rab7 for retromer recruitment.
- Identify the palmitoyltransferase that Palmitoylates Rab7 for Retromer recruitment.
- Evaluate the effect of the depletion of the kinase in palmitoylation to establish a link between the two PATs.
- Asses the impact of phosphorylation at serine 72 in Retromer recruitment and lysosomal functioning.
- Give insights into the CLN5 function in Rab7 Palmitoylation and posterior retromer recruitment.

3. LITERATURE REVIEW

3.1. Endolysosomal system.

Endocytosis involves internalizing molecules, nutrients, receptors, virus microorganisms, and toxins into the cells (Mukherjee et al., 1997). There are four different variants of endocytosis in eukaryotic cells. The most common is clathrin-mediated endocytosis, a name that comes from a critical component in this process, the clathrin protein. Despite this, more than 50 other proteins from the cell are involved in creating clathrin-coated endocytic vesicles (McMahon et al., 2011).

Clathrin-mediated endocytosis starts with the coat assembly when endocytic proteins gather from within the cell and cluster on the inner side of the cell membrane to concentrate specific molecules to a coated region on the cell membrane, which causes the membrane to bend, forming a clathrin-coated pit. The scission process then narrows and cuts the neck of the membrane invagination, separating the clathrin-coated vesicle from the cell membrane (Kaksonen & Roux, 2018). Actin polymerization works with the coat and scission proteins to shape the membrane during this process (Robenson et al., 2009). Finally, the uncoating step disassembles the endocytic protein machinery, releasing the newly formed cargo-filled vesicle and allowing it to move further within the cell (Kaksonen & Roux, 2018).

Clathrin-independent endocytosis includes every other intake pathway that does not include clathrin proteins. One alternative is caveolae formation associated with caveolins and cavins (Mayor & Pagano, 2007; Sandvig et al., 2011). Further types of clathrin-independent endocytosis are macropinocytosis and phagocytosis (Hansen et al., 2009). Macropinocytosis is a nonspecific form of endocytosis that allows the cell to intake high amounts of extracellular fluids and plasmatic membrane (Lin et al., 2020) initiated by membrane ruffles from polymerized actin that produces a circular vacuole (Lim & Gleeson, 2011). Phagocytosis is described as a cellular uptake of particulates (>0.5 m) within a plasma-membrane envelope (Flannagan, 2012; Gordon, 2016). The difference with macropinocytosis is that this mechanism requires receptor recognition at the cellular membrane to engulf foreign bodies or apoptotic cells (Flannagan, 2012).

All the mechanisms already explained are cellular ways to clean and collect extracellular materials, but the cell also has an intracellular degradation system. Autophagy is the degradative mechanism for cytoplasmic components such as damaged organelles and aggregated proteins (Vargas et al., 2023). Autophagy can be classified into microautophagy, macroautophagy, and chaperone-mediated autophagy. In microautophagy, the cargo is taken directly to the endosomes

and lysosomes by membrane invagination and protrusion (Wang et al., 2023). In macroautophagy, the damaged organelle or cargo is surrounded by a membrane fraction called phagophore that expands until it reaches a sphere-shaped complete double membrane, forming an autophagosome (Mizushima, 2007). These two types of autophagy can be selective and nonselective (Mijaljica et al., 2012), contrary to chaperone-mediated autophagy, where the chaperone protein recognizes the cytosolic proteins HSPA8 through KFERQ-like motifs and is delivered to lysosomes (Dice, 2007).

All the organelles formed by endocytosis and autophagy go through a degradation phase into the lysosomes (Huber & Tesis, 2016). The formed vacuoles fuse with the late endosomes and posteriorly and lysosomes to degrade and recycle compounds (Huber & Teis, 2016). In autophagosome fusion, the outer membrane fuses with the endosome and lysosome (Mizushima, 2007).

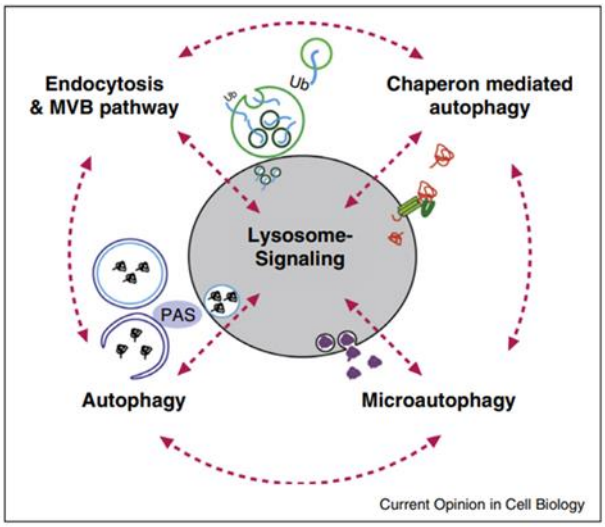


Figure 3.1 Cargo internalization pathway to the lysosomes

Autophagy, endocytosis, the multivesicular body (MVB) pathway, chaperone-mediated autophagy (CMA), and microautophagy (MA). Reprinted with permission from Huber, L. A., & Teis, D. (2016)

To grasp the cellular progression from early endosome/recycling endosome to late endosome, leading to the eventual fusion with the lysosome, it is crucial to have a clear understanding of the pathways involved in cargo degradation. For example, endocytosis permits receptor internalization to the early endosomes, regulating early endosome response as the first step in the endolysosomal pathway (Pasterkamp & Burk, 2021). Early endosomes and recycling endosomes internalize components from the plasma membrane to recycle them back to the plasma membrane or deliver the cargo to the late endosomes, recycling endosomes being a more

direct way for retrograde trafficking of different cellular compounds to the PM and other organelles (Scott et al., 2014; Elkin et al., 2016).

Early endosomes, late endosomes, and lysosomes have a distinctive identity formed by a molecular profile, cargos, and proteins. However, these organelles are recognized as fluid and dynamic. Early endosomes become late endosomes through endosomal maturation (Elkin et al., 2016). A change in the molecular profile of proteins, an increase in hydrolytic enzymes, and a decrease in luminal pH marks the transition from early to late endosomes. Subsequently, the maturation process continues to the lysosomes, where the pH decreases to approximately 4.5 (figure 2) (Elkin et al., 2016). This pH drop is facilitated by the H+ pump, which harnesses the energy derived from ATP hydrolysis in the membrane bilayer by v-type vacuolar to regulate the acidity within endosomes and lysosomes, maintaining an acidic lumen is crucial for properly functioning and activating hydrolytic enzymes (Fuchs et al., 1989; Alberts et al., 2002; Zeng et al., 2020).

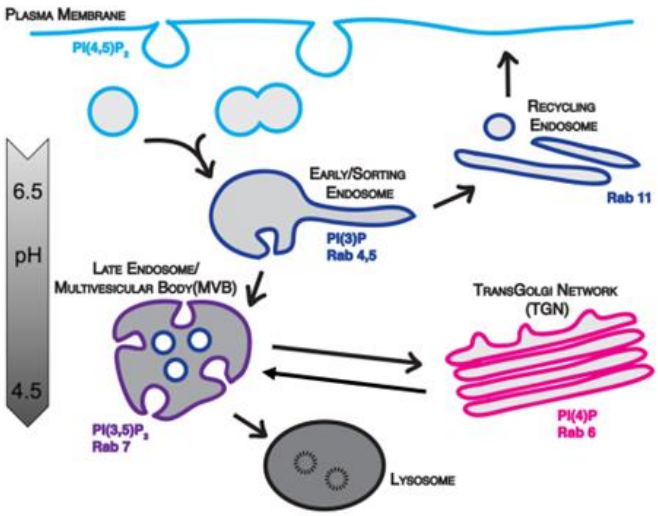


Figure 3.2 Endolysosomal trafficking

The process of internalized vesicles undergoes homotypic fusion to give rise to early/sorting endosomes, delivering the sorting of cargo. Subsequently, the internalized cargo has various options: it can undergo recycling back to the plasma membrane through the recycling endosome, it can be directed to the trans-Golgi network (TGN) via retrograde trafficking mechanisms, or it can be routed through the late endosome/multivesicular body (MVB) to the lysosome for degradation. As these endosomes mature, there is a progressive increase in acidification, represented in greyscale. Furthermore, each specific endosomal compartment is characterized by the distinctive Rab family GTPases. Adapted with permission from Elkin, S. R., Lakoduk, A. M., & Schmid, S. L. (2016).

The clathrin-coated vesicles deliver their cargo to endosomes, which ultimately completely mature into lysosomes by adding more lysosomal hydrolytic enzymes that travel from the Endoplasmic reticulum (ER) to the Golgi apparatus, where the enzymes are tagged for late-endosome delivery,

the low pH in the endosomal lumen allows the dissociation receptors from the hydrolytic soluble protein (cargo) (Olson et al., 2008; Olson et al., 2020).

Endosomes full of hydrolytic enzymes mature into lysosomes after the pH decreases to 4.5, where some hydrolases like cathepsin D are finally cleaved into a mature protein (Laurent-Matha et al., 2006). Within these newly formed lysosomes, the recently delivered hydrolases commence the digestion of endocytosed material, which is delivered by direct interaction with other organelles like endosomes (Mullock et al., 1998). When the lysosome is close to the endosomes containing material to degrade, the membranes get together and undergo fusion to exchange material between compartments, forming a hybrid larger organelle called an endo-lysosome (Desjardins, 1995; Bright et al., 1997; Mullock et al., 1998). Endolysosomes can undergo fission, which is the splitting or division of a single membrane into two separate membranes, leading to a reconstitution of lysosomes, encapsulating lysosomal proteins and functioning as storage compartments for enzymes until they undergo fusion with another organelle (Luzio et al., 2014).

Another mechanism is Kiss and Run, where the two organelles get into a partial fusion that allows them to interchange materials. The organelles “kiss” after the interchange is complete. The two organelles separate fast and maintain the individual compartments; in other words, they “run” (Desjardins., 1995). In the kiss and run mechanism, partial fusion is not always a requisite since, between organelles, it is possible to form an interconnecting tubule that is at a longer distance (Bright et al., 2005). Since the two mechanisms are not mutually exclusive, both may operate simultaneously in mammalian cells, indicating a complex interplay between these fusion processes (Bright et al., 2005).

3.2. Sorting of hydrolytic proteins to the late endosomes

Hydrolytic enzymes function within the lysosome to catalyze the breakdown of various macromolecules through hydrolysis reactions (Mellors & Tappel, 1967). In the lysosomes, more than 70 enzymes play a crucial role in cellular waste management, recycling, and maintaining cellular homeostasis by breaking down biomolecules (Meras et al., 2022). Examples of hydrolytic lysosomal enzymes include proteases, lipases, nucleases, and phosphatases (Bainton, 1981)

The correct localization of lysosomal protein is crucial for proper lysosomal function. Newly synthesized proteins in the ER travel through the Golgi to be delivered to different cellular compartments. As mentioned before, among the proteins that travel to the lysosomes include highly glycosylated membrane proteins such as LAMP1 and LAMP2 (Fukada, 1991), proteins implicated in the control of lysosomal pH, ion channels, membrane contact sites (Schwake et al., 2013) and hydrolytic enzymes (Mellors & Tappel, 1967).

The soluble proteins are often hydrolases dedicated to the lumen of lysosomes as their high degradative capacity in the delivery and maturation of these proteins is regulated. The traffic starts with a peptide with a 20 to 25 amino acid signal sequence that facilitates their movement accompanied by other proteins into the endoplasmic reticulum (Walter et al., 1984). An ER peptidase cleaves the signal peptide, while N-glycosylations are attached to asparagine residues within Asn-X-Ser/Thr motifs by the enzyme oligosaccharyl transferase (Mohorko et al., 2013). It is worth highlighting that some soluble lysosomal proteins, like CLN5, undergo an initial translation into a single-pass transmembrane protein and are cleaved. CLN5 is modified by intramembrane proteases from the SPPL family, found at the ER/Golgi interface—protein its cleavage results in the generation of a fully soluble protein (Jules et al., 2017).

At the ER, the lysosomal proteins are transported by CLN6 and CLN8 together to form a dimer that integrates the EGRESS complex (ER-to-Golgi relaying of enzymes of the lysosomal system). EGRESS recruits soluble lysosomal proteins and promotes their transport to the Golgi apparatus (Bajaj et al., 2020). This recruitment process is facilitated by the expansive luminal loop of CLN6 and the binding of lysosomal enzymes to the second luminal loop of CLN8 (Di Ronza et al., 2018). Soluble hydrolytic protein travels from ER to Golgi through coated vesicles facilitated by COPII, which selects the cargo and induces membrane deformation to form the new vesicles (Stephens, 2003; Zanetti et al., 2012). This complex is recruited by Sar1 GTPase (Kuge et al., 1994). The Sec24 subunit within the COPII complex interacts with CLN8 to identify cargo through motifs in the C-terminal region of cargo receptors, notably LMAN1/ERGIC53 and posterior package (Di

Ronza et al., 2018). This recognition mechanism is essential for sorting glycosylated proteins, such as the lysosomal enzyme cathepsin C. Moreover, it identifies the well-characterized ER export motif "XX" (where X can be any amino acid and XX signifies a hydrophobic amino acid) (Otsu et al., 2013). WD40b domains of Sec31 and Sec13 act to curve the membrane, facilitating the formation of vesicles (Stagg et al., 2018).

The vesicles travel with cargo to the Cis Golgi together with CLN8, while CLN6 remains in the ER (Bajaj et al., 2020). At the Cis side of the Golgi apparatus, soluble enzymes are delivered, and the process of recycling proteins, including CLN8, along with other ER resident proteins and receptors, from the Golgi to the ER occurs (Bajaj et al., 2020). The return journey is facilitated by receptors like KDELR, which can recognize the KDEL (Lys-Asp-Glu-Leu) motif present in ER-resident proteins (Tapia et al., 2019). Subsequently, KDELR is activated, changes its conformational structure, and recruits a COPI complex modulated by Arf1 GTPases and GEFs (Beck et al., 2009).

Within the Golgi apparatus, lysosomal proteins undergo maturation, marked by essential post-translational modifications (PTMs) such as phosphorylation, glycosylation, proteolytic cleavage, and sulfation to ensure accurate organelle localization and functionality (Potelle et al., 2015; Huang & Wang, 2017). The most relevant PTM for soluble lysosomal proteins is the addition of the mannose 6-phosphate (M6P) signal. The formation of the M6P recognition marker involves a sequential enzymatic reaction mediated by distinct enzymes: UDP-N-acetylglucosamine 1-phosphotransferase, α-N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase. Subsequently, the M6P groups generated are recognized by two distinct transmembrane M6P receptors in the trans-Golgi network: the cation-independent M6P receptor (CI-MPR) and the cation-dependent M6P receptor (CD-MPR), two different and non-redundant type I transmembrane proteins (Ludwig et al., 1993; Coutinho et al., 2012). These receptors play a crucial role in binding lysosomal hydrolases situated on the luminal side of the membrane, as well as adapting and facilitating the assembly of clathrin coats on the cytosolic side (Rodriguez & Müsch, 2005). This coordinated action enables the packaging of the hydrolase-MPR complex by a Clathrin-coated vesicle at the trans-Golgi network (TGN) for endosome delivery (Yang & Wang, 2021).

