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Mauricio Ramm

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“A good digestion turneth all to health.”
- George Herbert, 1633

For Jürgen, Nataliia and Sophia – my constant support.

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ABSTRACT

Autophagy is a lysosome-dependent degradation and recycling pathway essential for cellular homeostasis. Late-stage autophagy depends on lysosomal fusion machinery. Rab GTPases are master regulators of intracellular vesicle traffic, and many Rabs participate in the regulation of autophagy. This thesis combines compound screening, tissue-level Rab24 analysis, and mechanistic studies to investigate pharmacological autophagy modulation, Rab24 protein levels in tissues, and the role of Rab24 in lysosome-directed fusion events. Candidate autophagy modulators were evaluated in A549 cells by confocal microscopy for effects on autophagic flux, using the autophagy markers LC3 and p62. The tested compounds showed distinct, condition-dependent effects: famciclovir reduced autophagy flux under basal (fed) but not starvation conditions, while vioprolides suppressed the flux under both fed and starvation conditions. In a canine fibroblast model of neuronal vacuolation and spinocerebellar degeneration, slightly elevated basal LC3 flux and a blunted starvation response were observed. None of the tested compounds normalized flux to wild-type levels. Rab24 protein abundance was mapped across mouse tissues during postnatal development and adulthood and assessed across human cancers using tissue microarrays. Rab24 levels were tissue- and age-dependent, with enrichment in brain and several epithelial cell types. Rab24 protein levels also varied across tumor entities: elevated levels were detected in breast and skin cancers and in cancers of neuronal origin, while pancreatic neuroendocrine tumors showed reduced Rab24 levels. Finally, mechanistic studies in Neuro-2a cells linked Rab24 to lysosomal delivery: Rab24 knockout altered the balance of autophagic and degradative compartments without detectable defects in lysosomal activity. APEX2 proximity proteomics and co-immunoprecipitation identified the homotypic fusion and vacuolar protein sorting (HOPS) tethering complex and Rab7a as Rab24 interactors. The ataxia-associated Rab24-Q38P mutation weakened these interactions without loss of GTP binding. Together, the results support a model in which Rab24 promotes autophagic clearance by facilitating HOPS-dependent fusion events. The thesis places Rab24 function into tissue contexts relevant for neurodegeneration and cancer.

KEYWORDS: Autophagy, Rab24, Rab7a, HOPS complex, proximity biotinylation, lysosome, endolysosomal trafficking

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TIIVISTELMÄ

Autofagia on solujen homeostaasia tukeva, lysosomeihin johtava hajotus- ja kierrätysreitti. Rab-GTPaasit ovat solunsisäisen vesikkeliliikenteen säätelijöitä, ja monet Rab-proteiinit säätelevät autofagiaa. Tässä väitöskirjassa käytettiin yhdistekirjastojen seulontaa, kudostason Rab24-analyysia ja mekanistisia tutkimuksia, joiden avulla tutkittiin autofagian farmakologista modulointia, Rab24-proteiinitasoja kudoksissa ja syövissä sekä Rab24:n roolia lysosomeihin kohdistuvassa kalvoliikenteessä. Autofagiaa moduloivia yhdisteitä arvioitiin A549-soluissa; vaikutuksia autofagiareitin toimintaan selvitettiin kahden merkkiaineen (LC3 ja p62) avulla. Testatuilla yhdisteillä oli olosuhteista riippuvia vaikutuksia: famsikloviiri vähensi autofagiavirtausta ruokituissa mutta ei nälkiintyneissä soluissa, kun taas vioprolidit estivät virtausta sekä ruokituissa että nälkiintyneissä soluissa. Koiran fibroblastimallin avulla tutkittiin yhdisteiden vaikutuksia hermosolujen vakuolisaatio ja spinokerebellaarinen rappeutuminen -taudissa. Soluissa havaittiin hieman kohonnut perustason autofagiavirtaus ja heikentynyt nälkiintymisvaste. Mikään testatuista yhdisteistä ei normalisoinut virtausta villityypin tasolle. Rab24-proteiinin runsautta kartoitettiin hiiren kudoksissa postnataalisen kehityksen ja aikuisuuden aikana sekä ihmisen syövissä kudosmikrosirujen avulla. Rab24-tasot riippuivat kudoksesta ja iästä. Rab24:ää esiintyi runsaasti aivoissa ja useissa epiteelisolutyypeissä. Rab24-proteiinitasot vaihtelivat eri syövissä: kohonneita tasoja havaittiin rinta- ja ihosyövissä sekä hermosoluperäisissä syövissä, kun taas haiman neuroendokriinisissa kasvaimissa Rab24-tasot olivat alentuneet. Mekanistiset tutkimukset Neuro-2a-soluilla yhdistivät Rab24:n lysosomeihin kohdentuvaan kalvokuljetukseen: Rab24:n puute muutti autofagisten ja lysosomaalisten osastojen tasapainoa ilman havaittavia puutteita lysosomien aktiivisuudessa. Proximity biotinylation ja ko-immunopresipitaatio tunnistivat Rab24-vuorovaikuttajaksi HOPS-kompleksin (homotypic fusion and vacuolar protein sorting) ja Rab7a:n. Ataksiaa aiheuttava Rab24-Q38P-mutaatio heikensi näitä vuorovaikutuksia mutta ei vaikuttanut GTP:n sitoutumiseen. Tulosten mukaan Rab24 edistää myöhäistä autofagista kierrätystä avustamalla HOPS-riippuvaisia fuusiotapahtumia. Väitöskirjan tulosten mukaan Rab24:n toiminta sijoittuu konteksteihin, joilla on merkitystä neurodegeneraation ja syövän kannalta.

AVAINSANAT: autofagia, Rab24, Rab7a, HOPS-kompleksi, läheisyysbiotinylaatio, lysosomi, endolysosomaalinen kuljetus

Table of Contents

Abbreviations

ADP	Adenosine diphosphate
AIM	Atg8 interacting motif
Akt	Akt serine/threonine kinase
ALR	Autophagic lysosome reformation
Ambra1	Beclin 1-regulated autophagy protein 1
Ampa	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ampk	AMP-activated protein kinase
Anxa2	Annexin2
App	Amyloid beta precursor like protein
Arf	ADP ribosylation factor
Atg	Autophagy-related
AUTOTACs	Autophagy-Targeting Chimeras
Aβ	Amyloid-β
Bace1	Beta-secretase 1
Bag3	Bcl2-associated athanogene 3
Bdnf	Brain-derived neurotrophic factor
Bicd2	Bicaudal D cargo adaptor 2
Bloc-1	Biogenesis of lysosome-related organelles complex 1
Bnip3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
BORC	Bloc-1-related complex
Brca1	Breast cancer type 1 susceptibility protein
c-Abl	Abelson murine leukemia viral oncogene homolog 1
Calcoco2	Calcium-binding and coiled-coil domain-containing protein 2
CatD	Cathepsin D
$Cd4^+$	Cluster of differentiation 4-positive
CDR	Complementarity-determining regions
Ci-mpr	Cation-independent mannose-6-phosphate receptor
CMA	Chaperon-mediated autophagy
CORVET	Class C core vacuole/endosome tethering
Ctbp1	C-terminal-binding protein 1

Dfcp1	Double FYVE-containing protein 1, also called Zinc-finger FYVE domain-containing protein 1
Dnm1l	Dynamin-1-like protein
DQ-BSA	Dye-quenched BSA
Drs	Down-regulated by v-src
Eea1	Early endosome antigen 1
Epg5	Ectopic P granules protein 5 homolog
ER	Endoplasmic reticulum
Fgf21	Fibroblast growth factor 21
Fip200	Focal adhesion kinase family interacting protein of 200 kD (also called Rb1cc1)
Fis1	Mitochondrial fission 1 protein
Foxo3	Forkhead box protein O3
Fyco1	FYVE and coiled-coil domain-containing protein 1
FYVE	The first letters of four proteins (Fab1, Yotb, Vac1, Eea1) in which a zinc-finger domain was identified
Gabarap	Gamma-aminobutyric acid receptor-associated protein
Gabarapl1	Gamma-aminobutyric acid receptor-associated protein-like 1
GAP	GTPase-activating protein
GDF	GDI displacement factors
GDI	GDP dissociation inhibitor
GDP	Guanosine diphosphate
GEF	GDP-GTP exchange factor
GTP	Guanosine triphosphate
Hif-1α	Hypoxia-inducible factor 1-alpha
HOPS	Homotypic fusion and protein sorting
H-ras	GTPase HRas (Ras stands for rat sarcoma)
HVD	Hypervariable domain
Ide	Insulin-degrading enzyme
Ifnγ	Interferon gamma
IL-1β	Interleukin-1β
Jip	Jnk-interacting protein
Jnk	C-Jun N-terminal kinase
K-ras	GTPase KRas
Lamp	Lysosome-associated membrane protein
Lamtor1	Late endosomal/lysosomal adaptor, Mapk and mTor activator 1
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LIR	Light chain 3 (LC3)-interacting region, also called AIM
Lox	Lysyl oxidase
Lrrk2	Leucine-rich repeat serine/threonine-protein kinase 2

Map1LC3b	Microtubule-associated protein 1 light chain 3 beta, usually referred to as just 'LC3'
Map3k7	Mitogen-activated protein kinase kinase kinase 7
March1	E3 ubiquitin-protein ligase Marchf1
Marchf1	Membrane Associated Ring-CH-Type Finger 1
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MTOC	Microtubule organizing center
mTorc1	Mammalian/mechanistic target of rapamycin complex 1
Nbr1	Next to Brca1 gene 1 protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Ngf	Nerve growth factor
Nlrp3	NLR family pyrin domain containing 3
Nrbf2	Nuclear receptor-binding factor 2
Nsf	N-ethyl maleimide-sensitive fusion protein
NVSD	Neuronal vacuolation and spinocerebellar degeneration
Optn	Optineurin
Osbpl	Oxysterol binding protein
p115	General vesicular transport factor p115
p62	Ubiquitin-binding protein p62 (alternative name for Sqstm1)
Parkin	E3 ubiquitin-protein ligase parkin
Perk	Proline-rich receptor-like protein kinase
Pi3k	phosphoinositide 3-kinase
Pi3kc3	Class III phosphoinositide 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
Pi4kIIα	Phosphatidylinositol 4-kinase type 2-alpha
Picalm	Phosphatidylinositol-binding clathrin assembly protein
Pink1	PTEN-induced kinase 1
Plekhm	Pleckstrin homology domain-containing family M member
PLGA	Poly(lactic-co-glycolic-acid)
Psen1	Presenilin 1
Pten	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase Pten
Rab	Ras-related in brain
RabGGTase	Rab geranylgeranyl transferase
Ras	Rat sarcoma virus
Rb1cc1	RB1-inducible coiled-coil protein 1
REP	Rab escort protein
RhoE	Rho-related GTP-binding protein RhoE
Rilp	Rab-interacting-lysosomal protein

Ripk	Receptor-interacting serine/threonine-protein kinase 1
Sgk3	Serum- and glucocorticoid-induced kinase 3
Sipa1l2	Signal-induced proliferation-associated 1-like protein 2
SM	Sec1/Munc18
Snap29	Synaptosomal-associated protein 29
SNARE	Soluble Nsf attachment protein receptor
Sqstm1	Sequestosome-1 (also called p62)
Stat1	Signal transducer and activator of transcription 1
Stx17	Syntaxin-17
Tab3	TGF-β-activated kinase 1 and MAP3K7-binding protein 3
Tardbp	Transactive response DNA binding protein 43 kDa
Tax1bp1	Tax1-binding protein 1 homolog
TBC domain	Tre-2, Bub2, and Cdc16 domain
Tbc1d5	TBC1 domain family member 5
Tbk1	Tank-binding kinase 1
Tecpr1	Tectonin β-propeller repeat-containing protein 1
Tfeb	Transcription factor EB
Tgf-β	Transforming growth factor beta
TGN	Trans-Golgi network
Tia1	T-cell intracellular antigen 1
Tnfα	Tumor necrosis factor α
Tom	Translocase of the outer membrane
Tpc2	Two-pore channel 2
TrkB	Tropomyosin receptor kinase B
Ulk1	Unc-51-like autophagy-activating kinase 1
Vamp	Vesicle-associated membrane protein
V-ATPase	Vacuolar-type ATPase
Vcp	Transitional endoplasmic reticulum ATPase
Vegfa	Vascular endothelial growth factor A
Vegfr2	Vascular endothelial growth factor receptor 2
Vps	Vacuolar protein sorting-associated protein
Wipis	Tryptophan-aspartic acid (WD) repeat domain phosphoinositide-interacting proteins

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Fichtner J*, Beer YY*, Ramm HGM*, Mühlen S, Surup F, Herrmann J, Meister TL, Pfaender S, Bilitewski U, Brönstrup M, Müller R, Wirth M, Eskelinen EL, Schmitz I. Newly identified properties of known pharmaceuticals and myxobacterial small molecules revealed by screening for autophagy modulators. *The FEBS Journal*, 2025 (online ahead of print) https://doi.org/10.1111/febs.70243

II Ramm HGM, Ahmed F, Fazeli S, Bourgery M, Lopez MA, Tripathi L, Leivo I, Syrjä P, Eskelinen EL. Rab24 protein levels show dynamic changes in mouse tissues and human cancers. *Cell and Tissue Research*, 2026; 403(2):14. https://doi.org/10.1007/s00441-025-04043-4

III Ramm HGM*, Lopez AM*, Kotiniitty R, Nalbach K, Quoc Ngo B, Padzik A, Lampela O, Juffer AH, Behrends C, Eskelinen EL. Rab24 interacts with the homotypic fusion and vacuolar protein sorting complex. *Manuscript*

*These authors contributed equally.

1 Introduction

Cells continuously remodel their internal components to maintain homeostasis, adapt to fluctuating nutrient availability, and defend against stress. A major pathway supporting this adaptability is autophagy, a lysosome-dependent degradation route that delivers cytoplasmic material to acidic degradative compartments for breakdown and recycling. By turning over damaged organelles, misfolded or aggregation-prone proteins, and other unwanted cellular material, autophagy contributes to quality control and metabolic homeostasis. Autophagy is therefore relevant across many physiological contexts, including development, neuronal maintenance, epithelial homeostasis, and immunity, and its dysregulation has been linked to diverse diseases such as neurodegeneration, cancer, and metabolic disorders.

A defining feature of macroautophagy is the formation of a double-membrane autophagosome that sequesters cargo and ultimately fuses with endosomes and lysosomes to enable degradation. Because autophagy is a multi-step pathway, changes in commonly used markers, particularly LC3-positive vesicles, can reflect perturbations at different stages, from autophagosome biogenesis to vesicle transport and lysosomal fusion. This complexity is a central practical challenge for both basic research and therapeutic development: pharmacological “autophagy modulators” are often discovered through phenotypic assays, but assigning their effects to specific steps of the pathway requires careful validation using flux-based measurements and orthogonal readouts. At the same time, these challenges also represent an opportunity: systematic screening approaches, combined with mechanistically informative follow-up assays, can uncover unexpected activities of known drugs and natural products and provide new tools to investigate autophagy regulation in health and disease.

In parallel, understanding autophagy requires attention to be paid to the membrane trafficking machinery that positions and fuses organelles along the autophagic and endolysosomal systems. The events such as tethering and fusion depend on Rab-family small GTPases and multisubunit tethering complexes, including the homotypic fusion and vacuolar protein sorting (HOPS) complex, and proteins assisting in membrane fusion. While canonical pathways of endosome

maturation and lysosome fusion have been intensively studied, less is known about how the “atypical” Rab protein Rab24 interacts with tethering and fusion machinery, and how such interactions contribute to tissue-specific functions and disease vulnerability. Rab24 has been implicated in late stages of autophagic and endosomal pathways. Rab24 is also linked to neurodegeneration: mutations in Rab24 cause hereditary ataxia in dogs, characterized by Purkinje neuron degeneration with accumulation of autophagic vesicles. Despite these genetic and cell biological connections, the molecular partners and mechanisms through which Rab24 acts have remained poorly defined.

The purpose of this thesis is to combine disease-relevant cellular assays, tissue-level protein mapping, and mechanistic interaction studies to advance understanding of (i) how autophagy can be pharmacologically modulated and evaluated using rigorous, flux-measuring approaches, and (ii) how Rab24 functions in health and disease. Specifically, the thesis presents: (1) validation of small-molecule modulators of basal and starvation-induced autophagy using flux measurements and testing in an Atg4D-mutant fibroblast model of neurodegenerative vacuolar storage disease; (2) systematic analysis of Rab24 protein levels across mouse tissues during postnatal development and adulthood, complemented by assessment of Rab24 protein abundance across diverse human cancers; and (3) mechanistic dissection of Rab24 function in neuronal cells using proximity proteomics and biochemical interaction assays, revealing a functional connection between Rab24 and membrane fusion machinery. Together, the studies aim to provide both practical frameworks for evaluating autophagy inhibition and new mechanistic insight into how Rab24 interfaces with lysosome-proximal trafficking pathways relevant to neuro-degeneration and cancer.

2 Review of the Literature

2.1 Autophagy

Autophagy (Greek for ‘self-eating’) is a highly conserved intracellular degradation pathway first described in the 1960s (Deter, et al., 1967). It recycles intracellular material by delivering cytoplasmic cargo to the lysosome for degradation. Autophagy is a central mechanism for maintaining cellular homeostasis by eliminating damaged organelles, misfolded proteins and invading pathogens. Under basal conditions, autophagy continuously turns over cytoplasmic components, contributing to quality control, nutrient recycling, and organelle renewal (Zhang, et al., 2016b). Autophagy can be dynamically upregulated in response to stress signals such as nutrient deprivation (Mizushima, et al., 2004, Yabu, et al., 2012, Young, et al., 2009), hypoxia (Low, et al., 2013), accumulation of protein aggregates (Liu, et al., 2010, Wong, et al., 2012) or impaired mitochondrial function (Ashrafi, et al., 2014, Basak and Holzbaur, 2025, Gómez-Sánchez, et al., 2016). Through this adaptive response, autophagy protects cells from metabolic and proteotoxic stress and allows them to survive fluctuations of environmental conditions.

Central to the autophagy pathway is the generation of a double-membraned organelle called the autophagosome, which encapsulates cytoplasmic cargo for delivery to lysosomes. Autophagy proceeds through several coordinated steps: initiation, phagophore/isolation membrane formation, cargo sequestration, autophagosome closure, vesicle transport, and fusion of autophagosomes with lysosomes (Fig. 1). Key regulator proteins include the core autophagy related (Atg) machinery, which can be organized into functional modules that act in sequence during autophagosome biogenesis (e.g. the Unc-51-like autophagy-activating kinase 1 (Ulk1)/Atg1 kinase complex, Atg9 vesicle, the autophagy-specific class III PI3K complex, Atg2-Wipi/Atg18 factors, and the Atg12/Atg8 conjugation system; Nakatogawa, 2020). Once formed, autophagosomes are trafficked and tethered to lysosomes. Finally, fusion of the autophagosome outer limiting membrane with the lysosome enables enzymatic degradation and recycling of the cargo and the inner limiting membrane. Autophagosomes can also fuse with late endosomes before lysosome fusion, generating an intermediate referred to as an amphisome (Lahiri, et al., 2019).

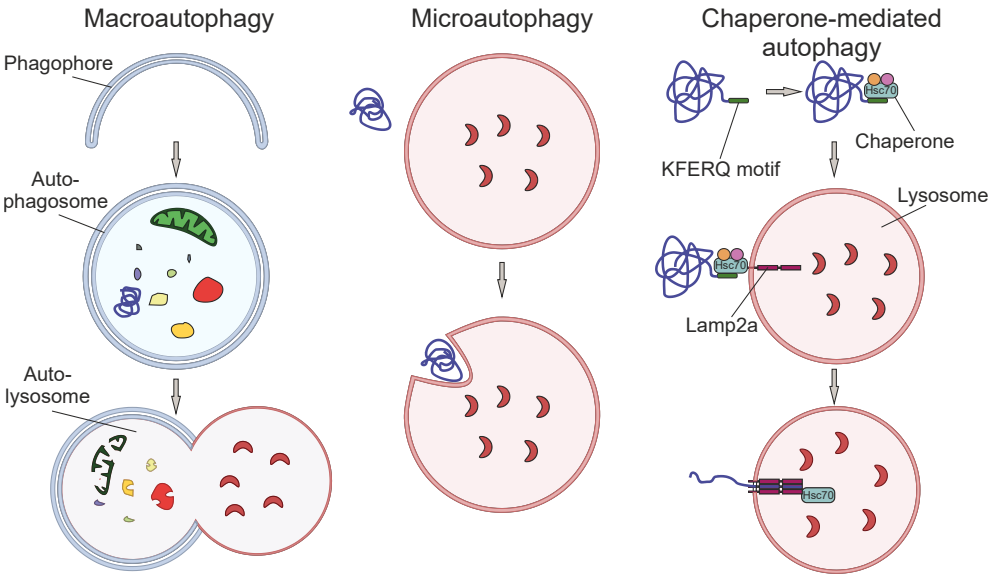

Figure 1. The three main types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy involves formation of a double-membrane phagophore that expands to enclose cytoplasmic cargo, generating an autophagosome that subsequently fuses with lysosomes for cargo degradation. Microautophagy entails direct invagination of the lysosomal membrane to engulf cytosolic material for degradation within the lysosomal lumen. Chaperone-mediated autophagy (CMA) selectively targets soluble proteins carrying a KFERQ-like motif, which are recognized by cytosolic chaperones and translocated across the lysosomal membrane via Lamp2a.

Multiple forms of autophagy coexist, tailored to distinct cellular contexts and cargo types. Macroautophagy, often simply called autophagy, is the best-characterized form and involves the sequestration of cytoplasmic material explained above (Fig. 1). In microautophagy, lysosomes directly invaginate and engulf surrounding cytosolic components without formation of autophagosomes (Kuchitsu and Taguchi, 2024). A third pathway, chaperone-mediated autophagy (CMA), selectively translocates proteins containing a KFERQ-like targeting motif across the lysosomal membrane through interactions with specialized cytosolic and lysosomal chaperones and the Lysosome-associated membrane protein 2a (Lamp2a) that acts as a receptor (Kaushik and Cuervo, 2018). Several specialized macroautophagy subtypes provide selective turnover of specific cargo, including mitophagy (mitochondria), pexophagy (peroxisomes), ER-phagy or reticulophagy (endoplasmic reticulum), lipophagy (lipid droplets), ribophagy (ribosomes), Golgiphagy (Golgi apparatus), nucleophagy (nuclear components), lysophagy (lysosomes), xenophagy (pathogens), chlorophagy (chloroplasts) and aggrephagy (protein aggregates). Despite mechanistic distinctions, all three forms of autophagy converge on lysosomal degradation and recycling, highlighting the role of autophagy as a

fundamental quality-control system. Hereafter, if not further specified, 'autophagy' will refer to macroautophagy.

Functionally, autophagy impacts a wide range of physiological systems. It contributes to neuronal survival (Fleming, et al., 2025), immune defense (Pereira, et al., 2025), stem cell maintenance (Zhao, et al., 2025), and organ development (Restrepo and Baehrecke, 2024), while its dysregulation has been implicated in diverse diseases including neurodegeneration (Nixon and Rubinsztein, 2024), cancer (Lu, et al., 2026b), and metabolic disorders (Vakili, et al., 2026). In tumor biology, autophagy may play dual roles: supporting cell survival under metabolic or treatment stress, but also limiting tumorigenesis by removing damaged components that would otherwise promote genomic instability (Lu, et al., 2026b). In the context of organ maturation and tissue remodeling, autophagy coordinates large-scale protein and organelle turnover required for functional transitions. Understanding the molecular mechanisms underlying autophagy, and particularly how autophagosome–lysosome fusion is regulated, is therefore central for elucidating its physiological and pathological roles.

2.1.1 Molecular machinery of autophagy

2.1.1.1 Autophagy initiation, phagophore nucleation and autophagosome maturation

Autophagy proceeds through a series of membrane remodeling events driven by the Atg proteins. The process starts with induction, typically following activation of AMP-activated protein kinase (Ampk) and/or inhibition of mammalian target of rapamycin complex 1 (mTorc1), which enables activation of the Ulk1/2 kinase complex, consisting of Ulk1, autophagy-related protein 13 (Atg13), Focal adhesion kinase family interacting protein of 200 kDa (Fip200, also called RB1-inducible coiled-coil protein 1 (Rb1cc1)) and Atg101 (Fig. 2; Hu and Reggiori, 2022). In selective autophagy, cargo binding by autophagy receptors can promote local activation of the Ulk1 complex by recruiting the Ulk machinery through Fip200, thereby coupling cargo recognition to local phagophore formation (Ravenhill, et al., 2019, Turco, et al., 2019, Zientara-Rytter and Subramani, 2020). The active Ulk1/2 kinase complex phosphorylates components of the class III phosphoinositide 3-kinase (Pi3kc3) complex I, thereby promoting PI3P production at early autophagic membranes (Fig. 2; Hu and Reggiori, 2022).

The autophagy-specific PI3K complex (complex I) is distinct from the related complex II that mainly functions in endosomal trafficking (Kihara, et al., 2001). In mammals, complex I comprises Vps34, p150 (Vps15), Beclin-1, Atg14l and nuclear factor erythroid 2 -related factor (Nrbf2) (Kihara, et al., 2001). This will lead to

production of the phospholipid phosphatidylinositol-3-phosphate (PI3P) at the omegasome, an omega (Ω)-shaped subdomain of the endoplasmic reticulum (ER) where autophagic membranes are generated in mammalian cells (Fig. 2; Axe, et al., 2008). PI3P enrichment leads to recruitment of WD repeat domain phosphoinositide-interacting proteins (Wipis) and Zinc-finger FYVE domain-containing protein 1 (Zfyve1, also called Dfcp1) by interaction with the PI3P-binding domains of these proteins (Fig. 2; Axe, et al., 2008, Muller and Proikas-Cezanne, 2015).

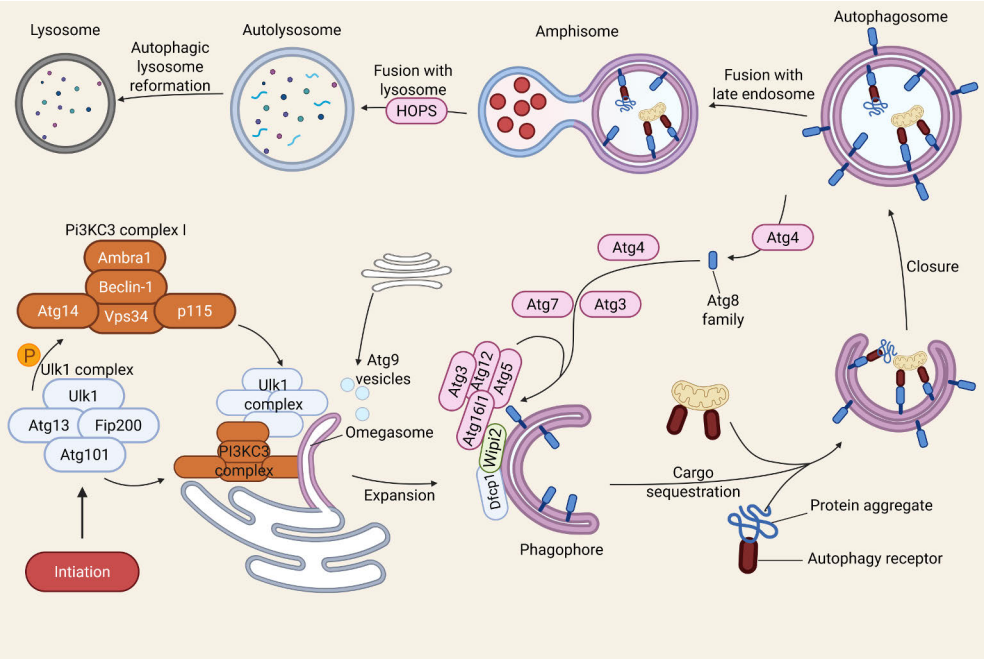

Figure 2. Core molecular machinery controlling autophagosome biogenesis, cargo sequestration and lysosomal delivery in macroautophagy. Autophagy is initiated by activation of the Ulk1 kinase complex (Ulk1–Atg13–Fip200–Atg101), which promotes recruitment/activation of the autophagy-specific class III Pi3k complex I (Beclin-1–Vps34–Atg14 and associated regulators) at ER subdomains termed omegasomes. Atg9-containing vesicles contribute membrane input to early autophagic intermediates. Local PI3P production by the class I PI3K complex supports recruitment of PI3P-binding factors (e.g., Dfcp1 and Wipi proteins) and assembly of the Atg conjugation machinery, including the Atg12–Atg5–Atg16l1 complex and Atg3, which together catalyze lipidation of Atg8-family proteins (LC3/Gabarap (blue bars)) on the growing phagophore. Cargo can be captured during phagophore expansion through selective autophagy receptors that link substrates (e.g., aggregates or damaged organelles like mitochondria) to LC3/Gabarap on the inner phagophore membrane. Phagophore closure yields a sealed autophagosome, which may fuse with a late endosome to form an amphisome before fusion with lysosomes. Autophagosome/amphisome–lysosome tethering and fusion is depicted as HOPS-dependent. Atg4-mediated deconjugation recycles Atg8-family proteins from the outer autophagosomal membrane. Following cargo degradation within the autolysosome, lysosomes are regenerated through autophagic lysosome reformation. Created in BioRender.

Atg9a is the only core Atg protein with multiple transmembrane segments. It cycles between intracellular membrane compartments and nascent autophagic structures (Fig. 2; Suzuki, et al., 2015), which develop into the phagophore, a crescent-shaped isolation membrane that expands to engulf cytoplasmic material during autophagosome formation. The membrane material for phagophore elongation is, at least in part, sourced from the ER. In addition, plasma membrane, mitochondria, recycling endosomes and the Golgi apparatus may also provide membrane material (Fig. 2). A major mechanism for phagophore expansion is lipid transfer from the ER, mediated by Atg2 proteins at ER–phagophore contact sites. *In vitro* reconstitution supports lipid-transfer and tethering activities for Atg2-family proteins (Valverde, et al., 2019). During the phagophore expansion, newly supplied lipids initially accumulate in the cytosolic leaflet and must be redistributed across the bilayer; Atg9a has therefore been proposed to act as a lipid scramblase that equilibrates lipids between the two leaflets of the growing phagophore membrane (Matoba, et al., 2020).

Further, Wipi2 recruits the Atg12-Atg5-Atg16l1 complex, which enables Atg3 to conjugate Atg8 family proteins to membrane-associated phosphatidyl-ethanolamine (PE) on the expanding phagophore (Fig. 2; Dooley, et al., 2014). Mammals have are seven Atg8 family proteins, formed by two sub-families: The Gamma-aminobutyric acid receptor-associated protein (Gabarap) subfamily consisting of Gabarap, Gabarapl1, and Gabarapl2. The light chain 3 (LC3) subfamily (also known as microtubule-associated proteins 1A/1B LC3, Map1LC3) consisting of Map1LC3a (LC3A), Map1LC3b (LC3B), Map1LC3b2 (LC3B2), and Map1LC3c (LC3C). During conjugation to PE, Atg8 family proteins are converted into their membrane-bound, lipidated form, e.g. LC3-I is converted into LC3-II during lipidation. Before lipidation, Atg8/LC3-family proteins must be proteolytically processed by Atg4 proteases to expose the C-terminal glycine that is required for conjugation to PE. The lipidation reaction is similar to ubiquitin conjugation, and executed by the Atg7 (E1-like) and Atg3 (E2-like) enzymes, and is promoted by the E3-like Atg12–Atg5–Atg16l1 complex (Fig. 2; Mizushima, 2020).

The lipidation and attachment to the phagophore membrane of Atg8 proteins serves multiple functions. Firstly, Atg8 proteins are needed for cargo sequestration via selective autophagy receptors containing LC3-interacting regions (LIRs), also called Atg8 interacting motifs (AIMs). Many autophagy receptors have been identified, among them Sequestosome-1 (Sqstm1, also called p62), Next to Brca1 gene 1 protein (Nbr1), Optineurin (Optn) and others. Selective autophagy is achieved by autophagy receptors that act as molecular bridges between cargo and the phagophore membrane. Many receptors are soluble adaptor proteins that recognize cargo tagged with ubiquitin through ubiquitin-binding domains and simultaneously bind lipidated Atg8-family proteins on the phagophore via LIRs/AIMs, thereby

mediating cargo specificity and promoting sequestration (Vainshtein and Grumati, 2020). In addition, some selective autophagy pathways use receptors that are embedded in the target organelle membrane (for example on mitochondria or the ER; Adriaenssens and Martens, 2025) and, upon activation by phosphorylation (Rogov, et al., 2017, Zhou, et al., 2021, Zhu, et al., 2013), directly recruit the autophagy machinery by binding Atg8-family proteins, enabling organelle-specific turnover without requiring a soluble adaptor.

Secondly, Atg8 proteins are needed for elongation of the phagophore. Specifically, Atg8 proteins are important for establishing and maintaining the membrane curvature of the phagophore together with a specific domain of Atg3 that can sense membrane curvature (Knorr, et al., 2014, Nath, et al., 2014). In addition, Atg8/LC3 proteins provide docking sites for multiple Atg and accessory factors through LIR/AIM motifs, integrating membrane growth with cargo capture during selective autophagy (Fig. 2; Lamark and Johansen, 2021). Lastly, Atg8 proteins also mediate closure of the phagophores resulting in the formation of a double-membrane vesicle, which is then called autophagosome. This process is explained in detail in the next paragraph.

The expanded phagophore becomes enriched in late-acting Atg proteins. The Atg8 family proteins on the inner and outer phagophore membranes facilitate membrane closure (Weidberg, et al., 2010). Gabarap and Gabarapl1 interact with the Soluble Nsf attachment protein receptor (SNARE)-associated protein Atg14l and are required for efficient sealing (Birgisdottir, et al., 2019, Padman, et al., 2017). Gabaraps further facilitate autophagosome maturation via recruitment of the lipid kinase Phosphatidylinositol 4-kinase type 2 (Pi4kIIα), while loss of Gabarap or Pi4kIIα leads to abnormally large autophagosomes (Wang, et al., 2015a).

Following the expansion, the phagophore membrane proceeds through a scission and sealing step to form the closed double-membrane autophagosome. The Endosomal sorting complex required for transport (ESCRT) system plays a central role in the sealing of the phagophore. In particular, the components of the ESCRT-III complex: Chmp2a,b, Chmp3, Chmp4a,b,c, Chmp5, Chmp6, Chmp7 and Ist1 are needed in this process. The ESCRT-III complex is recruited to the narrow membrane neck region of the closing phagophore surface. The complex mediates membrane constriction and fission from the cytosolic side; a process similar to the formation of luminal vesicles in multivesicular bodies (Knorr, et al., 2015, Takahashi, et al., 2018, Takahashi, et al., 2019, Zhen, et al., 2020). Depletion of ESCRT-III subunits has been shown to cause accumulation of unsealed autophagic membranes (Zhen, et al., 2020). The ATPase Vacuolar protein sorting-associated protein 4 (Vps4) is another important player in this process. Vps4 acts by constricting and cleaving ESCRT-III filaments, which leads to the formation of a membrane neck structure (Maity, et al.,

2019). Finally, Vps4 mediates disassembly and recycling of ESCRT-III filaments (Babst, et al., 1998, Yang and Hurley, 2010).

After autophagosome completion, maturation involves release of most Atg proteins from the outer limiting membrane and turnover of PI3P (e.g., by myotubularin-family phosphatases). Atg8/LC3 on the outer autophagosomal membrane is removed by Atg4-mediated delipidation (Fig. 2; Mizushima, 2020, Wu, et al., 2014). During phagophore expansion, Atg4 activity is restrained by Ulk1/Atg1-dependent regulation to prevent premature Atg8/LC3 removal, whereas Atg4-mediated delipidation becomes important again after autophagosome closure (Pengo, et al., 2017, Sánchez-Wandelmer, et al., 2017).

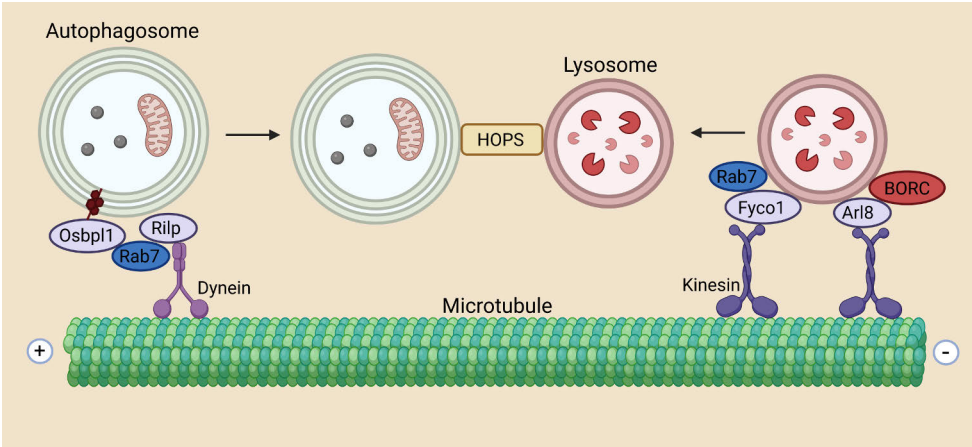

Figure 3. Microtubule-dependent transport of autophagosomes and lysosomes facilitates their encounter and fusion. An autophagosome is shown engaging the retrograde motor Dynein via Rab7 and its effectors Rilp and Osbpl1, supporting movement toward the microtubule minus-end and promoting delivery to the perinuclear region. Lysosomes are depicted as undergoing anterograde transport toward the microtubule plus-end through coupling to Kinesin via Rab7–Fyco1 and via the lysosome-positioning machinery Arl8-BORC, which also recruits Kinesin. Convergent transport of both compartments increases the probability of encounter; tethering of an autophagosome to a lysosome is illustrated as HOPS-dependent, preceding membrane fusion (not shown). Created in BioRender.

