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Toxoplasma gondii inhibits the expression of autophagy-related genes through AKT-dependent inactivation of the transcription factor FOXO3a

By

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Abstract

Toxoplasma gondii (*T. gondii*) is an intracellular protozoan parasite that can infect virtually any nucleated cell and a wide variety of warm-blooded vertebrate hosts, including humans, causing toxoplasmosis. While many cases are asymptomatic, symptoms may include fever, lymphadenopathy, and myalgia. In the United States, toxoplasmosis is a notable contributor to foodborne disease-associated mortality. Immunocompromised individuals are susceptible to severe ocular, pulmonary, and cerebral complications, and behavioral changes can occur. The disease results in an annual average of 71 deaths, with toxoplasmic encephalitis significantly impacting AIDS patients.

It is estimated that 30-50% of the world population is seropositive for *T. gondii*, making it one of the most prevalent infections among humans. *T. gondii* can be transmitted through ingestion of oocysts shed in felines feces or undercooked meat from infected animals. *T. gondii* undergoes various life stages, such as tachyzoites and bradyzoites, that are capable of persisting in the host's tissues; additionally, secreted virulence factors play a key role in different stages of infection and are linked to *T. gondii* virulence. Toxoplasmosis is an important public health concern, as there is no available treatment against all forms of the parasite or vaccine for humans. Treatment with drugs can have side effects and is unable to eliminate *T. gondii* cysts from the host.

T. gondii manipulates the host cell to create a safe environment for replication. To do this, the parasite scavenges essential nutrients, alters host metabolism, inhibits apoptosis, and manipulates host autophagy, immune response, and cell cycle progression. *T. gondii* has developed complex mechanisms to create a non-fusogenic parasitophorous vacuole (PV) that helps acquire nutrients while preventing contact with host cytoplasmic components and lysosomal content, that could trigger parasite destruction. The parasite also uses virulence factors to target various aspects of the host cell's biology, such as gene expression, transcriptional machinery, and host protein post-translational modifications.

As a key defense mechanism against infections, autophagy, a cellular process that aids in maintaining cellular homeostasis, is involved in the capture and elimination of intracellular parasites. However, intracellular parasites, such as *T. gondii*, *Plasmodium spp*, *Trypanosoma spp and Leishmania spp,* have developed several evasion mechanisms to manipulate the host

cell autophagy. The PI3K/AKT pathway, is activated in response to physiological circumstances such as growth factor signaling, insulin binding, cell survival signals, nutrient availability, and even stress conditions. It plays a crucial role in regulating cell survival, growth, and metabolism in various cell types. PI3K/AKT pathway also plays a role in inhibiting autophagy while promoting cell growth, differentiation, and survival. Inhibition of this pathway can lead to a significant increase in autophagy. Interestingly, *T. gondii* exploits the host's PI3K/AKT signaling to evade the killing effects of autophagy. This evasion strategy is particularly activated during the early stages of the invasion, where the parasite activates AKT. By doing so, *T. gondii* prevents the accumulation of autophagosome and lysosome components around its PV, which ultimately reduces parasite replication. However, the specific targets of AKT responsible for *T. gondii*'s ability to hinder the host's autophagy machinery are not yet fully understood.

Amongst the different AKT targets, FOXOs (Forkhead Box O subfamily) have been shown to be a major node regulating autophagy. FOXO transcription factors (TFs) are essential regulators of cellular homeostasis and autophagy that are controlled by the PI3K/AKT signaling pathway. When AKT phosphorylates FOXO TFs, it causes them to move out of the nucleus inhibiting their transcriptional activity and, in some cases, promoting their degradation in the cytoplasm or enabling cytoplasmic functions such as binding with other proteins or their entry into other organelles like the mitochondria. Interestingly, autophagy dysregulation is linked to various human diseases, with the FOXO-autophagy axis playing a crucial role in several of these diseases. Therefore, different pathogens manipulate FOXO proteins. As a result, various intracellular parasites use the manipulation of the host PI3K/AKT/FOXO pathway to survive within the host cell.

Using a combination of pharmacological and genetic approaches, herein we investigated whether *T. gondii* hijacks the PI3K/AKT pathway to suppress FOXO3a-regulated autophagy-related transcriptional programs, hindering the activation of the host autophagic response against the parasite. To corroborate this, we first investigated the molecular mechanisms responsible for AKT-dependent phosphorylation of FOXO3a. Then, we determined whether AKT-dependent repression of FOXO3a during *T. gondii* infection affects autophagy-related transcriptional programs. Finally, we established whether FOXO3a-regulated autophagy-related functions are altered during *T. gondii* infection.

We found that *T. gondii* triggers a gradual and sustained AKT-dependent phosphorylation of FOXO3a at residues S253 and T32 in both (human foreskin fibroblasts, HFF) and murine 3T3 fibroblasts. Additionally, live infection is necessary to manipulate and activate the PI3K/AKT signaling pathway, leading to the phosphorylation of FOXO3a. Interestingly, this phosphorylation process is independent of the plasma membrane receptor EGFR and the kinase PKCα. Furthermore, *T. gondii* causes nuclear exclusion of FOXO3a in infected HFF, which is directly correlated with its phosphorylation. However, the parasite cannot drive cytoplasmic localization of FOXO3a when AKT is blocked pharmacologically or when an AKT-insensitive mutant form of FOXO3a is overexpressed. During *T. gondii* infection, the transcription of a subset of autophagy-related target genes of FOXO3a is reduced in an AKT-dependent manner because FOXO3a is inactivated. However, the parasite's ability to repress autophagy-related genes remains unaffected by AKT inhibition in cells where *Foxo3* transcripts are silenced (knockdown). As a result, *T. gondii* fails to inhibit the recruitment of acidic organelles and LC3, an autophagy marker, to the PV when FOXO3a is chemically or genetically retained in the nucleus. In all, we provide evidence that *T. gondii* suppresses FOXO3a-regulated transcriptional programs to prevent autophagy-mediated killing.

In conclusion, autophagy is a cellular process that can be hijacked by several pathogens for replication and survival, but the host has developed countermeasures to prevent its survival. The complex interplay between autophagy and microbial adaptations determines the outcome of host-pathogen encounters. The FOXO family of TFs is crucial for regulating autophagy, and T. gondii manipulates host PI3K/AKT signaling to promote its survival and replication while subverting the host's autophagy process, partly by inhibiting the transcriptional activity of FOXO3a. However, inhibition of the PI3K/AKT pathway or expression of an AKT-resistant form of FOXO3a can promote host cell autophagy and target *T. gondii*, making autophagy an attractive therapeutic strategy against the parasite. Our findings indicate that *T. gondii* hijacks the PI3K/AKT pathway to suppress autophagy-related transcriptional programs under the control of FOXO3a, thereby hindering the activation of the host autophagic response against the parasite. Further characterization of altered transcriptional networks under the control of FOXO3a, and potentially other FOXO family members, during *T. gondii* infection will yield invaluable health-related knowledge to develop effective and safe host-directed strategies for better treatment or prevention of toxoplasmosis.

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List of Abbreviations

14-3-3: 14-3-3 proteins

AIDS: Acquired Immunodeficiency Syndromes

AKT: Protein Kinase B

AMPK: AMP-activated protein kinase

APDS: Activated PI3Kδ syndrome

ATF2: activating transcription factor 2

ATF6: Activating transcription factor 6.

ATG: Autophagy-Related Gene

ATG12-ATG5-ATG16L: Autophagy-related protein 12-5-16 complex

Avt3/Avt4: Amino Acid Transporters

Bad: Bcl-2-associated death promoter

Bax: Bcl-2-associated X protein

BCG: Bacille Calmette-Guérin

BCL-2: B-cell lymphoma 2

Beclin1: Bcl-2-Interacting Protein 1

BIM: Bcl-2 interacting mediator of cell death

BMDMs: Bone Marrow-Derived Macrophages

CaMKKβ: Calcium/calmodulin-dependent protein kinase kinase beta

CAMLG: Calmodulin-binding protein

CAT: Catalase

CBP: CREB-Binding Protein

CCR7: C-C Chemokine Receptor Type 7

CD4: Cluster of differentiation 4

CD40: Cluster of Differentiation 40

CD80: Cluster of differentiation 80

CD86: Cluster of differentiation 86

CDK1/2: Cyclin-Dependent Kinase 1/2

CK1: Casein Kinase 1

c-Myc: Cellular Myelocytomatosis oncogene

CNS: Central nervous system

CREB: cAMP response element-binding protein

DAF: Dauer formation abnormal

DBD: DNA-binding domain

DHFR: Dihydrofolate reductase

DHPS: Dihydropteroate synthetase

dTOR: Drosophila Target of Rapamycin

DYRK: Dual-Specificity Tyrosine-Phosphorylation-Regulated Kinase

EBV: Epstein-Barr virus

EGF: Epidermal growth factor

EGFR: Epidermal Growth Factor Receptor

EGR-1: Early growth response protein 1

eIF2*α*: Eukaryotic translation initiation factor 2 alpha

EL4: Murine T-cell lymphoma cell line

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinase

FAF1: Fas-associated factor 1

FAK: Focal Adhesion Kinase

Fas: Fatty acid synthase

FH: Forkhead

FHRE: Forkhead response element

FIP200: FAK family kinase-interacting protein of 200 kDa

FOXO: Forkhead box O protein

FOXO1: Forkhead box O1

FOXO3a: Forkhead box O3a

FOXO4: Forkhead box O4

FOXP3: Forkhead box P3

GABARAPL1: GABA type A receptor-associated protein like 1

GABARAPL2: GABA type A receptor-associated protein like 2

GADD45A: Growth arrest and DNA damage-inducible protein alpha

GBPs: Guanylate-binding proteins

GCN4: General Control Non-repressible 4

Gi: guanine nucleotide-binding protein

GKS IRGs: Guanylate binding protein (GBP), IFN-inducible GTPase (IRG) superfamily

GKS: p65 guanylate-binding protein

GPCR: G protein-coupled receptor

GRA: Dense granule protein

GSK-3β: Glycogen synthase kinase 3 beta

GTPases: Guanosine Triphosphatases

H2O2: Hydrogen peroxide

HATs: Histone Acetyltransferases

HAUSP: Herpesvirus-associated ubiquitin-specific protease

HBV: Hepatitis B virus

HCMV: Human cytomegalovirus

HCV: Chronic hepatitis C virus

HDACs: Histone Deacetylases

HFF: Human foreskin fibroblasts

HIF-1: Hypoxia-Inducible Factor 1

HIV: Human immunodeficiency virus

HIV-1: Human immunodeficiency virus 1

HNF4α: Hepatocyte nuclear factor-4α

HT-29: Human colon cancer cell line

IAVs: Influenza A viruses

ICAM-1: Intercellular Adhesion Molecule-1

ICP34.5: Herpes simplex virus type 1-encoded neurovirulence protein

IFNγ: Interferon-γ

IKK: Inhibitor of kappa B kinase

IL-10: Interleukin-10

IL-12: Interleukin-12

IL1β: Interleukin-1 beta

IL-23: Interleukin-23

IL-6: Interleukin-6

INK4B: Inhibitor of cyclin-dependent kinase 4B

iNOS: Inducible nitric oxide synthase

IRE1: Inositol-requiring enzyme 1.

IRF: interferon regulatory factor

IRF-3: Interferon regulatory factor 3

IRGs: Immunity-related GTPases

ISGs: Interferon-stimulated genes

IκB: Inhibitor of kappa B

IκB*α*: Inhibitory κB alpha

JAK/STAT: Janus kinase/signal transducer and activator of transcription

JNK: c-Jun N-terminal kinase

LAMP1: lysosomal-associated membrane protein 1

LAMP2: Lysosome-Associated Membrane Protein-2

LC3: Microtubule-Associated Protein Light Chain 3

LC3A: Microtubule-associated protein 1A/1B-light chain 3 alpha

LC3B: Microtubule-associated protein 1A/1B-light chain 3 beta

LC3C: Microtubule-associated protein 1A/1B-light chain 3 gamma

LCMV: Lymphocytic choriomeningitis virus

LKB1: Liver kinase B1

LMP1: Latent membrane protein 1

LMP2A: Latent membrane protein 2A

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinases

MHCI: Major histocompatibility complex class I

MHC-II: Major histocompatibility complex class II

Mi-2/NuRD complex: Nucleosome remodeling and deacetylase complex subunit Mi-2/NuRD

MIC: Microneme protein

MIF: Macrophage inhibitory factor

miRNA: microRNA

MOI: Multiplicity of infection

MST1: Mammalian Ste20-like Kinase 1

mTOR: Mammalian target of rapamycin

mTORC1: Mechanistic target of rapamycin complex 1

mTORC2: Mammalian target of rapamycin complex 2

MYD88: Myeloid differentiation primary response 88

NAD: Nicotinamide Adenosine Dinucleotide

NDPR52: Nuclear dot protein 52

NES: nuclear export signal

NF-κB: Nuclear Factor kappa B

NK: Natural killer cells

NLS: nuclear localization signal

NO: Nitric oxide

Nox4: NADPH oxidase 4

NRF2: Nuclear factor erythroid 2-related factor 2

NSF: N-Ethylmaleimide-Sensitive Factor

p38: p38 Mitogen-Activated Protein Kinase

p53: Tumor protein p53

p62: Sequestosome-1

PAMPs: Pathogen-associated molecular patterns

PARP-1: Poly (ADP-ribose) polymerase-1.

PARylation: Poly-ADP-Ribosylation

PD-1: Programmed death-1

PDK1: 3-Phosphoinositide-dependent protein kinase-1

PDNF: Parasite-derived neurotrophic factor

PE: Phosphatidylethanolamine

PEP4/PRB1: Proteinases A and B

PERK: PKR-like ER kinase

PGRP: Peptidoglycan-recognition protein.

PH: Pleckstrin homology

PI3K: Phosphatidylinositol 3-kinase

PI3KC3: Phosphatidylinositol 3-kinase catalytic subunit type 3

PI3Klll: Class III phosphatidylinositol 3-kinase

PIP2: Phosphatidylinositol 4,5-bisphosphate

PIP3: Phosphatidylinositol 3,4,5-trisphosphate

PKA: Protein Kinase A

PKCα: Protein kinase C alpha

PKCβ: Protein kinase C beta

PKR: Protein kinase R

PP2A: Protein Phosphatase Type 2A

PP2A-B55: Protein phosphatase 2A regulatory subunit B55

PtdIns3K: Phosphatidylinositol 3-Kinase

PTEN: Phosphatase and tensin homolog

PTM: Post-Translational Modification

PV: Parasitophorous Vacuole

PVM: PV membrane

PYR: Pyrimethamine

Rab7: Ras-related protein Rab-7a

rhEGF: Recombinant human EGF

RITK: RhoB-interacting serine/threonine kinase

RON: Rhoptry neck protein

ROP: Rhoptry neck protein

ROS: Reactive oxygen species

SGK: Serum and Glucocorticoid-Regulated Kinase

siRNA: Small interfering RNAs

SIRT1/2/3: Sirtuin 1/2/3

Sit4: 2A-Related Protein Phosphatase

SNARE: Soluble NSF attachment protein receptor

Snf1: Sucrose Non-Fermenting 1

SOCs1: Suppressor of cytokine signaling molecule-1

SOD2: Superoxide dismutase 2

SQSTM1: Sequestosome 1

Src: Proto-oncogene tyrosine-protein kinase Src

STAT-1: Signal Transducer and Activator of Transcription 1

STAT-3: Signal Transducer and Activator of Transcription 3

STAT-6: Signal Transducer and Activator of Transcription 6

T cells: T lymphocytes

TAD: Transactivation domain

TFEB: Transcription factor EB

TFs: Transcription factors

TgESA: *T. gondii* excretory/secretory antigen

TGF-β: Transforming growth factor–β

TgIST: *T. gondii Inhibitor* of STAT1 Transcriptional activity

TGN: Trans-Golgi Network

Th1: T-helper cell type 1

Th17: T helper 17

TLR: Toll-like receptor

TMP: Trimethoprim

TNF: Tumor necrosis factor

TNFR: Tumor necrosis factor receptor

TNF-α: Tumor necrosis factor-alpha

TOR: Target of Rapamycin

TORC1: Target of Rapamycin Complex 1

TORC1: Target of rapamycin complex 1.

TRAF6: TNF receptor-associated factor 6

TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

Tregs: Regulatory T cells

TSC1/2: Tuberous sclerosis complex 1/2

TSC2: Tuberous sclerosis complex 2

ULK1/ULK2: Unc-51-like kinase 1/2

ULK1: Unc-51 Like Autophagy Activating Kinase 1

ULK2: Unc-51 like autophagy activating kinase 2

VPS/HOPS: Vesicle Protein Sorting/Homotypic Fusion and Protein Sorting

VPS34: Vacuolar protein sorting 34

WIPI1: WD repeat domain phosphoinositide-interacting protein 1

WIPI4: WD repeat domain phosphoinositide-interacting protein 4

ZNF306: Zinc finger protein 306

CHAPTER 1 :

Introduction

1. *Toxoplasma gondii*

1.1 The Etiologic Agent of Toxoplasmosis

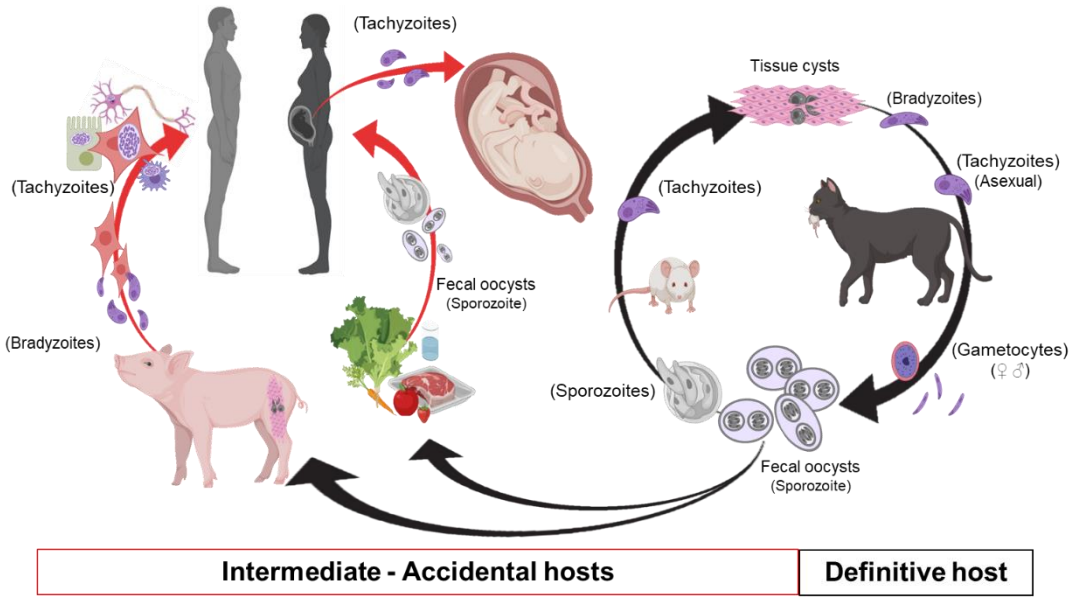
Toxoplasma gondii (*T. gondii*), the etiologic agent of toxoplasmosis, is an intracellular protozoan parasite that invades virtually any nucleated cell, and infects a wide variety of warm-blooded vertebrate hosts, including humans, cats, mice and birds (Innes, Hamilton et al. 2019). *T. gondii*, is a facultatively heteroxenous, polyxenous protozoon that has developed several potential routes of transmission within and between different host species. It can be transmitted vertically by tachyzoites that are passed to the foetus via placenta and horizontally by the ingestion of infectious oocysts from the environment as well as tissue cysts or tachyzoites which are contained in meat of many different animals (Tenter, Heckeroth et al. 2000). Transmission may also occur via tachyzoites contained in blood products, tissue transplants or unpasteurized milk. Due to its plasticity, diversity of life forms and ability to adapt to different environments and hosts, *T. gondii* is prevalent in most areas of the world and is considered of veterinary and medical importance. Because of its great importance as a causative agent of zoonosis, *T. gondii* has been studied most intensively among intracellular protozoan parasites, however, there are still many aspects of its biology, natural life cycle, and the epidemiology which should be studied in more detail (Tenter, Heckeroth et al. 2000). Intracellular parasitism is a strategy used by many eukaryotic microorganisms. Some, like *Plasmodium* and *Leishmania*, are limited in their ability to invade specific types of cells and establish a replicative cycle. Others, such as *Trypanosoma cruzi* and *T. gondii*, can invade nearly all types of cells. This gives these parasites a greater chance of transmission based on the epidemiological dynamics and prevalence among their hosts (Portes, Barrias et al. 2020). Another distinctive characteristic that makes *T. gondii* a successful parasite is its ability to induce long-term chronic infections through its interactions with the host, leading to conversion of the prolific tachyzoite stage to the quiescent bradyzoite parasite stage (Aliberti 2005). This stage is not usually harmful in immunocompetent individuals, although in immunodeficient individuals, they reconvert into cytolytic tachyzoites, resulting in severe toxoplasmosis and distant dissemination (Zhu, Li et al. 2019). In this sense, a non-generalist cell invasion strategy as well as a wide range of hosts and a

prolonged permanence within them, plus a large repertoire of mechanisms to avoid its destruction by the host, makes of *T. gondii* one of the most successful intracellular parasites.

1.2 Life cycle

Felids are the only definitive host and the only host species where the sexual life cycle of the parasite can occur resulting in the shedding of *T. gondii* oocysts in feces (Sukthana 2006, Dubey, Lago et al. 2012). Young cats usually become infected for the first time when hunting and eating birds or rodents infected with *T. gondii*. Tissue cysts containing *T. gondii* bradyzoites are ingested by the cat and the digestive enzymes in the gut break down the cysts wall releasing bradyzoites that invade the epithelial cells in the intestine enabling the parasite to develop within the host cells. The sexual cycle of *T. gondii* occurs in the gut of the cat with the female macrogametes and the male microgametes being found throughout the small intestine, most commonly in the ileum. The microgametes fertilize the macrogametes resulting in zygote formation and an oocyst wall is formed around the parasite (Dubey, Lago et al. 2012). The epithelial cells containing the oocysts rupture and release the oocysts into the gut of the cat where they are excreted in the faeces. The oocysts have a very tough outer shell and can survive for long periods of time in the environment, particularly in temperate and moist regions. However, they are not infectious to a new host until they undergo sporulation, which occurs within a few days outside the cat in the environment. Each oocyst is made up of two sporocysts, each containing four sporozoites. This persistence in the environment, despite vulnerability to desiccation and ultraviolet light, poses a risk for the transmission of *T. gondii* to humans and other animals (Lelu, Villena et al. 2012). Sporulated oocysts are infectious for new hosts if they are consumed either by grazing animals or through consumption of contaminated food or water (Dubey, Lago et al. 2012). In other animals or human hosts, transmission of *T. gondii* may occur through consumption of oocysts in contaminated food or water or through eating raw or undercooked meat from other animals persistently infected with *T. gondii* and harboring tissue cysts containing bradyzoites. Following consumption of oocysts by intermediate hosts, sporozoites excyst and invade the enterocytes and cells of the ileal epithelium and convert to tachyzoites, the rapidly multiplying stage of the parasite (Dubey, Lago et al. 2012). The tachyzoites can penetrate most nucleated host cells forming a PV and multiply using a process called endodyogeny.

The parasite will keep dividing within host cells until the host cell ruptures releasing the tachyzoites that will go on and invade and multiply within other host cells. A key stage in the parasite life cycle within the host is the conversion from tachyzoite to bradyzoite (slow multiplying stage) which enables the parasite to "hide" from the immune system of the host within an infected cell where there is a modification of the PV to form a cyst wall (Ferguson and Hutchison 1987). The bradyzoites multiply very slowly within these tissue cysts and in most host species are thought to persist for the lifetime of the host where they can be found in brain, eyes, heart and skeletal muscles (Dubey, Lago et al. 2012), **Figure 1.1**.



*Adapted from Center for disease control and prevention CDC
(Innes EA et al., 2019 Food Waterborne Parasitol).

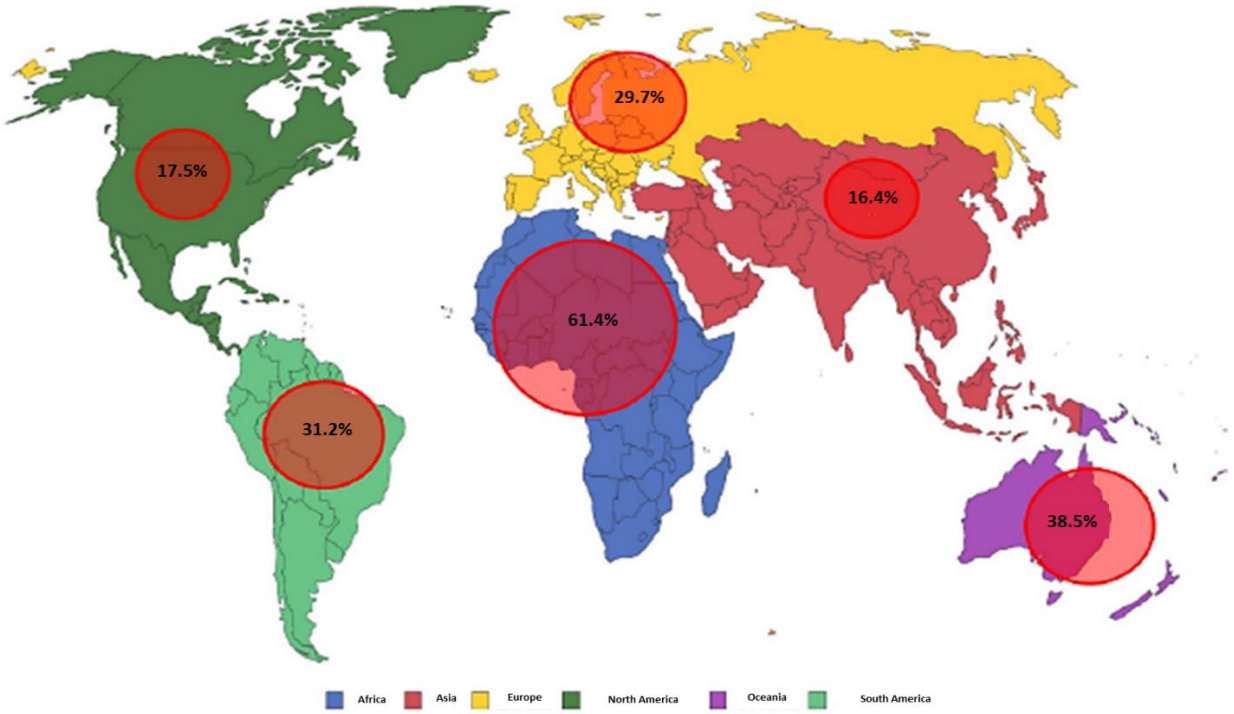
Figure 1.1 *T. gondii* Life Cycle Model

T. gondii has a complex life cycle involving sexual and asexual reproduction. Cats shed oocysts containing sporozoites, which can infect intermediate hosts like rodents or humans. The parasite can form tissue cysts in the host's muscles and brain, persisting for the host's lifetime. If ingested by a cat, the parasite reproduces sexually and sheds new oocysts. In addition, *T. gondii* can be transmitted vertically from an infected mother to her fetus through the placenta, causing congenital toxoplasmosis in the newborn.

1.3 Morbidity and Mortality

It is estimated that about 30-50% of the world population is seropositive for *T. gondii*, making toxoplasmosis the most prevalent infection among humans (Montazeri, Sharif et al. 2017). Worldwide, over 1 billion people are estimated to be infected with *T. gondii* (Hoffmann, Batz et al. 2012). The global prevalence rates of this parasite ranges from less than 10% to over 90% depending on social habits, climate condition, hygienic standards, and geographical regions (Molan, Nosaka et al. 2019). The complex life cycle and diverse hosts of *T. gondii* have resulted in numerous epidemiological studies to determine disease prevalence and explore prevention and control strategies in various clinical states. These include pregnancy, congenital toxoplasmosis, Alzheimer's disease, cancers, diabetes, foodborne illnesses, mood disorders, conditions related to acquired immunodeficiency syndromes (AID) and organ transplantation (Dubey 2008). One of the most recent systematic reviews of the epidemiological status of human toxoplasmosis around the world, showed the overall range of seroprevalence was determined to be 0.5 – 87.7%. African countries had the highest average seroprevalence rate of 61.4%, followed by Oceania with 38.5%, South America with 31.2%, Europe with 29.6%, USA/Canada with 17.5%, and Asia with 16.4%. Numerous environmental and human factors affect the differences in *T. gondii* seroprevalence rates observed between the various countries and continents (Molan, Nosaka et al. 2019). **Figure 1.2**. Thus, toxoplasmosis constitutes a serious public health concern worldwide (Flegr, Prandota et al. 2014). Furthermore, *T. gondii* is considered as one of the most important foods borne pathogens in Europe and USA, despite this, and the great impact that the disease has in congenitally infected children who suffer brain damage, eye and auditory disease for life, there are no drugs or treatments available to cure persistent *T. gondii* infection and efforts should be made to develop a vaccine to prevent human infection and reduce the disease (Innes, Hamilton et al. 2019).

Global Prevalence of *Toxoplasma gondii* Infection in Humans by Continent: A Graphical Summary



Global Status of *T. gondii* Seroprevalence

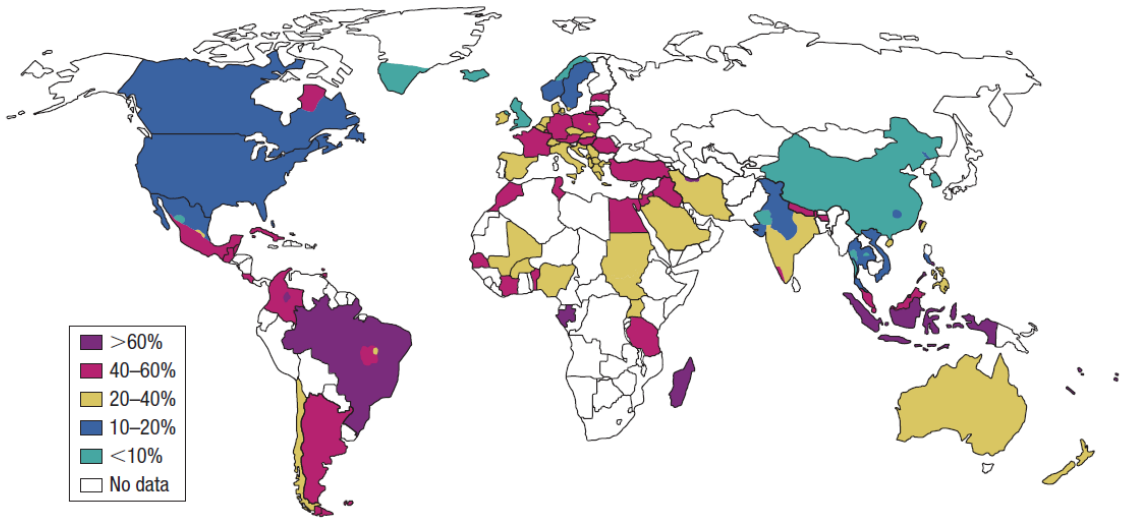


Figure 1.2 An Overview of Global Prevalence Data for *T. gondii*

This figure shows the continental and geographical trends in the global prevalence of *T. gondii* infection, based on a systematic examination of currently available epidemiological data. Adapted from (Molan, Nosaka et al. 2019). https://doi.org/10.1016/B978-0-323-55512-8.00106-X.

1.4 Clinical Signs

Toxoplasmosis presents a diverse array of clinical manifestations, categorized into four groups: acquired in immunocompetent patients, acquired or reactivated in immunodeficient patients, ocular, and congenital. Diagnosis and treatment can vary for each category, with serologic tests being the primary diagnostic method due to the difficulty in isolating the parasite. In immunocompetent individuals, acquired infection is often asymptomatic or may lead to mild symptoms like cervical lymphadenopathy or flu-like illness. However, emerging data suggest potential associations between *T. gondii* infection and neurologic or psychiatric conditions, such as schizophrenia and Alzheimer's disease. Immunodeficient patients, especially those with AIDS, may experience toxoplasmic encephalitis, often due to reactivation of latent infection. Ocular toxoplasmosis is a significant cause of chorioretinitis, and congenital toxoplasmosis can result in various clinical manifestations, with severity inversely related to the gestational age at maternal infection. While certain clinical features, like chorioretinitis, intracranial calcifications, and hydrocephalus, are indicative of congenital toxoplasmosis, some infected children may develop sequelae later in life, including visual impairments, developmental delay, epilepsy, or deafness. Toxoplasmosis exhibits a broad range of symptoms across different organ systems, and its clinical outcome depends on multiple factors, including the parasite strain, host, immune response, and various yet-unanswered questions about the disease's variability among individuals. Furthermore, while much research has been conducted using murine models and laboratory-adapted strains, these findings don't always directly translate to natural infections in humans. A deeper understanding of the host-parasite relationship in toxoplasmosis is crucial, especially given the potential for new vaccine or drug targets and the importance of investigating other clinical forms, such as ocular toxoplasmosis and congenital infections, to uncover the relevant host and parasite factors influencing infection outcomes (McAuley 2014, Mukhopadhyay, Arranz-Solis et al. 2020, Dubey 2021).

Clinical manifestations of *T. gondii* infection include various neurological symptoms such as headaches, fever, hemiparesis, ataxia, and seizures. Additionally, chronic *T. gondii* infection has been associated with intriguing behavioral changes in different hosts, particularly in mice. These behavioral changes range from impaired movements, deficits in spatial learning and memory, increased activity, reduced anxiety, and most notably, a decreased aversion to

feline odors. This phenomenon, often referred to as "fatal attraction," is believed to enhance the likelihood of *T. gondii* transmission to its definitive feline host. Recent findings indicate that *T. gondii* -infected mice lose their innate fear of various animal odors and unknown objects as well. However, the mechanisms underlying these behavioral changes are complex and may involve both direct neural tissue damage caused by parasite multiplication and neuroinflammation. Pro-inflammatory cytokines produced during infection can alter the levels of neuromodulators such as dopamine, glutamate, and serotonin, impacting behavior. Furthermore, *T. gondii* itself may influence neurotransmitter levels, contributing to hyperactivity and novelty seeking in infected rodents. While murine models provide insights, it's important to recognize that the human immune response to *T. gondii* differs, and epidemiological studies have linked *T. gondii* infection to various mental illnesses. However, the relationship between *T. gondii* and mental illnesses in humans remains complex, with multiple contributing factors and regional variations in prevalence (Mukhopadhyay, Arranz-Solis et al. 2020).

1.5 Treatments and Alternative Therapies for Toxoplasmosis

Despite the development of new experimental drugs for the treatment of toxoplasmosis, none of them have been approved to prevent congenital infection while minimizing teratogenic effects (Smith, Goulart et al. 2021). Most drugs are active only against the tachyzoite forms of the parasite, and treatment does not eradicate the infection. Specific treatment is not indicated for a healthy person with mild symptoms who is not pregnant, as they usually resolve within a few weeks. Treatment is usually recommended for severe illness in the immunocompetent host, infection acquired during pregnancy, clinical manifestations compatible with toxoplasmosis in immunosuppressed patients, congenital infection, or persons with active ocular disease or severe illness (Smith, Goulart et al. 2021). Treatment for Toxoplasmosis is restricted by limited options. Anti-Toxoplasma drugs mainly target the folate pathway, which plays a crucial role in DNA synthesis via the dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) enzymes. Two major drugs, Pyrimethamine (PYR) and Trimethoprim (TMP), are used to treat acute toxoplasmosis by acting on the parasite DHFR. However, these drugs cannot distinguish between the parasite and the human host's enzyme (Konstantinovic, Guegan et al. 2019). Therefore, current treatment regimens have side effects due to myelotoxicity, and require discontinuation of therapy, or, more

frequently, induce lack of compliance (Maldonado, Read et al. 2017). Most of all, no current drug is able to eliminate *T. gondii* cysts from the infected host, which remain quiescent, provided that the immune system is strong enough to hamper their reactivation into tachyzoites (El-Zawawy, El-Said et al. 2015). In comparison to adverse events, drug resistance in *T. gondii* is a relatively insignificant issue. Nonetheless, reports have suggested the failure of long-term Pyrimethamine (PYR)-based treatment for congenital toxoplasmosis, which may be attributed to the development of drug-resistant *T. gondii* strains (Doliwa, Xia et al. 2013).

No effective human vaccines have been developed against Toxoplasmosis (Innes, Hamilton et al. 2019). Most animals, including humans, establish a good protective immunity following infection, involving cell mediated immune responses to protect against *T. gondii*. Despite the significant numbers of research papers looking at vaccination against *T. gondii* over the last 30 years, there is currently only one vaccine available worldwide which is licensed for use in sheep to protect against abortion due to *T. gondii* infection (Buxton and Innes 1995). After a first infection with *T. gondii*, it is possible for the host to become protected against subsequent exposure to the parasite suggesting that vaccination is a feasible approach to help prevent and control disease (Innes, Bartley et al. 2011). Although there are drugs available that can restrict the fast-multiplying tachyzoite stage of the parasite, none have been found to be effective in eliminating parasites within tissue cysts. Nevertheless, most animals and humans infected with *T. gondii* develop a strong immune response, which provides protection against future disease. Therefore, the combination of vaccination and chemotherapy treatment is a justifiable strategy for combating toxoplasmosis (Innes, Hamilton et al. 2019).

1.6 Strains and Virulence Factors

The clinical outcome of toxoplasmosis depends on a complex balance between the host immune response and parasite virulence factors. Susceptibility to the disease is thus determined by both parasite strains and host species (Sanchez and Besteiro 2021). While a single species has been described for the genus *Toxoplasma*, there are several clonal lineages that differ in their pathogenicity. In Europe and North America the population structure of *T. gondii* is dominated by three main genetic lineages (I, II and III) (Howe and Sibley 1995, Khan, Dubey et al. 2011). In other parts of the world, the situation is more contrasted. In

South America, for example, there is a much greater genetic diversity, suggesting a greater occurrence of recombination (Lorenzi, Khan et al. 2016). Strain virulence is typically defined by the outcome of infection in the mouse model, in which type I strains are much more virulent, than type II and III strains (Sibley and Boothroyd 1992). Upon infection by *T. gondii*, disease development also depends on the type of host, its genetic background, and of course its immune status.

Parasite lineages are separated by asexual reproduction, regardless of geographical or ecological locality. For example, *T. cruzi*, has six major evolutionary lineages. Some of these lineages may have resulted from ancestral hybridization events. Parasite strains can occur jointly. There are several clonal lineages of *T. gondii* that differ in their pathogenicity. These lineages differ in a number of phenotypes, such as growth, migration, and transmigration. The best described of these is virulence in laboratory mice. The outcome of Toxoplasmosis in patients is also variable and the molecular basis for these differences in pathogenesis, especially in strains other than the clonal lineages, remains largely unexplored. These lineages are closely related. Most isolates from North America and Europe belong to one of these three lineages (Minot, Melo et al. 2012).

Toxoplasma is a genus of parasites that possess distinctive secretory organelles that are specialized in the production of virulence factors. These virulence factors are critical in determining the progression of the infection and can vary depending on the strain of the parasite (Sanchez and Besteiro 2021). *T. gondii* has two different types of specialized apical secretory organelles called micronemes and rhoptries, that secrete virulence factors that are essential for its dissemination and survival. Dense granules are another type of specialized secretory organelle that will later release important factors for the intracellular establishment of the parasite (Mercier and Cesbron-Delauw 2015, Dubois and Soldati-Favre 2019, Sparvoli and Lebrun 2021).

The invasion process, and subsequent steps of intracellular asexual replication and exit (or egress) from the host cell, collectively referred to as the "lytic cycle" has been mostly studied for tachyzoites (Sanchez and Besteiro 2021). Host cell invasion mechanism involves the formation of a PV that constitutes a unique replicative niche, providing some protection from the host cell and access to nutrient sources (Martin, Liu et al. 2007). Invasion and subsequent establishment of the parasite in the PV is due to the sequential exocytosis of the three

specialized secretory organelles: micronemes, rhoptries, and dense granules (Carruthers and Sibley 1997).

Strain-specific differences in *T. gondii* virulence can be linked to the fact that many effectors are highly polymorphic and thus have different outcomes depending on the strain (Secreted *T. gondii* ROP: Rhoptry neck protein and GRA: Dense granule protein effectors and their strain-specific impact on the host are summarized on **Table 1.1**) (Sanchez and Besteiro 2021). Among *T. gondii* virulence effectors, microneme proteins (MICs) secreted by the microneme play a basic role in the recognition, adhesion, and invasion of parasites into host cells during the invasion process (Meissner, Reiss et al. 2002).

During the initial stage of infection, *T. gondii* parasites secrete MICs from their micronemes. These proteins help the parasite to attach to the host cell membrane by recognizing specific receptors on the surface of the host cells. This adhesion process is crucial for the parasite to invade the host cells and establish an infection. Therefore, MICs play an important role in the early stages of *T. gondii* infection. There are currently at least 19 different types of MICs known, which can be found in **Table 1.2**. Some of these MICs, including TgMIC1-4, TgMIC6-9, TgMIC12, and SPATR, contain adhesion domains that are similar to the ones found in eukaryotic cells. These adhesion domains include integrin-like domains, thrombospondin type-1 repeats (TSR), epidermal growth factor (EGF)-like domains, chitin binding-like (CBL) domains, and Apple domains(Wang and Yin 2015).

The increasing number of effector proteins characterized over the recent years highlights the very complex nature of the host–pathogen interactions underlying *T. gondii* virulence in the mouse model. The current state of knowledge shows there is a diversity of mechanisms governing the balance of both pro- and anti-inflammatory modulation by various parasite proteins. While studies in the murine models have revealed a variety of parasite defense mechanisms and intricate relationship between *T. gondii* and its host, they need to be extended further to human cells. This may provide new insights into potential therapeutic strategies (Niedelman, Gold et al. 2012, Sanchez and Besteiro 2021).

Table 1.1 Secreted *T. gondii* ROP and GRA Effectors and Their Strain-specific Impact on the Host

Effector name	ToxodB Accession Number (Type I)	Localization	Target host protein	Role	Type I	Type II	Type III	Ref
ROP effectors								
ROP5	TGGT1_308090	PVM	Irga6	Inhibition of PVM IRG coating and activation of ROP18	Active	Less active (ROP18 activity not enhanced)	Active	(Behnke, Khan et al. 2011, Murillo-Leon, Muller et al. 2019)
ROP16	TGGT1_262730	Nucleus	STAT3/6	Activation of STAT3/6 inducing a decrease of IL-12 expression and suppressing TH1 response. Reduces the PVM coating of GBPs	Active	Less active (no sustained STAT3/6 activation)	Active	(Saeij, Boyle et al. 2006, Saeij, Coller et al. 2007, Jensen, Hu et al. 2013)
ROP17	TGGT1_258580	PVM	Irga6/Irgb6	Enhances ROP18 activity and binds IRGs (preferentially Irgb6) for disassembly	Active	Not studied	Not studied	(Etheridge, Alaganan et al. 2014)
ROP18	TGGT1_205250	PVM/ER	IRGs, ATF6β	Binds IRGs for disassembly (preferentially Irga6). Also targets host TF ATF6β, reducing antigen presentation	Active	Active	Less active (low expression)	(Saeij, Boyle et al. 2006, Taylor, Barragan et al. 2006, Zhao, Ferguson et al. 2009, Fentress, Behnke et al. 2010, Khaminets, Hunn et al. 2010, Yamamoto, Ma et al. 2011, Steinfeldt, Konen-Waisman et al. 2015, Hermanns, Muller et al. 2016)
ROP38	TGGT1_242110	IVN/PVM	Unknown	Inhibits MAPK/NF-κB pathways, controlling apoptosis in infected cells and IL-18 secretion. Expression levels vary between *T. gondii* strains	Potentially less active (low expression)	Active	Active (high expression)	(Peixoto, Chen et al. 2010, Melo, Nguyen et al. 2013, Fox, Rommereim et al. 2016, Xu, Wang et al. 2018)
ROP54	TGGT1_210370	PVM	GBP2	Inhibits GBP2 coating at the PVM	Less active? (no virulence phenotype for the KO)	Active	Not studied	(Kim, Nadipuram et al. 2016)

Effector name	ToxodB Accession Number (Type I)	Localization	Target host protein	Role	Type I	Type II	Type III	Ref
GRA effectors								
GRA6	TGGT1_275440	IVN/PVM	CAMLG	Activates host TF NFAT4 via CAMLG, leading to the expression of CCCL2/CXCL2 and neutrophil/monocyte recruitment. In type II parasites, has an epitope eliciting T-cell response	Active	Less active but has an epitope inducing a strong T-Cell response	Active	(Blanchard, Gonzalez et al. 2008, Ma, Sasai et al. 2014)
GRA7	TGGT1_203310	PVM	Irga6, TRAF6, ASC	Accelerates the turnover of Irga6 by interacting with the ROP5/ROP18 complex. The GRA7 protein can also stimulate the immune system though TRAF6/ NF-κB activation and inflammasome activation through the ASC adaptor.	Active	NF-κB pathway and macrophage activation	Not studied	(Alaganan, Fentress et al. 2014, Hermanns, Muller et al. 2016, Yang, Yuk et al. 2016, Ihara, Fereig et al. 2020)
GRA12	TGGT1_288650	IVN/PVM	Unknown	Inhibits IFN-γ mediated parasite killing	Active	Active	Not studied	(Fox, Guevara et al. 2019)
GRA14	TGGT1_239740	PV/PVM/IVN	Unknown	Activation of NF-κB pathway and recruitment of macrophages in type II parasites, potentially in other strains too	Active	Active	Not studied	(Rome, Beck et al. 2008, Ihara, Fereig et al. 2020)
GRA15	TGGT1_275470	PVM	TRAF2/TRAF6 GBP1	In type II parasites, activation of NF-κB pathway via TRAF2/6 interaction leading to IL-12 and IL-1B expression. Also linked to the inhibition of lysosomes fusion and GBP loading to the PVM	Truncated (and thus inactive) in some strains	Active	Active, but less than type II parasites	(Rosowski, Lu et al. 2011, Virreira Winter, Niedelman et al. 2011, Yang, Farrell et al. 2013, Sangare, Yang et al. 2019, Ihara, Fereig et al. 2020)
GRA16	TGGT1_208830	Nucleus	PP2A-B55, HAUSP	Modulates the expression of host cell genes involved in the control of cell-cycle progression, p53 signaling, steroids and lipids metabolism	Less active? (no virulence phenotype for the KO)	Active	Not studied	(Bougdour, Durandau et al. 2013)
GRA18	TGGT1_288840	Cytoplasm	PP2A,GSK3,β-catenin	Activation of β-Catenin inducing upregulation of IFN-B1,CCL24 and anti-inflammatory chemokines CCL22 and CCL17	Active (secreted, but no functional study)	Active	Not studied	(He, Brenier-Pinchart et al. 2018)

GRA24	TGGT1_230180	Nucleus	p38α/ MAPK	Activation of p38α/MAPK, inducing TH1/M1 polarization and cytokines/chemokines secretion	Active	Active	Not studied	(Braun, Brenier-Pinchart et al. 2013)
GRA25	TGGT1_290700	PV	Unknown	Allow secretion of CXCL1 and CCL2 chemokines by infected macrophages	Not studied	Active	Less active	(Shastri, Marino et al. 2014)
GRA28	TGGT1_231960	Nucleus	Unknown	Induces CCL22 secretion	Active	Likely active	Likely active	(Rudzki, Ander et al. 2021)
GRA60	TGGT1_204270	PVM	Unknown	Inhibits Irga6 and Irgb10 recruitment at the PVM	Active	Active	Not studied	(Nyonda, Hammoudi et al. 2021)
HCE1/TEEGR	TGGT1_239010	Nucleus	E2Fs/DP1	Inhibits NF-κB induced cytokines by interacting with host TFs and controls host Cyclin E expression by interacting with DP1	Active	Active	Not studied	(Braun, Brenier-Pinchart et al. 2019, Panas, Naor et al. 2019)
TgIST	TGGT1_240060	Nucleus	NuRD, STAT1/2	Blocks signaling through type I interferon by recruiting the NuRD repressor and binding to STAT1/STAT2 heterodimers	Active	Active	Not studied	(Gay, Braun et al. 2016, Olias, Etheridge et al. 2016)
TgNSM	TGGT1_235140	Nucleus	NCoR	Inhibits interferon-regulated genes involved in cell death	Not studied	Active	Not studied	(Rosenberg and Sibley 2021)
MAG1	TGGT1_270240	PVM/Cytoplasm	Unknown	Inhibits IL-1β secretion in macrophages	Not studied	Active	Not studied	(Tomita, Mukhopadhyay et al. 2021)
MAF1b	TGGT1_220950	PVM	MIB complex	Induces host mitochondria association with the PVM and modulates the cytokine response	Active	Inactive	Active	(Pernas, Adomako-Ankomah et al. 2014, Blank, Xia et al. 2021)

Abbreviations for cellular localizations: ER, endoplasmic reticulum; IVN, intravacuolar membrane network; PV, parasitophorous vacuole; PVM, PV membrane; Acronyms for host target proteins: ASC, Apoptosis-associated Speck-like protein containing a CARD (caspase activation and recruitment domain); ATF, activating TF; CAMLG, calcium-modulating ligand; CCCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand, DP1, E2F dimerization partner 1; E2F, E2 TF; GBP, guanylate-binding protein; GSK3, glycogene synthase kinase 3; HAUSP, herpesvirus-associated ubiquitin-specific protease; IRG, immunity-related GTPase; MAPK, mitogen-activated protein kinase; MIB complex, mitochondrial intermembrane space bridging complex; NCOR1, nuclear receptor corepressor 1; NuRD, nucleosome-remodeling deacetylase; PP2A, protein phosphatase 2A; STAT, signal transducers, and activators of transcription; TRAF; TNF receptor associated factor (Sanchez and Besteiro 2021).