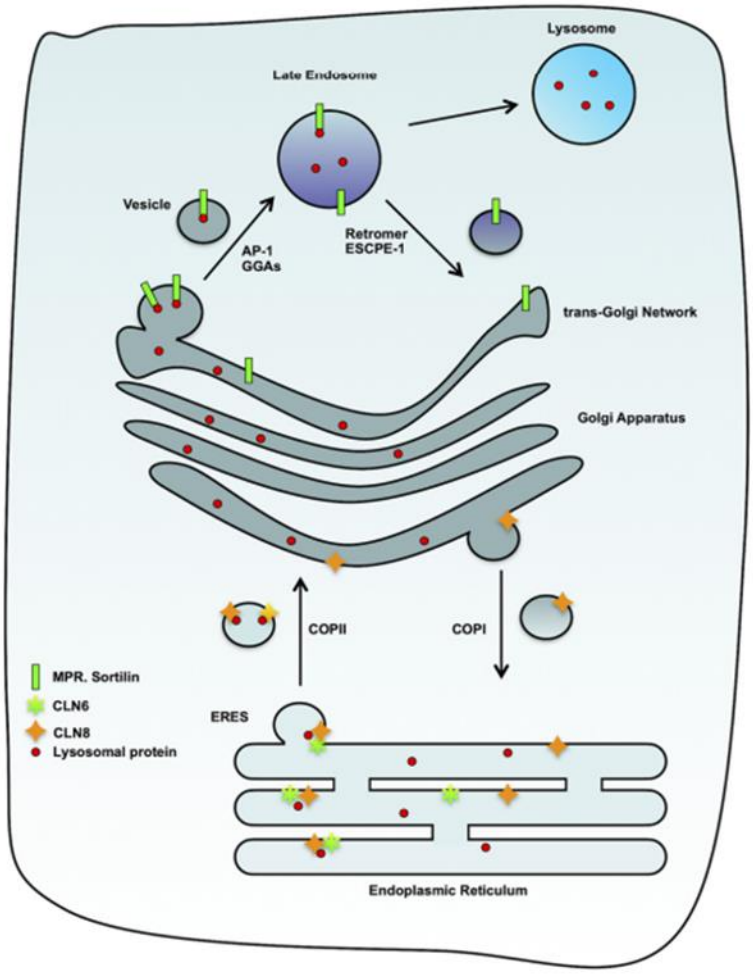


Figure 3.3 Soluble lysosomal enzymes pathway

Hydrolytic enzyme trafficking from the endoplasmic reticulum to lysosomes shows the interplay of CLN6 and CLN8 at cargo trafficking from the endoplasmic reticulum to Cis-Golgi. It is reprinted from Meras et al., 2022. (free access article).

Not all soluble enzymes and non-enzymatic proteins adhere to the same route facilitated by M6PR; some follow an M6P-independent trajectory (Bonifacino & Traub, 2003; Braulke & Bonifacino, 2009). Alternative receptors, like LIMP-2 or Sortilin, guide the unique transport of hydrolytic enzymes (e.g., cathepsin D and H), shaping a distinctive cellular mechanism. (Canuel et al., 2008). At the trans-Golgi Network (TGN), the lysosomal sorting receptors (LSRs) sort and direct soluble lysosomal resident proteins, commonly called cargo, towards lysosomes. For example, cathepsin D is sorted by MPR and prosaposin by Sortilin (Pohlmann et al., 1995; Lefrancois et al., 2003). The ensemble between cargo and LSR is undertaken by an adaptor protein AP-1 (adaptor protein 1) recruited by Arf1, a small GTPase, AP-1 posteriorly binds Clathrin (Morales, 2006 Evans et al., 2011; Nielsen et al., 1999; Metcalfe et al., 2008; Li, 2017).

Clathrin-coated vesicles and clathrin adaptors mediate the trafficking of cargo proteins from Golgi to the endosome. The Golgi-localized proteins known as Golgi-localized, γ-ear-containing, Arf, ADP-ribosylation factor-binding proteins (GGAs), characterized by their γ-ear domain and Arf-binding capability, serve as monomeric clathrin adaptors. Mammals possess three variants: GGA1, GGA2, and GGA3 (Takatsu et al., 2000). Current understanding suggests that GGAs and AP-1 (Adaptor protein) that is composed of 4 different subunits (Braulke & Bonifacino., 2009) function independently, yet in tandem, to create specialized vesicles containing the mannose 6-phosphate receptor (MPR) or sortilin at the trans-Golgi Network (TGN) (Puertollano et al., 2001; Takantsu et al., 2001). This process facilitates targeted delivery to various endosomal compartments. Moreover, GGAs are likely to facilitate MPR/sortilin entry into Clathrin-coated vehicles by engaging with AP-1 (Doray et al., 2002) by binding the tail of the receptors by the μ1 subunit to the tyrosine motif (YXXØ, where X is any amino acids and Ø is a bulky hydrophobic amino acid r σ1 γ t the dileucine motif ([D/E]XXXL[L/I]) (Ohno et al., 1998; Zhu et al., 2001; Doray et al., 2007).

Like the soluble lysosomal proteins, highly glycosidase integral lysosomal proteins LAMPS and LIMPS have a cytosolic domain with sorting motifs containing di-leucine motifs and tyrosine motifs recognized by adaptor complex (APS) establishing interactions with Clathrin coat components like GGAs or adaptor protein complexes at the trans-Golgi network targeting lysosomes (Winchester, 2001; Bonifacino & Traub, 2003). Like M6P, Sortilin receptors at the late endosomes are recycled to TGN for an additional round of sorting (Cullen & Steinberg, 2018). All the receptors are recycled back to TGN at late endosomes, guaranteeing another round of sorting of hydrolytic enzymes; receptor recognition and recycling at late endosomes are regulated by the retromer complex (Braulke & Bonifacino, 2009).

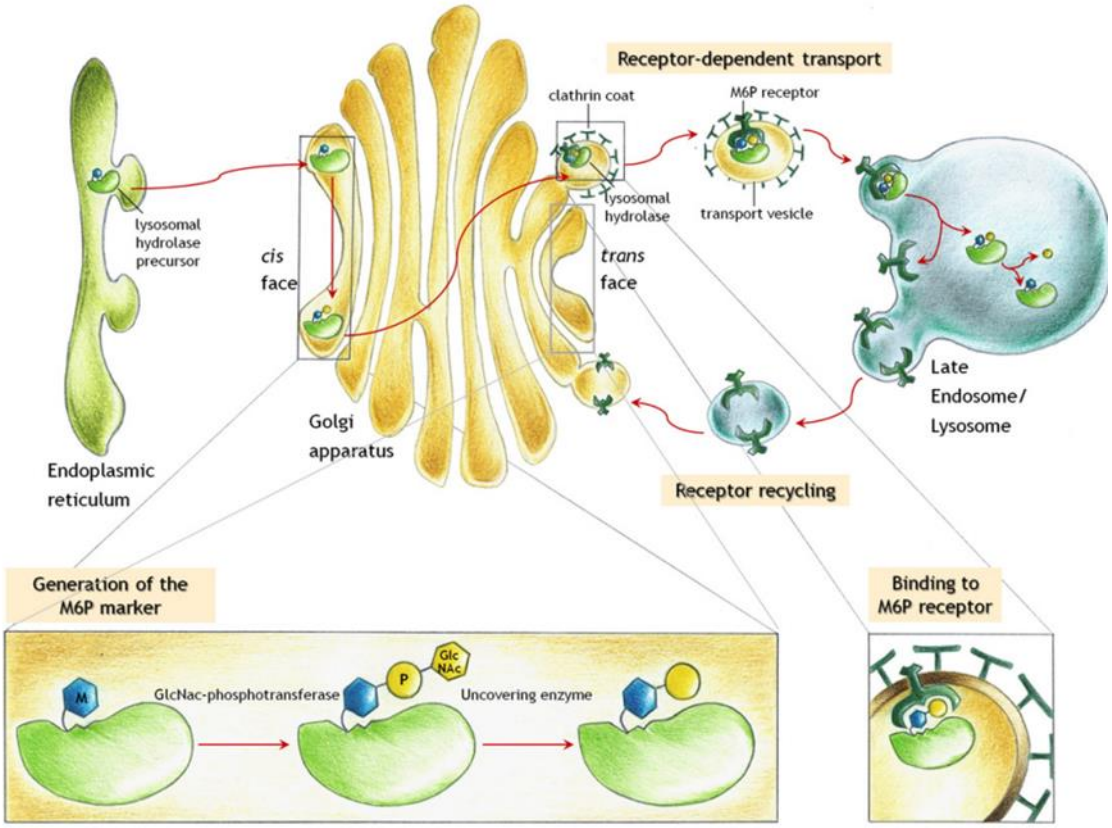


Figure 3.4 Proteins are modified with mannose-6-phosphate (M6P) groups.

Adding the M6P receptor to the hydrolytic enzymes process involves two lysosomal enzymes: GlcNac-phosphotransferase and the uncovering enzyme. GlcNac-phosphotransferase adds a GlcNAc-1-phosphate residue from UDP-GlcNAc to specific mannose residues, while the uncovering enzyme removes the terminal GlcNAc, exposing the M6P signal. In the trans-Golgi network, M6P signals allow lysosomal hydrolases to bind selectively to M6P receptors, facilitating their sorting into clathrin-coated vesicles for transport to late endosomes. Within the late endosome's acidic environment, hydrolases detach from M6P receptors, which are recycled back to the Golgi apparatus. Figure reprinted with permission from (Coutinho et al., 2012).

3.3. Sorting receptors for soluble protein trafficking from TGN to Endosomes

3.3.1. Sortilin.

Sortilin is a receptor that modulates the travelling of hydrolytic enzymes from the Golgi to the endosomes. Its β-propeller structure can bind to more than 50 proteins involved in different functions such as cellular death and lipid processing (Nykjaer et al., 2004; Zhong et al., 2016). Its involvement spans various pathological conditions, including hypercholesterolemia, Alzheimer's disease, prion diseases, Parkinson's disease, and inflammation syndromes (Mitok et al., 2022).

Sortilin functions as a cargo receptor responsible for sorting various proteins, including acid sphingomyelinase, prosaposin, as well as cathepsins D and H, directing them to lysosomes (Lefrancois et al., 2003; Canuel et al., 2008). The sorting of sortilin out of the Golgi apparatus depends upon AP-1 (Canuel et al., 2008). The abbreviated intracellular domain of Sortilin interacts with numerous cytoplasmic adaptor proteins, including the AP-1 complex for export out of the Golgi apparatus (Canuel et al., 2008) and Golgi-localizing,γ-adaptin ear homology domain, ARF-binding (GGA1 GGA2 and GGA3) (Nielsen et al., 2001). These adaptors primarily recognize two distinct motifs: a C-terminal acidic cluster dileucine motif and a YXXΦ motif positioned in the proximal third of the domain. Both motifs play pivotal roles in endocytosis and Golgi-to-endosome trafficking of Sortilin (Pallesen et al., 2020). Notably, the C-terminal acidic cluster, containing a phosphorylatable serine residue targeted by casein kinase, regulates adaptor binding to Sortilin. Furthermore, the cytoplasmic domain of Sortilin also associates with Rac-p21-activated kinases 1 to 3 (PAK1-3), influencing the affinity for AP-1 binding and consequently altering the intracellular localization of sortilin through modified trafficking pathways. Phosphorylation also modulates interaction with the GGAs (Cramer, 2010; Pallesen et al., 2020; Meras et al., 2022).

The cytosolic tail of sortilin is palmitoylated (McCormick et al., 2008). Palmitoylation is a PTM that consists of adding a fatty acid chain to the cysteine residue of a protein, modulating protein function and localization (Jiang et al., 2018). DHHC15 palmitoylates sortilin, a modification required for retrograde trafficking from the endosome to the Golgi (McCormick et al., 2008). When sortilin is not palmitoylated, it is ubiquitinated by Nedd4 ubiquitin ligase and degraded, serving as a modulator of hydrolase sorting (Dumaresq-Doiron et al., 2013).

Sortilin functions not only as a transporter of hydrolytic protein out of Golgi but also as a receptor for neurotrophic factors and neuropeptides and a co-receptor for various other molecules, including cytokine receptors, tyrosine receptor kinases, G-protein coupled receptors, and ion

channels (Mazella et al., 1998; Morris et al., 1998). It is identified as an essential receptor in neurons of the central and peripheral nervous systems (Sarret et al., 2003); in certain organs, it has specific roles; in the liver, Sortilin regulates the release of lipoprotein into circulation (Musunuru et al., 2010). The diverse functions attributed to Sortilin stem from its localization within the cell, spanning from the cell surface to different organelles; through this spatial distribution, Sortilin orchestrates the fate of target proteins, facilitating processes such as cell surface exposure, signal transduction, regulated secretion, endocytic uptake, and anterograde and retrograde sorting (Nykjaer & Willnow., 2012).

3.3.2. LIMP-2

LIMP-2, known as SCARB2, is a type II integral membrane protein primarily localized to late endosomes and lysosomes (Gonzales et al., 2014). Its primary function involves the transportation of hydrolases, such as β-glucocerebrosidase, directly from the ER to the lysosomes, unlike Sortilin and M6PR, where the transport starts at Golgi, where the glucocerebrosidase is processed under low pH conditions (Reczek et al., 2007). β-glucocerebrosidase binding is mediated by a helical loop localized in the apex of the luminal domain of LIMP-2, which aids in its transit to the lysosomes (Zunke et al., 2016) β-glucocerebrosidase activity is reduced in PD, detriment in the stability or transportation of β-glucocerebrosidase to lysosomes is related with accumulation of α-synuclein. (Parnetti et al., 2017).

Additionally, LIMP-2 serves as a phospholipid receptor, a discovery prompted by its prolonged residence within lysosomes (Conrad et al., 2017). The structure of LIMP-2 creates a cavity conducive to transporting cholesterol and other lipids from the lysosomes to the plasma membrane. Furthermore, the transport of this specific enzyme by LIMP-2 also influences cholesterol transport to the lysosomal membrane and lipid droplets (Heybrock et al., 2019).

LIMP-2 facilitates the transportation of proteins such as CD36 or CD8 to the lysosomes via a motif located within the cytoplasmic tail of this receptor, characterized by the absence of tyrosine residues (Vega et al., 1991; Ogata & Fukada, 1994). In concordance with the LIMP- 2 function in lipid trafficking, it was shown that this receptor also influences the conformation of endosomes and lysosomes. Overexpression of LIMP-2 leads to the enlargement of endosomes and impairs the retrograde trafficking from the endosomes and an increase of cholesterol into the enlarged endosomes; this phenotype is rescued by the over-expression of the early endosome GTPase Rab5 (Kuronita et al., 2002).

3.3.3. Mannose 6-phosphate receptor

The mannose 6-phosphate receptor (MPR) refers to 2 types of receptors that are both type-1 integral membrane glycoproteins, the 46-kDa cation-dependent MPR (CD-MPR) and the 300-kDa cation-independent MPR/insulin-like growth factor-II (IGF-II) receptor (CI-MPR). These receptors perform different and not redundant functions in the cells along with some redundant functions. Both are in charge of the transportation of hydrolytic enzymes out of the Trans Golgi to lysosomes (Ghosh et al., 2003).

The extracytoplasmic segment of CI-MPR showcases a repetitive pattern comprising 15 consecutive repeats, each approximately 147 amino acids in length. These repeats, with sequence identity ranging from 14% to 38% and similar cysteine distribution, suggest potential similarities in disulfide-bonding and tertiary structures (Lobel et al., 1988). Correspondingly, the extracytoplasmic domain of the CD-MPR, spanning 159 residues, reflects the repetitive nature observed in CI-MPR's units. Notably, while the CI-MPR's extracytoplasmic domain houses two M6P-binding sites and a lone IGF-II (Hancock et al., 2002), the CD-MPR features a solitary M6P-binding site and lacks affinity for IGF-II, showing their substrate independence. As such, CI-MPR can bind IGF-II (insulin-grown factor) at the cell surface and internalizes it for degradation in lysosomes (Thong et al., 1988). Both receptors' cytoplasmic tails carry numerous sorting signals, some of which undergo phosphorylation or palmitoylation (Rosorus et al., 1993; Schweizer, 1996). A structural alignment based on sequences between the CD-MPR and domains 3 and 9 of the CI-MPR provides compelling evidence that both receptors employ a comparable mechanism for carbohydrate recognition (Roberts et al., 1998).

3.4. Retrograde Trafficking of the receptors back to TGN

The receptors mentioned earlier are recycled back to the TNG for another round of sorting, avoiding its degradation at the endosomal lumen; this process is mediated by the retromer (Braulke & Bonifacino, 2009).

3.4.1. Retromer

Retromer is a protein complex comprising by a trimer of vacuolar protein sorting VPS26, VPS29, and VPS35 (Attar & Cullen, 2010) that work together with membrane sorting nexins SNX adaptors (Lucas & Hierro, 2017; Cullen & Steinberg, 2018). This protein complex plays a vital role in the endosome-to-Golgi retrieval of both CI-MPR and Sortilin, preventing their degradation within lysosomes (Braulke & Bonifacino, 2009; Staudt et al., 2016). Notably, at endosomes, the VPS35 retromer subunit interacts with the cytosolic domain of CI-MPR and facilitates its transportation via tubular vesicular structures back to the TGN (Arighi et al., 2004).