2.1.1.2 Autophagosome transport

After autophagosome completion, the cargo of the mature vesicles must be delivered to lysosomes for degradation. Autophagosomes can originate at any location within the cytoplasm, but lysosomes are concentrated in the perinuclear region of most mammalian cells (Ba, et al., 2018). Therefore, directed movement of autophagosomes towards the cell center along microtubules is required (Fig. 3).

Autophagosome movement is mediated primarily by microtubule-based motor proteins. The Dynein-Dynactin motor complex drives retrograde transport of autophagosomes towards the minus ends of microtubules located near the microtubule-organizing center (MTOC), while Kinesin motors mediate anterograde movement toward the cell periphery (Fig. 3; Boda, et al., 2025, Kimura, et al., 2008). Motor proteins are recruited to the autophagosome membrane by adaptor and scaffold proteins that recognize lipidated LC3. FYVE and coiled-coil domain-containing protein 1 (Fyco1) associates with LC3 and the small GTPase Rab7a to promote kinesin-dependent anterograde trafficking, whereas Jnk-interacting proteins 3 and 4 (Jip3, Jip4) and Rab-interacting-lysosomal protein (Rilp) links autophagosomes to Dynein and regulates retrograde movement (Fig. 3; Cason and Holzbaur, 2023, Khobrekar, et al., 2020, Pankiv, et al., 2010).

The interplay between these adaptor–motor systems produces bidirectional motion, allowing autophagosomes to traverse the intracellular space until fusion-competent lysosomes are encountered (Fig. 3). Disruption of these trafficking steps results in impaired autophagic flux, accumulation of autophagic intermediates, and defective cargo clearance, highlighting the importance of microtubule-based transport for cellular homeostasis.

Neuron-specific adaptations and constraints of these transport processes are described in 2.1.3.1.1 *Neuron-specific organization and challenges of the autophagy-lysosome system.*

2.1.1.3 Amphisomes

Once the autophagosome has fully matured and begun its journey to the lysosome, it can fuse with late endosomes (LE) or multivesicular bodies (MVB). The intermediate organelle formed in this fusion process is called amphisome (Gordon, et al., 1992, Liou, et al., 1997). This fusion step can be seen as an additional step in autophagosome maturation as the autophagosome is supplied with additional molecular machinery to mediate transport and fusion with the lysosome including motor proteins, SNARE complexes, tethering factors and Rab GTPases.

Amphisome formation is mediated by the same machinery that mediates vesicle fusion with the lysosome and is described in section 2.1.1.4 *Fusion with the lysosome*. An interesting exception is the HOPS complex – depletion of HOPS subunits causes formation of enlarged, non-acidified hybrid endosomal compartments that contain both early and late endosome markers and also accumulate LC3 and p62, indicating that amphisome-like compartments can form even when HOPS-dependent lysosomal fusion is impaired (van der Beek, et al., 2024). Autophagosome-LE fusion requires is Syntaxin-17, a Q/t-SNARE that is recruited to the autophagosome via its LIR domain (Itakura, et al., 2012b, Takats, et

al., 2013). Prevention of amphisome formation by RNA interference (RNAi) of Syntaxin-17 in rat dorsal root ganglion (DRG) neurons causes accumulation of autophagic vacuoles in neurites and synaptic terminals (Cheng, et al., 2015). Likewise, in zebrafish neurons, a HOPS-mediated fusion event is needed for initiation of autophagosome transport from axon terminals to lysosomes in the soma (Wisner, et al., 2024).

In addition, amphisomes provide an intersection between the autophagic and endocytic pathways. In recent years, amphisomes have been shown to be more than just an intermediate organelle on the way to lysosomal degradation. For example, ESCRT complexes regulate late endosome maturation by mediating intraluminal vesicle formation and cargo sorting in multivesicular bodies, thereby influencing which endocytic cargoes are delivered for degradation (Henne, et al., 2011). However, ESCRTs have been reported to be required also for autophagosome closure (Filimonenko, et al., 2007, Lee, et al., 2007, Rusten, et al., 2007). Mutation in CHMP2 that is associated with frontotemporal dementia has been shown to interfere with autophagic and endolysosomal degradation, which leads to protein aggregation (Clayton, et al., 2015, Skibinski, et al., 2005). This phenotype is most likely linked to the function of ESCRT III in autophagosome closure (described above).

Another neuronal function where amphisomes have been implicated is retrograde neurotrophic signaling. Brain-derived neurotrophic factor (Bdnf) is a member of the neurotrophin family of growth factors that governs synaptogenesis, synaptic plasticity and axonal outgrowth through retrograde signaling mechanisms (Colucci-D'Amato, et al., 2020, Deinhardt, et al., 2006, Villarroel-Campos, et al., 2026). Bdnf binds to its receptor, the Tyrosine receptor kinase (Trk), which is endocytosed into a special class of endosomes termed signaling endosomes that undergo retrograde axonal transport to relay signals from the axon terminal to the soma. This process has recently been linked to amphisomal trafficking. Studies using Botulinum neurotoxin (BoNT) as a tracer that co-travels with signaling endosomes carrying the Neurotrophin receptor $p75^{NTR}$, have shown that autophagosomes can sequester BoNT and mediate its retrograde movement along axons. The presence of endocytic cargo within these autophagic vesicles suggests that they are amphisomes (Wang, et al., 2016a, Wang, et al., 2015b). Moreover, it was recently shown that in hippocampal neurons, Bdnf/TrkB receptors are trafficked within amphisomes that not only mediate retrograde transport toward the soma but also engage in local presynaptic signaling. This signaling–transport coordination is regulated by the Rab GTPase-activating protein (GAP) Signal-induced proliferation-associated 1-like protein 2 (Sipa1l2), which links TrkB amphisomes to Dynein and modulates their activity to promote Bdnf-dependent presynaptic plasticity (Andres-Alonso, et al., 2019).

Classically, amphisomes have been defined as an intermediate organelle of the autophagy pathway and hence as degradative. However, mounting evidence suggests that amphisomes can also be secretory. In goblet cells of the colonic epithelium, functional autophagy was shown to be required for maintaining mucus secretion. Notably, approximately 70% of LC3 puncta in this cell type were found to co-localize with the Early endosome antigen 1 (Eea1) and therefore identified as amphisomes (Patel, et al., 2013). Similarly, defective autophagy prevented the Interferon γ (Ifnγ)-induced secretion of Annexin2 (Anxa2) in lung epithelial cells, which was shown to be dependent on amphisome fusion with the plasma membrane (Chen, et al., 2017a).

2.1.1.4 Fusion with the lysosome

After the autophagosome or amphisome has reached the lysosome, the two organelles are tethered together by the HOPS complex (Fig. 4; Jiang, et al., 2014, Takáts, et al., 2014). The recruitment of HOPS to the autophagosomal membrane is coordinated through Rab7a-positive late endosomal/lysosomal compartments and Rab7a-dependent adaptor/effector pathways, thereby supporting spatial coordination of fusion sites (Ganley, et al., 2011, Hickey, et al., 2009, Jiang, et al., 2014). Once tethering is established, HOPS facilitates the assembly of the SNARE machinery necessary to drive membrane fusion (Fig. 4B-D).

Rab7a has been viewed as a key regulator of the terminal fusion steps of macroautophagy, based on studies in which Rab7a perturbation by knockdown and dominant-negative construct caused accumulation of autophagic vacuoles and impaired formation of fully degradative organelles (Gutierrez, et al., 2004, Jäger, et al., 2004). Rab7a knockout studies refined this view by demonstrating that, under nutrient-rich conditions, Rab7a loss led to accumulation of LC3-positive, Lamp-positive autolysosome-like structures, indicating that autophagosome–late endosome/lysosome fusion can still occur but that efficient progression of autolysosomes (i.e., maturation and/or clearance) is compromised (Kuchitsu, et al., 2018). Moreover, in Rab7a-deficient cells, starvation conditions could rapidly clear the accumulated amphisomal/autolysosomal structures through Rab7a-independent mechanisms, suggesting that the relative requirement for Rab7a differs between nutrient-rich/basal conditions and starvation-induced autophagy states (Kuchitsu, et al., 2018). Thus, in mammalian systems Rab7a can be rate-limiting for autolysosome maturation under basal conditions without being universally required for the fusion event itself (Kuchitsu, et al., 2018, Matsui, et al., 2018).

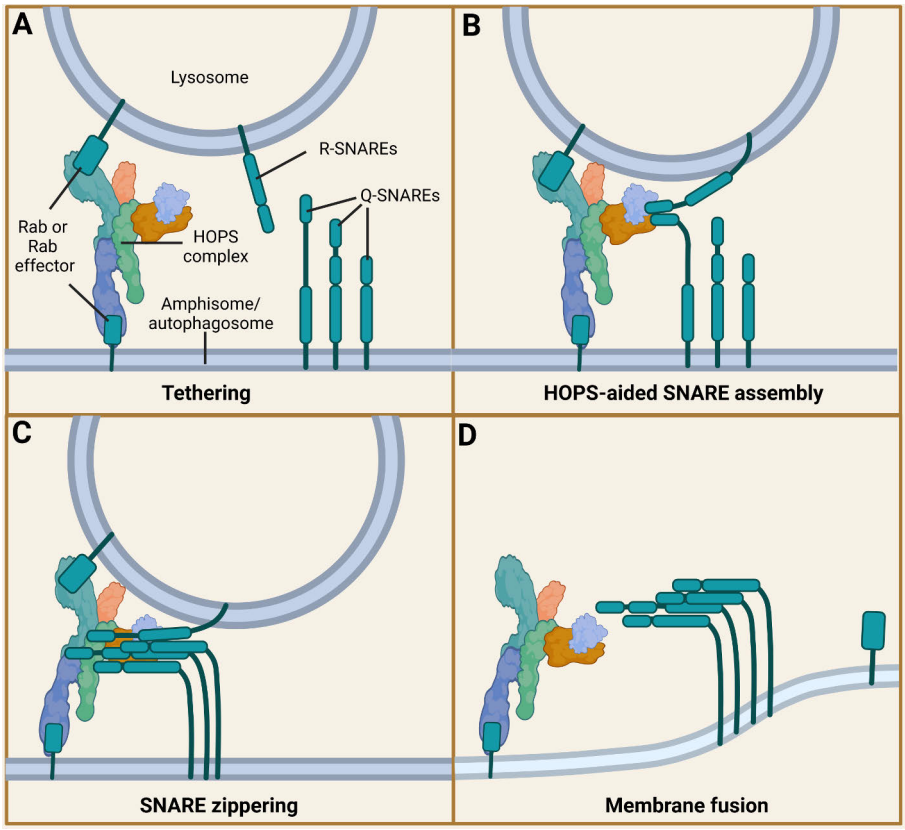

Figure 4. Model of HOPS-mediated tethering and SNARE-driven fusion at the lysosome. Schematic overview of the sequential steps leading to fusion of a lysosome with an incoming vesicle (e.g., late endosome, autophagosome or amphisome). (A) The multisubunit HOPS tethering complex is recruited to the two membranes through interactions with membrane identity markers such as Rab GTPases and/or Rab effector proteins, establishing an initial tethered contact. (B) HOPS facilitates assembly of a trans-SNARE complex by promoting productive pairing of vesicle- and lysosome-associated SNAREs. (C) Progressive SNARE "zippering" draws the two membranes into close apposition and provides the driving force for bilayer rearrangement. (D) Following membrane fusion, a single continuous membrane is formed. The SNARE complex and HOPS complex remain associated with the fused membrane prior to downstream disassembly and recycling steps (not depicted). Created in BioRender.

The canonical SNARE complex in autophagosome-lysosome fusion comprises Syntaxin-17 (Stx17) and Synaptosomal-associated protein 29 (Snap29), which assemble on the outer membrane of the autophagosome, and the lysosomal R-SNARE Vesicle-associated membrane protein 8 (Vamp8) or its close homolog Vamp7 on the opposing lysosomal membrane (Itakura, et al., 2012a, Jiang, et al., 2014, Orr, et al., 2017, Shen, et al., 2021). The interaction among these SNAREs forms a tight trans-complex that brings the membranes into close proximity, promoting lipid bilayer merger and content mixing.

Pleckstrin homology domain-containing family M member 1 (Plekhm1) acts as a critical adaptor coordinating the interactions between Rab7a, HOPS and the SNARE proteins (McEwan, et al., 2015; see also 2.3.2 *Recruitment of HOPS in metazoan cells*). In addition to the Rab7a–Plekhm1–HOPS complex, other cofactors such as Ectopic P granule protein 5 homolog (Epg5) and Tectonin β-propeller repeat-containing protein 1 (Tecpr1) function in autophagosome–lysosome fusion. Epg5 acts as a Rab7a effector that bridges mature autophagosomes with late endosomes and lysosomes by simultaneously binding LC3/Gabarap family proteins and the SNARE components Stx17–Snap29–Vamp7/8, thereby stabilizing trans-SNARE assembly and preventing aberrant vesicle fusion (Nam, et al., 2021, Wang, et al., 2016b). Mutations in Epg5 disrupt this tethering process and underlie Vici syndrome, highlighting its essential role in selective autophagosome maturation (Nam, et al., 2021, Wang, et al., 2016b). Complementing this function, Tecpr1 interacts with the Atg12–Atg5 conjugate and PI3P, recruiting Atg5 to autolysosomal membranes and initiating autophagosome maturation. Loss of Tecpr1 leads to autophagosome accumulation and impaired LC3-II and p62 turnover (Chen, et al., 2012).

2.1.1.5 Autophagic lysosome reformation

Autophagic lysosome reformation (ALR) is essential for the regeneration of functional lysosomes after autophagy, sustaining lysosomal homeostasis and proteostasis. ALR regenerates lysosomes from autolysosomes during prolonged starvation via the formation of Lamp1-positive tubules that bud to form proto-lysosome intermediates (Chen and Yu, 2018). ALR starts when mTor signaling is reactivated by release of amino acids via lysosomal degradation (Yu, et al., 2010). Rab7a was identified as a key regulator of this process: Rab7a associates with autolysosomes early during starvation but must dissociate from the lysosomal tubules/proto-lysosome fractions for lysosome reformation to proceed. Constitutively active, persistently membrane-bound Rab7a mutants block ALR, resulting in enlarged long-lasting autolysosomes (Yu, et al., 2010). Disruption of ALR through impaired mTor-Vps34 signaling or defective phosphoinositide metabolism causes abnormal lysosomal tubulation, vacuolization, and cell death under stress (Munson, et al., 2015, Uwada, et al., 2025).

2.1.2 Basal and stress-induced autophagy

The constitutive form of autophagy, also called basal autophagy, operates continuously. Opposed to this is the robust induction of autophagy under stress conditions termed (stress-)induced autophagy. Both forms largely rely on the core

autophagic machinery described above, but differ in their regulatory inputs, magnitude of activity, and cargo selectivity.

Under nutrient-rich and homeostatic conditions, basal autophagy continuously turns over long-lived proteins, excess membranes and damaged organelles. Basal autophagy acts as a housekeeping pathway critical for proteostasis, organelle quality control and metabolic balance. Basal autophagy removes misfolded or aggregated proteins before they accumulate and supports lipid and glycogen metabolism by mobilizing intracellular stores when metabolic demand fluctuates (Mizushima and Komatsu, 2011, Singh, et al., 2009).

The Ulk1 complex is canonically activated under nutrient stress, especially under amino-acid starvation. However, localized Ulk1 activation can also appear under basal conditions. The recruitment of Ulk1 has been observed even in the absence of starvation, where adaptor proteins such as Tax1-binding protein 1 homolog (Tax1bp1) can facilitate Ulk-complex recruitment and promote local autophosphorylation-driven kinase activity (Mercer, et al., 2021, Sarraf, et al., 2020, Torggler, et al., 2016). This suggests that micro-domains of Ulk1 activation can form independently of nutrient cues, providing a mechanism for maintaining basal autophagy in response to localized damage or misfolded protein accumulation. There are other cues that can drive microdomains of autophagic turnover without global pathway activation. Autophagic turnover can be driven by local variations in signaling inputs, such as transient activation of subcellular Ampk in response to local energetic stress and reactive oxygen species (ROS) gradients on mitochondrial microdomains, which in turn stimulate autophagy initiation at specific sites (Drake, et al., 2021, Koren, et al., 2023, Zhang, et al., 2016c).

In multicellular organisms, basal autophagy is not uniform across tissues but is regulated in a tissue-specific manner. In particular, neurons sustain constitutive autophagic flux under physiological conditions. Genetic ablation of core autophagic genes Atg5 and Fip200 in neurons of mice leads to neurodegeneration even without starvation, specifically of Purkinje neurons of the cerebellum (Hara, et al., 2006, Liang, et al., 2010). Similarly, neuronal deletion of Atg7 and Beclin-1 causes degeneration of hippocampal neurons in mice housed under normal conditions (Komatsu, et al., 2006, Pickford, et al., 2008). This suggests a high demand for continuous autophagy in the post-mitotic neuronal cells, especially in Purkinje cells.

Likewise, hepatocytes show high basal autophagic protein turnover under non-stress conditions. Hepatocyte-specific deletion of Atg7 lead to the development of hepatomegaly associated with parenchymal injury, pericellular fibrosis and ductular reduction (Kwanten, et al., 2016). Further, it was shown that systemic mosaic deletion of Atg5 and liver-specific deletion of Atg7 in mice causes the development of benign liver adenomas originating from autophagy-deficient hepatocytes

(Takamura, et al., 2011). The relevance of basal autophagy in the liver is further supported by finding from Haspel et al., who assessed LC3B protein turnover in the liver, heart, lung, kidney and spleen of mice and found the highest rate of basal autophagy in the liver (Haspel, et al., 2011). Together, these findings suggest a constitutive requirement for autophagic turnover in the liver to maintain metabolic and quality-control functions.

Another tissue in which autophagy plays a central and context-dependent role is skeletal muscle. Autophagy functions both as a mediator of protein degradation during atrophy and as a quality-control mechanism required for myofiber homeostasis. In catabolic conditions such as denervation (the loss of nerve supply to a tissue), fasting, and systemic disease, autophagic flux is transcriptionally upregulated as part of a coordinated atrophy program, with key autophagy-related genes including LC3 and Gabarap among the commonly induced genes (Bodine, et al., 2001, Franco-Romero, et al., 2025, Gomes, et al., 2001, Mammucari, et al., 2007, O'Leary, et al., 2012, Zhao, et al., 2007). This autophagy activation contributes to enhanced proteolysis and myofiber atrophy, as demonstrated by genetic models in which suppression of autophagy-related genes, such as LC3, attenuates muscle loss induced by factors like Forkhead box protein O3 (Foxo3) activation or oxidative stress (Dobrowolny, et al., 2008, Mammucari, et al., 2007). Mechanistically, transcription factors such as Foxo3 orchestrate the response by upregulating multiple components of the autophagy machinery, including Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3), Vps34, and Atg proteins, thereby sustaining autophagic degradation during prolonged stress (Mammucari, et al., 2007, Tracy and Macleod, 2007).

However, basal autophagy is equally essential for maintaining muscle integrity, as muscle-specific deletion of core autophagy genes such as Atg7 or Atg5 results in accumulation of protein aggregates, dysfunctional mitochondria, oxidative stress, and progressive myofiber degeneration (Masiero, et al., 2009, Raben, et al., 2008). In this context, selective autophagy pathways mediated by adaptor proteins such as Sqstm1/p62 and co-chaperone systems (e.g., Bcl2-associated athanogene 3 (Bag3)-dependent targeting of damaged filamin) ensure the removal of misfolded proteins and damaged organelles (Arndt, et al., 2010, Homma, et al., 2006). Furthermore, proper autophagosome maturation and lysosomal fusion are critical for efficient cargo clearance, and their disruption leads to pathological accumulation of undegraded autophagic vesicles and myopathy (Ju, et al., 2009). Collectively, these findings highlight that tight regulation of autophagy is required in skeletal muscle, as both excessive activation and impaired autophagic flux can contribute to muscle wasting and disease.

2.1.3 Autophagy in disease

Autophagy plays a central role in cellular homeostasis by balancing catabolic and anabolic processes. Given its role in protein and organelle quality control, metabolism, immune responses and cell survival, disturbances in autophagic flux are linked to numerous human diseases. The outcome of altered autophagy is context- and cell-type-dependent. Further, insufficient autophagic activity compromises cellular clearance and adaptive stress responses (Nixon and Rubinsztein, 2024), while excessive or dysregulated autophagy may lead to cell death (Huang, et al., 2025). Consequently, both loss and gain of functional autophagy have been implicated in pathogenesis.

At the mechanistic level, disease-related alterations in autophagy often stem from mutations of genes encoding core Atg components, lysosomal enzymes, or regulators of nutrient- and energy-sensing pathways such as mTor, Ampk, and Transcription factor EB (Tfeb; Deneubourg, et al., 2021, Fraiberg and Elazar, 2020). These defects can disturb all steps of the autophagic pathway, ranging from autophagosome formation to lysosomal fusion and degradation and lead either to accumulation of undegraded substrates or inappropriate turnover of essential cellular constituents. In addition, signaling pathways activated by inflammation (Djavaheri-Mergny, et al., 2006, Yuan, et al., 2018), metabolic imbalance (Li, et al., 2024, Yamamoto, et al., 2017), or DNA damage (Liu, et al., 2018, Torii, et al., 2016) can either suppress or induce autophagy. The resulting shifts in autophagic activity are closely tied to disease mechanisms across multiple systems, including the nervous system, liver, cardiovascular tissues, and the immune system.

Autophagy can serve both cytoprotective and cytotoxic roles, depending on the temporal and physiological context and cell type. In some settings, upregulated autophagy allows cells to adapt to stress by removing damaged organelles and providing metabolic substrates. In others, prolonged excessive autophagic activity, a process termed autosis, can lead to cell death (Kheloufi, et al., 2015, Liu, et al., 2013b, Nah, et al., 2020). Hence, understanding when, where and to what extent autophagy is regulated is essential for interpreting its diverse roles in pathology.

Among the best-characterized contexts are neurodegenerative diseases and cancer, which exemplify the dualistic nature of autophagy. In neurodegeneration, impaired autophagic clearance of misfolded proteins or dysfunctional mitochondria results in toxic intracellular accumulation and neuronal loss (Nixon and Rubinsztein, 2024). In cancer, autophagy may function either as a tumor-suppressive mechanism by limiting genomic instability and oncogenic stress or as a survival mechanism that enables tumor cells to endure metabolic and therapeutic stresses (Verma, et al., 2021). The following subsections therefore focus on these two major disease categories, highlighting how context-specific modulation of autophagy can shape disease onset, progression, and therapeutic response.

2.1.3.1 Autophagy in neurodegenerative diseases

Neurons face unique challenges for cellular homeostasis due to being postmitotic, extremely polarized, and having an exceptionally long lifespan. Unlike dividing cells, neurons cannot dilute damaged components through cell division, which makes them heavily reliant on degradative pathways such as autophagy and the proteasome system to maintain proteostasis and organelle quality control over decades. Consequently, even subtle disruptions to autophagic or lysosomal function can have cumulative and irreversible effects on neuronal health (Menzies, et al., 2017, Nixon and Rubinsztein, 2024).

Defective autophagy has emerged as a central pathogenic feature across a spectrum of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and frontotemporal dementia (Hara, et al., 2006, Komatsu, et al., 2006, Nixon and Rubinsztein, 2024). In these disorders, the failure to efficiently degrade misfolded or aggregation-prone proteins such as Amyloid-β (Aβ), Tau, α-Synuclein, and mutant Huntingtin leads to accumulation and subsequent formation of toxic inclusions, which triggers cellular stress, synaptic dysfunction, and eventual neurodegeneration.

A unifying concept among these conditions is autophagic–lysosomal insufficiency, in which the balance between autophagosome formation and lysosomal degradation is disrupted (Nixon and Rubinsztein, 2024). This imbalance can arise from impaired autophagosome biogenesis, defective trafficking and fusion with lysosomes, or compromised lysosomal clearance capacity, including loss of hydrolase activity and compromised acidification (Bae, et al., 2015). Over time, these disturbances lead to autophagic vacuole accumulation, mitochondrial dysfunction and metabolic stress, as well as selective neuronal loss. Understanding how normal autophagy fails in the ageing and diseased brain is therefore critical for elucidating the mechanisms of neurodegenerative disorders and identifying targets for therapeutic intervention.

2.1.3.1.1 Neuron-specific organization and challenges of the autophagy-lysosome system

Neurons present unique demands on autophagy to maintain homeostasis over the lifetime. Unlike dividing somatic cells, neurons are post-mitotic and strongly polarized with axons and dendrites that can extend over vast distances (Maday, et al., 2012). This specialized cell shape limits when and where degradative trafficking can take place. Autophagic flux must be coordinated between distal sites, e.g. axon terminals, and the soma, in which the majority of mature lysosomes are located (Stavoe and Holzbaur, 2019). As described in section 2.1.1.2 *Autophagosome*

transport, efficient long-range retrograde transport along microtubules is therefore indispensable to sustain neuronal proteostasis.

Recent imaging studies have revealed that autophagy in neurons operates within an integrated endosomal–lysosomal–autophagy network, dynamically interacting with the secretory system (Kulkarni, et al., 2022). Autophagosomes form close to the axon terminals, and are then transported towards the soma where the lysosomes localize (Maday, et al., 2012). Amphisome formation occurs during this transport (Cheng, et al., 2015) and provides an effective platform to deliver additional tethering and motor proteins that facilitate long-distance transport and tethering to lysosomes in the soma (Cheng, et al., 2015). This spatial organization allows autophagic compartments formed at distal synapses/axon terminals to be loaded with endosomal components during transport, while the final degradative step still mainly occurs after delivery to lysosome-rich regions in the soma..

However, this complexity makes neurons particularly sensitive to perturbations in vesicular trafficking and lysosome positioning. Spatial separation between autophagosome formation and lysosomal degradation introduces vulnerability. Any disruptions in axonal transport (Tammineni, et al., 2017), fusion efficiency (Luo, et al., 2024), or pH homeostasis within lysosomes (Lee, et al., 2011) lead to the progressive accumulation of undegraded autophagic vacuoles, a pathological hallmark observed early in multiple neurodegenerative diseases. In many disease states, autophagic vacuoles accumulate in axonal spheroids (Nixon, et al., 2005, Sanchez-Varo, et al., 2012). Moreover, neuron-specific demands such as neuro-transmission, local energy fluctuations, and region-specific mitochondrial turnover require continuous feedback between the autophagy pathway and other metabolic networks (Lu, et al., 2026a). Evidence from ageing models indicates that decline in lysosomal acidification capacity (Burrinha, et al., 2023) and impaired autophagosome clearance (Pareja-Cajiao, et al., 2021, Stavoe, et al., 2019, Tsong, et al., 2023) intensify with age, further compromising synaptic integrity and neuronal survival.

2.1.3.1.2 Defects in early steps of autophagy across neurodegenerative diseases

Early steps of the autophagy pathway, including autophagosome initiation, phagophore expansion, and cargo recognition represent crucial control points in neuronal proteostasis. Disruption of these early steps of autophagy has emerged as a pathogenic feature in several neurodegenerative disorders. Because neurons depend heavily on basal autophagy to clear damaged organelles and misfolded proteins, even partial loss of efficiency in the initiation or sequestration steps can be sufficient to initiate a cascade of toxic events that can result in neurodegeneration.

A prominent example of selective cargo recognition coupled to early autophagy steps is Pink1/Parkin-mediated mitophagy, which is centrally regulated by Pten-induced kinase 1 (Pink1)-E3 ubiquitin-protein ligase (Parkin) and strongly linked to Parkinson's disease. Under basal conditions, Pink1 is imported into mitochondria and rapidly degraded, while Parkin remains largely autoinhibited in the cytosol (Narendra, et al., 2008, Narendra, et al., 2010). Upon mitochondrial damage, Pink1 accumulates at the outer mitochondrial membrane (via the Translocase of the outer membrane (Tom) complex), undergoes activation, and phosphorylates ubiquitin and Parkin, thereby relieving Parkin autoinhibition and triggering its E3 ligase activity (Kane, et al., 2014, Kazlauskaite, et al., 2014, Koyano, et al., 2014). Parkin then ubiquitinates multiple outer mitochondrial membrane proteins, generating ubiquitin chains that can be further phosphorylated by Pink1 (Kane, et al., 2014, Ordureau, et al., 2014). This ubiquitin signaling recruits autophagy receptors such as Optn, Sqstm1/p62 and Calcium-binding and coiled-coil domain-containing protein 2 (Calcoco2, also called Ndp52), which bind both ubiquitin and Atg8 proteins and thus connect damaged mitochondria to LC3-positive phagophores and promote lysosomal degradation (Heo, et al., 2015, Lazarou, et al., 2015, Wong and Holzbaur, 2014). Autosomal recessive mutations in Pink1 or Parkin disrupt this cascade, resulting in inefficient mitochondrial clearance, increased oxidative stress and selective vulnerability of dopaminergic neurons, consistent with early-onset Parkinsonian neurodegeneration (Kitada, et al., 1998, Pickrell and Youle, 2015, Valente, et al., 2004).

In Parkinson's disease and Huntington's disease, autophagic failure is frequently caused by defects in autophagosome biogenesis rather than lysosomal dysfunction. Mutations in genes such as Leucine-rich repeat serine/threonine-protein kinase 2 (Lrrk2) (Saha, et al., 2015), Vps35 (Zavodszky, et al., 2014), and Atg5/7 (Hara, et al., 2006, Komatsu, et al., 2006, Komatsu, et al., 2007, Nishiyama, et al., 2007) affect phagophore formation and membrane elongation, thereby reducing autophagic flux. Another example in Parkinson's disease is the accumulating α-Synuclein aggregates, which activate Signal transducer and activator of transcription 1 (Stat1) in microglia via Toll-like receptor 4, which in turn suppresses transcription of the autophagy-initiating kinase Ulk1. Reduced Ulk1 levels impair autophagy initiation, leading to disrupted microglial clearance capacity, increased neuroinflammation, and exacerbation of dopaminergic neuron loss (Pei, et al., 2024). Similarly, in a Huntington's disease model, impaired Ulk1-dependent phosphorylation of Atg14 and Beclin-1 lowers the activity of Pi3kc3, leading to defective autophagosome formation and reduced clearance of mutant Huntingtin aggregates (Wold, et al., 2016).

Defects in autophagy receptors and their regulatory kinases play a central role in several neurodegenerative diseases by disrupting selective autophagy and

mitochondrial quality control. Tank-binding kinase 1 (Tbk1) phosphorylates multiple autophagy receptors, including Optn, Calcoco2, Tax1bp1, and Sqstm1/p62, thereby enhancing their ability to recognize ubiquitinated cargo and link it to the autophagic machinery (Deng, et al., 2020). Amyotrophic lateral sclerosis and frontotemporal dementia-associated mutations in Tbk1 or Sqstm1 reduce this phosphorylation, impairing selective autophagy and leading to inefficient clearance of ubiquitinated proteins and damaged organelles (Goode, et al., 2016, Richter, et al., 2016). Moreover, Tbk1 mutations can interfere with dimerization, recruitment to damaged mitochondria, or phosphorylation of the mitophagy receptor Optn, all of which compromise mitophagy and promote mitochondrial stress in neurons (Harding, et al., 2021).

Beyond inherited mutations, mitophagy can also decline with age and disease. In Alzheimer's disease, impaired mitophagy reduces removal of dysfunctional mitochondria, increasing oxidative damage and promoting both Aβ and Tau pathology, whereas pharmacological restoration of mitophagy has been reported to ameliorate cognitive deficits in experimental models (Fang, et al., 2019)

Suppression of autophagy induction plays a central role in the progression of neurodegenerative diseases by disrupting proteostasis and promoting the accumulation of toxic protein aggregates. In Alzheimer's disease models, Tau over-expression has been shown to suppress autophagosome formation by engaging T-cell intracellular antigen 1 (Tia1), an RNA-binding stress granule protein: Tau binding to the prion-related domain of Tia1 was associated with increased intracellular amino acid levels and consequenct activation of mTorc1 signaling, leading to inhibitory Ulk1 phosphorylation and reduced LC3 puncta, thereby exacerbating Tau accumulation (Li, et al., 2022). Similarly, altered regulation of the Akt serine/threonine kinase (Akt)–mTorc1 and Ampk pathways impairs autophagy induction in neurons, contributing to increased Aβ secretion and proteotoxic stress (Benito-Cuesta, et al., 2021a). Because Ampk normally promotes autophagy through inhibition of mTorC1 and activation of Ulk1, age-related decline in Ampk responsiveness further compromises autophagic clearance, elevates oxidative stress, and promotes chronic inflammation (Salminen and Kaarniranta, 2012). Pharmacological activation of Ampk, in contrast, can restore autophagic flux and protect against oxidative stress-induced senescence, supporting its role as a key metabolic regulator in neuronal survival (Han, et al., 2016). Combined with impaired insulin (Chow, et al., 2019, Liao, et al., 2025) and calcium signaling (Maimaiti, et al., 2017, Thibault, et al., 2013), which accompany neuronal aging, these disruptions disturb the balance between nutrient sensing and protein homeostasis.

In addition to Tau-driven suppression of autophagy initiation, Alzheimer's disease provides a clear example of how early autophagy steps can have bidirectional effects on pathogenic proteins. On one hand, Aβ and products derived from its

precursor can be degraded via the autophagy–lysosomal system, and pharmacological or genetic enhancement of autophagy has been reported to reduce Aβ accumulation in several experimental models (Cai and Ganesan, 2022, Nixon and Rubinsztein, 2024). On the other hand, autophagy can contribute to amyloidogenesis: autophagosomes have been described to be enriched in Amyloid β precursor like protein (App) and components of the amyloidogenic processing machinery, including β-secretase 1 (Bace1) and Presenilin 1 (Psen1), providing a compartment that can support intracellular Aβ generation (Chau, et al., 2024, Feng, et al., 2017, Jaeger, et al., 2010, Nilsson, et al., 2013, Pickford, et al., 2008, Yu, et al., 2005). Moreover, autophagy has been implicated in unconventional secretion of Aβ, as inhibition of neuronal autophagy was associated with increased intracellular Aβ but reduced extracellular plaque deposition, suggesting that secretory autophagy can contribute to amyloid release (Nilsson, et al., 2013). Further, dysregulation of autophagosome formation and maturation, for example linked to Phosphatidylinositol-binding clathrin assembly protein (Picalm) and Beclin-1 has been associated with impaired Aβ clearance and increased accumulation (Liang, et al., 1999, Moreau, et al., 2014, Pickford, et al., 2008, Rohn, et al., 2011, Small, et al., 2005, Swaminathan, et al., 2016, Xiao, et al., 2012). In neurons, impaired retrograde transport of autophagic vesicles can promote their accumulation at presynaptic sites, where they may act as reservoirs for Bace1 and thereby enhance local Aβ production (Feng, et al., 2017, Tammineni, et al., 2017). Collectively, these findings emphasize that early autophagy steps can contribute both to Aβ clearance and, under conditions of dysregulation, to Aβ production and release in Alzheimer's disease.

Finally, defects in early autophagy can also manifest through age- and state-dependent changes in non-neuronal cells. In Parkinson's disease, senescent glial cells have been reported to exhibit reduced autophagy–lysosome activity and diminished clearance of aggregated α-Synuclein, thereby sustaining neuroinflammation and contributing to neuronal death (Hong, et al., 2024).

In addition, autophagy in microglia and astrocytes can actively shape neurodegenerative disease progression by regulating neuroinflammation and the clearance of extracellular aggregates. In Alzheimer's disease models, microglial autophagy has been reported to restrain inflammasome activation and pro-inflammatory cytokine production, as microglia-specific Atg7 deletion shifts microglia toward a pro-inflammatory phenotype (Xu, et al., 2021) and LC3-positive vesicles have been observed to associate with NLR family pyrin domain containing 3 (Nlrp3) aggregates in Beclin-1+/– microglia (Houtman, et al., 2019). Moreover, microglia employ LC3-associated phagocytosis and LC3-associated endocytosis to support uptake, receptor recycling and lysosomal degradation of amyloid-β, with Atg5/Atg7-dependent LC3 recruitment enhancing phagosome maturation and cargo

clearance (Heckmann, et al., 2019, Lee, et al., 2019, Martinez, et al., 2011, Sanjuan, et al., 2007). In Parkinson's disease models, loss of key autophagy genes in microglia has been reported to increase neuroinflammation and dopaminergic neuron loss (Cheng, et al., 2020, Choi, et al., 2022, Qin, et al., 2021), whereas selective clearance of misfolded α-synuclein by p62/Sqstm1-dependent synucleinphagy can limit α-synuclein accumulation (Choi, et al., 2020, Panicker, et al., 2019). Astrocytes also contribute to extracellular Aβ clearance by internalization and autophagy/lysosome-dependent degradation (Pomilio, et al., 2016), and astrocyte autophagy has been linked to release of Insulin-degrading enzyme (Ide), a protease that degrades extracellular Aβ (Son, et al., 2016). Consistently, astrocyte-selective Tfeb overexpression enhances lysosomal function and reduces Aβ plaque deposition in AD models (Xiao, et al., 2014). Hence, autophagy dysfunction in glial cells can contribute to neurodegeneration both by impairing aggregate clearance and by amplifying inflammatory signaling pathways.