Table 1.2 Secreted *T. gondii* MIC Effectors and Their Impact on the Host

MICs	Domains/homologies	Major putative functions	Ref
MIC1	2 TSRs	Transport/folding of MIC4 and MIC6. Binding to host cells	(Cerede, Dubremetz et al. 2005, Saouros, Edwards-Jones et al. 2005)
MIC2	1 Integrin, 5 TSRs, and 1 TM	Transport M2AP and adhesion	(Wan, Carruthers et al. 1997, Jewett and Sibley 2004)
MIC3	1 CBL and 5 EGF	Adhesion	(Garcia-Reguet, Lebrun et al. 2000, Brecht, Carruthers et al. 2001, Cerede, Dubremetz et al. 2005)
MIC4	6 Apples	Adhesion	(Brecht, Carruthers et al. 2001, Reiss, Viebig et al. 2001)
MIC5	Parvulin-like PPIase motif	Suppressing TgSUB1 activity	(Brydges, Sherman et al. 2000)
MIC6	3 EGF-like	Escorter	(Reiss, Viebig et al. 2001)
MIC7	5 EGF-like and 1 TM	unkown	(Meissner, Reiss et al. 2002)
MIC8	1 CBL, 10 EGF-like, and 1 TM	Escorter	(Meissner, Reiss et al. 2002)
MIC9	3 EGF and 1 TM	unkown	(Meissner, Reiss et al. 2002)
MIC10		unkown	(Hoff, Cook et al. 2001)
MIC11		unkown	(Harper, Zhou et al. 2004)
MIC12	31 EGF, 4 TSRs, and 1 TM	unkown	(Opitz, Di Cristina et al. 2002)
AMA1	Cysteine rich	Inhibiting secretion of the rhoptries	(Mital, Meissner et al. 2005)
M2AP	Beta and coil	Transport/folding of MIC2	(Rabenau, Sohrabi et al. 2001, Huynh, Rabenau et al. 2003)
SUB1	Subtilase and GPI	Proteolysis	(Miller, Binder et al. 2001)
ROM1	Rhomboid	Proteolysis	(Brossier, Jewett et al. 2005, Dowse and Soldati 2005)
SPATR	1 thrombomodulin EGF, 2 TSRs	Trafficking of the protein	(Huynh, Boulanger et al. 2014)
PLP1		Cytolysis and parasite egress	(Kafsack, Pena et al. 2009, Roiko and Carruthers 2013)
TLN4		unkown	(Laliberte and Carruthers 2011)

Known types of MICs containing different adhesion domains similar to the adhesion molecules in eukaryotic cells (integrin-like domain, thrombospondin type-1 repeat (TSR), epidermal-growth factor (EGF)-like domain, chitin binding-like (CBL) domain, and the Apple domain) (Wang and Yin 2015).

1.7 Parasite Replication: Strategies and Mechanisms

To efficiently replicate, *T. gondii* must first manipulate and modify their host cells to create a secure environment. *T. gondii* specifically modifies three primary cellular processes through infection: metabolism, membrane and cytoskeletal architecture formation, and cell death (Blader and Koshy 2014). To survive within the intracellular environment, *T. gondii* must withstand various pressures while also scavenging essential nutrients from the host cell. These nutrients, such as carbon sources (glucose and glutamine) for energy, specific amino acids, lipids, and other vital compounds, are necessary to meet the parasite's nutritional requirements (Denton, Roberts et al. 1996). The host plasma membrane is a critical interface between *T. gondii* and its host cell, both during and after invasion, and a key target for the parasite's regulation of the host cell. A primary role of the plasma membrane is to trigger intracellular signaling pathways in response to extracellular cues. The interactions between MICs and parasite surface antigens with the host plasma membrane suggest that the parasite may regulate host cell signaling by binding to host plasma membrane receptors. This hypothesis is supported by the discovery that the addition of parasite-secreted factors, predominantly composed of MICs, to uninfected host cells, results in changes in gene expression (Stutz, Kessler et al. 2012). Furthermore, *T. gondii* establishes its PV while evading the endolysosomal pathway, as this pathway may lead to autophagy-mediated degradation of the PV and the parasite (Jones, Yeh et al. 1972, Sibley, Weidner et al. 1985, Ling, Shaw et al. 2006).

The inhibition of tyrosine kinase and the identified target, EGF receptor, have been shown to prevent *T. gondii* from avoiding the phagolysosome. In a study by (Muniz-Feliciano, Van Grol et al. 2013), treatment of cells with tyrosine kinase inhibitors resulted in attenuated parasite growth. Moreover, transfection with small interfering RNAs (siRNA) targeting the EGF receptor resulted in PVs undergoing autophagic destruction. The EGFR and AKT signaling is critical in regulating various physiological processes in uninfected cells. This pathway is primarily involved in cell growth, survival, and proliferation and it is triggered in response to extracellular signals. Its functions in uninfected cells are cell growth and proliferation, upon EGF binding to EGFR, a signaling cascade is initiated, leading to the activation of AKT. Activated AKT promotes cell growth and proliferation by regulating the activity of various downstream effectors involved in the cell cycle; cell survival, the

EGF/AKT pathway plays a crucial role in promoting cell survival by inhibiting apoptosis. Activated AKT can phosphorylate and inactivate pro-apoptotic factors, thereby preventing cell death; metabolism, AKT regulates cellular metabolism by promoting glucose uptake and glycolysis, which provide energy and substrates for cell growth and survival; angiogenesis, in some contexts, EGF/AKT signaling is involved in angiogenesis, the formation of new blood vessels. This process is important for tissue growth and repair; cell motility and migration, AKT can influence cell motility and migration, which is crucial in processes like wound healing and tissue regeneration, and gene expression, the EGF/AKT pathway can also modulate gene expression by activating transcription factors, which influence the expression of genes related to cell growth and survival.

In uninfected cells, this pathway is tightly regulated and is activated in response to specific physiological signals, typically when the cell needs to grow, divide, or respond to growth factors in its environment. It's an essential part of normal cellular functions and contributes to tissue development, maintenance, and repair. However, dysregulation or abnormal activation of this pathway can also be associated with various diseases, including cancer, where uncontrolled cell growth and survival are characteristic features (Freudlsperger, Burnett et al. 2011).

In infected cells, AKT kinase activation of phosphatidylinositol-3 kinase (PI3K) appears to be the critical downstream target of the EGF receptor signaling. These data suggest that EGF/AKT signaling either prevents the invading parasite from entering the endolysosomal pathway or prevents lysosomal recruitment to the PV. These data also suggest that the receptor is not activated by parasites with knockout mutations in the MIC1 and MIC3 micronemal proteins, indicating a potential role for these proteins in its activation (Muniz-Feliciano, Van Grol et al. 2013). Another way for *T. gondii* to establish a secure replicative niche is to ensure that its host cell remains alive long enough for the parasite to replicate and then egress and invade the next host cell. Apoptosis is a form of programmed cell death that has been implicated as an innate defense mechanism to eliminate intracellular pathogens There are two major pathways inducing apoptosis: the intrinsic and the extrinsic. *T. gondii* has been reported to renders the host cells resistant to stimuli that activate either the intrinsic pathway or the extrinsic pathway (Nash, Purner et al. 1998). One important aspect of T. gondii's establishment of a replicative niche is the regulation of host nuclear cell functions,

modulating host cell gene expression. This is achieved in great part by the activation or inactivation of host TFs such as STAT3/6, NF-κB, and HIF-1(Saeij, Coller et al. 2007).

To conclude, *T. gondii* modifies host cell processes, scavenges nutrients, avoids lysosomal degradation, and inhibits host cell death to establish a replicative niche. The parasite regulates host cell signaling through plasma membrane interactions and the activation or inactivation of host TFs. Additionally, targeting the EGF receptor signaling pathway has been shown to be important in preventing *T. gondii* from avoiding lysosomal degradation.

1.8 The Parasitophorous Vacuole

To establish a safe replicative niche, *T. gondii* forms a nonfusogenic PV that facilitates nutrient acquisition while preventing contact with host cytoplasmic components that could trigger parasite destruction (Coppens, Dunn et al. 2006, Coppens and Romano 2018). The PV is a unique replicative compartment for *T. gondii*, derived from the host plasma membrane. The PV is rendered nonfusogenic with the host endolysosomal system, The PV is essential for the survival and replication of the parasite because it separates the parasite from the host cell's endolysosomal system, which is crucial for immune defense and degradation of foreign material. To render the PV nonfusogenic with the host endolysosomal system, *T. gondii* deploys various strategies: molecular alterations, *T. gondii* secretes specific proteins into the PV space. Some of these proteins interact with host cell proteins or modify the PV membrane to inhibit fusion with host endosomes and lysosomes. For example, *T. gondii* secretes ROP proteins that play a role in preventing fusion. Maintenance of low pH, the PV maintains a lower pH than the host endolysosomal system. This acidic environment inhibits the activity of enzymes typically found in lysosomes, making it difficult for host cells to degrade the parasite. PV membrane (PVM) composition, the PVM itself has a distinct composition, which is different from that of the host endolysosomal compartments. This composition prevents the recognition and fusion of the PV with host organelles. Evasion of host defense mechanisms, *T. gondii* can also manipulate host immune responses to avoid detection by the host's immune system and its antimicrobial defenses (Mordue, Hakansson et al. 1999).

By employing these strategies, *T. gondii* can create and maintain a safe replicative niche within the PV, shielding itself from the host cell's endolysosomal system and thus promoting its intracellular survival and replication.

Rhoptry and dense granule proteins secreted by *T. gondii* modify the forming vacuole, enable nutrient uptake, and set up mechanisms of host subversion (Clough and Frickel 2017). Interestingly, *T. gondii* can also generate selective nutrient exchange through the PVM, for example, uptake of host-derived nutrients: *T. gondii* can actively transport specific host-derived nutrients across the PVM. The parasite can acquire amino acids, such as arginine and tryptophan, from the host cell. These amino acids are essential for the parasite's metabolism and growth. Secreting effector proteins: *T. gondii* secretes effector proteins into the PV space, some of which may interact with the PVM. These effectors can manipulate the permeability of the PVM and promote the uptake of nutrients while restricting the release of harmful substances. For example, the protein GRA17 was identified as playing a role in nutrient acquisition. Modulating the PVM composition: *T. gondii* can modify the lipid composition of the PVM, making it more permissive for the selective exchange of nutrients. This lipid remodeling can influence the transport of molecules across the membrane. Avoiding immune recognition: *T. gondii* can also employ strategies to evade the host's immune system, as an immune response can negatively affect nutrient exchange. The parasite can minimize the activation of host immune pathways, such as the interferon-gamma response, which can lead to a more permissive environment for nutrient uptake. Co-opting host transporters: *T. gondii* may exploit host cell transporters to facilitate the acquisition of specific nutrients. By co-opting host transporters, the parasite can actively transport nutrients across the PVM (Zhu, Li et al. 2019, Kloehn, Hammoudi et al. 2021).

Furthermore, *T. gondii* recruits host cell mitochondria and endoplasmic reticulum (ER) to its PVM soon after invasion. This ability to modify the PV and recruit host cell organelles highlights the intricate relationship between *T. gondii* and its host cell. *T. gondii* recruits host cell mitochondria and ER for several specific purposes related to its intracellular survival and replication, for example, nutrient Acquisition: *T. gondii* requires various nutrients to sustain its growth and replication. By positioning itself close to the host cell's mitochondria and ER, the parasite can access critical metabolites and nutrients generated by these organelles. The host cell's mitochondria are involved in ATP production, and the ER plays a role in lipid synthesis and calcium storage, all of which can be beneficial for the parasite's metabolic needs. Calcium Regulation: The ER is a key organelle for calcium storage and signaling. *T. gondii* can manipulate calcium signaling within the host cell to its advantage. By being in

proximity to the ER, the parasite can potentially influence calcium release, which may aid in various intracellular processes, including egress and host cell invasion. Protection from host defenses: By positioning itself close to host mitochondria and ER, *T. gondii* can potentially shield itself from host immune defenses. It has been suggested that the close association with host mitochondria can help protect the parasite from host cell apoptosis (programmed cell death), which is an immune response mechanism. Metabolic support: The recruitment of host mitochondria may provide *T. gondii* with additional metabolic support, including access to ATP and other metabolites generated by the host cell's energy-producing machinery (Sinai, Webster et al. 1997, Walker, Hjort et al. 2008, Wang, Weiss et al. 2009, Pernas, Bean et al. 2018).

Although the PVM shields the parasite from acidification caused by endocytic vesicle fusion, this non-fusogenic state also deprives *T. gondii* of access to the host's abundant source of nutrients from the endocytic and exocytic systems. However, the parasite compensates for this by acquiring small molecules that cross the PVM through pores. This allows *T. gondii* to establish a bidirectional molecular exchange with host cells, a process that is remarkably independent of energy.

Overall, *T. gondii* has evolved multiple mechanisms to ensure its survival and proliferation, even in the face of isolation from the host (Schwab, Beckers et al. 1994, Sinai, Webster et al. 1997, Walker, Hjort et al. 2008, Wang, Weiss et al. 2009, Pernas, Bean et al. 2018). Moreover, during cell invasion, *T. gondii* creates a subcellular compartment that serves as an interface between the parasite and the host cytoplasm. This compartment not only supports parasite replication and infection but also serves as a platform for the modulation of several host cell functions. Recent studies have shown that *T. gondii* manipulates host cell signaling pathways through the deployment of parasite kinases and phosphatases. These molecules infiltrate the host nucleus and control several cellular pathways, enabling the parasite to establish an anti-apoptotic environment that favors its survival. To conclude, *T. gondii* has evolved sophisticated mechanisms to exploit host cell functions for its own benefit, allowing it to successfully infect and proliferate within host cells (Laliberte and Carruthers 2008).

2. Protective Immunity Against *T. gondii*: Insights into Innate Immune Responses, PRRs, PAMPs, and Adaptive Immunity

The detection of protozoan parasites by the mammalian immune system is a complex process due to the absence of classical microbial and viral molecules commonly sensed by innate immune receptors in these pathogens. Instead, the innate immune system has evolved to recognize *T. gondii* by identifying a unique set of molecules specific to protozoan parasites. Additionally, *T. gondii* presence is indirectly detected through the recognition of tissue damage events associated with parasitic infection (Yarovinsky 2014).

Profilin is a protein associated with *T. gondii* that plays a key role in activating the innate immune system. The recognition of *T. gondii* and its profilin protein involves a cooperative interplay between endosomal Toll-like receptor 11 (TLR11) and TLR12. Several models have been proposed to elucidate this process. In one model, TLR11 directly interacts with *T. gondii* profilin in endolysosomes, leading to MYD88 recruitment and downstream immune signaling cascades, with TLR12 contributing to this interaction. An alternative model suggests that both TLR11-TLR12 heterodimers and TLR12 homodimers are responsible for dendritic cell (DC) responsiveness to *T. gondii* profilin. Lastly, a third model posits that TLR7 and TLR9 could compensate for the absence of TLR11, reducing the dependence on the TLR11-TLR12 heterodimer. Importantly, these mechanisms are specific to mice, as humans lack functional TLR11, and the presence of TLR12 in the human genome remains unknown. While the precise mode of action of the TLR12 signaling receptor complex is still not entirely clear, its role in cytokine production in the absence of MYD88 recruitment is a subject of ongoing investigation (Koblansky, Jankovic et al. 2013, Yarovinsky 2014).

The innate immune response acts as the initial defense against *T. gondii* infection, aiming to detect and restrict its invasion and dissemination. Macrophages, DCs, and natural killer (NK) cells contribute to limiting parasite multiplication during the early stages of acute infection. Notably, IL-12-producing DCs not only stimulate the innate immune response but also shape adaptive immunity, crucial for long-term control of the infection. CD4 T cells, capable of producing IFNγ, help control the early stages, while CD8 T cells are vital for sustained protection and maintaining chronic infection. Several studies emphasize the importance of CD8 T cells in controlling *T. gondii* infection, with their ability to produce IFNγ during the late stages. However, achieving complete parasite eradication remains a challenge, as a

robust CD8 T cell response may not guarantee sterile immunity. In susceptible mouse models, vigorous CD8 T cell effector immunity is observed, but memory responses are compromised (Yarovinsky 2014).

Engagement of TLRs and nucleotide-binding oligomerization domain-like receptors (NLRs) by microbial-associated molecular patterns (MAMPs) initiates downstream signaling pathways. These pathways involve processes like phosphorylation, ubiquitination, and protein-protein interactions, ultimately leading to the activation of host cell transcription factors. These factors, in turn, upregulate genes related to inflammation and antimicrobial defenses. The key signaling pathways in TLR and NLR-induced immune responses are nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPKs), and interferon regulatory factors (IRFs). They are central in the production of various cytokines, including interleukin (IL)-12, IL-1β, tumor necrosis factor α (TNFα), and interferons (IFNs) type I and type II. Activation of these pathways results in the recruitment of immune cells to the infection site through chemokine secretion, and these recruited cells can combat *T. gondii* through the upregulation of effector molecules. It's worth noting that the innate immune response to *T. gondii* differs among host species, both in terms of detection and the subsequent elimination of the parasite through IFNγ-induced anti-parasitic activities (Yarovinsky 2014, Mukhopadhyay, Arranz-Solis et al. 2020).

Critical to the host's defense against *T. gondii* is the production of IL12 by DCs (DCs) and macrophages, as it primes various immune cells like NK cells, CD4 T cells, and CD8 T cells to secrete IFNγ. While DC-derived IL12 is crucial for IFNγ secretion in vivo, it alone cannot ensure the host's survival, as Myd88-deficient mice that received recombinant IL12 post-infection did not survive the disease. Inflammasome-activated pro-inflammatory cytokines IL18 and IL1β, secreted by monocytes and macrophages, also enhance IFNγ production and, together with IL12, contribute to host resistance against *T. gondii*. The specific lack of Myd88 in DCs or macrophage lineages in mice does not lead to rapid mortality, indicating the involvement of other factors and cytokines regulating IL12 and IFNγ secretion. Neutrophils recruited to the infection site secrete IL12 and IFNγ, while monocytes secrete IL1β and TNFα, inducing IFNγ secretion by neutrophils, NK cells, and *T. gondii* in a MyD88-dependent manner. The recruitment of immune cells to the infection site via chemokine secretion is a crucial step in controlling the parasite, with different chemokine receptors

facilitating the recruitment of various immune cell types. The cooperative interaction between DCs and NK cells further promotes IL12 and IFNγ production, and the significance of CD8α DCs and NK cells in host defense against *T. gondii* infection is evident in mice lacking certain receptors and transcription factors, which succumb rapidly to the disease (Yarovinsky 2014, Mukhopadhyay, Arranz-Solis et al. 2020).

IFNγ, either alone or in conjunction with TNFα, plays a crucial role in initiating the direct anti-parasitic activity within hematopoietic and non-hematopoietic cells upon *T. gondii* infection. However, humans with IFNγ-receptor deficiency don't exhibit increased susceptibility, possibly due to the induction of host-protective autophagy by TNFα and CD40-CD40L interactions in infected macrophages and non-hematopoietic cells. This autophagic process leads to parasite elimination. Neutrophils, abundant at the infection site, form neutrophil extracellular traps (NETs) composed of nuclear DNA fibers, histones, and antimicrobial peptides, which immobilize and kill trapped parasites. This NET-based defense mechanism is conserved across various species. After initial sensing and immune activation, the CD8 T cell response is triggered when they encounter MHC I-presented epitopes by antigen-presenting cells (APCs). Effective CD8 T cell activation relies on the elimination of intracellular parasites within the APCs, followed by the presentation of immunodominant epitopes derived from specific *T. gondii* proteins. In vitro studies highlight the importance of NLRP3 for the CD8 T cell-mediated immune response. Depletion of CD8 T cells renders mice more susceptible to the chronic phase of infection, with CD4 and CD8 T cells restraining parasite growth through various mechanisms such as IFNγ production, CD40-CD40L interaction-mediated autophagy, and perforin-mediated lysis of infected cells (Yarovinsky 2014, Mukhopadhyay, Arranz-Solis et al. 2020).

The host defense against *T. gondii* infection is multifaceted, involving both Th1 and Th2 immune responses. CD4+ cells produce high levels of Th1 cytokines (IL2 and IFNγ) upon interaction with tachyzoites, contributing to resistance against the parasite. Th1 cell-mediated immunity is essential, particularly due to IFNγ production by Th1 effector cells. Th1 cytokines, including IFNγ, IL12, and TNFα, play crucial roles in host resistance to toxoplasmosis. The absence of any of these pro-inflammatory mediators increases susceptibility to the infection. Additionally, various cytokines such as IL2, IL6, IL7, IL15, IL18, and IL23 are linked to the development of a robust immunogenic response. Recent

findings reveal TLR-11-independent activation of inflammasomes, promoting CD4+ T-cell-derived IFNγ-mediated host resistance to *T. gondii*. GRA24-driven protective immunity, mediated through p38 MAPK activation and IL12 production, is independent of the MyD88 pathway. In contrast, the cytokines associated with the Th2 response, notably IL4 and IL10, enhance susceptibility to *T. gondii* infection in the early stages. However, their regulatory functions have also been unveiled, as evidenced by decreased susceptibility in IL4-knockout mice and increased resistance in IL10 knockout mice when exposed to *T. gondii* infection. These complex interactions highlight the importance of balancing Th1 and Th2 responses in host defense against *T. gondii* (Yarovinsky 2014, Mukhopadhyay, Arranz-Solis et al. 2020, Sana, Rashid et al. 2022).

3. Hijacking Host Signaling and Its Immune Responses: *T.gondii'*s strategy for intracellular survival

Recent research on the interaction between the host and *T. gondii*, has revealed significant changes to various host signaling pathways during infection. These changes can be attributed to a range of parasite effectors, such as the ROP kinases and pseudokinases that are secreted during the invasion process. These effectors have been found to block host immune pathways and aid in the parasite's survival within the host. Additionally, the secretion of GRA proteins by the parasite has been found to significantly impact host gene expression. This is achieved through various mechanisms, such as the activation of host TFs, modification of chromatin, and induction of small noncoding RNAs (Hakimi and Bougdour 2015, Hakimi, Olias et al. 2017).

The secretion and release of the different virulence factors of the parasite is a highly regulated and synchronized process throughout the infection time. ROP proteins can be secreted into cells by a noninvading parasite, and hence, the delivery of effectors may alter host cell function even in the absence of invasion (Koshy, Dietrich et al. 2012). Also, several GRA proteins anchor in the PVM and extend at least partly into the host cytosol, where they interact with host proteins; therefore, more than one population of GRA proteins that occupy the lumen or PVM are released in a single dose early in invasion, while those that traffic outside the PV are released more slowly over time (Mercier and Cesbron-Delauw 2015).

When *T. gondii* infects a host, it releases its virulence factors in a specific sequence. However, research has uncovered that this process is not simply a matter of the parasite lacking the necessary machinery. Rather, the parasite actively reprograms the host cell's gene expression by manipulating its transcriptional machinery. This is accomplished using a collection of molecular hijackers, which give it the ability to take control of the host cell's gene expression in a sequential and orderly manner during infection (Hakimi, Olias et al. 2017). With this repertoire, *T. gondii* specifically can target gene expression at both the transcriptional and post-transcriptional levels (Zeiner, Norman et al. 2010, Cannella, Brenier-Pinchart et al. 2014). Likewise, *T. gondii* can manipulate host proteins by controlling their amount and post-translational modifications, enabling the parasite to activate or inactivate them as necessary. Additionally, the parasite selectively reprograms host mRNA translation, contributing to its survival (Leroux, Lorent et al. 2018).

3.1 Metabolic Manipulation

T. gondii is auxotrophic for several key metabolites which must be scavenged from the host cell, as a result, *T. gondii* depends on its host for the salvage of energy sources, building blocks, vitamins, and cofactors to survive and replicate (Kloehn, Hammoudi et al. 2021).

Studies have reported that *T. gondii* can alter metabolite abundance in various metabolic pathways, such as the tricarboxylic acid cycle, the pentose phosphate pathway, glycolysis, amino acid synthesis, and nucleotide metabolism, resulting in changes to the host's energetic and biosynthetic pathways during infection (Olson, Martorelli Di Genova et al. 2020). Some of these changes have been attributed to the transcriptional regulation of the parasite. Likewise, changes in metabolic pathways such as pentose phosphate, are provoked by the transcriptional regulation of both the parasite and the host (Olson, Martorelli Di Genova et al. 2020). Additionally, host metabolites directly impact the parasite life cycle development by triggering or blocking differentiation (Kloehn, Hammoudi et al. 2021). However, further research is required to understand the mechanisms that facilitate the comprehensive restructuring of host metabolism for optimal growth of the parasite.

T. gondii tachyzoites can rapidly and continuously induce c-Myc, a key regulator of host cell transcription. This tightly controlled TF is involved in essential cellular processes, such as metabolism. Upregulation of c-Myc may be a mechanism used by the parasite to manipulate the host’s metabolic pathways, ensuring that it has access to sufficient nutrients (Franco,

Shastri et al. 2014). The hypothetical effector involved in c-Myc upregulation could act either directly or indirectly via manipulation of many pathways that *T. gondii* is known to impact, such as STAT-3, NF-κB, PI3 kinase, and MAP kinases (Franco, Shastri et al. 2014). A previous study showed that infection with *T. gondii* tachyzoites specifically upregulates microRNAs that belong to transcriptional loci miR-17∼92 and miR-106b∼25 (Zeiner, Norman et al. 2010). Therefore, studies have shown that c-Myc is regulated by miR-17∼92 and miR-106b∼25. This suggests that *T. gondii* may induce the transcriptional regulation of these microRNAs to upregulate c-Myc to facilitate its intracellular survival (Franco, Shastri et al. 2014, Valinezhad Orang, Safaralizadeh et al. 2014).

3.2 Apoptosis Manipulation

Programmed cell death or apoptosis, is a common mechanism used by the host to combat a wide range of pathogens, from viruses, bacteria and intracellular protozoan parasites, mainly by decreasing the replication rate of pathogens and activating the innate and adaptive immune system (Labbe and Saleh 2008). In that sense, during infection, the host cell undergoes apoptosis as a means of limiting the replication and spread of the intracellular parasite. This response involves both innate and adaptive immune mechanisms that work to restrict parasite replication and limit its spread within the host. Additionally, apoptosis has regulatory functions that help modulate host immune responses to infection. By inducing an apoptotic response in infected cells, the host is able to reduce the replication and spread of the pathogen, ultimately contributing to its own survival (Laliberte and Carruthers 2008).

T. gondii has evolved strategies to evade elimination by the host's apoptotic response. This helps the parasite to avoid rapid clearance by macrophages, which are activated by signals emitted by apoptotic cells. The parasite must also ensure the integrity of the host cell to obtain nutrients, as apoptotic cells undergo self-catabolism to release their macromolecules for neighboring cells and phagocytes. By evading apoptosis, *T. gondii* can maintain a stable niche within the host, allowing it to scavenge nutrients without activating the immune response. These tactics illustrate the sophisticated and multifaceted approach that the parasite takes to survive within the host cell (Nash, Purner et al. 1998).

Consequently, *T. gondii* acts on common downstream apoptotic effectors or inhibits apoptotic elements of various pathways simultaneously (Laliberte and Carruthers 2008). There are two basic apoptotic signaling pathways: the extrinsic and the intrinsic pathways,

which converge on a common “execution” phase, driven by proteases, known as caspases. Consequently, it has been reported that *T. gondii* is capable of regulating apoptosis by causing negative regulation of the activity of caspases. The parasite can inhibit the release of cytochrome c from mitochondria to the host cell cytosol (Keller, Schaumburg et al. 2006), affect the balance of pro- and anti-apoptotic BCL-2 family members (Carmen, Hardi et al. 2006), or directly interfere with caspase processing and function (Vutova, Wirth et al. 2007). It can also block upstream signaling pathways through positive or negative regulation of TFs that can activate or inactivate apoptotic processes (Molestina, Payne et al. 2003).

T. gondii has been shown to manipulate various TFs that are involved in the regulation of apoptosis pathways, including NF-κB and p53(Hwang, Quan et al. 2010, Besteiro 2015). However, the exact mechanisms by which the parasite achieves this manipulation are not yet clear.

Further studies are needed to fully understand the intricate interplay between *T. gondii* and the host cell signaling pathways involved in both immune response and apoptosis regulation.

3.3 Circumventing the Host's IFN-γ

To establish a successful infection, *T. gondii* must manipulate the host's signaling pathways, particularly the activation of the interferon-γ (IFNγ) immune response, which can trigger parasite clearance. IFNγ is a pro-inflammatory cytokine that plays a critical role in initiating and maintaining both innate and acquired immunity against *T. gondii*. It is considered the main cytokine induced by T cells (CD4+ and CD8+), γδT cells, and NK cells, and is vital for protective immunity against both the acute and chronic phases of *T. gondii* infection. Therefore, IFNγ is a key factor in restricting the proliferation of *T. gondii* and is crucial for preventing the development of disease (Sana, Rashid et al. 2022). After induction of acquired immunity, host immune cells robustly produce the proinflammatory IFN-γ, which activates a set of IFN-γ-inducible proteins, including GTPases (Sasai and Yamamoto 2019).

IFN-γ inducible Guanosine Triphosphatases (GTPases) are essential for cell-autonomous immunity and are specialized for effective clearance and growth inhibition of *T. gondii* by accumulating in PVM (Sasai and Yamamoto 2019). IFN-γ-stimulated cells express hundreds of IFN-γ -stimulated genes, including GTPases family members, which are strongly involved in the immune response against *T. gondii* (Sana, Rashid et al. 2022).

The parasite can inhibit IFNγ signaling by curtailing the function of the TF STAT-1 and by augmenting the levels of IFNγ signaling suppressor molecules, named as suppressor of cytokine signaling molecule-1 (SOCs1) (Zimmermann, Murray et al. 2006).

In both mice and humans, the innate immune system responds to *T. gondii* infection by producing high levels of IL-12 and IFN-γ through the adaptor protein MyD88 (Sasai and Yamamoto 2019). The initial recognition of the parasite involves host receptors such as TLR2, TLR4, and TLR11, which detect specific parasite molecules. However, the deletion of individual TLRs has only modest effects on susceptibility to infection, suggesting that multiple TLRs synergistically are involved in recognizing *T. gondii*. Furthermore, triple deficiency mice, which lack proper TLR3/7/9 trafficking and TLR11 signaling, are highly susceptible to *T. gondii* infection, highlighting the importance of these receptors in the host defense (Hunter and Sibley 2012). Interestingly, while TLR11 plays a dominant role in sensing *T. gondii*, it is represented only by a pseudogene in humans, leaving unanswered questions about how innate and adaptive immune responses occur in the absence of TLR11in humans.

Furthermore, MyD88 is essential for host resistance against *T. gondii*, its activation being necessary for induction of pro-inflammatory cytokines and the establishment of strong Th1 responses (Pifer and Yarovinsky 2011). In addition, MyD88 functions in various immune cells, including neutrophils, macrophages, DCs, and T cells, influencing IL-12 production and T cell activation. TLR signaling in DCs is critical for restricting *T. gondii* expansion and promoting IFN-γ production by NK cells, which subsequently activates inflammatory monocytes to eliminate the parasites.

Consequently, the innate recognition, particularly through TLR activation in DCs, contributes to host resistance against *T. gondii* (Pifer and Yarovinsky 2011, Sasai and Yamamoto 2019).

Mouse models have been instrumental in understanding the cellular and molecular mechanisms of innate and adaptive immune responses to *T. gondii* (Pifer and Yarovinsky 2011). However, there are significant differences between mice and humans in their responses to the parasite. Additionally, mice have a more robust effector system involving multiple immune-related GTPases (IRGs), whereas humans have a limited effector system. Mice have a more robust effector system against *T. gondii* because they have multiple IRGs.

Humans have only one full-length IRG gene, which contains a testis-specific promoter. Mice have 23 IRG genes in their genome and IL-12 initiates IFN-γ dependent induction of multiple IRGs. However, this effector system is largely non-functional in humans and is limited to expression of only one protein (Pifer and Yarovinsky 2011).

TLR11 recognizes *T. gondii* through released microbial profilin, allowing innate sensing of the parasite in non-infected cells (Pifer and Yarovinsky 2011). This recognition strategy enables mouse innate immune cells, particularly DCs, to initiate immune responses while avoiding the immunosuppressive effects observed occurring in infected macrophages and DCs. The co-evolution of *T. gondii* virulence factors and host defenses in mice has led to spatially separated detection and effector functions to combat the parasite (Wang and Jiang 2023). In contrast, humans, being accidental hosts, may not possess such elaborate defenses. Humans are accidental hosts for *T. gondii* because they lack the elements that allow mice to detect and control the parasite. Humans lack of TLR 11 and TLR121 and a complete set of IRGs. Additionally, humans are dead-end hosts for *T. gondii* because they are rarely eaten by felines (Pifer and Yarovinsky 2011, Wang and Jiang 2023).

TLR11 deficiency in humans may be compensated by other recognition systems that directly sense the parasite or rely on other stimuli, including commensal bacteria (Pifer and Yarovinsky 2011). Genetic association studies have identified candidate genes, such as TLR9, purinergic receptor P2X7, and NOD-like receptor NALP1, which may be involved in human responses to *T. gondii*. NALP1, located within the rat genome susceptibility/resistance region, shows potential as an intracellular sensor for the parasite. The purinergic receptor P2X7 has also been associated with congenital and ocular toxoplasmosis, and it may regulate the killing of the parasite and enhance lysosomal fusion with the PV (Pifer and Yarovinsky 2011).

In summary, *T. gondii* manipulates the host's immune response by inhibiting IFNγ signaling. IFNγ and IFNγ-inducible GTPases are critical for protective immunity against the parasite. These GTPases accumulate in the PVM to effectively clear and inhibit the growth of *T. gondii*. However, the parasite has developed strategies to evade the host's immune system, such as inhibiting IFNγ signaling. The immune response to *T. gondii* infection involves the production of IL-12 and IFN-γ, with TLR signaling through MyD88 playing a crucial role. However, while mice have robust defense mechanisms, including TLR11 recognition,

humans have differences in their immune response. In conclusion, investigating the immune response to *T. gondii* in both mice and humans reveals intriguing differences and highlights the need for further exploration to fully understand host-pathogen interactions and develop effective strategies against this common pathogen.

3.4 Manipulating the Host's Cell Cycle

T. gondii modulates various functional aspects of its host cell, including the cell cycle. It was first observed that the parasite can influence the host cellular cell cycle, leading to a reduction in host cell division (Velasquez, Conejeros et al. 2019). Mammalian cells infected with *T. gondii* are characterized by a deep reprogramming of gene expression and the parasite-dependent reprogramming of gene expression correlates with the modulation of transcripts that regulate cell proliferation such as TF Egr-1 which has been shown to activate the expression of growth factors participating in the amplification of a mitogenic stimulus (Molestina, El-Guendy et al. 2008). While there isn't a significant amount of evidence detailing the advantages of modulating the cell cycle during parasitic infections such as *T. gondii*, changes in host cell transcription - particularly those that impact the cell cycle machinery - can lead to the modulation of the intracellular environment and ultimately affect parasite development (Molestina, El-Guendy et al. 2008). *T. gondii* has developed strategies to both induce a growth signal and prevent cell cycle progression in the infected cell. The perturbations in the host cell cycle machinery may influence how the parasite adapts to its own replication scheme during intracellular residence (Velasquez, Conejeros et al. 2019). In addition, *T. gondii* affects the cell cycle of not only the cells it directly invades, but neighboring cells as well. Thus, the parasite induces neighboring cells to enter S-phase, allowing more rapid invasion by extracellular *T. gondii* and providing a possible selective advantage for the parasite (Lavine and Arrizabalaga 2009).

To conclude, *T. gondii* alters host cell function, including the cell cycle, to promote its own growth. Changes in host transcription can affect parasite development, and the perturbations in the host cell cycle machinery may impact the parasite's replication, potentially giving it a selective advantage.

3.5 Manipulating the Host's Transcription Factors

T. gondii like other pathogens, has evolved strategies to promote its survival by dramatically modifying the transcriptional profile and protein content of host cells, either by the activation of the host's immune response or by the initiation of the mechanism used by the parasite to circumvent immunity or defenses. Notably, *T gondii* infection result in changes in expression of specific genes, such as those encoding TFs (Silmon de Monerri and Kim 2014). For instance, GRA proteins, preferentially target cell-specific TFs, such as NF-κB, interferon regulatory factor (IRF), and JAK/STAT, which act as coordinating hubs in host defenses. By differentially phosphorylating these TFs, GRA proteins can modulate their intrinsic activities and expression levels, leading to further changes in host gene expression altering the host's immune response against the parasite (Hakimi, Olias et al. 2017). *T. gondii* employs these effectors, such as TgIST and GRA24, that translocate directly to the host nucleus to modify transcriptional activity, negatively or positively regulating the host's immune response. TgIST translocate to the nucleus and recruits the Mi-2/NuRD complex to STAT1-dependent promoters. This leads to chromatin alteration and signal blockage of the IFN response (Matta, Olias et al. 2019), while GRA24 forms a complex with p38α to activate TFs responsible for the expression of proinflammatory genes and for stimulation of a Th1 response, modulating the early immune response to infection (Braun, Brenier-Pinchart et al. 2013). Others, like GRA16, control p53 levels by binding to PP2A-B55 and HAUSP while translocating to the host nucleus where it positively modulates genes involved in cell-cycle progression and the p53 tumor suppressor pathway (Braun, Brenier-Pinchart et al. 2013). In type II strains, GRA15 localizes to the PVM, activates the NF-κB pathway by activating TRAF6 and subsequently IKK, leading to the phosphorylation and degradation of IκB which promotes the activation of the immune response of the host against the parasite. GRA15 interaction with TRAF6 determines parasite strain differences in susceptibility to IFNγ (Sangare, Yang et al. 2019). Lastly, GRA6, which partially interacts with the host cell cytosol while residing in the PV, interacts with CAMLG to activate calcineurin and stimulate the TF NFAT4 (Ma, Sasai et al. 2014). These diverse mechanisms of manipulation demonstrate the complex strategies *T. gondii* employs to evade and manipulate host responses to infection.

Although some virulence factors have been identified for certain host TFs, such as TgIST for STAT1 and GRA24 for p38α, others remain unknown (Hakimi, Olias et al. 2017). Host TFs

such as hypoxia-inducible factor (HIF), cAMP response element-binding protein (CREB), and activating transcription factor 2 (ATF2) play crucial roles in promoting the optimal growth of *T. gondii* under hypoxic conditions and the proliferation, survival, and differentiation of host cells (Spear, Chan et al. 2006, Wen, Sakamoto et al. 2010, Braun, Brenier-Pinchart et al. 2013, Watson, Ronai et al. 2017). Identifying the virulence factors that regulate these TFs could provide valuable insights into the mechanisms of *T. gondii* infection and lead to the development of novel therapeutic strategies.

T. gondii can manipulate multiple cellular processes in the host cell by altering the function of single TFs, through the alteration of chromatin accessibility and remodeling (Leng, Butcher et al. 2009, Falvo, Tsytsykova et al. 2010). This strategy is advantageous for the parasite, as the regulation of a single TF can lead to the regulation of multiple processes with very few induction or inhibition steps. For example, inhibition of host cell apoptosis can be a concomitant consequence of the regulation of TFs related to the immune response stimulated by IFNγ. Understanding the mechanisms by which *T. gondii* manipulates host TFs can provide valuable insights into the pathogenesis of *T. gondii* infection, **Figure 1.3.**

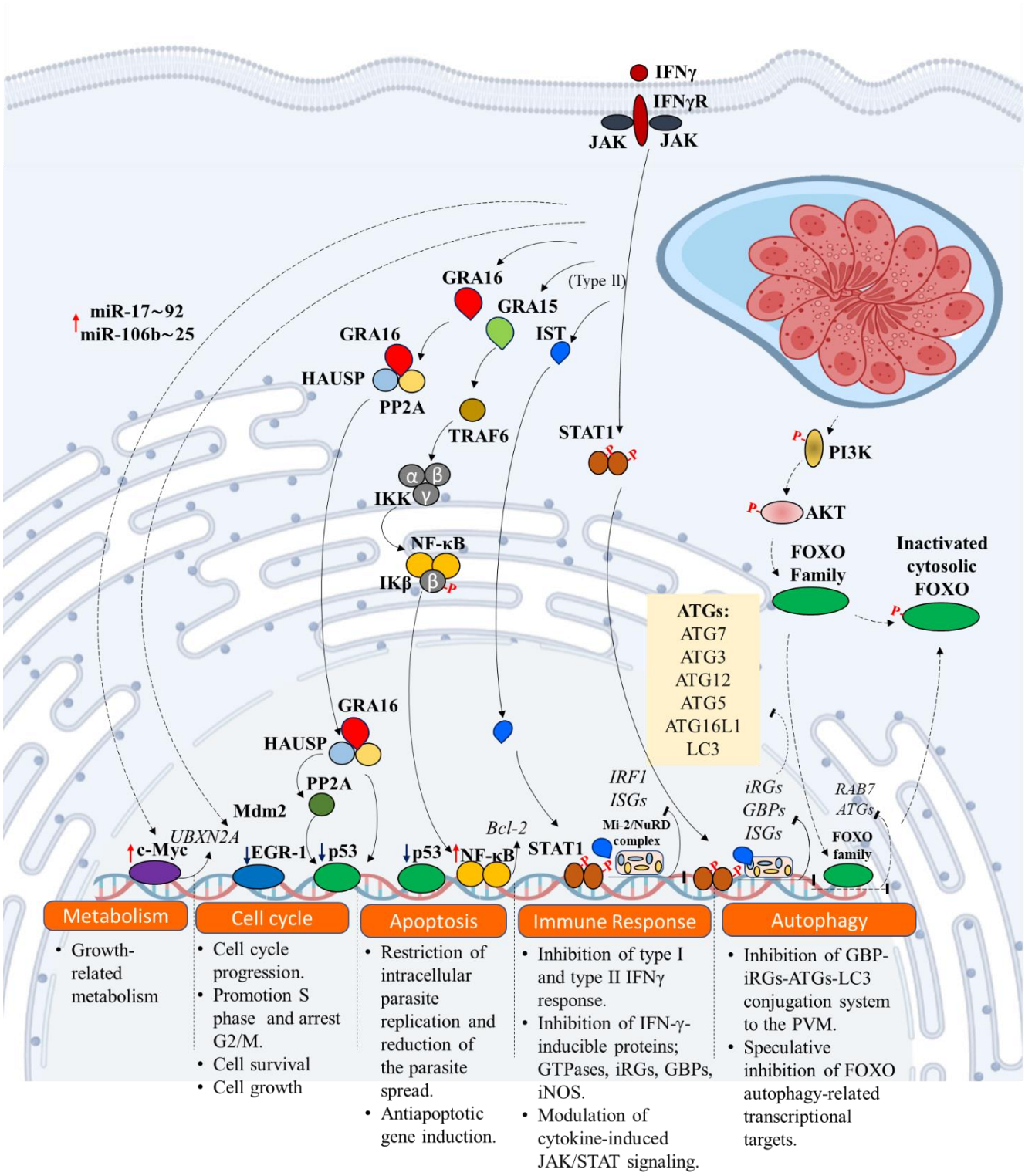


Figure 1.3 *T. gondii* Manipulation of Host Transcription Factors

T. gondii actively reprograms host gene expression by subverting host transcriptional machinery and manipulating TFs responsible for regulating various biological processes. During infection, *T. gondii* upregulates some TFs, such as c-Myc, and downregulates others, such as EGR-1, to promote the alteration of

metabolic processes related to host growth and cell cycle progression. Additionally, *T. gondii* secretes effectors from dense granules, such as GRA16 and TgIST, which inhibit host TFs P53 and STAT1, respectively, altering the host cell cycle, decreasing apoptosis, and globally blocking the IFN-JAK/STAT response through the recruitment of the Mi-2/NuRD repressor complex. In type II strains, *T. gondii* activates the NF-κB pathway through the activation of TRAF6, leading to the phosphorylation and degradation of IκB, promoting the upregulation of NF-κB transcriptional targets such as Bcl-2, which inhibits apoptosis. Adapted from (Hakimi, Olias et al. 2017)

4. Autophagy

4.1 Definition and Function in Steady State Conditions

Autophagy, which means "self-eating," is an evolutionarily conserved catabolic process that facilitates the degradation of cytoplasmic materials, including organelles and proteins, by delivering them to the lysosome. There are four main types of autophagy: autophagosome-mediated macroautophagy, microautophagy, chaperone-mediated autophagy, and non-canonical autophagy. Macroautophagy, commonly referred to as autophagy, involves the fusion of double membrane autophagosomes with lysosomes to break down cargo. Microautophagy is less understood but appears to entail the direct uptake of materials by lysosomes, resembling pinocytosis. Chaperone-mediated autophagy is a biochemical process that transports specific proteins into lysosomes, relying on a signature sequence and chaperone interactions. Non-canonical autophagy is an alternative pathway of autophagy that can operate without the involvement of certain key autophagy-related proteins, offering potential therapeutic possibilities. It represents a deviation from the traditional autophagic process, which relies on a hierarchy of autophagy-related (ATG) proteins for autophagosome formation, the central organelle in macroautophagy, a lysosomal degradation pathway essential for cell survival, pathogen elimination, and various physiological functions (Codogno, Mehrpour et al. 2011, Das, Shravage et al. 2012).

Autophagy involves the formation of membranes and fusion events, beginning with the initiation of an isolation membrane or phagophore from a source known as the phagophore assembly site. The source of the autophagosome membrane can be the smooth endoplasmic reticulum, although other membrane sources like mitochondria and the plasma membrane can contribute under different conditions. The isolation membrane engulfs the cargo, forming the double-membrane autophagosome, which subsequently fuses with lysosomes to create

autolysosomes for cargo degradation by lysosomal hydrolases. The process is cyclical, as lysosomes must reform to allow subsequent rounds of autophagy (Codogno, Mehrpour et al. 2011, Das, Shravage et al. 2012).

Mammalian autophagy serves as a fundamental biological process with multifaceted roles, contributing to cytoplasmic quality control, cellular metabolism, and innate and adaptive immunity. These three crucial missions - defense, metabolic regulation, and quality control - are interlinked in the context of immunity. Dysregulation of autophagy often leads to inflammatory disorders in both animal models and human diseases. The term "autophagy" encompasses a spectrum of lysosomal processes that maintain intracellular homeostasis, involving the turnover of proteins, membranes, organelles, and the generation of metabolic precursors, especially during periods of nutrient scarcity (Deretic 2021).

Autophagy is a multi-step process, encompasses induction, cargo recognition and selection, vesicle formation, autophagosome-vacuole fusion, and cargo breakdown. At each of these steps, ATG proteins play crucial roles. In yeast and Drosophila, mTOR negatively regulates Atg1 kinase, an essential autophagy initiator, under nutrient-rich conditions. Upon mTOR inhibition, Atg1's kinase activity is activated, promoting autophagosome formation. In mammals, ULK1 and ULK2, along with FIP200, serve as homologs of yeast Atg1 and Atg17, involved in autophagy initiation. Selective autophagy relies on the recognition of specific cargos by receptor proteins, such as p62. Cargo recognition triggers the assembly of autophagosomes, which are double-membrane vesicles formed at the phagophore assembly site through the coordination of various Atg proteins. Nucleation and membrane assembly require the class III phosphatidylinositol 3-kinase (PtdIns3K) complex, Atg12–Atg5-Atg16, and Atg8–PE conjugation systems. After vesicle formation, autophagosomes fuse with lysosomes or vacuoles, followed by the degradation of inner vesicle content by lysosomal hydrolases. This degradation process releases small molecules like amino acids back into the cytosol, aiding in nutrient recycling during starvation conditions (He and Klionsky 2009).