Dysfunction of the retromer leads to impaired sorting of soluble cargo and lysosomal dysfunction (Seaman et al., 2004; Arighi et al., 2004), a phenotype reflected in different nervous system pathologies such as Parkinson's disease (PD). Several mutations in the VPS35 subunit of retromer have been identified in PD, resulting in changes of distribution, size, and disruptions in cathepsin D trafficking responsible in degradation of α-synuclein, a causative agent of PD (Follett et al., 2014; Follet et al., 2016), Alzheimer disease where the levels of cellular Aβ peptide are increased in VPS35 and VPS26 downregulated expression in AD patients (Small et al., 2005; Muhammad et al., 2008), in hippocampal neurons from a VPS35KO mice model shows that retromer can modulate microglia morphology and this regulation is not related to Tau the most studied protein in Alzheimer, giving insights of the role of endosomal trafficking and late-onset sporadic AD (Qureshi et al., 2022). It has also been shown that retromer core organization in neurons is organized around VPS26b. The brain cortex is the most affected by VPS26 depletion, and the high demand for the endosomal recycling process in this region appears to be one of the mechanisms that lead to AD (Simoes et al., 2021). Retromer is also related to amyotrophic lateral sclerosis; the VPS35-VPS29 expression in ALS mice model increases retromer stability, increasing its survival rates, increasing VPS35 expression, and mitigating locomotion impairment (Muzio et al., 2020).

In concordance with the studies related to retromer implication in neurological diseases, Cui et al. (2019) found that Vps35 causes alteration in lysosomal structure and function reflected in impaired autophagy, in addition to reduced proteolytic activity; these phenotypes are not dependent on other sorting mechanism such SNX mediated sorting.

It is essential to mention that retrograde sorting from endosomes can be mediated by other than retromer, and alternative mechanisms exist to retrieve receptors from the endosomes to TGN and the plasma membrane. The endosomal sorting complex for promoting exit 1 (ESCPE-1) is a combination of different SNX proteins and is promoted by (BAR) domain (SNX–BAR). This complex can interact with retromer through SNX27, one of the endosomal cargo adaptors (Simonetti et al., 2019; Yong et al., 2020). The capacity for both complexes to recycle M6PR emerges as a controversy; it is not clear why two mechanisms exist and how interplay together exists a spatial separation that could modulate the sort of proteins in different carriers (Tu et al., 2021). Models have been proposed where SNX27–Retromer captured cargo proteins are transferred into ESCPE-1 transport carriers to promote endosome-to-plasma membrane recycling (Yong et al., 2020)

Recruitment of retromer to the endosomal membranes depends on the active form (GTP-bound) of the small GTPases Rab7 (Rojas et al., 2008; Seaman et al., 2009). Its depletion causes an accumulation of the receptors at the endosomal membrane but not its degradations since Rab7 is involved in different pathways at the endosomal network, including lysosome degradation (Rojas et al., 2008).

3.5. Rab GTPs Family

GTPases are small proteins that function as molecular switch modulating different cellular functions. GTPases are divided into five families: Ras, Rab, Rho, Ran, and Arf (Rojas et al., 2012). Rab proteins regulate the interchange of lipids and proteins between membrane-bounded organelles, facilitated by their location on the cytosolic side of the intracellular membranes (Stenmark, 2009). Rabs are proteins with molecular weights between 20 and 40 kDa that belong to the Ras superfamily of small GTPases (Stenmark & Olkkonen, 2001). There are approximately 70 Rab proteins in humans, which are phylogenetically classified based on amino acid alignments in the switch II region where GAP proteins turn Rabs on or off. However, it is not well known if there is any paralogous relationship between similar Rabs (Kawasaki et al., 2005).

The Rab family protein is typically attached to the target membrane by prenylation. Rabs are bound to a geranyl transferase or a farnesyl transferase that modifies the C-terminus cysteines using geranyl pyrophosphate or farnesyl pyrophosphate (Zhang & Casey,1996; Zhang et al., 2022). When Rab is attached to the membrane, it can be switched from an inactive state (GDP bound) to an active state (GTP bound). This cycle is catalyzed by GTPase-activating proteins GAPs and guanine nucleotide exchange factors GEFs generating conformational changes at the Rab structure (Dumas et al., 1999; Barr & Lambright, 2011) and allowing Rab7 interactions to bind in a specific membrane context (Homma et al., 2021). Among the possible events regulated by Rabs are membrane tethering, vesicle movement, vesicle formation, and membrane fusion (Bröcker et al., 2010; Horgan, year, 201; Pfeffer, 2017).

Within the Rabs associated as a regulator of endocytic trafficking, Rab4 is at early endosomes to recycle receptors to the plasma membrane (Van Der Sluijs et al., 1991), Rab11 is at the recycling endosome (Ullrich et al., 1996), Rab5 is at the early endosome, and Rab7 is at late endosomes. Other Rab proteins associated are Rab9 at the late endosome and Rab10 and Rab35 (Zhang et al., 2022). Understanding retrograde trafficking from the late endosomes to the trans-Golgi network requires biological knowledge of the critical regulator Rab7.

Inactive Rab7 (GDP-bound) is located in the cytosol. It is activated by vacuolar fusion proteins Mon1–Ccz1 complex by GTP loading to posterior recruitment in the endosomal membrane (Kinchen et al., 2021) C5orf51 has been identified as a distinct binding partner of GDP-locked RAB7A. It also exhibits interactions with key components of the RAB7A guanine nucleotide exchange factor (GEF) complex, including MON1 and CCZ1. When C5orf51 is absent, the proper

localization of RAB7A on depolarized mitochondria is disrupted, leading to its degradation via the proteasome pathway (Yan et al., 2022).

The deactivation of Rab7 is mediated by two GTPase-activating proteins (GAPs), TBC1D5 or TBC1D15. These GAPs catalyze the hydrolysis of GTP back into GDP, thereby inducing the shutdown of Rab7 (Seaman et al., 2009). TBC1D15 acts as a Rab7 GTPase-activating protein (GAP), diminishing Rab7's interaction with its effector protein, RILP. This action leads to lysosome fragmentation and protects against cell death induced by growth factor withdrawal. Notably, TBC1D15's GAP activity exhibits selectivity towards Rab7, as evidenced by its lack of inhibition on processes such as transferrin internalization or recycling, which are Rab7-independent and rely on the activation of Rab4, Rab5, and Rab11 (Yan et al., 2022). Inhibiting the GTPase-activating protein TBC1D5 can amplify Rab7 activation, resulting in a heightened functionality of the retromer (Seaman et al., 2018).

3.5.1. Rab7 and its effectors in different pathways.

Rab7 plays a central role in modulating various functions within the endocytic pathway, depending on which effector Rab7 interacts with. Late endosome-lysosome fusion initiates with the assembly of the SNARE complex, a process regulated by the homotypic fusion and vacuole protein sorting (HOPS) complex (Zick & Wickner, 2013). Comprising six subunits (VPS11, VPS16, VPS18, VPS33, VPS39, and VPS41), HOPS acts as a bridge between membranes from different compartments, facilitating their proximity and fusion mediated by C-terminal residues of the synaptobrevin2 and syntaxin 1A transmembrane domains of the SNAREs complex (Sharma & Lindau, 2018; Zhao & Zhang, 2019; Zhang et al., 2023). Rab7 does not directly mediate the recruitment of HOPS subunits to endosomal membranes. Instead, Rab7 plays a role by modulating its effectors, RILP and PLEKHM1 (Van der Kant et al., 2013; McEwan et al., 2015). PLEKHM1, an adaptor protein, promotes the recruitment and stabilization of HOPS subunits, facilitating the tethering of endosome and autophagosome membranes with lysosomes (McEwan et al., 2015). Furthermore, Rab7 indirectly influences endosome fusion by interacting with RILP and the cholesterol sensor ORP1L. The Rab7-RILP-ORP1L complex regulates endosome movement, fusion coordination, and the transition from transport machinery to fusion machinery (Wijdeven et al., 2016; Van der Kant et al., 2013).

Rab7 also modulates lysosomal maturation; when Rab7 interacts with WDR91, it downregulates the interaction with PtdIns3P, which promotes Rab switching from Rab5 in the early endosome to the late endosome (Liu et al., 2017). The WDR91/Rab7 interaction also inhibits the HOPS

complex from interacting with Rab7, controlling the fusion dynamics between endosomes and lysosomes (Xing et al., 2021).

Rab7/RILP (interacting lysosomal protein) interaction modulates the endosomal and lysosomal distribution within the cell through the recruitment of dynein-dynactin motor complexes for endosomal lysosomal microtubule-transportation (Jordens et al., 2001). This pathway requires the recruitment of two Rab7 effectors at the same time RILP and ORP1L (oxysterol binding protein) creating RILP-Rab7-ORP1L complex (Johansson et al., 2007). RILP also modulates the degradation of EGF (epidermal grow factor) and low-density lipoproteins (Cantalupo et al., 2001).

Retromer recruitment by Rab7 modulates retrograde transport from the endosome to TGN (Rojas et al., 2008; Seaman et al., 2007). Rab7 recruits the retromer to the endosomal membrane through direct interaction (Rojas et al., 2008; Seaman, 2007). Once the complex is on the late endosome membrane, the retromer identifies and recycles the receptor back to Golgi, while the cargo is delivered to the luminal site of the endosome (Seaman, 2021). Rab7 dysfunction causes retromer to dissociate from the endocytic membrane, though SNX is not dissociated. Decreased retromer recruitment inhibits CI-MPR retrieval and cathepsin D missorting (Rojas et al., 2008). Retromer is negatively modulated by a Rab GAP protein TBC1D5, causing Rab7 to dissociate from the membrane (Seaman et al., 2009). Retromer interacts with Rab7 directly to the VPS35 N-terminal domain. However, the VPS26 subunit appears to be critical in the interactions as the contact site between the subunits VPS26 and VPS35 impairs the membrane recruitment by Rab7 suggesting that VPS26 and VPS35 mediate interaction (Priya et al., 2015).

Regulation between Rab7 and different effectors in the endocytic pathway is regulated by post-translational modifications (PTM). Rab7 is ubiquitinated (Sapmaz et al., 2019), phosphorylated (Heo et al., 2018; Hanafusa et al., 2019), and palmitoylated (Modica et la., 2017). Like in many other Rabs, phosphorylation and lipid modifications are pivotal in regulating the recognition and trafficking of lysosomal membrane proteins (Modica & Lefrancois, 2020). The interplay between cytosolic and luminal signals and their interaction with recognition proteins ensures proteins' precise and directed transportation to lysosomes (Braulke & Bonifacino, 2009). This elaborate system underscores the sophistication of cellular processes involved in maintaining the functionality of lysosomes within the cell (Campbell & Philips, 2021).

3.6. Post-translational (PTM) modifications of Rab7 as modulators of its functions.

3.6.1. Rab7 Phosphorylation.

In general, phosphorylation has been shown to act as a modulator of Rab proteins; they can regulate, for example, the interaction with GTPase-activating proteins (GAPs) and effector proteins (Müller & Goody, 2018). Rab7 can be phosphorylated on the serine72 (S72) and at tyrosine 183 (Y183), and its phosphorylation modulates different functions under different cellular conditions (Sharma et al., 2014; Modica & Lefrancois, 2020). When Rab7 is stimulated by epidermal growth factor (EGF), Rab7 is phosphorylated by Src kinase at Y183 (Lin et al., 2017), prompting EGF receptor (EGFR) degradation. Otherwise, those receptors are recycled to the plasma membrane. Y183 phosphorylation also can prevent Rab7 interactions, as with RILP (Francavilla et al., 2016; Shinde et al., 2016), inhibiting EGFR degradation (Lin et al., 2016).

At S72, Rab7 is phosphorylated by TBK1, modulating autophagy via PINK1 and PARKIN The latter ubiquitinates the outer membrane of damaged mitochondria to recruit cargo receptors with TBK1 (Harper et al., 2018). Heo et al., 2018 found in a proteomic assay that expressing the phospho-null Rab7 mutant inhibits the recruitment of FLCN-FNIP1, required in damaged mitochondria recruitment, while a phosphomimetic mutant increases their interaction, negatively modulating Rab7 GDP dissociation inhibitor (Heo et al., 2018). Leucine-rich repeat kinase (LRRK1) also mediates phosphorylation of Rab7 at S72 repeat, which orchestrates specific cargo trafficking within endosomes. This phosphorylation event enhances the interaction between Rab7 and RILP (Rab-interacting lysosomal protein), thereby facilitating the recruitment of the dynein-dynactin complex. Consequently, EGF-positive endosomes are efficiently transported to the perinuclear region of the cell, thereby regulating their spatial distribution and subsequent cellular signalling pathways. (Hanafusa et al., 2019).

NEK7, a Serine/Threonine Kinase (Fry et al., 2012), plays a crucial role in inflammasome activation, particularly through its interaction with NLRP3, an inflammasome sensor (Xu et al., 2016; Sharif et al., 2019; Anderson et al., 2023). NLRP3 responds to various stimuli such as reactive oxygen species (ROS), potassium efflux, lysosomal destabilization, NF-κB signalling, protein misfolding aggregation, and toxicants. Interestingly, the inflammasome activation mediated by NEK7 and NLRP3 appears independent of NEK7's catalytic activity (Xu et al., 2016; Sharif et al., 2019; Anderson et al., 2023). However, NEK7's catalytic function is essential for regulating microtubule dynamics and promoting spindle assembly, particularly during interphase (He et al., 2016).

In the context of endocytic trafficking, Zhang et al. (2023) uncovered a complex interaction involving NEK7 and NLRP3. Their study revealed that disruptions in binding sites between the endoplasmic reticulum and endosome membranes lead to phosphatidylinositol 4-phosphate (PI4P) accumulation within endosomes. This accumulation results in the retention of retrograde transport cargo, such as CI-MPR and TGN46, within endosomes, facilitating NLRP3 recruitment. Notably, NEK7's role in this process remains undefined. Joseph et al. (2023) further investigated the retrograde trafficking pathway from endosomes to the Trans-Golgi network (TGN). Knocking down NEK6 or NEK7 was found to cause missorting and degradation of CI-MPR from endosomes, implicating a potential role of NEK7 in retrograde trafficking related to retromer function (Arighi et al., 2004). However, the involvement of NEK7 in lysosomal pathways or its connection with Rab7 remains unexplored in these studies.

3.6.2. Rab7 Palmitoylation

Palmitoylation, also known as S-acylation, is a post-translational modification characterized by the addition of an extended fatty acid group derived from acyl-CoA (Lee et al., 2022), which us attached by a thioester linkage to a cysteine residue of a target protein (Schroeder et al., 1997; Levental et al., 2010; Rana et al., 2018). Palmitoylation can increase hydrophobicity to modulate protein complex assembly, stability, trafficking, aggregation, localization, and activity or a combination of all these changes at the same time (Rocks et al., 2005; Holland et al., 2016; Campbell et al., 2021). This modification, among other lipid modifications, exhibits a characteristic that is reversible by thioesterases, allowing the palmitoyltransferases (PAT) to modulate dynamic cyclic functions within the cell and shuttle between membranes and microdomains (Schroeder et al., 1997; Levental et al., 2010).

Palmitoylation is orchestrated by PATs, a group of 22 integral membrane proteins localized to organellar membranes such as the endoplasmic reticulum, the plasma membrane, and the Golgi apparatus, where most of the PATs are found (Ohno et al., 2006). The literature does not agree on the cellular location of most of the PATs, and it has been reported that PATs can cycle between organelles (Greaves et al., 2011).

PATs are referred to as ZDHHCs because of the conserved aspartate–histidine–histidine–cysteine motif between them. They are differentiated by variations in the N - and C -terminal echoed in vastly different protein sizes across the DHHC family ranging from 263 residues in DHHC22 to DHHC8 with more than 750 residues (Stix et al., 2020; Mitchell et al., 2006). ZDHHCs are characterized by four to six transmembrane domains, with the C-terminal and the N-terminal facing the cytosol (Politis et al., 2005), where the acyl-transfer occurs by a transfer of a palmitate group from a palmitoyl coenzyme A (CoA) to the target protein. All the DHHC proteins are auto-acylated at the cytosolic loop of the protein in a cysteine motif crucial for protein functioning (Jiang et al., 2018).