2.1.3.1.3 Lysosomal dysfunction in neurodegeneration

Once cargoes have been sequestered into autophagosomes, their efficient degradation depends on the fusion of autophagosomes with lysosomes and functionality of lysosomes. Neurons, with their exceptionally long lifespan and post-mitotic state, are uniquely dependent on a robust lysosomal system to sustain proteostasis and quality control of organelles. Defects in lysosomal acidification, ion homeostasis, membrane integrity, or enzyme activity are therefore critical pathogenic features of neurodegenerative diseases.

Acidification of the lysosomal lumen, mediated primarily by the vacuolar H^+-ATPase (V-ATPase), is essential for activating resident hydrolases (Yoshimori, et al., 1991). Defects in lysosomal acidification impair autophagic degradation and contribute to the accumulation of pathogenic proteins in neurodegenerative diseases. In Alzheimer's disease, loss or mutation of presenilin-1 disrupts targeting of the V-ATPase V0a1 subunit to lysosomes, preventing proper acidification of the lysosomal lumen and inhibiting cathepsin activation. As a result, autolysosomes fail to degrade their cargo, leading to defective proteolysis, autophagosome accumulation, and neurodegeneration (Lee, et al., 2010b). Similarly, in Parkinson's disease, mutations in ATP13A2—a lysosomal P-type ATPase—produce analogous lysosomal defects, including impaired acidification, defective processing of lysosomal hydrolases, and incomplete degradation of substrates. These defects inhibit autophagosome clearance and promote dopaminergic neuronal death (Dehay, et al., 2012).

Another important factor of lysosomal homeostasis that contributes to neuronal health is lysosomal calcium homeostasis, which has emerged as a central contributor

to neurodegenerative disease mechanisms. The lysosomal calcium channel Mucolipin-1 mediates calcium- and iron-dependent signaling required for lysosomal activation, membrane fusion, and degradative function. Loss-of-function mutations in Mucolipin-1 cause mucolipidosis type IV, a lysosomal storage disorder characterized by neurodevelopmental and retinal degeneration (Dong, et al., 2008, Vergarajauregui, et al., 2008). Mechanistically, Mucolipin-1 deficiency disrupts lysosomal Fe^{2+} release and leads to iron accumulation in the lysosomal lumen, impaired lysosomal degradation, and build-up of lipofuscin-like material. In neurons and patient-derived fibroblasts, loss of Mucolipin-1 or impaired calcium release cause defective autophagosome–lysosome fusion, constitutive activation yet ineffective completion of autophagy, and accumulation of ubiquitinated protein aggregates, all of which promote neurodegeneration (Vergarajauregui, et al., 2008). Beyond mucolipidosis type IV, Mucolipin-1–mediated Ca^{2+} release can trigger autophagosome-lysosome fusion and lysosomal acidification by mobilizing SNARE carrier vesicles containing Syntaxin-7 and Vamp7, linking calcium signaling to vesicular trafficking and hydrolytic activation (Bhattacharjee, et al., 2024). Dysregulated Mucolipin-1 activity by genetic loss or pathological hyperactivation in presenilin-1-deficient neurons and Alzheimer's disease neurons, induces aberrant lysosomal calcium efflux, activates stress kinases such as c-Jun N-terminal kinases (Jnks), and impairs retrograde transport of late endosomes and amphisomes, which leads to axonal dystrophy (Lie, et al., 2022). Similarly, disruption of other lysosomal Ca^{2+} channels such as Two-pore channel 2 (Tpc2) alters autophagosome-lysosome fusion and lysosomal pH, compromising degradative capacity (Sun and Yue, 2018). In hippocampal neurons, inhibition of Mucolipin-1 by the Ragulator subunit Late endosomal/lysosomal adaptor, Mapk and mTor activator 1 (Lamtor1) maintains dendritic lysosomal trafficking and synaptic plasticity, while loss of Mucolipin-1 inhibition enhances Ca^{2+} efflux, leading to excessive lysosomal motility, altered α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (Ampa) receptor turnover, and cognitive deficits (Sun, et al., 2022).

Loss of lysosomal membrane integrity and subsequent leakage of lysosomal contents are key pathological mechanisms contributing to neuronal dysfunction in neurodegenerative diseases such as Alzheimer's disease. Accumulation of undegraded Aβ within late endosomes and lysosomes leads to oxidative damage of lysosomal membranes, collapse of the proton gradient, and leakage of lysosomal hydrolases into the cytosol (Ditaranto, et al., 2001). The permeabilization of lysosomal membranes induces intralysosomal free-radical generation and membrane oxidation (Ditaranto, et al., 2001), precedes neuronal death and is specific for the $Aβ_{1–42}$ isoform. Once lysosomal integrity is compromised, proteases such as Cathepsin D leak into the cytosol, where they contribute to protein carbonylation, Tau phosphorylation, and neuronal degeneration (Wang, et al., 2020). Elevated

cytosolic Cathepsin D activity has been detected in Aβ-treated cortical neurons, the brain cortex of Alzheimer's disease model mice, and post-mortem Alzheimer brains, correlating with regional neurodegeneration (Wang, et al., 2020). In contrast, restoration of lysosomal membrane integrity, for example by treatment with lysosome-targeting poly(lactic-co-glycolic-acid) (PLGA) nanoparticles, attenuates Aβ-induced toxicity, reduces oxidative damage, and normalizes Cathepsin D distribution (Wang, et al., 2020).

In addition to disease-specific mechanisms, ageing itself predisposes lysosomes to instability: studies of aged rat brains show increased total Cathepsin D activity, enrichment of Cathepsin D and β-Glucuronidase in the cytosolic fraction, and enhanced leakage of these enzymes from isolated lysosomes upon incubation, indicating a global decline in lysosomal membrane stability similar to that induced by chemical lysosomotropic stressors (Nakamura, et al., 1989). The progressive loss of lysosomal integrity with age likely lowers the threshold for membrane permeabilization under oxidative or proteotoxic conditions. Consistently, oxidative stress amplifies lysosomal fragility, and under hyperoxic conditions neuronal lysosomes accumulate large Aβ-positive inclusions, demonstrating impaired degradation capacity and heightened vulnerability to lysosomal membrane permeabilization (Zheng, et al., 2006).

Altered synthesis, trafficking, or activation of lysosomal hydrolases is another major cause of neuronal vulnerability. In Alzheimer's disease, reduced expression and improper processing of Cathepsin D impairs protein degradation and reflects broader lysosomal dysfunction (Urbanelli, et al., 2008). In Parkinson's disease and dementia with Lewy bodies, diminished activities of key hydrolases such as Glucocerebrosidase and Cathepsin D are observed across vulnerable brain regions, leading to α-Synuclein accumulation and neuronal loss (Moors, et al., 2019, Wong and Krainc, 2016). Mutations in the *Gba1* gene, encoding Glucocerebrosidase, further lower glucocerebrosidase and Cathepsin D activity, thereby promoting α-Synuclein buildup, while restoration of these enzymes alleviates pathology (Yang, et al., 2020a). Parkinson's disease brains show deficiencies in multiple sphingolipid hydrolases and accumulation of glycosphingolipids. These findings support the idea that impaired lysosomal hydrolase activity contributes to disease pathology and progression. However, it remains possible that the observed enzyme insufficiency is not solely a primary driver but can also arise secondary to other lysosomal or trafficking defects, thereby creating a self-reinforcing vicious cycle that exacerbates neurodegeneration (Huebecker, et al., 2019).

In Parkinson's disease, Glucocerebrosidase deficiency inhibits ALR by reducing mTor activity and impairing Rab7a-regulated lysosomal recycling dynamics, leading to loss of mature lysosomes and accumulation of α-Synuclein aggregates (Magalhaes, et al., 2016). A more detailed mechanistic discussion of Rab7a in ALR

and late autophagic maturation is provided in section 2.1.1.5 *Autophagic lysosome reformation*. Thus, failure of ALR undermines lysosomal renewal and contributes to neuronal dysfunction and degeneration in neurodegenerative disease.

2.1.3.1.4 Ageing and autophagy

Ageing is accompanied by a gradual decline in cellular maintenance systems that leads to loss of physiological integrity and heightened vulnerability to disease (López-Otín, et al., 2013). Among the nine recognized hallmarks of ageing, including genomic instability, mitochondrial dysfunction, and loss of proteostasis, impairment of autophagy plays a central role in neuronal ageing and degeneration (López-Otín, et al., 2013). As outlined above, functional autophagy and structural and functional integrity of lysosomes are indispensable for neuronal survival. Cellular senescence suppresses autophagic flux, leading to accumulation of Aβ in neurons and selective neuronal loss in models combining accelerated senescence with Alzheimer-like pathology (Suelves, et al., 2023).

In summary, age-related decline in autophagy and lysosomal integrity creates a permissive environment for neurodegeneration. The interplay between mitochondrial dysfunction, lysosomal fragility, and reduced proteostatic capacity links the normal ageing process to heightened neuronal vulnerability as well as the onset of neurodegenerative diseases.

2.1.3.2 Autophagy in cancer

Autophagy plays multifaceted and sometimes opposing roles in cancer, functioning as a “double-edged sword”. Autophagy’s impact on cancer depends on tumor type and stage, genetic context, and microenvironmental pressures (Levy and Thorburn, 2020, Thorburn, et al., 2014).

During the early stages of tumorigenesis, basal autophagy prevents malignant transformation by limiting oxidative stress, mitigating genomic instability, and restraining chronic inflammation (Degenhardt, et al., 2006, Maheshwari, et al., 2025). This tumor-suppressive capacity is closely linked to core autophagy regulators such as Beclin-1. Reduced Beclin-1 levels (commonly through mono-allelic loss) compromise basal autophagy and predispose tissues to tumor development, supporting its classification as a haploinsufficient tumor suppressor (Liang, et al., 1999).

Once tumors are established, autophagy often shifts from a tumor-suppressive mechanism to a cell-survival pathway that enables cancer cells to adapt to hostile microenvironments characterized by nutrient deprivation, hypoxia, and cytotoxic stress (Amaravadi, et al., 2007, Levy and Thorburn, 2020, Pan, et al., 2014). In this

setting, autophagy can provide metabolic substrates, preserve mitochondrial function, and buffer proteotoxic damage, thereby promoting tumor cell fitness and contributing to resistance to cytotoxic and targeted therapies (Amaravadi, et al., 2007, Pan, et al., 2014). Importantly, the consequences of autophagy inhibition are also context-dependent: while blocking autophagy can sensitize tumor cells to treatment, combined disruption of autophagy and apoptosis may shift stressed cells toward necrotic death, potentially amplifying inflammation and influencing tumor progression (Degenhardt, et al., 2006).

Accordingly, understanding autophagy in cancer requires an integrated view that spans (i) its dual tumor-suppressive and tumor-promoting functions, (ii) its interplay with cell death pathways, (iii) its regulation by, and impact on, the tumor microenvironment (TME), including hypoxia and inflammatory/immune cues, and (iv) the therapeutic opportunities and risks associated with autophagy modulation (Levy and Thorburn, 2020, Thorburn, et al., 2014). These themes are developed in the following sections, with emphasis on the mechanistic basis of context dependence and the implications for targeting autophagy in cancer therapy.

Recent attention has also turned towards the relationship between autophagy and cancer stem cells (CSCs), a cell population critical for tumor initiation, metastasis, and relapse. Autophagy contributes to CSC maintenance and therapeutic resistance by providing metabolic flexibility and enabling survival under stress (Jia, et al., 2025). Conversely, in certain contexts, enhanced autophagy can drive differentiation or trigger cell death in CSCs, revealing its dual regulatory nature. Pharmacological modulation – either inhibition with agents like chloroquine or hydroxychloroquine or controlled enhancement when autophagy becomes cytotoxic – shows promise for increasing CSC sensitivity to standard treatments and for preventing recurrence (Jia, et al., 2025).

2.1.3.2.1 Dual role of autophagy in cancer

Autophagy has emerged as an important intrinsic mechanism for tumor suppression, functioning to maintain cellular and genomic integrity. Genetic and functional studies identified Beclin-1 as a critical autophagy regulator with tumor-suppressive properties. Beclin-1 is deleted in one allele in a significant proportion of human breast, ovarian, and prostate cancers, and its reduced expression correlates with diminished autophagic activity and increased cellular proliferation (Liang, et al., 1999, Qu, et al., 2003). Mouse models further confirm that Beclin-1 heterozygosity increases the incidence of spontaneous malignancies, establishing autophagy as a tumor-suppressor pathway (Qu, et al., 2003, Yue, et al., 2003). Mechanistic studies show that defective autophagy sensitizes epithelial cells to metabolic stress, enhances DNA damage, and promotes genomic instability, thereby facilitating

tumorigenesis (Karantza-Wadsworth, et al., 2007). Similar findings are observed when essential autophagy genes such as *Atg5* or *Atg7* are disrupted: autophagy-deficient hepatocytes accumulate damaged mitochondria, reactive oxygen species (ROS), and the autophagy adaptor protein Sqstm1/p62, leading to oxidative stress and DNA damage responses that contribute to tumor formation (Takamura, et al., 2011). More broadly, autophagy supports genomic stability by regulating redox homeostasis and limiting ROS-mediated DNA damage, thus maintaining chromosomal integrity and suppressing malignant transformation (Bu, et al., 2020). This shows that functional autophagy safeguards against oncogenic initiation by restraining oxidative stress, preserving genomic stability, and limiting the pro-tumorigenic consequences of cellular damage.

Conversely, in established tumors, autophagy is frequently exploited as a survival mechanism that enables cancer cells to adapt to metabolic and microenvironmental stress. Cancer models driven by mutated oncogenes such as the GTPases KRas and HRas show that elevated basal autophagy maintains mitochondrial function and tricarboxylic acid (TCA) cycle activity, thereby sustaining energy production and biosynthetic metabolism under nutrient-limiting conditions (Guo, et al., 2011). Inhibition of core autophagy genes such as *Atg7* in these models results in decreased respiration, depletion of nucleotide pools, and impaired starvation survival, demonstrating that autophagy provides substrates necessary for tumor cell viability during metabolic stress (Guo, et al., 2016). Similarly, cells with defective apoptosis rely on autophagy to compensate for nutrient deprivation. When both pathways are compromised, metabolic collapse leads to necrosis and inflammation, processes that can paradoxically drive tumor progression (Degenhardt, et al., 2006, Mathew, et al., 2007). Hypoxia, a hallmark of the tumor microenvironment, further induces autophagy, allowing cancer cells to remove damaged organelles and mitigate cellular stress under limited oxygen availability (Zaarour, et al., 2021).

Collectively, although autophagy can preserve genomic integrity in early disease, its sustained activation in advanced or oncogene-activated cancers provides a critical adaptive advantage for cancer cells: supporting metabolic flexibility, survival during therapeutic stress, and ultimately promoting tumor maintenance and progression.

2.1.3.2.2 Autophagy and the tumor microenvironment

The tumor microenvironment is a highly dynamic and interactive ecosystem composed of malignant cells, stromal fibroblasts, immune cell, vascular cells, extracellular matrix components, soluble mediators, and even microbiota. Together, these elements orchestrate cancer progression, metastasis, immune evasion, and

therapeutic resistance through intricate molecular and metabolic crosstalk (Almazrouei, et al., 2025). Autophagy is a crucial regulator in this niche, influencing both the tumor and stromal compartments by modulating nutrient exchange, angiogenesis, immune responses, and fibroblast activation.

In the stromal compartment, autophagy in cancer-associated fibroblasts fuels tumor growth through the selective degradation of mitochondria and other cellular components, a process called the "autophagic tumor stroma model" or "reverse Warburg effect", in which oxidative stress initiated by cancer cells provokes mitophagy in adjacent fibroblasts. Through this metabolic coupling, tumor cells can use recycled metabolites such as lactate and ketones as substrates that sustain anabolic proliferation and metastasis (Martinez-Outschoorn, et al., 2011, Martinez-Outschoorn, et al., 2010). Hypoxia and oxidative stress further amplify this process by driving the autophagic degradation of Caveolin-1 in fibroblasts via Hypoxia-inducible factor 1-α (Hif-1α) and Nuclear factor κ-B p105 subunit (NF-κB) pathways. This leads to a tumor-promoting stroma that protects cancer cells from apoptosis and promotes therapeutic resistance (Martinez-Outschoorn, et al., 2010). Moreover, stromal autophagy can support fibrotic remodeling of the tumor stroma by promoting Collagen I secretion. Genetic inhibition of autophagy in fibroblasts disrupts the fibrotic matrix formation and impairs mammary tumor progression in vivo (Rudnick and Debnath, 2021).

Autophagy is also important for the vasculature and immune-control of the tumor microenvironment. In tumor endothelial cells, autophagy maintains an immune-suppressive and anti-inflammatory phenotype by dampening NF-κB-driven activation. Loss of autophagy in tumor endothelial cells induces a transcriptionally inflamed and immunostimulatory endothelial state, enhancing T-cell infiltration, effector function, and responsiveness to immunotherapy in melanoma models (Verhoeven, et al., 2023). In addition, endothelial autophagy regulates angiogenesis and vascular adaptation under hypoxic and nutrient-deprived conditions, allowing tumor endothelial cells to balance redox stress and cover the elevated demand for energy of the tumor milieu (Schaaf, et al., 2019).

Hence, autophagy does not act in isolation within tumor cells but rather operates as an intercellular regulatory mechanism that shapes the metabolic, structural, and immunologic framework of the tumor microenvironment. By coordinating energy transfer between cancer and stromal compartments, orchestrating extracellular matrix remodeling, and controlling vascular inflammation, autophagy serves as a master modulator of tumor growth, metastasis, and therapeutic response.

2.1.3.2.3 Autophagy and immune modulation

Autophagy shapes antitumor immunity by controlling both antigen presentation and inflammatory signaling, with outcomes that differ by cell type and micro-environmental context. In antigen-presenting cells, macroautophagy delivers endogenous cytosolic and nuclear proteins to lysosomes, thereby expanding the peptide pool available for major histocompatibility complex (MHC) class II loading and Cluster of differentiation 4-positive ($CD4^+$) T-cell priming (Crotzer and Blum, 2009, Münz, 2012, Münz, 2016). Beyond this "classical" role in presenting intracellular antigens, components of the autophagy machinery also regulate processing of extracellular antigens. In dendritic cells, Atg5-dependent machinery supports efficient phagosome-to-lysosome fusion and optimal MHC-II presentation of phagocytosed material (including cargo containing Toll-like receptor stimuli), whereas MHC-I presentation can remain largely intact in the absence of Atg5, showing that there are selective requirements for autophagy proteins in specific presentation routes (Lee, et al., 2010a). More broadly, LC3-associated phagocytosis links autophagy-related LC3/Atg8 lipidation to the phagosomal limiting membrane, which enhances phagosome maturation. This preocess can influence extracellular antigen processing for MHC-I and MHC-II presentation (Münz, 2016). Thereby, autophagy or autophagy proteins determine how tumors and microbes are "seen" by the adaptive immune system through $CD4^+$ and, in some contexts, $CD8^+$ T-cell responses (Crotzer and Blum, 2009, Münz, 2016).

Autophagy also modulates innate immune tone through reciprocal crosstalk with inflammasomes and cytokine programs. By removing endogenous danger signals (e.g., damaged mitochondria), inflammasome components, or even cytokine substrates, autophagy can restrain inflammasome activation and limit excessive Interleukin-1β (IL-1β)/IL-18-driven inflammation. Conversely, inflammasome pathways can feed back to alter autophagic activity, creating a regulatory feedback loop (Sun, et al., 2017). Importantly, autophagy can additionally tune cytokine secretion via inflammasome-independent mechanisms. In human peripheral blood mononuclear cells, inhibiting basal autophagy enhances Toll-like receptor (Tlr)-induced IL-1β production while reducing tumor necrosis factor α (Tnfα), whereas starvation-induced autophagy suppresses IL-1β. In this case the regulation appears to act at the transcriptional level (potentially involving p38 Mapk signaling) rather than by directly changing inflammasome processing (Crisan, et al., 2011). Thus, autophagy can either dampen or reprogram inflammatory outputs depending on which immune pathway and regulatory layer dominates (inflammasome vs transcriptional control; Crisan, et al., 2011, Sun, et al., 2017).

Within cancer cells, autophagy frequently promotes immune escape by limiting antigen display. In pancreatic ductal adenocarcinoma, MHC-I molecules can be

selectively targeted for autophagy-dependent lysosomal degradation through the cargo receptor Nbr1, reducing surface MHC-I and impairing $Cd8^+$ T-cell recognition. Autophagy inhibition restores surface MHC-I, enhances antitumor T-cell responses, and synergizes with dual checkpoint blockade (treating with two immune checkpoint inhibitors to produce a stronger T-cell response) *in vivo* (Yamamoto, et al., 2020). Microenvironmental hypoxia provides another route to autophagy-mediated immune evasion: hypoxia can activate autophagy via the Proline-rich receptor-like protein kinase (Perk) arm of the unfolded protein response, downregulating MHC-I abundance and shrinking the MHC-I immunopeptidome. Blocking autophagy under hypoxia improves antigen presentation, linking stress-adaptive autophagy to reduced tumor visibility to T cells (Estephan, et al., 2025). Mechanistically, autophagy-related LC3/Atg8 lipidation has also been implicated in enhancing MHC-I internalization and degradation, further diminishing surface antigen display in certain contexts (Loi, et al., 2016). These studies highlight a recurrent theme: autophagy can convert tumor stress adaptation into reduced immunogenicity by lowering MHC-I-dependent antigen presentation (Estephan, et al., 2025, Loi, et al., 2016, Münz, 2016, Yamamoto, et al., 2020).

Autophagy in bone marrow (myeloid) compartments adds another layer of immune modulation in the tumor microenvironment, particularly through myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages. In melanoma, MDSCs exhibit elevated autophagy, and genetic inhibition of autophagy in myeloid cells delays tumor growth and strengthens antitumor immunity. Notably, autophagy-deficient monocytic MDSCs show impaired lysosomal degradation and increased surface MHC-II expression, enabling more effective activation of tumor-specific $CD4^+$ T cells. Targeting the E3 ubiquitin-protein ligase Membrane Associated Ring-CH-Type Finger 1 (Marchf1), which promotes lysosomal degradation of MHC-II, similarly weakens MDSC suppressive function and enhances antitumor immunity (Alissafi, et al., 2018). In macrophages, autophagy is tightly connected to polarization states, but the directionality can be context dependent. Loss of macrophage autophagy (e.g., Atg5 deletion) can skew polarization toward heightened inflammatory M1 features with reduced anti-inflammatory M2 programs in metabolic inflammation models, consistent with an overall anti-inflammatory role for autophagy in some settings (Liu, et al., 2015). In contrast, in hepatocellular carcinoma–associated macrophage polarization, inhibition of macrophage autophagy has been reported to promote M2-like polarization via NF-κB pathway dampening (through Tgf-β-activated kinase 1 and Map3k7-binding protein 3 (Tab3) destabilization), with correlations observed in human hepatocellular carcinoma (HCC) samples and in vivo experiments (Gao, et al., 2023). Reviews of tumor-associated macrophage biology

emphasize that autophagy can influence macrophage production, recruitment, differentiation, and polarization, making it a potentially druggable target in the myeloid compartment, albeit one requiring careful context-specific interpretation (Chen, et al., 2014, Vitaliti, et al., 2025).

Taken together, autophagy acts at the tumor–immune interface as a double-edged regulator: it can support antigen processing pathways that promote T-cell priming (particularly via MHC-II in APCs; Crotzer and Blum, 2009, Lee, et al., 2010a, Münz, 2012, Münz, 2016), while in tumors and certain suppressive myeloid subsets it can reduce antigen display or reinforce immunosuppressive functions (e.g., MHC-I degradation in cancer cells and MDSC suppressive programming; Alissafi, et al., 2018, Estephan, et al., 2025, Yamamoto, et al., 2020). This duality helps explain why modulating autophagy can either enhance or impair antitumor immunity depending on which cell population is targeted and which immune pathway is dominant. Combining autophagy inhibition with immunotherapies in settings where autophagy primarily drives immune escape is a potential treatment option (Wang, et al., 2025, Yamamoto, et al., 2020).

2.1.3.3 Pharmaceutical targeting of autophagy

Pharmacological modulation of autophagy is widely used both as an experimental strategy to map rate-limiting steps in autophagic flux and as a potential therapeutic approach in diseases linked to defective proteostasis, infection control, or stress adaptation. In principle, small molecules can either enhance autophagy (e.g., by activating nutrient-stress signaling pathways) or inhibit it (either by blocking autophagosome biogenesis or by impairing lysosomal fusion or degradation). However, translating compound effects into clear mechanistic conclusions is not trivial because autophagy is a dynamic multistep pathway: an increase in LC3-positive puncta or LC3-II abundance can reflect either increased autophagosome formation or impaired turnover at later stages, or both. For this reason, interpretation generally requires flux-aware readouts (for example, comparisons in the presence and absence of lysosomal inhibitors and complementary markers such as the cargo protein Sqstm1/p62) rather than reliance on LC3 measurements alone (Galluzzi, et al., 2017).

A second key complication is specificity. Many widely used “autophagy drugs” act on signaling nodes or lysosomal functions that control multiple aspects of cell physiology and membrane trafficking. For example, mTor- and Pi3k-pathway inhibitors influence cell growth and metabolism in addition to autophagy. Lysosomotropic agents are lipophilic and therefore can penetrate membranes, and have a weak base character, which causes them to become protonated and trapped in the low-pH environment of late endosomes and lysosomes. E.g., chloroquine and

hydroxychloroquine perturb the endo-lysosomal system broadly rather than selectively blocking autophagic degradation (Kocak, et al., 2022, Mauthe, et al., 2018). As a result, drug-induced phenotypes may combine effects on autophagy with changes in endocytosis, lysosome function, organelle identity, and immune signaling. This is particularly relevant for studies focused on the late steps of autophagy (autophagosome–lysosome fusion and cargo degradation), because pharmacological interference with lysosomal acidity, protease activity, or vesicle fusion competence can secondarily alter the abundance and composition of autophagic intermediates.

Finally, the therapeutic rationale for autophagy modulation is strongly context dependent. In some disease settings, stimulating autophagosome formation can enhance clearance of toxic material, whereas in others, especially when lysosomal degradation is compromised, further upstream induction may primarily increase the load of undegraded autophagic vacuoles. Conversely, lysosomal inhibition can be useful to sensitize tumors to therapy in certain contexts, yet may be limited by off-target effects, incomplete target engagement, and the absence of robust translational biomarkers that report pathway modulation in patient tissues (Kocak, et al., 2022). Together, these considerations motivate a pathway-aware view of autophagy pharmacology, in which compounds are interpreted based on where they act along autophagic pathway and which additional endo-lysosomal or other processes they are likely to affect.

2.1.3.3.1 Intervention points and major classes of pharmacological modulators

Pharmacological compounds that stimulate or disrupt autophagy can be grouped by the phase they primarily affect:

1. Initiation and signaling.
2. Phagophore nucleation and early membrane remodeling.
3. Autophagosome maturation, transport and fusion.
4. Lysosomal degradation.

Fig. 5 summarizes major drug classes according to their dominant intervention points, with emphasis on practical interpretation.

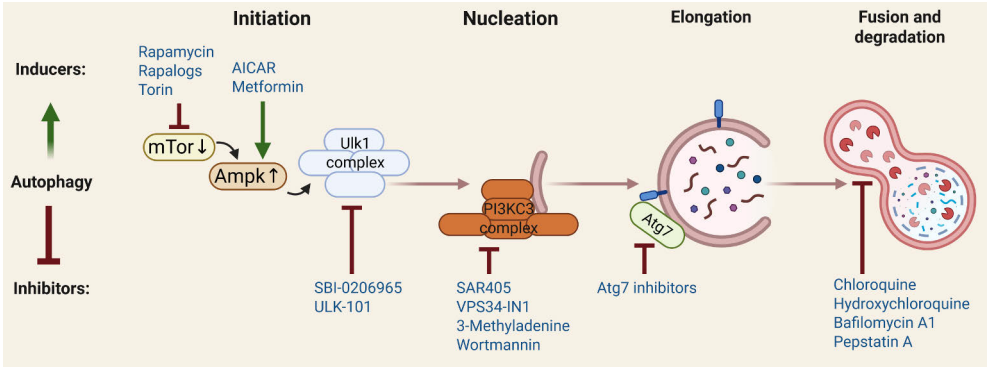

Figure 5. Autophagic flux with major druggable nodes. Schematic overview of macroautophagy pathway from initiation to lysosomal degradation, highlighting pharmacological intervention points. The pathway is organized into four stages: (1) initiation (Ulk1 complex regulated by Ampk activation and mTorc1 inhibition), (2) nucleation/PI3P production (Pi3kc3 complex), (3) phagophore elongation and closure (Atg7 mediated LC3 lipidation), and (4) fusion and degradation (fusion with lysosomes and lysosomal hydrolase–mediated cargo breakdown). Green arrows indicate activating inputs, red blunt-ended lines indicate inhibitory inputs, and blue labels denote example compounds targeting specific nodes. The figure emphasizes that increased or decreased LC3-positive vesicle accumulation can reflect either altered autophagosome biogenesis and/or impaired late-stage clearance, underscoring the importance of flux-based interpretation. Created in BioRender.

A large group of autophagy inducers act upstream by manipulating nutrient and energy sensing signaling pathways. Inhibition of mTorc1 is a canonical route to enhance autophagy induction, exemplified by rapamycin and rapalogs, which are widely used to trigger autophagy but also affect other mTor-dependent processes such as protein synthesis and immune regulation (Kim and Guan, 2015, Wang, et al., 2018). Pharmacological activation of Ampk can likewise promote autophagy, either by acting on Ampk directly or indirectly by shifting cellular energy status. Metformin is a prominent example used to induced autophagy in preclinical disease contexts, diabetes treatment and clinical repurposing efforts, although Ampk-independent effects have also been described (Fig. 5; Fontaine, 2018, Wang, et al., 2018). From an experimental perspective, the upstream autophagy inducers are useful for testing whether a system has “autophagy capacity” and whether boosting its initiation ameliorates accumulation of toxic substrates. From a translational perspective, the compounds require cautious interpretation because modulation of mTor/Ampk influences broad metabolic and signaling programs beyond autophagy.

By contrast, early-stage autophagy inhibitors reduce autophagosome biogenesis. Inhibitors of the Pik3c3 suppress PI3P production required for recruitment of PI(3)P-binding autophagy proteins and early autophagic membrane organization. Historically, widely used agents such as 3-methyladenine and wortmannin inhibit autophagosome formation, but their lack of selectivity across Pi3k classes can

complicate interpretation (Fig. 5; Peng, et al., 2014, Powis, et al., 1994, Rao, et al., 2019, Ronan, et al., 2014, Thelen, et al., 1994, Vakifahmetoglu-Norberg, et al., 2015, Wu, et al., 2013, Zhang, et al., 2018). More selective Pik3c3 inhibitors such as Vps34-IN1 and SAR405 provide improved tools to inhibit autophagy nucleation with better kinase selectivity profiles (Fig. 5). They have been used to demonstrate pathway dependence in cancer and other models (Bago, et al., 2014, Bashirah, et al., 2026, Noman, et al., 2020, Ronan, et al., 2014). Another early intervention point is the Ulk1/Ulk2 kinase complex. Ulk1 inhibitors such as SBI-0206965 and ULK-101 (Ahwazi, et al., 2021, Egan, et al., 2015, Martin, et al., 2018, Tang, et al., 2017) can suppress initiation-associated phosphorylation events and reduce autophagy in cellular assays, though the contribution of Ulk1/2 can vary by stimulus and cell type. In addition, Ulk1 inhibition may be partially buffered in some settings because Ulk2 and other upstream signaling routes can compensate for reduced Ulk1 activity, complicating predictions of therapeutic efficacy (Cheong, et al., 2011). In general, early-stage autophagy inhibitors are most informative when the experimental question is whether phenotypes depend on autophagosome formation and/or maturation/degradation.

Another major category comprises late-stage autophagy inhibitors that impair lysosomal fusion and/or function and thereby prevent completion of autophagic flux. Lysosomotropic weak bases such as chloroquine and hydroxychloroquine accumulate in acidic compartments and raise lysosomal pH, reducing hydrolase activity and indirectly perturbing multiple lysosome-dependent processes (Fig. 5; Verbaanderd, et al., 2017). In addition, bafilomycin A1 is widely used as a research tool to inhibit the vacuolar H+-ATPase, thereby preventing lysosomal acidification and blocking autophagic cargo degradation, which makes it particularly useful for flux measurements in cell-based assays (Klionsky, et al., 2021). The clinical application of autophagy-modulating compounds is summarized in section 2.1.3.3.3 *Autophagy in cancer therapy*.

Finally, newer compounds attempt to target core autophagy enzymes more directly, with the goal of increasing mechanistic specificity. These include inhibitors of Atg4 proteases, which are required for LC3/Gabarap processing and lipidation, and thus can block autophagosome formation and/or maturation and cargo sequestration (Akin, et al., 2014, Qiu, et al., 2016). Another emerging target is Atg7, the E1-like enzyme required for both Atg12–Atg5 and LC3/Gabarap-PE conjugation systems, for which small-molecule inhibitors have been reported as candidate compounds to inhibit autophagy (Fig. 5; Huang, et al., 2020). While these agents are not yet clinically established, they provide a route to assess autophagy dependence while reducing the confounding effects of broad lysosomal alkalization or global mTor inhibition.

2.1.3.3.2 Autophagy as a therapeutic target in neurodegeneration

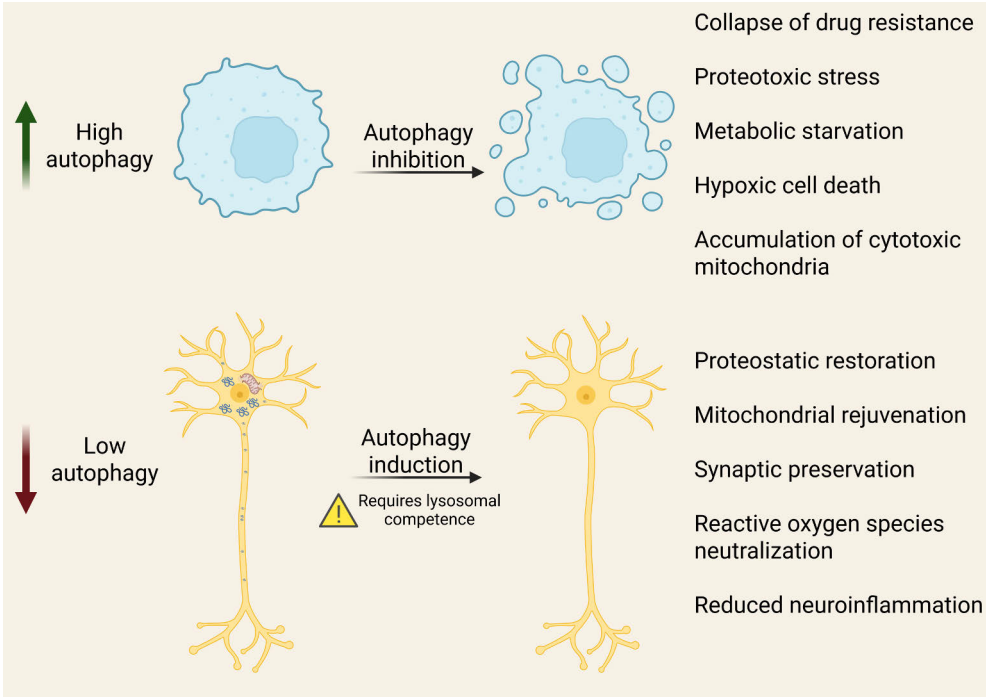

Figure 6. Therapeutic direction of autophagy modulation differs between cancer and neurodegeneration. Conceptual schematic illustrating how the same pathway can represent opposite therapeutic opportunities depending on disease context. Top: In many cancers, elevated or stress-induced autophagy can support tumor cell fitness; pharmacological autophagy inhibition is therefore proposed to increase vulnerability. Bottom: In neurodegenerative disease settings, insufficient autophagy can contribute to accumulation of protein aggregates and damaged mitochondria; autophagy induction may support neuronal homeostasis. A key constraint is highlighted: therapeutic autophagy induction is expected to be beneficial only when lysosomal competence is preserved, as increased upstream autophagosome formation without effective lysosomal degradation may worsen accumulation of autophagic intermediates. Created in BioRender.

Given the central role of impaired autophagy and lysosomal dysfunction in age-related neurodegeneration, therapeutics have increasingly focused on pharmacological and genetic modulation of the autophagy–lysosomal pathway. Inhibition of mTor with agents such as rapamycin enhances autophagic clearance of mutant Huntingtin, Tau, and Aβ, reducing aggregate toxicity in Huntington's disease and Alzheimer's disease models (Fig. 6; Majumder, et al., 2011, Sarkar, et al., 2009). Furthermore, a similar neuroprotective benefit from autophagy inducers has been demonstrated in α-synuclein-based Parkinson's disease models, where enhancing autophagic flux accelerates the clearance of toxic aggregates and rescues

dopaminergic neurons (Jiao, et al., 2025, Renna, et al., 2010, Webb, et al., 2003). Importantly, early intervention yields stronger neuroprotective effects, and optimized, intermittent dosing, as shown with brain-penetrant mTor inhibitors like PQR530, can limit Tau pathology without causing excessive autophagy activation (Morawe, et al., 2022). Parallel strategies activating Ampk also promote cellular homeostasis; metformin and resveratrol confer neuroprotection in Parkinson's disease and cerebral ischemia models respectively, through Ampk-dependent autophagy and mitigation of oxidative stress and neuroinflammation (Lu, et al., 2016, Pineda-Ramírez, et al., 2020). Nonetheless, Ampk's effects on amyloidogenic processing appear context-dependent. Ampk activation can variably influence Aβ secretion in neurons, highlighting the importance of pathway- and cell-specific modulation (Benito-Cuesta, et al., 2021b).