4.2 Mechanisms of Induction and Regulation

Autophagy is a tightly regulated cellular process critical for maintaining homeostasis. Its intricacy is reflected in the eukaryotes' elaborate control mechanisms, where both insufficient and excessive autophagy can be detrimental. Several signaling pathways orchestrate this regulation. The TORC1 pathway, primarily through its nutrient-sensing function, plays a

central role in autophagy control. In nutrient-rich conditions, active TORC1 inhibits autophagy, while nutrient deprivation leads to its inactivation, allowing autophagy to surge. Additionally, TORC1 exerts influence by regulating the Atg1–Atg13–Atg17 kinase complex. The Ras/cAMP-dependent PKA pathway also negatively regulates autophagy, especially in nutrient-rich environments. Furthermore, eIF2α kinase signaling and the GCN4 general control pathway respond to amino acid starvation and impact autophagy. In addition to these, several other signaling pathways like Snf1 and Pho85 influence autophagy, although their precise mechanisms are still under investigation. These intricate regulatory networks are critical for understanding the role of autophagy in various physiological contexts and diseases (Yang and Klionsky 2009).

Autophagosome formation increases during nutrient deprivation. Two key signaling pathways, TOR and Ras-cAMP-PKA, control this process in response to nutrient levels. TORC1 inhibits autophagy under nutrient-rich conditions, but its inhibition by rapamycin stimulates autophagy. Amino acids are essential regulators, activating mTOR either directly or through class III PtdIns3K. This complex interplay between pathways and amino acids modulates autophagy to adapt to varying nutrient availability. In yeast, TORC1 also suppresses autophagy by phosphorylating Tap42, which activates the negative regulator PP2A, potentially impacting Atg proteins and autophagy regulation (He and Klionsky 2009). The Ras/PKA signaling pathway, along with the TOR and Sch9 pathways, contributes to the regulation of autophagy in response to nutrient conditions. In nutrient-rich environments, active Ras1 and Ras2 stimulate cAMP production, which in turn releases PKA's inhibitory effect on autophagy. This inhibitory action may involve PKA phosphorylating Atg1, causing it to remain cytosolic. In contrast, under starvation conditions, Atg1 is dephosphorylated and localized to the phagophore assembly site, promoting autophagy initiation. The interplay between these pathways provides a complex network for autophagy modulation, allowing cells to adapt to varying nutrient availability. Autophagy can also be regulated at the transcriptional level by Msn2/4 and Rim15, particularly when both PKA and Sch9 pathways are simultaneously inactivated. This intricate regulation ensures cells respond appropriately to nutrient fluctuations (He and Klionsky 2009).

4.2.1 Insulin and Growth Factor Pathways

When it comes to autophagy regulation, growth factors and nutrients converge on the TOR pathway, but their signaling mechanisms differ. In higher eukaryotes, hormones like insulin and insulin-like growth factors regulate mTOR through class I PtdIns3K. Insulin binding leads to receptor autophosphorylation and recruitment of downstream proteins, including class I PtdIns3K subunits. This results in the generation of PIP3, which activates AKT. Activated AKT then phosphorylates TSC2, leading to mTORC1 activation. The absence of hormones inactivates mTOR, relieving its inhibitory effect on autophagy (He and Klionsky 2009, Manning and Toker 2017).

Ras signaling also plays a role in autophagy regulation by growth factors. In certain contexts, activated Ras inhibits autophagy through class I PtdIns3K, while in others, it positively regulates autophagy through the Ras-Raf-1-ERK1/2 pathway. The balance between these pathways, influenced by amino acids and cell-specific factors, determines the role of Ras in autophagy regulation in response to growth factors and amino acid availability (He and Klionsky 2009).

4.2.2 Energy Sensing

In times of intracellular metabolic stress, the activation of autophagy plays a crucial role in ensuring cell viability. In mammalian cells, low ATP levels trigger AMPK (5′-AMP-activated protein kinase), activated by the decreased ATP/AMP ratio through LKB1 kinase. Active AMPK phosphorylates and activates the TSC1/2 complex, inhibiting mTOR via Rheb. This leads to autophagy induction, promoting ATP production by recycling nutrients. Additionally, the LKB1-AMPK pathway activates p27kip1, causing cell cycle arrest, preventing apoptotic cell death, and promoting autophagy to cope with bioenergetic stress during growth factor withdrawal and nutrient deprivation (He and Klionsky 2009, Manning and Toker 2017).

4.2.3 Stress Response

Autophagy is induced in response to various forms of stress. In cases of endoplasmic reticulum (ER) stress, triggered by factors like protein misfolding, autophagy is vital for maintaining cell viability. Hypoxic conditions, characterized by low oxygen levels, also prompt autophagy in mammalian cells, with pathways like HIF-1 involved in mitophagy, the

selective removal of mitochondria. However, the exact pathways may vary according to cell type. In the context of ROS-induced autophagy, the protease Atg4 and hydrogen peroxide-activated poly (ADP-ribose) polymerase-1 (PARP-1) have been identified as contributing factors. DNA damage resulting from oxidative stress could also be implicated in PARP-1 activation and the induction of autophagy (He and Klionsky 2009, Manning and Toker 2017).

4.3 Regulation by AKT

Autophagy occurs at low basal levels in virtually all cells to perform homeostatic functions such as protein and organelle turnover. It is rapidly upregulated when cells need to generate intracellular nutrients and energy, for example, during starvation, growth factor withdrawal, or high bioenergetic demands. Autophagy is also upregulated when cells are preparing to undergo structural remodeling such as during developmental transitions or to eliminate damaging cytoplasmic components, for example, during oxidative stress, infection, or protein aggregate accumulation (Levine and Kroemer 2008).

AKT is a well-known survival kinase that plays a significant role in regulating autophagy. Research shows that inhibitors of the PI3K/AKT pathway or AKT knockdown can lead to a marked increase in autophagy (Degtyarev, De Maziere et al. 2008). The class I PI3K/AKT signaling molecules play a crucial role in the activation of mTOR, considered the major inhibitory signal that suppresses autophagy in the presence of growth factors and abundant nutrients; mTOR inhibits autophagy in response to insulin-like and other growth factor signals (Lum, Bauer et al. 2005, Levine and Kroemer 2008).

AKT can also inhibit autophagy through mTOR-independent mechanisms. This process depends on the regulation of downstream substrates, whose functions have not been fully described. An example are FOXO TFs, which participate in diverse cellular and physiological processes, including cell proliferation, apoptosis, ROS response, longevity, regulation of the cell cycle, metabolism, and autophagy (Martins, Lithgow et al. 2016).

Early genetic and biochemical studies have identified AKT as a primary regulator of FOXO function in various organisms. AKT mediates this regulation through phosphorylation on

three predicted sites, T32, S253, and S315, both in vitro and in vivo, resulting in the nuclear exclusion of these TFs (Tzivion, Dobson et al. 2011).

FOXO proteins play a crucial role in inducing autophagy, not only through the transactivation of autophagy genes but also through direct interaction with autophagy proteins and by epigenetic regulation via histone modifications and miRNA, which regulate autophagy activity (Cheng 2019). As a consequence, the nuclear exclusion of these TFs, induced by their phosphorylation by AKT, can contribute to the inhibition of the transactivation of its autophagy genes, **Figure 1.4**.

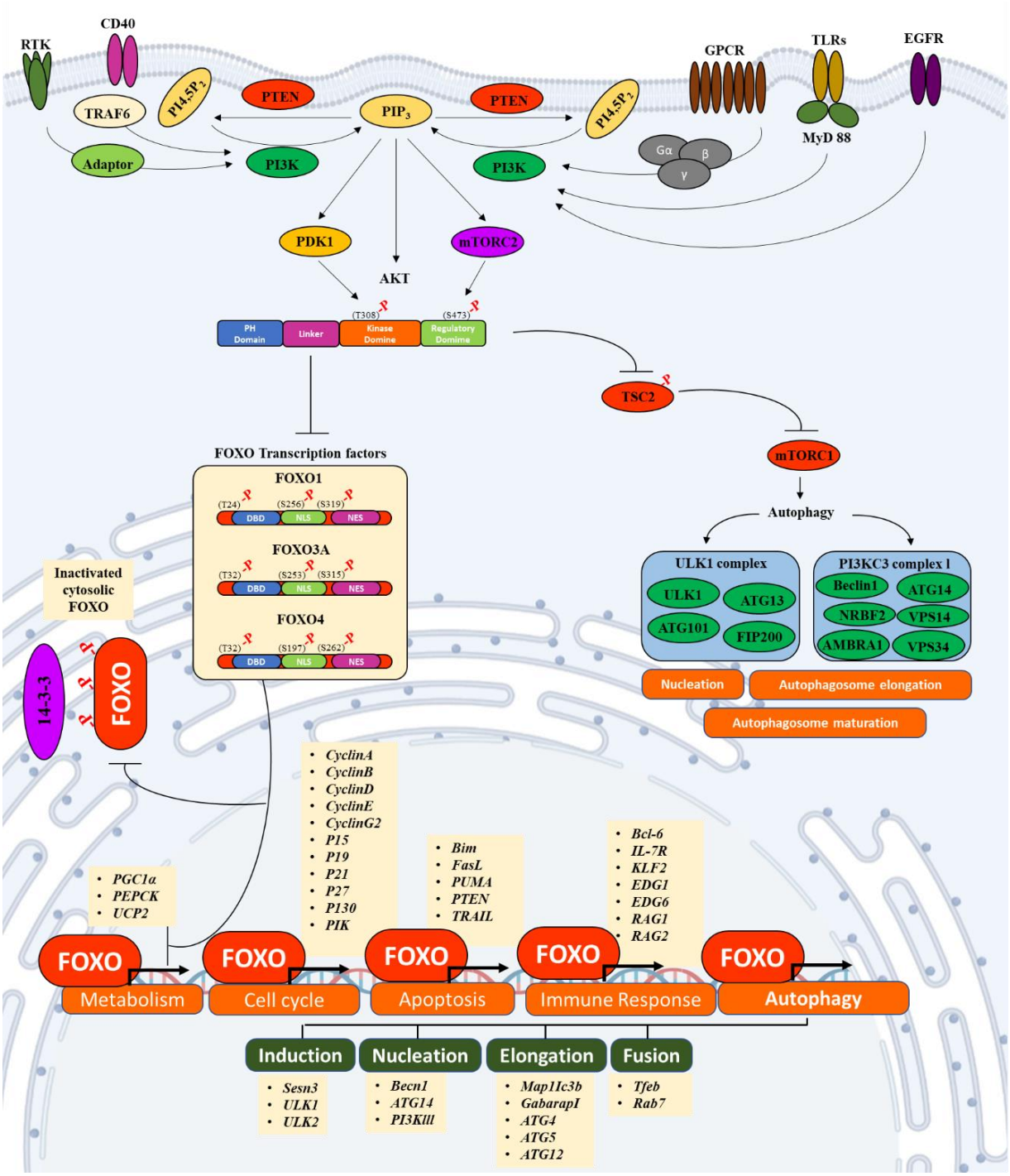


Figure 1.4 Regulation of Autophagy by AKT and FOXO

Regulatory pathways by which AKT and FOXO control autophagy and other cellular processes at the level of transcriptional regulation. AKT is activated by PI3K through phosphorylation on Thr-308 and Ser-473. The binding of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3] to AKT's pleckstrin homology domain is necessary for this activation and also directly activate an upstream kinase. AKT is a central player in signal transduction pathways activated by various stimuli and is involved in controlling diverse cellular functions,

including proliferation, survival, metabolism, and autophagy. AKT phosphorylates FOXO TFs, leading to their cytoplasmic retention and degradation, thereby inhibiting their transcriptional activity. FOXO proteins regulate autophagy by transactivating genes involved in autophagy formation and fusion of autophagosomes with lysosomes and by directly interacting with autophagy proteins. Additionally, FOXOs regulate cell cycle arrest, oxidative stress response, and DNA damage repair. Other proteins such as RITK, PTEN, TSC2, and 14-3-3, as well as mTORC1 and mTORC2 complexes, also play important roles in the regulation of AKT and FOXO activity and downstream signaling. These proteins act in concert to finely tune AKT-FOXO signaling to achieve proper cellular responses to various stimuli. The information provided in the figure is based on the sources (Lum, Bauer et al. 2005, Levine and Kroemer 2008, Tzivion, Dobson et al. 2011, Martins, Lithgow et al. 2016, Cheng 2019).

4.4 Protective Role Against Intracellular Pathogens

Autophagy serves as a vital cell-autonomous defense mechanism against intracellular pathogens, such as bacteria and viruses. Autophagy's role in innate and adaptive immunity has been increasingly recognized. In Drosophila, the peptidoglycan-recognition protein (PGRP) family, particularly PGRP-LE, plays a critical role in detecting intracellular bacteria and initiating autophagy. In mammals, TLRs on the cell surface and endosomes trigger autophagy in response to pathogen-derived ligands. For example, TLR7 responds to viral single-stranded RNA, TLR2 is activated by zymosan, and TLR4 is stimulated by LPS from Gram-negative bacteria. Adaptor proteins like MyD88 and TRIF mediate the signaling cascade leading to autophagy. Additionally, during viral infections, the antiviral eIF2α kinase signaling pathway, involving eIF2α and PKR, is activated to promote autophagy. Some viruses have evolved strategies to counter autophagy, like the herpes simplex virus type 1 protein ICP34.5, which inhibits autophagy by sequestering Beclin 1. Moreover, viral infections, including hepatitis C virus, induce ER stress, triggering autophagy through the unfolded protein response pathways (IRE1, ATF6, and PERK). Autophagy's multifaceted role in antimicrobial defense underscores its significance in maintaining cellular health in the face of intracellular pathogens (He and Klionsky 2009, Manning and Toker 2017, Deretic 2021).

4.5 Autophagy as a Host Defense Against *T. gondii*

As noted earlier, previous studies have established autophagy as a crucial defense mechanism against *T. gondii*, but recent advancements have uncovered novel insights into the regulatory pathways involved in autophagy and the ways in which *T. gondii* attempts to evade

autophagic responses (Deretic, Saitoh et al. 2013, Ghartey-Kwansah, Adu-Nti et al. 2020). Originally described as a recycling route to obtain nutrients in conditions of starvation, autophagy is as well a fundamental cell biological pathway affecting immunity. Through the control of inflammation via regulatory interactions with innate immunity signaling, by removing endogenous inflammasome agonists, and through effects on secretion of immune mediators, autophagy contributes to antigen presentation, T cell homeostasis, and affects T cell repertories and polarization including Th17 inflammation (Deretic, Saitoh et al. 2013).

Macroautophagy is initiated by the regulator serine/threonine protein kinases ULK1 and ULK2 (Wang and Li 2020), as well as the lipid kinase activity of PI3Klll, which forms a complex with BECLIN1 and ATG14L (Mizushima 2020). These factors integrate upstream signals to induce the downstream ATG conjugation cascade, which includes the association of mammalian paralogues of ATG8: LC3A, LC3B, LC3C, GABARAPL1, and GABARAPL2 (Mizushima 2020). Lipidated mammalian paralogues of ATG8 then function together with other factors to assemble, elongate, and lead to the closure of nascent autophagic organelles.

The lipid kinase VPS34 produces PI3P, which is recognized by WIPI1-WIPI4, and these factors cooperate with ATG2 and ATG9 to form the phagophore. The attachment of a tail-anchored SNARE, syntaxin17, to the autophagosome membrane enables fusion with lysosomes during the final maturation of autophagosomes into autolysosomes (Yao, Ren et al. 2021) **Figure 1.5**. This process is highly regulated and coordinated, with transcriptional adjustments being necessary for sustained autophagy. Transcription-positive regulators, such as TF TFEB, FOXO3a, NRF2 (Mizushima, Yoshimori et al. 2011, Mizushima 2020), and transcription-negative regulators, such as ZNF306 (Di Malta, Cinque et al. 2019), coordinate autophagy with lysosomal, proteasomal, lipolytic, and oxidative stress response systems.

While autophagy induced by starvation and that induced by the presence of parasites share some similarities, there are also some notable differences. For example, in the case of intracellular parasitic infections, clearance can start with the recognition of pathogen-associated molecular patterns (PAMPs) via Toll-like receptors, marking the first line of defense. Pathogens may also be ubiquitinated before being recognized by autophagy receptors and labeled with LC3 protein.

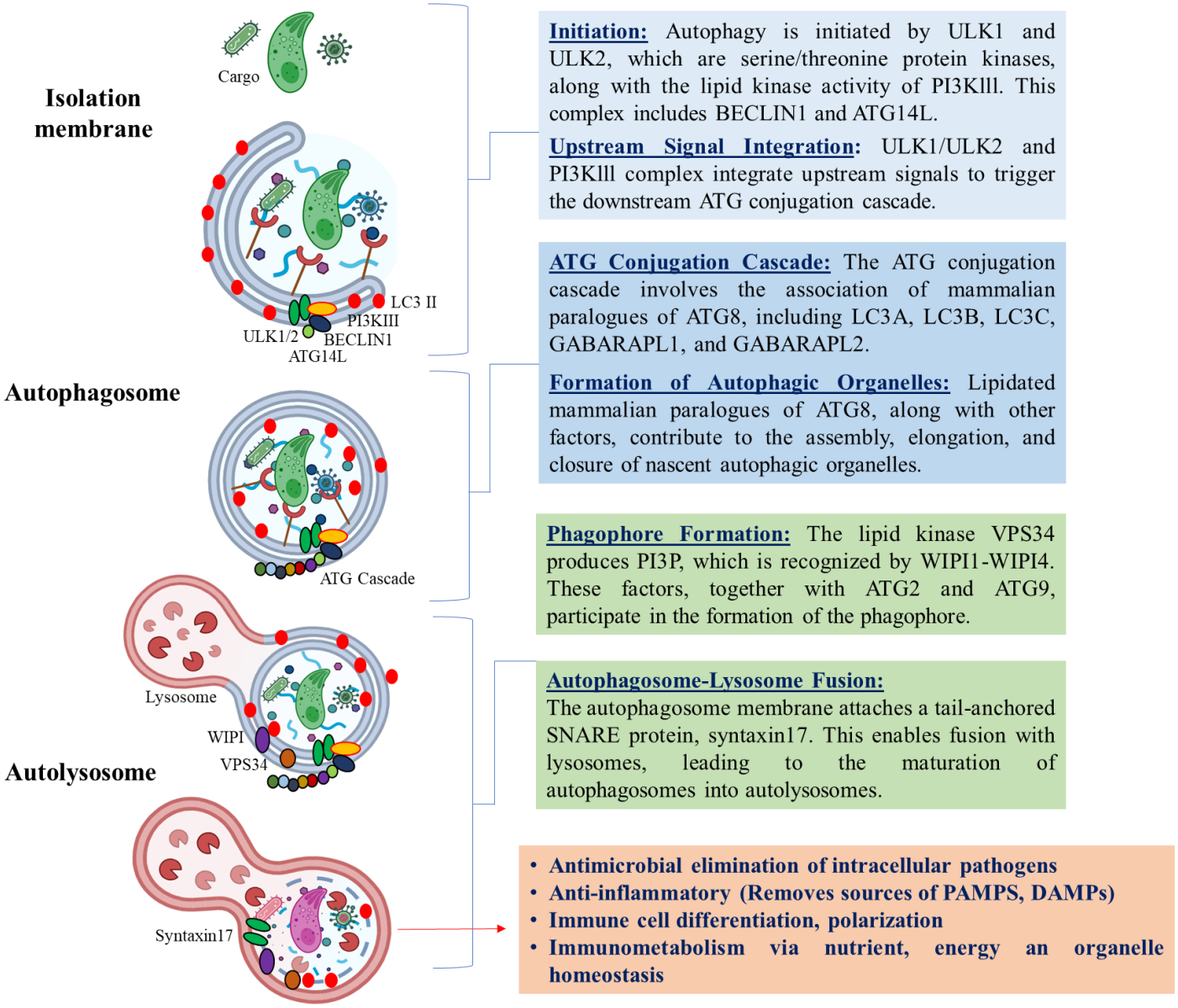


Figure 1.5 Autophagy: Insights into Initiation, Conjugation Cascade, and Organelle Maturation

Schematic Representation of Autophagy Pathway: The autophagy process involves the initiation, followed by the ATG conjugation cascade and formation of the phagophore. The final maturation involves fusion with lysosomes. The information provided in the figure is based on the sources (Wang and Li 2020), (Mizushima 2020), and (Yao, Ren et al. 2021).

Interestingly, LC3-associated phagocytosis can occur without the involvement of the ULK initiation complex, with LC3 protein recruited directly into the vacuole membrane. Ultimately, the activation of the ULK complex, composed of ATG13, FIP200, and ATG101, is crucial for initiating the host's autophagic response and clearing the infection (Ghartey-Kwansah, Adu-Nti et al. 2020).

The subversion of phagolysosomal degradation is a common strategy used by cytosolic pathogens and some parasites have developed the ability to impair the autophagic machinery of phagocytes through this strategy (Frank, Marcu et al. 2015). *Leishmania*, for instance, can utilize macrophage autophagy to evade detection and digestion. During early infection—and via activation of the AKT pathway—*Leishmania* actively inhibits the induction of autophagy. However, by 24 h, *Leishmania* switched from being an inhibitor to an overall inducer of autophagy in host macrophages. Interestingly, induction of autophagy in infected macrophages has been linked to increased growth and parasite load of *Leishmania amazonensis* (Pinheiro, Nunes et al. 2009). Similarly, *T. gondii* has developed several strategies to preserve its integrity, as well as that of the PV through the inhibition of autophagy induced by membrane receptors such as CD40 and IFN-γR (Van Grol, Muniz-Feliciano et al. 2013). The ligation of CD40 triggers autophagy activation by activating ULK1, which depends on the calcium sensor CaMKKβ that stimulates the energy/nutrient sensor AMPK. Besides activating ULK1, AMPK inhibits mTORC1, which is an inhibitor of autophagy.

Moreover, CD40 stimulates autophagy by triggering the activation of pro-autophagy proteins PKR and eIF2α, upregulating autophagy protein BECLIN1, and releasing it from its negative regulator Bcl-2, after Bcl-2 phosphorylation induced by TNF receptor (TNFR)-JNK signaling. CD40 induces recruitment of LC3 around PVM, followed by Rab7-dependent fusion with lysosomes and the killing of the parasite. This process requires ULK1, BECLIN1, PI3KC3, ATG5, ATG7, and lysosomal enzymes (Subauste 2021), **Figure 1.6.**

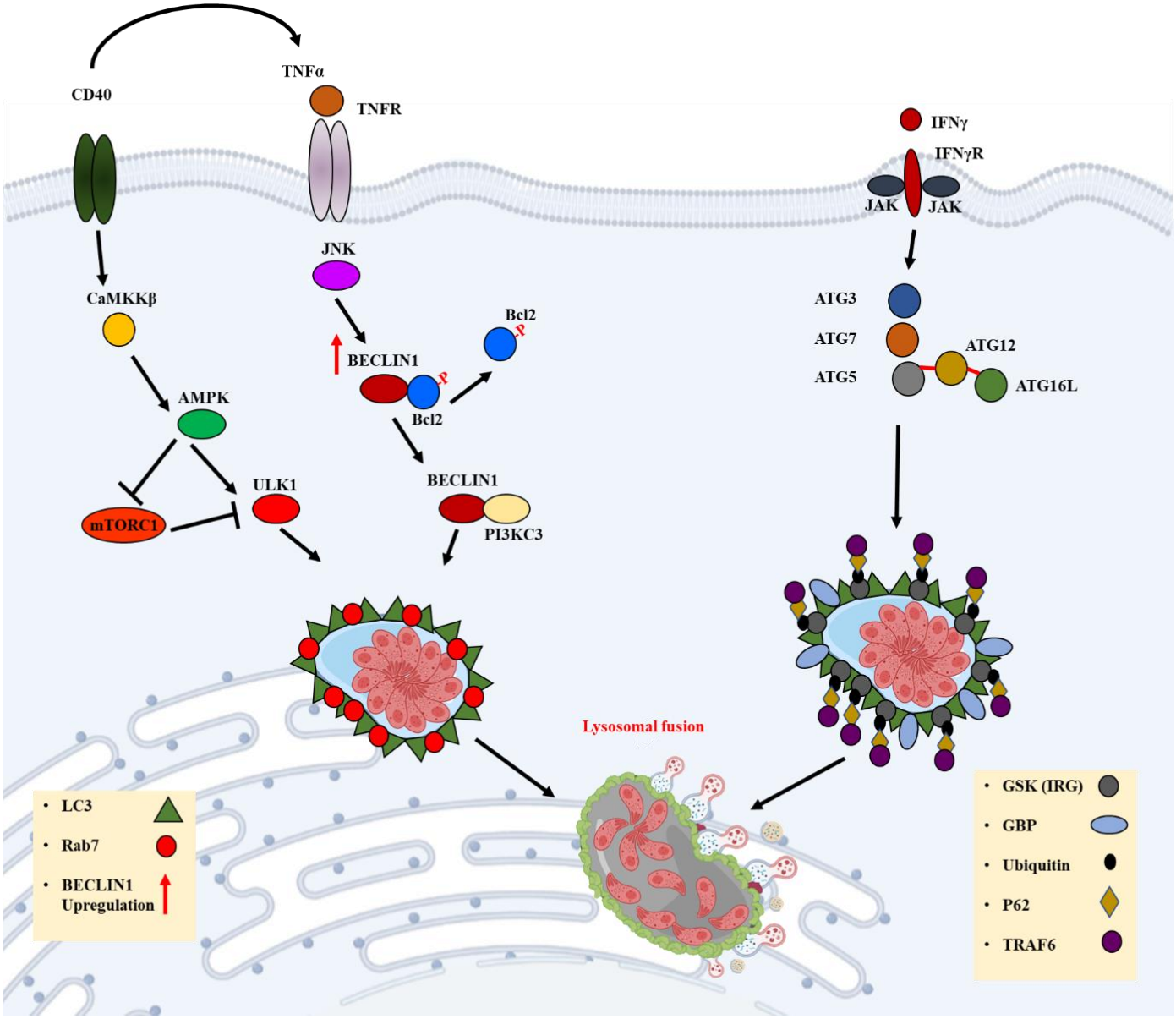


Figure 1.6 Mechanisms of *T. gondii* killing Via Autophagy: CD40 and IFN-γ Pathways

CD40-induced autophagy leads to the killing of *T. gondii* through the recruitment of LC3 around the PVM, which is followed by Rab7-dependent fusion with lysosomes and lysosomal degradation of the parasite. CD40 ligation activates ULK1 and upregulates pro-autophagy proteins such as Beclin 1, while inhibiting mTORC1, an inhibitor of autophagy, and releasing Beclin 1 from its negative regulator Bcl-2. This process depends on the calcium sensor CaMKKβ, which activates the energy/nutrient sensor AMPK. The killing of type II and III strains of *T. gondii* by IFN-γ involves the recruitment of GKS IRGs to PVM, LC3, and ATG8 orthologs, leading to the deposition of ubiquitin, p62, and TRAF6. However, type I strains of *T. gondii* prevent IRGs from loading onto PVM by inactivating IRGs with ROP5/ROP18/GRA7. Both CD40-induced autophagy and IFN-γ-induced killing of *T. gondii* require ULK1, Beclin 1, PI3KC3, ATG5, ATG7, and lysosomal enzymes, as well as ATG3, ATG12-ATG5-ATG16L, and lysosomal degradation. The information provided in the figure is based on the source (Subauste 2021)

Additionally, IFN-γ has been reported to induce killing of type II and III strains of *T. gondii* in mouse cells, triggering recruitment of GKS, a member of the subfamily of immunity-related GTPases (IRG), a process that requires ATG3, ATG7, and ATG12–ATG5–ATG16L (Sturge and Yarovinsky 2014, Chandrasekaran, Kochanowsky et al. 2022). LC3 and ATG8 orthologs are recruited to PVM, and IRGs promote deposition of ubiquitin, p62, and the E3 ubiquitin ligase TRAF6 (Traver, Henry et al. 2011). In the same way, p62 and TRAF6 further promote sustained targeting of the PVM with ubiquitin, which leads to delivery of guanylate-binding proteins (GBPs) to the PVM (Besteiro 2019). The result is the disruption of PVM and clearance of the parasite (Subauste 2021). It should be noted that this process does not occur in cells infected with type I strains of *T. gondii* because ROP5/ROP18/GRA7 inactivate IRGs preventing their loading onto PVM (Etheridge, Alaganan et al. 2014), **Figure 1.6.**

5. Forkhead Box O (FOXO) Transcription Factors

5.1 Family

FOXO proteins are a subclass of the Forkhead family of TFs possessing a conserved DNA-binding domain called the 'Forkhead box' or FOX. In humans, the FOX family is composed of more than 100 members, which are classified from FOXA to FOXR based on their sequence's similarity. Notably, members of class 'O' are regulated by the PI3K/AKT signaling pathway (Calnan and Brunet 2008). In mammals, the FOXO family comprises FOXO1, FOXO3a, FOXO4, and FOXO6. FOXO1 and FOXO3a are expressed in almost all tissues, FOXO4 is highly expressed in muscle, kidney, and colorectal tissue, and FOXO6 is primarily expressed in the brain and liver (van der Vos and Coffer 2011).

5.2 Structure and Domains

FOXO proteins possess four functional domains, including a forkhead DNA binding domain (DBD) that binds to two different DNA-response elements: the DAF-16 binding element and the insulin-responsive element. The core sequence is recognized by all forkhead family members. FOXO also have a nuclear export signal (NES), a nuclear localization signal (NLS), and a transactivation domain (TAD) (Obsil and Obsilova 2008), **Figure 1.7A**.

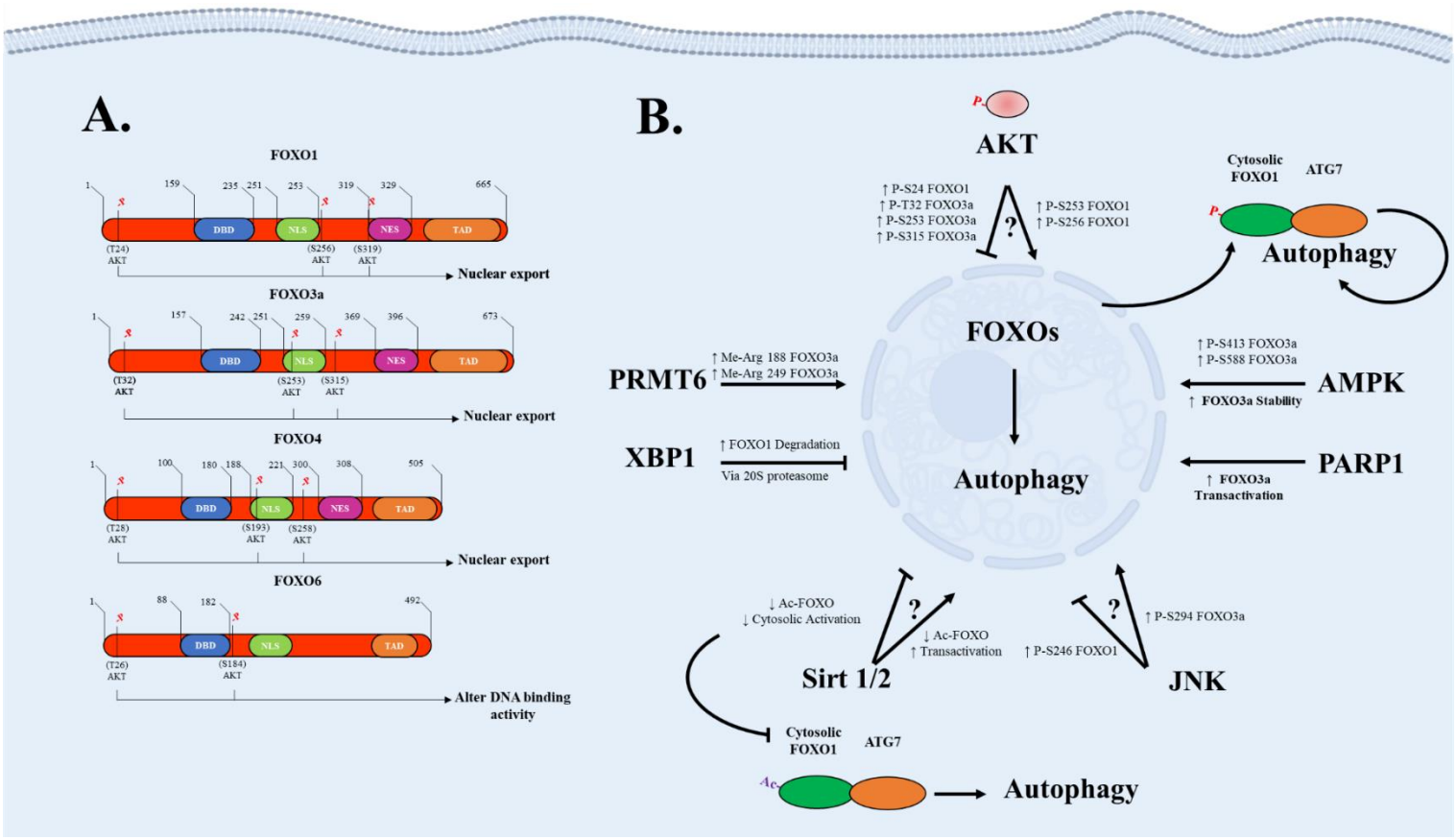


Figure 1.7 Post-translational Regulation of the FOXO–Autophagy Axis

This figure illustrates the structure of the FOXO proteins and post-translational regulation of the FOXO-autophagy axis. **A** show the domain structure of FOXO proteins, highlighting the AKT phosphorylation sites and the effect that AKT phosphorylation has on FOXO proteins. **B** highlights additional post-translational modifications that regulate autophagy, including phosphorylation, acetylation, methylation, and PARylation. These modifications impact FOXO turnover, nuclear localization, and transactivation activity, thereby controlling autophagy gene expression. Acetylation (Ac); phosphorylation (P); Methylation (Me), and the addition of poly (ADP-ribose) chains (PARylation). The information provided in the figure is based on the sources (Obsil and Obsilova 2008, Wang, Yu et al. 2016, Calissi, Lam et al. 2021).

5.3 Functions as Transcription Factors

FOXO proteins play a significant role in regulating cellular homeostasis by coordinating responses to environmental changes, including growth factor deprivation, metabolic stress, and oxidative stress. They are involved in numerous cellular processes, such as glucose and lipid metabolism, apoptosis, autophagy, cell cycle inhibition, stress resistance, DNA repair, angiogenesis, inflammation, immune response, pluripotency, and differentiation (Eijkelenboom and Burgering 2013, Calissi, Lam et al. 2021), **Figures 1.3 and 1.4**. Knockout mice studies have shown that FOXO1 is fundamental for embryonic angiogenesis, FOXO3 is important for fertility in female mice, whereas FOXO4 knockout does not exhibit an overt phenotype. In addition, FOXO6 regulates memory consolidation and synaptic function (Hosaka, Biggs et al. 2004, Salih, Rashid et al. 2012). Although FOXO3 and FOXO1 share similarities in structure and function, they have different tissue-dependent expression patterns, with FOXO1 highly expressed in adipose tissues and FOXO3 in brain tissue in mammals. Furthermore, the promoter of FOXO1 contains a consensus Forkhead response element (FHRE) that is not present in FOXO3 (Berry, Skarie et al. 2008).

The post-translational modifications (PTMs) that regulate the subcellular distribution, stability, and transcriptional activity of FOXO proteins include phosphorylation, acetylation, ubiquitylation, and methylation (Wang, Yu et al. 2016, Calissi, Lam et al. 2021), **Figure 1.7B**. Phosphorylation is the main PTM regulating the activity of FOXO proteins. It is carried out by several kinases, which can be classified into two categories: FOXO-inactivating kinases (such as AKT, SGK, ERK, p38, DYRK, IKK, CDK1/2, CK1) and FOXO-activating kinases (including AMPK, JNK, and MST1) (Brunet, Park et al. 2001, Woods, Rena et al. 2001, Rena, Woods et al. 2002, Hu, Lee et al. 2004, Huang, Regan et al. 2006, Yang, Zong et al. 2008, Ho, McGuire et al. 2012, Brown and Webb 2018).

FOXO proteins are also acetylated by histone acetyltransferases (HATs) such as CBP and p300 in response to oxidative stress, and deacetylated by histone deacetylases (HDACs) including members of the sirtuin family of proteins (SIRT1, SIRT2, and SIRT3) (Motta, Divecha et al. 2004, van der Horst, Tertoolen et al. 2004). Moreover, FOXO proteins are substrates for ubiquitylation, by ubiquitin ligases, leading to their degradation (Huang, Regan et al. 2005). In addition to these modifications, other important PTMs regulate FOXO

proteins, such as glycosylation and poly-ADP-ribosylation (PARylation) (Kuo, Zilberfarb et al. 2008, Lu, Zhang et al. 2016).

AKT-mediated phosphorylation of FOXO1 and FOXO3a has been extensively described, with three highly conserved residues targeted by AKT (S253, T32, S315, and T24, S256, S319 respectively) (Brunet, Bonni et al. 1999). Phosphorylation of these residues in the cytosol leads to the binding of the 14-3-3 chaperone protein, which masks the NLS and prevents FOXO3a from entering the nucleus (Brunet, Bonni et al. 1999). Conversely, when AKT-dependent phosphorylation occurs in the nucleus, it exposes FOXO NES, promoting its translocation to the cytosol (Biggs, Meisenhelder et al. 1999, Calissi, Lam et al. 2021). **Figures 1.3, 1.4** and **1.7**.

5.4 Others Functions in Regulation

Recent investigations have unveiled an intriguing dimension of FOXO proteins, particularly FOXO1 and FOXO3a, beyond their nuclear transcriptional functions. These proteins exhibit significant activities in the cytoplasm, which are distinct from their traditional roles as transcription factors. Notably, cytosolic FOXO1 has been identified as a key player in inhibiting tumor growth through its ability to induce autophagy, and programmed cell death. This newfound cytoplasmic role adds a fresh perspective to our comprehension of how FOXO proteins participate in diverse cellular responses. It sheds light on their contributions to cellular processes influenced by factors like growth signals, nutrient availability, and oxidative stress. Specifically, in the cytoplasm, FOXO1 targets several autophagy-related genes to sustain autophagic processes in various scenarios, such as muscle atrophy and cellular stress (Medema and Jaattela 2010, Zhao, Wang et al. 2010, Zhao, Yang et al. 2010).

FOXO1 plays a unique role in the cell's cytoplasmic domain, distinct from its conventional transcriptional function. When confronted with stressors like serum starvation and oxidative stress, cytoplasmic FOXO1 undergoes acetylation following its disassociation from SIRT2. This acetylated FOXO1, in turn, forms a critical partnership with Atg7, a key player in the autophagic process (Zhao, Yang et al. 2010). Together, they trigger autophagy for the degradation of impaired proteins and organelles. Significantly, this newly unveiled function of cytoplasmic FOXO1 isn't just limited to autophagy, it has relevance to lipophagy, a specialized form of autophagy that targets lipid droplets for degradation (Lettieri Barbato,

Tatulli et al. 2013). This process intertwines with the regulation of lipid metabolism, shedding light on how FOXO1's multifaceted functions contribute to cellular responses. Furthermore, this autophagic role of FOXO1 in the cytoplasm is independent of its transcriptional activities. The mechanism hinges on the acetylated FOXO1's interaction with Atg7, a critical regulator involved in the formation of autophagosomes and the progression of lipophagy. The implications of these findings extend beyond cellular responses to stress; they also have implications in the context of tumor suppression and open up new avenues of research in understanding the intricate connections between FOXO1, lipophagy, and lipid metabolism (Medema and Jaattela 2010, Zhao, Wang et al. 2010, Zhao, Yang et al. 2010).

Within mammals, FOXO3a orchestrates a coordinated transcriptional program that governs cell cycle control, cell death, cell metabolism, redox balance, DNA repair, and autophagy (Celestini, Tezil et al. 2018). These genes, which share a common regulatory motif, ensure expression specificity through phosphorylation-dependent subcellular localization. While some kinases, like AKT and IKKß, trigger FOXO3a exclusion from the nucleus and its subsequent degradation in the cytoplasm, others, such as p38 and AMPK, facilitate nuclear localization and transcriptional activation. This "molecular FOXO code" plays a crucial role in finely regulating FOXO's diverse functions (Calnan and Brunet 2008, Wang, Yu et al. 2016).

Recent discoveries also unveil a role for FOXO3A in the mitochondria. In response to glucose restriction, FOXO3A, in collaboration with AMPK, accumulates in the mitochondria, where it forms a transcriptional complex with SIRT3 and mitochondrial RNA polymerase (mtRNAPOL). This complex activates mitochondrial gene expression, leading to an increase in oxygen consumption. This phenomenon is an essential recovery mechanism to sustain cellular metabolism during nutrient shortage and metabolic stress (Celestini, Tezil et al. 2018, Fasano, Disciglio et al. 2019).

In cancer cells, FOXO3A's mitochondrial role is particularly intriguing. When these cells experience metabolic and genotoxic stress due to chemotherapeutic agents, FOXO3A is recruited to the mitochondrial surface, depending on the activation of the MEK/ERK and AMPK pathways. Once inside the mitochondria, FOXO3A can induce a transcriptional program that promotes cancer cell survival. Moreover, mitochondrial FOXO3A seems to be

a crucial factor in apoptosis induction under metformin treatment in cancer cells. This dual role of FOXO3A, both within and outside the nucleus, showcases its adaptability as a central player in maintaining cellular homeostasis under varying conditions of cellular stress. Given its significance in age-related diseases, cancer, and other pathological conditions, FOXO3A holds promise as a potential therapeutic target, although its intricacies require further exploration(Mei, Zhang et al. 2009, Cao, Jiang et al. 2013, Stefanetti, Voisin et al. 2018, Fasano, Disciglio et al. 2019, Kodani and Nakae 2020, Cheng 2022).

In summary, recent research has unveiled diverse roles for FOXO1 and FOXO3a beyond their traditional transcription functions. They now exhibit cytoplasmic and mitochondrial activities, demonstrating remarkable adaptability under various cellular stresses. FOXO1 participates in autophagy and lipid metabolism independently of its nuclear role, while the "molecular FOXO code" governs their precise functions. FOXO3a orchestrates multiple cellular processes and contributes to cellular recovery in the mitochondria. Understanding how FOXO proteins function outside the nucleus could shed light on their roles in host-parasite interactions, with the possibility of developing new strategies to modulate these interactions for therapeutic purposes.

6. The FOXO–Autophagy Axis

FOXO proteins have a central role in proteostasis and cellular stress responses, and emerging evidence has identified them as key transcriptional regulators of autophagy in both healthy and diseased states. These proteins transactivate genes that control the formation of autophagosomes and their fusion with lysosomes (Zhou, Liao et al. 2012, Zhu, Tong et al. 2015, Lee, Nam et al. 2018, Fasano, Disciglio et al. 2019, Guo, Li et al. 2022). Regulation of autophagosome-mediated autophagy via the PI3K/AKT pathway occurs through repression of FOXO family members (Zhou, Liao et al. 2012, Lee, Nam et al. 2018, Audesse, Dhakal et al. 2019). Interestingly, recent studies have shown that FOXO1 and FOXO3 specifically promote autophagy (Juhasz, Puskas et al. 2007, Zhao, Yang et al. 2010), highlighting the complex regulatory mechanisms involved in autophagy regulation. The transactivation activity of FOXO proteins is tightly controlled by several domains, including a conserved NLS and NES domains, a DBD, and a C-terminal transactivation domain (Cheng and White 2011, Martins, Lithgow et al. 2016, Cheng 2019), **Figure 1.7A**.

In response to oxidative stress, altered nutrient status, or growth factor signaling, FOXO proteins undergo PTMs in the NLS and NES domains, and translocate from the cytoplasm to the nucleus, or vice versa, to regulate the expression of genes across tissues, including those related to autophagy **Figure 1.3; 1.4 and 1.7A** (Daitoku, Sakamaki et al. 2011, Klotz, Sanchez-Ramos et al. 2015, Webb, Kundaje et al. 2016). FOXO proteins can regulate autophagy independently of their interactions with DNA. For example, FOXO1 can be excluded from the nucleus, and to bind to ATG7, an E1-like enzyme, in the cytoplasm to upregulate autophagy (Zhao, Yang et al. 2010, Wang, Xia et al. 2016).

Acetylation of FOXO1 and AKT-induced phosphorylation play an important role in FOXO1 translocation into the cytoplasm and its interaction with ATG7 for autophagy induction, **Figure 1.7B** (Hariharan, Maejima et al. 2010, Zhao, Yang et al. 2010, Zhou, Liao et al. 2012). Questions remain regarding how autophagy is sustained or enhanced regardless of loss of FOXO transactivation of autophagy genes, and how FOXO proteins are stabilized versus AKT-mediated phosphorylation that promotes their proteasomal degradation. Despite the description of the regulation of autophagy independent of transcriptional activity, increasing evidence has linked FOXOs to macroautophagy through their binding to promoter regions and activation of autophagy genes (Fullgrabe, Klionsky et al. 2014, Cheng 2019). The transactivation of FOXO proteins increases the expression of genes encoding autophagy proteins involved in multiple stages of the autophagic process (e.g., induction, nucleation, elongation, and fusion, **Figure 1.5**), and PTM modifications can promote or prevent the transactivation activity by mediating their nuclear translocation or exclusion; consequently, inhibitory PTMs induce nuclear exclusion (e.g., Akt-induced phosphorylation) whereas activating PTMs promote the nuclear accumulation (e.g., AMPK-induced phosphorylation) (Cheng 2019), **Figure 1.7**. To conclude, the FOXO family members that are important downstream targets of AKT could be responsible for *T. gondii*-driven repression of the host autophagy machinery through the regulation of the PI3K/AKT/FOXO axis.

6.1 The Interplay of FOXO and Autophagy in Pathologies

Dysregulated autophagy has been implicated in several human diseases, including obesity, diabetes, cardiovascular disease, neurodegenerative diseases, and cancer (Cheng 2019). In a related way, the FOXO-autophagy axis maintains tissue homeostasis in several organs,

including the liver, brain, kidney, cartilage, and intervertebral disk (Chen, Lv et al. 2016, Li, Zviti et al. 2017, Matsuzaki, Alvarez-Garcia et al. 2018, Schaffner, Minakaki et al. 2018). For instance, studies have shown that ablation of the hepatic FOXO-autophagy axis in mice through the inhibition of FOXO3a with FOXO3a-siRNA, dysregulates lipid metabolism, whereas restoration of autophagy protects against alcohol-induced hepatotoxicity (Chen, Lv et al. 2016). Additionally, the FOXO-autophagy axis enhances neuronal integrity and downregulates age-dependent axonal degeneration (Hwang, Oh et al. 2018). However, increased activity of the FOXO-autophagy axis can be pathogenic, leading to cardiac and skeletal muscle atrophy (Cao, Jiang et al. 2013, O'Neill, Bhardwaj et al. 2019). Interestingly, autophagy regulates the turnover of FOXO proteins, thereby modulating the regulation of many of its transcriptional targets related to cellular processes such as apoptosis (Fitzwalter, Towers et al. 2018). Moreover, ablation of autophagy upregulates FOXO proteins and downstream proapoptotic factors, thereby potentiating anticancer effects (Fitzwalter, Towers et al. 2018). These findings highlight the multifaceted role of FOXO TFs in autophagy regulation and its implications in several human diseases.

6.2 FOXO Transcription Factors and Viral Infections

FOXO TFs have been shown to play both protective and pathogenic roles during viral, bacterial, and parasitic infections. FOXO1 has been found to promote human cytomegalovirus (HCMV) replication by binding directly to the promoters of the major immediate-early genes. Indeed, FOXO1 expression is strongly induced by HCMV infection in cells of fibroblast origin, and suppression of FOXO1 significantly inhibits HCMV growth and replication. On the other hand, overexpression of FOXO1 promotes an overexpression of viral early/late transcripts from the major immediate early locus (MIE) (Hale, Collins-McMillen et al. 2020, Sleman 2022). Interestingly, during HCMV infection of lytically permissive cells, AKT accumulates in an inactive state promoting the nuclear localization of FOXO3a. The expression of constitutively active AKT in these cells causes substantial viral replication defects with corresponding reductions in viral gene expression and viral DNA synthesis. However, when FOXO3a nuclear localization is decoupled from its negative regulation by AKT, the viral replication defects observed in the presence of constitutively active AKT are reversed. This strongly implies that HCMV inactivates AKT to promote the

nuclear localization of FOXO TFs, which then transactivate cellular and/or viral genes during infection (Zhang, Domma et al. 2022).