The 22 PATs expressed in humans are classified into subfamilies according to structural familiarities. It is generally hypnotized that a correlation between the protein structure and the substrate specificity could exist, which implies that DHHC proteins have different target proteins but could overlap. Palmitoylation is highlighted as a relevant PTM in the modulation of cellular function and cell homeostasis, especially in neurons, since it is related to neurological and neuropsychiatric conditions (Fukata & Fukata, 2010; Mukai et al., 2004). One example of the relevance of PATs in neurological conditions is DHHC17, also known as HIP14. It is primarily

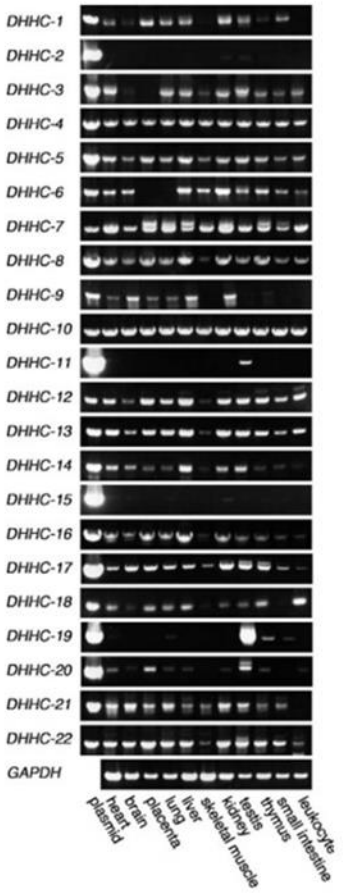
expressed in neurons (Huang et al., 2004) and is associated with Huntington's disease (Singaraja et al., 2011; Young et al., 2012). It localizes at vesicular structures such as the Golgi apparatus and sorting recycling endosomal structures (Huang et al., 2004). Studies indicate that depletion of DHHC17 impairs neuronal axon outgrowth during neuronal development and is necessary for forming TrKA-tubulin (Shi et al., 2015). Additionally, DHHC17 has been identified as a critical protein involved in ischemic stroke, in cell death regulation (Yang & Cynader, 2011), and plays a role in Mg2+ transport (Goytain et al., 2008), and Ca2+ and Potassium (BK) channels regulation (Hines et al., 2010; Tian et al., 2010).

DHHC5 is a PAT associated with bipolar disorder and learning difficulties (Fallin et al., 2004; Li et al., 2010). It has been related to the palmitoylation of different proteins for its cellular trafficking and immune responses. Some of the substrates identified for this enzyme are NOD1/2 intracellular scaffolding proteins required for caspase activation in cellular death activation (Lu et al., 2019); DHHC5 interacts with GOLGA7 at Golgi apparatus for protein transportation; it also colocalized with ER, together with DHHC9 has been associated with this pathway too (Ko et al., 2019).

DHHC8 is another PAT highlighted in the literature in normal neuronal functioning. Knockout (KO) of DHHC8 in neurons leads to a reduction in dendritic spines and glutamatergic synapses and a reduction of PSD95 (postsynaptic density protein) (Mukai et al., 2008). DHHC5 and DHHC8 target the trafficking of GRIP1 (glutamate receptor interacting protein) to dendritic endosomes in neurons to modulate AMPA trafficking (Thomas et al., 2012). Like DHHC17, DHHC5 plays a critical role in maintaining Ca (2+) through NCX palmitoylation, showing the redundancy of the PATS for different substrates (Vesli & Soboloff., 2020). Nonetheless, there is no consensus in the literature about the substrate specificity of each PAT and whether the localization of each enzyme is associated with its substrate. Huang et al. (2009) demonstrated that in the case of Huntingtin protein, it can be only palymitoylated by DHHC17 and DHHC13, proteins with 57% similarity between them, but is not palmitoylated by DHHC3 and DHHC8, showing specificity in the substrate. The determinant of the PAT with the substrate is not known yet since all the proteins evaluated are highly expressed in the same compartment at the Golgi apparatus.

The localization of the proteins and the tissue-specific expression also differ in the literature, and a study by Ohno et al. (2006) estimated the tissue expression and their localization into the cell (figure 3). In that study, the colocalization with specific markers other than ER and Golgi was not assessed. They found that some PATs are ubiquitously expressed, while others are tissue-specific. However, some of the PATs for each tissue are not in concordance with other studies.

In the study by Miles et al. (2021), they reported that in the heart, DHHC1, DHHC2, DHHC4, DHHC5, DHHC7, DHHC8, DHHC9, DHHC17, DHHC18, DHHC20, and DHHC21 are highly expressed. This was different than what was reported in Ohio et al., 2006. The differences in the study could be due to the technique used, as they validated different antibodies for the PATs. The lack of excellent and specific antibodies has been a limitation in the study of PATs.



Protein	Localization
Akr1	Golgi
Akr2	Golgi
Swf1	ER
Pfa3	Vacuole
Pfa4	ER
Pfa5	Plasma membrane
Erf2	ER
DHHC-1	ER
DHHC-2	ER/Golgi
DHHC-3	Golgi
DHHC-4	Golgi
DHHC-5	Plasma membrane
DHHC-6	ER
DHHC-7	Golgi
DHHC-8	Golgi
DHHC-9	ER/Golgi[a]
DHHC-10	ER
DHHC-11	ER
DHHC-12	ER/Golgi
DHHC-13	ER
DHHC-14	ER
DHHC-15	Golgi
DHHC-16	ER
DHHC-17	Golgi
DHHC-18	Golgi
DHHC-19	ER
DHHC-20	Plasma membrane
DHHC-21	Plasma membrane
DHHC-22	ER/Golgi[a]

Figure 3.5 DHHC's expression by PCR amplification in different tissues.

DHHCs are expressed by PCR amplification in different tissues and cellular localization to different organelles. Reprinted with permission from Ohno et al., 2006.

Palmitoylation highly regulates Rab family GTPases. In general, Loss in the cysteine motif in Ras proteins results in its missorting of the proteins from the correct membrane organelle, for example, the displacement from the recycling endosomes to the plasma membrane, Golgi, or endoplasmic reticulum (Taguchi et al., 2011). Diaz-Rohrer et al. (2023) show that Rab3, a small GTPase

exclusively expressed in the brain, as a mediator of association of different sorting proteins to ordered cellular microdomains, the reversible association and the association with rafts domains were modulated by Rab3 palmitoylation.

Rab3gap1 is palmitoylated by DHHC9 to modulate atrial morphology (Essandoh et al., 2023), and the Rab11a α-helix 8 binding domains are regulated by palmitoylation (Reid et al., 2010). Rab7 is palmitoylated in two Cysteine residues, C83 and C84. This modification is required for retromer recruitment and cargo retrieval to Golgi, but not for Rab7 membrane binding. Interactions with other effectors like RILP do not seem altered by lack of palmitoylation, which suggests that palmitoylation modulates some, but not all, Rab7 functions (Modica et al., 2017). Retromer is not palmitoylated, but other proteins involved in trans-Golgi trafficking, like the receptors sortilin and CI-M6PR, are palmitoylated by DHHC15. This modification is required for retrograde trafficking from endosome to Golgi as non-palmitoylatable receptors are degraded at the endosomes due lack of interaction with retromer (McCormick et al., 2008). Since palmitoylation is a modification common in several proteins in this pathway, we hypothesize that palmitoylation regulates endosome-to-Golgi trafficking by localizing proteins together into a specific membrane microdomain.Until now, no PAT has been identified for interaction or palmitoylation of Rab7.

3.7. Other mechanism that regulates retromer recruitment, CLN proteins

Neuronal Ceroid Lipofuscinoses (NCLs), also known as Batten Disease, are rare inherited lysosomal disorders that primarily affects children and stands as the most prevalent neurodegenerative disease during childhood, with an incidence of 1 in one in 12,500 (Cotman & Staropoli, 2012). Clinically, NCL manifests through seizures, vision loss, deterioration of motor and cognitive abilities, a bedridden state, and premature death. At the cellular level, a defining characteristic is the accumulation of auto-fluorescent ceroid material (lipofuscins) within the lysosomal lumen (Gomez-Giro et al., 2019).

CLN3 and CLN5 mutations are implicated in NCL, along with several other CLN genes. These genes encode various degradative enzymes (such as CLN1, CLN2, CLN10/Cathepsin D), ER-resident integral membrane proteins (CLN8 and CLN6), as well as other transmembrane proteins (e.g., CLN3, CLN7, and CLN12), along with proteins involved in the secretory pathway like CLN11, and two cytosolic proteins, CLN4 and CLN14. Mutations in any of these proteins result in a very similar disease, though with variations in the age of onset. A comprehension of the functions of CLN proteins remains elusive, primarily due to the lack of knowledge regarding their roles in modulating homeostasis through the endo-lysosomal pathway (Butz et al., 2020).

CLN3 causes Juvenile NCL with onset between four and eight years of age (Cotman & Staropoli, 2012); CLN3 is a 438 amino acid protein with six transmembrane domains with N- and C-termini facing the cytosol and three lumenal loops (Nugent et al., 2008), which is located at Golgi, endosomes, and lysosomes (Calgagni et al., 2023). CLN3 has been associated with different cellular dynamics from the lysosomal biogenesis process, promoting lysosomal tubulation for new proto-lysosome formation (Calgani et al., 2023) to Calcium response regulation (Chang et al., 2007). Previous studies related CLN3 to cell structure, actin cytoskeleton regulation in endocytosis, autophagy vesicular trafficking, cell migration, and membrane tethering to it by the interaction of ß-fodrin and myosin-II-b (Getty et al., 2011). CLN3 interaction and modulation of GTPases are demonstrated for Rab7 (Yasa et al., 2020; Yasa et al., 2021), and Cdc42. Cdc42 shows a less active phenotype due to a decrease of recruitment to the GTPase activating protein ARHGAP21 by Arf1 in $CLN3^{KO}$ mouse brain cells causing increasing actin polymerization reducing macropinocytosis (Schultz et al., 2014), and altered lysosomal positioning size, abnormal mitochondria, and less resistance to oxidative stress have also reported in mutant mice (Fossale et al., 2004). CLN3 depletion leads to a deficient sorting of the microdomain associated protein Caveolin-1 and syntaxin-6 traffic from the Golgi to the plasmic membrane, which leads to

an impediment in endocytosis in brain cells and to abnormalities in the blood-brain barrier (Tecedor et al., 2013). All data suggest that CLN3 plays several roles in cellular structure conformation, trafficking, and endocytic dynamics.

It has been shown that CLN3 could have a role in maintaining lysosomal pH (Holopainen et al., 2001). Loss of CLN3 affects cathepsin D processing due to lysosomal alkalinization (Golabek et al., 2000); however, different experiments show different results in the lysosomal pH in CLN3KO cells (discussed at Cotman & Lefrancois, 2021) and the Cathepsin D defect can also be attributed by the effect of CLN3 depletion in cargo transport from trans-Golgi to endosomes and its retrograde recycling. CLN3 also plays a role in calcium sensing by interaction with presenilin and downstream interaction with Calselinin, negatively regulating Ca2+-induced cellular death (Chang et al., 2007). CLN3 is also known as an antiapoptotic gene and appears to be dysregulated in certain types of cancer (Zhu et al., 2014). Modified mouse brains lacking CLN3 show that lysosomes accumulate glycerophosphodiesters (GPDs), showing a catabolic problem (Laqtom et al., 2022).

CLN3 at the endosomes interacts with several proteins involved in Trans-Golgi trafficking, such as Retromer, SNAREs, Sortilin, and CI-MPR (Yasa et al., 2020; Calgani et al., 2023). CLN3 depletion leads to CI-MPR miss trafficking (Metcalf et al., 2008) and alterations in 14 degradative enzymes at the proteome lysosomal like GAA alpha-glucosidase and GusB beta-glucuronidase (Schmidtke et al., 2019; Calgani et al., 2023). CLN3 also appears to have a role in the proper transport from the Golgi of the CI-MPR since CLN3 depletion leads to a decrease in the maturation and cellular activity of lysosomal cathepsins (Metcalf et al., 2008).

CLN3 appears to play a role in facilitating interactions between Rab7 and retromer, and the sorting receptors function as a scaffold protein to modulate interactions between Rab7, retromer, and sortilin. CLN3 is also involved in other endosomal functions like autophagy by regulating Rab7-PLEKHM1 interaction, required for autophagosome to lysosome fusion (Yasa et al., 2020) which causes high levels of the protein marker for autophagy LC3-II correspondeing to the increased number of autophagosomes (Chandrachud et al., 2015). Like Rab7, CLN3 is also palmitoylated (unpublished data). It is unknown how this post-translational modification could modulate any of the mentioned functions; our first approach to this question is by identifying possible PATs interacting with CLN3.

CLN5 is a glycosylated protein comprising 407 amino acids, initially synthesized as a type II integral membrane protein spanning residue 1–407. Subsequently, SPPL3, an intermembrane protease, cleaves CLN5, yielding a mature soluble form (residues 93–407), which is then

conveyed to endolysosomes (Jules et al., 2017). The precise mechanism responsible for transporting CLN5 to the lysosome remains un-elucidated. Notably, neither the mannose-6-phosphate receptor (MPR)-mediated pathway nor the Sortilin-mediated pathway appeared indispensable for CLN5 transport. This conclusion is drawn from observations of lysosomal localization of CLN5 in both MPR-deficient fibroblasts (Schmiedt et al., 2010) and immunoprecipitation of CLN5 with sortilin at different pH (Mamo et al., 2012), even when sortilin do not transport CLN5 interacts with it.

Cells lacking CLN5 expression show a degradation of the CI-MPR and sortilin at the lysosomes. This phenotype is caused by a defect in the recycling machinery for retrograde transport of the receptors back to Golgi; in CLN5KD cells, Rab7 is not capable of recruiting retromer to the membrane; this incapacity of retromer recruitment is not due to an alteration in SNX dimer recruitment, the cause of the recruitment impairment of retromer is less GTP loaded a prerequisite to switch Rab7 on (Mamo et al., 2012). Although the complete function of CLN5 is not well elucidated, it is well known that CLN5 works as part of the endosomal switch that controls whether the receptors are recycled to Golgi or degraded at the lysosomes in the activation machinery of Rab7 rather than acting as an effector itself (Mamo et al., 2012).

In $CLN5^{KO}$ HeLa cells, the result is consistent with the $CLN5^{KD}$ cells; the retromer is less recruited to the membrane; this phenomenon is a consequence of a reduction in Rab7 palmitoylation (Yasa et al., 2021). PTM is required for retromer recruitment by Rab7 to the endosomal membranes (Modica et al., 2017). The disruption in the recruitment of retromer Is accompanied by a degradation of Sortilin at the endosomes by a reduction in interaction between CLN3 and Sortilin with a consequent reduction in interaction between Rab7/retromer and retromer/Sortilin, CLN3 also lose the interactions with all these proteins all these alterations are reflected in changes in the lysosomal function by alterations in hydrolytic enzymes processing like cathepsin D, cathepsin B activity, and β-glucuronidase activity (Yasa et al, 2021). Since CLN5 is a soluble luminal protein, it must be a pathway that allows it to modulate retrograde trafficking in the cytosolic side of the endosome membranes. CLN3 works as a scaffold protein (Yasa et al., 2020) for Rab7/Retromer/Sortilin interactions, and CLN5 interacts with CLN3 to modulate these interactions, forming a complex. While the exact process of Rab7 palmitoylation remains unclear, it is plausible to suggest that CLN5 potentially influences the interaction between the palmitoylation apparatus and CLN3, thereby facilitating Rab7 palmitoylation.

The $CLN5^{KO}$ also showed impairments in the efficacy of the lysosomal degradation of EGFR through weaker alterations of the RILP effector interaction with Rab7 (Progida et al., 2007; Yasa

et al., 2021). This interaction was previously discussed and is necessary to move vesicles by dynein motors to transport lysosomes (Van der Kant et al., 2013; Johanson et al., 2016; Wijdeven et al., 2016), which is a requisite for autophagosomes-lysosomal fusion (Cabukusta et al., 2018). After starvation to induce autophagy in WT cells, lysosomes retrogradely move to the cellular periphery; in CLN5KO cells, the lysosomes do not move as efficiently as in WT, resulting in fewer lysosomes to the periphery; the changes in lysosomal distribution are reflected in autophagy fusion defects (Adams et al., 2019; Yasa et al., 2021).