To overcome the limitations of broad pathway modulation, next-generation strategies are shifting toward targeted cargo degradation. A notable example is the development of Autophagy-Targeting Chimeras (AUTOTACs). AUTOTACs are bifunctional molecules that bring a chosen target protein into proximity of the autophagy receptor p62. One end of the AUTOTAC binds the target, while the other binds the p62 ZZ domain in a way that conformationally activates p62 (promoting p62 oligomerization and LC3 binding), thereby driving sequestration of the target–p62 complex into autophagosomes and subsequent lysosomal degradation. (Ji, et al., 2022). In parallel, several multi-target drugs are currently in clinical trials for Alzheimer's and Parkinson's diseases that stimulate autophagic flux as part of their broader therapeutic profile. These include blarcamesine, a sigma-1 receptor agonist that upregulates autophagy (Macfarlane, et al., 2025), and Abelson murine leukemia viral oncogene homolog 1 (c-Abl) kinase inhibitors such as nilotinib and bosutinib, which indirectly promote aggregate clearance alongside their primary kinase-inhibiting effects (Hebron, et al., 2013, Nobili, et al., 2023). Together, these findings show that precise, balanced activation of autophagy via core signaling pathways, targeted degradation, or indirect clinical candidates represents a promising therapeutic direction for neurodegenerative disorders. More broadly, pharmacological induction strategy requires careful control of dose, schedule, and disease context, because excessive or prolonged autophagy activation can be detrimental rather than uniformly protective in some settings (Galluzzi, et al., 2015, Liu, et al., 2013a).Alongside the upstream autophagy-inducers, lysosome-targeted strategies are emerging to boost cellular degradative capacity directly. The disaccharide trehalose enhances autophagic clearance of toxic substrates by inducing mild lysosomal stress, elevating lysosomal pH, and activating Tfeb, the master regulator of autophagy–lysosomal gene networks (Jeong, et al., 2021). In enzyme-deficient mucopolysaccharidosis IIIB mouse models, trehalose treatment reduced inflammation, preserved neuronal integrity, improved behavior and vision, and

extended lifespan through Tfeb-mediated stimulation of lysosomal biogenesis (Lotfi, et al., 2018). Tfeb activation enhances both autophagic activity and lysosomal capacity and has been reported to improve the clearance of Amyloid- and Tau-associated aggregates. Consistently, pharmacological approaches that boost lysosomal function (including compounds that activate the Tfeb pathway) show benefits in experimental models of Alzheimer's, Parkinson's and Huntington's diseases (Xue, et al., 2023). Complementing these approaches, gene therapy restoring *Gba1* function in Gaucher's and Parkinson's models augments glucocerebrosidase activity, reduces glycolipid and α-Synuclein accumulation, and protects dopaminergic neurons (Okai, et al., 2025, Rocha, et al., 2015). Activation of autophagy by Beclin-1 overexpression alleviates α-Synuclein pathology in Lewy body and Parkinson's disease models (Spencer, et al., 2009). Finally, in amyotrophic lateral sclerosis/frontotemporal dementia, loss of Transactive response DNA binding protein 43 kDa (Tardbp) function downregulates autophagy-related genes such as Atg7, and genetic enhancement of autophagy by Atg7 overexpression rescues neuronal survival (Donde, et al., 2020). The lysosome-supporting approaches are particularly relevant in cases where lysosomal degradation is rate-limiting, because increasing autophagosome formation alone may be insufficient or even exacerbate accumulation of non-degraded autophagic vacuoles when downstream clearance is impaired (Lee, et al., 2010b).

Although macroautophagy is largely viewed as a cytoprotective process, excessive or dysregulated activation can drive neuronal demise under pathological stress. Chronic restraint stress in mice induces autophagic cell death of hippocampal neural stem cells via Serum- and glucocorticoid-induced kinase 3 (Sgk3) signaling, which can be prevented by Atg7 deletion (Jung, et al., 2020). Similarly, oxidative stress, ischemia, and corticosterone exposure trigger non-apoptotic, autophagy-dependent neuronal death that can be mitigated by pharmacological or genetic suppression of autophagy-related genes such as Beclin-1 and Atg7, implicating overactivated autophagy in neuronal vulnerability to redox and metabolic insults (Higgins, et al., 2011, Querfurth and Lee, 2021, Shi, et al., 2012). These findings underscore a dual role of autophagy in the nervous system, acting as both a survival mechanism and, when excessive, a driver of neurodegeneration.

Recent advances in imaging and biomarker discovery are transforming the ability to monitor autophagy in living systems and translate its modulation into personalized therapies. Novel approaches now enable non-invasive monitoring of autophagic flux *in vivo* using engineered nanoparticle probes that are taken up into autophagosomes and undergo cathepsin-dependent processing after delivery to lysosomes, producing MRI- and near-infrared–detectable signals that reflect flux (Chen, et al., 2022). Adeno-associated viral reporters expressing tandem mCherry-GFP-LC3 enable dynamic assessment of neuronal autophagy in animal models

(Castillo, et al., 2013). In parallel, analyses of cerebrospinal fluid and serum reveal disease-specific patterns of autophagy and mitophagy biomarkers, for example altered Pink1, Ulk1, and Tfeb levels distinguishing Alzheimer's disease and frontotemporal lobar degeneration (Veverová, et al., 2025), and peripheral autophagy–lysosomal protein signatures aiding early Parkinson's disease diagnosis (Lee, et al., 2025). These advances enable more precise evaluation of therapeutic efficacy and bring autophagy-based interventions closer to clinical translation.

2.1.3.3.3 Autophagy in cancer therapy

Autophagy is frequently engaged as an adaptive stress-response program in cancer. In many cancer models it functions predominantly as a cytoprotective mechanism that helps tumor cells tolerate treatment-induced metabolic, oxidative, and proteotoxic stress (Fig. 6). Autophagy is induced by chemotherapy, radiation, and targeted agents via stress-sensing pathways such as Ampk-Ulk1/mTor signaling and is further reinforced by microenvironmental pressure like hypoxia, which can sustain autophagic flux during treatment and promote persistence of viable cells (Amaravadi, et al., 2007, DeVorkin, et al., 2017, Min, et al., 2014, Notte, et al., 2013). Accordingly, genetic or pharmacologic autophagy inhibition (e.g., Atg5 knockdown, chloroquine-class lysosomal inhibitors) sensitizes tumor cells to diverse therapies in multiple preclinical settings (Fig. 6; Amaravadi, et al., 2007, Min, et al., 2014). Hypoxia can strengthen the protective autophagy phenotype by sustaining autophagic flux during treatment, thereby promoting resistance and persistence of viable cells after therapy (DeVorkin, et al., 2017, Notte, et al., 2013). Beyond therapy-induced stress adaptation, some tumors display an intrinsic dependence on basal autophagy to restrain ROS/DNA damage and maintain mitochondrial metabolism, suggesting that autophagy can support both baseline tumor fitness and post-therapy survival (Yang, et al., 2011).

The impact of therapy-induced autophagy is strongly context dependent. Autophagy is cytoprotetive in many settings. This is demonstrated by reactivation of the tumor suppressor protein p53 in an apoptosis-impaired Myc lymphoma model, where autophagy is induced in the surviving cells. In this model, autophagy inhibition increases tumor cell killing by p53 activation or alkylating chemotherapy (Amaravadi, et al., 2007). However, autophagy can also contribute to cell death when apoptosis is blocked: Bax/Bak-deficient cells undergo an autophagy-dependent programmed death (Shimizu, et al., 2004), and Caspase-8 inhibition has been reported to switch cells into an Atg7/Beclin-1–dependent death program involving Receptor-interacting serine/threonine-protein kinase 1 Ripk/Jnk signaling (Yu, et al., 2004). Finally, autophagy can be induced but have a limited impact on cell fate ("non-protective" autophagy). For instance, therapy-induced senescence and sub-

sequent proliferative recovery can proceed despite pharmacologic or genetic inhibition of autophagy (Saleh, et al., 2020). Crosstalk between autophagy, apoptosis, and metabolism is therefore a major determinant of the outcome. Notably, combined impairment of apoptosis and autophagy can shift stressed cells toward necrosis with inflammatory consequences (Degenhardt, et al., 2006).

The above observations have motivated combination strategies that pharmacologically inhibit autophagy to sensitize tumors to chemotherapy, radiation, and targeted therapy. Clinically, the most widely used agents are chloroquine and hydroxychloroquine, which block late-stage autophagy. Importantly, chloroquine and related lysosomotropic agents perturb lysosomal and endo-lysosomal function broadly, and their anticancer effects can include autophagy-independent components, complicating mechanistic interpretation and clinical target-engagement assessment (Maycotte, et al., 2012). While chloroquine/hydroxychloroquine can enhance antitumor efficacy in multiple preclinical models (Amaravadi, et al., 2007, Bryant, et al., 2019, Rebecca, et al., 2014), translation to patients has been more challenging. For instance, hydroxychloroquine monotherapy in metastatic pancreatic cancer produced inconsistent pharmacodynamic evidence of autophagy inhibition (e.g., variable LC3-II changes in peripheral lymphocytes) and limited clinical activity (Wolpin, et al., 2014). Further, determinants of chloroquine sensitivity are heterogeneous and difficult to predict (Morgan, et al., 2014). These outcomes highlight several recurrent challenges in clinical translation, including the fact that chloroquines are lysosomotropic agents that broadly perturb lysosomal function. Therefore, pharmacodynamic confirmation of autophagy inhibition in patient tumors can be difficult (Hassan, et al., 2024, Wolpin, et al., 2014). Major drug classes and their main points of action along the autophagy pathway are described in section 2.1.3.3.1 *Intervention points and major classes of pharmacological modulators*.

A major complication for autophagy-targeted therapy is that autophagy is active not only in cancer cells but also in immune, stromal, and endothelial compartments. Thus, systemic autophagy inhibition can remodel the tumor microenvironment in ways that are not uniformly beneficial. In tumor cells, autophagy can reduce immunogenicity by lowering MHC-I surface display through LC3/Atg-dependent internalization and lysosomal degradation (Loi, et al., 2016, Yamamoto, et al., 2020). Conversely, autophagy machinery can support key antigen-presentation functions in professional antigen-presenting cells: dendritic cell Atg5 contributes to processing of phagocytosed antigens and efficient MHC-II presentation and thereby raises the possibility that broad inhibition could impair aspects of antitumor immunity depending on timing and cellular target (Lee, et al., 2010a, Strawbridge and Blum, 2007). Beyond immunity, autophagy in stromal and endothelial cells can sustain hypoxia/oxidative-stress adaptations that promote tumor persistence. For example, endothelial Atg5 supports mitochondrial function and pro-angiogenic vascular

endothelial growth factor receptor 2 (Vegfr2) signaling during hypoxia and reoxygenation-driven pathological neovascularization (Sprott, et al., 2019). Further, hypoxia-induced autophagy in cancer-associated fibroblasts can promote Caveolin-1 loss and a metabolically supportive, therapy-resistant stromal state that protects adjacent cancer cells from apoptosis (Martinez-Outschoorn, et al., 2010). Thus, the net effect of autophagy modulation reflects both tumor-intrinsic changes and microenvironmental rewiring (Lee, et al., 2010a, Loi, et al., 2016, Martinez-Outschoorn, et al., 2010, Sprott, et al., 2019, Strawbridge and Blum, 2007, Yamamoto, et al., 2020).

Collectively, autophagy represents a therapeutically actionable pathway in cancer, but its modulation requires careful, context-specific action. Successful clinical translation will depend on (i) defining when autophagy is functionally cytoprotective versus cytotoxic, (ii) selecting appropriate combinations with chemotherapy, targeted therapy, radiotherapy, or immunotherapy, (iii) developing robust biomarkers to confirm autophagy inhibition in tumors, and (iv) accounting for the effects of autophagy modulation across tumor, stromal and immune compartments (Hassan, et al., 2024, Xia, et al., 2021). In parallel, improved patient stratification, i.e., identifying tumors with high basal autophagy/lysosomal dependency or therapy-induced autophagy reliance, will likely be essential for matching autophagy-modulating agents to settings where they provide meaningful benefit.

2.2 Rab GTPases in vesicular trafficking

2.2.1 G proteins

Guanine nucleotide binding proteins or G proteins are ubiquitously-expressed proteins that function as molecular switches to control a multitude of biological processes. The function of G proteins relies on their ability to cycle between two states: their active (“on”) state bound to guanosine triphosphate (GTP) and their inactive (“off”) state bound to guanosine diphosphate (GDP).

G proteins are enzymes with an intrinsic GTPase activity, which allows them to hydrolyze bound GTP to GDP and phosphate. The superfamily of G proteins is divided into two classes: The heterotrimeric, large G proteins and the monomeric, small G proteins. The large G protein consist of 3 subunits – the α, β and γ subunits and are generally associated with G protein-coupled receptors to translate extracellular signals into an intracellular response. In contrast, the small GTPases are monomers, which function autonomously in diverse intracellular locations to regulate processes ranging from cell growth to vesicular transport.

2.2.2 Small GTPases

Small GTPases or small G proteins are monomeric enzymes that can bind GTP and form the Ras superfamily. These proteins are small in size with a molecular weight of 20-25 kDa. They share a conserved structural core, which is called the G domain that facilitates nucleotide binding and hydrolysis (Pai, et al., 1990). Small GTPases are homologous to the α subunit of large G proteins and can hydrolyze GTP independent of other subunits, which is accelerated by GAPs (Fig. 7).

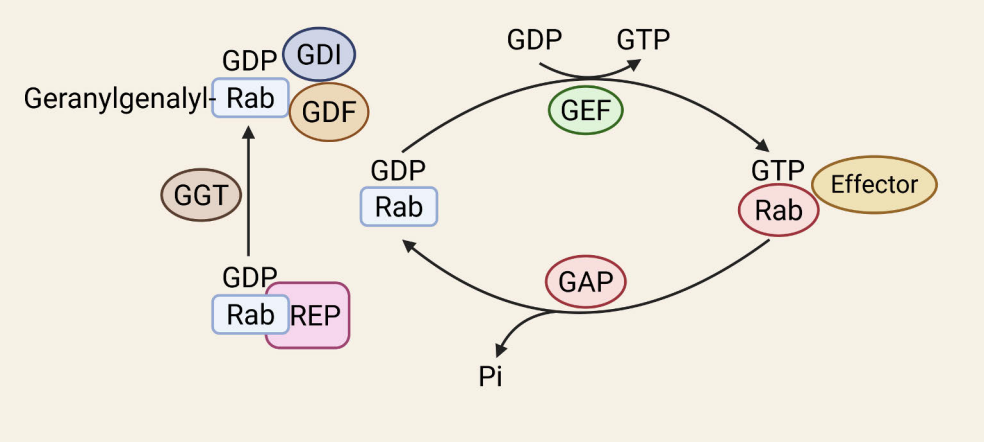

Figure 7. Rab GTPase activation cycle and prenylation-dependent membrane targeting. Schematic representation of the Rab nucleotide cycle (right) and the Rab prenylation/solubilization pathway (left). In the nucleotide cycle, inactive Rab–GDP is converted to active Rab–GTP by a guanine nucleotide exchange factor (GEF), enabling recruitment of downstream effector proteins. Active Rab–GTP is returned to the GDP-bound state by a GAP, which stimulates GTP hydrolysis and release of inorganic phosphate (Pi). In the prenylation pathway, newly-synthesized Rab–GDP associates with Rab escort protein (REP) and is modified by Rab geranylgeranyl transferase (GGT) to install C-terminal geranylgeranyl lipid groups. Prenylated Rab can be bound and solubilized by GDP dissociation inhibitor (GDI) for cytosolic transport, and delivered to target membranes where GDI displacement factor (GDF) promotes Rab membrane insertion and recycling. Created in BioRender.

Similar to the large G proteins, the small GTPases are active in their GTP-bound state and inactive in their GDP-bound state. Their activation cycle is tightly controlled by specific regulatory proteins. Guanine nucleotide exchange factors (GEFs) catalyze the release of GDP and the binding of GTP to activate the protein (Fig. 7; Klebe, et al., 1995, Lenzen, et al., 1998), while GAPs facilitate the intrinsic GTP hydrolysis rate to return the protein to its inactive state (Fig. 7; Makler, et al., 1995, Randazzo and Kahn, 1994). Additionally, GDIs act as chaperones that can sequester specific small GTPases in the cytosol and thereby prevent their activation (Fig. 7).

The rat sarcoma virus (Ras) superfamily of small GTPases is divided into five major subfamilies based on sequence homology and functional specialization: Ras, Rho, ADP ribosylation factor (Arf), and Ras-related proteins in brain (Rab). Each subfamily carries out distinct functions of cell biology. For instance, Ras proteins are pivotal in gene expression and cell proliferation, Rho proteins regulate cytoskeletal organization, and Rab proteins are involved in intracellular membrane traffic. The majority of these proteins require a post-translational lipid modification (prenylation) to associate with cellular membranes, a localization that is essential for their biological activity.

2.2.3 Rab proteins

The Rab family forms the largest branch of the Ras superfamily with approximately 70 members in humans, reflecting the complexity of membrane transport pathways in eukaryotic cells (Diekmann, et al., 2011, Klöpper, et al., 2012, Pereira-Leal and Seabra, 2001). Rab proteins are key determinants of membrane identity and act as primary organizers of intracellular vesicle traffic by coordinating cargo sorting, vesicle formation, motility, tethering, and fusion with high fidelity (Grosshans, et al., 2006, Hutagalung and Novick, 2011, Pfeffer, 2001, Stenmark, 2009, Zerial and McBride, 2001). Rab GTPases are positioned as master regulators across endocytic, autophagic, and exocytic routes and regulate protein complexes that catalyze vesicle fission and fusion between subcellular compartments (Lu, et al., 2021). The Rab family is evolutionarily old but has undergone extensive expansion and diversification across eukaryotic lineages, supporting the idea that Rab paralog number and specialization contribute to the evolution of compartmentalization and trafficking complexity (Hutagalung and Novick, 2011, Klöpper, et al., 2012, Lu, et al., 2021, Porfirio-Sousa, et al., 2022).

Although Rab-family members share a conserved GTPase fold (“G domain”), they have acquired sequence and structural features that enable selective interactions with specific regulatory proteins and effectors, thereby supporting Rab-specific functions at defined membrane compartments (Dumas, et al., 1999, Stroupe and Brunger, 2000, Vetter and Wittinghofer, 2001, Zhen and Stenmark, 2015). In particular, Rab proteins have conserved nucleotide-binding elements required for guanine nucleotide binding, GDP/GTP exchange, and GTP hydrolysis, which is in line with their membership in the Ras superfamily (Bourne, et al., 1990). The Rab G-domain has a compact globular shape, which is typically 180-200 amino acids long, forming a central β-sheet surrounded by α-helices. Together, this conserved fold provides the structural scaffold for nucleotide binding and GTP hydrolysis (Barr and Lambright, 2010, Hutagalung and Novick, 2011).

2.2.3.1 Rab GTPase cycle

Like other small monomeric GTPases, Rabs function as nucleotide-dependent molecular switches. In the Rab family this switch is functionally linked to controlling membrane traffic by coupling nucleotide state to effector recruitment and downstream transport events. Rab proteins alternate between an inactive GDP-bound conformation and an active GTP-bound conformation, with GTP binding stabilizing the switch I and switch II regions that form the main interaction surface for effectors. In the GDP-bound state, the switch regions are more flexible/disordered, whereas the GTP-bound state adopts a more ordered "ON" conformation that supports selective partner binding (Dumas, et al., 1999, Lee, et al., 2008, Milburn, et al., 1990, Stroupe and Brunger, 2000, Sultana, et al., 2011, Vetter and Wittinghofer, 2001). Although the overall switch mechanism is conserved, Rab-family members show substantial diversity in switch-region conformations and dynamics, which contributes to partner selectivity and functional specialization across Rab subfamilies (Lee, et al., 2008, Milburn, et al., 1990, Muller and Goody, 2018, Sultana, et al., 2011, Vetter and Wittinghofer, 2001). Through repeated rounds of activation and inactivation, Rabs provide a timed, compartment-specific platform for assembling trafficking machinery that executes vesicle budding, transport, tethering and fusion (Hutagalung and Novick, 2011).

The Rab cycle is controlled by three major classes of regulators:

1. GEFs catalyze GDP release and promote GTP loading, thereby activating Rabs on membranes (Fig. 7; Blümer, et al., 2013, Chin, et al., 2009, Gerondopoulos, et al., 2012, Guo, et al., 2005, Klebe, et al., 1995, Langemeyer, et al., 2014).
2. GAPs accelerate GTP hydrolysis to switch Rabs off (Fig. 7; Bernards, 2003, Langemeyer, et al., 2014). Most known Rab GAPs contain a Tre-2, Bub2, and Cdc16 (TBC) domain and employ a conserved catalytic mechanism involving trans-acting catalytic residues ("dual finger" mechanism; Albert, et al., 1999, Du, et al., 1998, Frasa, et al., 2012, Pan, et al., 2006, Strom, et al., 1993).
3. Effectors bind preferentially to the GTP-bound state and execute downstream trafficking functions (Fig. 7; Grosshans, et al., 2006, Pylypenko, et al., 2018)

2.2.3.2 Prenylation, cytosolic recycling, and membrane targeting

Membrane association of Rab GTPases depends on C-terminal prenylation, typically the addition of one or two geranylgeranyl groups to cysteine residues, which anchors Rabs to membranes and is essential for function (Andres, et al., 1993, Guo, et al.,

2008, Leung, et al., 2007). Prenylation of newly-synthesized Rabs requires Rab escort protein (REP), which presents Rab substrates to Rab geranylgeranyl transferase (RabGGTase) and subsequently participates in delivery to membranes (Andres, et al., 1993, Guo, et al., 2008, Pylypenko, et al., 2003, Rak, et al., 2004, Thomä, et al., 2001Fig. 7; , Wu, et al., 2009). In the cytosol, GDP-bound prenylated Rabs are chaperoned by GDI, which shields the lipophilic geranylgeranyl moieties and enables extraction from membranes after inactivation (Fig. 7; Muller and Goody, 2018, Ullrich, et al., 1993, Wu, et al., 2007). Release of Rab proteins from GDI and transfer back to membranes can be facilitated by GDI displacement factors (GDFs), although the mechanistic basis of GDF action remains incompletely understood (Fig. 7; Gavriljuk, et al., 2013, Sivars, et al., 2003, Voss, et al., 2019). Multiple studies support the idea that membrane-localized GEFs play a key role in coupling membrane recruitment with activation and thereby control when and where Rabs are localized and active (Blümer, et al., 2013, Gerondopoulos, et al., 2012, Wu, et al., 2010).

2.2.3.3 Rab effectors: coupling transport, tethering, and fusion

Once activated on membranes, Rab GTPases recruit diverse effector proteins that collectively coordinate successive vesicle trafficking steps. Rab effectors include cargo sorting adaptors, lipid-modifying enzymes, molecular motors, and tethering factors/complexes that establish long-range membrane contacts preceding fusion (Grosshans, et al., 2006, Wandinger-Ness and Zerial, 2014). The effectors regulate multiple stages of trafficking, including vesicle formation, movement along actin and microtubule networks, and membrane tethering and fusion at target compartments (Parray, 2025, Vazquez-Martinez and Malagon, 2011).

At the molecular level, many Rab–effector interaction interfaces are located on the switch/interswitch region (interswitch is the region between the switch I and switch II regions) and frequently involve a set of three highly conserved aromatic amino acids termed the invariant hydrophobic triad (phenylalanine in switch I, tryptophan in the interswitch and tyrosine or phenylalanine in switch II). The triad is a hallmark of Rab and Arf GTPases distinguishing them from other members of the Ras superfamily (Chavas, et al., 2008, Kukimoto-Niino, et al., 2008, Merithew, et al., 2001).

In addition to a canonical interaction surface, a secondary effector-binding site has been described for Rab11, enabling simultaneous binding of distinct effectors, supporting coordinated regulation of trafficking events and showcasing that in principle multiple simultaneous effector interactions are possible in some Rab proteins (Burke, et al., 2014, Vetter, et al., 2015). Further, also effectors can be multivalent. They can interact with and thereby integrate signals from multiple Rab

and/or Arf-family GTPases, which enables crosstalk between trafficking pathways (De Renzis, et al., 2002, Fielding, et al., 2005, Merithew, et al., 2003, Miserey-Lenkei, et al., 2007, Vitale, et al., 1998). Rab cascades can also arise when one Rab recruits the GEF for another Rab, supporting transitions in compartment identity as in the Rab5-Rab7a cascade described in the next chapter.

2.2.3.4 Rab-defined membrane identity, Rab domains, and Rab conversion during organelle maturation

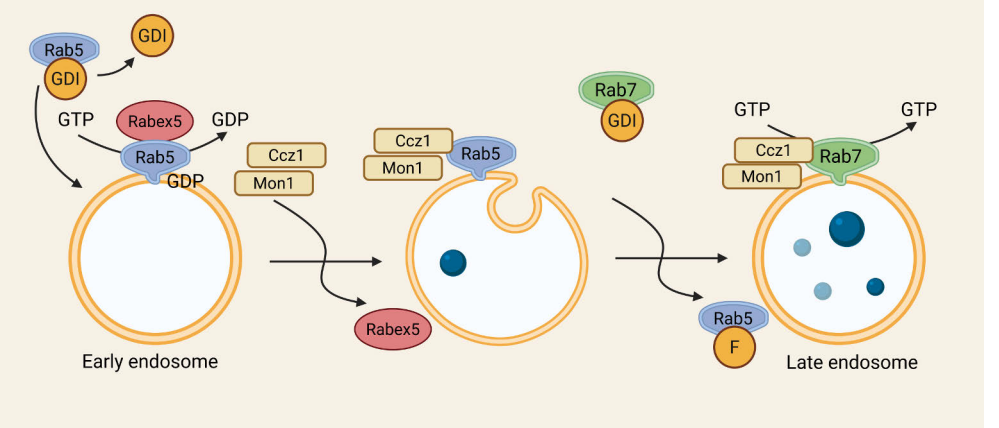

Figure 8. Rab5-Rab7a conversion during endosome maturation (Rab5-Rab7a cascade). Schematic overview of the Rab GTPase cascade that drives the transition from early to late endosomes. Cytosolic Rab5–GDI is delivered to early endosomal membranes, where GDI is dissociated and Rab5 is activated by its GEF Rabex5, generating membrane-associated Rab5–GTP. During endosome maturation, recruitment of the Mon1–Ccz1 complex promotes removal of Rab5-associated factors and supports establishment of late-endosomal identity. Mon1–Ccz1 functions as a Rab7a GEF, enabling activation and accumulation of Rab7a–GTP on the maturing endosome, while Rab5 is removed and returned to the cytosol as Rab5–GDI. The result is a late endosome enriched in Rab7a and Rab7a-dependent trafficking functions. Created in BioRender.

A defining property of Rab proteins is their contribution to establishing membrane identity. Specific Rabs are enriched on distinct intracellular compartments (e.g., Rab5 on early endosomes; Rab7a on late endosomes and lysosomes). The localization of Rabs is regulated by localized GEF and GAP activities as well as Rab effector-mediated feedback mechanisms that can either stabilize or remodel Rab-enriched membrane territories (membrane patches that carry a specific Rab and its associated effector machinery; Blümer, et al., 2013, Gerondopoulos, et al., 2012, Grosshans, et al., 2006, Pfeffer, 2005, Wu, et al., 2010). GEFs control Rab activation, whereas GAPs mediate timely Rab inactivation and extraction from the

membrane by GDI, thereby restricting Rab residence times on membranes. Importantly, effectors recruited by Rab-GTP can give feedback onto Rab distribution by modulating the local activation state of the Rab itself and/or by shaping the membrane environment and recruiting additional regulatory factors (Langemeyer, et al., 2020). A well-characterized example of positive feedback is Rab5 on early endosomes: Rab5-GTP recruits the effector Rabaptin-5, which forms a complex with the Rab5 GEF Rabex-5; Rabaptin-5 binding relieves Rabex-5 autoinhibition and thereby amplifies Rab5 activation on the same membrane, reinforcing a Rab5-positive domain (Delprato and Lambright, 2007, Horiuchi, et al., 1997, Zhang, et al., 2014). Conversely, negative feedback can contribute to Rab domain resolution and maturation. For instance, after the Rab5-to-Rab7 transition (Fig. 8), the Rab7a effector Retromer can recruit the Rab7a GAP TBC1 domain family member 5 (Tbc1d5), promoting Rab7a inactivation on endosomal subdomains and facilitating tubule formation and/or scission events that eventually lose Rab7a (Distefano, et al., 2018, Jimenez-Orgaz, et al., 2018, Jongsma, et al., 2020, Kvainickas, et al., 2019, Rojas, et al., 2008, Seaman, et al., 2009). Thus, Rab domains can function as self-organizing membrane platforms, which are controlled by coupled regulatory loops between Rabs, their effectors, and the lipid/protein composition of the membrane.

Increasing evidence suggests that membranes can be organized into overlapping Rab domains with distinct functional properties (Parray, 2025). This organization supports compartment maturation, directional trafficking, and separation of pathway stages, and can be reinforced by cascade mechanisms that limit overlap between sequential Rabs (Vazquez-Martinez and Malagon, 2011). A well-understood example is the Rab5-to-Rab7a conversion during endosome maturation, where early endosomal Rab5 is replaced by late endosomal Rab7a in a highly coordinated transition (Fig. 8; Poteryaev, et al., 2010, Rink, et al., 2005). Rab5 helps recruit and activate the Rab7a GEF complex Mon1-Ccz1 on Rab5- and PI3P-positive endosomes, thereby linking early endosome identity to subsequent Rab7a activation and late endosome biogenesis (Langemeyer, et al., 2020, Nordmann, et al., 2010, Poteryaev, et al., 2010). Recent reconstitution studies further indicate that Mon1–Ccz1 GEF activity toward Rab7a shows a strong dependence on membrane-bound Rab5-GTP, providing a mechanistic explanation for how Rab5 activity can trigger Rab7a domain formation on the membranes of maturing endosomes (Langemeyer, et al., 2020). In parallel, maturation can be promoted by switch-like network behavior (“cut-out switch”), in which Rab5-driven Rab7a activation suppresses further Rab5 activation, promoting an ordered and directional progression from early to late endosomal identity (Borchers, et al., 2021, Del Conte-Zerial, et al., 2008).

2.2.3.5 Determinants of specificity of Rab interactions

Despite broad conservation of the Rab GTPase fold, Rab family members interact with specific regulators and effectors. Structural analyses indicate that specificity is mediated by sequence and conformational variation within switch/interswitch regions and adjacent variable elements, including complementarity-determining region (CDR)-like surfaces that can shape binding to effectors and regulatory factors (Guo, et al., 2013, Müller, et al., 2010, Ostermeier and Brunger, 1999). In addition, sequence analyses have defined Rab-family motifs (RabF regions) that distinguish Rab proteins from other Ras superfamily members, and Rab subfamily-specific motifs (RabSF regions) that are conserved within subfamilies and contribute to functional specialization (Parray, 2025). The conserved switch/interswitch surface shared across Rabs enables use of universal machinery for prenylation and recycling (REP/GDI), while surrounding variable amino acids control affinity and specificity toward particular GEFs, GAPs, and effectors (Alory and Balch, 2001, Andres, et al., 1993, Guo, et al., 2008, Leung, et al., 2007, Muller and Goody, 2018, Pylypenko, et al., 2006, Wu, et al., 2007).

The C-terminal hypervariable domain (HVD) has been implicated in mediating the specificity of the localization of some Rab proteins. This indicates that in some cases the C-terminal HVD can have roles beyond mediating membrane anchoring via the prenylation sites (Li, et al., 2014, Pylypenko, et al., 2018).

2.2.3.6 Rab24

Rab24 is an evolutionarily old ("primordial") Rab GTPase that already existed in the last eukaryotic common ancestor (Klöpper, et al., 2012). Rab24 is conserved in many metazoans, including zebrafish and mammals, but has been lost in several commonly used model organisms such as *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans* (Elias, et al., 2012). This evolutionary heritage has been interpreted to connect Rab24 to fundamental features of the endocytic pathway that emerged early in eukaryote evolution (Elias, et al., 2012). In mammalian cells, Rab24 has been linked to late endocytic compartments and autophagic organelles and functional studies support a role in late, lysosome-associated degradative processes, with Rab24 being required for efficient completion of basal autophagy (Ylä-Anttila, et al., 2015) and endocytic degradation (Amaya, et al., 2016). Rab24 has also been suggested to support normal cell division (Militello, et al., 2013).

2.2.3.6.1 Discovery and subcellular localization of Rab24

Rab24 was first characterized by Olkkonen and colleagues, who reported Rab24 mRNA expression across mouse tissues (highest in brain) and described Rab24

protein as predominantly perinuclear and associated with multiple membrane compartments (Olkkonen, et al., 1993). In immunofluorescence-based localization experiments, Rab24 was observed at the cis-Golgi/ER-facing side, the endoplasmic reticulum (ER), the ER–Golgi intermediate compartment (ERGIC), and late endosomal vesicles (Olkkonen, et al., 1993).

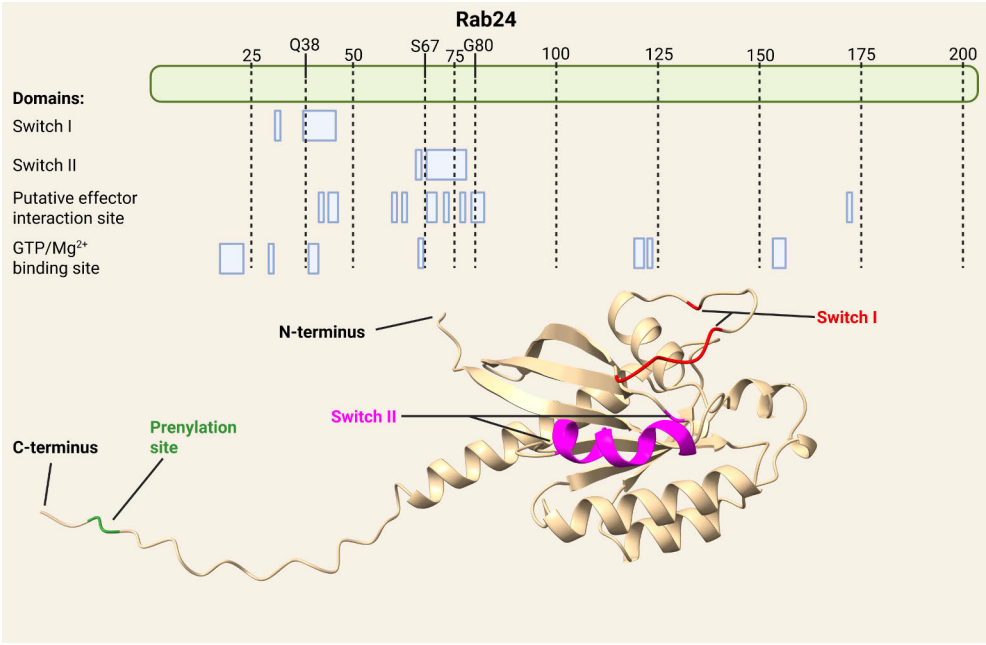

Figure 9. Predicted domain architecture and tertiary structure of Rab24. Top: Schematic representation of the Rab24 primary sequence with residue numbering and positions of amino acids 38, 67, and 80 indicated. Functional regions predicted by InterPro are mapped onto the sequence, including switch I, switch II, putative effector interaction sites, and the GTP/Mg^{2+}-binding site. The corresponding residue ranges are shown as light blue boxes. Bottom: predicted Rab24 tertiary structure generated by AlphaFold3, with N- and C-termini indicated. The switch I region is highlighted in red, switch II in magenta, and the C-terminal prenylation site in green. Created in BioRender.

2.2.3.6.2 Rab24 as an “unusual” Rab GTPase

Rab24 has been described as an atypical Rab protein because several of its biochemical and sequence properties diverge from canonical Rab features and this may influence both its regulation and cellular behavior. Firstly, Rab24 has aa unusually low intrinsic GTPase activity and therefore has been reported to occur predominantly in the GTP-bound state in cells (Erdman, et al., 2000). This has been linked to a non-canonical amino acid in the conserved nucleotide-binding region: at position 67 Rab24 contains serine (S67), whereas most other Rab proteins have

glutamine at the equivalent position, which is often critical for efficient GTP hydrolysis (Fig. 9; Erdman, et al., 2000). In line with this, restoring the "typical" amino acid by introducing the conversion of S67 to glutamine (S67Q) increased Rab24 GTPase activity (Erdman, et al., 2000).

Interestingly, a conceptually similar serine amino acid is found in another Ras-superfamily GTPase, Rho-related GTP-binding protein RhoE (also known as Rnd3), which contains a serine at the position corresponding to the catalytic glutamine. RhoE has been reported to lack detectable GTP hydrolysis activity and to be insensitive to GAP stimulation, thereby remaining constitutively GTP-bound (Foster, et al., 1996). While Rab24 is not thought to be completely hydrolysis-deficient, the RhoE example supports the broader principle that substitution of the conserved glutamine can strongly impair GTP hydrolysis and shift the equilibrium toward near-persistent GTP loading (Erdman, et al., 2000, Foster, et al., 1996).