Chronic hepatitis C virus (HCV) infection has been associated with dysregulation of various host cellular pathways. For example, despite no significant changes in the mRNA and protein levels of FOXO1, the phosphorylation of FOXO1 at Ser319 is reduced in HCV-infected cells, leading to increased nuclear accumulation of FOXO1 and sustained transcriptional activity. The decreased phosphorylation of FOXO1 concurrent to AKT inactivation, induced by HCV infection. Interestingly, HCV promotes hepatic gluconeogenesis through an AKT/FOXO1-dependent pathway, which involves in regulating glucose metabolism during HCV infection (Deng, Shoji et al. 2011).

In the case of influenza, A viruses (IAVs) infection, maintaining normal levels of FOXO1 can reduce the pro-apoptotic effects of the virus. Studies have shown that manipulating anti-inflammatory and anti-apoptotic responses via FOXOs in response to IAVs infection could be a potential target for the treatment of IAVs infection (Wu, Zhang et al. 2019). FOXO proteins can have therapeutic potential for COVID-19 treatment by activating an anti-inflammatory approach and restoring redox and inflammatory homeostasis, repairing damaged tissue, and activating lymphocyte effector and memory cells (Cheema, Nandi et al. 2021). However, oncoviruses, such as Epstein-Barr virus (EBV), can deregulate FOXO proteins through various mechanisms, including alterations in posttranslational modifications, cellular localization, and virus-encoded miRNAs, leading to downstream effects on proliferation, metastasis, chemotherapy resistance, and immunosuppression in virus-induced cancers.

In EBV, inactivation of FOXO1 expression is mediated by two latent proteins, LMP1 and LMP2A, through the induction of the PI3K signaling pathway in B cells. This deregulation is associated with downregulation and upregulation of the FOXO1 downstream target genes expression, Bcl-6, and cyclin D2, respectively, which may contribute to the distinctive apoptotic resistance of immortalized B cells in malignancies caused by EBV (Ramezani, Nikravesh et al. 2019).

In general, viral infection triggers the activation of the TF IRF3, which is essential to produce type I interferons (IFNs) and display innate antiviral immune responses.

FOXO1 has been identified as a negative regulator of virus-induced IFN-β induction: its overexpression inhibits virus-induced IFN-β induction and cellular antiviral response, while its ablation has the opposite effect.

Furthermore, FOXO1 interacts with IRF3 in a viral infection-dependent manner, promoting K48-linked polyubiquitination and degradation of IRF3 in the cytosol. Therefore, FOXO1 negatively regulates cellular antiviral response by promoting IRF3 ubiquitination and degradation (Lei, Zhang et al. 2013). During chronic murine lymphocytic choriomeningitis virus infection, the activation of AKT/mTOR pathway is impaired in antiviral cytotoxic T lymphocytes, resulting in enhanced activity of FOXO1. As a result, FOXO1-null cytotoxic T lymphocytes fail to persist and control chronic viral infection. Hence, FOXO1 is necessary to sustain cytotoxic T lymphocyte responses and control chronic viral infection (Staron, Gray et al. 2014). FOXO1 also regulates survival of cytotoxic T cells through sustained expression of the inhibitory PD-1 receptor and the consequent survival of antiviral CD8+ T Cells during chronic infection, demonstrating that FOXO TF contribute to control the intensity of the responses (Staron, Gray et al. 2014). However, it is unclear whether viruses are really using this TF to immunosuppress their host.

In airway epithelial cells FOXO3 seems to play an important regulatory role in the control of viral infections. Studies have shown that FOXO3a knockout mice infected with rhinovirus exhibit enhanced lung inflammation, elevated levels of pro-inflammatory cytokines, and viral persistence. This is attributed to the attenuated IFN response to rhinovirus infection, which is associated with a conformational change in mitochondrial antiviral signaling proteins. Therefore, FOXO3a is necessary for optimal viral clearance and preventing excessive lung inflammation following rhinovirus infection (Gimenes-Junior, Owuar et al. 2019). Additionally, FOXO3a has been linked to the inhibition of T cell-mediated adaptive immune responses during HIV infection, with the phosphorylated FOXO3a playing a role in controlling viremia and slowing disease progression. Interestingly, the FOXO3a transcriptional pathway is related to the survival of memory CD4+ T cells in HIV elite

controller subjects, contributing to their immune protection (van Grevenynghe, Procopio et al. 2008). In LCMV infections, FOXO3 deficiency leads to a marked increase in the expansion of effector CD8 T cells by reducing cellular apoptosis. Therefore, FOXO3 is a critical regulator of CD8 T cell memory, and therapeutic modulation of FOXO3 may enhance vaccine-induced protective immunity against intracellular pathogens (Sullivan, Kim et al. 2012).

Activation of AKT plays a dynamic role during the HIV-1 life cycle in macrophages. In the early stages of HIV-1 infection, AKT activation leads to the inactivation of FOXO3a, resulting in resistance to cell death and promoting viral replication and accumulation within the cell. As viral replication increases, the PI3K/AKT pathway is downregulated, lifting the restriction on FOXO3a, and allowing it to play a role in cell death through other factors such as TRAIL or Fas. These PI3K/AKT/FOXO3a-mediated mechanisms also make HIV-1 infected macrophages important viral reservoirs during HIV latency (Cui, Huang et al. 2008, Cui, Huang et al. 2009, Zhang, Tang et al. 2011, Pasquereau and Herbein 2022).

Similarly, in the case of Hepatitis B virus (HBV) infection, FOXO4 protein is significantly downregulated, while FOXO1 and FOXO3a are largely unaffected. FOXO4 has been shown to have an inhibitory effect on HBV transcription and replication both in vitro and in vivo by suppressing hepatocyte nuclear factor-4α (HNF4α) expression via activation of the ERK pathway. HBV is capable of downregulating FOXO4 expression, indicating that it may have developed strategies to escape from FOXO4-mediated antiviral effects. Activation of FOXO4 presents a novel therapeutic target against HBV infection (Li, Li et al. 2019).

6.3 FOXO Transcription Factors and Bacterial Infections

Although there is insufficient evidence on the regulation of host FOXO proteins by different pathogens, regarding immune-evasion or the activation of the innate or adaptive immune responses, it is common for these TFs to be activated during infection to mount an appropriate immune response that is dependent on the cell type and tissue. For instance, lipopolysaccharide (LPS) present in the cell wall of Gram-negative bacteria, is known to stimulate FOXO1 expression, nuclear localization, and FOXO1-mediated gene transcription.

In this sense, LPS-induced inflammatory cytokine expression is mediated in part through FOXO TFs (Su, Coudriet et al. 2009, Wang, Zhou et al. 2014).

FOXOs play an essential role in the innate immune response to microbial infections. For instance, neutrophils are critical in clearing bacterial infections, and FOXO1 deletion negatively affects several critical aspects of neutrophil function, including mobilization, recruitment, and clearance of bacteria (Dong, Song et al. 2017). Furthermore, FOXO1 activation is also required for the activation of DCs, where it regulates target genes such as ICAM-1, CCR7, and integrin αvβ3, leading to the phagocytosis, migration, homing to lymph nodes, stimulation of CD4+ T cells and resting B cells, and antibody production (Cabrera-Ortega, Feinberg et al. 2017).

Interestingly, *in vitro* studies have shown that FOXO1 also regulates neutrophil chemotaxis and bacterial killing during *Porphyromonas gingivalis* infection. FOXO1 activation by bacterial challenge is needed to mobilize neutrophils to transit from the bone marrow to peripheral tissues in response to infection and for bacterial clearance (Dong, Song et al. 2017).

Immunofluorescence has shown that FOXO1 accumulates in the nucleus of *P. gingivalis*-infected gingival epithelial cells, inducing the transcription of genes involved in protection against oxidative stress (Cat, Sod2, Prdx3), inflammatory responses (IL1β), and anti-apoptosis (Bcl-6) (Wang, Sztukowska et al. 2015).

Uropathogenic *Escherichia coli* (*E. coli*) is another example of bacteria inducing innate immune responses through the host cell FOXO1 and FOXO3a proteins. Infection with uropathogenic *E. coli* results in abrogation of activation of the host AKT signaling pathway, leading to activation of FOXO TFs. FOXO proteins of uropathogenic *E. coli* infected testes are localized in the nucleus and show increased DNA-binding activity, which results in the activation of FOXO TFs (Zhang, Wang et al. 2016). Interestingly, the protein and messenger RNA expression levels of the FOXO target gene superoxide dismutase 2 and catalase were not regulated by *E. coli* infection. Furthermore, the protein/mRNA expression levels of other known FOXO target genes such as p15INK4B, p27Kip1, Cyclin D1, and the DNA repair gene Gadd45α in Sertoli cells were not changed by *E. coli* infection (Zhang, Wang et al.

2016). Nevertheless, uropathogenic *E. coli* infection decreases the mRNA expression of the FOXO target gene BIM, epigenetically blocking caspase 3 activation (Zhang, Wang et al. 2016).

In the case of *Mycobacterium tuberculosis*, FOXO3a induces macrophage polarization towards an M1 phenotype, which is pro-inflammatory and enhances the adaptive immune response by modulating IL-10 secretion in *mycobacteria*-infected macrophages. Knocking down FOXO3a resulted in decreased IL-10 production and M1 polarization, whereas activation of FOXO3 has an opposite effect. This suggests that FOXO3 plays an important role in regulating the immune response to *M. tuberculosis* infection (Bouzeyen, Haoues et al. 2019). On the other hand, *Citrobacter rodentium* induces FOXO3a translocation from the nucleus to the cytosol, leading to its degradation in intestinal epithelial cells. In infected colon cells, FOXO3a is expressed along the crypts and located mainly in the cytosol, suggesting its inactivation. Additionally, the PI3K pathway inactivates FOXO3a, leading to the upregulation of IL-8 by suppressing inhibitory IκBα (Snoeks, Weber et al. 2008).

In conclusion, FOXO proteins, especially FOXO3a plays an important role in controlling infections caused by intracellular pathogens such as *S. typhimurium*, a bacterium that causes various diseases including gastroenteritis, typhoid, sepsis, inflammatory bowel disease and colon cancer (Joseph, Ametepe et al. 2016). During the chronic stage of infection, FOXO3a helps limit the persistence of oxidative stress and promote a proinflammatory state. FOXO3a has been shown to regulate extracellular signal-regulated kinase (ERK) signalling in macrophages, leading to the maintenance of a proinflammatory state without affecting cell proliferation or death. In this model of infection, FOXO3a promotes the termination of ERK signalling to induce inflammatory immune responses that are necessary to control infection with virulent intracellular pathogens (Joseph, Ametepe et al. 2016). While the exact mechanisms of FOXO-mediated regulation of immune responses are still being elucidated, these findings highlight the importance of FOXO TFs in host defense against pathogens.

6.4 FOXO Transcription Factors and Intracellular Protozoa Parasite Infections

Recent studies have investigated the activation of the host PI3K/AKT/FOXO pathway in *Leishmania* spp. (Gupta, Das et al. 2022), but the surface molecule on the host cell that triggers the PI3K/AKT pathway when interacting with the parasite remains unknown. *L. donovani* has been shown to utilize the SIRT1/FOXO1 axis to overactivate the programmed death-1 (PD-1) signaling pathway in macrophages, contributing to parasite survival within the host cell. The infection-mediated PD-1 pathway induction during late phases of infection (48-72h), both in vitro and *in vivo*, leads to downregulation of pro-inflammatory cytokines, ROS, and NO, allowing for parasite survival (Roy, Gupta et al. 2017). Interestingly, AKT is deactivated during the late phases of infection, leading to increased nuclear retention of FOXO1. However, SIRT1-dependent deacetylation-mediated inactivation of FOXO1 is responsible for the inhibition of apoptosis in infected in macrophages, preventing nuclear FOXO1 from inducing apoptosis. PD-1 signaling, which is upregulated during the late phase of *L. donovani* infection, leads to the inactivation of AKT, followed by nuclear sequestration of FOXO1. Concurrently, SIRT1 expression is induced during the late phase of infection, deacetylating nuclear FOXO1 and inactivating it to prevent apoptosis (Roy, Saha et al. 2019). In contrast an independent study conducted on *L. donovani*-infected RAW264.7 and bone marrow-derived macrophages (BMDMs) revealed that *L. donovani* triggered AKT activation during the early stages of infection (0-24h) to regulate the GSK-3β/β-catenin/FOXO1 axis, inhibiting both host cell apoptosis and the immune response, which are essential for its intra-macrophage survival by reducing macrophage apoptosis, the pro-inflammatory response and therefore increasing parasite survival. The activation of AKT signaling helped *L. donovani* shift the cytokine balance from a pro-inflammatory to an anti-inflammatory response and inhibited host cell apoptosis. Following AKT phosphoactivation during infection, GSK-3β is phosphorylated at the Ser9 residue, resulting in inhibition of Ser33/37 phosphorylation-mediated degradation of β-catenin. Phosphorylation of β-catenin at Ser552 led to its nuclear localization, and phosphorylation of FOXO1 at Ser256 caused its cytosolic translocation, leading to parasite survival (Gupta, Srivastav et al. 2016, Saha, Basu et al. 2018).

Trypanosoma cruzi, the causative agent of Chagas' disease, undergoes differentiation in the cytosol of host cells and replicates to spread infection. These processes require the long-term survival of infected cells. It has been reported that parasite-derived neurotrophic factor (PDNF), a trans-sialidase located on the surface of *T. cruzi*, is both a substrate and activator of AKT in the cytosol of host cells. After contact with the host cell surface, PDNF increases the expression of the gene that encodes AKT while suppressing the transcription of genes that encode pro-apoptotic factors, such as members of the FOXO family. As a result, PDNF elicits a sustained functional response that protects host cells from apoptosis induced by oxidative stress and proinflammatory cytokines like tumor necrosis factor–α and transforming growth factor–β (Chuenkova and PereiraPerrin 2009).

Although FOXO3a is not regulated during infection by parasites such as *Plasmodium* spp, it plays a role in controlling the immune response during infection. For instance, the FOXO3a gene variant rs12212067 has been associated with differential severity of infectious diseases like malaria, where FOXO3a is associated with increased inflammatory responses to *Plasmodium falciparum*. This suggests a role for the FOXO3-dependent pathway in malaria (Lee, Espeli et al. 2013, Nguetse, Kremsner et al. 2015).

In 2013, Wei Zhou and colleagues conducted a study that demonstrated how *T. gondii* infection or excretory/secretory protein (ESP) treatment of the human retinal pigment epithelium cell line ARPE-19 results in the activation of the PI3K/AKT signaling pathway, which leads to the reduction of intracellular ROS levels and enhanced proliferation of *T. gondii* in host cells (Zhou, Quan et al. 2013). Specifically, the study revealed that *T. gondii* infection or ESP treatment significantly reduced the expression of NADPH oxidase 4 (Nox4), the main source of ROS in ARPE-19 cells. Importantly, this study was the first to suggest the possible involvement of FOXO proteins in the regulation of ROS and proliferation during infection with *T. gondii*. The researchers found that *T. gondii* infection possibly induces the phosphorylation of FOXO and its relocation from the nucleus to the cytosol, which correlated with intracellular ROS levels; although the researchers proposed that FOXO proteins could potentially be downregulated during *T. gondii* infection, they did not provide any supporting evidence for this hypothesis. These findings suggest that FOXO TFs may be involved in

regulating Nox4 transcription and the PI3K/AKT pathway during *T. gondii* infection (Zhou, Quan et al. 2013).

In 2019, Karanovic and colleagues reported the first cases of disseminated and congenital toxoplasmosis in a mother and child who share a pathogenic mutation in PIK3R1 and discussed the mechanisms underlying susceptibility to severe *T. gondii* infection in activated PI3Kδ syndrome (APDS) as well as in other forms of primary immunodeficiency (Karanovic, Michelow et al. 2019, Choi, Gao et al. 2020). Their findings suggest that the loss of regulation of FOXO proteins may be a key factor in controlling *T. gondii* replication in these patients. Although there is no direct experimental evidence, Karanovic et al. proposed that active AKT phosphorylates and inactivates the TF FOXO1, thus preventing p22phox transcription. FOXO1 transcription activity controls the expression of p22phox, and the activation of the NOX4/p22phox complex allows the generation of ROS, activation of MAPK, NF-κB signaling, and production of the pro-inflammatory macrophage inhibitory factor (MIF).

In patients with APDS, increased PI3K signaling induces AKT phosphorylation, which mediates phosphorylation of FOXO1, impairing p22phox gene expression, and resulting in reduced production of ROS, defective activation of MAPK and NF-κB, and impaired production of pro-inflammatory macrophage inhibitory factor (MIF) in response to *T. gondii* infection, ultimately promoting its replication (Karanovic, Michelow et al. 2019).

In 2021, Gao FF. and collaborators reported downregulation of Fas-associated factor 1 (FAF1) expression in *T. gondii* infected ARPE-19 cells, an effect reversed by PI3K/AKT inhibitors. In this same study, overexpression of FOXO1 led to increased FAF1 expression, whereas its deletion had the opposite effect. *T. gondii*-induced FAF1 downregulation was concurrent to enhanced IRF3 transcriptional activity, nuclear import, and the transcription of interferon-stimulated genes (ISGs). These results indicate that *T. gondii* can downregulate host FAF1 in a PI3K/AKT/FOXO1-dependent manner, which is essential for IRF3 nuclear translocation and the transcriptional activation of ISGs. However, the regulation of *T. gondii* over FOXO1 or how the overexpression of ISGs promotes the proliferation of the parasite is not completely understood (Zhou, Quan et al. 2013, Gao, Quan et al. 2021). Of note, the expression of ISGs plays an important role in the host defense against *T. gondii* infection.

ISGs are activated by interferons, which are produced by host cells in response to infection (Steinfeldt, Konen-Waisman et al. 2010). However, recent studies have suggested that some ISGs may also promote the proliferation of *T. gondii*. For example, the ISG GBP1 (guanylate-binding protein 1) has been shown to facilitate the formation of the *T. gondii* PV, which provides a protected environment for its replication. Other ISGs, such as IRG1 (Immunity-Related GTPase family M protein 1) and IDO1 (Indoleamine 2,3-dioxygenase 1), have been shown to promote the survival and proliferation of *T. gondii* by regulating the host cell metabolism and nutrient availability (Steinfeldt, Konen-Waisman et al. 2010).

As was previously mentioned, in a study published by Lee et al. in 2022, it was reported that BMDMs are protected against *T. gondii* through the activation of the SIRT1-FOXO1/FOXO3a-autophagy axis via the AMPK and PI3K/AKT signaling pathways. The authors found that SIRT1 contributes to autophagy activation and regulation of the FOXO-autophagy axis (Lee, Kim et al. 2022). In SIRT1-deficient BMDMs, *T. gondii*-induced FOXO1 phosphorylation, acetylation, and cytosolic translocation were enhanced, additionally, Myeloid-specific SIRT1-deficient mice exhibited an increased cyst burden in brain tissue compared to wild-type mice following infection with the avirulent ME49 strain. Consistently, the intracellular survival of *T. gondii* was markedly increased SIRT1-deficient BMDMs. In addition, the pharmacological inhibition of PI3K/AKT signaling reduced the cytosolic translocation of FOXO1 in BMDMs infected with *T. gondii* ultimately inducing antiparasitic autophagy. This is the first report that clearly describes post-translational modifications of a FOXO protein that trigger its export to the cytoplasm during *T. gondii* infection. The study also shows that *T. gondii* RH infection of BMDMs results in FOXO3a export from the nucleus. The authors suggest that SIRT1 promotes FOXO3a deacetylation and its CaMKK2/AMPK-dependent phosphorylation, leading to nuclear accumulation and transactivation.

7. Mechanisms Used by *T. gondii* to Circumvent Autophagy-Dependent Elimination

Autophagy is a highly conserved homeostatic process allowing cells to degrade and recycle damaged cytosolic components, which is upregulated in response to stressors such as nutrient

deprivation or infection (Deretic, Saitoh et al. 2013). Autophagy constitutes a central mechanism of host defense against intracellular pathogens, including *T. gondii* (Deretic, Saitoh et al. 2013, Ghartey-Kwansah, Adu-Nti et al. 2020). There are four main types of autophagy: autophagosome-mediated macroautophagy, microautophagy, chaperone-mediated autophagy, and non-canonical autophagy (Deretic, Saitoh et al. 2013).

Macroautophagy, involves the formation of a PV-containing autophagosome, which fuses with the lysosome, resulting in the degradation of the target if the parasite can not counteract its activation; this type of autophagy can effectively inhibit *T. gondii* infection (Cheng, Zhang et al. 2022).

In addition, the PV can also be targeted via autophagosome-independent processes orchestrated by autophagy proteins in IFN-γ-activated cells (Ma, Sasai et al. 2014). The host cell autophagic response against *T. gondii* is mainly triggered by CD40- and IFN-γ-dependent signals that induce the expression and activation of autophagy-related proteins (e.g., ULK1, Beclin-1, LC3, etc.), a process that culminates with the destruction of the parasite by lysosomal enzymes (Ling, Shaw et al. 2006, Van Grol, Muniz-Feliciano et al. 2013).

Interestingly, some studies have reported that infection by *T. gondii* has a dual behavior where the parasite induces host cell autophagy in both HeLa cells and primary fibroblasts via a mechanism that is dependent on host Atg5, but independent of host mTOR suppression. Additionally, autophagy-dependent parasite growth is correlated with the consumption of host cell mass that is dependent on parasite growth progression. These findings suggest a role for autophagy as a pathway through which the parasites may effectively compete with the host cell for limiting anabolic resources (Wang, Weiss et al. 2009).

Studies have shown as well, that *T. gondii* utilizes host lipophagy, the autophagy of lipid droplets, to acquire the cellular fatty acids necessary for its proliferation (Pernas, Bean et al. 2018). These findings suggest that *T. gondii* must regulate autophagy in a dual manner to survive. On one hand, it must evade the recruitment of autophagy effectors to the PV, but on the other hand, it must enhance host autophagy to obtain a source of nutrients that will facilitate its growth and proliferation. This implies that *T. gondii* has developed a sophisticated mechanism for manipulating host autophagy to ensure its survival and proliferation.

As mentioned before, several defense mechanisms are employed by the host to clear *T. gondii* infection, including CD40-mediated autophagy and IFN-γ-induced clearance through autophagy proteins, which effectively eliminate the parasites within host cells.

One of the best described mechanisms involves activating the host cell signaling cascade of the EGFR via PI3K, which prevents the targeting of autophagy effectors to the PV through PI3K-mediated AKT phosphorylation. This activation can then stimulate mTORC1, a negative regulator of autophagy, leading to the inhibition of autophagy. These evasion mechanisms demonstrate *T. gondii*'s ability to manipulate host cell signaling pathways to its advantage during infection (Portillo, Okenka et al. 2010, Muniz-Feliciano, Van Grol et al. 2013, Coppens 2017, Wu, Cudjoe et al. 2020).

From what has been mentioned earlier, it is evident that the PI3K-AKT-EGFR signaling pathway plays a critical role in promoting the survival of *T. gondii*. During the parasite's intracellular phase, prolonged PKCα/PKCβ-Src signaling ensures EGFR autophosphorylation, which triggers AKT phosphorylation, and enables the parasite to evade host autophagy (Lopez Corcino, Gonzalez Ferrer et al. 2019).

In the regulation of TFs related to autophagy during *T. gondii* infection, IFN-γ and its downstream molecule STAT1 are required for protection in mice (Gavrilescu, Butcher et al. 2004). STAT1 signaling can be blocked by *T. gondii* through the action of an effector called Inhibitor of STAT Transcription (TgIST), which blocks responses to IFN-γ. *T. gondii* can also prevent the dissociation of STAT1 from DNA, which limits its ability to transcribe other STAT1-dependent genes (Gavrilescu, Butcher et al. 2004). In the context of infection, STAT1 plays a pivotal role in the regulation of interferon-responsive genes (iRGs), guanylate-binding proteins (GBPs), and autophagy-related genes (ASGs) (Selleck, Fentress et al. 2013). Upon infection, STAT1 becomes activated and translocates to the nucleus, where it promotes the transcription of iRGs, enhancing the host's immune response. This leads to the upregulation of GBPs, which are essential for the host's defense against intracellular pathogens like *T. gondii* (Selleck, Fentress et al. 2013). Additionally, STAT1 contributes to the regulation of ASGs, which are crucial for autophagy, a process that can help eliminate the pathogen (Besteiro 2019, Ahmadpour, Babaie et al. 2023). However, it is noteworthy that *T. gondii* can negatively regulate the transcription of STAT1 transcriptional targets through the NURD complex and the virulence factor TgIST. This negative regulation can potentially

impact the autophagic process and serve as a protective mechanism for the parasite, allowing it to evade elimination by the host's immune response (Huang, Liu et al. 2022), **Figure 1.3**.

T. gondii's downregulation of STAT1 results in the downregulation of iNOS and MHC-II, as well as other IRGs and GBPs (Hakimi, Olias et al. 2017). Autophagy proteins such as Atg7, Atg5, Atg16, and Atg12 play a crucial role in recruiting IRGs and GBPs to the *T. gondii*-containing PV (Choi, Park et al. 2014) and LC3's early delivery to parasite-containing vacuoles also aids in parasite clearance, indicating a direct involvement of Atg proteins' direct involvement in recruiting the IRG/GBP effectors to the PV membrane, **Figure 1.3**.

IFN-γ plays a crucial role in promoting the deposition of ubiquitin, adaptor proteins (p62, NDP52, and optineurin), and LC3 around the PVM of human epithelial cells. This process is dependent on STAT1 and ISG15 upregulation, resulting in the formation of a multilayer membrane structure around the PVM that entraps the parasite and inhibits its growth. In human endothelial cells infected with a type II strain of *T. gondii*, IFN-γ induces the deposition of ubiquitin, p62, and NDP52, followed by the recruitment of Rab7, lysosome fusion, and parasite killing (Subauste 2021). However, the inhibition of STAT1 transcription and IFN-γ-dependent gene expression by the recruitment of the Mi-2/NuRD complex can trigger the inhibition of autophagy mechanisms that protect against *T. gondii*, as reported by (Olias, Etheridge et al. 2016).

Recent studies have demonstrated that Sirtuin 1 (SIRT1), a nicotinamide adenosine dinucleotide (NAD)-dependent protein deacetylase, plays a critical role in autophagy activation via the AMP-activated protein kinase (AMPK) and PI3K/AKT signaling pathways, leading to enhanced parasite clearance during *T. gondii* infection. Resveratrol-mediated activation of SIRT1 promotes autophagy, resulting in a significantly increased anti-*T. gondii* effect (Lee, Kim et al. 2022). Moreover, SIRT1 has been reported to regulate the FOXO family, which play a key role in various cellular processes, including the autophagy axis (Di Malta, Cinque et al. 2019). Interestingly, *T. gondii*-induced phosphorylation, acetylation, and cytosolic translocation of FOXO1 in SIRT1-deficient BMDMs resulted in a negative regulation of their autophagy-related transcriptional activity (Lee, Kim et al. 2022). However, it remains unclear whether *T. gondii* can directly downregulate the transcriptional activity of FOXO1 or other members of the FOXO family proteins to block autophagic parasite clearance.

7.1 EGFR-PI3K-AKT Signaling and Its Role in the Inhibition of Autophagy by *T. gondii*

AKT plays a critical role in numerous cellular processes, including oncogenesis, metabolism, apoptosis, cell growth, proliferation, and cell survival (Scheid and Woodgett 2001). Given its central roles, AKT is an attractive target for *T. gondii* to manipulate during infection. As previously mentioned, accumulating evidence suggests that *T. gondii* activates host AKT to evade autophagy-mediated killing (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019, Cheng, Zhang et al. 2022).

In the canonical membrane localized PI3K/AKT pathway, AKT is activated through phosphorylation, at either T308 or/and S473 by PDK1 and mTORC2 after being recruited to the plasma membrane by PIP3 via respective PH domains. The availability of PIP3 at the plasma membrane is tightly regulated by the opposing processes of PI3K, which phosphorylates PIP2 to PIP3, and PTEN, which dephosphorylates PIP3 and abrogates AKT recruitment (Stokoe, Stephens et al. 1997, Scheid and Woodgett 2001). **Figure 1.5**.

An active form of AKT can also be found in the nucleus; but the mechanisms, and functions of nuclear AKT activation are still not well understood (Wang and Brattain 2006, Martelli, Tabellini et al. 2012).

The mTOR pathway is a downstream target of PI3K and AKT that plays an essential role in promoting cell growth, differentiation, and survival, while simultaneously downregulating apoptotic signals. The activation of the PI3K/AKT/mTOR pathway can lead to the inhibition of autophagy by activating mTOR. Conversely, induction of autophagy, which occurs through mTOR inhibition, may compromise the parasite's survival by promoting sustained autophagy-mediated killing (Heras-Sandoval, Perez-Rojas et al. 2014).

During *T. gondii* infection, PI3K/AKT pathway is essential for parasite invasion and proliferation (Mammari, Halabi et al. 2019).Macrophage invasion by *T. gondii* activates the PI3K/AKT pathway in different ways. Firstly, via toll-like receptors TLR2 and TLR4, where the lectins MIC1 and MIC4 interact with N-linked glycans on TLR2 and TLR4, respectively, activating NF-κB and inducing a pro-inflammatory response and IL-12, IL-23, TNF-α, and IL-6 secretion (Quan, Chu et al. 2015). MIC1 and MIC4 also trigger the secretion of the anti-

inflammatory cytokine IL-10, presumably through the internalization of TLR4 from the cell surface. Secretion of IL-10 is advantageous for the parasite's survival by strategically regulating the host's immune response (Quan, Chu et al. 2015, Ricci-Azevedo, Mendonca-Natividade et al. 2021); this leads to the activation of PI3K/AKT and subsequently, the activation of IRF-3-dependent gene transcription, which results in the expression of IL-10 (Quan, Chu et al. 2015, Ricci-Azevedo, Mendonca-Natividade et al. 2021).

Overall, the activation of the PI3K/AKT pathway is required to manipulate different processes and to prevent killing of *T. gondii* (Tarassishin, Suh et al. 2011, Aziz, Kang et al. 2020, Ricci-Azevedo, Mendonca-Natividade et al. 2021).

In mouse macrophages, *T. gondii* infection renders cells resistant to multiple pro-apoptotic signals, and this resistance is attributed to the activation of the PI3K/AKT pathway (Kim and Denkers 2006). Interestingly, this activation occurs independently of the Toll-like receptor adaptor protein MyD88 but depends on G-protein-mediated signaling. The PI3K inhibitors wortmannin and LY294002 block parasite-induced AKT phosphorylation, indicating the importance of the PI3K/AKT pathway in this phenomenon.

T. gondii infection induces the phosphorylation of AKT and Bcl-2-associated death promoter (Bad), which promotes PI3K/AKT-dependent Bad phosphorylation. This leads to the inhibition of Bcl-2-associated X protein (Bax) translocation to the nucleus, downregulating apoptosis both in vitro and in vivo (Mammari, Halabi et al. 2019).

Infection of the human retinal pigment epithelium cell line ARPE-19 by *T. gondii* or exposure to T. gondii TgESA activates the AKT pathway. PI3K inhibitors have also proven effective in reducing *T. gondii* proliferation within host cells (Quan, Cha et al. 2013, Zhou, Quan et al. 2013). This effect is achieved by *T. gondii* or TgESA through the activation of the PI3K/AKT signaling pathway, leading to a significant reduction in the expression of NADPH oxidase 4 (Nox4) and, consequently, a decrease in intracellular levels of reactive oxygen species (ROS) within the host cells (Zhou, Quan et al. 2013).

DCs are among the first innate immune cells that encounter *T. gondii* after it crosses the host intestinal epithelium, and the parasite uses them as a carrier to infiltrate the host central nervous system (CNS) undetected.

In DCs, *T. gondii* or TgESA can reduce hydrogen peroxide (H2O2)-induced ROS and host endogenous ROS via the activation of PI3K/AKT, thus creating a favorable environment for parasite growth (Choi, Gao et al. 2020). Moreover, within the PI3K/AKT signaling pathway, FOXO TFs play a crucial role in regulating reactive oxygen species (ROS) by controlling the expression of antioxidant enzymes and genes involved in ROS detoxification. In silico analysis has revealed the presence of two potential FOXO TF-specific binding sequences in the murine NOX4 promoter site, suggesting that *T. gondii* may reduce host ROS production via PI3K/AKT/FOXO signaling activation to create a favorable environment for its growth (Choi, Gao et al. 2020).

The activation of EGFR/PI3K/AKT signaling pathway is crucial in preventing the accumulation of autophagosome and lysosome components around the PV and reducing parasite replication (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019). This activation occurs early during host cell invasion and continues throughout the intracellular stage of the parasite. The adhesins MIC3 and MIC6, which possess EGF-like domains, induce phosphorylation of tyrosine residues in the C-terminal end of EGFR, leading to autophosphorylation (Y1068, Y1148) and activation of AKT (Degtyarev, De Maziere et al. 2008). Furthermore, the formation of the moving junction during parasite penetration results in FAK phosphorylation (Y397), followed by Src phosphorylation (Y416), which transactivates EGFR (Y845) and triggers early STAT3 signaling, preventing PKR activation (T451) and subsequent autophagic targeting of the PV (Muniz-Feliciano, Van Grol et al. 2013, Portillo, Muniz-Feliciano et al. 2017).

T. gondii also activates the PKCα/PKCβ–Src signaling pathway, which sustains EGFR autophosphorylation and maintains the inhibition of autophagic targeting during the parasite's intracellular stage, potentially mediated by AKT (Masek, Fiore et al. 2006, Lopez Corcino, Gonzalez Ferrer et al. 2019) **Figure 1.8**. Interestingly, the genetic ablation of ULK1, Beclin1, or ATG7, key components of the autophagy machinery, impairs parasite elimination when EGFR, PI3K, or AKT activity is pharmacologically blocked, indicating the involvement of autophagy-related proteins in *T. gondii* infection (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019).

In non-hematopoietic cells like endothelial cells, retinal pigment epithelial cells, and microglia, *T. gondii* activates the EGFR/PI3K/AKT pathway through MIC proteins containing EGF domains (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019). This rapid activation is independent of GPCR and TLR signaling and helps maintain the non-fusogenic nature of the PV.

Inhibition of EGFR/PI3K/AKT signaling results in the accumulation of autophagy proteins LC3 and LAMP1 around the parasite, vacuole-lysosome fusion, and autophagy-mediated killing of *T. gondii* via ATG7, ULK1, and Beclin1 (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019).

Additionally, an alternative signaling pathway involving FAK activation during invasion prevents the targeting of the parasite by autophagy proteins (Portillo, Muniz-Feliciano et al. 2017). FAK is activated at the host cell-parasite junction site associated with RON4. Interestingly, FAK/Src-mediated EGFR transactivation is not essential for AKT activation induced by *T. gondii*, indicating an alternative pathway for autophagy inhibition that is independent of AKT (Portillo, Muniz-Feliciano et al. 2017).

Although the effects of EGFR/PI3K/AKT/mTOR activation on autophagy after *T. gondii* infection have been well documented, it remains unclear which downstream targets of AKT are responsible for the parasite's ability to repress the host autophagy machinery. Further research is needed to identify these targets and gain a deeper understanding of the mechanisms behind *T. gondii* infection.

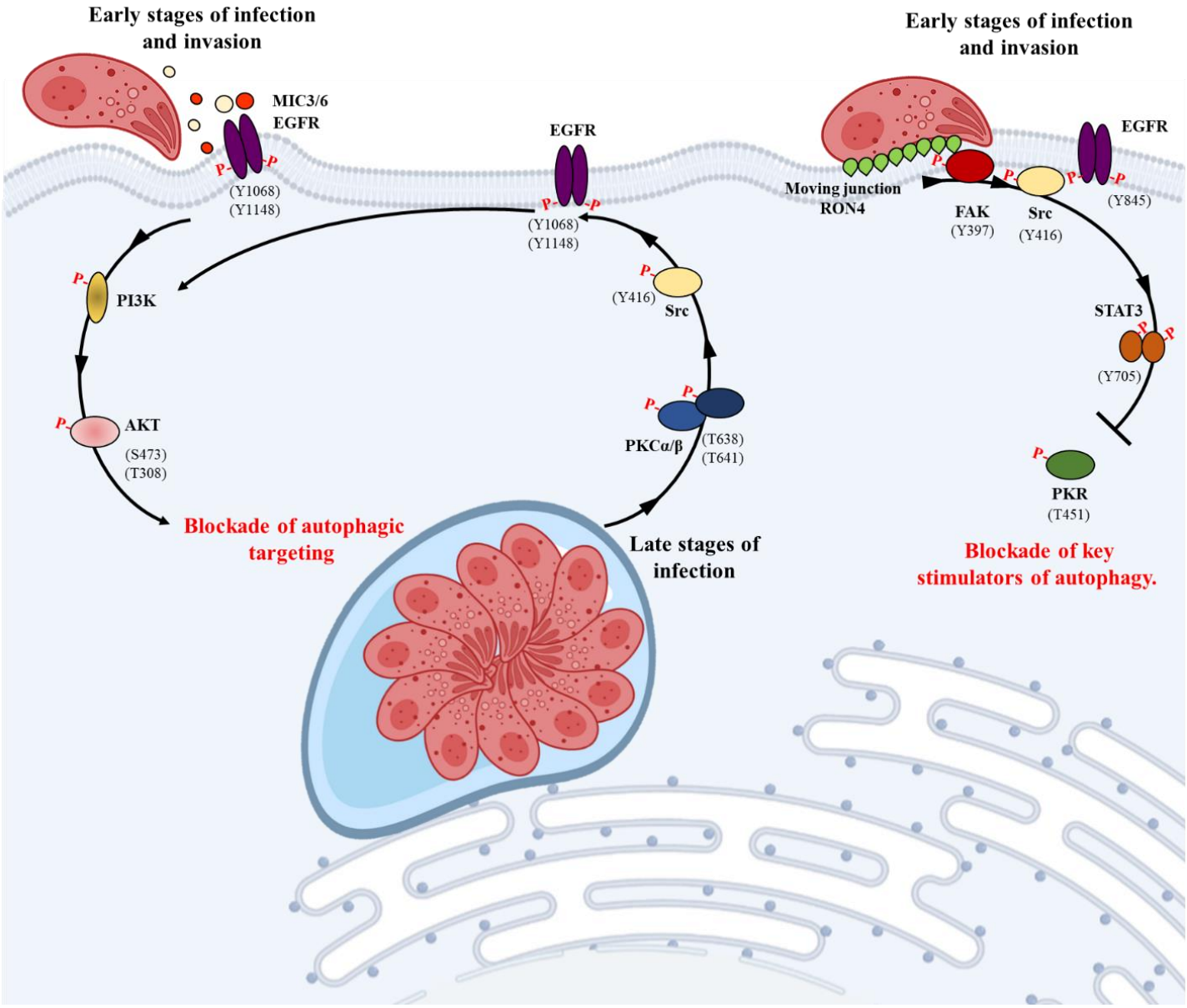


Figure 1.8 Current Understanding of EGFR/PI3K/AKT Signaling During *T. gondii* Infection

T. gondii has evolved to manipulate host cell signaling pathways to prevent autophagic targeting. Parasite adhesins MIC3 and MIC6 trigger EGFR autophosphorylation, which activates AKT, an inhibitor of autophagy. During invasion, FAK is activated at the moving junction, leading to Src-dependent transactivation of EGFR and STAT3 signaling that blocks activation of pro-autophagy proteins such as PKR. *T. gondii* maintains this blockade by activating PKCα/PKCβ kinases, which sustain Src signaling, EGFR autophosphorylation, and AKT activation. RON4 expression during invasion leads to unique EGFR transactivation and STAT3 signaling that prevents PKR activation. In later stages, PKCα/PKCβ-Src signaling maintains EGFR autophosphorylation, and a complex of GRA7 and ROP proteins permanently inactivates IRGs and GBPs to ensure the parasite's survival. Blocking any of these signaling molecules leads to autophagic targeting and parasite killing replication. The information provided in the figure is based on the sources (Masek, Fiore et al. 2006, Degtyarev, De Maziere et al. 2008, Muniz-Feliciano, Van Grol et al. 2013, Portillo, Muniz-Feliciano et al. 2017, Lopez Corcino, Gonzalez Ferrer et al. 2019).

CHAPTER 2:

Hypothesis and Objectives

T. gondii is a protozoan parasite that can infect a wide range of warm-blooded vertebrate hosts, including humans, cats, and mice (Montazeri, Sharif et al. 2017). Although generally asymptomatic, toxoplasmosis can cause serious health problems, especially in immunocompromised individuals and during pregnancy (Smith, Goulart et al. 2021). Currently, there are no effective human vaccines to prevent toxoplasmosis, and current treatments rely on anti-parasitic drugs, which may not be effective against drug-resistant strains (Innes, Hamilton et al. 2019). To establish a safe replicative niche, *T. gondii* forms a protective PV that prevents contact with host cytoplasmic components that could trigger parasite destruction. *T. gondii* also targets host cell signaling pathways and TFs to evade antimicrobial responses (Deretic, Saitoh et al. 2013, Hakimi, Olias et al. 2017, Ghartey-Kwansah, Adu-Nti et al. 2020).

Autophagy, a central mechanism of host defense against intracellular pathogens, inhibits *T. gondii* infection by the formation of a PV-containing autophagosome and its fusion with lysosomes (Coppens, Dunn et al. 2006, Coppens and Romano 2018). However, *T. gondii* can prevent this process by activating the host serine/threonine kinase AKT (Zhou, Liao et al. 2012, Lee, Nam et al. 2018, Audesse, Dhakal et al. 2019, Choi, Gao et al. 2020). FOXO3a, a crucial TF in autophagy regulation, is regulated by the PI3K-AKT pathway (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019). AKT phosphorylates FOXO3a at three highly conserved residues, which results in the binding of chaperone protein 14-3-3, masking its nuclear localization signal and preventing FOXO3a from entering the nucleus. Conversely, when AKT-dependent phosphorylation occurs in the nucleus, it exposes FOXO3 nuclear export signal, promoting its translocation to the cytosolic compartment (Brunet, Bonni et al. 1999). FOXO3a plays an essential role in the maintenance of cellular and tissue homeostasis and modulation of stress responses (Zhou, Liao et al. 2012, Zhu, Tong et al. 2015, Lee, Nam et al. 2018, Fasano, Disciglio et al. 2019, Guo, Li et al. 2022). It is involved in the regulation of autophagy, controlling the formation of autophagosomes and their fusion with lysosomes.

Dysregulated FOXO3a activity has been linked to inhibition of T cell-mediated adaptive immune responses during viral and bacterial infections (Snoeks, Weber et al. 2008, van Grevenynghe, Procopio et al. 2008, Sullivan, Kim et al. 2012, Bouzeyen, Haoues et al. 2019, Gimenes-Junior, Owuar et al. 2019). Interestingly, a recent report suggests decreased nuclear

FOXO3a levels in murine *T. gondii*-infected macrophages (Lee, Kim et al. 2022), but the role and regulation of FOXO3a during *T. gondii* infection are yet to be investigated.

Thus, the working hypothesis of this research is that *T. gondii* hijacks the PI3K-AKT pathway to suppress FOXO3a-regulated autophagy-related transcriptional programs, hindering the activation of the host autophagic response against the parasite.

- To test this hypothesis, the general objective was to determine whether AKT-dependent repression of the transcriptional activity of FOXO3a affects autophagy-related transcriptional programs that contribute to promote *T. gondii* infection.
    - The first objective was to determine whether the activation of AKT leads to the phosphorylation and nuclear exclusion of FOXO3a during *T. gondii* infection.
    - The second objective was to investigate the molecular mechanisms responsible for the activation of AKT-dependent phosphorylation and nuclear exclusion of FOXO3a during *T. gondii* infection.
    - The third objective was to establish whether FOXO3a-regulated autophagy-related transcriptional programs and functions are altered during *T. gondii* infection.

CHAPTER 3

Publication No.1

Toxoplasma gondii inhibits the expression of autophagy-related genes through AKT-dependent inactivation of the transcription factor FOXO3a

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Running Title: Inhibition of FOXO3a-mediated autophagy by *T. gondii*

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3.1 Author Contributions

Conceptualisation : AFD (Andres Felipe Diez), LPL (Louis-Philippe Leroux), SC (Sophie Chagneau), MJ (Maritza Jaramillo). **Formal analysis:** AFD, LPL, AP (Alexandra Plouffe), MG (Mackenzie Gold), VC (Visnu Chaparro). **Funding acquisition :** LPL, MJ. **Investigation :** AFD, LPL, AP, MG, VC. Project administration: MJ. Supervision: LPL, VC, MJ. **Writing – original draft:** AFD, LPL, MJ. **Writing – review & editing:** AFD, LPL, MJ. Author Contributions in the Article "*Toxoplasma gondii* inhibits the expression of autophagy-related genes through AKT-dependent inactivation of the transcription factor FOXO3a" (Diez, Leroux et al. 2023).

Andres Felipe Diez: Conceptualization, writing and revision of the manuscript, preparation of the figures. Realization of the experiments for figures: 3.1A, 3.1B, 3.1C; S3.3; S3.4; 3.2A, 3.2B, 3.2C, 3.2D; S3.5; S3.6; 3.3A, 3.3C, 3.3D; 3.4A, 3.4B, 3.4C; S3.8A, S3.8B; 3.5A, 3.5B, 3.5C, 3.5D; S3.9.A; S3.9.B; 3.6A, 3.6B, 3.6C, 3.6D.

Louis-Philippe Leroux: Conceptualization, writing and revision of the manuscript, preparation of the figures. Realization of the experiments for figures: 3.1A, 3.1B, 3.1C; S3.1; S3.2; S3.4; S3.5; 3.3A, 3.3B, 3.3C; S3.7; 3.4A, 3.4B, 3.4C; S3.8B; S3.9.A; S3.9.B.

Sophie Chagneau: Conceptualization and Revision of the manuscript.

Alexandra Plouffe: Revision of the manuscript and realization of the experiments for figures: 3.1A, 3.1B; 3.2A, 3.2B, 3.2C, 3.2D; S3.6.

Mackenzie Gold: Revision of the manuscript and realization of the experiments for figures: 3.1C.

Visnu Chaparro: Revision of the manuscript and Realization of the experiments for figures: 3.B.

Maritza Jaramillo: Conceptualization, writing and revision of the manuscript.

3.2 Abstract

The intracellular parasite *Toxoplasma gondii* induces host AKT activation to prevent autophagy-mediated clearance; however, the molecular underpinnings are not fully understood. Autophagy can be negatively regulated through AKT-sensitive phosphorylation and nuclear export of the TF Forkhead box O3a (FOXO3a). Using a combination of pharmacological and genetic approaches, herein we investigated whether *T. gondii* hinders host autophagy through AKT-dependent inactivation of FOXO3a. We found that infection by type I and II strains of *T. gondii* promotes gradual and sustained AKT-dependent phosphorylation of FOXO3a at residues S253 and T32 in human foreskin fibroblasts (HFF) and murine 3T3 fibroblasts. Mechanistically, AKT-sensitive phosphorylation of FOXO3a by *T. gondii* required live infection and the activity of PI3K but was independent of the plasma membrane receptor EGFR and the kinase PKCα. Phosphorylation of FOXO3a at AKT-sensitive residues was paralleled by its nuclear exclusion in *T. gondii*-infected HFF. Importantly, the parasite was unable to drive cytoplasmic localization of FOXO3a upon pharmacological blockade of AKT or overexpression of an AKT-insensitive mutant form of FOXO3a. Transcription of a subset of bona fide autophagy-related targets of FOXO3a was reduced during *T. gondii* infection in an AKT-dependent fashion. However, parasite-directed repression of autophagy-related genes was AKT-resistant in cells deficient in FOXO3a. Consistent with this, *T. gondii* failed to inhibit the recruitment of acidic organelles and LC3, an autophagy marker, to the PV upon chemically- or genetically-induced nuclear retention of FOXO3a. In all, we provide evidence that *T. gondii* suppresses FOXO3a-regulated transcriptional programs to prevent autophagy-mediated killing.

3.3 Importance

The parasite *Toxoplasma gondii* is the etiological agent of toxoplasmosis, an opportunistic infection commonly transmitted by ingestion of contaminated food or water. To date, no effective vaccines in humans have been developed and no promising drugs are available to treat chronic infection or prevent congenital infection. *T. gondii* targets numerous host cell processes to establish a favorable replicative niche. Of note, *T. gondii* activates the host AKT signaling pathway to prevent autophagy-mediated killing. Herein we report that *T. gondii* inhibits FOXO3a, a TF that regulates expression of autophagy-related genes, through AKT-dependent phosphorylation. The parasite's ability to block the recruitment of the autophagy

machinery to the PV is impeded upon pharmacological inhibition of AKT or overexpression of an AKT-insensitive form of FOXO3a. Thus, our study provides greater granularity in the role of FOXO3a during infection and reinforces the potential of targeting autophagy as a therapeutic strategy against *T. gondii*.