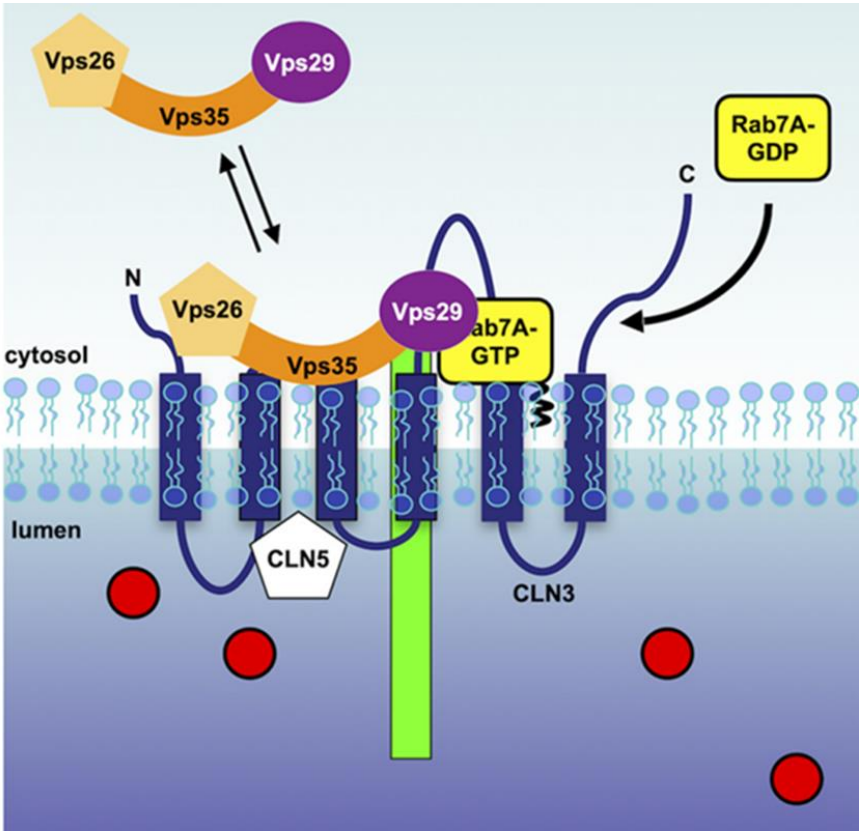


Figure 3.6 Recapitulation image of the association of proteins involved in the retrograde trafficking from the late endosome membrane.

An image showing the role of CLN3 as a scaffold protein shows that CLN5 is represented as a soluble protein that modulates interactions from the luminal side of the endosomal membrane through CLN3. GTP-bound Rab7 recruits a retromer at the cytosolic face that recognizes the endosomal receptor (Sortilin, M6P-R)—reprinted from Meras et al. (2022) (free access article).

4. METHODS

4.1. Cell culture and transfections

HeLa and HEK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Wisent et al.) at 37°C in a humidified chamber at 95% air and 5% CO2. The cells were placed into 12-well plates with a seeding density of 2 × 10^5 cells per well and into 6-well plates with a seeding density of 5 × 10^5 cells per well 24 hours before the transfection procedure. Transfections were performed with polyethylenimine (PEI). Solution 1 was prepared by diluting the plasmid into Opti-MEM (ThermoFisher Scientific). Solution 2 was prepared by diluting PEI (1 µg/µl) into Opti-MEM in a ratio of 1:4 with the DNA to be transfected. After a 5-minute incubation, the two solutions were mixed, vortexed for 3 s, incubated at room temperature (RT) for 15 min, and added to the cells. Subsequent experiments were performed 48 hours after transfection.

4.2. CRISPR/CAS9

We used CRISPR/Cas9 technology to engineer knockout HEK or HeLa cells. Lines already generated include NEK7 knockout ($NEK7^{KO}$), $DHHC8^{KO}$, and $ZDHHC11^{KO}$ cells. Briefly, cells were transfected with an all-in-one plasmid containing Cas9, the guide RNA, and a fluorescent section marker. Forty-eight hours post-transfection, cells were sorted based on fluorescence expression (one cell per well) and left to grow. The KO efficiency was tested using Western blotting and confirmed using sequencing. $CLN5^{KO}$ and $Rab7^{KO}$ were previously described (Yasa et al., 2021; Yasa et al., 2020).

4.3. Stable cell lines generation

For rescue experiments, HEK293 $NEK7^{KO}$ cells were transfected with 500ng of HA-NEK7 plasmid and HA-$NEK7^{K64M}$ plasmid using PEI in a concentration of 1:4. Forty-eight hours after transfection; the cells were exposed to G418 antibiotic at a 500 ng/ml concentration in DMEM 10% SBF. Non-transfected cells were used as a reference point to measure the lethal dose. After the treated cells that were not positively transfected died, the cell was kept in the culture at a concentration of half of the lethal dose (250ng/ml). These stable cell lines were used in Cathepsin L and separation membrane assays since an elevated percentage of transfected cells were required to rescue phenotypes.

4.4. Bimolecular fluorescence complementation (BiFC).

HEK cells were seeded in 12-well plates and co-transfected with plasmids VN-Rab7 and CC-DHHCs and, in an alternative way, CC-Rab7 with VN-DHHCs. Forty-eight hours post-transfection, cells were washed in PBS, detached with five mM EDTA in PBS, and collected in 500 ml of PBS. The cell quantity was adjusted using a spectrophotometer; cells were transferred to black 96-well plates in triplicates, and total fluorescence was first measured with the Tecan Infinite M1000 Pro plate reader with the excitation and emission set at 510 nm and 530 nm, respectively. Cells transfected with salmon sperm were used to control the fluorescence background. The final fluorescence was calculated by subtracting the cellular background from the initial fluorescence.

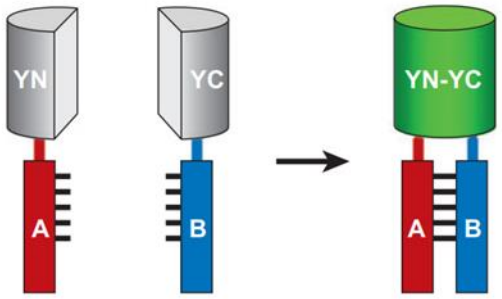


Figure 4.1 BiFC assay illustration.

BiFC assay illustration of Two inactive fragments (YN and YC) of the yellow fluorescent protein (YFP) joined with interacting proteins (A and B). A dual-component fluorescent complex is created when these interaction partners come together (Kerppola, 2008).

4.5. Bioluminescence resonance energy transfer (BRET)

HEK cells were seeded in 12-well plates and co-transfected with plasmids Rluc-Rab7, VPS26-Venus, and Rluc-Rab7 with Sortilin-YFP. Forty-eight hours post-transfection, cells were washed in PBS, detached with five mM EDTA in PBS, and collected in 500 ml of PBS. Cells were transferred to white 96-well plates in triplicates. Total fluorescence was first measured with the Tecan Infinite M1000 Pro plate reader with the excitation and emission set at 500 and 530 nm for BRET1. The substrate h-coelenterazine was added to all wells (5 mM final concentration), and luminescence signals were measured 2 minutes later. The BRET signals were calculated as a ratio of the light emitted at 525 ±15 nm over the light emitted at 410 ±40 nm. The BRETnet signals were calculated as the difference between the BRET signal in cells expressing both fluorescence and luminescence constructs and the BRET signal from cells where only the luminescence fused construct was expressed, as explained in Yasa et al. (2021).

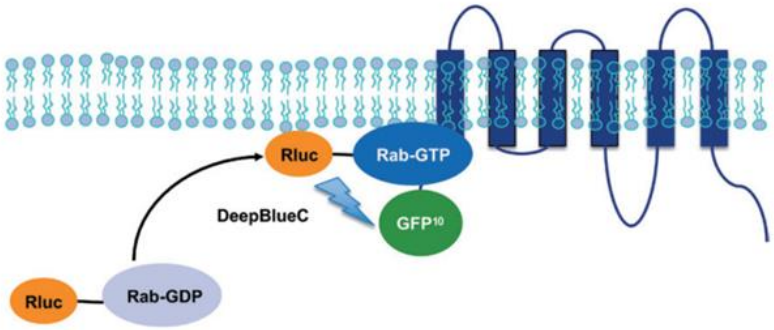


Figure 4.2. BRET assay illustration.

Representation of Rab Rluc-tagged protein interacting with a transmembrane protein with the transference of energy product of the interaction. Take from Savageau and Lefrancois (2019).

4.6. Western blot

For Western blotting analysis, cells were first detached using a solution containing five mM EDTA in PBS, followed by a rise in 1× PBS and subsequent collection through centrifugation. To lyse the cells, a TNE buffer (composed of 150 mM NaCl, 50 mM Tris at pH 7.5, 2 mM EDTA, 0.5% Triton X-100, and a protease inhibitor cocktail) was utilized, with incubation on ice for 30 minutes. Afterward, the lysates underwent high-speed centrifugation for 10 minutes, and the resulting supernatants (comprising the whole cell lysate) were prepared for Western blotting analysis. The prepared samples were combined with a 3× sample buffer to reach a final concentration of 1× (containing 62.5 mM Tris–HCl at pH 6.5, 2.5% SDS, 10% glycerol, and 0.01% bromophenol blue). These samples were then heated to 95°C for 5 minutes and subjected to separation through SDS–PAGE, followed by wet-transfer onto nitrocellulose membranes. The subsequent detection was carried out by immunoblotting using the specified antibodies. Quantification was performed using ImageJ.

The following mouse monoclonal antibodies were used: Anti- tubulin (Wb: 1:8000, Sigma T9026); anti-Lamp2 (Wb: 1:1000, source), anti-HA (Wb: 1:1000, Origen TA180128) anti-Cathepsin D (1:100, source). The following rabbit monoclonal antibodies were used: anti-Rab7 (Wb 1:1000, Abcam ER7589). The following rabbit polyclonal antibodies were used: anti-VPS26A (Wb: 1:1000, Abcam ab23892), anti-phospho-S72 Rab7 (Wb 1:1000 Abcam Ab302494), anti-NEK7 (Wb: 1:1000, Cell Signaling C34C3).

4.7. Membrane separation assay

After a 24-hour transfection in 6 healthy plates, cells were harvested using a solution containing five mM EDTA in PBS. These cells were rapidly frozen by immersion in liquid nitrogen and thawed at room temperature for 5 minutes. The cells were resuspended in Buffer 1 (0.1 M Mes-NaOH at pH 6.5, 1 mM MgAc, 0.5 mM EGTA, 200 µM sodium orthovanadate, and 0.2 M sucrose), followed by centrifugation at 10,000 g for 5 minutes at 4°C. The resulting supernatant containing the cytosolic proteins (S, soluble fraction) was collected.

The remaining pellet was resuspended in Buffer 2 (50 mM Tris, 150 mM NaCl, one mM EDTA, 0.1% SDS, and 1% Triton X-100) and centrifugated at 10,000 g for 5 minutes at 4°C. Equal volumes of the samples were loaded into SDS–PAGE gels. The intensity of the bands was quantified using ImageJ software. To determine the protein distribution, the intensity of each fraction was calculated and divided by the total intensity.

4.8. Lysosomal activity

4.8.1. Cathepsin B and L:

To evaluate Cathepsin D and L activity, cells were cultured in 12 healthy plates and then collected to a density of 3 x 106 cells/ml. The cells were then transferred to a 96-well black plate in triplicate. The Magic Red reagent (Bio-Rad) was added to PBS for 60 min at 37 °C and protected from light. The fluorescence intensity was measured with a Tecan Infinite M1000 Pro plate reader (Tecan et al., Switzerland) with the excitation and emission set at 592 nm and 628 nm, respectively. signals were standardized using Hoechst stain.

4.9. Acyl-rac (Resin-Assisted Capture)

Cells were lysed with RIPA buffer 30min at four °C supplemented with 50nM N-ethylamine (NEM) to block the free thiol groups. Samples were then centrifugated for 10 min at 13000 RPM at 4°C. The supernatants were incubated for two hours at RT on a rotating wheel. Samples were precipitated overnight with two volumes of cold acetone at −20°C. The next day, the samples were washed with cold acetone and centrifugated. The pellet was air dried and resuspended in binding buffer (BB) (100 mM HEPES, one mM EDTA, 1% SDS) with 250 mM hydroxylamine (Sigma–Aldrich) (NH2OH) pH7.5 to cleave the thioester bond between the palmitoyl group and the modified cysteine residues. Control samples were resuspended in BB containing 250 mM NaCl but no hydroxyl amine and incubated for 2 hours, rotating at room temperature until the

pellets were completely diluted. 50 µl was saved as input. Thiopropyl sepharose beads were added, and samples were incubated for two hours at RT on a rotating wheel. Beads were washed four times with BB, and captured proteins were eluted with a sample buffer containing 100 mM DTT.

4.10. Statistical analysis.

Statistical analysis was conducted using GraphPad Prism Version 8.2.1 (GraphPad Software, San Diego, CA, USA; www.graphpad.com). The data was analyzed as described in each figure the type of statistical analysis was performed according to the data nature, of the statistics used are Ordinary one-way ANOVA and Two-way ANOVA with a Dunnett's or Turkey post hoc test and nonparametric T-test.

5. RESULTS

Previous work in our laboratory suggests that Phosphorylation at Serine72 is required for Rab7 retromer recruitment. The involvement of previously known interacting kinases LRRK1 and TBK1 was tested by chemical inhibitors, siRNA, and CRISPR-cas9 without any effect on the retromer membrane distribution (Unpublished data Modica et al., 2024). We hypothesize that this pathway is controlled by a different kinase that has not been described before as an interacting protein of Rab7. In this study, we assess NEK7, a mitotically active kinase, as a potential candidate responsible for phosphorylating Rab7 and facilitating subsequent recruitment of retromer proteins. Additionally, previous research has hinted at the involvement of the mitotic kinase CDK1 in phosphorylating Rabs, exemplified by the early findings of Rab1 phosphorylation (Bailly et al., 1991).

NEK7 is a Serine/Threonine Kinase that is a member of the NIMA-related Kinases (Never in mitosis) family characterized by a lack of C-terminal domain (NEK) (Fry et al., 2012). The protein structure is limited to a kinase domain with a brief N-terminal region, which could hold significance in substrate recognition, like in the NEK6 protein structure, which is similar at 87 percent. Nevertheless, the two proteins do not share the same interactome (Vaz et al., 2010; De Souza et al., 2014). NEK7 is involved in different cellular functions according to its interacting protein and kinase-dependent or independent activity. Most of the literature about NEK7 surrounds the fact that this protein is implicated in the assembly and activation of NLRP3 inflammasome during mitotic events. NLRP3 works as an inflammasome sensor that interacts directly with NEK7 to start an inflammatory response to stimuli such as ROS, potassium efflux, lysosomal destabilization, NF-κB signalling, protein misfolding aggregation, and toxicants (Xu et al., 2016; Sharif et al., 2019; Anderson et al., 2023). This pathway does not appear to require NEK7 catalytic activity. However, regulating the microtubule dynamic instability and facilitating spindle assembly necessitate the catalytic activity of NEK7, especially in interphase (He et al., 2016). NEK7, in its mitotic role, interacts with NEK9, competing and regulating mitosis and inflammation events; when NEK7 interacts with NLRP3, it can no longer interact with NEK9 for mitosis (Shi et al., 2016; Sharif et al., 2019).

Zhang et al. (2023) revealed the intricate connection between endocytic trafficking involving the NEK7 interactor NLRP3. Their study demonstrated that destabilizing binding sites between the endoplasmic reticulum and endosome membrane results in an accumulation of phosphatidylinositol 4-phosphate (PI4P) within endosomes. This accumulation subsequently

leads to the retention of retrograde transport of CI-MPR and TGN46 cargo with most of the protein located at the endosomes, ultimately facilitating NLRP3 recruitment to the endosomes. However, in this study, it is unclear how NLRP3 disrupts the trafficking, and NEK7 was unrelated to the process.