However, interpretations of "atypical" Rab residues and presumed activating mutations require caution. Key work on Rab GAP mechanisms showed that, unlike Ras GAPs, many Rab GAPs employ a "dual-finger" mechanism in which catalytic residues are contributed by the GAP protein rather than relying on a single conserved glutamine residue in the Rab itself (Pan, et al., 2006).Therefore, the conserved switch II glutamine in many Rabs is not necessarily the catalytic residue that directly orients the hydrolytic water, but can instead be important for interaction with a specific GAP (Nottingham and Pfeffer, 2014, Pan, et al., 2006). Consistent with this, mutation of the switch II glutamine can have a relatively modest effect on GAP-catalyzed hydrolysis in some Rab–GAP pairs, whereas mutation of the catalytic "finger" residues in the GAP produces much larger decreases in catalytic efficiency (Nottingham and Pfeffer, 2014, Pan, et al., 2006). Importantly, Langemeyer et al. tested the assumptions underlying canonical "constitutively active" Rab mutants and showed that switch II glutamine mutations in several Rabs can impair activation by their respective GEFs, meaning that mutants widely assumed to be GTP-locked may in some cases fail to become activated at all in cells (Langemeyer, et al., 2014, Nottingham and Pfeffer, 2014). Importantly, different GEFs and GAPs can impose distinct structural requirements on the same Rab and the functional outcome of a mutation therefore could reflect altered regulator recognition rather than simple GTP hydrolysis impairment (Nottingham and Pfeffer, 2014).

These considerations are directly relevant for Rab24 because the "unusual" residue at position 67 (S67) lies within the broader nucleotide-binding/switch machinery that supports interactions with both regulators and effectors. While Erdman et al. linked S67 to reduced intrinsic GTPase activity and increased GTP-binding (Erdman, et al., 2000), later reports suggest that changes at conserved positions can also alter how a Rab is activated by GEFs or inactivated by GAPs, and

can therefore reshape the cycling behavior in ways that are not predictable from hydrolysis considerations alone (Langemeyer, et al., 2014, Nottingham and Pfeffer, 2014). In this context, Rab24-S67 mutants may primarily perturb regulator recognition and membrane targeting rather than simply shift the steady-state nucleotide state.

A related example is provided by Rab25, another atypical Rab in which a non-canonical leucine residue at the position corresponding to the conserved glutamine led to the assumption of constitutive activation. However, experimental work has shown that Rab25 can hydrolyze GTP (Gaudreault, et al., 2025). This supports the point that residues at conserved positions do not unambiguously define nucleotide state or functional output of Rab proteins, and that mutant-based inferences should always be validated in the relevant cellular context (Gaudreault, et al., 2025, Nottingham and Pfeffer, 2014).

Notably, Rab24 also differs from many other Rab proteins in how it responds to the "classical" activating mutations. In many Rabs, mutation of the conserved glutamine to leucine (e.g., Rab7-Q67L) leads to a constitutively active phenotype by reducing GTP hydrolysis. In contrast, Rab24-S67L binds GTP less efficiently than wild type Rab24, shows diffuse cytosolic distribution, does not detectably localize to membranous organelles, and has been interpreted as a dominant-negative mutant when overexpressed (Gutierrez, et al., 2005, Munafó and Colombo, 2002, Ylä-Anttila, et al., 2015). In line with this, Rab24-S67L fails to colocalize with LC3-positive autophagic structures, unlike wild-type Rab24 (Ylä-Anttila, et al., 2015). This behavior is consistent with Langemeyer et al., who reported that mutations assumed to generate constitutive activity can instead prevent activation by the relevant GEF, leading to loss of membrane association and downstream function (Langemeyer, et al., 2014, Nottingham and Pfeffer, 2014).

Additional unusual sequence features have also been proposed to influence Rab24's membrane cycling. Rab24 contains two histidines C-terminally adjacent to the prenylated cysteines. This pattern was suggested to be atypical among prenylated proteins and potentially relevant for Rab24 prenylation and/or association with GDIs (Erdman, et al., 2000). Indeed, Erdman et al. reported inefficient prenylation and weak association with GDI, implying that Rab24 might deviate from canonical Rab recycling mechanisms (Erdman, et al., 2000). However, subsequent work observed Rab24 interactions with both Gdi1 and Gdi2 in immunoprecipitation experiments (Behrends, et al., 2010), and Rab24 prenylation has been reported to be comparable to Rab7a (Ylä-Anttila, et al., 2015). Functionally, Rab24 targeting to autophagic compartments requires prenylation and GTP binding (Ylä-Anttila, et al., 2015). Thus, while Rab24 has unusual primary sequence features, available data overall support the importance of the canonical membrane-anchoring and nucleotide-binding properties for Rab24 localization and function. Nevertheless, there are

discrepancies across studies (Behrends, et al., 2010, Erdman, et al., 2000, Ylä-Anttila, et al., 2015).

Rab24 has also been reported to undergo tyrosine phosphorylation. Ding et al. identified phosphorylation at Y17 within the P-loop region and at Y172 in the hypervariable domain, with higher phosphorylation in cytosolic than membrane-associated Rab24 pools (Ding, et al., 2003). While phosphorylation at these sites was proposed to potentially influence GTP hydrolysis (Y17) and/or membrane targeting (Y172; Ding, et al., 2003), Rab24 recruitment to autophagosomes was reported not to require phosphorylation or dephosphorylation at Y17 or Y172 (Ylä-Anttila, et al., 2015). The physiological function of Rab24 tyrosine phosphorylation therefore remains to be clarified.

2.2.3.6.3 Rab24 in autophagy

Multiple Rab GTPases have been implicated at different steps of the autophagy pathway, from autophagosome formation to endolysosomal fusion (Fader, et al., 2008, Gutierrez, et al., 2004, Hirota and Tanaka, 2009, Itoh, et al., 2008, Itoh, et al., 2011, Jager, et al., 2004, Longatti, et al., 2012, Pilli, et al., 2012, Ravikumar, et al., 2008, Talaber, et al., 2014, Zoppino, et al., 2010). Rab24 has long been linked to autophagy, initially through localization-based studies and later through functional perturbation studies. Early work suggested that Rab24 relocalizes upon amino acid starvation to colocalize with LC3-positive vesicles and acidic compartments (Munafó and Colombo, 2002). Subsequent studies reported Rab24 colocalization with autophagosome markers including Gabarap and LC3-associated structures under additional stress and proteostasis-challenging conditions (Marambio, et al., 2010, Tambe, et al., 2009, Wu, et al., 2006). Importantly, ultrastructural analyses detected Rab24 on both the inner and outer membranes of autophagosomes and subcellular fractionation placed endogenous Rab24 in LC3-II and Sqstm1/p62-containing fractions (Ylä-Anttila, et al., 2015).

Rab24 was demonstrated to be selectively required for the late steps of basal autophagy. In HeLa and NRK cells, Rab24 depletion did not impair formation, maturation, or clearance of amino-acid starvation-induced autophagosomes, indicating that Rab24 is dispensable for starvation-induced autophagy under the conditions tested (Ylä-Anttila, et al., 2015). In contrast, under basal conditions Rab24 depletion caused accumulation of acidic, single-membraned autophagic vacuoles consistent with amphisomes or autolysosomes. Flux experiments supported the interpretation that this reflected defective clearance rather than enhanced formation (Ylä-Anttila, et al., 2015). Rab24 depletion also slowed basal clearance of a mutant Huntingtin-polyglutamine probe and slightly reduced the degradation of long-lived proteins (Ylä-Anttila, et al., 2015). These findings position Rab24 as a

regulator of late stages of basal autophagy, acting downstream of autophagosome formation and amphisome/autolysosome formation, which resemble the role of Rab7a in autophagy (Gutierrez, et al., 2004, Jager, et al., 2004). Several other Rab proteins primarily act earlier in autophagosome biogenesis or in starvation-dependent autophagy (Hirota and Tanaka, 2009, Itoh, et al., 2008, Longatti, et al., 2012, Ravikumar, et al., 2008, Talaber, et al., 2014, Zoppino, et al., 2010).

The molecular mechanism of the late basal-autophagy phenotype in Rab24-depleted cells remained unresolved. Two main routes have been described for autolysosome clearance: lysosome reformation from autolysosomes and fusion of autolysosomes with the plasma membrane (Chen and Yu, 2017, Chen and Yu, 2018, Dai, et al., 2019, Magalhaes, et al., 2016). Whether Rab24 participates directly in either pathway, or instead regulates an as-yet uncharacterized basal-autophagy-specific route of amphisome/autolysosome turnover, remained to be determined.

Work in endothelial cells further supports the notion that Rab24 can promote cargo turnover via basal autophagy. Neill et al. showed that intracellular Vegfa behaves as a basal autophagic substrate whose clearance can be enhanced by Decorin signaling via a Vegfr2/Ampk/Peg3 axis, and that Rab24 is required for efficient Vegfa catabolism in this pathway (Neill, et al., 2020). While the precise step at which Rab24 acts was not defined at the molecular level, these findings are consistent with Rab24 supporting late-stage autophagic degradation and/or turnover of autophagic/lysosomal compartments under basal conditions (Neill, et al., 2020, Ylä-Anttila, et al., 2015).

2.2.3.6.4 Rab24 in endolysosomal traffic

In addition to autophagy, Rab24 has been implicated in late endosomal/lysosomal traffic and degradative capacity. Amaya et al. reported that Rab24 co-precipitated with Rab7a and the Rab7a effector Rilp, colocalized with Rab7a-positive structures, and was required for Rab7a localization to vesicular compartments (Amaya, et al., 2016). Functionally, Rab24 was required for efficient degradation of endocytic cargo, supporting a role in late endocytic traffic and/or lysosomal degradation (Amaya, et al., 2016). On this basis, Rab24 was proposed to cooperate with Rab7a/Rilp on late endosomal/lysosomal compartments to support transport and/or fusion events necessary for endocytic degradation (Amaya, et al., 2016). These findings align with Rab24's proposed role in basal autophagy (Ylä-Anttila, et al., 2015).

2.2.3.6.5 Putative interaction partners of Rab24

At present, bona fide Rab24 effectors have not been conclusively established, and most reported interactions should be considered candidates that require independent

validation. Nevertheless, several reported interaction partners cluster around membrane tethering/fusion and SNARE-associated machinery. Rab24 has been reported to co-precipitate with Snap29 (Schardt, et al., 2009), a SNARE-associated protein implicated in autophagosome–endosome/lysosome fusion (see also section 2.1.1.4 Fusion with the lysosome; Itakura, et al., 2012b, Itakura and Mizushima, 2013, Morelli, et al., 2014, Schardt, et al., 2009, Takats, et al., 2013). Snap29 is required for autophagosome-lysosome fusion in both basal and starvation-induced autophagy, whereas Rab24 was suggested to be selectively required for late basal autophagy (Ylä-Anttila, et al., 2015). Rab24 depletion preferentially led to accumulation of acidic single-membrane amphisome/autolysosome-like structures rather than unfused double-membrane autophagosomes. Therefore, Rab24 is unlikely to function as a core SNARE component assisting membrane fusion (Itakura, et al., 2012b, Takats, et al., 2013, Ylä-Anttila, et al., 2015).

Proteomics-based interaction mapping of autophagy proteins placed Rab24 in an N-ethyl maleimide-sensitive fusion protein (Nsf)-centered subnetwork together with multiple SNARE components, and suggested direct interactions with Gdi1, Gdi2, Nsf, and Plakophilin-1, with Nsf potentially mediating indirect connectivity to additional SNARE proteins including Gosr1 and Snap29 (Behrends, et al., 2010). In addition, Rab24 has been reported to co-precipitate with the tumor suppressor Down-regulated by v-src (Drs; Tambe, et al., 2009), weakly bind Bicaudal D cargo adaptor 2 (Bicd2, also called Bicdr2) in GST pulldown assays (Schlager, et al., 2010), and interact with mutant forms of C-terminal-binding protein 1 (Ctbp1) (Fukuda, et al., 2008). While the functional significance of these interactions remains to be clarified, the recurrent appearance of SNARE- and Nsf-associated components among Rab24 candidate partners is consistent with a possible role for Rab24 in regulating events that depend on membrane fusion machinery (Amaya, et al., 2016, Behrends, et al., 2010, Schardt, et al., 2009).

2.2.3.6.6 Other functions of Rab24

In addition to its established roles in basal autophagy and late endolysosomal degradation described above, Rab24 has been implicated in other cellular processes, including functions in cell division, cytoskeletal organization, and organelle homeostasis. Rab24 has been reported to relocalize dynamically during mitosis: to the mitotic spindle in metaphase, midbody in telophase, and cleavage furrow during cytokinesis (Militello, et al., 2013). Further, Rab24 partially colocalized with tubulin and associated with microtubules. Both Rab24 overexpression and Rab24 depletion were associated with mitotic abnormalities, including aberrant spindle formation, chromosome misalignment/lagging, chromatin bridges, and increased frequencies of bi-/multinucleated cells, which indicates a requirement for appropriate Rab24 levels

for faithful chromosome segregation and cytokinesis (Militello, et al., 2013). Consistent with these mitotic findings, Rab24 has also been implicated in mammalian oocyte meiosis: Rab24 depletion impaired the progression of oocyte maturation, increased the abnormalities of meiotic apparatus, disturbed kinetochore–microtubule attachments, and promoted chromosome segregation errors and aneuploidy (Qiu, et al., 2019).

Rab24 has also been linked to mitochondrial morphology and metabolic homeostasis in the liver. Rab24 was identified as a regulator of mitochondrial fission through interaction with the fission factor mitochondrial fission 1 protein (Fis1) and modulation of the recruitment of Dynamin-1-like protein (Dnm1L, also known as Drp1). These interactions influenced mitochondrial network connectivity, respiration, and mitophagy-related phenotypes in hepatocytes. *In vivo*, liver-specific knockdown of Rab24 improved glucose tolerance and serum lipid levels and reduced hepatic steatosis in diet-induced obesity and non-alcoholic steatohepatitis mouse models. Rab24-depletion was accompanied by increased mitochondrial mass/connectivity, and altered autophagic flux. Notably, Rab24 knockdown was also reported to increase hepatic expression and secretion of the hepatokine Fibroblast growth factor 21 (Fgf21), and genetic evidence in Fgf21-deficient mice suggested that the improvement in glucose homeostasis and peripheral insulin signaling upon Rab24 knockdown depended on Fgf21 (Seitz, et al., 2019). In humans, hepatic Rab24 expression was reported to be elevated in obesity and non-alcoholic fatty liver disease and non-alcoholic steatohepatitis cohorts and to correlate with clinical/metabolic parameters, supporting potential disease relevance (Seitz, et al., 2019). These observations suggest that Rab24 can influence mitochondrial plasticity in the liver with systemic metabolic consequences, providing a conceptual link between trafficking regulators, mitochondrial dynamics, and metabolic adaptation.

Beyond the cytoskeleton- and mitochondria-related roles, Rab24 has emerged in screens for additional membrane trafficking functions. Systematic knockdown screening of Rab GTPases under Rab32/38-null conditions identified Rab24 and Rab10 to contribute to tyrosine transport, implicating Rab24 in endosome-to-melanosome traffic (the melanosome is a lysosome-related organelle) in cells where canonical Rab32/38-dependent transport is absent (Nishizawa, et al., 2022).

Finally, several studies have reported nuclear localization of Rab24 in particular experimental settings, highlighting context-dependence in Rab24 localization and interaction behavior. Rab24 constructs bearing substitutions in a conserved nucleotide-binding motif accumulated in ubiquitin- and Hsp70-positive nuclear inclusions and disrupted nuclear envelope integrity, with evidence showing that Rab24's unique C-terminal domain contributes to targeting of the mutant protein to inclusions (Maltese, et al., 2002). Likewise, Wu and colleagues reported predominant nuclear localization of Rab24 in COS-7 cells and identified interaction

candidates including Cyclophilin A and Gabarap (Wu, et al., 2006). While such nuclear/inclusion observations require cautious interpretation given potential effects of cell type, expression level and mutant constructs, they underscore that Rab24 has unusual features that may enable non-canonical localization patterns and protein interactions under specific conditions.

2.2.3.6.7 Rab24 in disease

The strongest evidence for a pathogenic connection of Rab24 comes from canine hereditary ataxia. A recessively inherited missense variant p.Gln38Pro (Q38P), located in the switch I region (Fig. 9), was shown to cause cerebellar ataxia in Gordon Setters and Old English Sheepdogs (Agler, et al., 2014a). Purkinje neuron loss with accumulation of ubiquitin-positive aggregates and late autophagic vacuoles (amphisomes/autolysosomes) in axonal spheroids was reported in the affected brains, consistent with defective clearance at late stages of the autophagy–lysosome pathway (see also section 2.2.3.6.3 *Rab24 in autophagy*). More recently, Schwarz et al. reported a second, independent pathogenic variant in Rab24, identified by whole-genome sequencing in a family of random-bred dogs with slowly progressive cerebellar ataxia. The variant (c.239G>T; G80V) showed segregation consistent with recessive inheritance and was absent from a large control dataset (Fig. 9). Notably, G80V locates in the conserved RabF4 motif and introduces a hydrophobic side chain at a position putatively exposed to the aqueous environment of the cytosol, providing a plausible structural basis for altered Rab24 function (Schwarz, et al., 2025). While functional validation was not performed in that study, the close clinical similarity to the previously-described Rab24-associated ataxia strengthens the conclusion that Rab24 dysfunction can cause neurodegenerative disease *in vivo* and supports considering Rab24 as a candidate gene in unresolved human ataxias (Schwarz, et al., 2025).

In addition to the genetic data, Rab24 expression has been reported to be stress-responsive in neuronal injury contexts, which may be relevant for understanding how Rab24-linked pathways are engaged in nervous system pathology. Rab24 mRNA and protein were upregulated in rat hypoglossal motor neurons following nerve transection, with a transient induction peaking in the early post-injury phase. In differentiated PC12 cells, Rab24 mRNA was selectively induced by proteasome inhibition (MG132) but not by several other stressors (Egami, et al., 2005). MG132 treatment increased LC3 mRNA and LC3-II accumulation, accompanied by partial colocalization of Rab24 and LC3-positive puncta. *In vivo*, nerve injury in rats also increased LC3 expression and LC3-II levels in the hypoglossal nucleus, with partial Rab24–LC3 colocalization by immunohistochemistry. The coordinated induction of Rab24 and autophagy markers supports a model in which Rab24 participates in

cellular remodeling and proteostasis responses triggered by neuronal injury, potentially by facilitating trafficking events required for autophagy and/or lysosome function under stress (Egami, et al., 2005). These observations provide physiological context for Rab24's function in neurons, although they do not by themselves demonstrate a causal role in disease.

Rab24 has been linked to liver cancer. The microRNA miR-615-5p is downregulated in HCC tissues and cell lines due to promoter hypermethylation, and restoration of miR-615–5p suppresses malignant phenotypes. Rab24 was identified as a direct functional target of miR-615–5p. Functionally, Rab24 overexpression phenocopied, whereas Rab24 knockdown opposed, the effects of miR-615–5p: elevated Rab24 promoted HCC cell growth by accelerating cell-cycle progression and reducing apoptosis, increased migration and invasion, and enhanced metastatic behavior *in vivo* (Chen, et al., 2017b). Moreover, Rab24 was linked to epithelial–mesenchymal transition, invasion-associated traits including cell–matrix adhesion (associated with modulation of β1-integrin levels) and vasculogenic mimicry (Chen, et al., 2017b). In addition, Rab24 has been reported to be upregulated in HCC/liver cancer expression profiling studies (Chen, et al., 2017b, Gu, et al., 2025, He, et al., 2002).

A recent study has also provided evidence for a pro-tumorigenic role of Rab24 in clear-cell renal cell carcinoma (ccRCC). *Rab24* mRNA and protein were reported to be upregulated in ccRCC compared with normal kidney tissue, and high *Rab24* expression was associated with advanced clinical stage, metastasis and poorer overall survival, with multivariate analyses supporting *Rab24* as an independent prognostic factor (Xu, et al., 2026). Functionally, *Rab24* overexpression promoted proliferation, migration and invasion in ccRCC cell lines, whereas shRNA-mediated knockdown suppressed these malignant traits and reduced xenograft tumor growth (Xu, et al., 2026). The same study further linked high *Rab24* expression to an immunosuppressive tumor microenvironment, reporting reduced $CD8^+$ T-cell infiltration in high-Rab24 tumors and increased CD8 staining in xenografts upon *Rab24* knockdown (Xu, et al., 2026). Interestingly, modulation of *Rab24* did not measurably change autophagy marker levels (p62, LC3, Beclin-1) or overall autophagy intensity by electron microscopy in this model, suggesting that Rab24's contribution to ccRCC progression may not be linked to alterations in autophagy (Xu, et al., 2026).

Changes in Rab24 expression have been reported across multiple human diseases, including cancer, inflammatory conditions, and neurological disorders, most commonly based on transcriptomic or multi-gene signature approaches. It should be noted that such association-level datasets can identify disease-linked expression patterns but typically do not resolve whether Rab24 is a driver of pathogenesis, a secondary marker of altered autophagy-lysosome function and/or

other cellular stress responses, or plays no role in the pathogenesis. In cancer, Rab24 has been included in autophagy-related prognostic models in prostate cancer (Hu, et al., 2020) and pancreatic cancer (Deng, et al., 2022, Yu, et al., 2021). In neurodegeneration, Rab24 appears in blood-based autophagy-related diagnostic models for Alzheimer's disease (Li, et al., 2023, Qin, et al., 2022). In immune-mediated disease, Rab24 has been proposed as a susceptibility-associated gene in rheumatoid arthritis by transcriptome-wide association analysis (Wu, et al., 2021) and shows altered expression alongside other autophagy-related genes in multiple sclerosis blood samples (Igci, et al., 2016). Rab24 has also been incorporated into diagnostic and immune-microenvironment-linked signatures in other conditions, including intervertebral disc degeneration (Hai, et al., 2022, Wang, et al., 2023) and low-grade glioma methylation-based prognostic modeling (Qiao, et al., 2022). Collectively, these studies suggest that Rab24 expression can track with disease state and prognosis in multiple contexts.

Overall, Rab24 is currently best supported as a disease-relevant trafficking regulator in three settings: (i) canine neurodegenerative ataxia, where genetics and neuropathology suggest disrupted proteostasis and accumulation of autophagic or autolysosomal structures, (ii) hepatocellular carcinoma and clear-cell renal cell carcinoma, where Rab24 has been functionally validated as a driver of malignancy and (iii) fatty liver and non-alcoholic steatohepatitis, where Rab24 expression has been reported to be elevated. Many additional reported disease associations remain to be functionally resolved, and the extent to which they converge mechanistically on Rab24's roles in basal autophagy and late endolysosomal trafficking remains an open question.

2.2.3.7 Rab7a

Rab7a is a highly conserved "housekeeping" Rab GTPase that localizes predominantly to late endosomes and lysosomes and coordinates trafficking steps at the late endocytic-lysosomal interface (Bucci, et al., 2000, Diekmann, et al., 2011). New research has identified another variant of Rab7, called Rab7b (Yang, et al., 2004), while the canonical Rab7 was renamed to Rab7a. Rab7a is introduced here primarily because it intersects with Rab24-regulated late degradative pathways: Rab24 co-precipitates with Rab7a and the Rab7a effector Rilp and is required for robust Rab7a localization to vesicular compartments in at least one study, suggesting functional coupling at late endosomal/lysosomal membranes (Amaya, et al., 2016; see also section 2.2.3.6.4 *Rab24 in endolysosomal trafficking*). In addition, Rab7a contributes to late stages of autophagic flux (Gutierrez, et al., 2004, Jäger, et al., 2004). Rab7a knockout data in mammalian cells support a prominent role in autolysosome maturation/clearance under nutrient-rich conditions rather than an

absolute requirement for autophagosome-lysosome fusion (Kuchitsu, et al., 2018; see section 2.2.3.7.3 *Rab7a in autophagy*).

2.2.3.7.1 Rab7a localization and roles in late endosome–lysosome function

Rab7a localizes predominantly to late endosomes and lysosomes and is a regulator of the late endosomal identity and function (Bucci, et al., 2000, Diekmann, et al., 2011). Rab7a acquisition on maturing endosomes accompanies the loss of Rab5 in the Rab5-to-Rab7a conversion process, marking the transition from early/sorting endosomal identity to late endosomal identity and enabling recruitment of late endosome–specific trafficking machinery (Poteryaev, et al., 2010, Rink, et al., 2005; see also section 2.2.3.4 (*Rab-defined membrane identity, Rab domains, and Rab conversion during organelle maturation*) and Fig. 8). In mammalian cells, Rab7a-positive compartments typically accumulate in the perinuclear region, suggesting that regulated positioning of late endosomes and lysosomes facilitates efficient maturation and cargo delivery to degradative compartments (Bucci, et al., 2000).

Functionally, Rab7a supports endocytic cargo delivery to degradative compartments and contributes to lysosome homeostasis (Bucci, et al., 2000). Perturbation of Rab7a function (by dominant-negative mutants or depletion) can disrupt late endosomal trafficking, alter perinuclear organization of late endosomes/lysosomes and autophagic vacuoles (Jäger, et al., 2004), and impair degradative flux, consistent with Rab7a acting at multiple points in late endosome maturation and lysosome-directed transport (Guerra and Bucci, 2016). Rab7a is also central to establishing and maintaining degradative capacity of late endosomal/lysosomal and autophagic compartments, thereby coupling endosomal and autophagic maturation to efficient proteolysis and cargo turnover (Guerra and Bucci, 2016).

Rab7a further influences cellular physiology by shaping the trafficking lifetime of internalized cell-surface receptors and transporters. By promoting progression of receptor-containing endosomes toward degradation or recycling pathways, Rab7a impacts both signal termination and the ability of cells to maintain appropriate levels of plasma-membrane proteins under changing environmental conditions (Ceresa and Bahr, 2006, Rojas, et al., 2008).

In addition, Rab7a contributes indirectly to sustained lysosomal function by supporting endosome-to-trans Golgi network (TGN) retrieval routes required for delivery of lysosomal hydrolases. For example, Rab7a-dependent regulation of the retromer pathway is required for efficient recycling of the Cation-independent mannose-6-phosphate receptor (Ci-mpr) between endosomes and TGN, and disruption of this retrieval can compromise lysosomal enzyme sorting and the degradative capacity of lysosomes (Rojas, et al., 2007, Rojas, et al., 2008).

The molecular basis of the Rab7a outputs is mediated by distinct Rab7a effectors controlling motility and positioning, tethering and fusion, luminal conditions, and endosome-to-TGN retrieval. These mechanisms are summarized separately in section 2.2.3.7.2 *Rab7a effectors*. Notably, Rab7a–retromer–GAP feedback via Tbc1d5 was introduced as an example of Rab domain regulation in section 2.2.3.4 (*Rab-defined membrane identity, Rab domains, and Rab conversion during organelle maturation*). The present subsection therefore focuses on Rab7a's compartment-level functions that have not been described earlier.

2.2.3.7.2 Rab7a effectors

Rab7a executes its late endosomal, autophagic and lysosomal functions by recruiting distinct effectors, which:

1. Coordinate organelle positioning and long-range transport.
2. Promote tethering/fusion with lysosomes.
3. Establish and maintain the degradative potential of lysosomes.

The Rab7 effectors most relevant to endosome and lysosome organization and lysosomal degradation are summarized in this subsection. Rab7a functions in autophagosome transport and autophagic maturation are discussed in sections 2.2.3.7.3 *Rab7a in autophagy*; see also section 2.1.1.2 *Autophagosome transport*, 2.1.1.4 *Fusion with the lysosome* and 2.1.1.5 *Autophagic lysosome reformation*.

A central Rab7a effector is Rilp, which links Rab7a-positive membranes to the dynein–dynactin complex and thereby promotes minus-end–directed movement toward the perinuclear region (Cantalupo, et al., 2001, Jordens, et al., 2001). Rilp can form a tripartite complex with Rab7a and the cholesterol-sensing protein oxysterol-binding protein-related 1 long (Osbpl1, also called Orp1l),. Osbp1l integrates lipid status with organelle positioning and regulates dynein-dependent transport and late endosome/lysosome clustering (Johansson, et al., 2005, Johansson, et al., 2007). Conversely, Rab7a can also mediate microtubule plus-end motility via Fyco1, a Rab7a effector that supports kinesin-dependent transport of Rab7a-positive compartments toward the cell periphery (Pankiv, et al., 2010). Fyco1 also couples to LC3 and participates in autophagosome motility; its autophagy-relevant role is discussed in section 2.1.1.2 *Autophagosome transport*. Together, these two Rab7a effectors provide a mechanistic basis for how Rab7a can control the spatial distribution of degradative organelles, which is important for both cargo delivery and fusion efficiency.

Rab7a-positive late endosomes and lysosomes undergo homotypic and heterotypic fusion events as they mature and exchange content. In yeast, the HOPS tethering complex is a core component of late endosome/vacuole fusion and can bind

the Rab7a orthologue Ypt7, coupling Rab identity to tethering and SNARE engagement (Bröcker, et al., 2012, Lürick, et al., 2015). In mammals, the precise relationship between Rab7a and HOPS recruitment appears more complex and HOPS recruitment to target organelles may not be strictly Rab7a-dependent. Several studies support roles for additional small GTPases and adaptor proteins in HOPS assembly and targeting (Khatter, et al., 2015, Lin, et al., 2014b). Nevertheless, Rab7a effectors, particularly Rilp, can interact with HOPS components, thereby linking Rab7a-positive compartments to tethering/fusion machinery at lysosomes (Jiang, et al., 2014, Lin, et al., 2014b). Section 2.3.1 *HOPS composition and its relationship to CORVET* describes further details on HOPS recruitment in metazoan cells.

Rab7a effectors also contribute to establishing the biochemical environment required for lysosomal degradation. Rab7a, via Rilp, has been linked to regulation of lysosomal pH by controlling the assembly and/or function of the vacuolar-type ATPase (V-ATPase), including interactions involving the V1G1 subunit (De Luca and Bucci, 2014). Because V-ATPase-dependent acidification is essential for the activity of lysosomal hydrolases and for ligand–receptor dissociation within late endocytic compartments, Rab7a–Rilp coupling to V-ATPase provides a direct route by which a Rab7a can influence lysosomal degradative efficiency beyond membrane transport alone (Collins and Forgac, 2020).

Another Rab7a effector is the retromer complex, which mediates endosome-to-TGN retrieval of sorting receptors including Ci-mpr and thereby sustains lysosomal enzyme delivery. Rab7a interacts with components of the retromer cargo-recognition complex including Vps26 and Vps35. Rab7a loss-of-function disrupts retromer membrane association and impairs retrieval of Ci-mpr, leading to missorting of lysosomal hydrolases and compromised lysosomal proteolytic capacity (Rojas, et al., 2008, Seaman, et al., 2009). Thus, the interaction with the retromer complex also provides a mechanistic link between Rab7a-regulated endosome maturation and long-term lysosomal maintenance. The Rab7a–retromer relationship is dynamically regulated by the Rab7a GAP Tbc1d5, illustrating how Rab7a switch-on/switch-off cycling can finetune retrieval pathways and the composition/function of late endosomes (Seaman, et al., 2009). In neurons, Rab7a–retromer coupling is of particular interest because it intersects with trafficking decisions for signaling receptors and the long-term maintenance of degradative capacity in highly polarized cells (Rahman and Morrison, 2019). Rab7a–retromer–GAP feedback was introduced conceptually in section 2.2.3.4 *Rab-defined membrane identity, Rab domains, and Rab conversion during organelle maturation*.

In summary, Rab7a effectors can be viewed as partially separable modules that together define late endosome/lysosome behavior: motor coupling (Rilp/Osbpl1 and Fyco1), tethering/fusion competency (HOPS complex), luminal acidification (V-ATPase-linked regulation), and retrieval-dependent lysosome maintenance

(retromer). This modular organization provides a useful framework for comparing Rab7a-linked late degradative phenotypes with those attributed to Rab24 at lysosome-proximal steps (sections 2.2.3.6.3 *Rab24 in autophagy* and 2.2.3.6.4 *Rab24 in endolysosomal trafficking*).

2.2.3.7.3 Rab7a in autophagy

Rab7a has long been implicated in late steps of macroautophagy, consistent with its localization to late endosomes/lysosomes and its general role in maturation of degradative compartments (Kuchitsu and Fukuda, 2018). This subsection describes the roles of Rab7a in neuronal cells. Autophagosome transport and the core tethering and fusion machinery of autophagosome–lysosome fusion are described in sections 2.1.1.2 *Autophagosome transport* and 2.1.1.4 *Fusion with the lysosome* respectively. Rab7 also participates in maintaining lysosome homeostasis after sustained autophagic activity through ALR (see section 2.1.1.5 *Autophagic lysosome reformation*).

The requirement for temporally controlled Rab7a membrane association provides a framework for interpreting late-stage clearance phenotypes in autophagy, where impaired autophagic cargo clearance may reflect impaired autphagosome-lysosome fusion or impaired recycling of autolysosomes by ALR or both.

2.2.3.7.4 Rab7a in neuronal trafficking and disease

Rab7a is ubiquitously expressed and essential for viability. Rab7a knockout is embryonically lethal in mice (Kawamura, et al., 2012). Neurons place particularly stringent demands on Rab7a-regulated endosomal trafficking because of their extreme polarity, long axons, and need to sustain proteostasis and signaling over the lifetime of the organism (Boecker, et al., 2020, Saxena, et al., 2005). In neuronal processes, Rab7a-positive late endosomes and lysosome-related compartments are generally enriched more proximally. Rab7a-dependent retrograde movement contributes to the delivery of endocytic and autophagic cargo toward somatic lysosomes, thereby supporting both receptor turnover and proteostatic homeostasis (Yap, et al., 2018, Yap, et al., 2022). This subsection focuses on Rab7a-linked neuronal trafficking concepts that are most directly relevant to disease. Neuron-specific organization of the autophagy-lysosome system and long-range transport constraints are discussed in section 2.1.3.1.1 *Neuron-specific organization and challenges of the autophagy-lysosome system*, and amphisome-based neurotrophin signaling contexts are introduced in section 2.1.1.2 *Amphisomes*.

An important neuronal function of Rab7a is neurotrophin receptor trafficking. Nerve growth factor (Ngf) binding to cell-surface TrkA at distal axons triggers

endocytosis into signaling endosomes that undergo long-range retrograde transport to communicate survival and differentiation signals to the soma. Multiple studies suggest that a significant fraction of these endocytic carriers acquire Rab7a during maturation, and perturbation of Rab7a can alter TrkA trafficking dynamics and signaling output (Howe and Mobley, 2005, Saxena, et al., 2005). In this context, Rab7a primarily contributes to the maturation and routing of neurotrophin receptor–containing endosomes for degradation, thereby influencing where and for how long signaling persists (Barford, et al., 2017, Ye, et al., 2018).

The strongest direct genetic link between Rab7a dysfunction and human disease is Charcot–Marie–Tooth disease type 2B, an autosomal dominant axonal peripheral neuropathy caused by missense mutations in Rab7a (including K126R, L129F, K157N, N161T/I, and V162M; Cogli, et al., 2009, Verhoeven, et al., 2003). These mutations cluster in conserved nucleotide-binding regions and are commonly described as "rapid-cycling" alleles with increased nucleotide exchange and elevated GTP loading, leading to altered membrane association and spatiotemporal control of effector engagement (McCray, et al., 2010, Spinosa, et al., 2008). A prevailing model is that Charcot–Marie–Tooth disease type 2B is not caused by complete loss of Rab7a function but rather by dysregulated Rab7a activity that perturbs effector balance and endosomal progression in long peripheral axons, thereby disrupting neurotrophin receptor trafficking or signaling and/or broader degradative homeostasis (Bucci and De Luca, 2012, Liu and Wu, 2017). Consistent with this, studies in cell and animal models of Charcot–Marie–Tooth disease type 2B have reported altered axonal transport behavior of Rab7a-positive endosomes and changes in receptor trafficking kinetics, though the directionality and magnitude of these effects can vary across experimental systems and specific alleles (Cioni, et al., 2019, Zhang, et al., 2013). Overall, Charcot–Marie–Tooth disease type 2B highlights that comparatively subtle perturbations in a late endosomal Rab can produce selective vulnerability of long peripheral neurons, supporting the principle that precise regulation of endosome and lysosome trafficking and signaling is particularly critical in the nervous system (Boecker, et al., 2020).

2.3 HOPS tethering complex

Endocytic, autophagic, and biosynthetic trafficking routes converge on lysosomes for macromolecule breakdown and recycling. The multisubunit HOPS complex functions as a conserved membrane-tether that operates at late stages of the endo-lysosomal and autophagic pathways. Because the delivery of cargo into highly proteolytic, acidic compartments ultimately commits the cargo for degradation, the fusion reactions that connect upstream organelles to lysosomes require stringent

regulation. In this setting, HOPS has emerged as a central coordinator of several lysosome-directed fusion events, thereby contributing to both efficient degradative flux and preservation of compartment identity within the endo-lysosomal and autophagic network (van der Beek and Klumperman, 2025).