3.4 Introduction

Toxoplasma gondii (*T. gondii*), the etiologic agent of toxoplasmosis, is an intracellular protozoan parasite that invades virtually any nucleated cell, and infects a wide variety of warm-blooded vertebrate hosts, including humans, cats, and mice (Innes, Hamilton et al. 2019). It is estimated that about 30-50% of the world population is seropositive for *T. gondii* (Montazeri, Sharif et al. 2017). The infection can be acute, chronic, or latent (Flegr, Prandota et al. 2014); however, symptoms, or lack thereof, at the time of infection do not predict disease manifestation later in life (Innes, Hamilton et al. 2019). Toxoplasmosis is generally asymptomatic, but reactivation of encysted parasites can lead to life-threatening consequences in immuno-compromised individuals, and cause abortions or birth defects if contracted in primo-infected women during pregnancy (Smith, Goulart et al. 2021). No effective human vaccines have been developed (Innes, Hamilton et al. 2019), and despite the development of new experimental drugs, none of them have been approved to prevent congenital infection while minimizing teratogenic effects (Smith, Goulart et al. 2021). Thus, toxoplasmosis constitutes a serious public health concern worldwide (Flegr, Prandota et al. 2014).

To establish a safe replicative niche, *T. gondii* forms a nonfusogenic PV that facilitates nutrient acquisition while preventing contact with host cytoplasmic components that could trigger parasite destruction (Coppens, Dunn et al. 2006, Coppens and Romano 2018). In addition, *T. gondii* targets host cell signaling pathways and TFs to evade antimicrobial responses (Hakimi and Bougdour 2015, Hakimi, Olias et al. 2017). Autophagy is a highly conserved homeostatic process that allows cells to degrade and recycle damaged cytosolic components; however, it can also be upregulated in response to stressors such as nutrient deprivation or infection (Deretic, Saitoh et al. 2013). Hence, autophagy constitutes a central mechanism of host defense against intracellular pathogens, including *T. gondii* (Deretic, Saitoh et al. 2013, Ghartey-Kwansah, Adu-Nti et al. 2020). There are four main

types of autophagy: autophagosome-mediated macroautophagy, microautophagy, chaperone-mediated autophagy, and non-canonical autophagy (Deretic, Saitoh et al. 2013). Macroautophagy (hereinafter referred to as autophagy) inhibits *T. gondii* infection through the formation of a PV-containing autophagosome and its fusion with the lysosome (Cheng, Zhang et al. 2022). In addition, the PV can also be targeted via autophagosome-independent processes orchestrated by autophagy proteins in IFN-γ-activated cells (Ma, Sasai et al. 2014). The host cell autophagic response against *T. gondii* is mainly triggered by CD40- and IFN-γ-dependent signals that induce the expression and activation of autophagy-related proteins (e.g., ULK1, Beclin-1, LC3, etc.), a process that culminates with the destruction of the parasite by lysosomal enzymes (Ling, Shaw et al. 2006, Van Grol, Muniz-Feliciano et al. 2013). Accumulating evidence indicates that activation of the host serine/threonine kinase AKT constitutes one of the strategies developed by *T. gondii* to avoid autophagy-mediated killing (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019, Cheng, Zhang et al. 2022). In this regard, it was reported that early and prolonged activation of EGF receptor (EGFR)-dependent AKT phosphorylation by *T. gondii* was required to prevent accumulation of autophagosome and lysosome components around the PV (i.e., LC3 and LAMP-1, respectively) and reduce parasite replication (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019). Of note, genetic ablation of ULK1, Beclin-1 or ATG7 hampered parasite elimination upon pharmacological blockade of EGFR, PI3K, or AKT activity (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019), hinting at regulation of autophagy-related proteins during *T. gondii* infection. However, the downstream targets of AKT that are responsible for *T. gondii*-driven repression of the host autophagy machinery are yet to be identified.

Regulation of autophagosome-mediated autophagy via the PI3K-AKT pathway is accomplished in part through the repression of the TF Forkhead box O3a (FOXO3a; formerly Forkhead In Rhabdomyosarcoma-Like 1, FKHRL1) (Zhou, Liao et al. 2012, Lee, Nam et al. 2018, Audesse, Dhakal et al. 2019). FOXO3a is a core regulator of cellular and tissue homeostasis (e.g., cell cycle, proteostasis, proliferation, stem cell maintenance, longevity, fertility, etc.), but it also functions as a critical modulator of stress responses (e.g., nutritional, energetic, oxidative, and genotoxic stress, etc.) (Morris, Willcox et al. 2015, Arts, Joosten et al. 2018, Fasano, Disciglio et al. 2019, Calissi, Lam et al. 2021). The subcellular distribution,

stability, and transcriptional activity of FOXO3a are mainly regulated through post-translational modifications; namely, phosphorylation, acetylation, ubiquitylation, and methylation (Wang, Yu et al. 2016, Calissi, Lam et al. 2021). AKT phosphorylates FOXO3a at three highly conserved residues (i.e., S253, T32, and S315) (Brunet, Bonni et al. 1999). Phosphorylation of cytoplasmic FOXO3a at AKT-sensitive residues induces binding of the chaperone protein 14-3-3, which prevents FOXO3a from entering the nucleus by masking the nuclear localization signal (NLS) (Brunet, Bonni et al. 1999). Conversely, when AKT-dependent phosphorylation occurs in the nucleus, it exposes FOXO3 nuclear export signal (NES) thereby promoting its translocation to the cytosolic compartment (Biggs, Meisenhelder et al. 1999, Calissi, Lam et al. 2021).

In line with its central role in proteostasis and cellular stress responses, FOXO3a has emerged as a key transcriptional regulator of autophagy in healthy and diseased states by transactivating genes that control the formation of autophagosomes and their fusion with lysosomes (Zhou, Liao et al. 2012, Zhu, Tong et al. 2015, Lee, Nam et al. 2018, Fasano, Disciglio et al. 2019, Guo, Li et al. 2022).

Positive and negative deregulation of FOXO3a activity has been detected in multiple pathologies including various types of cancers, neurodegenerative diseases, and muscle dystrophy (Liu, Ao et al. 2018, Cheng 2019). Moreover, FOXO3a activity has been associated with either host protective or pathogenic roles during viral and bacterial infections (e.g., LCMV, HIV, rhinovirus, *Mycobacterium tuberculosis*, *Citrobacter rodentium*, etc.) (Snoeks, Weber et al. 2008, van Grevenynghe, Procopio et al. 2008, Sullivan, Kim et al. 2012, Bouzeyen, Haoues et al. 2019, Gimenes-Junior, Owuar et al. 2019). For instance, FOXO3a promotes type I IFN antiviral innate immune responses while limiting tissue damage during rhinovirus infection (Gimenes-Junior, Owuar et al. 2019). Similarly, FOXO3a confers protection against *M. tuberculosis* by inducing macrophage polarization towards an M1 (i.e., pro-inflammatory) phenotype (Bouzeyen, Haoues et al. 2019). In stark contrast, FOXO3a activity is linked to the inhibition of T cell-mediated adaptive immune responses during HIV and LCMV infections (van Grevenynghe, Procopio et al. 2008, Sullivan, Kim et al. 2012). Interestingly, it was recently reported that nuclear FOXO3a levels decrease in *T. gondii*-infected murine macrophages at 18h post infection (Lee, Kim et al. 2022). However, the role and the regulation of FOXO3a during *T. gondii* infection have yet

to be investigated. Here, we report that *T. gondii* hijacks the PI3K-AKT pathway to suppress autophagy-related transcriptional programs under the control of FOXO3a, thereby hindering the activation of the host autophagic response against the parasite.

3.5 Results

3.5.1 *Toxoplasma gondii* induces AKT-sensitive phosphorylation of host FOXO3a

The subcellular localization and transcriptional activity of FOXO3a are tightly controlled through post-translational modifications, including AKT-dependent phosphorylation (Brunet, Bonni et al. 1999, Wang, Yu et al. 2016, Calissi, Lam et al. 2021). *T. gondii* infection leads to the activation of AKT signaling (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019, Cheng, Zhang et al. 2022); hence, we postulated that *T. gondii* modulates FOXO3a phosphorylation in an AKT-dependent fashion. To address this, we monitored changes in the phosphorylation status of AKT and FOXO3a in human foreskin fibroblasts (HFF) infected with type I or type II *T. gondii* strains (i.e., RH and ME49, respectively) (**Fig. 3.1A**). Both strains were included to evaluate any strain-specific differences in the modulation of host signaling pathways (Mukhopadhyay, Arranz-Solis et al. 2020). Increased phosphorylation of AKT at residues S473 and T308 was detected more rapidly in HFF infected with RH than ME49 (8 and 12 h post-infection (h p.i.), respectively) but was maintained up to 32 h p.i. in cultures infected with either strain. Accordingly, a pronounced and sustained phosphorylation of FOXO3a at AKT-sensitive residues S253 and T32 was observed in RH- and ME49-infected HFF (**Fig. 3.1A**). Importantly, the kinetics of FOXO3a phosphorylation matched closely those observed for AKT. Parasite extracts (i.e., devoid of any host cell ["Tg only"]) were probed in parallel to rule out the possibility that the observed changes in signaling were due to cross-reactivity of the antibodies against parasite proteins. AKT and FOXO3a phosphorylation patterns appeared to be dependent on the parasite load since increasing MOI (multiplicity of infection) ratios led to higher phosphorylation levels as observed by western blotting analyses (**Fig. S3.1**). Similar results were obtained in mouse fibroblast cell line 3T3 upon infection with type I and type II *T. gondii* strains (**Fig. S3.2**). To confirm that *T. gondii*-inducible phosphorylation of FOXO3a was mediated by AKT, HFF cultures were treated with MK-2206, a pan-AKT inhibitor (Hirai, Sootome et al. 2010), or vehicle and infected with RH *T. gondii* or left uninfected. As expected, AKT phosphorylation at S473 and T308 was abrogated in presence of MK-2206

regardless of the infection status (**Fig. 3.1B**) without affecting host cell viability (**Fig. S3.3**). Of note, pharmacological inhibition of AKT prevented FOXO3a phosphorylation at S253 and T32 in *T. gondii*-infected cells (**Fig. 3.1B**). In line with previous reports (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019), *T. gondii* infection rates remained unaffected by chemical blockade of AKT activity (**Fig. S3.4**) whereas parasite replication was significantly reduced (**Fig. 3.1C**). In all, this set of experiments indicates that *T. gondii* hijacks host cell signaling to drive AKT-dependent phosphorylation of the TF FOXO3a which appears to favor parasite replication.

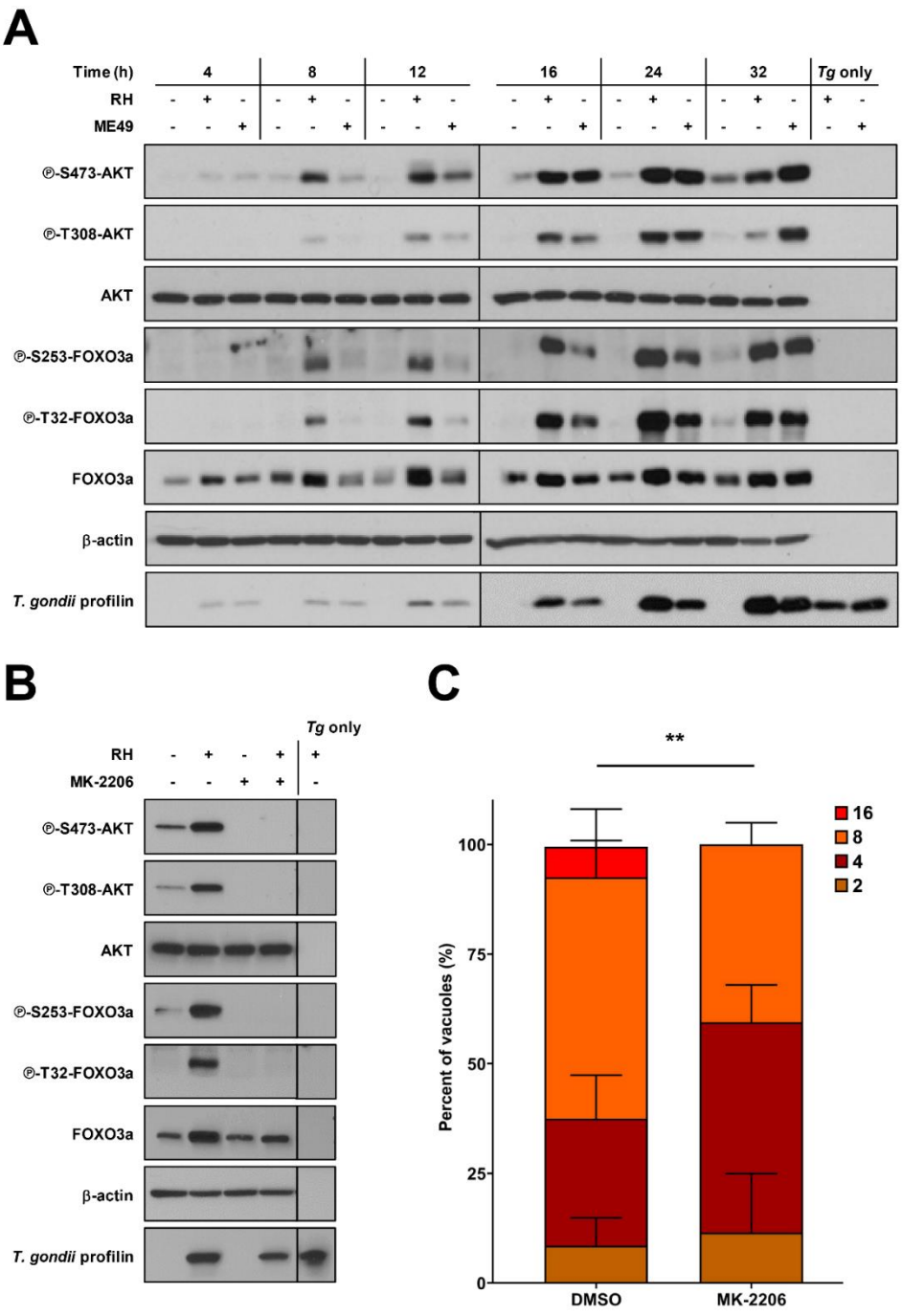


Figure 3.1 *T. gondii* induces phosphorylation of host FOXO3A and promotes its own replication within HFF in an AKT-dependent fashion

(A) HFF cultures were inoculated with either RH or ME49 *T. gondii* tachyzoites or left uninfected for the indicated times then processed for western blot analyses. **(B, C)** HFF cultures were pretreated with 2 μM MK-2206 or an equivalent volume of vehicle (i.e., DMSO) for 1 h then inoculated with RH *T. gondii* parasites. Cells were cultured up to 32 h following infection in the presence or absence of MK-2206. **(A, B)** Phosphorylation

and expression levels of indicated proteins were monitored by western blotting. Total amounts of β-actin were used as a loading control, and an antibody raised against *T. gondii* profilin-like protein was used to assess infection of HFF cultures. Total protein extracts from extracellular tachyzoites ("Tg only") were used to control for any cross-reactivity of the antibodies against *T. gondii* proteins. Data are representative of at least three biological replicates. **(C)** Cultures were fixed post-infection then processed for epifluorescence microscopy analyses. The number of parasites per PV in at least 50 infected cells in different fields of view were enumerated. Data collected from 2 independent experiment were compiled. **p < 0.01.

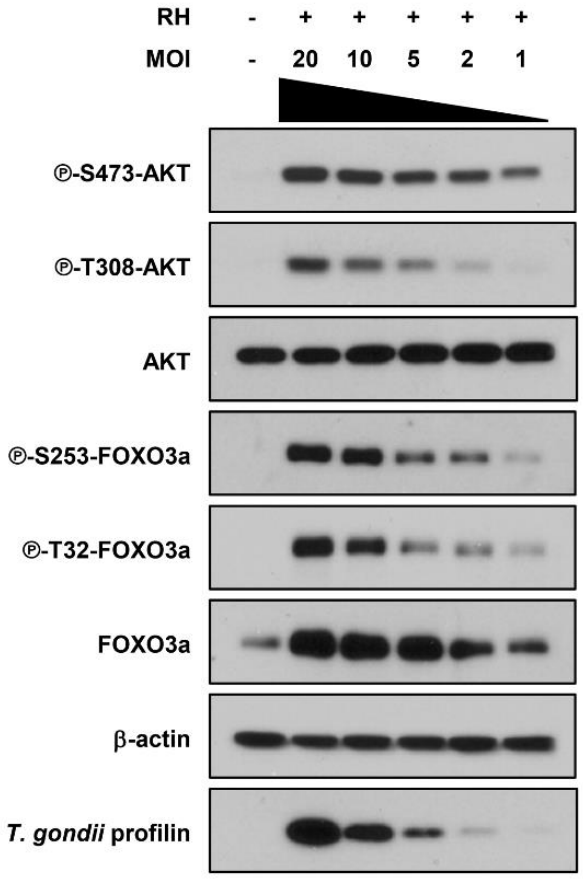


Figure S3.1 The multiplicity of infection (MOI) influences phosphorylation levels of AKT and FOXO3a in *T. gondii*-infected cells

HFF cultures were inoculated with RH *T. gondii* tachyzoites at the indicated MOI or left uninfected. Samples were collected 24 h after infection. Phosphorylation and expression levels of indicated proteins were monitored by western blotting. Total amounts of β-actin were used as a loading control, and an antibody raised against *T. gondii* profilin-like protein was used to assess infection of HFF cultures.

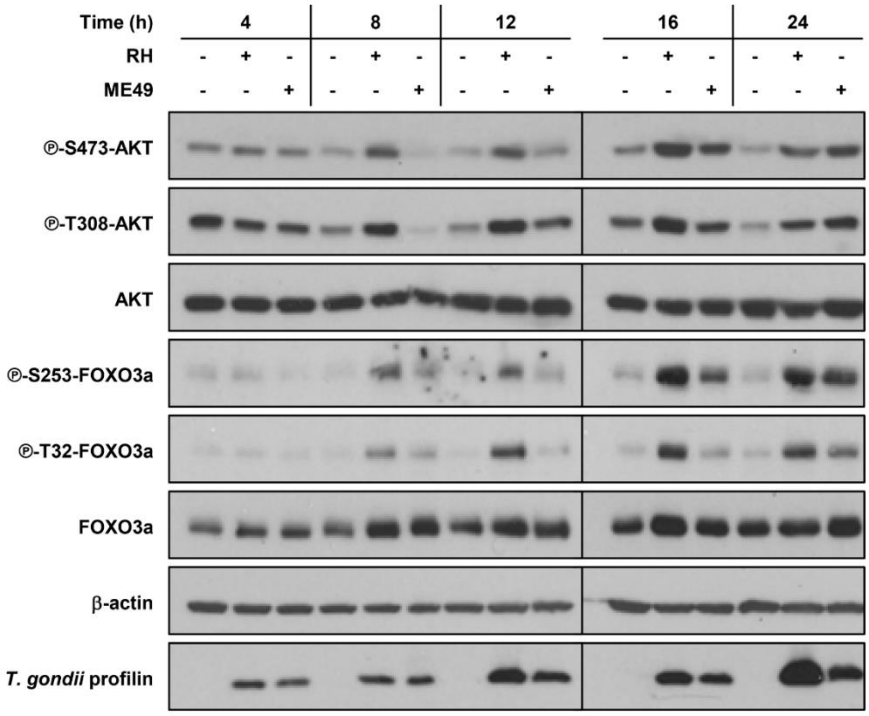


Figure S3.2 Infection of 3T3 by *T. gondii* induces phosphorylation of host AKT and FOXO3a

3T3 cultures were inoculated with either RH or ME49 T. gondii tachyzoites or left uninfected for the indicated times. Phosphorylation and expression levels of indicated proteins were monitored by western blotting.

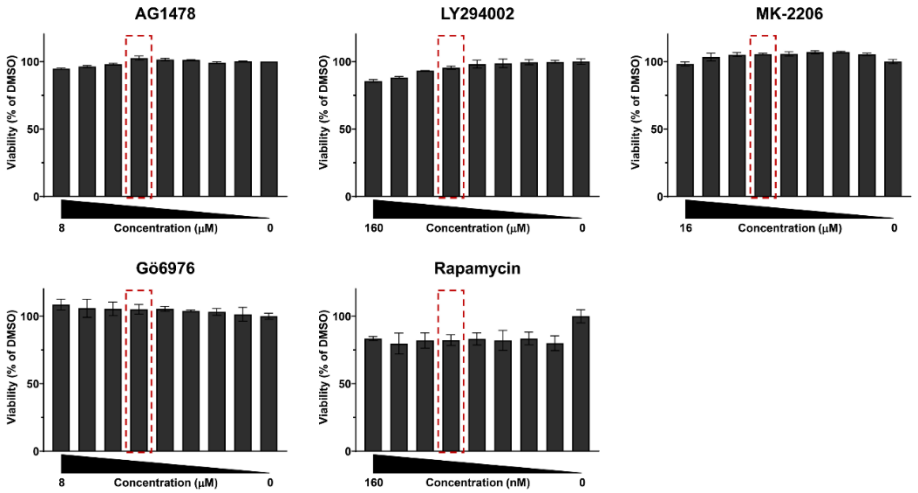


Figure S3.3 Selected inhibitors do not display acute toxicity in HFF cells

HFF cultures were treated with increasing (two-fold) concentrations of different inhibitors (AG1478, LY294002, MK-2206, Gö6976, and rapamycin) or an equivalent volume of vehicle (i.e., DMSO), as indicated, for 32 h. Cell viability was measured by colorimetric-based resazurin assays. The concentration used in

subsequent experiments is identified by a red dashed box for each inhibitor. OD values were normalized to DMSO-treated samples. Results are presented as mean (SD); all samples were performed in technical triplicates. No statistically significant differences in cell viability were observed between the various inhibitors and their respective controls.

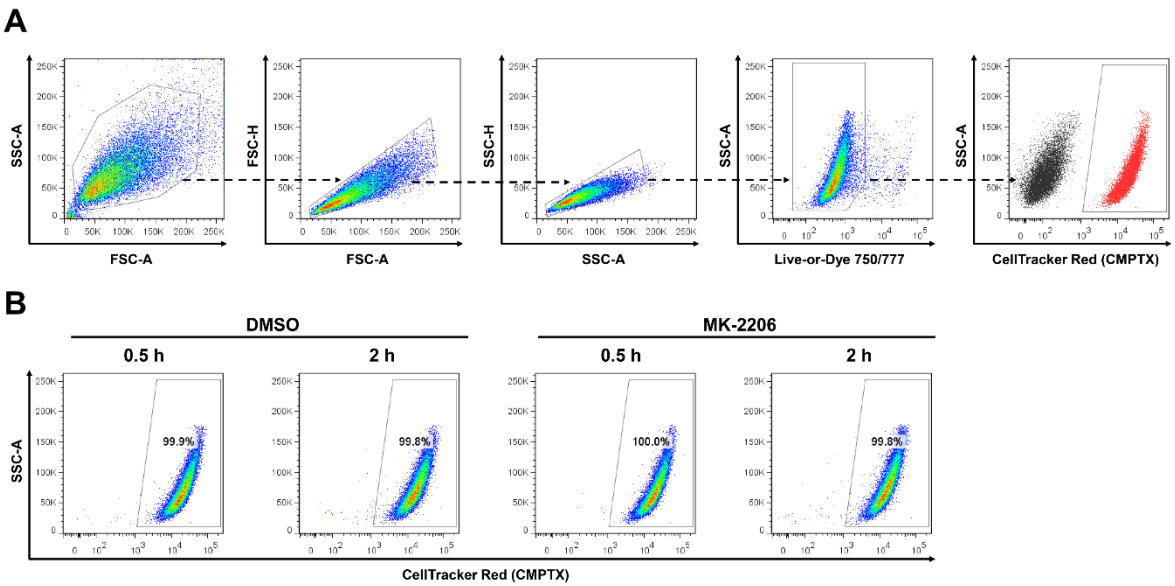


Figure S3.4 MK-2206 treatment of HFF does not affect infection rates by *T. gondii*

(A, B) HFF cultures were pre-treated with 2 μM MK-2206 or an equivalent volume of vehicle (i.e., DMSO) for 1 h then inoculated with CellTracker Red (CMPTX)-stained RH *T. gondii* parasites. Cells were collected by trypsinization 0.5 and 2 h post-infection, then processed for flow cytometry analyses to monitor infection rates. **(A)** Shown here, gating strategy utilized to measure infection rates. First, cells were identified according to FSC-A and SSC-A scatter profiles. Then, singlets were gated based on FSC-A vs. FSC-H and SSC-A vs. SSC-H. Dead cells were gated out, and only live cells were considered according to low staining with Live-or-Dye 750/777. Infected cells were identified based on positive signal for CellTracker Red. **(B)** Infection rates for cultures reported for DMSO- (left panels) and MK-2206-treated (right panels) cells at the indicated timepoints. Data and data analyses are representative of two biological replicates.

3.5.2 *Toxoplasma gondii* promotes nuclear export of host FOXO3a in an AKT-dependent fashion

Given that AKT-dependent phosphorylation leads to either cytosolic retention or nuclear export of FOXO3a (Biggs, Meisenhelder et al. 1999, Brunet, Bonni et al. 1999, Calissi, Lam et al. 2021), we next set out to assess the subcellular localization of FOXO3a over the course of the infection by confocal microscopy. In uninfected HFF, FOXO3a was detected mostly in the nucleus throughout the entire time course (**Fig. 3.2A and 3.2B**) presumably due to the lack of phosphorylation of FOXO3a at AKT-sensitive residues as observed by western

blotting (**Fig. 3.1A**). Conversely, FOXO3a was gradually exported from the host nucleus upon RH infection (**Fig. 3.2A and 3.2B**) in an MOI-dependent fashion (**Fig. S3.5**). Significant differences in the subcellular localization of FOXO3a were detected 24 h p.i. and beyond, as evidenced by the decreased co-localization coefficient of FOXO3a with the nuclear staining (**Fig. 3.2B**). Similarly, nuclear exclusion of FOXO3a was observed in HFF cultures upon ME49 infection (**Fig. S3.6**). Consistent with our western blot data on the effect of AKT inhibition on FOXO3a phosphorylation (**Fig. 3.1B**), HFF treatment with MK-2206 prevented *T. gondii*-inducible nuclear export of FOXO3a (**Fig. 3.2C and 3.2D**). Taken together, these data indicate that infection by *T. gondii* leads to AKT-sensitive FOXO3a nuclear export.

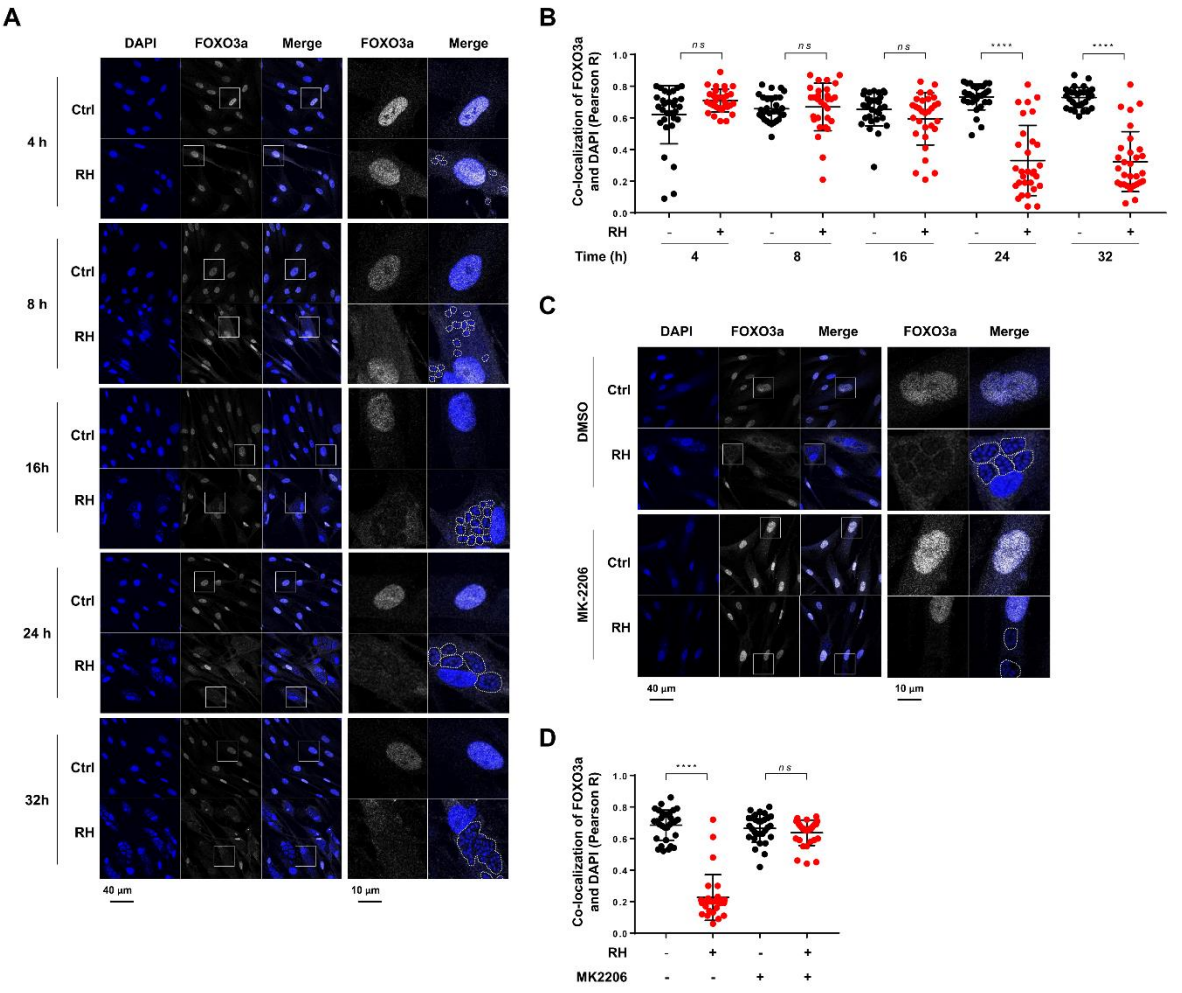


Figure 3.2 *T. gondii* infection leads to AKT-dependent FOXO3a export from the host nucleus

(**A, B**) HFF cultures were inoculated with RH *T. gondii* tachyzoites or left uninfected and fixed at the indicated times and processed for confocal immunofluorescence microscopy. (**C, D**) HFF cultures were pretreated with 2 μM MK-2206 or an equivalent volume of vehicle (i.e., DMSO) for 1 h then inoculated with RH *T. gondii* parasites. Cells were cultured up to 32 h following infection in the presence of absence of MK-2206. (**A, C**) Samples were stained with DAPI (shown in blue), used as a nuclear marker, and for total FOXO3a (shown in white). Images are representative of at least two independent experiments. Original magnification (left panels), 4 times-enlarged insets (right panels). PVs are outlined with dashed lines to indicate the presence of parasites within infected cells. (**B, D**) Co-localization of FOXO3a and DAPI was quantified using Pearson R coefficient. Data are compiled from two independent experiments (n = 2) in which at least 25 cells were analyzed in different fields of view. Each data point represents the Pearson R coefficient of a single cell. ****p < 0.0001; and ns, not significant.

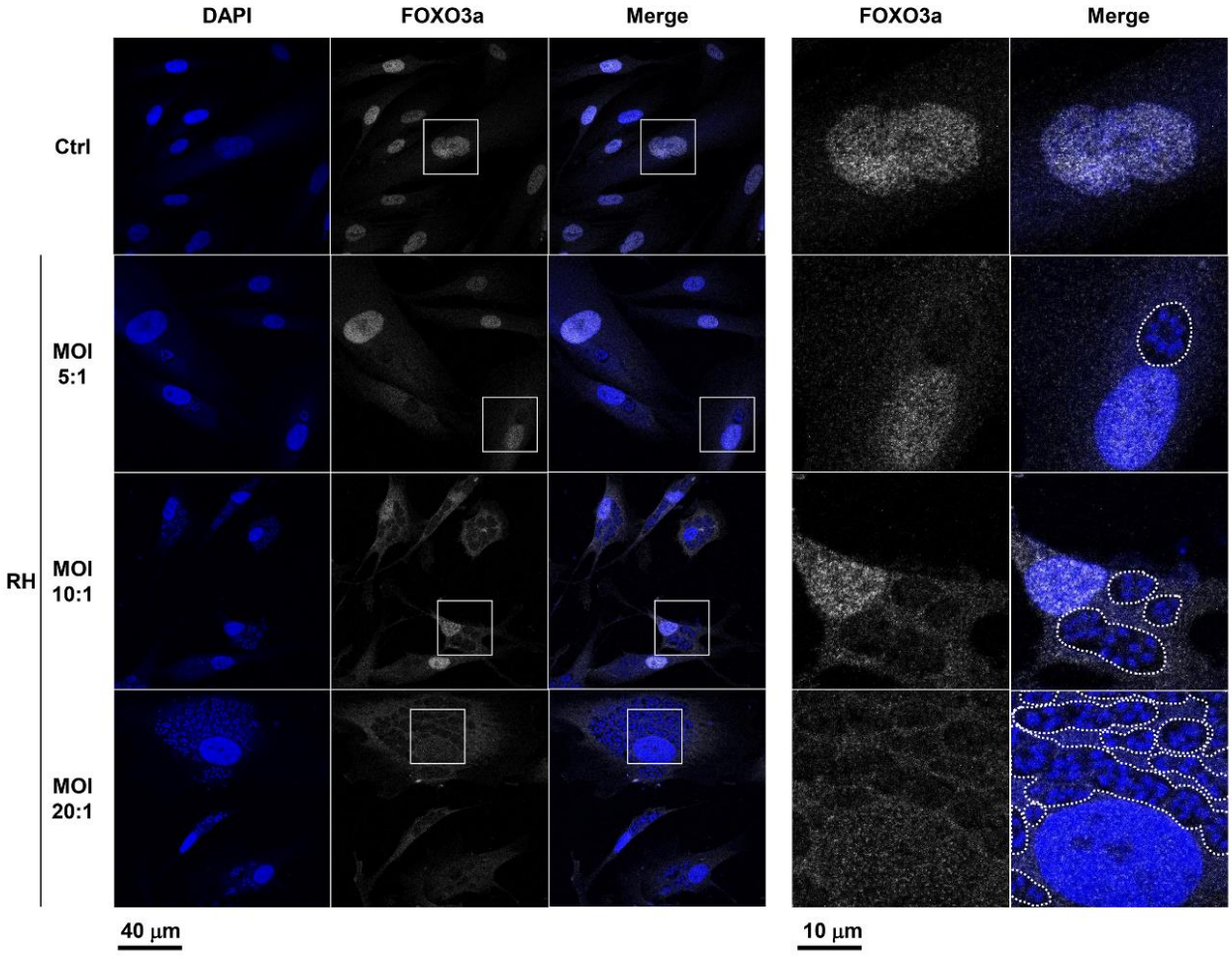


Figure S3.5 The multiplicity of infection (MOI) influences nuclear export of FOXO3a in *T. gondii*-infected cells

HFF cultures were inoculated with RH *T. gondii* tachyzoites at the indicated MOI or left uninfected. Cultures were fixed 32 h after infection and processed for confocal immunofluorescence microscopy. PVs are outlined with dashed lines to indicate the presence of parasites within infected cells. Data are representative of two biological replicates.

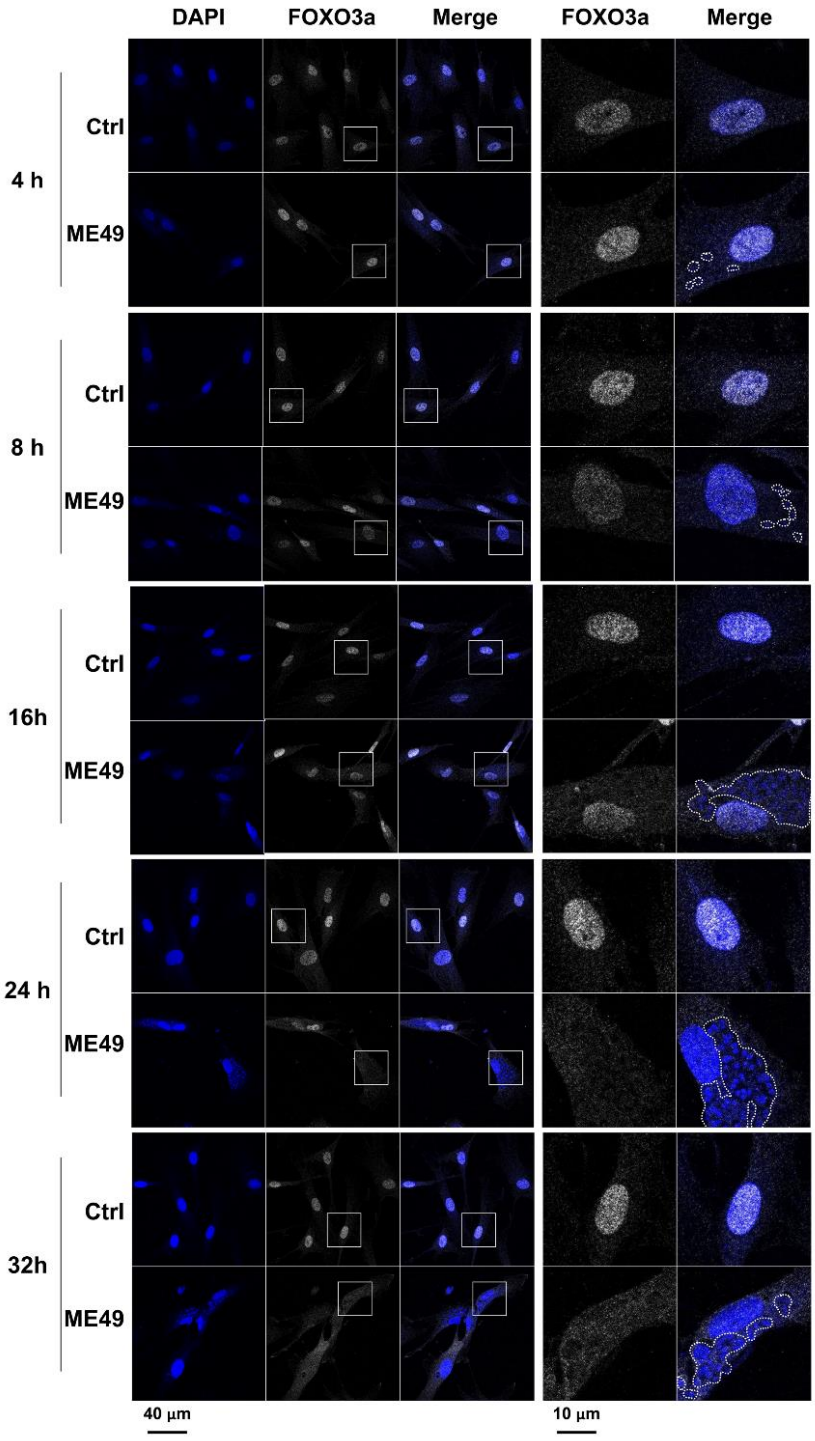


Figure S3.6. Infection with type II *T. gondii* strain ME49 leads to FOXO3a nuclear export from the host nucleus

HFF cultures were inoculated with ME49 *T. gondii* tachyzoites or left uninfected and fixed at the indicated times and processed for confocal immunofluorescence microscopy. Samples were stained with DAPI (shown

in blue) and for total FOXO3a (shown in white). Original magnification (left panels), 4 times-enlarged insets (right panels). PVs are outlined with dashed lines to indicate the presence of parasites within infected cells. Images are representative of two biological replicates.

3.5.3 *Toxoplasma gondii*-driven phosphorylation and nuclear export of FOXO3a requires live infection and EGFR-independent PI3K-AKT signaling

We next sought to determine whether live infection was required to induce phosphorylation of AKT and FOXO3a. Unlike infection with live parasites, treatment of HFF cultures with soluble RH or ME49 *T. gondii* antigens (STAg) or heat-killed (HK) parasites failed to induce phosphorylation of AKT and FOXO3a (**Fig. 3.3A**). Of note, STAg did not lead to AKT and FOXO3a phosphorylation regardless of the STAg concentrations tested (**Fig. S3.7**).

It was previously reported that phosphorylation of AKT upon *T. gondii* infection was in part regulated through the early activation of EGFR (Muniz-Feliciano, Van Grol et al. 2013). To test the involvement of EGFR on the phosphorylation of AKT and FOXO3a, we pre-treated or not HFF cells with AG1478, an EGFR inhibitor (Daub, Weiss et al. 1996), then inoculated cultures or not with RH *T. gondii* tachyzoites, and collected samples at early timepoints (i.e., 15-, 30-, and 60-min post-inoculation). Infection did not induce noticeable levels of AKT and FOXO3a phosphorylation at these timepoints (**Fig. 3.3B**). In parallel, HFF cultures were stimulated with recombinant human EGF (rhEGF) for 10 min as a positive control for EGFR activation. Treatment with rhEGF strongly induced the phosphorylation of EGFR (Y1068) as well as AKT and FOXO3a. Moreover, pre-treatment with AG1478 abrogated rhEGF-induced phosphorylation of EGFR, AKT, and FOXO3a, confirming the efficacy of the inhibitor at the tested concentration. These data indicate that *T. gondii*-driven phosphorylation of FOXO3a requires live infection and is independent of early EGFR and AKT activation.

Despite the absence of early EGFR activation during *T. gondii* infection, we assessed its role in the gradual and sustained phosphorylation of AKT and FOXO3a observed at later timepoints p.i. To do so, we first infected or not HFF cultures with either RH or ME49 *T. gondii* tachyzoites. Then, 4 h after inoculation, cultures were treated with AG1478 or vehicle and further incubated for an additional 20 h. This approach helped avoid any effects of the compound on parasite's ability to invade host cells. Pharmacological inhibition of EGFR activity did not affect the parasite's ability to induce the phosphorylation of AKT and FOXO3a (**Fig. 3.3C**). Confocal immunofluorescence microscopy analyses confirmed that

infection by *T. gondii* still led to the nuclear export of FOXO3a despite concomitant treatment with AG1478 (**Fig. 3.3D**). Considering these results, we sought for alternative molecular mechanisms responsible for *T. gondii*-induced phosphorylation of FOXO3a. It has been previously shown that *T. gondii* can phosphorylate AKT through the activation of its upstream regulator phosphatidylinositol 3-kinase (PI3K) (Wang, Weiss et al. 2009, Muniz-Feliciano, Van Grol et al. 2013, Zhou, Quan et al. 2013, Quan, Chu et al. 2015). Treatment with LY294002, a potent inhibitor of PI3K (Vlahos, Matter et al. 1994), and MK-2206 completely inhibited AKT and FOXO3a phosphorylation (**Fig. 3.3C**). In addition to EGFR and PI3K, PKCα and mTOR have been shown to upregulate AKT activity in *T. gondii*-infected cells (Wang, Weiss et al. 2010, Lopez Corcino, Gonzalez Ferrer et al. 2019). To evaluate the potential contribution of these regulators, we treated HFF cultures with either Gö6976 or rapamycin, a PKCα and an mTOR complex 1 (mTORC1) inhibitor, respectively (Martiny-Baron, Kazanietz et al. 1993, Leroux, Lorent et al. 2018). These inhibitors failed to prevent infection-induced phosphorylation of AKT and FOXO3a (**Fig. 3.3C**). Of note, none of these inhibitory compounds displayed overt toxicity on HFF cells at the tested concentrations (**Fig. S3.3**). Taken together, these results suggest that the PI3K-AKT signaling axis is required to induce the phosphorylation of FOXO3a during *T. gondii* infection but independently of EGFR, PKCα, or mTORC1 activity.

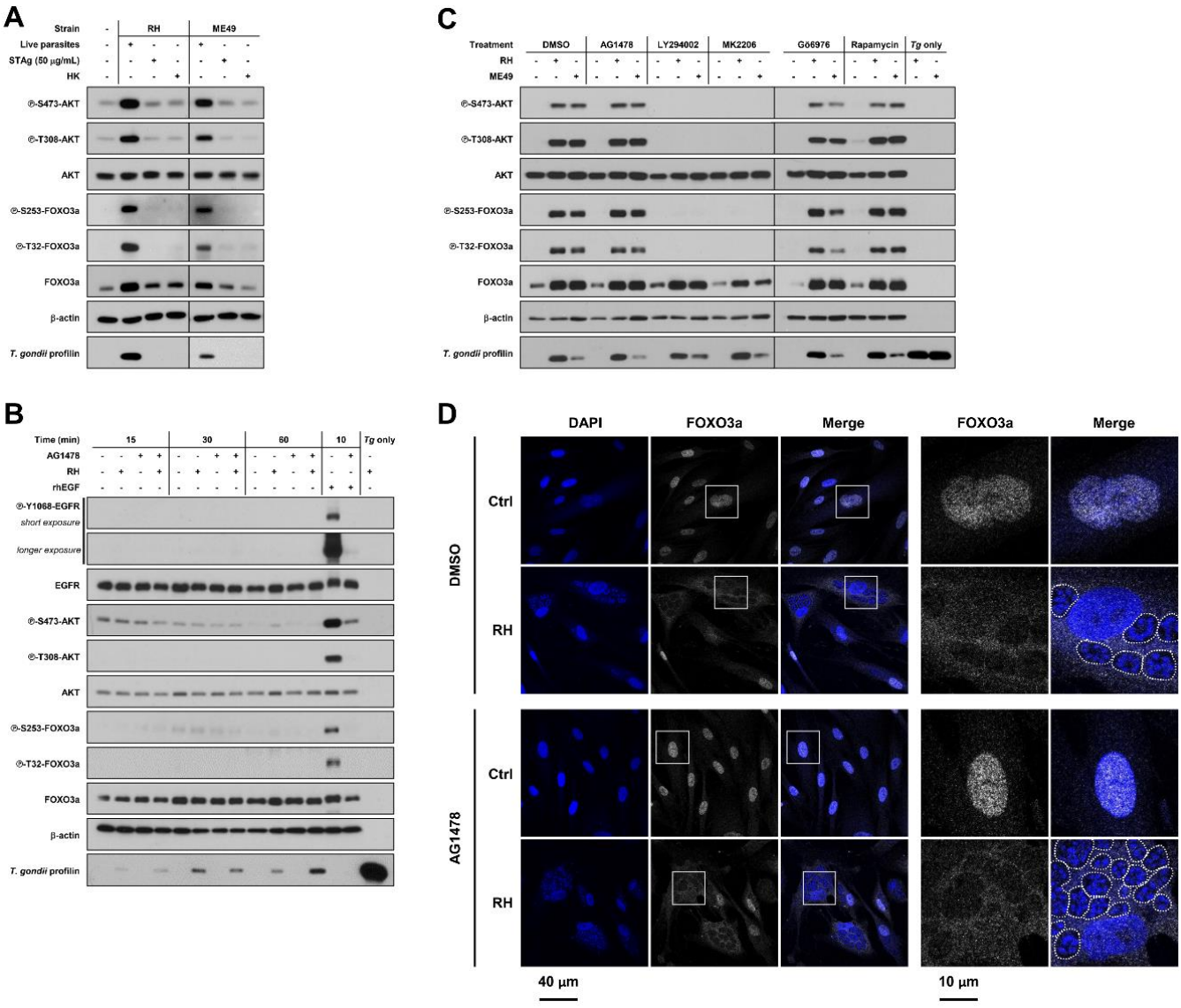


Figure 3.3 *T. gondii*-driven phosphorylation and nuclear export of FOXO3a requires live infection and EGFR-independent PI3K-AKT signaling

(A) HFF cultures were inoculated with live or heat-killed (HK) RH or ME49 *T. gondii* tachyzoites, treated with 50 μg/mL STAg concentration, or left uninfected and untreated for 24 h. Phosphorylation and expression levels of indicated proteins were monitored by western blotting. **(B)** HFF cultures were pre-treated with 1 μM AG1478 or an equivalent volume of vehicle (i.e., DMSO) for 1 h. Then, cultures were inoculated with RH *T. gondii* tachyzoites or left uninfected. Samples were collected at the indicated times following inoculation and processed for western blotting analyses. As a positive control for the induced phosphorylation of EGFR, cells were treated with 100 ng/mL recombinant human EGF for 10 min. **(C)** HFF cultures were inoculated with RH or ME49 *T. gondii* tachyzoites or left uninfected for 4 h. Then, cultures were treated with the indicated inhibitors (1 μM AG1478, 20 μM LY294002, 2 μM MK-2206, 1 μM Gö6976, and 20 nM rapamycin), or an equivalent volume of DMSO (i.e., vehicle) for 20 h. Phosphorylation and expression levels of indicated proteins were monitored by western blotting. **(D)** HFF cultures were inoculated with RH *T. gondii* tachyzoites or left uninfected for 4 h. Then, cultures were treated with 1 μM AG1478 or an equivalent volume of DMSO (i.e., vehicle) for 20 h. Samples were processed for confocal immunofluorescence microscopy. Fixed cells were

stained with DAPI (shown in blue), used as a nuclear marker, and for total FOXO3a (shown in white). Images are representative of two independent experiments. Original magnification (left panels), 4 times-enlarged insets (right panels). PVs are outlined with dashed lines to indicate the presence of parasites within infected cells. Data are representative of at least two biological replicates.