In general, it has been shown that NIMA-related Kinases could have an essential role in controlling intracellular trafficking as knockdown of NEKl-3, C. elegant ortholog of NEK7, generates defects in endocytosis that NEK7 and NEK6 can rescue in NIMA mutant worms. These functions are conserved between species since human protein can rescue the phenotype (Joseph et al., 2020). A recent study shows more than 50 percent colocalization between NELKL-3, Rab7, and Rab5, which leads to the association of human NEK7 with endosomal structures. Other NEKL homologous proteins to NEK8 have a colocalization mainly at the early endosomes, and its depletion generates an increase in early endosome size but not late endosomes, which suggests that different NEK proteins could be involved in different steps of endocytic trafficking. Moreover, there is no colocalization between NEKL-2 and NEKL-3, suggesting different functions, contrary to NEK-2 depletion NEKL-3 depletion led to substantial defects at both early and late endosomes (Joseph et al., 2023).

Joseph et al., 2023 found that changes in the retrograde pathway from endosomes to the Trans-Golgi network seem to be affected by knocking down either NEK6 or NEK7, leading to the missorting and degradation of CI-MPR from endosomes, which can be attributed to a problem related to retromer (Arighi et al., 2004). However, how NEK7 depletion leads to trafficking defects was not shown. The depletion of NEK7 also increases the size and number of endosomes, possibly affecting fusion/fission events. However, this has not been tested directly (Joseph et al., 2023).

Considering this information, we decided to generate $NEK7^{KO}$ Cell lines using CRISPR-Cas9 technology, test if NEK7 phosphorylates Rab7 at S72, and evaluate its role in retromer recruitment. Forty-two single-clone cells were evaluated using an NEK7-specific antibody (Figure 5.1A). Only the clones in which its tubulin control was expressed were considered KO positive; we got 28 out of 42 KO positives, from which we selected clone number 11 for the following experiments (Figure 5.1B)

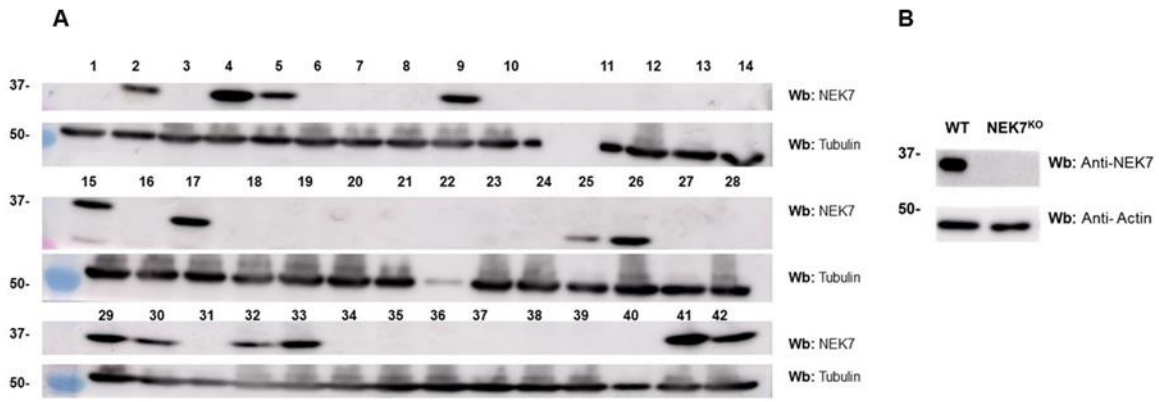


Figure 5.1. Screening for NEK7^{ko} cell line.

The figure shows the screening of different single-cell clones cell lines after CRISPR-cas9 treatment (A). The final clone selected for the experiments showed the non-expressed protein NEK7.

5.1. NEK7 Phosphorylates Rab7 at S72.

We tested if NEK7 was required for Rab7 phosphorylation using an antibody raised to detect phosphorylated Rab7 at serine 72. Using this antibody, we found a decrease of phosphorylated Rab7 in NEK7^{KO} cells (Figure 5.2), which was rescued by expressing wild-type NEK7 (HA-NEK7), but not by expressing the kinase-dead mutant (HA-NEK7^{K64M}). The signal of phosphorylated Rab7 does not disappear entirely, most likely due to the other kinases that modulate Rab7 at serine72. Rab7 as a target for NEK7 phosphorylation was not reported previously.

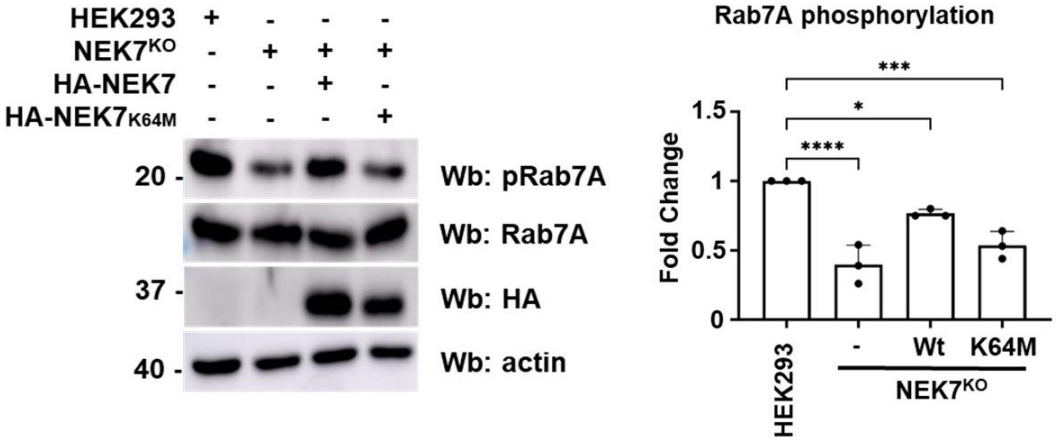


Figure 5.2 Rab7 Phosphorylation levels at Serine72

Cell lysates derived from HEK293, NEK7^{KO} and NEK7^{KO} cells expressing either wild-type HA-NEK7 or the kinase-dead mutant HA-NEK7 ^{K64M} were analyzed via Western blot (Wb) using specific antibodies against phosphorylated Rab7A at serine 72 (pRab7A), total Rab7A, HA-tag (to detect HA-NEK7 variants), and actin (used as a loading control). Rab7A serine 72 phosphorylation was quantified across three independent experiments and is presented as mean ± standard deviation

(SD). Statistical significance was determined using one-way ANOVA with Tukey's post-hoc test, where significance levels are indicated as * ($P < 0.05$), *** ($P < 0.001$), and **** ($P < 0.0001$). Rab7 Phosphorylation levels at Serine72 with a decrease in phosphorylation in NEK7ko vs. WT HEK, the phenotype was rescued by transient transfection of Ha-NEK7 in NEK^{KO} cells. In contrast, the kinase death mutant Ha-$NEK7^{K64M}$ does not rescue the phenotype.

5.2. Phosphorylation of S72 in Rab7 is required to recruit Retromer.

To confirm that NEK7 regulates endosome-to-TGN retrieval through retromer, we evaluated the membrane distribution of retromer in $NEK7^{KO}$ cells. We found a 20 percent increase in the retromer subunit VPS26 in the cytosolic fraction in $NEK7^{KO}$ cells compared to WT cells (Figure 5.3 A, B), this increase in the cytosolic fraction was comparable with the retromer distribution in $Rab7^{KO}$ cells suggesting a similar impact on retromer localization between NEK7 and Rab7 knockout cells. To investigate the impact of NEK7 kinase activity, we introduced either wild-type HA-NEK7 or the kinase-dead mutant, HA-$NEK7^{K64M}$, into NEK7 knockout HEK293 cells and established stable cell lines through G418 selection. Subsequently, we conducted a membrane separation assay to assess the effects of these NEK7 variants on cellular membrane dynamics. The phenotype was rescued in a cell line stably expressing HA-NEK7 while expressing the kinase-dead mutant (K64M) did not rescue the membrane distribution of retromer (Figure 5.3 C, D).

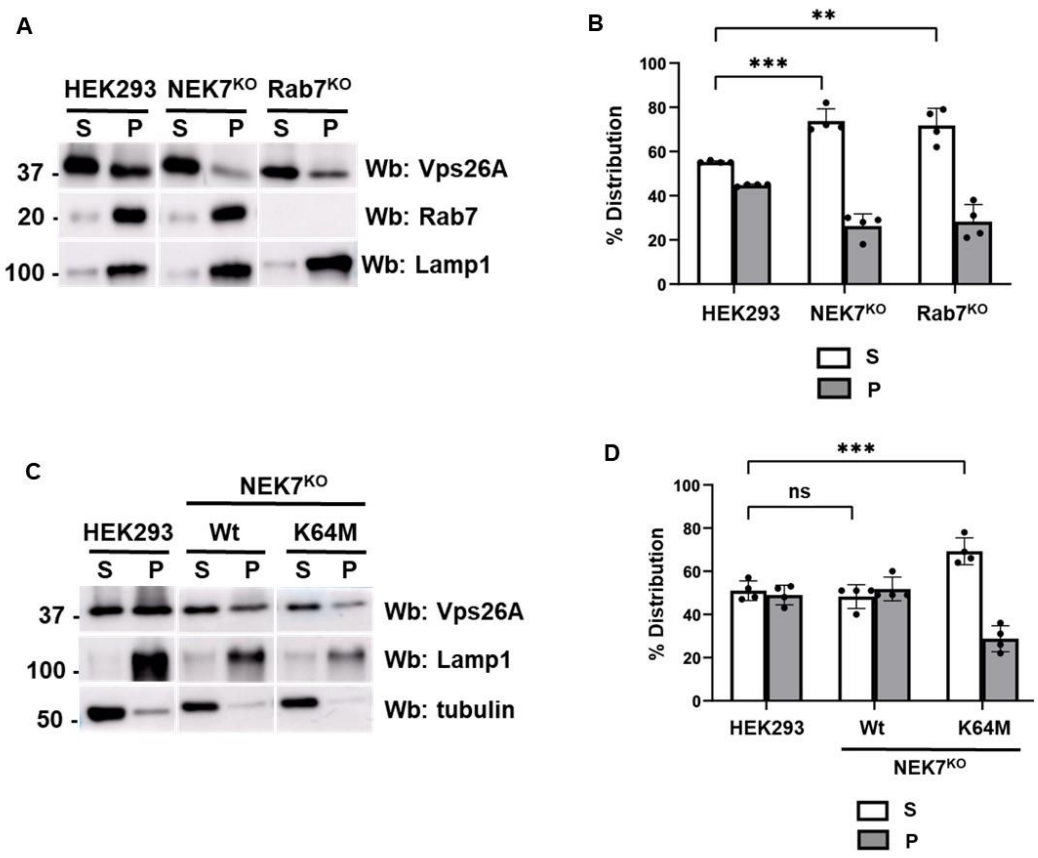


Figure 5.3 VPS26 Retromer subunit distribution at cytosol with Rab7 in NEK7^{ko} cells and rescues with stable cell lines.

VPS26 Retromer subunit distribution between the cytosolic space and the membrane-bound protein, staining for the membrane control LAMP1, and tubulin for the cytosolic fraction. (A, B) Quantitative analysis based on three independent experiments shows that NEK7^{KO} HEK293 cells exhibited reduced membrane-bound retromer levels (26.25% in the pellet fraction) compared to wild-type HEK293 cells (44.75% in the pellet fraction). NEK7^{KO} cells showed comparable levels of membrane-bound retromer to Rab7^{KO} HEK293 cells (28.25% in the pellet fraction), (C, D) Quantitative analysis based on three independent experiments NEK7^{KO} HEK293 cells expressing HA-NEK7 exhibited retromer distribution comparable to wild-type HEK293 cells (51.64% in the pellet fraction). In contrast, NEK7^{KO} HEK293 cells expressing HA-NEK7^{K64M} showed no significant improvement in retromer distribution (30.32% in the pellet fraction) data analysis two ways ANOVA with Dunnett post-hoc test, where significance levels are indicated as * ($P < 0.05$), *** ($P < 0.001$), and****($P<0.0001$).

We also evaluated the interactions between Rab7 and VPS26 by BRET to confirm this data. BRET is a technique that evaluates protein-protein interactions (PPI) in live cells without destroying the cellular context, like membranes where some interactions occur. In contrast to immunoprecipitation and other pull-down methods where cell lysates are necessary. The strength of the interaction is measured by calculating the BRET50, which is inverse to the probability of

two proteins interacting. We found lower BRET50 in NEK7KO cells (Figure 5.4 A, B), representing a weaker interaction; the expression of NEK7 rescues the interaction (Savageau et al., unpublished). This data suggests that NEK7 modulates retromer recruitment mediated by Rab7.

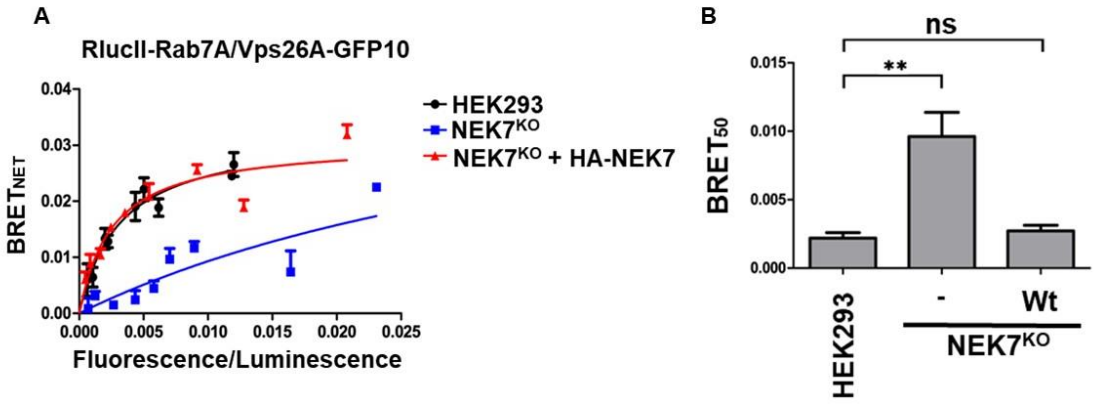


Figure 5.4. Evaluation of the interactions between Rab7 and VPS26 in live cells by BRET.

Evaluation of the interactions between Rab7 and VPS26 in live cells by BRET. BRET50 is inverse to the probability of two proteins interacting; a lower BRET50 represents a more vital interaction, A 4.3-fold increase in BRET50 was observed in NEK7^{KO} HEK293 cells for the interaction between RlucII-Rab7A and VPS26A-GFP10 compared to wild-type cells (BRET50 values of 0.0096 ± 0.0039 and 0.0022 ± 0.0009, respectively), indicating a substantially weaker interaction. This weaker interaction was restored by expressing wild-type HA-NEK7 (BRET50 value of 0.0027 ± 0.0009). The mean BRET50 values derived from three separate experiments are presented, along with the corresponding standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey's post-hoc test, where significance levels are denoted as ** ($P < 0.01$) and NS (not significant). Sauvageau et al. (Unpublished data).

5.3. NEK7 is Required for Rab7 Palmitoylation

Since Rab7 Palmitoylation is required for recruiting retromer (Modica et al., 2017), we wonder if Rab7 palmitoylation could be affected by the depletion of NEK7; using acyl-rac, we found a decrease of Rab7 palmitoylation in NEK7^{KO} cells, a phenotype that was rescued by the transient transfection of HA-NEK7 but not by the kinase death mutant (Figure 5.5). This data shows an interplay between phosphorylation at Serine72 and Rab7 palmitoylation.