Mechanistically, HOPS functions within the canonical membrane fusion governed by Rab-family GTPases, tethering factors, and SNARE proteins. As a multisubunit tethering complex, HOPS can integrate multiple binding interactions on opposing membranes to stabilize close-range contacts in a process termed tethering. The HOPS complex promotes efficient SNARE complex assembly via its Sec1/Munc18 (SM)-family subunit, which couples membrane tethering to fusion catalysis (Ungermann and Moeller, 2025). In metazoan cells, loss of HOPS consistently delays or blocks late endosome–lysosome cargo transfer, resulting in accumulation of late endosomal intermediates that are poorly connected to degradative lysosomes (Pols, et al., 2013a, Rieder and Emr, 1997, van der Beek, et al., 2024, Wartosch, et al., 2015). HOPS is also required for late-stage autophagic traffic, with recent experiments supporting a particularly stringent requirement at the amphisome–lysosome fusion step (Miao, et al., 2021, van der Beek, et al., 2024). In addition, emerging work indicates that HOPS subunits, most notably Vps41, participate in tethering or fusion of specific TGN-derived biosynthetic carriers that deliver lysosomal membrane proteins such as Lamps to late endosomal compartments (Pols, et al., 2013b, Sanzà, et al., 2025, Schoppe, et al., 2020). At the organismal level, disruption of HOPS subunits is incompatible with normal development and is linked to severe, often neurodegenerative disease phenotypes, emphasizing the importance of HOPS-controlled lysosome delivery pathways for tissue homeostasis (Monfrini, et al., 2021b, van der Beek, et al., 2019).

The following subsections outline HOPS composition and architecture, summarize current mechanistic models for how HOPS promotes membrane tethering and SNARE-dependent fusion, and discuss how HOPS connects distinct lysosome-directed trafficking pathways in metazoan cells.

2.3.1 HOPS composition and its relationship to CORVET

HOPS is part of a set of endo-lysosomal multisubunit tethering complexes that organize membrane fusion reactions by integrating Rab recognition with SNARE regulation. Its closest counterpart is the class C core vacuole/endosome tethering (CORVET) complex, which operates in the endocytic pathway. Both complexes were originally defined in yeast and are conserved across eukaryotes, sharing a common design principle in which a conserved core is combined with complex-specific subunits that mediate stage- and compartment-specific targeting (Fig. 10;

Peplowska, et al., 2007, Seals, et al., 2000). In their canonical form, HOPS and CORVET are heterohexamers with an identical four-subunit “class C” core composed of Vps11, Vps16, Vps18 and Vps33a (Fig. 10; Peplowska, et al., 2007, van der Kant, et al., 2015, Zhang and Hughson, 2021). Within this core, Vps33a is an SM-family protein and, together with Vps16, forms the conserved SNARE-interacting module, consistent with a direct role of these tethers in SNARE complex assembly and fusion catalysis rather than tethering alone (Fig. 10; Baker, et al., 2015, Zhang and Hughson, 2021). The remaining two subunits specify the complex: HOPS contains Vps39 and Vps41, whereas CORVET contains Vps3 and Vps8 (Fig. 10; Seals, et al., 2000, van der Kant, et al., 2015, Wurmser, et al., 2000).

Structural studies support an elongated organization of the HOPS and CORVET complexes, in which the Rab-interacting subunits are positioned toward the periphery, whereas the Vps16–Vps33a module is arranged more centrally, providing a geometry for bridging opposing membranes while simultaneously presenting a SNARE-assembly site (Fig. 10; Shvarev, et al., 2024, Shvarev, et al., 2022). Early negative-stain electron microscopy of yeast HOPS showed an extended architecture compatible with tethering across two membranes, and subsequent work suggested that HOPS can adopt alternative conformations, implying flexibility that may be relevant during engagement of membranes and SNAREs (Bröcker, et al., 2012, Chou, et al., 2016). Recent structural comparisons further indicate that, aside from the SM subunit Vps33, the individual subunits share a related domain arrangement that includes N-terminal β-propeller and α-solenoid regions, underscoring that HOPS and CORVET are built from repeated “building blocks” adapted into different tethering complexes (Ungermann and Moeller, 2025).

Despite shared architecture, CORVET and HOPS are recruited to different membranes and linked to different Rab-defined stages of endosome and autophagosome maturation. CORVET associates with Rab5-positive early endosomes and supports fusion at early endocytic compartments (Peplowska, et al., 2007, Perini, et al., 2014). By contrast, HOPS functions at late endosomes, amphisomes, and lysosomes. In yeast, HOPS is recruited by the homolog of mammalian Rab7a, the GTPase Ypt7 on late endosomes/vacuoles (Bröcker, et al., 2012, Seals, et al., 2000).

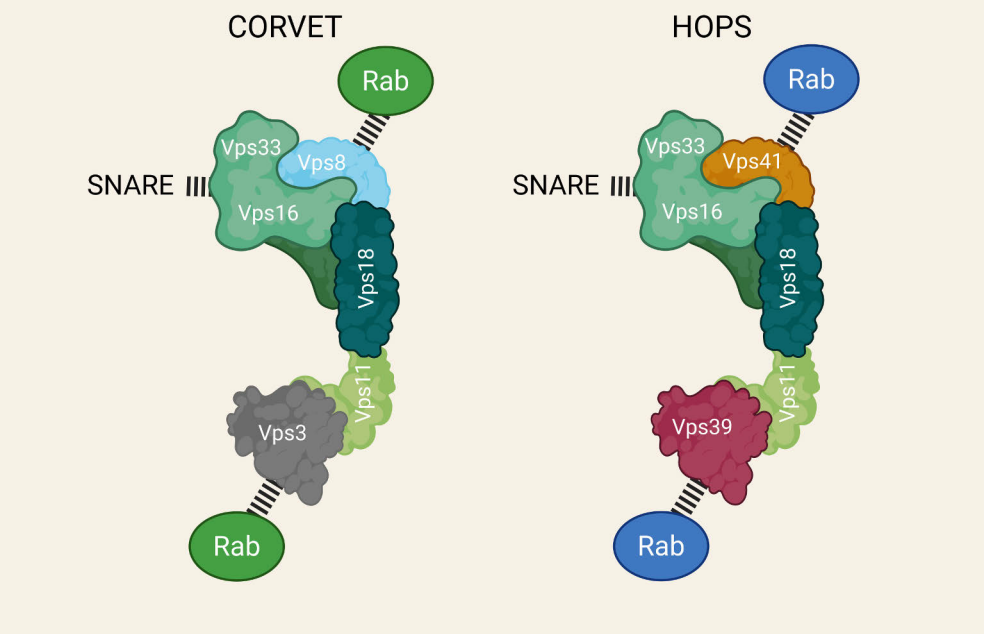

Figure 10. Subunit arrangement of the CORVET and HOPS tethering complexes and their interaction interfaces. Both complexes share a conserved four-subunit core consisting of Vps11, Vps16, Vps18 and the SM-family subunit Vps33, while they differ in their complex-specific subunits: Vps3 and Vps8 in CORVET, and Vps39 and Vps41 in HOPS. Rab/Rab effector-binding sites are depicted on the complex-specific subunits, reflecting recruitment to markers of membrane-compartment identity that can include Rab GTPases and/or Rab-associated effector proteins. A SNARE-binding/assembly interface is indicated on the Vps33–Vps16 region, consistent with the role of these subunits in coupling membrane tethering to SNARE-mediated fusion. Created in BioRender.

2.3.2 Recruitment of HOPS in metazoan cells

In metazoan cells, the determinants of HOPS recruitment to its target membranes are more diversified than in yeast: Vps39 and Vps41 can interact with several small GTPases, and recent work has highlighted Rab2 and Arl8b as key interactors for late endosomal and lysosomal targeting, respectively (Khatter, et al., 2015, Sanzà, et al., 2025, Schleinitz, et al., 2023). In particular, multiple lines of evidence support an Arl8b–Vps41-centered mechanism for positioning HOPS on lysosome-associated membranes. Khatter et al. found that Arl8b (rather than Rab7a) is required for membrane association of human Vps41, and that recruitment of additional HOPS subunits to Arl8b/Vps41-positive lysosomes follows from subunit–subunit interactions within the complex (Khatter, et al., 2015). In the same study, the Arl8b effector Plekhm2 (also called SKIP) was shown to interact with and recruit HOPS subunits to Arl8b-positive, Kinesin-associated peripheral lysosomes. Consistent with this, Plekhm2 depletion disrupted lysosome positioning/trafficking and delayed

the degradation of Epidermal growth factor receptor (Egfr; Khatter, et al., 2015). More recently, Vps41 was also implicated in recruiting TGN-derived Lamp-carrier intermediates in an Arl8b-dependent manner, expanding the Vps41–Arl8b axis beyond endosome–lysosome fusion (Sanzà, et al., 2025).

HOPS recruitment has also been linked to Rab effector proteins that connect Rab proteins to HOPS. In particular, the Rab7a effector Plekhm1 binds to GTP-bound Rab7a on late endosomes and lysosomes. It has also been proposed to bind directly to LC3/Gabarap family proteins on the autophagosome membrane, functioning as a molecular bridge that couples maturing autophagosomes to degradative compartments. Through its multiple domains, Plekhm1 recruits the HOPS complex to the autophagosomal surface, thereby promoting efficient SNARE complex assembly and membrane fusion. Loss of Plekhm1 disrupts this coordination, leading to stalled autophagosomes and impaired cargo degradation (McEwan, et al., 2015). Notably, Plekhm1 can also bind Arl8b, and Arl8b promotes HOPS recruitment to Plekhm1-positive vesicle contact sites, supporting delivery of both endocytic and autophagic cargo for lysosomal degradation (Marwaha, et al., 2017).

In addition to Plekhm1, the Rab7a effector Rilp has been reported to recruit HOPS components to late endosomes through direct interaction with the HOPS subunit Vps41. This interaction was mapped to the N-terminus of Rilp and the C-terminal region of Vps41, and found to be independent of Rab7a. Disruption of Vps41 function was found to delay Egfr degradation, consistent with a role for the Rilp–Vps41 axis in endocytic cargo delivery to lysosomes (Lin, et al., 2014a).

Recent studies of late endosome–lysosome tethering further refined the topology of the HOPS recruitment cues: Rab2a is enriched on late endosomes, whereas Arl8b, anchored by the BLOC-one-related (BORC) complex, defines the lysosomal membrane. HOPS can bridge Rab2a-positive late endosomes with Arl8-positive lysosomes. In this model, Rab7a primarily promotes late endosome motility towards lysosomes by coupling to dynein-driven transport but is not enriched at the actual HOPS-dependent tethering sites (Schleinitz, et al., 2023).

In autophagy, reconstitution experiments suggest that mammalian HOPS can assemble on membranes from two stable subcomplexes in a Rab-dependent manner (“hook-up” model). The model proposes two HOPS subcomplexes that dock on their target membranes via Rabs (Zhang, et al., 2023). Rab39a was identified as a Rab that recruits a HOPS subcomplex to lysosomes. Rab39a activation was promoted by the amyotrophic lateral sclerosis/frontotemporal dementia-associated factor C9orf72 (Zhang, et al., 2023). Rab2 was proposed to recruit the other HOPS subcomplex to autophagosomes. Rab2-HOPS-Rab39a was proposed to promote autophagosome-lysosome fusion.

The divergence in the models for HOPS recruitment suggests that, in metazoans, the same tethering complex can be positioned by multiple GTPase cues on distinct lysosome-directed membranes rather than relying on a single Rab as in yeast.

2.3.3 HOPS function in tethering and SNARE-mediated fusion

Membrane fusion at late endosomes and lysosomes is mediated by canonical Rab-directed fusion in which active Rab GTPases recruit effectors, including tethering factors, and thereby promote stable membrane contact that enables trans-SNARE complex assembly and bilayer mixing. As a multisubunit tethering complex, HOPS contributes to this process in at least two ways: it helps establish and stabilize the initial contact between membranes (tethering), and it promotes efficient SNARE assembly through its central SM family subunit Vps33a (Fig. 4; Baker, et al., 2015). This dual functionality distinguishes HOPS from purely structural tethers and supports the view that HOPS can actively facilitate fusion events once the correct membranes have been brought together.

At the targeting level, HOPS recognizes organelle identity by binding to membrane-associated proteins, primarily small GTPases. In yeast, HOPS membrane association and tethering depend on Ypt7, whereas CORVET preferentially recognizes Rab5-positive early endosomal membranes and shows sensitivity to particular charged phospholipids and membrane properties (Ungermann and Moeller, 2025). In metazoan cells, HOPS recruitment can be achieved through multiple GTPases or their effectors (section 2.3.2 *Recruitment of HOPS in metazoan cells*), with Vps39 and Vps41 engaging distinct GTPases on late endosomal or autophagic and lysosomal membranes, respectively, thereby enabling HOPS to bridge two apposing organelles at lysosome-proximal fusion steps (Ding, et al., 2019, Khatter, et al., 2015, Schleinitz, et al., 2023, Zhang, et al., 2023).

Following initial tethering, HOPS promotes formation of fusogenic SNARE complexes. Structural and biochemical studies in yeast indicate that HOPS contains a SNARE-assembly module formed by the SM protein Vps33 and its partner Vps16, positioned centrally within the tether, allowing HOPS to couple membrane proximity to SNARE engagement (Baker, et al., 2015). *In vitro*, HOPS can catalyze Rab- and SNARE-dependent fusion of proteoliposomes. Vps33 can bind SNAREs to facilitate assembly of trans-SNARE complexes between opposing membranes, organizing productive SNARE pairing at the tethered site (Stroupe, et al., 2006, Stroupe, et al., 2009, Zick and Wickner, 2016). Mechanistic models further suggest that the physical size and geometry of CORVET/HOPS can contribute to lowering the energetic barrier for fusion pore formation and expansion once a SNARE complex has engaged, thereby enhancing the efficiency of bilayer mixing

(D'Agostino, et al., 2018, D'Agostino, et al., 2017). While the mechanistic insights derive largely from yeast and reconstituted systems, they provide a conceptual framework for understanding how HOPS can act as a catalyst of membrane fusion rather than a passive tether.

In mammalian autophagy, HOPS-dependent fusion is mediated by a defined set of autophagy-linked SNAREs and cofactors. These components (including Stx17–Snap29–Vamp7/8 and adaptors such as Plekhm1), are introduced in section 2.1.1.4 *Fusion with the lysosome*.

2.3.4 HOPS in vesicle delivery to the lysosome

HOPS is required at lysosome-proximal fusion reactions that connect endosomes, autophagosomes/amphisomes, and selected biosynthetic carriers to the degradative compartments (van der Beek and Klumperman, 2025). This positioning makes HOPS a useful conceptual "hub" for understanding how cells maintain directional cargo delivery to lysosomes while limiting inappropriate mixing between upstream organelles.

In the endocytic pathway, HOPS is linked to late endosome–lysosome fusion. In metazoan cells depleted of HOPS subunits, late endosomes accumulate and cargo delivery to lysosomes is strongly delayed, indicating that fusion with the lysosome becomes inefficient when HOPS function is compromised (Caplan, et al., 2001, Pols, et al., 2013a, van der Beek, et al., 2024). Yeast studies established that the HOPS-dependent fusion is conserved, although the upstream recruitment cues differ between organisms (Seals, et al., 2000, Stroupe, et al., 2006).

In autophagy, HOPS contributes to delivery of autophagic cargo to lysosomes, but recent genetic evidence suggests that its requirement differs between sequential fusion steps. Multiple studies support that HOPS interacts with autophagy-linked SNARE machinery (Jiang, et al., 2014, Takáts, et al., 2014). However, analyses of HOPS knockout cells indicate that, while autophagic material can still enter endosomal compartments, fusion of the amphisome-like intermediates with lysosomes is strongly dependent on HOPS (Miao, et al., 2021, van der Beek, et al., 2024). In this scenario, amphisomes accumulate when HOPS function is disrupted, consistent with a model in which HOPS is dispensable or partially redundant for autophagosome–endosome fusion but becomes essential for the subsequent amphisome–lysosome fusion step (Miao, et al., 2021, van der Beek, et al., 2024). This two-step model of autophagosome fusions is consistent with the observation that disrupting HOPS function (for example via viral interference with Vps39) can lead to build-up of late autophagic intermediates (Miao, et al., 2021). Thus, within the autophagy pathway, HOPS appears to act most stringently at the lysosome-

directed fusion event that commits endosome-associated autophagic cargo to lysosomal degradation.

Beyond endocytosis and autophagy, accumulating evidence indicates that HOPS also intersects with biosynthetic delivery routes that provide lysosomal components to the endo-lysosomal system. In particular, work on TGN-derived “Lamp carriers”, vesicles that transport newly-made Lamps from the TGN to late endosomes, suggests that a subset of lysosomal membrane proteins are delivered directly from the TGN to late endosomes, and that fusion of these carriers with endosomal compartments is, at least in part, dependent on Vps41 (Pols, et al., 2013b, Sanzà, et al., 2025, Schoppe, et al., 2020). Such carriers can transport lysosomal membrane proteins (e.g., Lamp1/2) and, in some systems, additional lysosome-associated components, implying that biosynthetic input can help prime late endosomes for efficient lysosomal fusion by supplying membrane proteins and potentially fusion-related machinery (Pols, et al., 2013b, Sanzà, et al., 2025). Hence, the delivery of soluble hydrolases and the delivery of lysosomal membrane proteins are routed through distinct pathways, providing cells with additional regulatory control over how degradative capacity is assembled and maintained (Pols, et al., 2013b).

2.3.5 HOPS dysfunction in development and disease

Genetic disruption of HOPS subunits has severe consequences at both the cellular and organismal level, consistent with the central role of HOPS in lysosome-directed fusion steps. However, it is also possible that the HOPS subunits have biological functions that are independent of the whole HOPS complex. In metazoan cells, loss of HOPS function typically slows or prevents fusion steps that deliver endocytic and autophagic cargo into degradative lysosomes, resulting in accumulation of late endosomal and autophagy-related intermediates (Caplan, et al., 2001, Pols, et al., 2013a, van der Beek, et al., 2024, Wartosch, et al., 2015).

At the organismal level, complete loss of HOPS function impairs normal development. Full knockout of CORVET/HOPS core subunits or HOPS-specific subunits is frequently embryonic or early postnatal lethal in animal models, suggesting that HOPS-dependent fusion steps are required for tissue homeostasis from early stages onward (Aoyama, et al., 2012, Messller, et al., 2011, Peng, et al., 2012b). When milder alleles (genetic variants that reduce gene function only partially), hypomorphic variants, or conditional knockouts were examined, a recurrent theme was vulnerability of the nervous system, consistent with the high dependency of neurons on efficient lysosomal clearance and autophagic flux (Monfrini, et al., 2021b, Peng, et al., 2012b).

For example, neuron-specific loss of the core subunit Vps18 in mice causes severe neurodegeneration and neuronal migration defects, accompanied by blockade

of the lysosome-directed trafficking routes (autophagy, endocytosis and biosynthetic delivery) and altered surface levels of β1-integrin. Knockdown of β1-integrin partially rescued the migration defects (Peng, et al., 2012b). Additional work indicated that Vps18-dependent lysosomal degradation can be selectively critical for dendrite development in certain neuronal populations, with Vps18 deficiency impairing Purkinje cell dendritogenesis and leading to accumulation of Lysyl oxidase (Lox), proposed to be a lysosomal substrate in this context (Peng, et al., 2012a).

Across vertebrate and invertebrate models, mutations affecting the HOPS/CORVET core subunits have been associated with neurodegeneration (Zhang, et al., 2016a) and additional lysosome-related phenotypes such as pigmentation defects in zebrafish (Clancey, et al., 2013, Maldonado, et al., 2006, Pulipparacharuvil, et al., 2005, Thomas, et al., 2011) and in *Drosophila melanogaster* (Lloyd, et al., 1998, Pulipparacharuvil, et al., 2005, Sevrioukov, et al., 1999, Shestopal, et al., 1997, Warner, et al., 1998). Furthermore, HOPS defects can cause liver dysfunction in zebrafish models (Clancey, et al., 2013, Maldonado, et al., 2006, Pulipparacharuvil, et al., 2005, Sadler, et al., 2005, Schonthaler, et al., 2008, Thomas, et al., 2011). This reflects the broad requirement for lysosomal targeting in tissues.

In humans, variants in genes encoding HOPS subunits are increasingly linked to neurological diseases, particularly dystonia-spectrum phenotypes and progressive neurodevelopmental disorders. Patient-derived fibroblasts with defects in Vps16, Vps41 and Vps11 show ultrastructural abnormalities of lysosomal/autophagic compartments consistent with defects in trafficking to the lysosome and in autophagic flux (Cai, et al., 2016, Monfrini, et al., 2021a, Zhang, et al., 2016a). Heterozygous loss-of-function variants in Vps16 were identified in Vps16 in patients with early-onset progressive dystonia, and biallelic loss-of-function variants in Vps41 were found in severe infantile-onset disease (Steel, et al., 2020). Additional genetic studies established homozygous Vps41 variants as a cause of a progressive neurodevelopmental syndrome characterized by cognitive impairment, cerebellar atrophy/hypoplasia, and motor dysfunction with ataxia and dystonia. Zebrafish model of the disease supported widespread lysosomal dysregulation in the brain (Sanderson, et al., 2021). Mechanistic work with patient fibroblasts further indicated that Vps41 mutations can disrupt the formation of a functional HOPS complex and delay lysosomal delivery of endocytic and autophagic cargo (van der Welle, et al., 2021).

3 Aims

Autophagy is a central lysosome-dependent quality-control pathway with key roles in development, infection control, neurodegeneration and cancer. While the core molecular machinery of autophagosome formation and lysosomal degradation has been intensively studied, major gaps remain in (i) identifying pharmacological compounds that modulate autophagic flux in a mechanistically interpretable manner, (ii) understanding how autophagy-relevant trafficking regulators are expressed across tissues and disease contexts, and (iii) defining how the atypical small GTPase Rab24 connects to autophagosome/amphisome-to-lysosome fusion machinery that governs cargo delivery to degradative compartments. Addressing these questions is important both for improving autophagy-targeted therapeutic strategies and for clarifying how defects in the late-stages of autophagy contribute to neuro-degeneration and cancer-associated phenotypes. Therefore, this PhD thesis combines phenotypic screening, organism- and disease-relevant expression analysis, and mechanistic interaction studies to investigate pharmacological modulation of autophagy and the role of Rab24 in lysosome-directed trafficking.

The specific objectives of this thesis were:

1. To characterize small-molecule modulators of basal and stress-induced autophagy using microscopy, and to evaluate selected hits in a cellular model of neurodegenerative vacuolar storage disease linked to Atg4D mutation.
2. To determine Rab24 protein abundance across mouse tissues during postnatal development and adulthood, and to assess how Rab24 protein levels differ between human cancers and corresponding normal tissues, in order to identify tissue- and disease-associated contexts in which Rab24 may be functionally relevant.
3. To define the molecular link between Rab24 and lysosome-proximal fusion machinery, with a focus on the homotypic fusion and vacuolar protein sorting (HOPS) complex and Rab7a, and to assess how ataxia-associated Rab24 mutation alters these interactions and correlates with autophagy phenotypes.

4 Materials and Methods

The experimental procedures that were performed by the author of this PhD thesis are described below. Detailed methodology is available in the original publications (I–III).

4.1 Cell culture (I–III)

4.1.1 Cell lines and culture conditions (I–III)

The cell lines used in the experimental work are listed in Table 1 together with their culture media and the corresponding original publication(s).

A549 cells stably expressing GFP–LC3 were used for confocal analysis of compound effects on LC3- and p62-associated readouts and for LC3–Lamp2 colocalization analyses (I). Wild type (WT) and Rab24-knockout (KO) mouse neuroblastoma Neuro-2a cells were used for studies of Rab24 function, including proliferation and migration assays, RNA sequencing (II), ultrastructural analysis, lysosomal functional assays, proximity proteomics and interaction experiments (III). WT and Rab24-KO human HeLa cells were used for functional phenotyping in publication II. Primary fibroblasts derived from Lagotto Romagnolo dogs (WT and Atg4D-A430T mutant) were used to assess basal and starvation-induced LC3 phenotypes and compound responses in a disease-relevant genetic context (I).

All cell culture media were supplemented as described in the original publications. Where applicable, selection antibiotics were maintained only for routine propagation of stable lines and omitted during experimental treatments unless otherwise required.

Table 1. Cell lines and culture media used in this thesis (I–III).

Cell line	Species / type	Key modification/ genotype	Culture medium	Used in
A549 GFP–LC3	Human lung carcinoma	Stable GFP–LC3	Ham's F-12K Nutrient Mix + supplements	I
Neuro-2a WT	Mouse neuroblastoma	Wild type	MEMα + supplements	II, III
Neuro-2a Rab24-KO	Mouse neuroblastoma	CRISPR/Cas9 Rab24 knockout	MEMα + supplements	III
Neuro-2a Rab24-KO + rescue	Mouse neuroblastoma	Stable GFP–Rab24 (WT/mutants) or APEX2 constructs	MEMα + supplements	III
HeLa WT / Rab24-KO	Human cervical carcinoma	CRISPR/Cas9 Rab24 knockout	DMEM + supplements	II
Dog fibroblasts WT / Atg4D A430T	Canine primary fibroblasts	Wild type and NVSD model	MEMα + supplements	I

4.1.2 Starvation and inhibitor treatments for autophagy assays (I)

For autophagy phenotyping in A549 GFP–LC3 cells and canine fibroblasts, experiments were performed under nutrient-rich full culture medium ("fed") conditions or under serum and amino acid starvation conditions as described in publication I. Starvation lasted for 2 h. Where indicated, bafilomycin A1 was used to block lysosomal acidification to enable estimation of LC3- and p62-associated flux by comparing matched samples in the absence and presence of bafilomycin A1 (I). Compounds and concentrations were selected from the screening performed at Helmholtz Centre for Infection Research, Braunschweig, Germany, (anisomycin, aciclovir, famciclovir, vioprolides C/D).

4.1.3 Generation and maintenance of stable and knockout cell lines (II, III)

Rab24-KO Neuro-2a and HeLa cell lines were generated by CRISPR–Cas9 genome editing as described in publication II. Stable Neuro-2a Rab24-KO cell lines expressing GFP–Rab24 or APEX2-Rab24 constructs (Rab24-WT, Rab24-Q38P, Rab24-S67L) or APEX2–NES were generated by lentiviral transduction. APEX2-NES that expresses APEX2 with a nuclear export signal (NES) was used as a control in the proximity biotinylation assay (III). Fluorescence-activated cell sorting was used to obtain polyclonal populations with defined expression levels of the GFP-Rab24 cells lines (III). Stable lines were expanded and routinely maintained under

standard culture conditions. For experiments, cells were seeded to reach the indicated confluency at the time of treatment or labeling.

4.2 Proximity biotinylation and mass spectrometry (III)

APEX2-mediated proximity biotinylation followed by streptavidin-mediated enrichment of the biotinylated proteins and identification of the peptides by liquid chromatography-tandem mass spectrometry (LC–MS/MS) was used to identify proteins in the vicinity of Rab24 in Neuro-2a cells, and to compare the proximal proteomes of Rab24-WT and Rab24 mutant variants. APEX2 proximity labeling was performed in Rab24-knockout Neuro-2a cells stably expressing the APEX2–Rab24 constructs (APEX2–Rab24-WT, APEX2–Rab24-Q38P, APEX2–Rab24-S67L) or APEX2–NES as a diffuse cytosolic control (III). LC-MS/MS was performed at Ludwig-Maximilians-Universität München, Munich, Germany. The experimental workflow and MS acquisition/processing were performed as described in publication III. Comparative analyses and visualizations included in this thesis (e.g., volcano plots and gene ontology (GO) term summaries; Figs. 12,13) were performed by the author.

4.3 Fluorescence microscopy and image analysis (I, II, III)

Fluorescence microscopy was used to quantify autophagy-related structures and colocalization metrics in publication I, to assess Rab24 subcellular localization in publication III, and to perform additional imaging experiments (Figs. 11,14).

4.3.1 Immunofluorescence staining of cultured cells (I, III)

Cells grown on glass coverslips were fixed and processed for immunofluorescence staining using standard procedures. For publication III, Neuro-2a cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Cells were permeabilized with 0.2% saponin in PBS and blocked in PBS containing 3% bovine-serum albumin (BSA) and 0.2% saponin prior to antibody incubations. Primary antibodies were incubated as described in publication III and detected with species-appropriate Alexa Fluor–conjugated secondary antibodies. Nuclei were counterstained with DAPI and coverslips were mounted in Mowiol-based mounting medium containing DABCO antifade reagent.

To assess endogenous LC3 and p62 vesicle abundance in A549 GFP-LC3 cells and LC3 in canine fibroblasts in publication I, A549 GFP-LC3 cells, and WT and Atg4D-mutant fibroblasts were treated as indicated, fixed, and immunostained with

antibodies against endogenous Sqstm1/p62 (A549 GFP-LC3 cells) or LC3 (canine fibroblasts). Vesicular structures were quantified from confocal images using the image analysis workflow described in publication I, and flux estimates were derived from matched samples incubated in the absence or presence of bafilomycin A1. The abundance of GFP-LC3 vesicles in A549 cells was analyzed equally but using the GFP fluorescence without immunostaining (I).

To assess colocalization of GFP–LC3 and Lamp2 in A549 GFP–LC3 cells (I), cells were treated as indicated and fixed, followed by immunostaining for endogenous Lamp2 using a primary antibody and fluorophore-conjugated secondary antibody. Confocal images were acquired under identical settings for all samples within each experiment.

To assess Golgi-associated localization of Rab24 variants, Rab24-KO Neuro-2a cells expressing GFP–Rab24-WT or GFP–Rab24-Q38P were stained with an antibody against the cis-Golgi marker Golga2 (also called GM130) and imaged by confocal microscopy (III).

To visualize COPII-associated structures, WT Neuro-2a cells, Rab24-KO Neuro-2a cells, and Rab24-KO cells rescued by GFP–Rab24 expression were immunostained for Sec31a, a component of the COPII coat (Fig. 14). Staining was performed according to the general immunofluorescence workflow described above and imaged by confocal microscopy (Fig. 14).

For analysis of autophagosome maturation/acidification, Neuro-2a WT and Rab24-KO cells were transiently transfected with GFP–mRFP–LC3, fixed and imaged by confocal microscopy (Fig. 11).

4.3.2 Confocal microscopy acquisition (I, III)

Confocal imaging was performed as described in the original publications. For publication III, images were acquired using a Marianas CSU-W1 spinning-disk confocal microscope (3i) equipped with 63× (NA = 1.4, oil immersion) or 100× objectives (NA = 1.46, oil immersion; III). For publication I, confocal imaging of GFP–LC3 and immunostained endogenous markers (p62 and Lamp2) was performed using the confocal microscopy platform described in publication I. Channels were selected according to fluorophore spectra, and imaging settings were kept constant for all samples within each experiment to enable quantitative comparisons between treatments (I, III).

4.3.3 Colocalization and puncta quantification (I, III)

Colocalization between GFP–Rab24 variants and GM130 was quantified from confocal z-stacks using Fiji/ImageJ. Individual cells were segmented, background

subtraction was applied, and colocalization was quantified using the Coloc 2 plugin, reporting Pearson's correlation coefficient (Pearson's R) (III). For each condition, 30 cells were analyzed (III).

To evaluate association of LC3-positive structures with lysosome-associated compartments in A549 GFP–LC3 cells, overlap between GFP–LC3 and Lamp2 signals was quantified in ImageJ/Fiji (Schindelin, et al., 2012) using Mander's overlap coefficient as described in publication I.

For quantification of autophagosome acidification using the GFP-mRFP-LC3 reporter, mRFP-positive puncta were segmented to define total LC3-reporter puncta, and then classified according to whether they retained detectable GFP signal or not. Results were reported as the fraction of mRFP puncta that were also GFP-positive (Fig. 11).

4.4 Electron microscopy and ultrastructural quantification (III)

Transmission electron microscopy (TEM) was used to assess ultrastructural changes in autophagic and endolysosomal compartments in WT and Rab24-KO Neuro-2a cells. Sample preparation, imaging and quantification were performed as described in publication III. Briefly, WT and Rab24-KO Neuro-2a cells were grown to subconfluency and fixed *in situ* with 2% glutaraldehyde in 0.2 M HEPES (pH 7.4) for 2 h. After fixation, the cells were postfixed in osmium tetroxide, dehydrated through an alcohol series, embedded in epoxy resin and thin-sectioned using a diamond knife. Thin sections were imaged on a Jeol JEM-1400 Plus transmission electron microscope operated at 80 kV acceleration voltage .

For quantification, uniform random sampling was applied to acquire micrographs at 3000× original magnification. Organelle profiles were classified into the following categories based on ultrastructural criteria: autophagosomes (double-membrane vesicles containing cytoplasmic material), amphisome-/autolysosome-like compartments (single-membrane vacuoles containing partially degraded cytoplasmic material), and late endosomes/lysosomes (electron-dense, multivesicular or multilamellar endolysosomal profiles). For each image, the cytoplasmic area was estimated by point counting in ImageJ/Fiji, and organelle profiles were counted and normalized to cytoplasmic area to obtain organelle density measures.

4.5 Lysosomal functional assays by flow cytometry (III)

To assess whether Rab24-KO affects lysosomal acidity or lysosomal proteolytic activity, LysoSensor- and dye-quenched BSA (DQ-BSA)–based flow cytometry

assays were performed in WT and Rab24-KO Neuro-2a cells as described in publication III. In both assays, bafilomycin A1 (BafA1) was used as a control condition to inhibit vacuolar H+-ATPase activity, thereby increasing lysosomal pH and reducing lysosomal degradation.

4.5.1 LysoSensor-based acidity measurements (III)

WT and Rab24-KO Neuro-2a cells were seeded in 6-well plates. The following day, lysosomal acidity was monitored by incubating the cells with 1 µM LysoSensor™ Green DND-189 in culture medium for 1 h at 37 °C and 5% CO_2, either in the absence or presence of 100 nM BafA1 (co-incubated throughout the staining period). Cells were then detached by trypsinization and passed through cell strainer cap tubes to obtain single-cell suspensions. Samples were analysed on a BD LSRFortessa flow cytometer. LysoSensor Green DND-189 was excited with the 488-nm laser and emission was collected in the FITC/green channel. Data were analysed in FlowJo by gating to exclude cell debris (FSC/SSC) and selecting singlets (e.g., FSC-A vs FSC-H). Fluorescence was quantified as the median fluorescence intensity of the singlet population and compared across genotypes and treatment conditions.

4.5.2 DQ-BSA degradation assay (III)

To assess lysosomal delivery and proteolytic processing of endocytic cargo, WT and Rab24-KO Neuro-2a cells were incubated with 10 µg/mL DQ Red BSA for 6 h at 37 °C and 5% CO_2, either in the absence or presence of 100 nM BafA1. As a negative control for background fluorescence, cells were incubated with 10 µg/mL BSA. After incubation, cells were detached by trypsinization, filtered to obtain single-cell suspensions, and analyzed by flow cytometry (BD LSRFortessa). DQ Red BSA fluorescence was excited using the 561-nm laser and emission was collected in the PE–Texas Red channel. Data were analyzed in FlowJo using debris exclusion and singlet gating as above, and fluorescence was quantified as the median fluorescence intensity of the singlet population for comparison between WT and Rab24-KO cells and across treatment conditions.

4.6 Rab24 tissue expression and cancer-associated protein analysis (II)

Mouse tissues were collected at defined postnatal and adult ages spanning early development to mature adulthood. For microscopy sample collection, mice were anesthetized and fixed by perfusion. Organs were dissected, processed for either

protein extraction (immunoblotting) or formalin fixation and paraffin embedding (histology), and stored under conditions suitable for downstream analyses.

For tissue-level quantification of Rab24, protein extracts were prepared from the indicated organs and analyzed by SDS–PAGE and immunoblotting with a Rab24 antibody. Because housekeeping protein expression varies between tissues and during development, total protein normalization was used for densitometric quantification of the immunoblots. To facilitate comparisons across several immunoblots, a reference sample was included across gels, and Rab24 signals were quantified relative to total protein and the common reference.

Paraffin sections from mouse tissues were stained by immunohistochemistry (IHC) for Rab24 to determine cell-type and regional localization patterns. Where indicated, additional markers linked to autophagy and the endolysosomal system (e.g., LC3, Sqstm1/p62, Beclin-1, Rab5, Rab7, Lamp1/2) were stained on serial sections to enable qualitative comparison of cell-type distribution patterns. Stained slides were imaged using a slide scanner and 3D Histotech's slide converter.

4.6.1 Ethics approval (II)

All animal experiments were approved by the Project Authorization Board of the Regional State Administrative Agency of Southern Finland (ESAVI/613/2019) and conducted in accordance with the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

Sections from human multicancer and pancreatic neuroendocrine tumor (PNET) tissue microarrays (TMAs) were obtained from the Helsinki Biobank with approval from the Ethics Committee of the Hospital District of Helsinki and Uusimaa (HUS/697/2020). The samples were collected with informed patient consent, and no information regarding patient identity, tumor stage, or survival was provided. Neuroblastoma and brain tumor TMAs were purchased from Tissue Array (TissueArray.Com, USA) and supplied in an anonymized format, ensuring that individual donors could not be re-identified.

4.6.2 Human tissue microarrays, staining and digital pathology scoring (II)

To assess Rab24 protein levels across tumor entities, TMAs containing malignant and corresponding normal tissues were stained for Rab24 by IHC. Whole-slide images were acquired by slide scanning and analyzed using QuPath software to derive semi-quantitative H-scores for Rab24 stained intensity from tumor regions and normal tissues, enabling comparisons between tumor types, tumor versus normal tissues, and within selected cohorts.

4.7 Analysis of public cancer datasets (TCGA/UALCAN) (II)

Rab24 mRNA expression in tumor versus normal tissue and associations with overall survival were examined in selected TCGA (The Cancer Genome Atlas) cohorts using the UALCAN portal as described in publication II. Patients were stratified by expression level and survival curves were compared using the portal's built-in statistical framework.