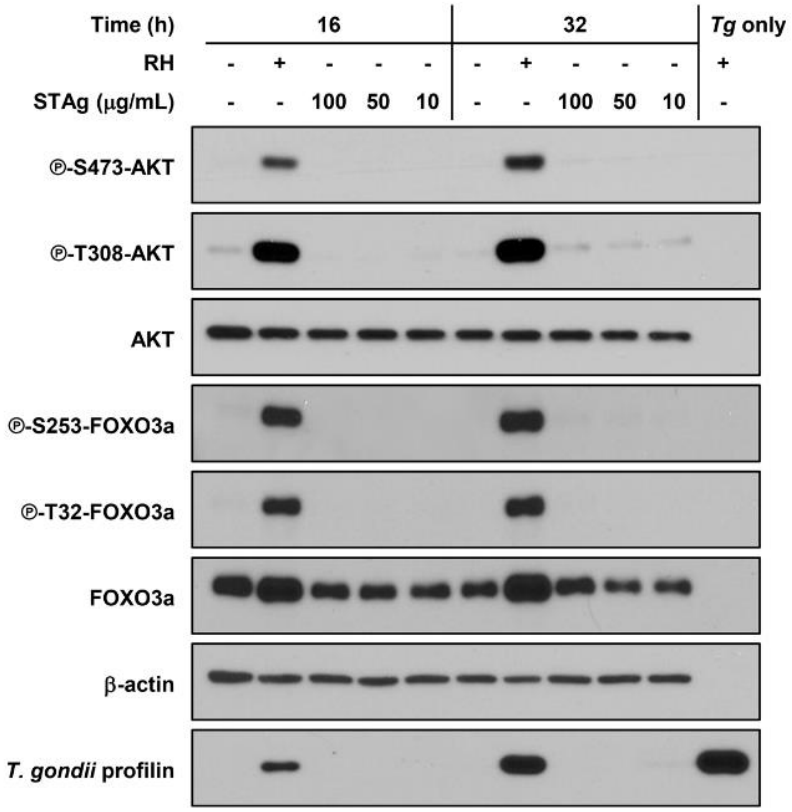


Figure S3.7 Soluble *T. gondii* antigens (STAg) fail to induce FOXO3a phosphorylation and phenocopy infection with live parasites

HFF cultures were inoculated with RH *T. gondii* tachyzoites, treated at the indicated STAg concentration, or left uninfected and untreated for the indicated times. Phosphorylation and expression levels of indicated proteins were monitored by western blotting.

3.5.4 *Toxoplasma gondii* inhibits the expression of autophagy-related genes through AKT-dependent inactivation of FOXO3a

Previous reports support the notion that *T. gondii* prevents autophagy-mediated killing through AKT-dependent signaling (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019, Cheng, Zhang et al. 2022); however, the AKT downstream effector(s) are yet to be identified. FOXO3a is an important TF of autophagy-related genes (Zhou, Liao et al. 2012, Lee, Nam et al. 2018, Audesse, Dhakal et al. 2019) that is under the control of AKT (Brunet, Bonni et al. 1999, Calissi, Lam et al. 2021). Hence, we postulated

that AKT blocks FOXO3a-dependent autophagy-related transcriptional programs during *T. gondii* infection. To test this, we induced transcription of autophagy-related genes in HFF cultures by serum starvation, a well-described approach to induce FOXO3a-dependent autophagy (Moruno, Perez-Jimenez et al. 2012). In addition, cells were treated with MK-2206 to prevent AKT-dependent phosphorylation and nuclear export of FOXO3a. Serum starvation led to an increase of all autophagy-related transcripts monitored by RT-qPCR in uninfected HFF cells compared to control cells not deprived of serum (**Fig. S3.8A**). Of note, infection by *T. gondii* significantly inhibited starvation-induced transcription of autophagy-related genes (i.e., ULK1, BECN1, PINK1, GABARAPL2, SQSTM1, and NBR1), as compared to uninfected serum-starved HFF cultures (i.e., "Control") (**Fig. 3.4A** and **Fig. S3.8A**, top panel). Conversely, transcription of several other autophagy-related genes was not inhibited by *T. gondii* in serum-starved HFF cells (i.e., ATG5, ATG7, ATG12, ATG16L, BNIP3L, and GABARAP) (**Fig. S3.8A**, bottom panel). Inhibition of AKT by MK-2206 treatment enhanced the expression of ULK1, BECN1, PINK1, GABARAPL2, SQSTM1, and NBR1 in infected cells (**Fig. 3.4A** and **Fig. S3.8A**, top panel), highlighting the requirement of intact AKT signaling for selective transcriptional repression of autophagy-related genes by *T. gondii*.

To resolve *T. gondii*-driven repression of autophagy-related genes through AKT-dependent inactivation of FOXO3a, we generated a FOXO3a knockdown (KD) HFF cell line by shRNA targeting. In parallel, control HFF cultures were transduced to express a non-targeting scrambled (Scr.) shRNA. The efficiency of the KD of FOXO3 and subsequent inhibition of FOXO3a protein expression were confirmed by RT-qPCR and Western blot analyses, respectively (**Fig. 3.4B**). Infection of either Scr. or FOXO3a KD HFF by *T. gondii* markedly inhibited transcription of ULK1, BECN1, PINK1, GABARAPL2, SQSTM1, and NBR1 as compared to uninfected serum-starved Scr. HFF cultures (i.e., "Scrambled Control") (**Fig. 3.4C** and **Fig. S3.8B**). In line with RT-qPCR experiments carried out in wildtype (WT) HFF (**Fig. 3.4A** and **Fig. S3.8A**, top panel), treatment with MK-2206 enhanced transcription of autophagy-related genes in *T. gondii*-infected Scr. HFF (**Fig. 3.4C** and **Fig. S3.8B**). In stark contrast, pharmacological blockade of AKT did not reverse the inhibitory effect of *T. gondii* on the expression of ULK1, BECN1, PINK1, GABARAPL2, and NBR1 mRNAs in FOXO3a KD HFF (**Fig. 3.4C**). Interestingly, *T. gondii*-driven repression of SQSTM1 transcription

appeared to be AKT-dependent but FOXO3a-independent since AKT inhibition led to an increase in SQSTM1 mRNA levels in both infected Scr. and FOXO3a KD HFF cultures (**Fig. S3.8B**). This set of experiments provides evidence *T. gondii* downregulates the expression of selected autophagy-related genes through AKT-dependent inactivation of FOXO3a transcriptional programs.

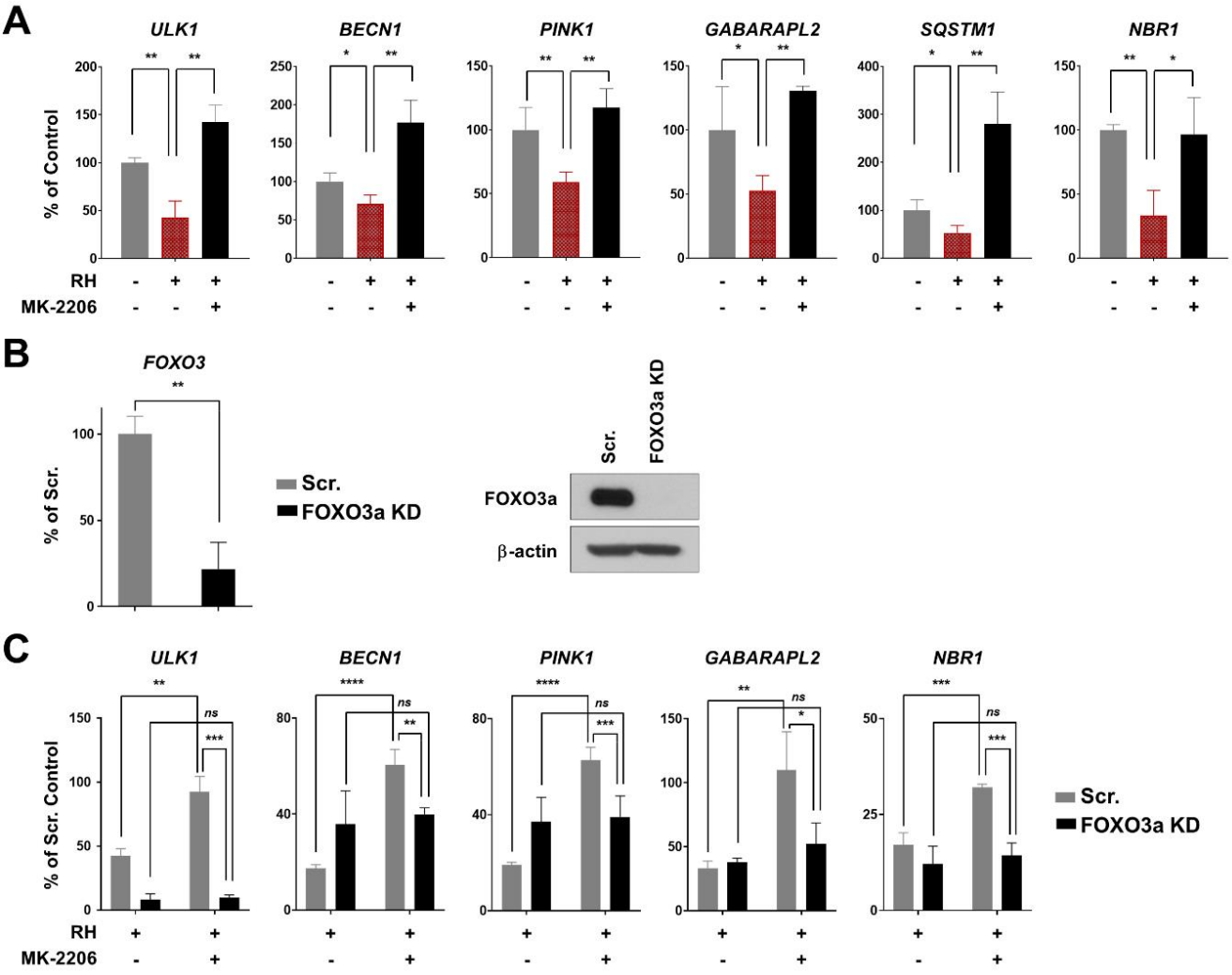


Figure 3.4 AKT-FOXO3a-sensitive transcription of autophagy-related host genes induced upon serum starvation is impeded following *T. gondii* infection

(A) HFF cultures were pretreated with 2 μM MK-2206 or an equivalent volume of vehicle (i.e., DMSO) for 1 h, then inoculated with RH *T. gondii* parasites or left uninfected. To induce autophagy, cultures were deprived of serum throughout the course of the infection (24 h). **(B)** Knock-down levels of FOXO3 mRNA and FOXO3a protein in lentivirus-transduced HFF was monitored by qPCR and Western blotting, respectively, and compared to cells transduced with scramble (Scr.) shRNA. **(C)** Scr. or FOXO3 shRNA-transduced HFF cultures were

pretreated with 2 μM MK-2206 or an equivalent volume of vehicle (i.e., DMSO) for 1 h, then inoculated with RH *T. gondii* parasites or left uninfected. Cultures were deprived of serum throughout the course of the infection (24 h). (A, B, left panel, and C) Relative expression of indicated genes was normalized to ACTB and was calculated as a percentage of (A) serum-starved DMSO-treated uninfected control (i.e., "Control"), (B) Scr. HFF cultures, or (C) serum-starved DMSO-treated uninfected Scr. HFF cultures. Data are presented as mean (SD) in technical triplicates and are representative of at least two biological replicates. **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; and ns, not significant.

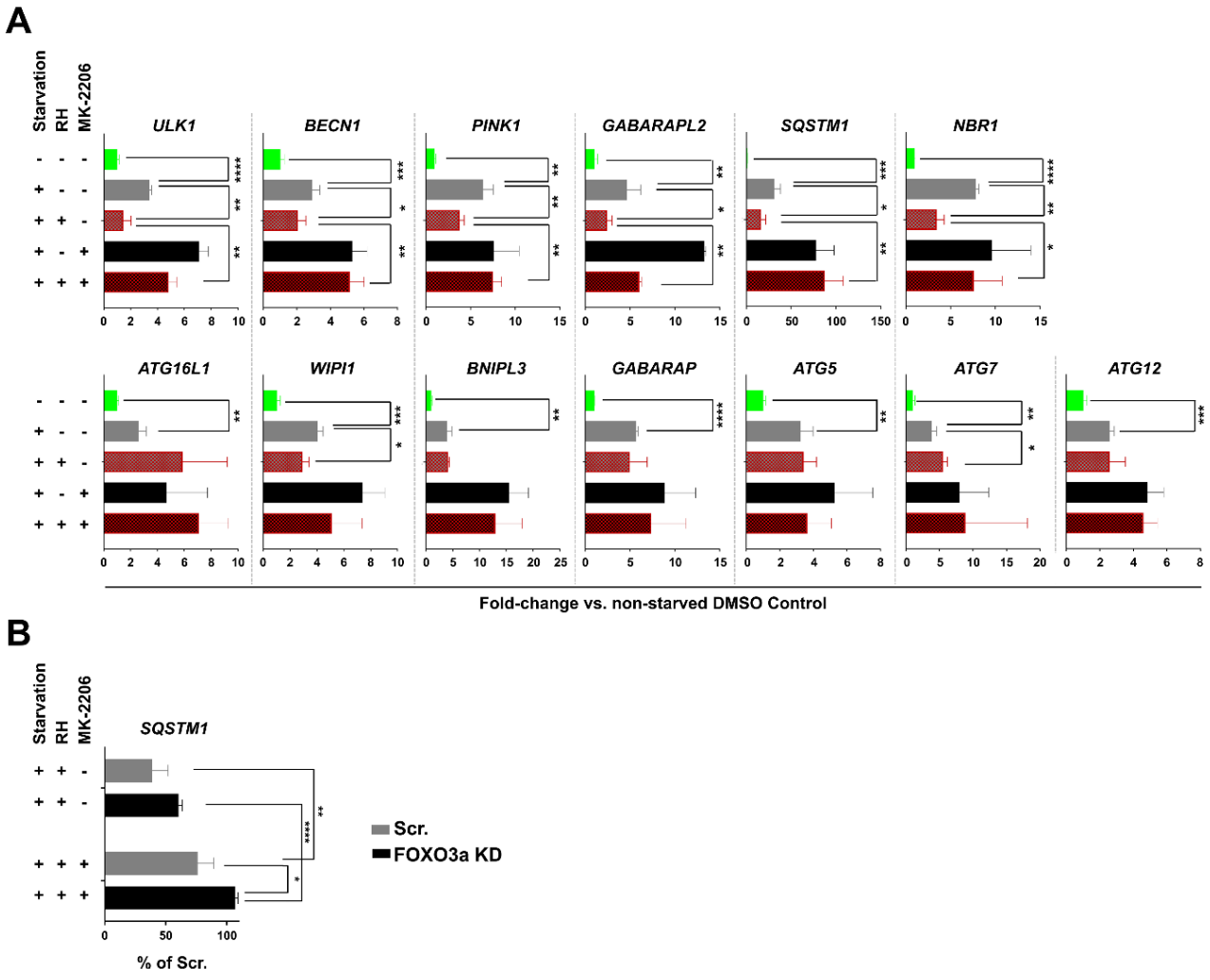


Figure S3.8 Infection by *T. gondii* inhibits serum starvation-induced expression of a subset of autophagy-related genes through AKT-FOXO3a-dependent mechanisms

(A) HFF cultures were pre-treated with 2 μM MK-2206 or an equivalent volume of vehicle (i.e., DMSO). Then, cells were inoculated with RH *T. gondii* tachyzoites or left uninfected for 24 h. Cultures were deprived of FBS (i.e., serum-starved) for the entire length of the experiment to induce autophagy or not, as indicated. (**A, B**) Samples were processed for qPCR analyses. Relative mRNA amounts of a subset of autophagy-related transcripts (normalized to ACTB) were compared to uninfected control cultures. Relative expression fold-

change of the indicated genes was calculated against (A) non-starved DMSO-treated uninfected control or (B) as a percentage of serum-starved DMSO-treated uninfected Scr. HFF cultures. Each sample was analyzed in technical triplicates, the averages (SD) of which are plotted. Data are representative of at least two biological replicates.

3.5.5 *Toxoplasma gondii* drives AKT-sensitive nuclear export of FOXO3a to prevent targeting of the parasitophorous vacuole by the host autophagic response

T. gondii can block the recruitment of host phagolysosomes and autophagy effectors to the PV to avoid elimination (Muniz-Feliciano, Van Grol et al. 2013, Portillo, Muniz-Feliciano et al. 2017, Lopez Corcino, Gonzalez Ferrer et al. 2019, Cheng, Zhang et al. 2022). As previously reported, this is mediated in part through the sustained activation of AKT (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019). To confirm this in our system, we infected serum-deprived HFF cultures treated or not with MK-2206 to inhibit AKT. We then stained cells with LysoTracker Red DND-99, which selectively stains acidic organelles, to monitor the presence and recruitment of lysosomal/autophagolysosomal structures. Consistent with data shown in **Fig. 3.2C**, FOXO3a was excluded from the host nucleus in infected cells but remained nuclear upon MK-2206 treatment (**Fig. 3.5A and 3.5C**). Pharmacological blockade of AKT also led to a pronounced increase in the staining intensity and recruitment of acidic structures around the PV as compared to DMSO-treated infected cells (**Fig. 3.5A and 3.5B**). In parallel, we assessed the expression and localization of LC3, a well-described autophagy effector protein (Deretic, Saitoh et al. 2013), in these cells. While staining was relatively weak and diffused in the entire cytoplasm of infected DMSO-treated HFF cultures, punctate staining patterns for LC3 were readily observed surrounding the PV in MK-2206-treated cells (**Fig. 3.5B and 3.5D**). In line with previous reports (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019), these data indicate that intact AKT activity in *T. gondii*-infected cells is required to prevent autophagolysosomal targeting of the PV.

To complement our pharmacological approach, HFF cultures were transduced to express either a N-terminus Myc-tagged WT or an AKT-resistant form of FOXO3a (i.e., Triple Mutant; TM), in which AKT-sensitive residues S253, T32, and S315 are mutated to alanine (i.e., S253A, T32A, S315A) (Brunet, Bonni et al. 1999, Zhu, Tong et al. 2015, Lee, Nam et

al. 2018). To ensure that the exogenous Myc-FOXO3a WT form behaved similarly to the endogenous protein vis-à-vis AKT-sensitive subcellular localization, we transduced HFF cells to express the Myc-FOXO3a WT form or with the Empty vector. Then, cells were cultured in nutrient-rich medium (i.e., 10% FBS) to promote nuclear export of FOXO3a under non-restrictive growth conditions and treated with MK-2206 or DMSO. Using an anti-Myc-tag-specific antibody, Myc-FOXO3a WT was readily observed in the cytoplasm of DMSO-treated cells but was predominantly nuclear upon AKT inhibition (**Fig. S3.9A**). No signal was detected in Empty vector transduced HFF cultures highlighting the specificity of the immunostaining (**Fig. S3.9A**). Staining cells using an anti-FOXO3a antibody revealed a similar subcellular distribution in DMSO versus MK-2006 treatment (**Fig. S3.9B**). Moreover, this latter approach confirmed higher expression of FOXO3a in Myc-FOXO3a WT transduced cells as compared to Empty vector-transduced control HFF.

Next, we compared recruitment of acidic organelles and LC3 to the PV in HFF cells expressing either Myc-FOXO3a WT or Myc-FOXO3a TM variants. Myc-FOXO3a WT was readily exported from the nucleus upon *T. gondii* infection while recruitment of Lysotracker-positive structures and LC3 was minimal (**Fig. 3.6A** and **3.6C**, top panels). These observations were in line with those made in WT HFF cultures (**Fig. 3.5A** and **3.5C**, top panels). In stark contrast, mutation of the AKT-sensitive residues precluded nuclear export of the Myc-FOXO3a-TM form despite infection by *T. gondii* (**Fig. 3.6A** and **3.6C**, bottom panels). Recruitment of autophagolysosomal structures and LC3 were markedly enhanced in infected cells expressing the TM form of FOXO3a (**Fig. 3.6A** and **3.6C**, bottom panels, and **3.6B** and **3.6D**). These results were reminiscent of those obtained with the use of MK-2206 in WT HFF cells (**Fig. 3.5A** and **3.5C**, bottom panels). Altogether, these data provide evidence that AKT-dependent nuclear export of FOXO3a is necessary to prevent recruitment of autophagy-related effectors to the PV, a likely strategy by which *T. gondii* promotes its intracellular survival.

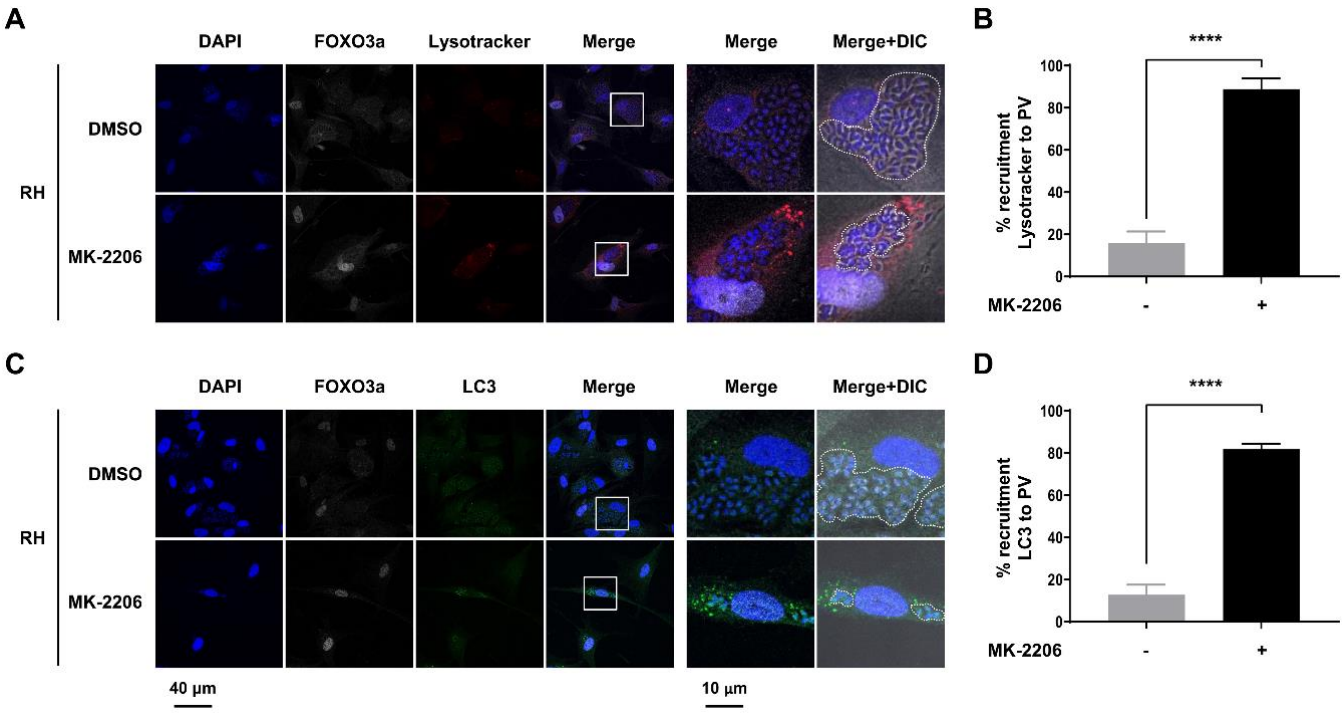
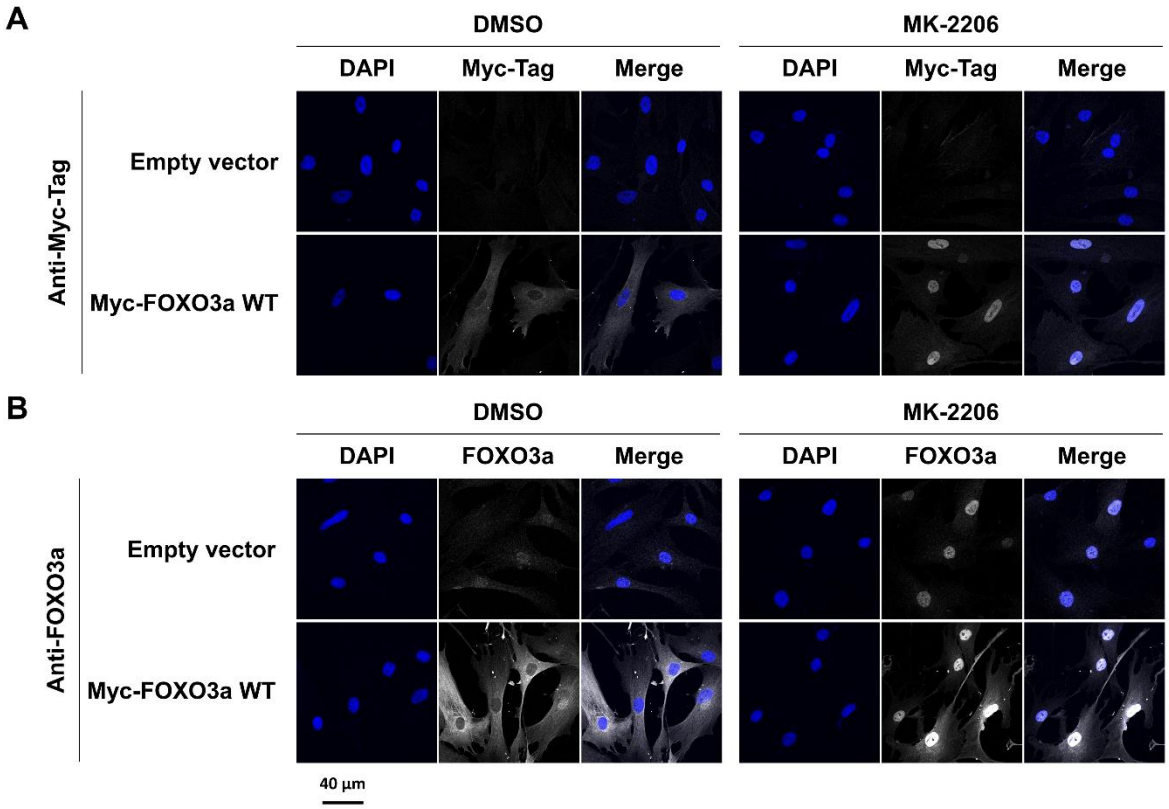


Figure 3.5. AKT inhibition leads to recruitment of autophagolysosomal markers at the parasitophorous vacuole

(**A, B**) HFF cultures were treated with 2 µM MK-2206 or an equivalent volume of vehicle (i.e., DMSO) for 1 h. Cells were then inoculated with RH *T. gondii* parasites or left uninfected for 24 h. Cultures were serum-starved for the entire length of the experiment. Samples were processed for confocal immunofluorescence microscopy. Shown here, cells were stained with DAPI, for FOXO3a, and (**A, B**) LysoTracker™ Red DND-99 (2 h prior to fixation) or (**C, D**) LC3. Original magnification (left panels), 4 times-enlarged insets (right panels). Data are representative of two biological replicates. (**A, C**) PVs are outlined with dashed lines to indicate the presence of parasites within infected cells. Recruitment of (**B**) lysosomal/autophagolysosomal structures (Lysotracker-stained) and (**D**) LC3 to the PV were quantified. Data are presented as mean (SD) are representative of three biological replicates. **** $p < 0.0001$.



22Figure S3.9. Exogenous Myc-FOXO3a WT protein behaves like the endogenous form and is sensitive to AKT activity

(**A, B**) HFF cultures were transduced to express an N-terminal Myc-tagged FOXO3a WT form or transduced with the empty vector. Cells were treated with 2 µM MK-2206 or an equivalent volume of vehicle (i.e., DMSO) for 4 h. Samples were processed for confocal immunofluorescence microscopy. Samples were stained with DAPI (shown in blue) and for Myc-tagged FOXO3a WT (shown in white) using anti-Myc-tag (**A**) or anti-FOXO3a (**B**) antibodies.

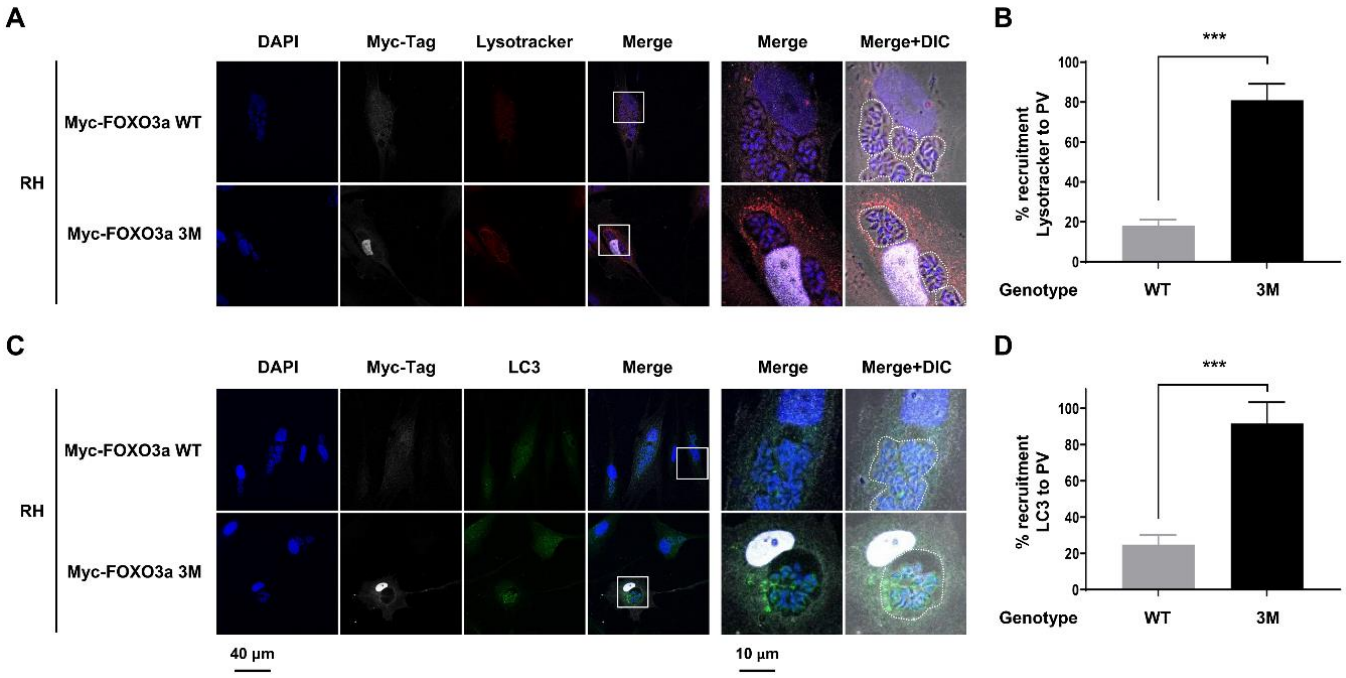


Figure 3.6. Recruitment of autophagy-related effectors to the parasitophorous vacuole is in part dependent on FOXO3a activity

(**A, B**) HFF were transduced to express either N-terminal Myc-tagged FOXO3a WT or TM forms. Cell cultures were inoculated with RH *T. gondii* parasites or left uninfected for 24 h. Cultures were deprived of FBS (i.e., starved) for the entire length of the experiment. Samples were processed for confocal immunofluorescence microscopy. Cells were stained with DAPI, for Myc-tagged FOXO3a, and (**A, B**) LysoTracker™ Red DND-99 (2 h prior to fixation) or (**C, D**) LC3. Original magnification (left panels), 4 times-enlarged insets (right panels). Data are representative of two biological replicates. (**A, C**) PVs are outlined with dashed lines to indicate the presence of parasites within infected cells. Recruitment of (**B**) lysosomal/autophagolysosomal structures (Lysotracker-stained) and (**D**) LC3 to the PV were quantified. Data are presented as mean (SD) are representative of three biological replicates. *** $p < 0.0001$.

3.6 Discussion

Autophagy is a highly regulated catabolic process that targets cytosolic material for autophagolysosomal degradation (Corona Velazquez and Jackson 2018). It has also evolved into an important host defense mechanism against viruses as well as intracellular bacteria and protozoan parasites such as *T. gondii* (Deretic, Saitoh et al. 2013, Ghartey-Kwansah, Adu-Nti et al. 2020). In this regard, it was previously described that CD40- and IFN-γ-mediated activation of autophagic response against *T. gondii* leads to parasite elimination (Ling, Shaw et al. 2006, Van Grol, Muniz-Feliciano et al. 2013). However, *T. gondii* has developed several subversion strategies to counteract autophagic targeting (Muniz-Feliciano, Van Grol et al. 2013, Portillo, Muniz-Feliciano et al. 2017, Lopez Corcino, Gonzalez Ferrer et al. 2019,

Nemati, Pazoki et al. 2021, Subauste 2021, Cheng, Zhang et al. 2022), including activation of host AKT signaling (Muniz-Feliciano, Van Grol et al. 2013, Lopez Corcino, Gonzalez Ferrer et al. 2019). Despite this body of evidence, AKT downstream targets implicated in parasite-driven dysregulation of autophagy remained elusive. Herein, using cell imaging in combination with pharmacological and genetic approaches, we demonstrate that AKT-dependent curbing of the autophagic response by *T. gondii* depends in part on the inactivation of transcriptional programs controlled by FOXO3a.

Reduced nuclear FOXO3a levels were detected in murine macrophages infected with *T. gondii* (Lee, Kim et al. 2022). In line with these observations, we provide evidence that *T. gondii* actively forces FOXO3a out of the nucleus via AKT to hamper transcriptional programs involved in host defense responses in HFF. Similarly, the bacterium *C. rodentium* was shown to induce translocation of nuclear FOXO3a into the cytosol of infected human HT-29 and mouse CMT-93 epithelial cells lines, and colonic epithelium of infected mice (Snoeks, Weber et al. 2008). Even though no experimental evidence was provided on the alteration of transcriptional programs downstream of FOXO3a, the observed phenotype was associated with exacerbated pro-inflammatory cytokine production and disease pathogenesis during *C. rodentium* infection (Snoeks, Weber et al. 2008). We observed a dramatic increase in AKT-sensitive phosphorylation and nuclear exclusion of FOXO3a in *T. gondii*-infected HFF. In stark contrast, HCV was shown to induce FOXO3a phosphorylation at S574, a novel JNK-sensitive residue that promoted FOXO3a nuclear translocation in human hepatoma-derived HuH-7 cell line (Tikhanovich, Kuravi et al. 2014). These data hint at increased FOXO3a-mediated transcriptional activity; however, whether FOXO3a exerts a pro-viral or a host protective role during HCV infection was not tested. These reports along with our data warrant further investigation on the biological consequences of pathogen-driven modulation of the phosphorylation and subcellular redistribution of FOXO3, two events that appear to be context-dependent.

FOXO3a subcellular localization and transcriptional activity do not seem to be modulated in response to other infectious agents such as *M. tuberculosis, rhinovirus*, and HIV; however, forward and reverse genetics approaches have provided evidence that FOXO3a-dependent transcriptional programs play a crucial role in the outcome of these infections (van Grevenynghe, Procopio et al. 2008, Bouzeyen, Haoues et al. 2019, Gimenes-Junior, Owuar

et al. 2019). For instance, KD of FOXO3a led to non-protective responses in M. tuberculosis-infected macrophages whereas overexpression of a constitutively active form of FOXO3a (i.e., FOXO3a TM) had the opposite effect (Bouzeyen, Haoues et al. 2019). Likewise, conditional KO of FOXO3a in epithelial airway cells prevented type I and III IFN production and efficient antiviral immune responses in mice infected with rhinovirus (Gimenes-Junior, Owuar et al. 2019). The same phenotype was reported in a stable FOXO3a KO human airway epithelial cell line (Gimenes-Junior, Owuar et al. 2019). Interestingly, overexpression of FOXO1, another member of the FOXO family of TFs, restored the expression of Fas-associated factor 1 (FAF1), prevented IRF3 nuclear translocation, and abrogated interferon stimulated gene (ISG) expression in human epithelial cells infected with *T. gondii* (Gao, Quan et al. 2021), suggesting a role for FOXO1 in the regulation of IFN-mediated responses during toxoplasmosis. In addition, AKT-sensitive phosphorylation at T24 and PI3K-dependent nuclear exclusion of FOXO1 were recently reported in *T. gondii*-infected macrophages (Lee, Kim et al. 2022). However, it remains to be established whether *T. gondii* alters FOXO1 transcriptional programs in the host cell. Future work is required to shed light on the regulation and the biological consequences of FOXO1 nuclear exclusion during *T. gondii* infection.

Our data indicate that AKT and AKT-sensitive phosphorylation of FOXO3a in *T. gondii*-infected HFF requires intact PI3K activity. These observations are in line with previous reports showing that either chemical blockade or genetic ablation of PI3K, using LY294002 or p110α siRNA, respectively, prevent AKT phosphorylation by *T. gondii* in both hematopoietic and non-hematopoietic human cells (i.e., monocytic THP-1 cells, primary brain microvascular endothelial cells (HBMEC), and human retinal pigment epithelial cells ARPE-19) (Muniz-Feliciano, Van Grol et al. 2013, Zhou, Quan et al. 2013, Quan, Chu et al. 2015). Activation of AKT at early stages of infection in HBMEC was shown to be triggered via EGFR autophosphorylation by *T. gondii* microneme (MIC) proteins MIC3 and MIC6, both adhesins harboring EGF-like domains (Muniz-Feliciano, Van Grol et al. 2013). Conversely, we did not detect early activation of EGFR-AKT signaling and AKT-mediated phosphorylation of FOXO3a in *T. gondii*-infected HFF. Prolonged PKCα/PKCβ–Src-dependent phosphorylation of EGFR has been implicated in sustained activation of AKT in *T. gondii*-infected human retinal pigment epithelial cells ARPE-19 (Lopez Corcino,

Gonzalez Ferrer et al. 2019). In stark contrast, HFF treatment with PKC and EGFR inhibitors (AG1478 and Gö6976, respectively) did not prevent prolonged phosphorylation of AKT and FOXO3a upon *T. gondii* infection. These reports, along with our data, suggest that *T. gondii* infection drives the activation of PI3K-AKT signaling through diverse mechanisms which appear to be in part time- and host cell type-dependent. Comparative in cellulo analyses and in vivo studies will shed light on the molecular underpinnings of the PI3K-AKT-FOXO3a axis during *T. gondii* infection in different cell types and tissues.

Pharmacological approaches employed in the present study also revealed that infection-induced phosphorylation of AKT and FOXO3a in HFF do not require mTOR activity. These data are reminiscent of those obtained in LPS-stimulated macrophages showing that unlike PI3K, mTOR is dispensable for AKT-dependent phosphorylation of FOXO3a (Lee, Nam et al. 2018). Furthermore, AKT activation appeared to require infection by live parasites in HFF since treatment with STAg or HK parasites failed to trigger AKT-sensitive phosphorylation of FOXO3a. This latter observation does not exclude the possibility that secreted virulence factors or other soluble factors are linked to AKT and FOXO3a phosphorylation but rather that certain events are required for these factors to mediate their effects within the host cell (e.g., formation and presence of the PV membrane, specific route of entry of these molecules, etc.). Interestingly, both type I and II *T. gondii* strains tested (i.e., RH and ME49, respectively) were able to modulate FOXO3a phosphorylation and nuclear translocation in an AKT-dependent fashion, suggesting that this is a core process favoring parasite persistence that does not depend on strain-specific virulence factors. Taken together, our results indicate that phosphorylation and subsequent inactivation of FOXO3a by *T. gondii* requires live infection and occurs in a PI3K-AKT-dependent fashion independently of EGFR, PKCα, and mTOR activity in HFF. Further investigation will enable the identification of potential host and/or parasite factors involved in sustained AKT activation and subsequent nuclear exclusion of FOXO3a.

Our results are in agreement with two independent studies showing that inhibition of AKT signaling dramatically reduces *T. gondii* replication but does not hinder infection rates (Muniz-Feliciano, Van Grol et al. 2013, Choi, Gao et al. 2020), hinting at a crucial role for AKT activity to evade host cell defense mechanisms triggered after parasite internalization. Accordingly, intact PI3K-AKT signaling was identified as an essential mechanism utilized

by *T. gondii* to hamper oxidative stress responses (Choi, Gao et al. 2020) and autophagy-mediated parasite clearance (Muniz-Feliciano, Van Grol et al. 2013). Confirming and extending the latter report, we provide evidence that FOXO3a represents a downstream effector of AKT targeted by *T. gondii* to prevent accumulation of the autophagy protein LC3 and recruitment of acidic organelles around the PV. This is achieved in part through transcriptional repression of a subset of autophagy-related genes previously identified as bona fide FOXO3a targets using a combination of chromatin immunoprecipitation (ChIP)-Seq and RNA-Seq analyses (Audesse, Dhakal et al. 2019). Parasite-directed silencing of FOXO3a-regulated autophagy transcripts identified in our screening may impede several steps of the host autophagic response, including initiation and nucleation and cargo recruitment (i.e., BECN1 and ULK1), and trafficking (e.g., NBR1 and GABARAPL2) (**Fig. 3.7**) (Audesse, Dhakal et al. 2019, Cheng 2019). In line with this notion, knockdown of BECN1 in *T. gondii*-infected cells was shown to abrogate CD40-induced autophagic targeting of the PV (Van Grol, Muniz-Feliciano et al. 2013) and prevent parasite killing upon chemical blockade of AKT signaling (Muniz-Feliciano, Van Grol et al. 2013). Similarly, knockdown of ULK1 restored parasite replication despite pharmacological downregulation of sustained AKT phosphorylation during *T. gondii* infection using an EGFR tyrosine kinase inhibitor (Lopez Corcino, Gonzalez Ferrer et al. 2019). Further supporting our hypothesis that downregulation of FOXO3a transcriptional programs contributes to the multifaceted strategy utilized by *T. gondii* to stave off autophagic targeting, recruitment of LC3 and acidic organelle structures to the PV was markedly increased in cells expressing a mutated AKT-resistant form of FOXO3a. These observations are consistent with previous studies showing that overexpression of FOXO3a TM increases the number of LC3 puncta and promotes autophagic activity in microglia and HEK293T cells (Zhou, Liao et al. 2012, Lee, Nam et al. 2018). It is noteworthy that *T. gondii* manipulates autophagy through the positive and negative regulation of the autophagy effectors ATG5, ATG12, and ATG7 to hinder cargo-recruitment and elongation steps around the PV thereby promoting parasite replication (Nemati, Pazoki et al. 2021). Importantly, our data indicate that not all bona fide autophagy-related FOXO3a-regulated genes are modulated following *T. gondii* infection while other genes, such as SQSTM1, seemed to be AKT-dependent but FOXO3a-independent. Therefore, *T. gondii*-driven transcriptional reprogramming of host autophagy genes cannot

be solely attributed to FOXO3a dysregulation and further investigation is required to identify additional players.

In sum, we report a novel mechanism employed by *T. gondii* to inhibit autophagic targeting through repression of FOXO3a transcriptional activity (**Fig. 3.7**). In recent years, promoting host cell autophagy has become an increasingly attractive therapeutic strategy against *T. gondii* (Cheng, Zhang et al. 2022). Interestingly, in addition to autophagy, other cellular processes that are transcriptionally regulated by FOXO3a (e.g., apoptosis, cell cycle, oxidative stress, etc.) (van Grevenynghe, Cubas et al. 2012, Calissi, Lam et al. 2021) are also targeted by *T. gondii* (Zhu, Li et al. 2019). Moreover, FOXO3a has emerged as promising druggable target for various pathological conditions (e.g., cancer, diabetes, cardiovascular disease, chronic neurological diseases, etc.) (Calissi, Lam et al. 2021). Hence, it is tempting to speculate that restoring transcriptional programs regulated by FOXO3a, including but not limited to autophagy-related genes, could represent a new therapeutic approach to treat toxoplasmosis. Further characterization of altered transcriptional networks under the control of FOXO3a, and potentially other FOXO family members, during *T. gondii* infection will yield invaluable health-related knowledge to develop effective and safe host-directed strategies for better treatment or prevention of toxoplasmosis and potentially other infectious diseases.

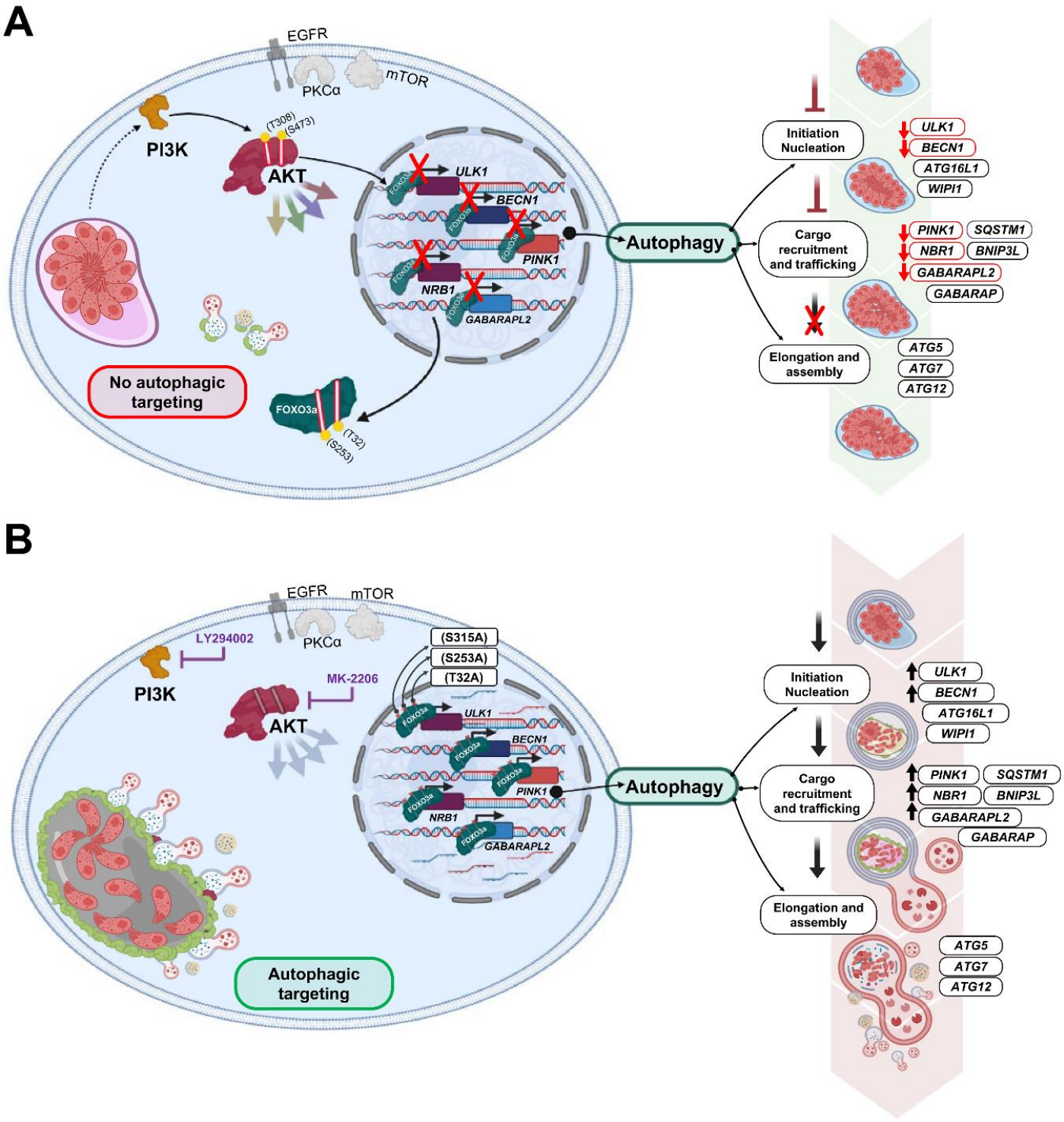


Figure 3.7. *T. gondi*i represses FOXO3a-driven transcriptional programs to hamper autophagic targeting of the PV (Proposed Model)

(A) Upon establishment and replication within the PV, *T. gondii* tachyzoites (shown in pink forming a rosette) activate the host cell PI3K-AKT signaling pathway independently of EGFR, PKCα, and mTOR. Phosphorylation of AKT (S473 and T308) leads to its activation, and in turn to the phosphorylation of FOXO3a at AKT-sensitive residues (S253 and T32). Phosphorylation of FOXO3a at these residues leads to its nuclear exclusion and its inactivation. As such, transcription of a subset of FOXO3a-dependent autophagy-related genes (i.e., ULK1, BECN1, NBR1, PINK1, and GABARAPL2) is downregulated (as indicated by the red "X" and the downward red arrows). Proteins encoded by this subset of transcripts are reported to participate in distinct steps of the autophagic response (right panel). Consequently, autophagic targeting of the PV is prevented, favoring parasite survival and replication. **(B)** Pharmacological inhibition of the PI3K-AKT pathway (i.e., treatment with LY294092 or MK-2206) precludes AKT-dependent phosphorylation and nuclear export of FOXO3a thus promoting transcription of autophagy-related genes (as indicated by upward black arrows) despite infection by *T. gondii*. Exogenous expression of an AKT-resistant form of FOXO3a harboring phosphosite mutations (S253A, T32A, and S315A) phenocopies chemical activation of FOXO3-driven autophagic targeting of *T. gondii*.