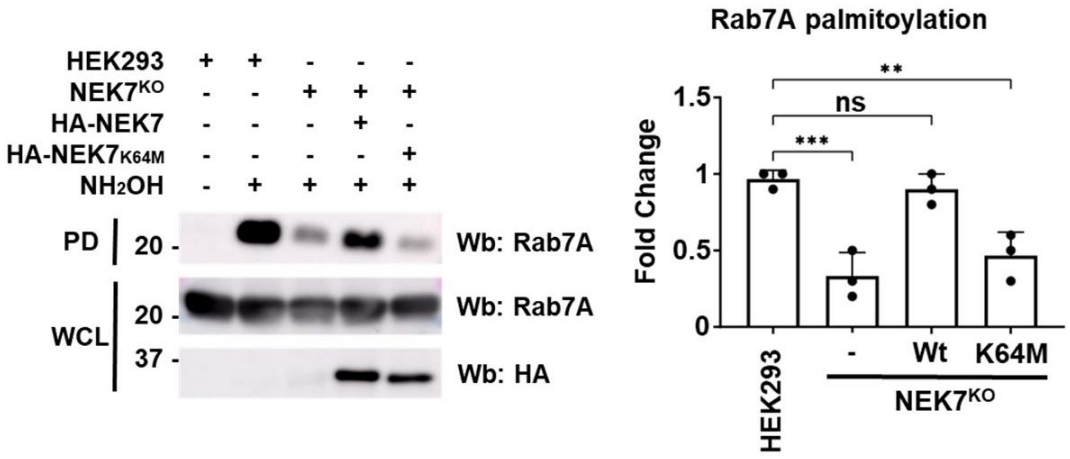


Figure 5.5 Rab7 Palmitoylation in NEK7KO cells vs HEK WT cells

HEK293 cell lysates, along with NEK7^{KO} and NEK7^{KO} cells expressing either wild-type HA-NEK7 or the kinase-dead mutant HA-NEK7^{K64M}, were subjected to Acyl-RAC to assess Rab7A palmitoylation levels. The procedure involved NH2OH treatment, pulldown (PD), and whole cell lysates (WCL) analysis. Quantitative analysis based on three independent Acyl-RAC assays was conducted to determine Rab7A palmitoylation levels. Data are expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey's post-hoc test, where significance levels are denoted as NS (not significant), ** ($P < 0.01$), and *** ($P < 0.001$).

5.4. Lysosomal function is disrupted in NEK7ko cells.

As we found before, NEK7 modulates the Endo-lysosomal pathways affecting the recruitment of retromer to the membrane, and in consequence, Sortilin recognition, we wonder if lysosomal function could be affected, we evaluate the maturation process of cathepsin D using specific antibodies and the lysosomal activity of cathepsin B and L using Magic red (MR), which is a Cell-permeant reagent that enters through the membranes to the cell without necessity of lysis. Specific target sequences confer the specificity for each cathepsin. After cleaving it, the reagent emits fluorescence and aggregates inside the lysosomes and other organelles with low pH. When the protease activity has a place in time, more Magic Red is cleaved, emitting more fluorescence.

We found an increase in intermediate cathepsin D in comparison to WT; we use Rab7ko cells to compare phenotypes, while in NEK7ko cells, the intermediate form increases in Rab7ko cells pro cathepsin D increases and the mature form decreases, which could be interpreted as a missorting of the iCatD reducing mCatD (Figure 5.6 A, B).

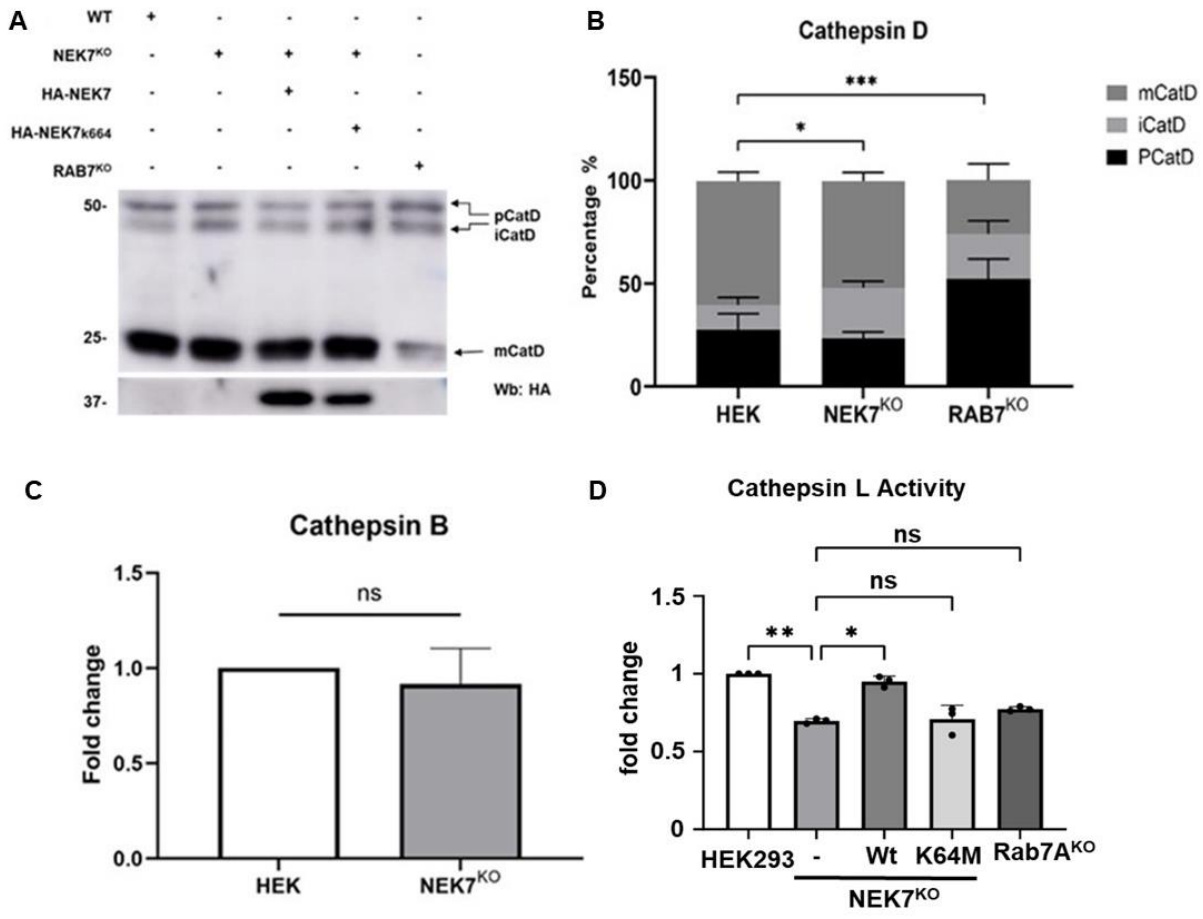


Figure 5.6 Cathepsin D processing (A, B), cathepsin B intensity signal (C), Cathepsin L signal

(A, B) Cathepsin D processing from total lysates from three individual experiments, data analyzed using Two-way ANOVA with Dunnett's post-hoc test significance levels are indicated as * ($P < 0.05$) and *** ($P < 0.001$) (C) Cathepsin D Magic Red substrate fluorescence was measured in three independent experiments data was analyzed using t-test, NS denotes no significance (P:0,4021) (D) WT, NEK7^{KO} and NEK7^{KO} cells expressing HA-NEK7 or HA-NEK7^{K64M}, and Rab7^{KO} HEK293 cells were treated with the Cathepsin L MagicRed substrate for 60 minutes. Quantitative analysis based on three independent experiments was conducted. Data are presented as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA with Dunnett's post-hoc test, where significance levels are indicated as *** ($P < 0.001$) and **** ($P < 0.0001$), while NS denotes no significance.

The difference in the cathepsin L phenotype could be compensated by cathepsin B, and this phenotype could explain the change in the processing in cathepsin D that requires cathepsin L. Reducing functional Cathepsin L into the lysosomes could alternate cathepsin D processing efficiency. However, the mature form of cathepsin D does not change in NEK7^{KO}, and the expected phenotype in the cathepsin B signal can explain this. The low activity of cathepsin L was rescued by transfection of WT NEK7 but not by the phosphonull mutant (Figure 5.6, D).

5.5. CLN5 and CLN3 are not required for Rab7 phosphorylation at Serine72.

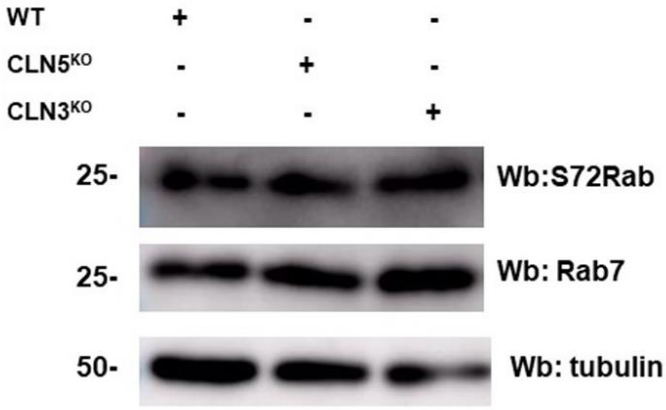


Figure 5.7 Rab7 Phosphorylation at S72 in CLN3^{KO} cells and CLN5^{KO} cells

Phosphorylation levels of Rab7 in CLN3^{KO} and CLN5^{KO} cells vs. Hela WT cells with the total Rab7 and the tubulin as control.

Since CLN5 is required for the Palmitoylation of Rab7 and CLN3 is required for the functioning of retromer (Yasa et al., 2021), we wondered if the modulation of retromer functioning could be by modulating phosphorylation and consequently showing an effect in Rab7 Palmitoylation. We did not find differences in the levels of Rab7 S72 phosphorylation in CLN5^{KO} and CLN3^{KO}, which suggests that CLN5 modulates retromer recruitment directly thought Rab7 palmitoylation after phosphorylation by NEK7.

5.6. ZDHHC11 Palmitoylates Rab7

Previous research in our lab showed that Rab7 needs to be palmitoylated to recruit retromer (Modica et al., 2017). In CLN5 knockout (CLN5^{KO}) HeLa cells, Rab7 is less palmitoylated than wild-type cells (Yasa et al., 2021). Those findings led us to propose that CLN5 plays a crucial role in the palmitoylation process of Rab7 and could mediate the Rab7/PAT interaction. To understand the Rab7 Palmitoylation and phosphorylation dynamics and their implications in Neuronal Ceroid Lipofuscinosis, we must identify the PAT that Palmitoylates Rab7. Our first approach was to evaluate the possible interactions in WT cells vs CLN5^{KO} cells since Palmitoylation of Rab7 is reduced in CLN5^{KO}, causing a reduction in retromer recruitment to the endosome membrane (Yasa et al., 2021). As an initial approach to test which PAT is palmitoylating Rab7, we used biomolecular fluorescence complementation (BiFC) to screen changes in Rab7-PAT interactions.

BiFC is a technique used to identify proteins that interact in live cells. It consists of complementing two halves of a fluorescent protein attached to two proteins of interest. When the two interacting proteins are close enough to interact, the halves of the fluorescent proteins get together, emitting a fluorescence signal. Among the 20 PATs tested, we observed strong interactions between Rab7 and DHHC2, DHHC6, DHHC8, and ZDHHC11. The interactions of ZDHHC11 were diminished in CLN5KO (Figure 5.8 A).

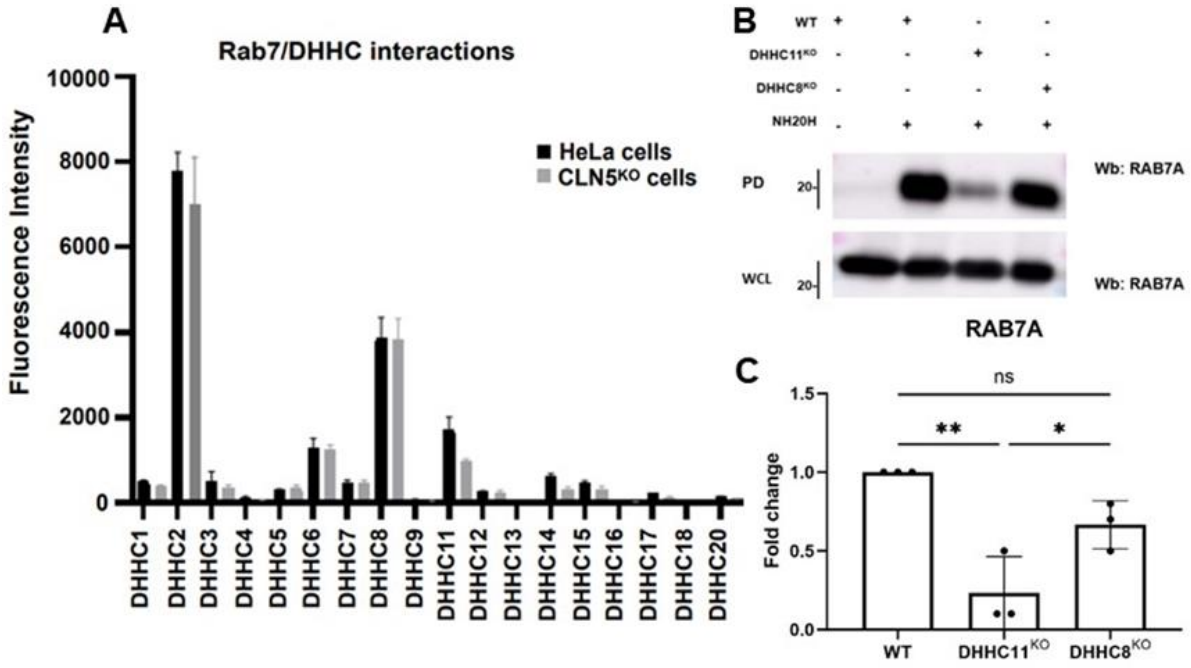


Figure 5.8 Rab7 Interactions with DHHCs proteins and Acyl Rac for Rab7 in DHHC11ko cells

(A) Fluorescence signal screening of interactions between Rab7 and different PATS in WT cells vs CLN5KO cells, (B, C) WT, ZDHHC11^{KO} cells, and ZDHHC8^{KO} cells were subjected to Acyl-RAC to assess Rab7A palmitoylation levels Pull down of Palmitoylated Rab7 quantification of Pull down palmitoylated Rab7 from three independent experiments data are expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey's post-hoc test, where significance levels are denoted as NS (not significant), * ($P < 0.05$) and ** ($P < 0.01$).

To further characterize these PATs, we used CRISPR-CAS9 to deplete DHHC8 and ZDHHC11. We found significantly less palmitoylated Rab7 in DHHC11^{KO} cells compared to the wild type, while loss of DHHC8 does not affect the levels of Rab7 palmitoylated protein. Several DHHCs appear to interact with Rab7; however, to confirm if the interaction Is not due to a random collision, further experiments must be done with the most precise method, such as BRET.

5.7. ZDHHC11 Palmitoylation is required to recruit a retromer.

To confirm if ZDHHC11 was the PAT involved in Retromer recruitment, we separated the membrane cellular fraction from the cellular cytosolic fraction and quantified the retromer subunit in ZDHHC11^{KO} cells and DHHC8^{KO} cells. We found an increase in the cytosolic fraction in the VPS26 subunit by 15% in DHHC11^{KO} cells, but no changes in DHHC8^{KO} cells. This suggests the possibility of more than one PAT modulating retromer recruitment by Rab7 palmitoylation (Figure 5.9, A and B). We quantified the Rab7 distribution to confirm that the changes in VPS26 distribution were not caused by an effect in Rab7 recruitment to the membranes (Figure 5.9, C). Our results show no changes between the different cell lines, attributing the changes to the capacity of Rab7 in the recruitment of retromer.