4.8 Functional assays in wild type and Rab24 knockout cells (II)

Scratch-wound assays were used to quantify collective cell migration of WT and Rab24-KO Neuro-2a and HeLa cells. Cells were seeded to generate confluent monolayers, linear wounds were introduced, and wound closure was monitored by time-lapse live-cell imaging. Relative wound confluency was quantified over time to compare migration dynamics between WT and Rab24-KO cells.

Cell proliferation was assessed by live-cell imaging–based growth monitoring of WT and Rab24-KO Neuro-2a and HeLa cells. Cells were seeded at defined densities and imaged over time, and growth curves were generated using confluency-based quantification to compare proliferation rates between genotypes.

4.8.1 RNA-sequencing and enrichment analysis (II)

Total RNA was isolated from WT and Rab24-KO Neuro-2a cells and subjected to RNA sequencing, which was performed at the Finnish Functional Genomics Center at Turku Bioscience Centre. Differential gene expression analyses were performed between WT and KO conditions, and GO term enrichment analyses were used to identify biological process categories significantly associated with gene sets altered by Rab24 loss. Enrichment results were compared across independent Rab24-KO clones to identify reproducible Rab24-dependent transcriptional programs (II).

4.9 Antibodies

Antibodies used in publications II and III are listed in the respective publications (II, Table S1 and III, Table 2). Further primary antibodies used for immunofluorescence are listed in Table 2.

Table 2: Antibodies used for immunofluorescence in publication I and the experiments presented in the Results section of this thesis.

Antigen	Company	Cat. No	Dilution
LC3	CosmoBio	CTB-LC3-2-IC	1:200
Sqstm1/p62	BD Bioscience	610832	1:100
Lamp2	Developmental Studies Hybridoma Bank	H4B4	1:100
Sec31a	BD Bioscience	612351	1:100
Anti-mouse IgG Alexa Fluor 488	ThermoFisher	A-11001	1:500

4.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism (version as indicated in the respective publications or figure legends). The statistical test applied for each dataset is stated in the corresponding figure legend. In general, two-group comparisons were analyzed using unpaired two-tailed Student's t-test for normally distributed data or Mann–Whitney U test for non-normally distributed data. For comparisons across multiple groups, one-way ANOVA (with appropriate post hoc correction) or Kruskal–Wallis test followed by Dunn's multiple comparisons test was used where applicable. For all analyses, n denotes the number of independent biological replicates unless otherwise specified (e.g., number of cells analyzed for colocalization). A threshold of $p < 0.05$ was considered statistically significant.

5 Results

5.1 Identification of autophagy-modulating compounds and evaluation in a disease model (I)

To identify small molecules that alter autophagy, a high-content fluorescence microscopy workflow was established using A549 cells stably expressing GFP–LC3, in the laboratory of Manfred Wirth and Ingo Schmitz, Helmholtz Centre for Infection Research, Braunschweig, Germany. Compounds were screened under full culture medium (fed) and amino-acid-starvation conditions to capture modulators of basal and induced autophagy, respectively (I, Fig. 1). Primary hits from commercial bioactives and microbial natural product libraries were subsequently prioritized for characterization by dose–response analysis and confocal microscopy–based flux measurements (± bafilomycin A1). Selected compounds were further tested in an Atg4D-mutant dog fibroblast model of neuronal vacuolation and spinocerebellar degeneration (NVSD) (I, Figs. 2–5, 8).

5.1.1 Effects of candidate compounds on autophagic flux and LC3-Lamp2 overlap (I)

Candidate compounds were selected from a preceding high-content microscopy screen performed by collaborators. A549 cells stably expressing GFP–LC3 were used to quantify changes in LC3-positive vesicle accumulation under fed and starvation conditions. In the primary screen, several treatments altered the GFP–LC3 vesicle readout beyond control variability, including known modulators as well as compounds not previously linked to autophagy regulation. Based on reproducibility in the primary screen and follow-up studies, anisomycin, aciclovir, famciclovir and the myxobacterial natural products vioprolide C and vioprolide D were chosen for further validation and flux-based analysis in our laboratory.

To distinguish whether the accumulation of GFP-LC3-positive compartments was due to increased formation or decreased clearance, the selected compounds were analyzed by confocal microscopy in the absence and presence of bafilomycin A1 (BafA1). BafA1 was used because it prevents lysosomal acidification and thereby

blocks turnover of autophagic cargo (Yoshimori, et al., 1991), allowing estimation of autophagic flux by comparing the amount of GFP-LC3-positive compartments in samples treated with and without BafA1 (I, Fig. 3). For quantification of GFP–LC3 puncta, several metrics were evaluated, and the product of puncta area and intensity, normalized to cell area, was used for subsequent analyses because it captured vesicle number, size and signal strength (I, Fig. 3A).

Using this approach, the effects of anisomycin, aciclovir, famciclovir, vioprolide C and vioprolide D were quantified under fed (full culture medium) and starvation (serum and amino-acid free medium for 2 h) conditions, each with and without BafA1 (I, Fig. 3B–C). Flux was calculated from paired conditions (±BafA1) for each treatment (I, Fig. 3C). Across conditions, anisomycin and both vioprolides reduced GFP–LC3 flux under fed and starved conditions, whereas famciclovir reduced GFP–LC3 flux primarily under fed conditions. In contrast, aciclovir did not measurably change GFP–LC3 flux relative to solvent controls (I, Fig. 3C). In parallel, endogenous Sqstm1/p62 puncta were quantified as a second autophagy-associated marker and analyzed in the same manner, including an estimate of p62 flux (I, Fig. 4). All tested compounds decreased p62 flux relative to controls under starvation conditions (I, Fig. 4B). Together, these experiments provided an assessment of how the compounds influenced LC3- and p62-associated dynamics across basal and starvation-induced conditions.

To probe whether the compound effects were linked to altered delivery of GFP-LC3-positive structures to late endosomal/lysosomal compartments, GFP–LC3 cells were additionally stained for endogenous late endosomal/lysosomal marker Lamp2 and analyzed for signal overlap using Mander's coefficient (I, Fig. 5). Under fed conditions, none of the tested compounds significantly changed the degree of GFP–LC3 colocalization with Lamp2, arguing against a block of autophagosome fusion with Lamp2-positive compartments under basal conditions (I, Fig. 5B). Under starvation, vioprolide C significantly reduced GFP–LC3–Lamp2 overlap (I, Fig. 5A–B). This indicates that, in addition to lowering LC3-associated readouts (I, Fig. 3C), this compound impaired the fusion of LC3-positive structures with Lamp2-positive compartments under starvation conditions. In contrast, anisomycin-treated cells showed a higher GFP–LC3–Lamp2 overlap under starvation compared with fed conditions (I, Fig. 5B). This is consistent with a shift in the distribution of LC3-positive structures toward Lamp2-positive compartments during starvation in the presence of anisomycin. Under starvation, anisomycin decreased both GFP-LC3 flux (I, Fig. 3C), and p62 flux (I, Fig 4B). These findings suggest that the clearance of amphisomes or autolysosomes may be retarded in anisomycin-treated cells.

Overall, combining GFP-LC3 and p62 flux estimation (±BafA1) with LC3–Lamp2 overlap provided a characterization of compound effects on the autophagy pathway. The follow-up analyses indicated that none of the tested compounds

induced autophagy in this assay setup; instead, they predominantly reduced autophagy-associated flux in a condition-dependent manner. Famciclovir (and to a lesser extent aciclovir) preferentially reduced basal (fed) LC3 flux with little effect on starvation-induced LC3 flux, whereas vioprolides reduced LC3 flux under both fed and starvation conditions. In parallel, all tested compounds reduced starvation-induced p62 flux. Finally, vioprolide C was the only compound that measurably decreased LC3–Lamp2 colocalization under starvation, suggesting impaired association of LC3-positive structures with Lamp2-positive compartments specifically under starvation conditions (I, Figs. 3–5). Thus, the decreased autophagy flux caused by vioprolide C under starvation was at least partially due to decreased fusion of GFP-LC3-positive compartments with Lamp2-positive compartments.

5.1.2 Candidate compounds modulate basal autophagy phenotypes in Atg4D-mutant fibroblasts (I)

To test whether the compounds identified in the GFP–LC3 screening also modulate autophagy phenotypes in a genetically defined disease context, candidate hits were evaluated in fibroblasts derived from Lagotto Romagnolo dogs carrying the Atg4D-A430T mutation associated with neurodegenerative vacuolar storage disease (NVSD). Autophagy was assessed by quantitative immunofluorescence of endogenous LC3 under fed and starvation conditions, with and without BafA1 to estimate LC3 flux (I, Fig. 8).

Compared with control (DMSO) WT fibroblasts, control Atg4D-mutant fibroblasts displayed elevated levels of LC3-positive vesicles under basal (fed) conditions (I, Fig. 8A, B). Upon starvation, the mutant cells showed a smaller increase in LC3-positive vesicles than WT cells, consistent with a reduced capacity to upregulate LC3-associated autophagic compartments under nutrient stress. The difference was also observed in flux quantifications: WT cells significantly increased LC3 flux during starvation, whereas Atg4D-mutant cells showed weak starvation-associated increase that did not reach statistical significance (I, Fig. 8A–B).

The candidate compounds were tested for their ability to alter both LC3 vesicle abundance and flux in both genotypes (I, Fig. 8). In WT fibroblasts, vioprolides C and D produced a strong reduction in LC3-positive vesicle levels under both fed and starved conditions. Consistent with this, flux estimates showed that vioprolides C and D also reduced basal (fed) LC3 flux in WT fibroblasts, whereas under starvation conditions a clear flux reduction was observed primarily with vioprolide D (I, Fig. 8A, bottom). In Atg4D-mutant fibroblasts, anisomycin and famciclovir lowered the elevated basal LC3 vesicle accumulation toward WT levels, while aciclovir showed modest effects under starvation (I, Fig. 8B). In the mutant cells, flux analysis indicated that all tested compounds decreased the elevated LC3 flux under fed

conditions, and under starvation conditions vioprolide D showed the most evident reduction in LC3 flux (I, Fig. 8B, bottom). However, none of the tested compounds normalized LC3 flux to wild-type levels. Together, these results indicate that compound-dependent changes in LC3 vesicle abundance in this model are accompanied by measurable effects on LC3 flux. The strongest and most consistent flux suppression was observed for the vioprolides, with basal flux in Atg4D-mutant cells being broadly sensitive to all tested compounds (I, Fig. 8).

Taken together, these experiments demonstrate that compounds emerging from the screening pipeline modulate LC3-flux in both WT cells and in a disease-relevant genetic background. Aciclovir and famciclovir reduced the elevated basal LC3 vesicle accumulation in Atg4D-mutant fibroblasts and also reduced LC3 flux, while vioprolides acted as strong inhibitors of LC3-associated vesicle accumulation and flux in WT fibroblasts (I, Fig. 8). However, none of the tested compounds was able to normalize the LC3 flux of Atg4D mutant cells to the levels observed in control WT cells.

5.2 Rab24 protein distribution in development and cancer and functional consequences of Rab24 loss (II)

To place Rab24 functions in autophagy- and trafficking-related phenotypes into a physiological context, Rab24 protein abundance was quantified across major mouse organs during postnatal development and adulthood using immunoblotting (II, Figs. 1,2). Cellular Rab24 localization was investigated using IHC (II, Figs. 3-6). Because Rab24 has also been implicated in tumor biology, Rab24 protein levels were additionally assessed across a broad panel of human cancers using tissue microarrays (TMAs) with semi-quantitative scoring (H-scores; II, Figs. 11–13). Finally, to gain functional insight beyond protein expression patterns, Rab24-deficient cell lines were generated by CRISPR–Cas9 and analyzed for transcriptional changes as well as migration and proliferation phenotypes (II, Figs. 8–10). Together, these results establish where Rab24 protein is most prominently expressed *in vivo*, identify cell types with high Rab24 protein abundance, and link Rab24 loss to altered cellular programs and behaviors relevant to neuronal maintenance and cancer-associated traits.

5.2.1 Rab24 protein levels vary across mouse tissues and change during postnatal development (II)

To determine how Rab24 protein abundance varies across organs and with age, Rab24 levels were analyzed by immunoblotting in eight mouse tissues (cerebral

cortex, heart, liver, lung, kidney, spleen, skeletal muscle and pancreas) collected from postnatal day 1 through 9 months of age (II, Fig. 1A). Total protein staining was used for normalization to account for tissue-specific differences in housekeeping protein abundance (II, Fig. 1B–H).

Rab24 protein levels were not uniform across tissues and displayed clear age-dependent shifts. In the earliest postnatal time points (days 1 and 7), Rab24 abundance ranged from low levels in several tissues to comparatively higher levels in heart and, to a lesser extent, brain and kidney (II, Fig. 1B–C). By postnatal day 14, Rab24 levels increased prominently in the brain and were also elevated in kidney, heart and skeletal muscle relative to other organs (II, Fig. 1D). In adult mice (3–9 months), the highest Rab24 protein levels were observed in the brain, with kidney consistently ranking as the second highest tissue, whereas multiple other organs maintained comparatively low levels (II, Fig. 1F–H).

To resolve within-tissue developmental trajectories with higher confidence, additional immunoblots were performed with all age group samples from a given tissue loaded on the same gel and normalized to the day 1 sample for that tissue (II, Fig. 2A–H). This analysis revealed distinct temporal patterns between organs. In the brain, Rab24 increased sharply around postnatal day 14 and remained elevated into adulthood (II, Fig. 2A). In contrast, heart, skeletal muscle, pancreas and liver showed transient postnatal elevations, with increased Rab24 during the first two weeks and reduced levels from one month onwards (II, Fig. 2B–E). Rab24 levels were comparatively stable across ages in the kidney, spleen and lung (II, Fig. 2F-H).

Collectively, these results demonstrate that Rab24 protein abundance is tissue- and age-dependent, with sustained high expression in adult brain and kidney and transient postnatal enrichment in the skeletal muscle, pancreas, and liver, tissues that undergo rapid maturation during the early postnatal period (II, Figs. 1–2).

5.2.2 Cell-type localization of Rab24 reveals neuronal and epithelial enrichment (II)

To identify which cell types account for the tissue-level Rab24 protein patterns observed by immunoblotting, Rab24 localization was determined by IHC in multiple organs at representative developmental and adult stages (II, Fig. 1A and Figs. 3–6). In the brain, where Rab24 levels rose strongly by postnatal day 14 and remained high in adulthood, IHC revealed that Rab24 immunoreactivity was most prominent in neuronal populations in 1-month-old and older animals (II, Fig. 4). In the cerebellum, Purkinje neurons showed clear Rab24 staining already in 7-day-old animals and maintained strong staining across the age groups analyzed, indicating a sustained Rab24 signal in this neuronal subtype (II, Fig. 3). In contrast, many other neuronal populations showed comparatively weak or absent staining at postnatal day 7 but

became distinctly positive by one month of age, in agreement with the developmental increase observed by immunoblotting (II, Fig. 2A and Fig. 4).

Outside the nervous system, Rab24 staining was also enriched in several epithelial cells, with patterns that differed by tissue and age (II, Fig. 5). Ependymal cells lining the brain ventricles displayed relatively strong Rab24 staining in the early postnatal stage, whereas in older brains the ependymal signal was less distinct relative to surrounding Rab24-positive neural tissue (II, Fig. 5A–B). In the kidney, Rab24 staining was strongest in proximal tubular epithelium compared with distal tubules and glomerular structures, and this epithelial distribution was broadly maintained between the juvenile and adult stages examined (II, Fig. 5C–D). In the lung, bronchiolar epithelial cells showed consistently strong Rab24 staining across ages, while higher Rab24 staining was observed in the alveolar lining of young animals than of adults (II, Fig. 5E–F). In the pancreas, endocrine islet cells showed markedly stronger Rab24 staining than surrounding exocrine tissue, and the exocrine compartment displayed higher Rab24 staining at the early postnatal stage than in adulthood (II, Fig. 5G–H).

Age-associated decreases in Rab24 staining were also evident in contractile tissues. In the heart, cardiomyocytes displayed moderate Rab24 staining in early postnatal tissue but substantially lower staining at one month and older ages, consistent with the postnatal decline observed in immunoblotting (II, Fig. 2B and Fig. 6A–B). A similar but less pronounced decrease was observed in skeletal muscle fibers (II, Fig. 6C–D). In the liver, Rab24 staining remained low across ages in the IHC analysis (II, Fig. 6E–F), whereas spleen showed moderate staining with relatively uniform distribution (II, Fig. 6G–H).

Together, the IHC analyses indicate that high Rab24 protein levels in adult brain largely reflect neuronal enrichment, while several epithelial cell types across organs also show robust Rab24 staining. These findings support the conclusion that Rab24 protein abundance is both tissue- and cell-type-selective, with particularly prominent enrichment in neuronal and epithelial compartments (II, Figs. 3–6).

5.2.3 Comparison of Rab24 with autophagy and endolysosomal markers suggests context-dependent overlap (II)

Because Rab24 has been linked to endosomal degradation and late stages of the autophagy pathway, Rab24 staining patterns were compared with marker proteins representing autophagic structures, endosomal compartments and lysosomes. Serial sections from selected tissues showing prominent age-dependent Rab24 expression (brain, kidney, lung and pancreas) were stained for LC3, Beclin-1 and Sqstm1/p62 (autophagosomes), as well as for Rab5 (early endosomes), Rab7a (late endosomes)

and Lamp1+Lamp2 (late endosomes/lysosomes), and the resulting patterns were evaluated qualitatively across the age groups 7 days and 1 month (II, Fig. 7; and Figs. S8–S12).

In the cerebellum, Purkinje cells showed Rab24 staining at both juvenile and adult stages. These neurons were also positive for several of the analyzed markers. Among the endosomal/lysosomal markers, Rab7a staining most closely resembled the Rab24 pattern in Purkinje cells, whereas LC3 and Beclin 1 were detected in Purkinje cells but also in additional cerebellar layers and not only in the Rab24-positive Purkinje population (II, Fig. 7). In other brain regions, neuronal Rab24 staining increased with age, and Rab7a similarly showed a shift toward stronger neuronal staining at the adult stage, suggesting partial agreement between Rab24 and late endosomal signatures in specific neuronal populations (II, Figs. S8–S9).

In peripheral tissues, the degree of overlap varied by organ and cell type. In the kidney, Rab24 staining was prominent in tubular epithelium but not in glomeruli. Several markers (including Rab7a and Lamp1/2) showed tubular patterns that aligned with adult Rab24 staining, whereas LC3 and Beclin-1 were detected more broadly across kidney structures, indicating that Rab24 distribution does not fully mirror global autophagy marker distribution (II, Fig. S10). In the lung, adult bronchiolar epithelium showed staining for multiple markers in addition to Rab24. In juvenile tissue, several markers were weak in the bronchiolar epithelium despite clear Rab24 staining (II, Fig. S11). In the pancreas, Rab24 staining was strongest in the endocrine islets, and this pattern showed the most consistent correspondence with multiple markers, including LC3, Beclin-1, p62, Rab5, Rab7a and Lamp1/2, which were all strongly positive in the islet cells relative to the surrounding acinar tissue (II, Fig. S12).

Overall, these comparisons indicate that Rab24 does not universally colocalize with autophagy or endolysosomal markers at the cell and tissue level, but instead shows context-dependent overlap that is most obvious in specific cell populations. In the brain, Rab24 distribution aligned most closely with Rab7a in several neuronal regions, whereas in the pancreatic endocrine islet cells, Rab24 showed strong parallel staining with a broad panel of autophagy and endolysosomal markers. These findings suggest that Rab24 is mainly enriched in certain cell types where late endosome–lysosome trafficking is particularly active. However, it is not a uniform marker of autophagy or endolysosomes across tissues (II, Fig. 7 and Figs. S8–S12).

5.2.4 Rab24 knockout alters transcriptional programs linked to neuronal functions and immunity (II)

To gain insight into cellular pathways influenced by Rab24, transcriptomic profiling was performed in a neuronal cell model using RNA sequencing of WT Neuro-2a

cells and two independent Rab24-KO clones generated by CRISPR–Cas9. Differentially expressed gene sets were analyzed by GO term enrichment, focusing on biological process terms (II, Fig. 8).

In both KO clones, the most consistent signals emerged from genes that were downregulated upon Rab24 loss, mainly because only a few genes were upregulated. When the results were compared between the two independent KO clones, a shared set of GO term categories was identified among the downregulated genes, indicating that Rab24 deficiency reproducibly suppresses specific transcriptional programs in this model (II, Fig. 8). GO biological process terms linked to neuronal and developmental functions including processes related to memory, regulation of membrane potential, brain and nervous system development, and cell differentiation were among the shared GO terms. In addition, angiogenesis-associated categories were enriched among the downregulated genes, suggesting that Rab24-dependent expression programs may extend to pathways involved in tissue remodeling or growth signaling (II, Fig. 8).

Beyond neuronal-function-associated terms, Rab24 KO also led to reduced expression of genes grouped under broader signaling and immune-related categories. Enrichment for terms inflammatory response, defense response to bacterium and response to xenobiotic stimulus suggested that Rab24 contributes, either directly or indirectly, to transcriptional states connected to cellular stress responses and innate immunity in Neuro-2a cells (II, Fig. 8). Finally, extracellular matrix organization was among the enriched categories shared between both Rab24 KO clones, consistent with Rab24 affecting genes that can influence cell adhesion- and migration-related processes (II, Fig. 8). Such pathways are frequently relevant in both development and tumor biology.

Together, the RNA sequencing results indicate that Rab24 loss in Neuro-2a cells is associated with coordinated downregulation of gene programs linked to neuronal function and development, signal transduction and immunity-associated responses. This provides insights into Rab24-dependent phenotypes in neuronal maintenance and disease contexts.

5.2.5 Rab24 supports migration and proliferation in neuronal and epithelial cell lines (II)

To test whether Rab24 influences cellular behaviors linked to development and cancer, the effect of Rab24-KO was examined in two cell models representing tissues with prominent Rab24 staining: Neuro-2a cells (neuronal origin) and HeLa cells (epithelial origin). WT and Rab24-KO cells were compared in scratch-wound migration assays and live-cell proliferation assays using automated imaging and quantitative confluency-based readouts (II, Figs. 9–10).

In Neuro-2a cells, Rab24 deficiency was associated with slower closure of the scratch wounds compared with WT cells (II, Fig. 9A–B). In parallel, Rab24-KO Neuro-2a cells expanded more slowly than WT cells in the proliferation assay, resulting in consistently lower confluency values at later time points (II, Fig. 9C–D). Thus, in a neuronal cell background, Rab24 supported both efficient migration in a wound-healing assay and population expansion under standard culture conditions (II, Fig. 9).

A similar phenotype was observed in HeLa cells. Rab24-KO HeLa cultures showed delayed wound closure relative to WT controls across the analyzed time points, indicating reduced capacity for migration in cells without Rab24 (II, Fig. 10A–B). In the proliferation assay, Rab24-KO HeLa cells also showed reduced confluency compared with WT cells, consistent with slower growth or reduced proliferation (II, Fig. 10C–D).

These results demonstrate that Rab24 contributes to efficient migration and proliferation in two distinct cellular contexts. The matching phenotypes in neuronal (Neuro-2a) and epithelial (HeLa) models support the interpretation that Rab24 can promote cellular behavior relevant for tissue remodeling and tumor-progression. These findings are in agreement with Rab24's context-dependent associations in cancer and development highlighted in the tissue and transcriptomic analyses (II, Figs. 9–10).

5.2.6 Rab24 protein levels differ across human cancers (II)

To elucidate whether Rab24 protein abundance is altered in malignant tissues, IHC staining for Rab24 was analyzed in human tissue microarrays (TMAs) representing a broad range of tumor entities as well as selected nervous system and pancreatic neuroendocrine tumors. Staining intensity was quantified by determining the H-score using a semi-automated image analysis pipeline, which was developed based on tissue scoring performed by an experienced pathologist. This enabled comparisons across tumor types and, where available, corresponding normal tissues (II, Figs. 11–13).

Across the multicancer TMAs, Rab24 staining in malignant samples most frequently fell within an intermediate range, with a subset of cancers displaying high H-scores. When malignant tissues were compared with matched normal tissue cores, cancer-type-specific differences were found. Several tumor types, particularly in the breast and skin cancer categories, showed increased Rab24 staining compared with corresponding normal tissues. A notable fraction of cancers originating from the digestive system and urinary tract showed reduced Rab24 staining in malignant tissue relative to normal tissue (II, Fig. 11; and Table S2). Thus, the multicancer

analysis indicated that alterations in Rab24 protein abundance occur in a context-dependent manner and differ based on the origin of the tissue (II, Fig. 11).

Given the neuronal enrichment of Rab24 in adult mouse brain, Rab24 levels were further analyzed in tumors of the brain and of neuronal origin. In a central nervous system tumor TMA, medulloblastoma showed significantly higher Rab24 H-scores compared with adjacent normal brain tissue, whereas several other brain tumor types did not show statistically significant differences relative to normal tissue in this cohort (II, Fig. 12A, C). In an independent neuroblastoma TMA, Rab24 staining was higher in neuroblastoma samples than in normal peripheral nerve tissue, indicating elevated Rab24 abundance in these peripheral neuronal tumors (II, Fig. 12B, D–G). Together, these data suggest that increased Rab24 staining is detectable in at least a subset of pediatric tumors of neuronal lineage (II, Fig. 12).

Finally, because Rab24 staining in mouse pancreas was strongest in endocrine islet cells, Rab24 protein levels were evaluated in pancreatic neuroendocrine tumors (PNETs), which arise from the endocrine islets. In this TMA, normal pancreatic islets displayed higher Rab24 staining than tumor tissue and tumor-edge regions. Stratification by tumor grade further indicated lower Rab24 H-scores in higher-grade PNETs compared with low-grade cases (II, Fig. 13D–E). Thus, in contrast to the medulloblastoma/neuroblastoma pattern, PNET samples showed reduced Rab24 abundance relative to their normal endocrine tissue of origin (II, Fig. 13).

Overall, the TMA analyses demonstrate that Rab24 protein levels can be increased, unchanged, or decreased depending on tumor type and tissue of origin. The observed heterogeneity across tumor entities suggests that Rab24 abundance is not a universal cancer marker, but may reflect differential regulation based on tumor origin and/or tumor state in selected cancers (II, Figs. 11–13).

5.2.7 Relationship between Rab24 mRNA and patient outcome in selected TCGA cohorts (II)

To place the results of the TMA-based protein levels into a broader clinical context and to determine whether RNA transcription patterns align with protein-level differences, publicly available TCGA datasets were analyzed using the UALCAN portal for selected cancer types in which we observed distinct Rab24 staining trends in the TMA. For each tumor type, *Rab24* mRNA levels in tumor versus normal tissue were compared, and overall survival of patients with either high or low/medium *Rab24* expression was analyzed (II, Fig. S16).

Across the selected TCGA cohorts, *Rab24* mRNA expression patterns were heterogeneous and broadly mirrored the context dependence observed at the protein level. For example, in pancreatic adenocarcinoma, *Rab24* mRNA levels were lower in tumors than in normal pancreas, consistent with reduced Rab24 staining observed

in several digestive system cancers in the TMAs (II, Fig. S16A and Table S2). In hepatocellular carcinoma, *Rab24* mRNA was increased in tumors compared to normal liver, aligning with prior reports of elevated *Rab24* mRNA and protein expression in this cancer type (Chen, et al., 2017b, Gu, et al., 2025, He, et al., 2002) and supporting the notion that *Rab24* regulation differs by tissue and tumor lineage (II, Fig. S16B). In other analyzed cohorts, *Rab24* mRNA changes ranged from no or modest differences to significant differences between the normal tissue and tumor depending on the tumor type (II, Fig. S16C–F).

Survival analyses further underscored that the clinical association of *Rab24* expression is not uniform. In some cancer types, higher *Rab24* expression correlated with improved overall survival (pancreatic adenocarcinoma, sarcoma), whereas in others it associated with poorer prognosis (hepatocellular carcinoma, glioblastoma multiforme, kidney chromophobe tumors) or showed no clear relationship with patient outcome (lung squamous cell carcinoma; II, Fig. S16).

Together, the TCGA-based analyses support two conclusions. First, *Rab24* mRNA level is cancer-type specific, consistent with the protein-level heterogeneity seen in the TMAs. Second, the relationship between *Rab24* expression and patient outcome varies across tumor entities, suggesting that possible prognostic or predictive utility of Rab24 mRNA and protein levels will be restricted to specific cancer contexts and will require tumor-type-specific validation (II, Fig. S16).

5.3 Rab24 is linked to lysosomal fusion machinery via interactions with HOPS and Rab7a (III)

To investigate how Rab24 contributes to late endo-lysosomal and autophagic trafficking steps, we analysed Rab24-deficient Neuro-2a cells using ultrastructural and functional assays, and mapped Rab24-associated proteins using APEX2 proximity biotinylation followed by mass spectrometry. Candidate interactions were validated biochemically by co-immunoprecipitation, and the effects of disease- and function-relevant Rab24 mutants (Q38P and S67L) were assessed in parallel. In addition to the data included in publication III, the PhD thesis includes complementary tandem-tag LC3 reporter experiments to probe autophagosome delivery to acidic compartments in Rab24 WT versus KO cells, proximity-proteomics comparisons (including an APEX2-NES negative control and an APEX2-Rab24-S67L mutant), a gene-ontology summary of the Rab24-proximal protein set, and qualitative analysis of coat protein II (COPII) marker distribution (Figs. 11-14).

5.3.1 Rab24 deficiency alters the balance between autophagic compartments and late endosomes/lysosomes (III)

To clarify whether loss of Rab24 affects the late-stages of the autophagy pathway in Neuro-2a cells, we performed ultrastructural analysis by quantifying organelles of the autophagic and endolysosomal system in WT and Rab24-KO Neuro-2a cells by transmission electron microscopy. In Rab24-KO cells, autophagosomes and autolysosome-/amphisome-like compartments showed a mild increase compared with WT cells, although the difference did not reach statistical significance (III, Fig. 1A–B). However, the number of late endosome/lysosome profiles was significantly reduced in Rab24-KO cells. Consequently, the ratio of autophagosomes to late endosomes/lysosomes was significantly increased in the KO background, consistent with an overall shift toward accumulation of autophagic intermediates relative to available degradative compartments (III, Fig. 1A–C). Together, the ultrastructural data are compatible with delayed clearance of autophagic cargo in Rab24-deficient Neuro-2a cells, in line with previous observations in other Rab24-silenced cell models (Ylä-Anttila, et al., 2015).

To further investigate autophagosome maturation and delivery of autophagic cargo to acidic, degradative compartments, WT and Rab24-KO Neuro-2a cells were transfected with a tandem-tagged LC3 reporter (GFP–mRFP–LC3). At neutral pH, both fluorophores are detectable, whereas upon delivery to acidic compartments the GFP signal is quenched while mRFP remains fluorescent, enabling discrimination of LC3-positive structures that have (mRFP-only) or have not (GFP+mRFP) reached an acidic environment, e.g. the lysosomal lumen (Fig. 10A). LC3 puncta were analyzed by assessing the proportion of red puncta that retained detectable green signal. In this assay, Rab24-KO cells showed a higher fraction of mRFP-positive puncta that remained GFP-positive compared with WT cells (36% versus 26%), consistent with a trend toward an increased pool of LC3-positive structures that have not progressed to an acidic environment where the GFP signal is efficiently quenched. However, the difference was not statistically significant and is therefore interpreted as a qualitative trend that aligns with, but does not independently prove, delayed late-stage progression of LC3-positive compartments in Rab24-deficient cells (Fig. 10B-C).

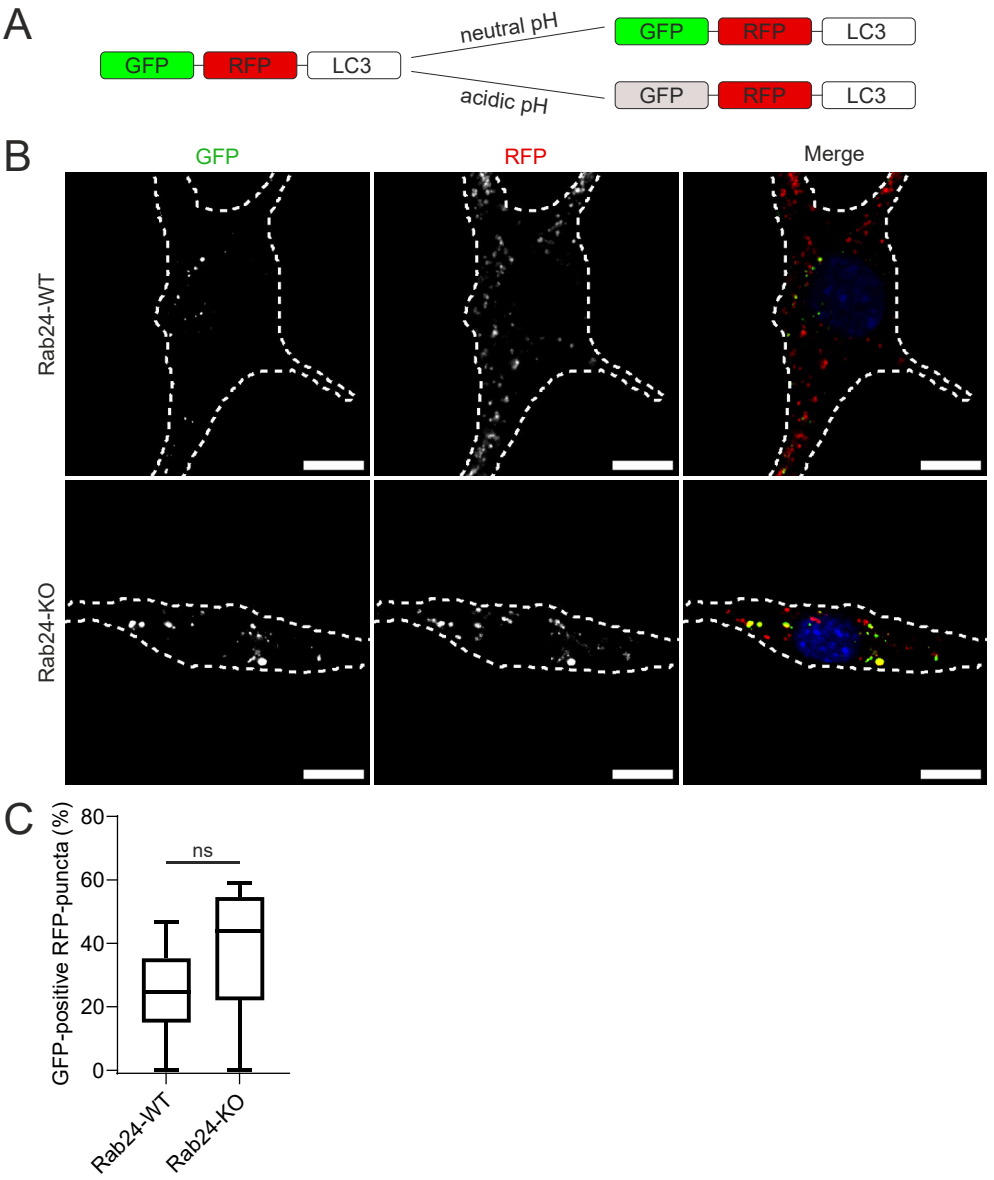

Figure 11. Tandem-tagged LC3 reporter suggests increased persistence of non-acidified LC3-positive structures in Rab24-KO Neuro-2a cells. (A) Schematic of the tandem fluorescent autophagy reporter GFP-mRFP-LC3. (B) Representative fluorescence microcopy images of Rab24-WT and Rab24-KO Neuro-2a cells cultured in full medium and transiently transfected with GFP-mRFP-LC3. Scale bar 10 µm. (C) Quantification of GFP-positive RFP puncta expressed as the percentage of mRFP puncta that retain detectable GFP-signal in Rab24-WT and Rab24-KO cells. ns, not significant.

5.3.2 Lysosomal degradative capacity is not impaired in Rab24-knockout cells (III)

To determine whether the delay on the autophagic pathway observed in Rab24-KO cells could be explained by a failure of lysosomal function, we assessed two core parameters of lysosomal competence in WT and Rab24-KO Neuro-2a cells: luminal acidity and degradative activity toward endocytosed cargo. Lysosomal pH was monitored using the pH-sensitive probe LysoSensor DND-189 followed by flow cytometry. LysoSensor fluorescence intensities were comparable across WT cells and two independent Rab24-KO clones, indicating that Rab24 loss did not measurably impair lysosomal acidification under the assay conditions (III, Fig. 2A–B).

To study lysosomal delivery and proteolytic degradation of endocytic cargo, cells were incubated with dye-quenched BSA (DQ-BSA), which becomes fluorescent upon proteolytic dequenching after trafficking via the endocytic route to degradative compartments. Flow cytometric quantification revealed no detectable differences in DQ-BSA fluorescence between WT cells and multiple Rab24-KO clones, suggesting that bulk endocytic transport to lysosomes as well as protease-dependent proteolysis within lysosomes remained intact despite Rab24 deficiency (III, Fig. 2C).

Together, these data argue against a model in which Rab24 KO causes defects of lysosomal acidity or general proteolytic capacity. Instead, they suggest that Rab24 more likely acts at late trafficking steps such as the organization, availability, or fusion competence of late endosomes or lysosomes with the autophagic compartments.