3.7 Materials and Methods

3.7.1 Reagents

Culture media and supplements were purchased from Wisent and Gibco; AG1478, Gö6976, and MK-2206 were obtained from Cayman Chemical; LY294002 was purchased from Camdea; rapamycin was obtained from LC Laboratories; and recombinant human EGF was a gift from Dr. Stéphane Lefrançois (INRS – Centre AFSB, Laval, QC, Canada).

3.7.2 Parasite Maintenance and Harvest

T. gondii tachyzoite cultures (RH and ME49 strains) were maintained by serial passages in Vero cells grown in DMEM culture medium supplemented with 5% heat-inactivated FBS, 2 mM L-glutamate, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin, and incubated at 37°C, 5% CO2, as previously described (Leroux, Lorent et al. 2018). For experimental infections, freshly egressed tachyzoites were harvested from Vero cultures, pelleted by centrifugation (1,300 × g, 7 min, 4°C), resuspended in ice-cold PBS (pH 7.2-7.4), and passed through a syringe fitted with a 27 G needle. Large cellular debris and intact host cells were pelleted by low-speed centrifugation (200 × g, 3 min, 4°C), and the supernatant containing parasites was filtered with a 3 µm-polycarbonate filter (Millipore). Tachyzoites were then washed twice in PBS and finally resuspended in the appropriate culture medium, according to the experiment.

3.7.3 Soluble *T. gondii* antigens (STAg) and heat-killed (HK) parasites

STAg were prepared from freshly egressed tachyzoites, as previously described (Leroux, Dasanayake et al. 2015). Briefly, parasites were resuspended in ice-cold PBS, subjected to three 5-min cycles of freezing/thawing using liquid nitrogen and a 37°C water bath, then sonicated on ice for 5 min (1 sec on/off pulses, 30% duty cycle) using a Sonic Dismembrator FB505 (ThermoFisher). Lysates were cleared by centrifugation (21,000 × g, 15 min, 4°C), and soluble material containing STAg was used for downstream experiments. Heat-killed (HK) parasites were prepared by incubating freshly egressed tachyzoites at 56°C for 10 min. After incubation, parasites were pelleted by centrifugation (1,300 × g, 7 min, RT) and resuspended in the appropriate culture medium, according to the experiment.

3.7.4 Infection of HFF and 3T3 fibroblasts

HFF and 3T3 cultures were plated one day before infection in DMEM culture medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamate, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C, 5% CO2. Cultures were serum-starved for 1 h and treated with inhibitors, when applicable. HFF cultures were then inoculated with live or HK parasites, treated with STAg, or left uninfected in fresh medium with 1% FBS unless otherwise indicated. Any remaining extracellular parasites were rinsed away with warm PBS (pH 7.2-7.4) 1 h following inoculation, fresh medium was added with inhibitors, when applicable, and cells were incubated until the end of the experiment. When needed, cultures were deprived of FBS (i.e., serum-starved) for the entire length of the experiment to induce autophagy.

3.7.5 Measurement of Infection Rates by Flow Cytometry

Parasite infection rates were determined by flow cytometry, as described (Leroux, Chaparro et al. 2020). In brief, HFF cultures were pre-treated with MK-2206 or an equivalent volume

of DMSO, then inoculated with *T. gondii* tachyzoites previously stained with 20 μM CellTracker Red (CMPTX) (Invitrogen). Cultures were harvested at the indicated times by trypsinization, stained for 30 min at RT with the viability dye Live-or-Dye 750/777 (Biotium), washed twice with FACS Buffer (PBS pH 7.2-7.4, 0.1% BSA), then fixed with 1% PFA in PBS (15 min, on ice). Samples were analyzed by flow cytometry using a BD Fortessa, and downstream analyses were performed with FlowJo.

3.7.6 Measurement of Parasite Replication

Parasite replication was evaluated by epifluorescence microscopy. Briefly, infected HFF cultures were fixed at the indicated times with PBS with 3.7% PFA (15 min, RT). Cells were permeabilized with PBS with 0.2% Triton X-100 (5 min, RT), stained with DAPI (5 min, RT), then mounted onto slides. The number of parasites in at least 50 vacuoles in different fields for each time point and treatment was counted by microscopy using a 40X oil-immersion objective.

3.7.7 Viability Assays

Viability of HFF cultures was determined by the resazurin assay as described (Chaparro, Leroux et al. 2020). Briefly, cells were treated with increasing concentrations of AG1478 (0.0625 – 8 μM), LY294002 (1.25 – 160 μM), MK-2206 (0.125 – 16 μM), Gö6976 (0.0625 – 8 μM), rapamycin (25 – 160 nM), or an equivalent volume of DMSO (vehicle) for 24 h at 37°C, 5% CO2. Medium was removed and replaced with fresh culture medium supplemented with 0.025% resazurin. Cultures were incubated for 4 h in presence of the inhibitors or DMSO at 37°C, 5% CO2. Optical density was measured using a Multiskan GO (Thermo-Fisher) at 600 and 570 nm. Absorbance at 600 nm was subtracted from readings at 570 nm. Experiments were performed in biological replicates (n =2); each sample was analyzed in a technical triplicate, the average of which was plotted against increasing concentrations of the respective inhibitor.

3.7.8 Western Blot Analysis

Following infection and other treatments, cultures were lysed directly with pre-warmed Laemmli loading buffer diluted in RIPA lysis buffer (25 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete EDTA-free protease inhibitor and PhosSTOP phosphatase inhibitor tablets (Sigma-Aldrich). Lysates were immediately heated at 95°C for 5 min, then stored at -80°C or processed immediately for SDS-PAGE. Resolved proteins were transferred onto PVDF membranes. Membranes were blocked for 1 h at RT in TBS 0.1% Tween-20 (TBS-T), 5% skim milk, then probed with the following primary antibodies: anti-phospho-EGFR (Y1068) (clone D7A5, #3777), anti-EGFR (clone D38B1, #4267), anti-phospho-AKT (S473) (clone D9E, #4060), anti-phospho-AKT (T308) (clone C31E5E, #2965), anti-AKT (clone C67E7, #4691), anti-phospho-FOXO3a (S253) (clone D18H8, #13129), anti-phospho-FOXO3a (T32) (polyclonal, #9464), anti-FOXO3a (clone 75D8, #2497), and β-actin (clone 8H10D10, #3700) were obtained from Cell Signaling Technologies; anti-*T. gondii* profilin-like protein (polyclonal, #AF3860) was purchased from R&D Systems. Membranes were then probed with either goat anti-rabbit, goat anti-mouse (Sigma-Aldrich), or rabbit anti-goat (R&D Systems) IgG horseradish peroxidase (HRP)-linked antibodies. Subsequently, proteins were visualized using the Clarity ECL Western blotting substrate (Bio-Rad) and exposing the membranes to autoradiography film.

3.7.9 Immunofluorescence and Confocal Microscopy

HFF were seeded onto glass coverslips in 24-well plates overnight. When applicable, cells were preloaded with the lysomotropic agent LysoTracker™ Red DND-99 (Invitrogen) diluted in DMEM (0.5 µM, final concentration) for 2 h at 37°C. At each timepoint, cells were rinsed with PBS three times and then fixed with 3.7% paraformaldehyde (PFA) in PBS for 15 min at RT. Cell were permeabilized with 0.2% Triton X-100 (in PBS) for 5 min at RT. Samples were kept in blocking solution (5% skim milk, 1% BSA, 5% normal goat serum in PBS) for 30 min at RT and incubated for 2 h at RT with the following primary antibodies diluted in PBS with 1% BSA: anti-FOXO3a (clone 75D8, #2497) and anti-Myc-Tag (clone 71D10, #2278) were obtained from Cell Signaling Technologies; anti-LC3 (clone 4E12, #M152-3) was purchased from MBL International. Samples were then incubated with the following fluorochrome-conjugated secondary antibodies for 1 h at RT: goat anti-rabbit IgG

(H+L) Alexa Fluor 647 (#A32733), donkey anti-rabbit IgG (H+L) Alexa Fluor 647 (#A-31573), and donkey anti-mouse IgG (H+L) Alexa Fluor 488 (#A-21202) were purchased from Invitrogen. Nuclei were stained with 300 nM 4',6-diamidino-2-phenylindole dilactate (DAPI) (Invitrogen) for 5 min at RT. Coverslips were mounted onto slides with Fluoromount G (Southern Biotech). Samples were visualized with the 40X objective of an LSM780 Zeiss confocal microscope, image acquisition was carried out using ZEN software, and image processing was performed with Icy Software from the Institut Pasteur (de Chaumont, Dallongeville et al. 2012).

3.7.10 Quantitative RT-PCR

RNA was extracted with Qiazol (Qiagen) according to the manufacturer's specifications. Purified RNA (500 ng) was reverse transcribed using LunaScript® RT SuperMix Kit (New England Biolabs). Quantitative PCR was performed with Luna® qPCR Master Mix (New England Biolabs). Relative quantification was calculated using the comparative Ct method (ΔΔCt) (Taylor, Wakem et al. 2010) and relative expression was normalized to human ACTB. Experiments were performed in independent biological replicates (n=3); each sample was analyzed in a technical triplicate, the average of which was plotted against the respective conditions used. Primers were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (**Table 3.1**).

Table 3.1 List of Primers

Target	Sense	Sequence (5'à 3')
ACTB	Forward	TGACCCAGATCATGTTTGAGACC
	Reverse	AGGGATAGCACAGCCTGGAT
FOXO3	Forward	CGTGCCCTACTTCAAGGATAA
	Reverse	ATTCTGGACCCGCATGAATC
ATG5	Forward	CACAAGCAACTCTGGATGGGATTG
	Reverse	GCAGCCACAGGACGAAACAG
ATG7	Forward	TCGAAAGCCATGATGTCGTCTT
	Reverse	CCAAAGCAGCATTGATGACCA
GABARAP	Forward	CTCCCTTATTCAGGACCGGC
	Reverse	TGCCAACTCCACCATTAC

ATG12	Forward	TGCTAAAGGCTGTGGGAGAC
	Reverse	ACTGTTCTGAGGCCACAAGTT
ATG16L1	Forward	GCATGACGTACCAAACAGGC
	Reverse	ACTCCCCACGTTTCTTGTGT
BECN1	Forward	CCACAGAAAGTGCCAACAGC
	Reverse	GACGTTGAGCTGAGTGTCCA
BNIP3L	Forward	GGACTCGGCTTGTTGTGTTG
	Reverse	TCGACTAGGTGGGACGAC
GABARAPL2	Forward	AGTCCCACAGTCCAGCCTAA
	Reverse	CGCAAAAGTGTTCTCTCCGC
NBR1	Forward	ATTCACCCCACAGGGATAGC
	Reverse	AACCTGTGGTTCCATGCTGT
SQSTM1	Forward	TGTGTAGCGTCTGCGAGGGAAA
	Reverse	AGTGTCCGTGGTTCACCTTCCCG
PINK1	Forward	CCTGGAGTGTGAAACGCTCT
	Reverse	CTCCCACCCTCACCATTCAC
ULK1	Forward	GGACACCATCAGGCTCTTCC
	Reverse	GAAGCCGAAGTCAGCGATCT
WIPI1	Forward	TGCACATCCCTAGCAACTGG
	Reverse	CTCCACGATGTAGACGTCGC

3.7.11 Lentivirus Production and HFF Transduction

Lentiviruses were produced in HEK293T cells using Lenti-Pac™ HIV Expression Packaging Kit, as per manufacturer's guidelines (GeneCopoeia, #LT001). Lentivirus titers were measured using Lenti-Pac™ HIV qRT-PCR Titration Kit (GeneCopoeia, #LT001) according to the manufacturer's protocol. HFF cells were transduced with the different lentivirus preparations for 3 days in culture medium supplemented with 5 μg/mL Polybrene (hexadimethrine bromide). Transduced cultures were either used immediately for downstream experiments or selected for 6 days using 2 μg/mL puromycin to generate stable cell lines. Plasmids, shRNA clones, and open reading frame (ORF) expression clones were purchased from GeneCopoeia (**Table 3.2**).

Table 3.2 List of shRNA and ORF Clones

shRNA clones		
Target	**Catalog # Genecopoeia**	**Description**
Scramble (Scr.)	CSHCTR001-LVRU6MP	shRNA scrambled control clone for psi-LVRU6MP
FOXO3	CS-HSH005759-LVRU6MP-02	shRNA clone set of 3 constructs against 3 variants for human *FOXO3* (ENST00000540898.1, NM_001455.4, and NM_201559.3) in lentiviral psi-LVRU6MP vector with U6 promoter, mCherry, puromycin
Vector and ORF clones		
Label	**Catalog # Genecopoeia**	**Description**
Empty Vector	EX-NEG-Lv107	Empty control vector for pReceiver-Lv107 vector
Myc-FOXO3a WT	EX-Z1129-Lv107	ORF expression clone for human *FOXO3* (NM_201559.2) in lentiviral pReceiver-Lv107 vector with CMV promoter, N-Myc tag, puromycin
Myc-FOXO3a TM	CS-Z1129-Lv107-01	Custom ORF expression clone for human FOXO3 (NM_201559.2) with T32A, S253A & S315A mutations in lentiviral pReceiver-Lv107 vector with CMV promoter, N-Myc tag, puromycin

3.7.12 Statistical Analysis

Where applicable, data are presented as the mean (standard deviation) (SD) of the mean. Statistical significance was determined by using one-way ANOVA followed by a Tukey post-hoc test or a two-tailed independent Student's T-Test followed by a Bonferroni post-hoc test; calculations were performed by using Prism 7 software package (GraphPad). Differences were considered significant when *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3.8 Acknowledgements

We thank Drs. Patrick Labonté and Stéphane Lefrançois (INRS-CAFSB, QC, Canada) for reagents and scientific insight. We are grateful to Jessie Tremblay for assistance with FACS, fluorescence microscopy experiments, and data analysis.

3.9 Data availability Statement

All relevant data are within the manuscript and its Supporting Information files.

3.10 Financial Disclosure Statement

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3.11 Competing Interests

The authors have declared that no competing interests exist.

CHAPTER 4

Discussion

4.1 Autophagy as a Double-Edged Sword in Pathogen Infection: Exploitation and Evasion Mechanisms

Several pathogens use their host autophagy, a cellular process that breaks down unwanted components for nutrients, to replicate and survive. One such pathogen, *T. gondii*, can use autophagy to acquire nutrients but it's not entirely clear how it out-competes the host for these resources (Deretic, Saitoh et al. 2013, Ghartey-Kwansah, Adu-Nti et al. 2020). Autophagy is a crucial pathway through which parasites effectively compete with the host for nutrients, but the host has also developed countermeasures to prevent pathogen survival (Ghartey-Kwansah, Adu-Nti et al. 2020). Research into the complex interplay between autophagy and pathogen evasion mechanisms may lead to new therapeutic strategies against infectious diseases, including toxoplasmosis (Ling, Shaw et al. 2006).

T. gondii has developed strategies to counteract autophagic targeting, such as activating host AKT signaling, which curbs the autophagic response (Muniz-Feliciano, Van Grol et al. 2013, Van Grol, Muniz-Feliciano et al. 2013, Portillo, Muniz-Feliciano et al. 2017, Lopez Corcino, Gonzalez Ferrer et al. 2019, Nemati, Pazoki et al. 2021, Subauste 2021, Cheng, Zhang et al. 2022). In this research work, we show that the inactivation of transcriptional programs controlled by FOXO3a is partly responsible for AKT-dependent curbing of the autophagic response by *T. gondii*.

Understanding the interaction between pathogens and host cells requires distinguishing between two forms of autophagy: starvation-induced autophagy or macroautophagy and other autophagic pathways such as xenophagy. In the context of xenophagy, pathogens first undergo ubiquitination before autophagy receptors recognize them. They are then labeled with the LC3 protein. In LC3-associated phagocytosis (LAP), LC3 is directly added to the vacuole membrane and operates independently of the ULK initiation complex (Ghartey-Kwansah, Adu-Nti et al. 2020).

Activation of the ULK complex, consisting of ATG13, FIP200, and ATG101, initiates the host autophagic response. Additionally, the recognition PAMPs by conserved pattern recognition receptors (PRRs), including TLRs, serves as the first line of defense against pathogens. Recent studies have shown that these crucial immune-related receptors play a role in regulating autophagy (Jiang, Tan et al. 2019). This knowledge is essential for

comprehending how parasites manipulate autophagy pathways for their benefit (Ghartey-Kwansah, Adu-Nti et al. 2020).

Nevertheless, some pathogens have mechanisms to block autophagy and/or use it for their own benefit, creating a complex interplay between autophagy and microbial adaptations that determine the outcome of host-pathogen encounters. In this context, the deployment of autophagic machinery by eukaryotic pathogens may also contribute to their pathogenesis (Deretic and Levine 2009).

Some bacteria have mechanisms to evade autophagy, while others use it to their advantage. For example, *Francisella tularensis* replicates within host cells by using autophagosomes, and whereas other bacteria induce autophagy to generate amphisomes, hybrid organelles that are formed through the fusion of autophagosomes with late endosomes or lysosomes and that provide nutrients for their growth (Steele, Brunton et al. 2013, Yu, Chen et al. 2018). Several pathogens depend on intracellular nutrients to survive and replicate and can utilize host autophagy to access the intracellular pool of nutrients by degrading macromolecules such as amino acids, lipids, and purines, including *T. gondii*, *Dengue virus*, *F. tularensis*, *Anaplasma phagocytophilum*, *Burkholderia pseudomallei*, *Coxiella burnetii*, and *L. amozonensis*. These pathogens induce autophagy and harvest the resulting nutrients while avoiding degradation. Interestingly, limited availability of nutrients, caused by inhibited autophagy in host cells may impair replication of certain intracellular pathogens (Wang, Weiss et al. 2009, Heaton and Randall 2010, Steele, Brunton et al. 2013, Riebisch, Muhlen et al. 2021).

T. gondii induces autophagy in a calcium-dependent, and mTOR-independent manner, inhibiting host cell's autophagy decreases *T. gondii* replication (Wang, Weiss et al. 2009). On the other hand, fusion of *T. gondii*-containing PVs with autophagosomes leads to parasite destruction (Wang, Weiss et al. 2009, Muniz-Feliciano, Van Grol et al. 2013).

In this study, we confirm that fusion of *T. gondii*-containing PVs with autophagosomes results in the destruction of the parasites. Inhibition of FOXO3a transcriptional activity and the activation of AKT were found to decrease the destruction of *T. gondii* within the PV because of diminished autophagy. Considering these findings, along with the results of the reported literature, it appears that *T. gondii* may rely on early autophagy activation for nutrient acquisition. However, during the later stages of infection, the parasite seems to inhibit autophagy as a strategy to evade its destruction.

Similarly, *Chikungunya virus*, *B. pseudomallei*, and *L. amazonensis* also exhibit enhanced replication when autophagy is induced and impaired intracellular replication when autophagy is inhibited. Other intracellular pathogens, including *S. typhimurium*, use autophagy to evade host defenses, but several other intracellular pathogens including *T. gondii* harvest autophagy-derived products for their replication (Gutierrez, Vazquez et al. 2005, Wang, Weiss et al. 2009, Heaton and Randall 2010, Krejbich-Trotot, Gay et al. 2011, Cyrino, Araujo et al. 2012, Muniz-Feliciano, Van Grol et al. 2013, Steele, Brunton et al. 2013, Newton, Kohler et al. 2014).

T. gondii triggers autophagy in host cells and consumes host cell mass to effectively compete with the host for nutrients (Wang, Weiss et al. 2009). Interestingly, the parasite requires host cell lipophagy to acquire essential fatty acids and promote its growth ,and host mitochondria fuse together during infection to restrict *T. gondii*'s uptake of fatty acids (FAs), thereby limiting the parasite's growth (Pernas, Bean et al. 2018). Lipophagy is crucial for *T. gondii*'s siphoning of FAs and growth, and in host cells lacking mitochondrial fusion or deficient in lipophagy or triglyceride depots reduced *T. gondii* growth is found. Mitochondrial fusion can act as a cellular defense mechanism against intracellular parasites by limiting their access to host nutrients obtained through lipophagy (Pernas, Bean et al. 2018).

Concerning lipophagy, AKT-dependent phosphorylation of FOXO1 or its deacetylation by SIRT1, results in its translocation from the nucleus to the cytoplasm, and its interaction with ATG7 to trigger lipophagy (Zhao, Wang et al. 2010, Zhao, Yang et al. 2010, Lettieri Barbato, Tatulli et al. 2013). This highlights the extranuclear role of FOXO proteins in inducing lipophagy and possibly generating FAs and carbon sources needed for *T. gondii*'s growth and replication.

In conclusion, lipophagy may provide the necessary energy for *T. gondii* to replicate within host cells, interestingly, this work coupled with the research conducted by (Lee, Kim et al. 2022), has shown that the parasite can induce the translocation of FOXO proteins such as FOXO3a and FOXO1, from the nucleus to the cytoplasm, inhibiting the clearance of the parasite through xenophagy, and possibly, promoting the availability of resources through lipophagy, preliminary data depicting the translocation and regulation of FOXO1 is illustrated in **Figure 4.1**. However, the complex interplay between autophagy and *T. gondii* adaptations that determine the outcome of host-pathogen encounters underscores the need

for more research in this area, particularly the role of FOXO1 in regulating lipophagy and its subsequent cytoplasmic functions.

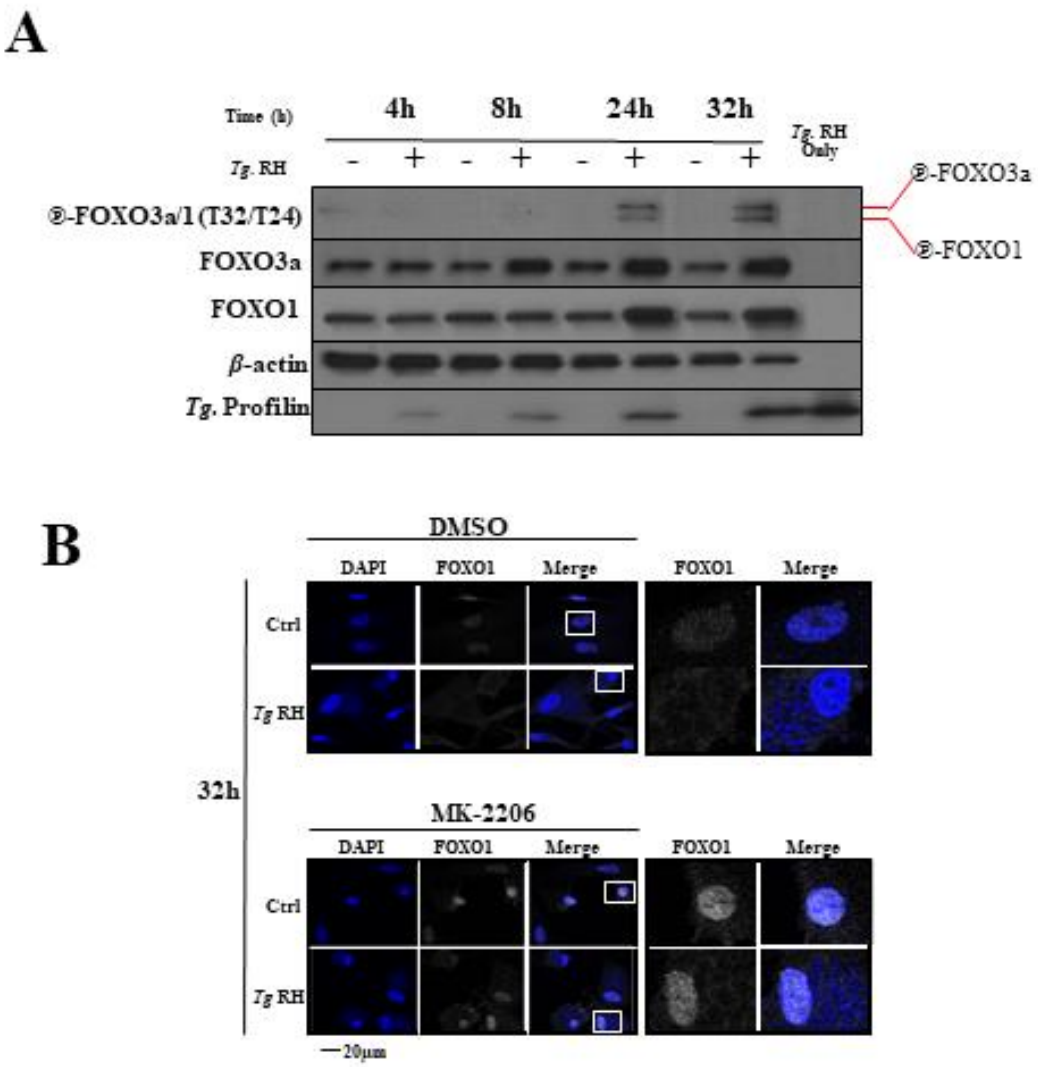


Figure 4.1 *T. gondii* induces phosphorylation of host FOXO1 and leads to AKT-dependent FOXO1 export from the host nucleus

(A) HFF cultures were inoculated with either RH *T. gondii* tachyzoites or left uninfected for the indicated times then processed for western blot analyses. Phosphorylation and expression levels of indicated proteins were monitored by western blotting. Total amounts of β-actin were used as a loading control, and an antibody raised against *T. gondii* profilin-like protein was used to assess infection of HFF cultures. Total protein extracts from

extracellular tachyzoites ("*Tg* only") were used to control for any cross-reactivity of the antibodies against *T. gondii* proteins. **(B)** HFF cultures were inoculated with RH *T. gondii* tachyzoites or left uninfected and fixed at the indicated times and processed for confocal immunofluorescence microscopy. HFF cultures were pretreated with 2 μM MK-2206 or an equivalent volume of vehicle (i.e., DMSO) for 1 h then inoculated with RH *T. gondii* parasites. Cells were cultured up to 32 h following infection in the presence or absence of MK-2206. Samples were stained with DAPI (shown in blue), used as a nuclear marker, and for total FOXO1 (shown in white). Original magnification (left panels), 4 times-enlarged insets (right panels). Primary antibodies: Anti-phospho-FOXO3/1a (T32/T24) (polyclonal, #9464) and anti-FOXO1 (clone C29H4, #2880) were obtained from Cell Signaling Technologies. These results should be interpreted as preliminary data and are representative of a single biological replicate.

4.2 The Intricate Role of FOXO Proteins in Host-pathogen Interaction: Complexities in Regulation and Altered Functions

FOXO TFs are a group of proteins that play crucial roles in several cellular processes such as metabolic adaptation, autophagy, oxidative stress response, and cell growth. In humans, FOXO genes have originated from successive gene duplications and have intrinsically disordered regions that interact with different proteins (Schmitt-Ney 2020). Their activity is regulated phosphorylation, acetylation, ubiquitination, methylation, and cysteine oxidation, which influence their cellular localization, DNA binding capacity, transactivation, as well as interaction with other proteins (Wang, Yu et al. 2016). FOXOs have broad expression and regulate the expression of their own paralogs. Also, the phenotype of knockout mice for a single FOXO member may be milder than expected due to compensation from remaining family members. The regulation and function of FOXOs are complex and involve multiple signaling pathways (Schmitt-Ney 2020).

In our research, we focused on investigating the negative regulation of FOXO3a transcriptional activity during *T. gondii* infection. We found that FOXO3a plays a crucial role in activating autophagy and in eliminating the parasite in late-stage infection in human fibroblasts. However, we acknowledge the possibility that other FOXO proteins, such as FOXO1, may have a greater impact on autophagy or parasite survival. There could be a compensatory mechanism at play, where other FOXO members compensate for the negative regulation exerted by *T. gondii*. Surprisingly, our preliminary data **Figure 4.1**, along with literature reports (Lee, Kim et al. 2022), suggest that both FOXO3a and FOXO1 are regulated in a similar manner during *T. gondii* infection. It is also likely that FOXO4 and FOXO6 follow the same regulation pattern. However, further studies involving knockout and

knockdown of these TFs, both individually and in combination, are necessary to determine if *T. gondii* can extend its regulation to the entire FOXO family or if there is a compensatory or redundant response between these proteins controlling autophagy. Additionally, each FOXO protein may play a distinct role in infected cells. For instance, the inhibition or activation of FOXO3a may impact mitochondrial metabolism, while the inhibition or activation of FOXO1 may influence lipid metabolism and the provision of energy resources for parasite growth.

However, and despite the majority of FOXO's sequence being comprised of intrinsically disordered regions, these regions contain conserved sites that mediate essential regulatory processes, including PTMs and protein-protein interactions (Wang, Marshall et al. 2015). While our research has primarily focused on phosphorylation as the primary PTM utilized by *T. gondii* to negatively regulate FOXO3a transactivation, we acknowledge the possibility of other PTMs, such as acetylation and methylation, occurring simultaneously with phosphorylation during infection. It is intriguing to consider whether the combination of various PTMs during infection might trigger distinct functions of FOXO proteins. For instance, in our study, we observed that FOXO3a undergoes phosphorylation, yet it does not appear to be degraded; instead, it accumulates in the cytoplasm. This leads us to speculate that after infection, FOXO3a is phosphorylated, sequestered in the cytoplasm, and not ubiquitinated for proteasomal degradation. Additionally, it is plausible that FOXO3a undergoes acetylation, potentially promoting its cytoplasmic functions, including migration to the mitochondria. Similarly, in the case of FOXO1, it is likely that acetylation is involved in lipophagy. Further research is needed to explore the specific interplay between different PTMs and the functional consequences for FOXO proteins during infection.

Additionally, FOXO TFs are implicated in responses caused by oxidative stress, DNA damage/repair, and tumor in angiogenesis. The activity of these processes is modulated through PTMs, such as phosphorylation, acetylation, ubiquitination, methylation, and glycosylation, which influence subcellular localization, transcriptional activity, and downstream target genes of FOXO proteins. Recent research suggests that different stimuli activate different FOXO PTMs, and that some PTMs may exert opposing effects to maintain FOXO protein activity at a specific level. Moreover, several enzymes that modify FOXO proteins are downstream target genes of FOXO TFs, forming a positive feedback loop.

Additionally, certain PTMs affect each other, influencing the activity of FOXO proteins directly and indirectly. This complex regulatory mechanism has implications for the relationship between disease and FOXO PTMs (Wang, Yu et al. 2016).

Furthermore, FOXO TFs play a vital role in regulating cellular quality control, cellular metabolism, and resistance to stress by promoting the expression of genes involved in autophagy and the ubiquitin-proteasome system, which are essential for maintaining proteostasis, an important process for aging and age-related metabolic and neurodegenerative diseases (Du and Zheng 2021). In mammalian neurons, FOXO TFs regulate autophagy and mitophagy, which may have implications for neurodegenerative diseases. FOXO3 promotes mitophagy by activating the PINK1 gene, while inducing apoptosis in dopaminergic neurons under conditions of oxidative stress. The paradoxical roles of FOXO TFs in dopaminergic neuron survival and death depend on the level of FOXO activation or the context in which FOXO is activated (Webb and Brunet 2014).

Dysregulation of FOXO activity caused by conditions such as obesity and insulin resistance can lead to various metabolic disorders, including type 2 diabetes. Understanding the complex mechanism of tissue-specific metabolic regulation by FOXO and the fine-tuning mechanism by the FOXO-binding protein may help prevent and treat metabolic disorders (Kodani and Nakae 2020). In our case, and as a promising avenue for future research, it is crucial to further explore the interaction of FOXO TFs with binding proteins, including 14-3-3, ATG7, and various members of the Sirtuin protein family. Investigating these protein interactions holds significant relevance and can provide valuable insights into the regulatory mechanisms and functional implications associated with FOXO TFs and the manipulation of the host cell during *T. gondii* infection.

Mitochondria are vital organelles for various cellular processes, and their dysfunction is implicated in several diseases. The maintenance of mitochondrial homeostasis is critical for metabolic health and tissue function. FOXOs can regulate mitochondrial processes such as biogenesis, fusion, and fission, as well as autophagy, but their effects may vary in different cells and tissues. Moreover, the complex interplay between mitochondrial processes may also influence the outcome of FOXO modulation (Cheng, Zhang et al. 2022, Cheng 2022).

For instance, the FOXO3 isoform2, an N-terminal truncated version of the full-length FOXO3 is involved in osteoclastogenesis and rheumatoid arthritis, but its biological function

has been unclear (Xu, Vitone et al. 2019). In this study it is suggested that FOXO3 isoform2 expression patterns and functions may be altered in response to different environmental settings and that future studies are needed to uncover its regulation and potential therapeutic implications (Xu, Vitone et al. 2019). It is crucial to investigate whether *T. gondii* infection can influence the regulation of FOXO3 isoforms and determine whether such alterations have any impact on the establishment of infection or the pathogenesis of toxoplasmosis.

Interestingly, depending on the subcellular context, activated FOXO3a induces specific sets of genes, including cell cycle inhibitors, pro-apoptotic genes, ROS scavengers, autophagy effectors, and gluconeogenic enzymes, and can also induce the transcription of OXPHOS genes in mitochondria (Fasano, Disciglio et al. 2019).

FOXO3a is an adaptable player in the dynamic homeostasis of normal and stressed cells, and its crosstalk between nuclear and mitochondrial functions is crucial for maintaining cellular homeostasis (Fasano, Disciglio et al. 2019). The possibility of *T. gondii* manipulating the subcellular localization of FOXO3a, translocating it from the nucleus to the cytoplasm, and subsequently directing it to the mitochondria to enhance its mitochondrial function presents an intriguing research question for future investigations. Examining the interplay between *T. gondii* and FOXO3a, particularly its impact on mitochondrial dynamics, holds promise for expanding our understanding of the intricate host-parasite interactions and may unveil novel avenues for therapeutic interventions.

The complex regulation of FOXO proteins, which is dependent on various factors such as PTMs, epigenetics, and interaction with accessory proteins, among others, suggests that their role in the host-pathogen interaction must be equally intricate. A study by (Snoeks, Weber et al. 2008) explored the effects of bacterial infection on FOXO3a in intestinal epithelial cells and its contribution to intestinal inflammation. The study found that bacterial lipopolysaccharide and infection with *Citrobacter rodentium* induce translocation of nuclear FOXO3a into the cytosol, where it degrades in human HT-29 and mouse CMT-93 cells. LPS was found to inhibit FOXO3a via PI3K pathway, leading to the upregulation of proinflammatory interleukin-8 by suppressing inhibitory IκBα. Similarly, *C. rodentium* was shown to induce translocation of nuclear FOXO3a into the cytosol of colonic epithelium of infected mice. While no experimental evidence was provided on the alteration of transcriptional programs downstream of FOXO3a, the observed phenotype was associated

with exacerbated pro-inflammatory cytokine production and disease pathogenesis during *C. rodentium* infection (Snoeks, Weber et al. 2008). In agreement with these investigations, our work showed that *T. gondii* also actively force FOXO3a out of the nucleus via PI3K/AKT to hamper transcriptional programs involved in host defense responses in human foreskin fibroblasts.

The regulation of FOXO proteins, specifically FOXO3a, during *T. gondii* infection is a complex process influenced by various factors. These factors include the diversity of PTMs that FOXO proteins undergo, the effects of different isoforms, specific stimuli, cell type variations, interactions with other proteins, and the balance between transcriptional and cytoplasmic activities. We have observed that the regulation of FOXO3a in mouse and human macrophages (BMDMs and THP1, respectively) differs slightly from that seen in HFFs. In fibroblasts, both FOXO3a and FOXO1 remain preserved outside the cell nucleus, with FOXO3a exerting a negative regulation on its transcriptional activity. However, our preliminary data indicate that in macrophages, FOXO3a tends to be downregulated with a progressive decrease in total protein throughout the infection. This suggests that the cytoplasmic functions of FOXO3a may not be required by the parasite in this context, **Figure 4.2**. These findings highlight the non-homogeneous and non-conserved nature of FOXO protein regulation during *T. gondii* infection, varying depending on the specific cell type being infected. It is evident that more studies are necessary to confirm the role of FOXO proteins in other cell types and gain a comprehensive understanding of their involvement in *T. gondii* infection. Moreover, an intriguing observation arises when examining the regulation of FOXO3a during *T. gondii* infection. There appears to be a lack of variation in the regulation between type I and type II strain parasites. Furthermore, this regulatory mechanism seems to be conserved across mouse and human cells, encompassing both fibroblasts and macrophages from both species.

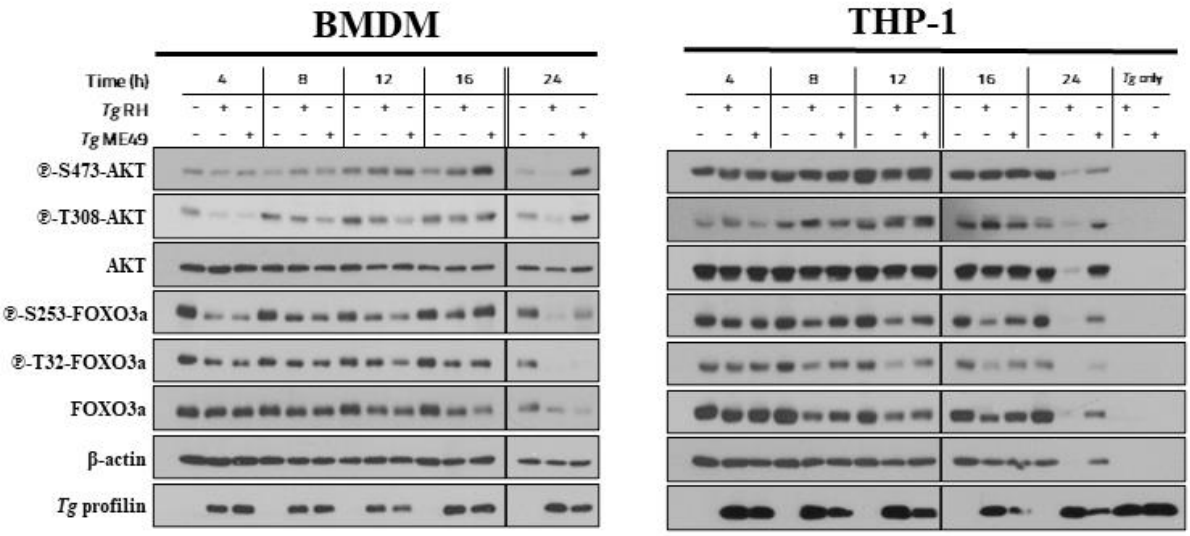


Figure 4.2 Impact of *T. gondii* infection on FOXO3a protein levels in BMDM and THP1 cells

BMDM cells or THP-1 cells were inoculated with either RH or ME49 *T. gondii* tachyzoites or left uninfected for the indicated times then processed for western blot analyses. Phosphorylation and expression levels of indicated proteins were monitored by western blotting. Total amounts of β-actin were used as a loading control, and an antibody raised against *T. gondii* profilin-like protein was used to assess infection of BMDM cells and THP-1 cells. Total protein extracts from extracellular tachyzoites ("Tg only") were used to control for any cross-reactivity of the antibodies against *T. gondii* proteins. These results should be interpreted as preliminary data and are representative of a single biological replicate, Leroux, LPL unpublished data.

As previously mentioned, FOXO proteins play a crucial role in maintaining host homeostasis. One notable example is the involvement of FOXO3a in the regulation of the hepatic antioxidant response system. Infection with hepatitis C virus (HCV) and alcohol consumption have been shown to disrupt this system, leading to chronic liver injury. A study by (Tikhanovich, Kuravi et al. 2014) examined the effects of HCV infection and alcohol on the regulation of FOXO3, and found that, in contrast to our findings in the *T. gondii* infection model, HCV-induced FOXO3 phosphorylation at a novel c-Jun N-terminal kinase (JNK) site promoted nuclear translocation and transcription. Conversely, alcohol suppressed arginine-methylation of FOXO3, leading to nuclear export and degradation of the JNK-phosphorylated form (Tikhanovich, Kuravi et al. 2014).

Although we show that *T. gondii* infection induce AKT-sensitive phosphorylation and nuclear exclusion of FOXO3a in human foreskin fibroblasts, the biological consequences of pathogen-driven modulation of FOXO3 are not fully understood and require further investigation.

To further our understanding of the impact of FOXO proteins deregulation, particularly FOXO3a, on the pathogenesis of toxoplasmosis, there is a need for future research studies. One crucial aspect involves establishing *in vivo* infection models to determine if *T. gondii* and its various infective forms can disrupt FOXO protein regulation in a similar manner to what has been observed in specific cell lines such as HFF and THP-1. Additionally, it is essential to explore more complex experimental models that encompass a broader range of cell and tissue diversity, such as organoids.

Examining the effects of FOXO protein manipulation during *T. gondii* infection through comprehensive omics approaches like transcriptomics or proteomics is also necessary. This analysis would determine if the alteration of FOXO proteins impacts various cellular functions beyond autophagy, ultimately leading to detrimental effects on the host and contributing to the pathogenesis of toxoplasmosis. It is worth noting that deregulation of a single TF can trigger alterations in a broader set of transcriptional targets. Therefore, experimental investigations are crucial to elucidate the specific clinical manifestations associated with toxoplasmosis, such as congenital toxoplasmosis, embryonic development, cerebral toxoplasmosis, and the disruption of host neuronal functions.

The subcellular localization and transcriptional activity of FOXO3a do not appear to be affected by various infectious agents, but studies using forward and reverse genetics approaches have shown that FOXO3a-dependent transcriptional programs play a critical role in the outcome of infections with *M. tuberculosis*, *Rhinovirus* (RV), and HIV. FOXO3a negatively regulates the expression of IL-10 in *M. tuberculosis*-infected macrophages, which can suppress the host immune response and contribute to Tuberculosis pathogenesis and persistence. Knocking down FOXO3 expression increased IL-10 production in BCG-infected macrophages, while activation of FOXO3 increased the expression of M1 markers and decreased the expression of IL-4 and IL-10 (van Grevenynghe, Procopio et al. 2008, Bouzeyen, Haoues et al. 2019, Gimenes-Junior, Owuar et al. 2019).

Airway epithelial cells play a critical role in antiviral responses to RV, and recent research has shown that FOXO3a is involved in regulating cell-type-specific antiviral responses in airway epithelial cells, and its ablation results in reduced interferon responses to RV and defective double-stranded RNA receptor signaling. In mice infected with RV, FOXO3a depletion leads to viral persistence, enhanced lung inflammation, and elevated pro-inflammatory cytokine levels. Treatment with MitoTEMPO, a mitochondrial-specific antioxidant, restores antiviral interferon responses and reduces pro-inflammatory cytokine responses to RV in FOXO3a knockout cells, suggesting that FOXO3a contributes to optimal viral clearance and limits excessive lung inflammation following RV infection (Gimenes-Junior, Owuar et al. 2019).

In a recent study by (Lee, Kim et al. 2022), it was reported that *T. gondii* infection in macrophages resulted in AKT-sensitive phosphorylation at T24 and PI3K-dependent nuclear exclusion of FOXO1. The authors also found that SIRT1 contributes to autophagy activation via the AMPK and PI3K/AKT signaling pathways, promoting anti-*T. gondii* responses. SIRT1- deficient BMDMs exhibited increased intracellular survival of *T. gondii* compared to wild-type cells, whereas activation of SIRT1 resulted in the induction of autophagy and increased anti-*T. gondii* effect. Additionally, the study describes that SIRT1 regulates the FOXO-autophagy axis in several human diseases, and the *T. gondii*-induced phosphorylation, acetylation, and cytosolic translocation of FOXO1 were enhanced in SIRT1- deficient BMDMs, nevertheless, future work is required to shed light on the regulation and biological consequences of FOXO1 nuclear exclusion during *T. gondii* infection (Lee, Kim et al. 2022).

The study revealed a previously unappreciated role for SIRT1 in *T. gondii* infection and its regulation of the FOXO-autophagy axis, highlighting its potential as a therapeutic target. Based on the results obtained in our research work and the studies regarding the regulation of FOXO proteins during *T. gondii* infection (Lee, Kim et al. 2022), we propose that the elimination or downregulation of FOXO3a during infection may enhance the parasite's replication within the host cell. To test this hypothesis, we employed the KD HFFs targeting FOXO3a and compared the replication rate of the parasite between these knockdown cells and control HFF transduced to express a non-targeting scrambled (Scr) shRNA. Surprisingly, contrary to our expectations, the preliminary data indicated that the absence of FOXO3a during infection does not contribute to an increase in the replication rate. In fact, it appears to have a negative impact on replication compared to the Scr HFF-infected cells, **Figure 4.3**. These findings lead us to confirm that *T. gondii* seems to derive benefits from maintaining FOXO3a outside the nucleus, while avoiding degradation in the cytoplasm, possibly contributing to its cytoplasmic functions.

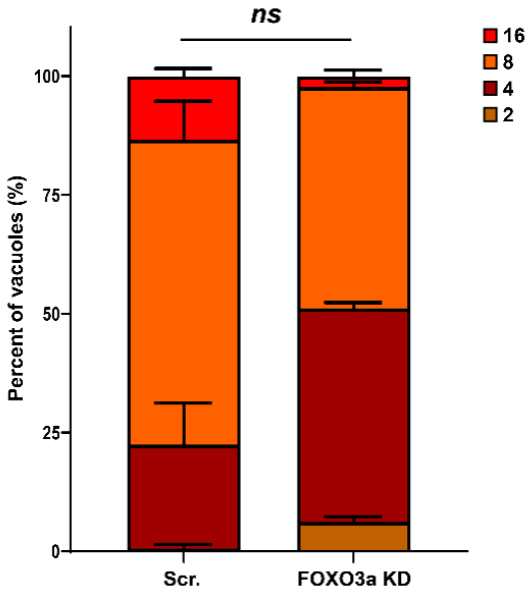


Figure 4.3 FOXO3a knockdown (KD) leads to a reduced replication rate of *T. gondii*

Scr or FOXO3 shRNA-transduced HFF cultures were inoculated with RH *T. gondii* parasites or left uninfected. Cultures were deprived of serum throughout the course of the infection (24 h). Cultures were fixed and processed for microscopy analyses. The number of parasites per PV in at least 50 infected cells in different fields of view were enumerated. Data collected from two independent experiment were compiled. *ns*, not significant.

In summary, FOXO TFs have a multifaceted role in infectious diseases and host-pathogen interactions. In the case of *T. gondii*, research suggests that the parasite can manipulate the host immune response by modulating the activity of FOXO TFs. Our study confirmed that *T. gondii* promotes the phosphorylation of FOXO3a, which inhibits its transcriptional activity and promotes the parasite's survival in the host cell. Conversely, the activation of FOXO3a might lead to the clearance of *T. gondii* infection. Furthermore, studies have demonstrated that *T. gondii* infection can induce the regulation of FOXO1, which may have implications for metabolic adaptation and inflammation. These findings highlight the importance of understanding the complex regulatory mechanisms of FOXO TFs in infectious diseases and host-pathogen interactions.

4.3 Beyond EGFR: Revealing a Novel Pathway for AKT Activation During *T. gondii* Infection

T. gondii is an obligate intracellular parasite that subverts host cell signaling pathways to promote its own survival and replication. Our data indicate that AKT dependent phosphorylation of FOXO3a in *T. gondii*-infected HFFs requires intact PI3K activity. These observations are in line with previous reports showing that either chemical blockade or genetic ablation of PI3K, using LY294002 or p110α siRNA, respectively, prevent AKT phosphorylation by *T. gondii* in both hematopoietic and non-hematopoietic human cells (i.e., monocytic THP-1 cells, primary brain microvascular endothelial cells (HBMEC), and human retinal pigment epithelial cells ARPE-19 (Muniz-Feliciano, Van Grol et al. 2013, Van Grol, Muniz-Feliciano et al. 2013, Zhou, Quan et al. 2013, Quan, Chu et al. 2015). *T. gondii* resides in a vacuole that excludes lysosomal degradation and autophagy-mediated killing. However, autophagy can re-route the PV to the lysosomes and cause parasite killing. To prevent autophagy, *T. gondii* activates EGFR in endothelial cells, retinal pigment epithelial cells, and microglia. The blockade of EGFR or AKT causes targeting of the parasite by LC3, vacuole-lysosomal fusion, lysosomal degradation, and killing of the parasite that are dependent on the autophagy proteins ATG7 and BECLIN1.