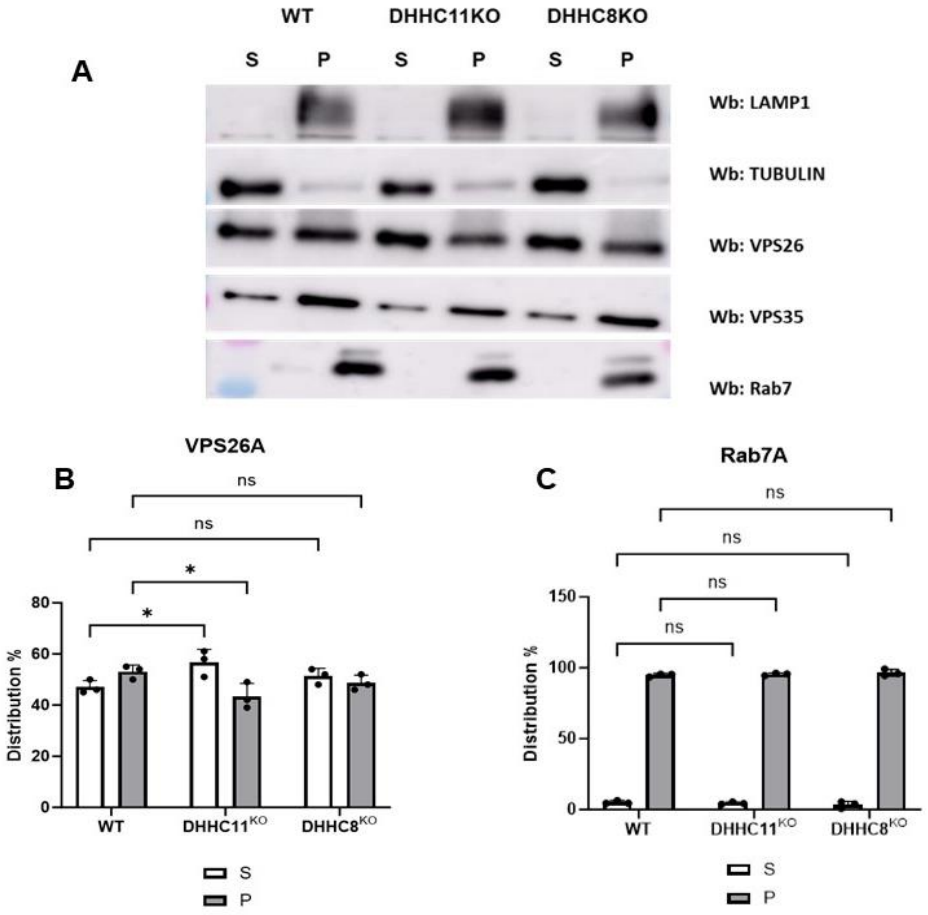


Figure 5.9 Retromer membrane distribution of VPS26 in ZDHHC^{KO} cells.

(A) Western blot in membrane assay for VPS26 control of the cytosolic fraction (tubulin), control for the Membrane fraction (LAMP), and Rab7. (B) Quantification of VPS26 distribution. (C) Quantification of Rab7 distribution. statistical analysis was performed with data from three independent experiments

using ANOVA two ways with Dunnet's post-hoc test where significance levels are denoted as NS (not significant), * ($P < 0.05$).

We will evaluate other possible PATs that could be redundant to ZDHHC11. Some of the options are based on evaluations by BRET in NEK7^{KO} cells WT and CLN5^{KO}. Then, using $_{Si}$RNA interference, we will realize double knockdowns to evaluate the membrane distribution in the retromer. Additionally, in the future, BRET is going to be used to evaluate interactions between ZDHHC11 and Rab7 in NEK7^{KO} cells, and the interactions between Rab7/Retromer, Retromer/Sortilin in ZDHHC11^{KO} or the double knockouts cell do ZDHHC11/DHHC8 and other combination of PATS.

5.8. NEK7 and ZDHHC11 interplay in retromer interaction with Rab7

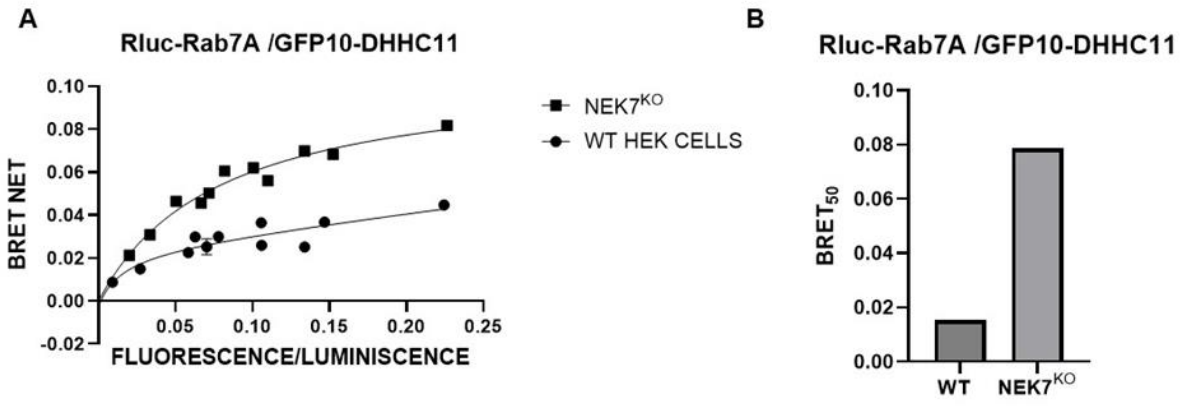


Figure 5.10 DHHC11/Rab7 interaction in NEK7^{KO} cells.

Using BRET, we evaluated the interaction between Rab7 and ZDHHC11. Since the depletion of NEK7 reduces Rab7 palmitoylation, we expected changes in interaction. We found an increase in the BRET50 between the proteins as a reflection of a weaker interaction; however, the BRETmax increases (Figure 5.10); this could be caused by a prolonged time of the ZDHHC11 protein trying to add the palmitate group to Rab7.

Since CLN3 binds Sortilin and Rab7, we wondered if it is also palmitoylated. This would enable all these proteins to localize into an endosomal subdomain, leading us to think that palmitoylation plays a role in locating all the interacting proteins in a specific subdomain at the late endosomes. However, the PATS that perform posttranslational modifications are not known. Using BiFC and bioluminescence resonance energy transfer (BRET), we have also identified DHHC7, DHHC14,

And DHHC15 as potential PATs for CLN3; future work must be done to figure out the role of these interactions in the endosomal network (Figure 5.11)

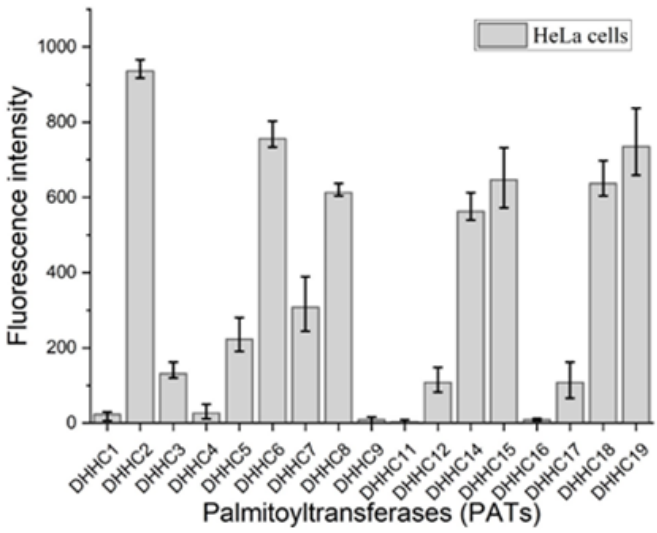


Figure 5.11 CLN3 Interactions with DHHC proteins

Fluorescence signal screening of interactions between CLN3 and different PATS in WT Hela cells.

6. DISCUSSION

Neurodegenerative disorders are frequently characterized by lysosomal dysfunction that could come from changes in pH regulation, enzymatic activity levels, and protein expression and function. Genes regulating lysosomal function are implicated in neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), and Huntington disease (HD) (Udayar et al., 2021; Hu et al., 2015). Correct functioning of the endo-lysosomal system to eliminate misfolding proteins and damaged organelles is required for neuronal survival, proper neuronal signalling, and synapses (Gegg & Schapira, 2022)

These diseases stem from genetic anomalies affecting proteins engaged in lysosomal transport and autophagy-related functions. They can potentially exacerbate protein aggregation, exemplified by the accumulation of Amyloid-β (Aβ) AD and α-synuclein in PD. This interference extends to the trafficking of signalling proteins to lysosomes, as in the case of GBA trafficking (Mazzulli et al., 2011; Das et al., 2013; Bras et al., 2015). In HD, a mutation in the huntingtin protein results in the aggregation of a polyglutamine protein (Walker, 2007). This aggregate hinders cargo recognition by impeding autophagosome biogenesis and transport (Jeong et al., 2009; Rui et al., 2015; Wong & Holzbaur, 2014). Aberrations in CLN12 (ATP13A2), a crucial player in lysosomal acidification, are linked to lysosomal dysfunction in PD (Ramirez et al., 2006) and mutated forms of CLN12 in individuals afflicted with NCL (Bras et al., 2012).

The retromer subunit, VPS35, harbours mutations identified in certain PD patients (Zimprich et al., 2011). ALS initiation might be linked to the disruption of autophagic flux and an accumulation of excessive autophagic vacuoles. (Song et al., 2012). Mutations in -RAB7 result in autosomal-dominant forms of neuromuscular disorder, CharcotMarieTooth disease; these mutations dysregulate endosomal trafficking (Lee et al., 2012) or modify endosomal mediated signalling (BasuRay et al., 2013).

Accurate control of protein assembly within distinct membrane domains is crucial for various cellular processes. Protein clustering initiation, maintenance, and regulation at the plasma membrane remain incompletely understood. Protein palmitoylation, a prevalent post-translational modification, is pivotal in directing proteins to the plasma membrane (Fukata et al., 2015). Consequently, these modified proteins exhibit enrichment within specialized membrane domains. Since Rab7, CLN3, CI-MPR, and Sortilin are palmitoylated, we propose palmitoylation as a common PTM that organizes functional domains in the lysosomal membrane for the correct functioning of the lysosomal trafficking.

This PTM is required for their efficient interaction of Rab7 with retromer (Modica et al., 2017) and, thus, endosome-to-TGN retrieval. The non-palmitoylated receptor is ubiquitinated and degraded in lysosomes (McCormick et al., 2008; Dumaresq-Doiron et al., 2013). However, the DHHCs that Palmitoylate Rab7 are unknown. We identified ZDHHC11 as a PAT involved in retromer recruitment, though the difference in VPS26 distribution is not as high as expected (Figure 8), it is possible that in ZDHHC11^{KO} cells the phenotype could be stabilized and normalized by the cells in time, another option is that there is a redundancy between PATs. There is evidence of specificity and preference in palmitoylate-specific substrates but not exclusivity since Knockdown of the PATs that are specific to the substrates does not diminish its palmitoylation or function (Ohno et al., 2012). Indeed, in yeast, single knockouts cannot affect the palmitate state of palmitoylated proteins an effect is notable when five or more are knockdown (Linder et al., 2013).

DHHCs are classified in families based on sequence similarity, assuming that the particularities in the C-terminal or N-terminal correspond to localization, recognition, and interaction of similar substrates (Gottlieb & Linder, 2017). Similarity and localization may play a role in the redundancy of these proteins masking the phenotype; we could expect that PATs with different cellular localization are less redundant; nevertheless, it is challenging to assume an exclusive localization according to the literature.

Scant information exists regarding the function of ZDHHC11 and most of the palmitoyl transferases (Indranil De, Sushabhan Sadhukhan, 2018). This gene is not ubiquitously expressed in all animal species. Avians, toads, and pigs do not encode ZDHHC11, and other palmitoyl transferases such as DHHC4, 19, and 18 species with fewer DHHC genes than humans do not possess the ZDHHC11 gene (Abdulrahman et al., 2021). However, it is not clear what its function could be regarding Rab7.

DHHCs expressed in HEK293T cells at intra-cellular localization revealed that most of these proteins localize to the ER and Golgi (Ohno et al., 2006). A study in silico by Shinichiro et al., 2013 found that Neurochodrin (Ncdn), a regulator of synaptic plasticity, is palmitoylated by DHHC1 and ZDHHC11 subfamily by microscopy; they found a localization with Rab5 at the early endosomes suggesting that ZDHHC11 may trap the protein to the early endosomes. Other palmitoyl transferases reported to be located at endosome vesicles are DHHC2 (Jia, 2011; Greaves, 2011) and DHHC13 (Singaraja et al., 2002; Huang et al., 2009). However, literature related to PAT localization is contradictory. In future experiments, we will examine the localization between ZDHHC11-Rab7 at the late endosomes and its position with the retromer.

We propose an interplay of palmitoylation and phosphorylation modulating receptor retrieval from endosomes to Golgi. The interplay between palmitoylation and phosphorylation, recognized as a regulatory mechanism in various neurology-related proteins, is exemplified in PDE10A2. The localization of PDE10A is modulated by phosphorylation at threonine 16. The membrane association of PDE10A relies on the palmitoylation of cysteine 11. Phosphorylation at threonine appears to manage PDE10A trafficking and localization by preventing the palmitoylation of Cys-11 (Charych et al., 2010).

NEK7 interaction with NLRP3 is enhanced by NLRP3 palmitoylation mediated by DHHC5 activating inflammasome response (Zheng et al., 2023). In this case, palmitoylation occurs first, facilitating phosphorylation; unlike the NEK7/Rab7 interaction, the relationship between phosphorylation by NEK7 and palmitoylation, in the end, lysosomal trafficking appears to be regulated positively. Our results suggest that Phosphorylation at serine 72 is necessary for Palmitoylation and consequent recruitment of Retromer. However, more experiments are necessary, and identifying the PATs involved in this pathway is required to understand the mechanism fully. Understanding this regulatory mechanism would be enriched by identifying the de-palmitoylation cycles and the thioesterases interacting with Rab7.

As the Sorting process to the endosomes is altered, we suggested that this can affect lysosomal functioning. We found no difference in the Cathepsin B signal, unlike Cathepsin L in $NEK7^{KO}$ (Figure 5.6). The literature suggests an interplay or redundancy between cathepsins, with similar functions and redundant substrates. CTSB and CTSL could compensate for each other's functions (Gocheva et al.,2006). Dosing of pro-cathepsin D, pro-cathepsin L, and pro-cathepsin B can clear the protein aggregates in the NCL10 mice model (Sevenich et al., 2006).

Cathepsin D is a lysosomal protease that undergoes a complex maturation process within the endosomal-lysosomal system. Initially translated as an inactive precursor, pro-cathepsin D, it is translocated to the endoplasmic reticulum and then trafficked to the endosomes (Saftig et al., 1993). Within the endosomes, the N-terminal propeptide is removed, resulting in the intermediate form of cathepsin D. Subsequently, this intermediate undergoes further proteolytic processing in the lysosomes, where the remaining propeptide is cleaved to yield the mature and enzymatically active cathepsin D (Conus et al., 2008). This maturation process is intricately regulated, involving multiple compartments and proteolytic events, ensuring the proper activation and localization of cathepsin D (Sloane, 1990; Brömme & Kaleta, 2002).

The maturation of cathepsin D requires cysteine proteases such as cathepsin L and cathepsin B; an increase in intermediate cathepsin D is notable using inhibitors (Wille et al., 2004; Laurent-

Martha et al., 2006). Mutations in cathepsin D significantly reduce activity and affect stability, post-translational processing, and targeting, resulting in NCL10 diseases (Koike et al., 2000: Steinfeld et al., 2006), like the disease caused by mutations in CLN3 and CLN5 proteins required for retromer recruitment and function (Yasa et al. 2020: Yasa et al., 2021).

Cathepsin D appears to be an essential lysosomal protease that malfunctions leads a strong phenotype of neuropathy mimicking the phenotype in NCL10, impaired autophagy (Koike et al, 2000; Steinfed, 2006; Saftig et al., 2021), the less severe phenotype is shown in cathepsin B and cathepsin L knockout mice, Cathepsin B knockout present no obvious phenotype (Reinheckel et al., 2001), while cathepsin L shows organ-specific phenotypes but not neuromuscular impairment. However, double knockout shows a phenotype that is like the NCL10 phenotype, severe hypotrophy and neuromuscular dysfunction, short life span, autophagy alterations, and neuronal cell death; this phenotype could be rescued by the genomic transgene of human cathepsin L (Sevenich et al., 2006).

7. CONCLUSIONS

We have identified NEK7 kinase and ZDHHC11 palmitoyl transferase as regulators of Retromer recruitment to the endosomal membrane. Phosphorylation of Rab7 at serine 72 is crucial for its palmitoylation and subsequent membrane recruitment. We found that ZDHHC11's interaction with Rab7 for palmitoylation depends on the phosphorylation of serine 72 by NEK7, suggesting a potential mechanism for modulating retromer recruitment. Further investigation is needed to determine if this modulation involves conformational changes in Rab7 that facilitate ZDHHC11's access to its cysteine motifs. Interestingly, CLN5's role in Rab7 palmitoylation appears independent of NEK7 phosphorylation, indicating a direct modulation via Rab7 palmitoylation.

Depletion of NEK7 results in impaired maturation of cathepsin D and reduced Cathepsin L activity due to the retromer's inability to recycle Sortilin. This underscores the importance of NEK7 in maintaining lysosomal function through retromer-mediated sorting mechanisms.

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