T. gondii microneme proteins containing EGF domains promote EGFR activation, and recombinant EGF-MICs cause EGFR-AKT activation. On the other hand, *T. gondii* down-

regulates host Nox4 expression via activation of the PI3K/AKT signaling pathway and thereby prevents innate immune defense (Zhou, Quan et al. 2013). This highlights *T. gondii*'s ability to manipulate host signaling pathways, which is crucial for its survival and proliferation within the host. Understanding these mechanisms of subverting host immunity is essential to develop new strategies to control infection. The PI3K/AKT signaling pathway is not only important for *T. gondii* to evade clearance by autophagy, but also for the control of the immune response against the parasite. By modulating the host immune response, *T. gondii* can establish a chronic infection and evade the host's defense mechanisms (Muniz-Feliciano, Van Grol et al. 2013, Van Grol, Muniz-Feliciano et al. 2013, Zhou, Quan et al. 2013, Quan, Chu et al. 2015).

Recent studies have been dedicated to investigating the early stages of the invasion process upon initial contact with *T. gondii* showing that the activation of AKT at the early stages of *T. gondii* infection in HBMEC is triggered through EGFR autophosphorylation by *T. gondii* proteins MIC3 and MIC6, which are adhesins harboring EGF-like domains (Muniz-Feliciano, Van Grol et al. 2013). *T. gondii*-induced activation of EGFR prevents autophagy protein-mediated killing of the parasite, as *T. gondii* resides in an intracellular compartment that excludes transmembrane molecules required for endosome-lysosome recruitment. However, *T. gondii* may deploy a strategy to prevent autophagic targeting to maintain the non-fusogenic nature of the vacuole (Muniz-Feliciano, Van Grol et al. 2013). In cells treated with autophagy stimulators (CD154, rapamycin), EGFR signaling inhibited LC3 accumulation around the parasite, whereas blockade of EGFR or its downstream molecule, AKT, caused killing of the parasite. Furthermore, disassembly of G-protein-coupled receptors or inhibition of metalloproteinases do not prevent EGFR-AKT activation. Additionally, parasites defective in EGF-MICs caused impaired EGFR-AKT activation (Muniz-Feliciano, Van Grol et al. 2013). However, in our research work the activation of EGFR-AKT signaling and AKT-mediated phosphorylation of FOXO3a was not detected in *T. gondii*-infected HFFs. These findings suggest that *T. gondii* employs different strategies to promote parasite survival in different host cell types, with EGFR activation being a crucial mechanism for maintaining the non-fusogenic nature of the PV in certain host cells.

It is noteworthy that the activation of the EGFR signaling pathway during *T. gondii* infection appears to be primarily and closely associated with the early stages of infection. Interestingly,

our model demonstrates that this pathway becomes dispensable for the parasite in later stages of infection once it has successfully established a secure replicative niche within the host cell. It is intriguing to observe that despite the need to evade autophagy activation during the lysis process near the time of parasite exit, the parasite employs alternative routes and potentially utilizes virulence factors other than MIC proteins with EGF-like domains. This suggests a complex mechanism employed by the parasite to persist and evade host defenses throughout the infection cycle.

T. gondii avoids being eliminated by autophagy by inducing prolonged host EGFR signaling. In a study conducted by (Lopez Corcino, Gonzalez Ferrer et al. 2019), it was found that prolonged PKCα/PKCβ-Src-dependent phosphorylation of EGFR leads to sustained activation of AKT in *T. gondii*-infected human ARPE-19 cells. While the parasite triggers EGFR signaling during invasion of host cells to avoid initial autophagic targeting, it may also use a strategy beyond invasion to maintain blockade of autophagic targeting. Inhibition of EGFR using gefitinib (EGFR tyrosine kinase inhibitor) in previously infected cells resulted in parasite entrapment by LC3 and LAMP-1 and pathogen killing dependent on the autophagy proteins ULK1 and BECLIN1 as well as lysosomal enzymes. Moreover, the administration of gefitinib to mice with ocular and cerebral toxoplasmosis led to disease control that was dependent on BECLIN1. In contrast, our study showed that treating HFF with PKC and EGFR inhibitors (AG1478 and Gö6976, respectively) did not prevent prolonged phosphorylation of AKT and FOXO3a upon *T. gondii* infection. These findings indicate that *T. gondii* infection drives the activation of PI3K-AKT signaling through diverse mechanisms which appear to be time- and host cell type-dependent. Comparative *in cellulo* analyses and *in vivo* studies will help to elucidate the molecular underpinnings of the PI3K-AKT-FOXO3a axis during *T. gondii* infection in different cell types and tissues (Chen, Hu et al. 2019, Lopez Corcino, Gonzalez Ferrer et al. 2019).

During the penetration of mammalian cells, the formation of the moving junction, which is accompanied by the expression of rhoptry neck protein RON4, activates FAK, leading to Src signaling. Src transactivates EGFR triggering early STAT3 signaling that prevents activation of PKR, thereby inhibiting autophagic targeting of the parasite (Portillo, Muniz-Feliciano et al. 2017). FAK/Src/(Y845)EGFR/STAT3 signaling axis within mammalian cells has been found to prevent activation of PKR and eIF2α, which are key stimulators of autophagy, and

enable the parasite to survive by avoiding autophagic targeting (Lebrun, Michelin et al. 2005, Portillo, Muniz-Feliciano et al. 2017, Subauste 2019). Although our study did not directly establish the link between MIC3, MIC9 and RON4 virulence factors and FOXO3a phosphorylation or localization, our observations suggest that these factors might not play a role in the transcriptional regulation of FOXO3a during *T. gondii* infection. The effect of these factors was not late in the infection process, and we observed that EGFR activation was not necessary for the phosphorylation of AKT and FOXO3a. Based on these findings, we speculate that other factors that are expressed later in the infection process and are independent of the *T. gondii* strain might be responsible for the regulation of FOXO3a phosphorylation and localization. Therefore, further studies are required to investigate the role of such factors in the transcriptional regulation of FOXO3a during *T. gondii* infection (Tables 1 and 2 present the virulence factors, highlighted in red, which may potentially be involved in the activation of FOXO3a through AKT phosphorylation during *T. gondii* infection).

In the present study, pharmacological approaches were used to investigate the involvement of mTOR in infection-induced phosphorylation of AKT and FOXO3a in HFFs. The results showed that mTOR activity was not required for the phosphorylation of AKT and FOXO3a, which is consistent with the findings in LPS-stimulated macrophages where mTOR was found to be dispensable for AKT-dependent phosphorylation of FOXO3a (Lee, Nam et al. 2018). TLR4 activation has been shown to suppress autophagy through inhibition of FOXO3 and impair phagocytic capacity of microglia (Lee, Nam et al. 2018). Microglia are phagocytic immune cells that reside in the central nervous system and have been shown to share many characteristics with macrophages. In the study by (Lee, Nam et al. 2018), it was demonstrated that LPS-induced TLR4 activation suppressed autophagy flux and expression of ATG genes in microglia, in contrast to the stimulation of autophagy in macrophages. Interestingly, inhibition of PI3K, but not mTOR, restored autophagy flux with concomitant dephosphorylation and nuclear translocation of FOXO3. This finding implies that FOXO3 might serve as a promising downstream candidate in the TLR4/PI3K pathway to regulate autophagy inhibition. It is particularly intriguing because previous research has firmly established *T. gondii*'s ability to activate TLR4 signaling during its invasion process (Ricci-Azevedo, Mendonca-Natividade et al. 2021).

Although *T. gondii* is known to activate TLR4 during early stages of infection (Ricci-Azevedo, Mendonca-Natividade et al. 2021), it cannot be concluded that this receptor is involved in the regulation of FOXO3a and autophagy through PI3K/AKT in HFFs, since this process is late and occurs mainly after the establishment of invasion and replication within the host cell. However, *T. gondii* has been shown to modulate host cell responses to favor its success in the early stage of infections by secreting MIC1 and MIC4 (Ricci-Azevedo, Mendonca-Natividade et al. 2021), which trigger pro-inflammatory host cell responses by interacting with N-linked glycans on TLR2 and TLR4, activating NF-κB and producing cytokines such as IL-12, TNF-α, and IL-6. Interestingly, MIC1 and MIC4 also induce the production of the anti-inflammatory cytokine IL-10 through mechanisms that are not yet fully understood; surprisingly, FOXO3a is a negative regulator of IL10 expression (Bouzeyen, Haoues et al. 2019). The ability of these MICs to induce macrophages to produce IL-10 depends on TLR4 internalization from the cell surface and macrophages subjected to blockade of endocytosis failed to produce IL-10 in response to MIC1 or MIC4 exposure, and MIC1- or MIC4-stimulated macrophages gained transient tolerance to LPS (Ricci-Azevedo, Mendonca-Natividade et al. 2021). This intriguing observation raises the possibility that the secretion of MIC1 and MIC4, leading to TLR4 activation and subsequent phosphorylation of FOXO3a, plays a role in expelling FOXO3a from the nucleus and facilitating the production of IL10. This mechanism could provide a partial explanation for *T. gondii*'s ability to manipulate the TLR4/FOXO3a/IL10 pathway, ultimately triggering an anti-inflammatory response. Nevertheless, further investigation is required to fully elucidate this research question in future studies.

In addition, our study showed that AKT activation require infection by live parasites in HFFs, indicating that certain events are required for the virulence factors to mediate their effects within the host cell (e.g., formation and presence of the PV membrane, specific route of entry of these molecules, etc.). The results also showed that both type I and II *T. gondii* strains tested were able to modulate FOXO3a phosphorylation and nuclear translocation in an AKT-dependent fashion, suggesting that this is a core process favoring parasite persistence that does not depend on strain-specific virulence factors.

Although our findings demonstrate that the phosphorylation of FOXO3a (T32/S253) and the total accumulation of FOXO3a protein are dependent on the MOI, the exclusion of FOXO3a

from the nucleus appears to be independent of *T. gondii* types I or II. However, there is a possibility that this phenomenon is partially attenuated (resulting in decreased phosphorylation and accumulation of FOXO3a) in *T. gondii* ME49. This attenuation could be attributed to the production of a virulence factor that is expressed to a lesser extent or is less active in type II strains, rather than solely due to *T. gondii* ME49 having a lower replication rate compared to *T. gondii* RH (Radke, Striepen et al. 2001, Khan and Grigg 2017). For instance, GRA15 exhibits higher activity in type I strains but is less active in type II strains (Rosowski, Lu et al. 2011, Virreira Winter, Niedelman et al. 2011, Yang, Farrell et al. 2013, Sangare, Yang et al. 2019, Ihara, Fereig et al. 2020). In contrast, other virulence factors, such as GRA16, have limited information available regarding their activity variation between strains. Future research is needed to elucidate the specific virulence factor responsible for regulating FOXO3a during *T. gondii* infection.

Taken together, our results indicate that phosphorylation and subsequent inactivation of FOXO3a by *T. gondii* requires live infection and occurs in a PI3K-AKT-dependent fashion independently of EGFR, PKCα, and mTOR activity in HFF. Further investigation will enable the identification of potential host and/or parasite factors involved in sustained AKT activation and subsequent nuclear exclusion of FOXO3a. Interestingly, the virulence factor GRA16 is a dense granule protein exported by *T. gondii* that targets the host cell nucleus and alters gene expression (Bougdour, Durandau et al. 2013). The protein binds to two host enzymes, HAUSP and PP2A phosphatase, to regulate the p53 tumor suppressor pathway and the cell cycle. GRA16 alters p53 levels in a HAUSP-dependent manner and induces nuclear translocation of the PP2A holoenzyme. Inhibition of HAUSP activity by small molecules or knockout of the enzyme induces p53 stabilization. The upregulation of p53 stability is believed to contribute to the formation of nuclear complexes that trigger the nuclear phosphoactivation of AKT and FOXO3a in response to stress stimuli (Chen, Choi et al. 2022). While the kinetics and yield of GRA16 secretion suggest continuous secretion throughout the intracellular life of the parasite, the mechanism of delivery from the vacuolar space to the host cell cytoplasm remains unclear. GRA16 represents an emerging subfamily of exported dense granule proteins that modulate host function and are crucial for pathogenesis. Given the limited understanding of the link between GRA16 and

PI3K/AKT/FOXO3a, further studies are necessary to validate this hypothesis and elucidate the underlying mechanisms.

4.4 *T. gondii* Inhibits Autophagy-Related Gene Expression through AKT-Dependent Inactivation of FOXO Transcription Factors: A Proposed Model and Future Perspectives

T. gondii can survive by residing within a PV in infected cells, which shields it from lysosomal degradation. However, it remains vulnerable to autophagy-mediated killing. Our results support two independent studies showing that inhibition of AKT signaling dramatically reduces *T. gondii* replication but does not hinder infection rates (Muniz-Feliciano, Van Grol et al. 2013, Choi, Gao et al. 2020). This suggests a crucial role for AKT activity in evading host cell defense mechanisms triggered after parasite internalization.

EGFR activation has been identified as a crucial strategy employed by *T. gondii* to ensure its survival within a non-fusogenic PV, particularly in the early stages of infection, however, our findings revealed a noteworthy discrepancy. In our study, HFF and 3T3 cells infected with *T. gondii* RH and ME49 strains did not exhibit phosphorylation of both EGFR and AKT during the early stages of infection (15 minutes to 4 hours). This outcome contradicts previous reports in the literature that emphasize the significance of EGFR-mediated AKT activation in various cell lines. It becomes evident from these results that the regulation of the PI3K/AKT/FOXO3a pathway is contingent upon the specific cell type and the timing of infection. This mechanism is regulated independently from the late stages, which was the focus of our study. In other cell lines and in the early stages, *T. gondii* MIC proteins containing EGF domains promote EGFR activation, and parasites defective in EGF-MICs cause impaired EGFR-AKT activation (Muniz-Feliciano, Van Grol et al. 2013). In DCs, *T. gondii* has been shown to induce AKT phosphorylation, and inhibition of PI3K, reduces *T. gondii* proliferation. Additionally, *T. gondii* has been found to activate the PI3K/AKT pathway and control NOX4 expression to reduce ROS levels, suggesting that manipulating the host PI3K/AKT signaling pathway and NOX4 expression is crucial for the parasite's survival and proliferation (Zhou, Quan et al. 2013). Despite this, our model suggests that fibroblasts infected with *T. gondii* do not appear to utilize the classical pattern of EGFR/AKT

activation and consequently, the regulation of FOXO3a does not occur early in the infection process.

Interestingly, the involvement of the PI3K/AKT pathway in Nox4 transcription in other cell lines implies that FOXO TFs may also play a role in regulating Nox4 transcription. FOXO TFs have been linked to redox signaling and the regulation of proliferation and apoptosis. These TFs can be inhibited and translocated out of the nucleus upon phosphorylation by AKT in the PI3K signaling pathway. Our data showed that *T. gondii* infection induces the phosphorylation of FOXO3a and its relocation out of the nucleus into the cytosol, which correlates with decreased autophagic flux during infection. This finding is consistent with the results of (Zhou, Quan et al. 2013), who also described a correlation between *T. gondii*-induced ROS reduction, PI3K/AKT pathway activation, and FOXO phosphorylation and translocation. In that sense, FOXO3a represents a downstream effector of AKT targeted by *T. gondii* to prevent the accumulation of the autophagy protein LC3 and recruitment of acidic organelles around the PV. Moreover, FOXO3a directly regulates an autophagy network to functionally regulate proteostasis in adult neural stem cells, contributing to the cell's response to oxidative stress (Audesse, Dhakal et al. 2019).

Taken together, these studies suggest that activation of PI3K/AKT/FOXO signaling is an essential mechanism utilized by *T. gondii* to hamper oxidative stress responses and autophagy-mediated parasite clearance (Muniz-Feliciano, Van Grol et al. 2013, Choi, Gao et al. 2020). Hence, *T. gondii* can manipulate the PI3K/AKT/FOXO signaling pathway to regulate autophagy and oxidative stress responses, and it may also be manipulating other biological processes in the host cells through the regulation of FOXOs, including apoptosis, metabolism, cell cycle, and immune response, to the benefit of the parasite. Further research should be directed towards understanding how *T. gondii*'s manipulation of these TFs affects the host cell.

Our screening identified FOXO3a-regulated autophagy transcripts that, when silenced by the parasite, may impede several steps of the host autophagic response, including initiation, nucleation, cargo recruitment (such as BECN1 and ULK1), and trafficking (e.g., NBR1 and GABARAPL2), **Figure 3.7** (Audesse, Dhakal et al. 2019, Cheng 2019). Interestingly, FOXO3a promotes autophagy in neural stem cells by regulating a direct network of autophagy genes critical for maintaining cellular quality control, preventing aggregate

accumulation, and reducing neurodegeneration in mammals (Audesse, Dhakal et al. 2019). Alteration of FOXO3a during infection may not only enable parasite survival but also partially account for the pathogenesis observed during clinical manifestations, including cerebral toxoplasmosis. In line with this, dysregulation of FOXO3a autophagy targets can result in neurodegeneration during aging (Audesse, Dhakal et al. 2019). Moreover, FOXO3 also regulates mitophagy, a selective form of autophagy in which mitochondria are recruited into the autophagy pathway, by targeting genes involved exclusively in mitophagy such as PINK1, BNIP3, and BNIP3L (Audesse, Dhakal et al. 2019). Our research findings indicate that FOXO3a transcriptional targets, including PINK1 and WIPI1, were downregulated during *T. gondii* infection in an AKT-dependent manner, providing novel insights into the mechanisms underlying the possible pathogenesis of this infection. PINK1 is a mitochondrial targeted serine/threonine kinase that directly phosphorylates the E3-ubiquitin ligase Parkin on the surface of mitochondria to target them to the autophagosome, which is critical for Parkinson's disease (Audesse, Dhakal et al. 2019). The interplay between FOXO3a and other transcriptional networks regulating autophagy is crucial for optimal cellular quality control under changing environmental conditions (Audesse, Dhakal et al. 2019). Further studies are necessary to investigate the connection between the modulation of mitophagy in *T. gondii* infection and the progression of cerebral toxoplasmosis.

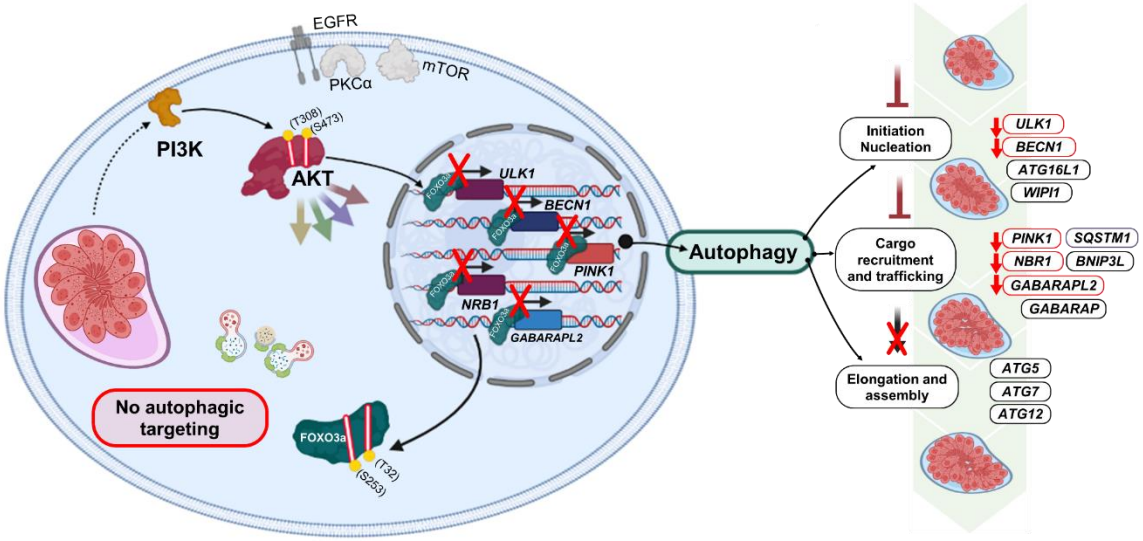
In line with this notion, knockdown of BECN1 in *T. gondii*-infected cells was shown to abrogate CD40-induced autophagic targeting of the PV and prevent parasite killing upon chemical blockade of AKT signaling (Van Grol, Muniz-Feliciano et al. 2013). Similarly, knockdown of ULK1 restored parasite replication despite pharmacological downregulation of sustained AKT phosphorylation during *T. gondii* infection (Muniz-Feliciano, Van Grol et al. 2013). In our work, the downregulation of FOXO3a transcriptional programs has been proposed to contribute to the multifaceted strategy utilized by *T. gondii* to stave off autophagic targeting. Recruitment of LC3 and acidic organelle structures to the PV was markedly increased in HFF cells expressing a mutated AKT-resistant form of FOXO3a. This is consistent with previous studies showing that overexpression of FOXO3a increases autophagic activity in microglia and HEK293T cells (Lopez Corcino, Gonzalez Ferrer et al. 2019). Furthermore, other FOXO protein family members, including FOXO1, have been shown to be involved in the modulation of autophagy (Lee, Nam et al. 2018), specifically,

FOXO3 induces a transcription-dependent autophagy, and FOXO1 is required for this process (Lee, Nam et al. 2018). The data also showed that FOXO3 promoted the translocation of FOXO1 from the nucleus to the cytoplasm, resulting in an increase in FOXO1-induced autophagy. Interestingly, depletion of FOXO1 attenuated FOXO3-induced autophagy (Lee, Nam et al. 2018). Together, these findings suggest that the regulation of FOXO1 and FOXO3a transcriptional programs plays a critical role in the control of autophagy during *T. gondii* infection (Zhou, Liao et al. 2012, Muniz-Feliciano, Van Grol et al. 2013, Van Grol, Muniz-Feliciano et al. 2013, Lee, Nam et al. 2018, Lopez Corcino, Gonzalez Ferrer et al. 2019).

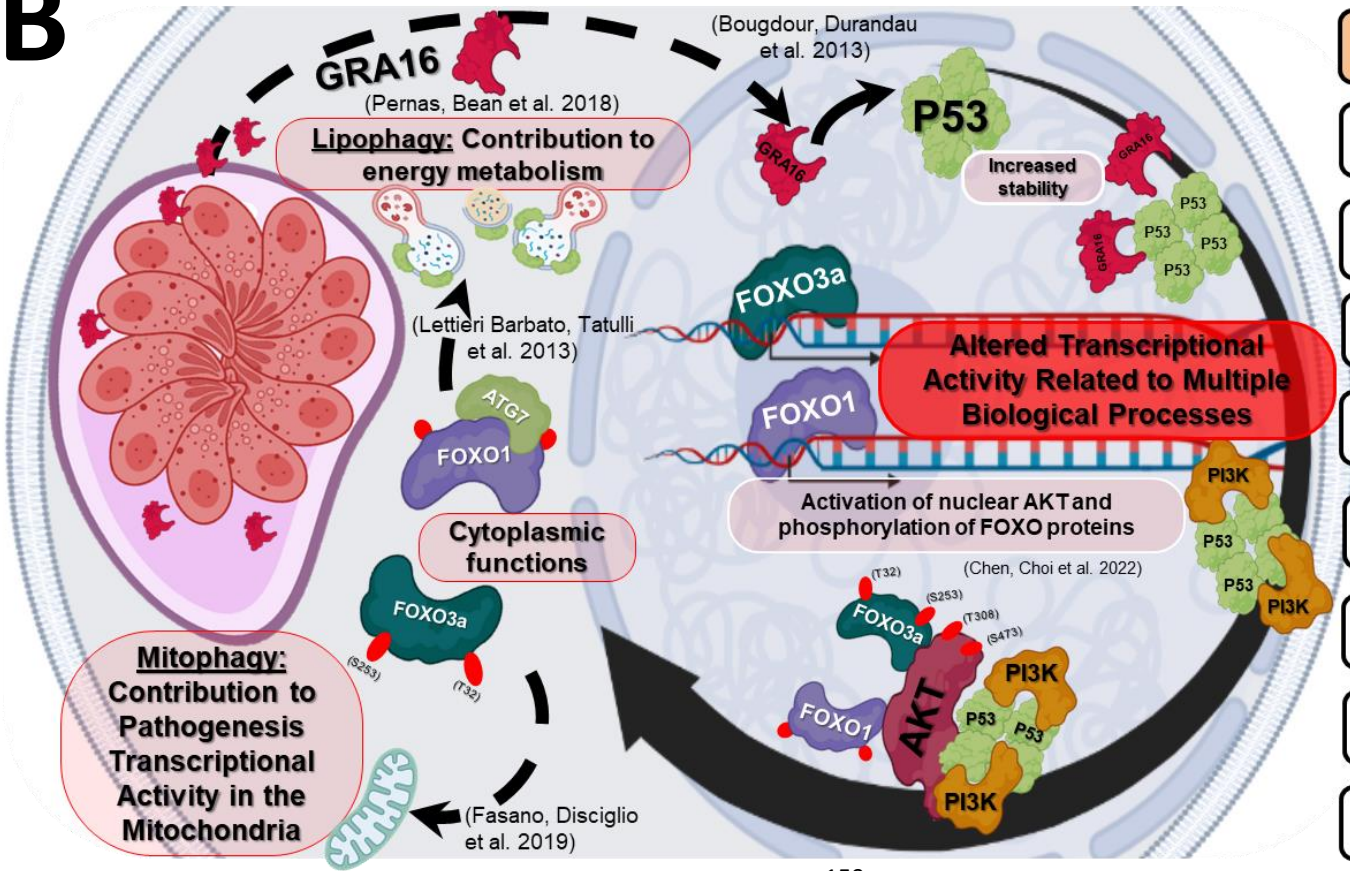
It is noteworthy that *T. gondii* is known to manipulate the autophagy process through the positive and negative regulation of autophagy effectors such as ATG5, ATG12, and ATG7, which hinders cargo-recruitment and elongation steps around the PV and promotes parasite replication (Nemati, Pazoki et al. 2021). Additionally, the expression of autophagy genes such as ATG5, ATG7, ATG12, and LC3b was evaluated in PMA-activated THP-1 cells incubated with *T. gondii* profilin and tachyzoites for 6 hours. The results showed that *T. gondii* profilin and tachyzoites downregulated the expression of ATG5 and ATG12 while upregulating ATG7, indicating that *T. gondii* and profilin may manipulate autophagy by preventing the formation of the ATG5-12-16L complex, thus facilitating the replication of *T. gondii* and the development of toxoplasmosis (Nemati, Pazoki et al. 2021), interestingly, our experimental model did not show negative regulation of ATG5 and ATG12 during *T. gondii* infection. However, we found a positive induction of ATG7, which appears to be independent of regulation through the PI3K/AKT/FOXO3a pathway. Notably, ATG7 has been identified as a cytoplasmic protein that, along with the cytoplasmic form of FOXO1, induces autophagy (Zhao, Yang et al. 2010). Moreover, ATG7 has been described as an essential protein for obtaining fatty acids by *T. gondii* during the induction of lipophagy (Pernas, Bean et al. 2018). However, it is important to note that not all autophagy-related FOXO3a-regulated genes are modulated following *T. gondii* infection, and additional investigation is needed to identify other players involved in *T. gondii*-driven transcriptional reprogramming of host autophagy genes. For example, it was also found that SQSTM1 seemed to be AKT-dependent but FOXO3a-independent.

In sum, we report a novel mechanism employed by *T. gondii* to inhibit autophagic targeting through repression of FOXO3a transcriptional activity (**Figure 4.4A)**. Upon establishment and replication within the PV, *T. gondii* tachyzoites activate the host cell PI3K-AKT signaling pathway independently of EGFR, PKCα, and mTOR. AKT phosphorylation (S473 and T308) leads to its activation, which, in turn, phosphorylates FOXO3a at AKT-sensitive residues (S253 and T32), resulting in its nuclear exclusion and inactivation. This downregulates transcription of FOXO3a-dependent autophagy-related genes (e.g., ULK1, BECN1, NBR1, PINK1, and GABARAPL2), whose encoded proteins participate in distinct steps of the autophagic response. As a result, autophagic targeting of the PV is prevented, promoting parasite survival and replication. However, pharmacological inhibition of the PI3K-AKT pathway (e.g., LY294092 or MK-2206 treatment) precludes AKT-dependent phosphorylation and nuclear export of FOXO3a, promoting transcription of autophagy-related genes despite *T. gondii* infection. Exogenous expression of an AKT-resistant form of FOXO3a harboring phosphosite mutations (S253A, T32A, and S315A) mimics chemical activation of FOXO3-driven autophagic targeting of *T. gondii*. Consequently, promoting host cell autophagy has become an increasingly attractive therapeutic strategy against *T. gondii*, and recent studies have identified potential new drug molecules from the DrugBank database that can modulate autophagy (Cheng, Zhang et al. 2022). The interplay between host cell autophagy and *T. gondii* is an emerging area with practical implications for the development of new therapeutic strategies.

A



B



Autophagy

- Cell cycle progression and proliferation
- Apoptosis in response to cellular stress or DNA damage
- Insulin signaling and glucose homeostasis
- Oxidative stress resistance
- Immune cell homeostasis and inflammation
- Stem cell self-renewal and differentiation
- Tissue regeneration and repair
- Longevity and healthy aging

Figure 4.4. *T. gondii* Inhibits Autophagy-Related Gene Expression through AKT-Dependent Inactivation of FOXO Transcription Factors: A Proposed Model and Future Perspectives

(A) Proposed Model. *T. gondii* tachyzoites (shown in pink forming a rosette) activate host cell PI3K-AKT signaling pathway independently of EGFR, PKCα, and mTOR upon establishment and replication within the PV. AKT phosphorylation leads to FOXO3a phosphorylation at AKT-sensitive residues (S253 and T32) and its subsequent nuclear exclusion and inactivation, resulting in downregulation of FOXO3a-dependent autophagy-related genes (i.e., ULK1, BECN1, NBR1, PINK1, and GABARAPL2). Consequently, autophagic targeting of the PV is prevented, favoring parasite survival and replication (as indicated by the red "X" and the downward red arrows). Proteins encoded by this subset of transcripts are reported to participate in distinct steps of the autophagic response (right panel). **(B) Speculative model and future research perspectives**. During late time points of infection, T. gondii secretes GRA16 from dense granules, which is exported through the PVM and reaches the host cell nucleus. GRA16 positively regulates the intracellular amounts of p53 in HFFs modulating genes involved in cell-cycle progression and the p53 tumor suppressor pathway. Genotoxic stress or in this case infection with *T. gondii* can activate nuclear AKT in a p53-dependent manner, leading to the phosphorylation of FOXOs and inhibition of FOXO-induced responses. The p53 scaffold is separate from the membrane, but it recruits and regulates the AKT pathway components that also function in a membrane-based compartment. Future research could corroborate the possible participation of GRA16 in the regulation of p53 and the activation of nuclear AKT, triggering the phosphorylation of FOXO proteins and their relocation outside the nucleus.

While our research work focuses solely on the regulation of FOXO3a and not on other members of the FOXO family, nor on the virulence factors involved in the phenotype, we propose a speculative model based on recent findings that shed light on the modulation of transcriptional activity by *T. gondii* during infection (**Figure 4.4B)**. This model includes new discoveries related to the cytoplasmic functions of FOXO proteins and their cytoplasmic location during infection. In addition, recent advances in nuclear AKT activation models through the regulation of P53, furthermore, we include the possibility that other cellular processes besides autophagy may be regulated by the manipulation of the PI3K/AKT/FOXO pathway, which could contribute to the survival of the parasite or the pathogenesis of the disease (**Figure 4.4B)**.

During late time points of infection, almost 20h p.i in HFFs, *T. gondii* secretes GRA16 from dense granules, which is exported through the PV membrane and reaches the host cell nucleus (Bougdour, Durandau et al. 2013). By modulating genes involved in cell-cycle progression and the p53 tumor suppressor pathway, GRA16 positively regulates the intracellular amounts of p53 in HFFs by forming a high-affinity complex with the ubiquitin-specific protease HAUSP. This represents a potentially emerging subfamily of exported dense granule proteins that modulate host function (Bougdour, Durandau et al. 2013). Additionally, genotoxic stress can activate nuclear AKT in a p53-dependent manner. This forms a complex with PI3,4,5P3 that activates AKT and phosphorylates FOXOs, leading to the inhibition of DNA damage-induced apoptosis. The p53 scaffold is separate from the membrane, suggesting a different structure and compartmentalization. Nevertheless, it recruits and regulates the AKT pathway components that also function in a membrane-based compartment (Chen, Choi et al. 2022). Future research could assess the possible participation of GRA16 in the regulation of p53 and the activation of nuclear AKT, which would trigger the phosphorylation of FOXO proteins and their relocation outside the nucleus (**Figure 4.4B)**.

Our research has uncovered an important finding regarding *T. gondii* infection in HFF cells. Specifically, we observed that FOXO3a protein levels did not decrease during infection and, in fact, showed an increase in signal intensity, however, as mentioned earlier, a notable distinction arises between BMDMs and THP-1 cells regarding this matter. In *T. gondii*-infected cells, both cell types displayed a decrease in the total abundance of FOXO3a compared to non-infected cells, **Figure 4.2**. This observation suggests that *T. gondii*

potentially exerts a negative influence on the FOXO3a protein specifically within macrophages. Unlike human and mouse fibroblasts, where FOXO3a tends to be preserved and accumulated in the cytoplasm upon infection, the regulatory mechanisms governing FOXO3a in macrophages appear to be distinct, leading to possible diverse biological consequences depending on the host cell type.

This suggests that in the case of HFFs, there may be alterations in the process of autophagy or in the turnover of FOXO proteins via a feedback loop (Zhu, Li et al. 2019). Additionally, it is possible that the parasite benefits from keeping the FOXO proteins intact to induce their cytoplasmic functions, such as FOXO1 interacting with ATG7 and activating lipophagy or FOXO3a inducing regulation of mitochondrial transcripts (Lettieri Barbato, Tatulli et al. 2013, Fasano, Disciglio et al. 2019). In either case, this could contribute to the induction of energy metabolism and the utilization of fatty acid carbon sources, as well as to disease pathology through the accumulation of proteins and materials that require removal by autophagy or the dysregulation of mitochondrial homeostasis (**Figure 4.4B**). These results shed light on the complex interplay between *T. gondii* and host cell signaling pathways and may pave the way for further investigation into the mechanisms underlying these observations. Interestingly, in addition to autophagy, other cellular processes that are transcriptionally regulated by FOXO3a (e.g., apoptosis, cell cycle, oxidative stress, etc.) are also targeted by *T. gondii* (van Grevenynghe, Cubas et al. 2012, Zhu, Li et al. 2019, Calissi, Lam et al. 2021). It has been observed as well that in infection models like *C. rodentium*, the nuclear FOXO3a translocates into the cytosol and subsequently degrades in the colonic epithelia of human HT-29 and mouse CMT-93 cells (Snoeks, Weber et al. 2008). In healthy mice, FOXO3a is present in both the nucleus and cytosol of the epithelia at the bottom of the crypts. However, in *C. rodentium*-infected colon, FOXO3a is mainly expressed along the crypts and located in the cytosol, indicating its inactivation (Snoeks, Weber et al. 2008). Given these findings, it is possible that *T. gondii*, particularly its other forms such as oocysts that come into contact with the intestinal epithelial tissue, may also negatively regulate the transcriptional activity of FOXO proteins by causing their translocation from the nucleus to the cytoplasm. To confirm this hypothesis, further research using suitable *in vivo* models or advanced *in vitro* models, such as organoids, is needed. It is worth mentioning that our laboratory has obtained preliminary data indicating significant findings. In these findings, the

infection of C57BL/6 mice with *T. gondii* ME49 cysts, specifically at 5- and 10-days post-infection, resulted in a notable reduction in FOXO3a transcripts within various sections of the intestinal tissue, including the ileum, duodenum, and ileum jejunum, when compared to uninfected mice. This suggests the possibility that *T. gondii* might exert a negative regulatory effect on FOXO3a, specifically at the transcriptional level, during *in vivo* infection. Further investigations are necessary to validate these preliminary findings and to elucidate the underlying molecular mechanisms through which *T. gondii* negatively modulates the expression of FOXO3a transcripts.

Finally, FOXO3a has emerged as promising druggable target for various pathological conditions (e.g., cancer, diabetes, cardiovascular disease, chronic neurological diseases, etc.) (Calissi, Lam et al. 2021). Hence, it is tempting to speculate that restoring transcriptional programs regulated by FOXO3a, including but not limited to autophagy-related genes, could represent a new therapeutic approach to treat toxoplasmosis. Further characterization of altered transcriptional networks under the control of FOXO3a, and potentially other FOXO family members, during *T. gondii* infection will yield invaluable health-related knowledge to develop effective and safe host-directed strategies for better treatment or prevention of toxoplasmosis and potentially other infectious diseases.

CHAPTER 5

Synthèse en français

5.1 Résumé

Toxoplasma gondii (*T. gondii*) est un parasite protozoaire intracellulaire capable d'infecter pratiquement n'importe quelle cellule nucléée et une grande variété d'hôtes vertébrés à sang chaud, y compris les humains, causant la toxoplasmose. Il est estimé que 30 à 50 % de la population mondiale est séropositive pour *T. gondii*, ce qui en fait l'une des infections les plus répandues chez l'Homme. *T. gondii* peut être transmis par l'ingestion d'ookystes excrétés dans les selles de félins ou de viande insuffisamment cuite provenant d'animaux infectés. *T. gondii* subit divers stades de vie, tels que les tachyzoïtes et les bradyzoïtes, capables de persister dans les tissus de l'hôte. De plus, des facteurs sécrétés jouent un rôle clé dans les différentes étapes de l'infection et sont liés à la virulence de *T. gondii*. La toxoplasmose est un problème de santé publique important, car il n'y a pas de traitement contre toutes les formes du parasite ou de vaccin disponible chez l' humain. Le traitement médicamenteux peut avoir des effets secondaires et est inefficace à éliminer les kystes de *T. gondii*.

T. gondii manipule la cellule hôte pour créer un environnement favorable à sa réplication. Pour ce faire, le parasite puise des nutriments essentiels, modifie le métabolisme de l'hôte, inhibe l'apoptose et manipule l'autophagie, la réponse immunitaire et la progression du cycle cellulaire de l'hôte. *T. gondii* a développé des mécanismes complexes pour créer une vacuole parasitaire (*parasitophorous vacuole*, PV) non-fusogénique qui aide à acquérir des nutriments tout en empêchant le contact avec les composants cytoplasmiques et contenu lysosomal de l'hôte qui pourraient déclencher la destruction du parasite. Le parasite utilise également des facteurs de virulence pour cibler divers aspects de la biologie de la cellule hôte, tels que l'expression génique, la machinerie transcriptionnelle et les modifications post-traductionnelles des protéines hôtes.

En tant que mécanisme de défense clé contre les infections, l'autophagie, un processus cellulaire qui aide à maintenir l'homéostasie cellulaire, est impliquée dans la capture et l'élimination des parasites intracellulaires. Cependant, les parasites intracellulaires, tels que *T. gondii*, ont développé plusieurs mécanismes d'évasion pour manipuler l'autophagie de la cellule hôte. La voie de signalisation PI3K/AKT joue un rôle dans l'inhibition de l'autophagie tout en favorisant la croissance, la différenciation et la survie cellulaire. L'inhibition de cette voie peut entraîner une augmentation significative de l'autophagie. Fait intéressant, *T. gondii* exploite la signalisation PI3K/AKT de l'hôte pour échapper aux effets destructeurs de

l'autophagie. Cette stratégie d'évasion est particulièrement activée pendant les premiers stades de l'invasion, où le parasite active AKT par phosphorylation dépendante de l'EGFR. Ainsi, *T. gondii* empêche l'accumulation de composantes d'autophagosomes et de lysosomes autour de sa PV pour ainsi éviter son élimination de la cellule hôte. Cependant, les cibles spécifiques d'AKT permettant à *T. gondii* d'entraver la machinerie autophagique de l'hôte ne sont pas encore entièrement caractérisées.

Parmi les différentes cibles d'AKT, les FOXO (sous-famille Forkhead Box O) ont été identifiées comme un nœud majeur régulant l'autophagie. Les facteurs de transcription FOXO sont des régulateurs essentiels de l'homéostasie cellulaire et de l'autophagie qui sont contrôlés par la voie de signalisation PI3K/AKT. La phosphorylation de facteurs de transcription FOXO par AKT mène à leur translocation du noyau vers le cytomplasme. Il est intéressant de noter que la dérégulation de l'axe FOXO-autophagy est liée à diverses maladies humaines. Par conséquent, les facteurs de transcription FOXO jouent un double rôle dans les infections virales, car ils peuvent être à la fois protecteurs et néfastes. Différents agents pathogènes manipulent les protéines FOXO de différentes manières. En conséquence, divers organismes intracellulaires pathogènes utilisent la manipulation de la voie PI3K/AKT/FOXO de l'hôte pour survivre à l'intérieur de la cellule hôte.

En utilisant une combinaison d'approches pharmacologiques et génétiques, nous avons examiné si *T. gondii* s'approprie la voie PI3K/AKT pour supprimer les programmes transcriptionnels liés à l'autophagie régulés par FOXO3a, entravant ainsi l'activation de la réponse autophagique de l'hôte contre le parasite. Pour corroborer cela, nous avons d'abord étudié les mécanismes moléculaires responsables de la phosphorylation dépendante de l'AKT de FOXO3a. Ensuite, nous avons déterminé si la répression dépendante de l'AKT de FOXO3a affecte les programmes transcriptionnels liés à l'autophagie. Enfin, nous avons établi si les fonctions régulées par FOXO3a et liées à l'autophagie sont altérées lors de l'infection par *T. gondii*.

Nous avons constaté que l'infection par *T. gondii* déclenche une phosphorylation progressive et soutenue de FOXO3a dépendante de AKT aux résidus S253 et T32 à la fois dans les fibroblastes préputiaux humains (*human foreskin fibroblasts,* HFF) et les fibroblastes murins 3T3. De plus, une infection active est nécessaire pour manipuler et activer la voie de

signalisation PI3K/AKT, ce qui entraîne la phosphorylation de FOXO3a. Curieusement, ce processus de phosphorylation est indépendant du récepteur de la membrane plasmique EGFR et de la kinase PKCα. De surcroît, *T. gondii* provoque l'exclusion nucléaire de FOXO3a dans les HFF infectés, un processus directement corrélé avec sa phosphorylation. Cependant, le parasite ne peut pas entraîner une localisation cytoplasmique de FOXO3a lorsque AKT est bloqué pharmacologiquement ou lorsque la forme mutante FOXO3a insensible à AKT est surexprimée. Au cours de l'infection par *T. gondii*, la transcription d'un sous-ensemble de gènes cibles liés à l'autophagie de FOXO3a est réduite de manière dépendante d'AKT. Cependant, la capacité du parasite à réprimer les gènes liés à l'autophagie reste inchangée par l'inhibition de AKT dans les cellules dans lesquelles les transcrits de *Foxo3* sont mis sous silence (*knockdown*). En conséquence, lorsque FOXO3a est maintenu dans le noyau, *T. gondii* ne peut pas empêcher le recrutement d'organites acides et de LC3 autour de la VP. En résumé, nos résultats indiquent que *T. gondii* supprime l'expression de gènes régulés par FOXO3a pour éviter la mort par autophagie.

En conclusion, l'autophagie est un processus cellulaire qui peut être détourné par plusieurs pathogènes pour leur réplication et leur survie, mais l'hôte a développé des contre-mesures pour empêcher leur survie. L'interaction complexe entre l'autophagie et les adaptations microbiennes détermine le résultat des rencontres hôte-pathogène. La famille des facteurs de transcription FOXO est cruciale pour la régulation de plusieurs processus cellulaires, y compris l'autophagie. *T. gondii* manipule le signal de signalisation PI3K/AKT de l'hôte pour promouvoir sa survie et sa réplication, et subvertit en partie le processus d'autophagie de l'hôte en inhibant l'activité transcriptionnelle de FOXO3a. Cependant, l'inhibition de la voie PI3K/AKT ou l'expression d'une forme résistante d'AKT de FOXO3a peut favoriser l'autophagie de la cellule hôte et cibler *T. gondii*, faisant de l'autophagie une stratégie thérapeutique attrayante contre le parasite. Nos résultats indiquent que *T. gondii* s'approprie la voie PI3K/AKT pour supprimer les programmes transcriptionnels liés à l'autophagie sous le contrôle de FOXO3a, entravant ainsi l'activation de la réponse autophagique de l'hôte contre le parasite. Une caractérisation plus poussée des réseaux transcriptionnels altérés sous le contrôle de FOXO3a, et potentiellement d'autres membres de la famille FOXO, pendant l'infection à *T. gondii* permettra d'obtenir des connaissances précieuses en matière de santé

pour développer des stratégies efficaces et sûres dirigées vers l'hôte pour le traitement ou la prévention de la toxoplasmose.

5.2 Sommaire du premier article: *Toxoplasma gondii* inhibe l'expression de gènes liés à l'autophagie par l'inactivation dépendante de AKT du facteur de transcription FOXO3a

Toxoplasma gondii est un parasite intracellulaire responsable de la toxoplasmose, une infection opportuniste qui peut être transmise à l'humain par ingestion d'aliments ou d'eau contaminés. Malgré l'impact significatif de *T. gondii* sur la santé publique, il n'existe actuellement aucun vaccin efficace pour l'être humain, et les options de traitement pour l'infection chronique ou la prévention de l'infection congénitale sont limitées. *T. gondii* a développé différentes stratégies pour manipuler les processus cellulaires de l'hôte afin d'établir une niche de réplication favorable, notamment en activant la voie de signalisation AKT de l'hôte pour prévenir l'élimination des parasites par autophagie.

L'autophagie est un processus cellulaire important caractérisé par la dégradation et le recyclage d'organites endommagés et de protéines par la machinerie lysosomale. De surcroît, l'activation de l'autophagie est un mécanisme de défense de la cellule hôte contre les pathogènes intracellulaires, y compris *T. gondii*. Cependant, *T. gondii* peut manipuler les voies de signalisation de la cellule hôte pour prévenir l'activation de l'autophagie et favoriser sa propre survie. L'une des voies ciblées par *T. gondii* est la voie de signalisation AKT, qui est critique pour la régulation de divers processus cellulaires, tel que l'autophagie.

Nos recherches ont mis en lumière comment *T. gondii* inhibe l'autophagie par l'inactivation dépendante de l'AKT du facteur de transcription Forkhead box O3a (FOXO3a). Nous avons constaté que l'infection par les souches de type I et II de *T. gondii* phosphoryle graduellement et de façon soutenue FOXO3a aux résidus S253 et T32 dans les fibroblastes de prépuce humain (HFF) et les fibroblastes murins 3T3. Cette phosphorylation est dépendante de l'activité de PI3K et d'AKT mais indépendante du récepteur de la membrane plasmique EGFR et de la kinase PKCα. Cette phosphorylation de FOXO3a conduit à son exclusion nucléaire dans les HFF infectés par *T. gondii*.

Nos recherches ont également révélé que *T. gondii* supprime les programmes transcriptionnels régulés par FOXO3a pour prévenir son élimination par l'autophagie. Le

parasite ne peut pas induire la translocation cytoplasmique de FOXO3a en bloquant pharmacologiquement AKT ou en surexprimant une forme mutante FOXO3a insensible à AKT. De plus, *T. gondii* est incapable d'inhiber le recrutement d'organites acides et de LC3 à la vacuole parasitophore lors de la rétention nucléaire de FOXO3a induite chimiquement ou génétiquement. Ces résultats suggèrent que *T. gondii* inhibe le recrutement de la machinerie d'autophagie à la vacuole parasitaire grâce à la phosphorylation dépendante d'AKT de FOXO3a.

Dans l'ensemble, ces résultats mettent en évidence le rôle critique de l'axe de signalisation AKT/FOXO3a dans l'infection par *T. gondii* et suggèrent que le ciblage de cette voie pourrait représenter une stratégie thérapeutique viable contre le parasite. Des études subséquentes sont nécessaires pour élucider davantage l'interaction complexe entre *T. gondii* et la machinerie d'autophagie de l'hôte et pour identifier des cibles supplémentaires pour les thérapies ciblant les fonctions cellulaires de l'hôte.

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