5.3.3 Proximity proteomics identifies Rab24-associated proteins and reveals mutation-dependent differences (III)

To identify candidate effectors and pathway components operating in Rab24-dependent trafficking, we mapped the Rab24 proximal proteome using APEX2-mediated proximity biotinylation followed by streptavidin enrichment and mass spectrometry. Rab24-KO Neuro-2a cells were used to create stable cell lines expressing APEX2–Rab24 (WT or mutant) for comparative analyses. Biotinylation was initiated by addition of biotin-phenol and hydrogen peroxide to label proteins in the immediate vicinity of the APEX2 fusion protein (III, Fig. 3A–B). Four cell lines stably expressing either APEX2–Rab24-WT, APEX2–Rab24-Q38P, APEX2–Rab24-S67L or APEX2-NES (a construct carrying a nuclear export signal as a diffuse cytosolic negative control), were included in the experiment (III, Fig. 3A; Fig. 12).

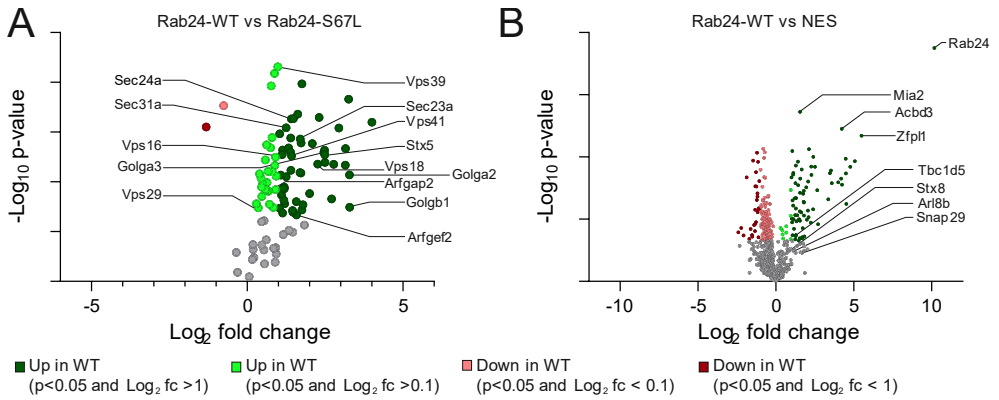

Figure 12. APEX2 proximity proteomics identifies mutation-dependent changes in the Rab24 proximal proteome. (A) Volcano plot comparing proteins enriched by APEX2 proximity labeling in Neuro-2a Rab24-knockout cells expressing either APEX2–Rab24-WT or APEX2–Rab24-S67L. Selected proteins highlighted on the plot include COPII coat components (Sec24A, Sec31A, Sec23A), Golgi-associated proteins (Golga2, Golga3, Golgb1), an ER–Golgi SNARE (Stx5), HOPS/CORVET-related subunits (Vps16, Vps18, Vps29) and HOPS-specific subunits (Vps39, Vps41), as well as proteins linked to ARF regulation (Arfgap2, Arfgef2). (B) Volcano plot comparing proteins enriched in APEX2–Rab24-WT versus the diffuse cytosolic control APEX2–NES. Highlighted proteins include Rab24 (fusion protein), Mia2, Acbd3, Zfpl1, Tbc1d5, Stx8, Arl8b, and Snap29. (A–B), proteins are plotted by $\log_2$ fold change (WT relative to comparison condition) and $-\log_{10}$ p-value. Color coding indicates direction and threshold of enrichment: dark green, up in WT ($p < 0.05$ and $\log_2FC > 1$); light green, up in WT ($p < 0.05$ and $\log_2FC > 0.1$); dark red, down in WT ($p < 0.05$ and $\log_2FC < -1$); light red, down in WT ($p < 0.05$ and $\log_2FC < -0.1$); gray, not meeting the indicated thresholds.

Comparison of the Rab24-WT and Q38P proximity proteomes revealed that multiple subunits of the HOPS tethering complex were reduced in proximity to the ataxia-associated Rab24-Q38P mutant relative to Rab24-WT. In particular, Vps18 and Vps41 were significantly less abundant in the Q38P proximal fraction, and Vps16 and Vps39 also showed reduced abundance but did not meet the statistical threshold in this comparison (III, Fig. 3C–D). These findings suggested that Rab24 associates with lysosome-proximal fusion machinery and that Q38P reduces this association.

To further explore how Rab24's GTP binding relates to its proximal interactome, the GTP-binding deficient mutant Rab24-S67L was analyzed in parallel. When the Rab24-WT proximal proteome was compared with Rab24-S67L, the WT condition showed a substantially larger set of enriched proteins than observed in the WT versus Q38P comparison: 77 proteins were enriched in the WT compared with S67L (Fig. 12A), whereas only 2 proteins were relatively enriched in the WT compared with Q38P (III, Fig. 3C-D). These results are consistent with Rab24-S67L having broadly reduced proximity to multiple Rab24-associated proteins under the assay conditions, in line with prior evidence that this mutant behaves as a dominant-negative form and

shows impaired association with membranous organelles (Munafó and Colombo, 2002, Ylä-Anttila, et al., 2015).

Finally, the APEX2–NES control was included to estimate background labeling of a soluble, cytoplasmic, non-organelle-targeted peroxidase. Comparison of Rab24 WT with APEX2–NES was used to distinguish Rab24-associated proximity signals from diffuse cytosolic labeling and to support prioritization of compartment- and pathway-enriched candidate proteins (Fig. 12B).

In publication III, downstream analyses focus on the Rab24 interaction candidates connected to late autophagic and endolysosomal fusion and trafficking, including HOPS components, which were consistently highlighted by the Rab24-WT proximal proteome and selectively reduced by Rab24-Q38P and Rab24-S67L (III, Fig. 3; Fig. 12). In addition, proteins highlighted in the volcano plots in Fig. 12 include multiple factors linked to ER–Golgi trafficking and Golgi organization (e.g., COPII coat components and Golgi-associated proteins) as well as additional candidates of mechanistic interest, including Arl8b, the Rab7a GAP Tbc1d5 and Snap29 (Fig. 12A). Moreover, several proteins implicated in Golgi/Arf regulation and lipid-transfer machinery at the ER–Golgi interface (including Arfgef2, Arfgap2 and Oxysterol binding protein (Osbpl) family members) were detected among Rab24-WT proximal proteins (Fig. 12A).

5.3.4 Rab24-proximal proteins are enriched for Golgi-associated and ER-to-Golgi trafficking factors, and Rab24 loss disperses COPII marker localization

To obtain a pathway-level overview of the APEX2 proximity proteomics dataset, a GO term analysis was performed on the set of proteins identified in the comparative analysis of Rab24-WT versus Rab24-S67L. This analysis indicated that a substantial fraction of the Rab24-proximal proteins mapped to compartments and processes of the early secretory pathway. In particular, 47.4% of the proteins were annotated to the cellular component term “Golgi membrane” (GO:0000139), and 30.3% were associated with the biological process “ER to Golgi vesicle-mediated transport” (GO:0006888) (Fig. 13A, B). These enrichments suggest that Rab24 is positioned in proximity to a protein network connected to Golgi-associated membrane traffic and COPII-mediated anterograde transport. This is consistent with the presence of multiple COPII and Golgi-associated proteins among the highlighted Rab24-WT proximal candidates (Figs. 12A, 13A-B, D).

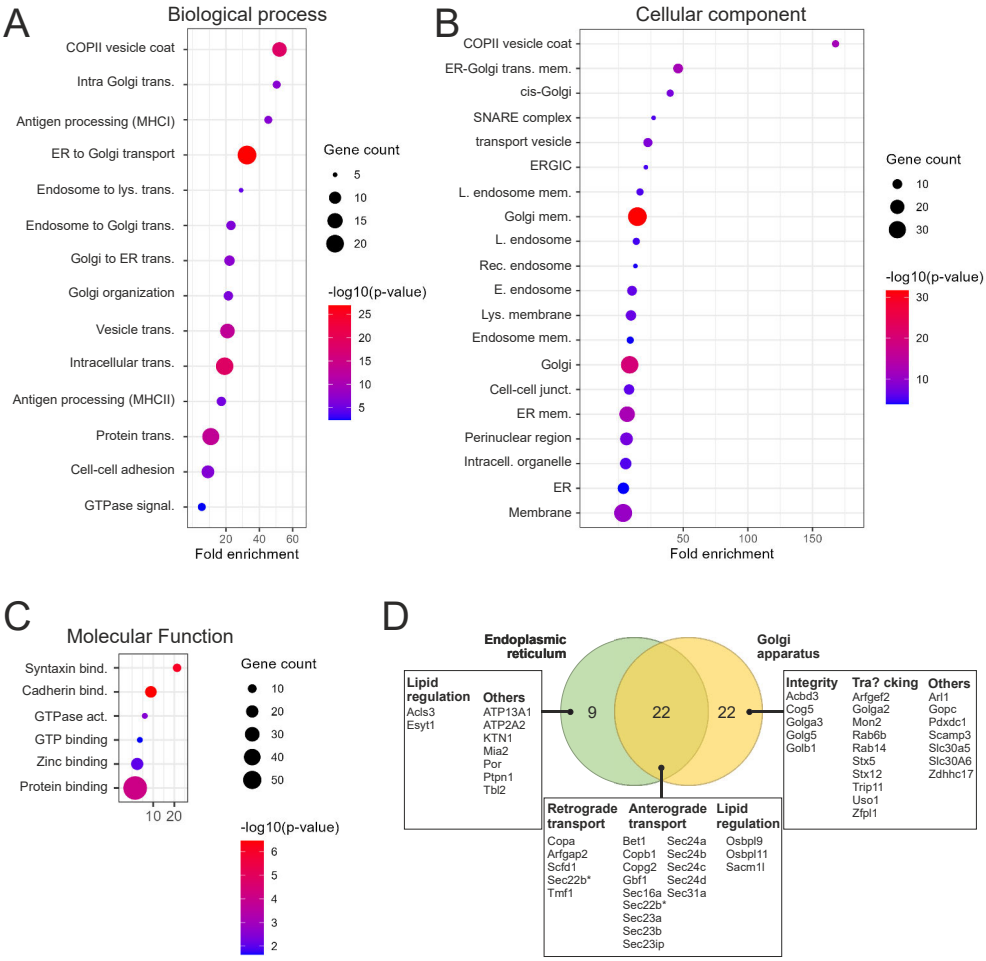

Figure 13. GO term enrichment and compartment annotation of Rab24 proximity proteomics hits. (A–C) Gene Ontology (GO) term enrichment analysis of proteins identified in the Rab24-WT vs Rab24-S67L proximity proteomics dataset, shown as the most enriched terms for Biological Process (A), Cellular Component (B) and Molecular Function (C). Abbreviations used in GO term labels: COPII vesicle coat = COPII vesicle coating; intra Golgi trans. = intra-Golgi vesicle-mediated transport; Antigen processing (MHCI) = antigen processing and presentation of peptide antigen via MHC class I; ER to Golgi transport = ER to Golgi vesicle-mediated transport; Endosome to lys. trans. = endosome to lysosome transport; Endosome to Golgi trans. = retrograde transport, endosome to Golgi; Vesicle trans. = intracellular protein transport; Antigen processing (MHCII) = antigen processing and presentation of exogenous peptide antigen via MHC class II; Protein trans. = protein transport; GTPase signal. = small GTPase mediated signal transduction; ER-Golgi trans. mem. = ER to Golgi transport vesicle membrane; cis-Golgi = cis-Golgi network; ERGIC = endoplasmic reticulum–Golgi intermediate compartment; L. endosome mem. = late endosome membrane; L. endosome = late endosome; Rec. endosome = recycling endosome; E. endosome = early endosome; Lys. membrane = lysosomal membrane; Endosome mem. = endosome membrane; Golgi = Golgi apparatus; Cell-cell junct. = cell-cell adherens junction; ER mem. = endoplasmic (continues on next page).

(Continued from previous page) reticulum membrane; Perinuclear region = perinuclear region of cytoplasm; Intracell. organelle = intracellular membrane-bounded organelle; ER = endoplasmic reticulum; Syntaxin bind. = syntaxin binding; Cadherin bind. = cadherin binding involved in cell-cell adhesion; GTPase act. = GTPase activity; Zinc binding = zinc ion binding. (D) Venn diagram summarizing the subset of proteins identified in the WT vs S67L comparison annotated to the endoplasmic reticulum (ER), Golgi, or both compartments. Numbers indicate proteins assigned to ER only (green), Golgi only (yellow), or annotated to both ER and Golgi categories (overlap).

Based on the observed enrichment pattern, we examined whether Rab24 influences the organization of COPII-coated vesicles in cells. COPII distribution was assessed by immunostaining for Sec31a, a core component of the COPII coat, in WT Neuro-2a cells, Rab24-KO cells, and Rab24-KO cells rescued by re-expression of GFP–Rab24. In WT cells, Sec31a-positive structures displayed a prominent perinuclear clustering pattern. A similar perinuclear enrichment was observed in Rab24-rescued KO cells. In contrast, Rab24-KO cells showed a more dispersed distribution of Sec31a-positive vesicular structures in the cytoplasm (Fig. 14).

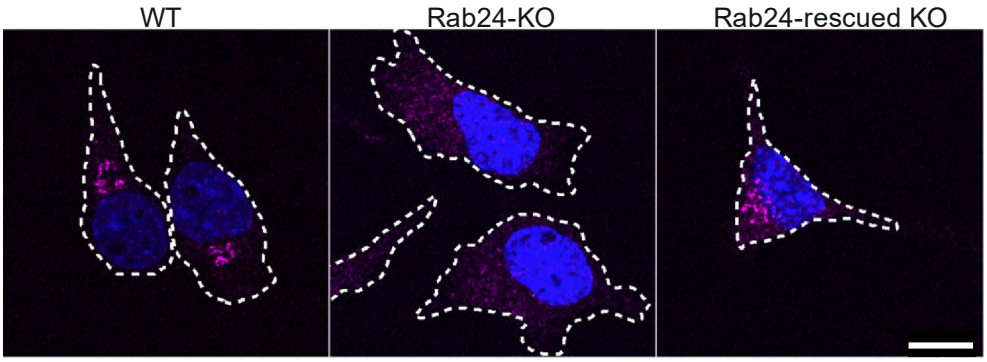

Figure 14. Rab24 KO is associated with a dispersed intracellular distribution of the COPII-coat marker Sec31a. Representative immunofluorescence images of wild-type (WT) Neuro-2a cells, Rab24-KO Neuro-2a cells, and Rab24-KO cells rescued by re-expression of GFP–Rab24, stained for Sec31a to visualize COPII-coated vesicular structures. Scale bar, 10 µm.

Taken together, the Rab24 proximity proteomics indicate that Rab24 contributes to the intracellular organization of COPII-associated structures that are known to mediate traffic from the endoplasmic reticulum to the cis-Golgi (Hauri and Schweizer, 1996). While the microscopic results are qualitative at this stage, together with the proximity proteome of Rab24 they support the hypothesis that Rab24 influences trafficking networks connected to the early secretory pathway. These findings motivate further mechanistic analyses of whether Rab24 links secretory pathway organization or function to downstream lysosome-directed trafficking and autophagy-related phenotypes (Figs. 11-14).

5.3.5 Rab24 physically associates with HOPS subunits and Rab7a, and the Q38P mutation weakens these interactions (III)

To validate candidate interactions suggested by the APEX2 proximity labeling results and to test whether Rab24 forms physical complexes with fusion machinery at the lysosome, co-immunoprecipitation (co-IP) experiments were performed in Rab24-KO Neuro-2a cells stably expressing GFP-tagged Rab24 variants. GFP–Rab24 was precipitated and co-precipitating proteins were detected by immunoblotting. These experiments confirmed that Rab24 associates with multiple components of the HOPS complex, including Vps18, Vps41, Vps16 and Vps33a (III, Fig. 4A–C, E–G). Independent pull-down experiments using FLAG-tagged Vps18 or Vps41 further showed that Rab24 can be recovered in complex with these HOPS subunits (III, Fig. S1).

Importantly, the ataxia-associated Rab24-Q38P mutation reduced Rab24 association with the HOPS components. Quantification of co-IP signals showed significantly diminished co-precipitation of Vps18, Vps41, and Vps33a with Rab24-Q38P compared with Rab24-WT (III, Fig. 4A–C, E-F). The reduction in Vps16 co-precipitation did not reach statistical significance (III, Fig. 4E, G). In contrast, Rab24-S67L—despite its reported diffuse cytoplasmic localization and differences observed in the proximity proteome—did not show a loss of HOPS binding in the co-IP assays (III, Fig. 4A-C, E-G). This indicates that Rab24-HOPS interactions are likely not dependent on Rab24 GTP binding, and that they can be partially uncoupled depending on the mutation and assay context. Control immunoblotting indicated that differences in co-IP were not explained by altered cellular abundance of the analyzed HOPS subunits across the Rab24-WT, Rab24-Q38P, and Rab24-S67L-expressing cell lines (III, Fig. S2).

Because Rab7a is a key late endosomal small GTPase implicated in lysosome-directed trafficking and HOPS-dependent fusion, Rab24 interactions with Rab7a were tested in parallel. Rab7a co-precipitated with Rab24-WT, confirming that these proteins can associate in cells. Both Rab24-Q38P and Rab24-S67L showed reduced Rab7a co-precipitation relative to Rab24-WT, with the strongest impairment observed for Q38P (III, Fig. 4A, D).

Together, the biochemical validation experiments establish that Rab24 can physically associate with multiple HOPS subunits as well as Rab7a, and that the disease-associated Q38P mutation selectively disrupts these interactions. These findings provide a mechanistic link between Rab24 and late autophagic and endolysosomal fusion machinery that is relevant for interpreting Rab24-dependent phenotypes in autophagy and neuronal health.

5.3.6 Rab24 and Rab7a show interdependence in HOPS association (III)

Rab24 interacts with both HOPS subunits and Rab7a, which raises the question if Rab24 influences the ability of Rab7a to associate with HOPS, and conversely whether Rab7a affects Rab24–HOPS interactions. To study the first question, GFP–Rab7a was expressed in WT and Rab24-KO Neuro-2a cells and precipitated with anti-GFP beads to assess co-recovery of the HOPS subunit Vps41. Rab7a-associated Vps41 was substantially reduced in the Rab24-KO background compared with WT cells, indicating that Rab24 supports efficient association of Rab7a with HOPS components under these conditions (III, Fig. 4H–I).

To test the opposite, Rab7a levels were reduced by siRNA in Neuro-2a cells expressing GFP–Rab24, and Rab24 precipitates were analyzed for HOPS subunits. Rab7a depletion increased the amount of Vps41 and Vps18 recovered with Rab24 (III, Fig. 5A–C), consistent with competition between Rab7a and Rab24 for HOPS association, or with Rab7a depletion redistributing HOPS into complexes that more readily associate with Rab24. In contrast, moderate Rab7a overexpression (approximately 71% increase in total Rab7a relative to mock-transfected cells) did not measurably change the amounts of Vps41 or Vps18 co-precipitating with Rab24(III, Fig. 5D–G). This suggests that within this expression range, Rab7a is not sufficient to displace Rab24 from HOPS or to otherwise detectably alter Rab24–HOPS association.

Together, these results support a model in which Rab24 is required for robust Rab7a–HOPS association, while Rab7a depletion increases Rab24–HOPS interaction. This pattern suggests that Rab24 and Rab7a contribute to the same HOPS-dependent trafficking pathway but interact with HOPS in a manner that is not simply additive or competitive. This raises the possibility of sequential engagement or partially overlapping binding sites of Rab24 and Rab7a to the HOPS complex. However, it should be noted that neither proximity biotinylation nor co-IP are able to differentiate whether Rab24 (and Rab7a) bind HOPS directly or via other unknown protein(s).

5.3.7 The Rab24-Q38P mutation only mildly affects GTP binding, but alters subcellular localization (III)

Because small GTPases typically recruit effector proteins in a GTP-dependent manner, we examined whether the ataxia-associated Q38P mutation compromises Rab24 function by affecting GTP binding. Recombinant Rab24 proteins were expressed in bacteria and analyzed using a GTP-agarose pull-down assay. In this assay, Rab24-Q38P showed a tendency toward reduced binding relative to Rab24-WT, but the decrease did not reach statistical significance (III, Fig. 6). In contrast,

mutations affecting residue 67 in the GTP-binding region (S67L and S67Q) reduced binding to GTP-agarose more strongly, consistent with pronounced impairment of nucleotide binding by these variants. Thus, the results confirm previous findings on the GTP-binding deficiency of Rab24-S67L (Ylä-Anttila, et al., 2015). Because Rab24-WT and Rab24-S67L co-precipitated the HOPS subunits equally well (III, Fig. 4), the results also confirm that the Rab24-HOPS interactions are not dependent on Rab24 GTP binding.

To complement the biochemical assay and evaluate whether a structure-based model can reproduce experimentally observed nucleotide-binding trends, molecular dynamics-based free-energy calculations were performed at the University of Oulu using a homology model of the Rab24–Mg^{2+}–GTP complex. In agreement with the pull-down data, the computed change in binding free energy indicated a clear destabilizing effect for the S67L mutation, whereas Q38P mutation produced only a small destabilization of the Rab24-Mg^{2+}-GTP complex (III, Table 1). Together, these analyses support the conclusion that the Q38P mutation does not substantially disrupt GTP binding, suggesting that the reduced association of Rab24-Q38P with HOPS subunits and Rab7a is unlikely to be explained by loss of GTP loading.

Because Rab24 mutations could affect protein targeting to or stabilization in subcellular compartments, the steady-state localization of GFP-tagged Rab24-WT and Q38P were examined by immunofluorescence. Rab24-KO Neuro-2a cells expressing GFP–Rab24-WT or GFP–Rab24-Q38P were stained for the cis-Golgi marker Golga2/GM130, and colocalization was quantified across confocal image stacks. Rab24-Q38P displayed increased overlap with GM130 compared with Rab24-WT (III, Fig. 7), indicating that the Q38P mutation shifts Rab24 distribution toward a cis-Golgi-associated pattern.

Taken together, these results indicate that the ataxia-associated Q38P mutation primarily affects Rab24 interaction networks and intracellular targeting rather than compromising GTP binding. This provides a mechanistic explanation for how the Q38P mutation could disrupt Rab24-dependent trafficking functions including autophagic transport while nucleotide binding is maintained at nearly normal levels.

6 Discussion

6.1 Evaluation of pharmacological modulation of autophagy

The first goal of this thesis was to identify and characterize compounds that modulate autophagy in a manner that is both experimentally tractable and biologically meaningful. Because autophagy is a multi-step pathway, changes in commonly used markers such as LC3 puncta can arise from perturbations of different steps of the pathway. A central outcome of the compound screening and validation work is therefore not only the identification of candidate inhibitors, but also an example of how LC3-based imaging assays can be interpreted rigorously by incorporating flux measurements, including additional autophagy markers, and by comparing basal and starvation-induced conditions (Klionsky, et al., 2021).

The initial high-content screen in A549 GFP–LC3 cells used vesicular LC3 accumulation as a primary readout. This choice is well suited for scalable screening, but it is ambiguous: a decrease in LC3-positive vesicles may reflect inhibition of early autophagosome formation, or accelerated clearance, whereas an increase can reflect either induction of autophagosome formation, association of LC3 with other membrane-bound organelles, LC3 aggregation (especially when LC3 is overexpressed), or blockade on the autophagic clearance pathway (Klionsky, et al., 2021). For this reason, follow-up characterization using bafilomycin A1 was essential, because comparing conditions ± lysosomal inhibition provides an estimate of LC3 flux and helps separate formation from turnover (Klionsky, et al., 2021, Tapper and Sundler, 1995, Yamamoto, et al., 1998). In addition, analysis of endogenous p62 puncta and p62 flux provided a second readout of autophagic activity. p62 is a selective autophagy receptor that is degraded together with the cytoplasmic cargo, but it can also participate in autophagosome initiation-associated condensate formation, meaning that its punctate distribution reflects more than a single step of the pathway (Kageyama, et al., 2021, Kishi-Itakura, et al., 2014, Lamark, et al., 2017). Finally, the colocalization analysis of LC3 and Lamp2 provided a readout for autophagosome fusion with Lamp2-positive compartments. Together, the three assays represent a practical workflow for moving from a

screening phenotype (“more or fewer LC3 puncta”) to a more mechanistic interpretation of the compound effects.

Across the tested compounds, the validation experiments emphasized that autophagy modulation is not a single uniform state but can arise through different points in the pathway. Aciclovir provided a clear example of this context dependence: in the GFP–LC3 screening assay, it reduced GFP-LC3 vesicles under fed conditions, yet showed little effect under starvation, and did not alter LC3 flux relative to controls under either fed or starved conditions. This suggests modulation of basal LC3-associated vesicle number that however did not significantly alter LC3 flux. In contrast, anisomycin and the vioprolides consistently reduced LC3 flux across the conditions in the confocal analysis, while famciclovir primarily reduced LC3 flux under fed conditions. Importantly, these compound-specific profiles underscore that “autophagy inhibition” should be described with respect to the experimental state (basal versus induced) and the readout (puncta abundance versus flux), rather than simply be classified as active or inactive (Klionsky, et al., 2021).

In addition to flux estimation, publication I included analysis of LC3–Lamp2 colocalization to evaluate if LC3-positive structures are efficiently delivered to Lamp2-positive late endosomal/lysosomal compartments. This readout is not a direct flux measurement, but it can help interpret flux changes by indicating whether LC3-positive compartments reach degradative organelles or remain spatially separated. Notably, vioprolide C was the only tested compound that reduced LC3–Lamp2 colocalization under starvation, whereas the other compounds did not significantly alter the overlap (I, Fig. 5). This pattern suggests a trafficking/fusion impairment specifically under induced conditions and therefore provides an additional mechanistic layer to the flux data: a reduction in LC3-associated flux could reflect not only reduced autophagosome generation and/or degradation but also reduced delivery of LC3-positive structures to Lamp2-positive compartments. Conversely, the unaltered colocalization for the other compounds suggests that their effects on LC3 and/or p62 flux may occur upstream of lysosomal delivery or through mechanisms that do not strongly change LC3–Lamp2 overlap at the time points measured, such as changes in lysosomal degradation capacity.

The importance of constitutively active basal autophagy is demonstrated in the Atg4D-mutant dog fibroblast model of NVSD, where the disease-associated LC3-vesicle accumulation phenotype is most evident under nutrient-rich conditions (Syrja, et al., 2017). In these cells under fed conditions, LC3-positive vesicle numbers, and LC3 flux to some extent, were elevated compared with WT fibroblasts. However, the starvation-induced increase in LC3 flux that was prominent in WT cells was strongly blunted in the mutant background. The separation between basal and induced responses provides a useful framework for interpreting the compound effects: interventions that reduce basal LC3 vesicle accumulation may be able to

normalize a disease-associated phenotype without necessarily restoring starvation responsiveness. In this context, anisomycin and famciclovir reduced the elevated basal LC3 vesicle levels in mutant fibroblasts toward WT levels, but none of the tested compounds normalized LC3 flux to WT levels. In fact, flux calculations under fed conditions indicated that the compounds reduced LC3 flux in mutant cells below the WT baseline. This observation highlights a key translational consideration: partial normalization of a disease phenotype at the level of vesicle abundance does not necessarily restore the turnover of autophagic cargo to normal physiological levels.

Finally, these results highlight a point that is relevant for autophagy-targeting strategies: LC3-positive vesicles represent a common point for several lysosome-directed pathways, and in some contexts LC3 association can represent processes that are not strictly equivalent to cargo degradation. LC3 can localize to autophagic structures but also to non-canonical LC3-associated pathways, which complicates interpretation of data in which LC3 puncta alone have been used as a measure of autophagy (Fletcher, et al., 2018, Florey, et al., 2015, Gardner, et al., 2023). Therefore, compound evaluation benefits from combining multiple measurements, e.g. LC3 flux, p62 flux (Bjorkoy, et al., 2005), and compartment overlap metrics such as LC3–Lamp1/2 colocalization (Pugsley, 2017) as used in publication I. Additional complementary approaches include LC3 pH-reporter probes (Farkas, et al., 2009, Kaizuka, et al., 2016, Kimura, et al., 2007), electron microscopy (Eskelinen, et al., 2011), and cytoplasmic cargo degradation assays (Klionsky, et al., 2021, Mortimore and Poso, 1987, Sun, et al., 2015).

6.2 Rab24 in physiological and disease context (II)

Our results showed that Rab24 protein abundance is not uniform across tissues, but instead follows pronounced tissue- and age-dependent patterns. This can be considered important for two reasons: First, it provides a physiological reference for interpreting Rab24-associated phenotypes observed in cell culture and in genetic disease settings. Second, it highlights that the consequences of altering Rab24 levels are likely to be context dependent, because Rab24 is naturally enriched in specific cell types and at certain points of postnatal development rather than being broadly expressed at comparable levels across organs.

At the tissue level, Rab24 protein levels were highest in adult brain and kidney, while several other organs showed comparatively low levels in adulthood. In multiple tissues, including the heart, skeletal muscle, pancreas and liver, Rab24 levels peaked during the early postnatal period and then declined. This suggests that Rab24 is regulated differently during early postnatal developmental and in adulthood, with transient developmental upregulation that is not maintained in

adulthood. In the brain, the marked increase in Rab24 abundance observed at postnatal day 14, together with the neuronal enrichment seen by IHC, aligns well with prior genetic evidence showing that Rab24 dysfunction is particularly detrimental for neuronal maintenance. Canine Rab24 mutations (Q38P and G80V) cause cerebellar ataxia with degeneration of Purkinje neurons (Agler, et al., 2014a, Schwarz, et al., 2025), and autophagy defects in neurons are known to cause progressive axonal pathology (Komatsu, et al., 2007). Thus, the expression atlas in publication II supports the idea that neurons, especially Purkinje cells, represent a cell type in which Rab24 function is required for survival.

At the cellular level, we showed that Rab24 staining was particularly prominent in neurons and in several epithelial cell types (e.g., kidney tubules, bronchiolar epithelium, pancreatic islets). This cell-type selectivity is notable because both neurons and epithelial cells are polarized and heavily rely on spatially organized membrane trafficking for homeostasis: neurons due to their extreme polarity and long-range transport demands (Horton and Ehlers, 2003, Karasmanis, et al., 2018, Kersten and Farias, 2023), and epithelial cells due to their strong endocytic, recycling, and often secretory activity (Stoops and Caplan, 2014, Thompson, et al., 2007). In this light, Rab24 enrichment in pancreatic islet cells is especially interesting, because islets displayed not only high Rab24 staining but also strong staining for multiple markers of autophagy and the endolysosomal system. This may reflect active lysosome-directed trafficking activity in the endocrine cells, potentially linked to secretory granule turnover, metabolic stress handling, and maintenance of organelle quality control. Rab24 protein abundance alone does not establish a functional role for Rab24. However, our observations of strong Rab24 staining in the islets, together with the pronounced Rab24 downregulation in pancreatic neuroendocrine tumors (PNETs), suggest that Rab24 levels may serve as a marker for endocrine cell differentiation state and could alter during tumor progression.

The cancer TMA analyses support the view that Rab24 regulation is not uniform across all malignancies. Rab24 staining was increased in several tumor types, including subsets of breast and skin cancers, while it was reduced in multiple cancers of the digestive system and urinary tract, and decreased in PNETs compared with normal islets. This heterogeneity is consistent with published observations showing that Rab24 can associate with either favorable or unfavorable clinical outcomes depending on tumor type. For example, Rab24 expression has been linked to tumor-promoting traits and poor prognosis in hepatocellular carcinoma (Chen, et al., 2017b, Gu, et al., 2025, Yang, et al., 2020b, Zhang, et al., 2021, Zhu, et al., 2020), and clear-cell renal cell carcinoma (Xu, et al., 2026), whereas in pancreatic adenocarcinoma it has been reported as an independent low-risk factor (Deng, et al., 2022). Such context dependence is not unusual for trafficking regulators, whose functional output depends on the cellular context (Xu, et al., 2024).

In our work, functional experiments in Neuro-2a and HeLa cells further indicated that Rab24 supports cell migration and proliferation. This is in line with previous work showing that Rab24 promotes malignant phenotypes in hepatocellular carcinoma models (Chen, et al., 2017b). These cellular phenotypes offer one mechanistic route by which increased Rab24 abundance could correlate with aggressive disease in some cancers. However, the opposite pattern of reduced Rab24 in certain tumor types suggests that Rab24 may also act as a differentiation-associated marker or participate in homeostatic trafficking programs that are diminished during transformation in specific tissues, such as endocrine pancreatic tumors. Distinguishing whether Rab24 acts primarily as a driver of tumor cell behavior or as a readout of lineage state, or plays no role in malignancy, will require integrating protein-level localization, disruption of Rab24 function, and analyses in physiologically relevant models.

Finally, in the brain, Rab7a staining patterns most closely resembled Rab24 in Purkinje neurons. Also, in several other neuronal regions Rab7a and Rab24 showed broadly similar distributions. Rab24 has been reported to interact with Rab7a and to influence endosomal degradation (Amaya, et al., 2016). The mechanistic work in publication III further supports functional overlap between Rab24 and Rab7a in association with late autophagic and late endosomal/lysosomal fusion machinery. While histological co-distribution does not prove that two proteins act together, the combination of neuronal co-enrichment (publication II) with biochemical and functional coupling (publication III; see also Amaya et al. 2016) suggests that Rab24 and Rab7a function may be particularly relevant in neuronal cells where autophagic and endosomal maturation and lysosome-directed trafficking are crucial for maintaining homeostasis.

The potential link between Rab24 and Rab7a is especially compelling for Purkinje neurons, which are selectively vulnerable in Rab24-mutant dogs (Agler, et al., 2014a) and are also sensitive to disruptions in lysosome-directed trafficking or lysosome function (Cook, et al., 2023, Feng, et al., 2022, Ko, et al., 2005, Sato, et al., 2021).

6.3 Rab24 in autophagy and endolysosomal trafficking

Our work provides mechanistic evidence that Rab24 connects to autophagic and endolysosomal fusion machinery through interactions with the HOPS complex and Rab7a. Disruption of these interactions offers a plausible explanation for the Rab24-Q38P-associated autophagy phenotype in canine Purkinje cells (Agler et al., 2014) and the accumulation of amphisomes/autolysosomes in Rab24-knockdown HeLa cells (Ylä-Anttila et. al., 2015).

Rab24 loss in Neuro-2a cells shifted the balance between autophagic structures and degradative compartments without causing a detectable change in lysosomal acidity or bulk proteolytic capacity. This argues against a model in which Rab24 is required for general lysosomal acidification or protease activity, and instead points toward a role in trafficking or fusion steps that determine how efficiently autophagic cargo reaches degradative compartments. At the same time, these results are informative in light of the emerging roles of Vps41 beyond canonical HOPS-mediated fusion events. Vps41 has been implicated in the trafficking of lysosomal membrane proteins from the trans-Golgi network to endo/lysosomes via Lamp-carrier intermediates (Sanzà, et al., 2025). Vps41-dependent delivery of lysosomal membrane proteins has also been linked to the maintenance of cellular cholesterol homeostasis through lysosomal targeting of the cholesterol transporter Npc1 (Ndoj, et al., 2025). Given that we observed Rab24 to interact with Vps41, it was conceivable that Rab24 loss could compromise Vps41-dependent lysosome-maintenance pathways and thereby cause lysosomal dysfunction. In Neuro-2a cells, we did not detect evidence for a generalized lysosomal defect based on the assays performed. Nevertheless, more specialized lysosomal functions (including lysosomal lipid export/metabolism and maintenance of lysosomal membrane protein composition) were not directly tested and are plausible targets for future investigation.

In co-immunoprecipitation, Rab24 interacted with multiple HOPS subunits (Vps18, Vps41, Vps16 and Vps33a) and the Q38P mutation weakened these interactions, most clearly for Vps18/Vps41 and Vps33a. Notably, the dominant-negative Rab24-S67L variant did not show a significant reduction in co-immunoprecipitation with the HOPS subunits compared with Rab24-WT, suggesting that Rab24–HOPS interaction is not strictly dependent on Rab24 being active/GTP-bound under the co-immunoprecipitation conditions.

The HOPS complex is a central tethering factor for lysosome-directed fusion events, including autophagosome/amphisome–lysosome fusion and fusion of endosomal intermediates with lysosomes (Jian, et al., 2025, Jiang, et al., 2014, van der Beek, et al., 2024, van der Beek and Klumperman, 2025). Therefore, Rab24–HOPS association provides a direct mechanistic link between Rab24 and the fusion step that is frequently rate-limiting for completion of autophagic degradation.

The disease relevance of the Rab24-HOPS connection is strengthened by the observation that loss-of-function perturbations in HOPS subunits can lead to vulnerability in neurons that is reminiscent of Rab24-mutant-associated ataxia. Vps18 deficiency disrupts Purkinje neuron dendritogenesis and can lead to neurodegenerative phenotypes in mice (Peng, et al., 2012a, Peng, et al., 2012b). In humans, bi-allelic Vps41 variants cause cerebellar ataxia and abnormal membrane trafficking (Sanderson, et al., 2021). In addition, neurodegenerative Vps41 variants

have been shown to impair HOPS function and perturb lysosome-associated signaling pathways (van der Welle, et al., 2021). These genetic data support the idea that compromised HOPS-dependent trafficking makes particularly neurons in the cerebellum susceptible. Further, the data provide a possible mechanism for how Rab24-Q38P might predispose to selective Purkinje-cell degeneration by weakening Rab24-HOPS interaction (Agler, et al., 2014b, Sanderson, et al., 2021). Notably, conditional ablation of Vps18 in the mouse nervous system disrupted multiple lysosome-directed trafficking routes (including autophagy, endocytosis and biosynthetic delivery) and was accompanied by upregulation of β1 integrin. Partial rescue of neuronal migration defects by β1 integrin knockdown suggested that altered integrin homeostasis can contribute functionally to the migration phenotype in this setting (Peng, et al., 2012b). This is relevant because Rab24 loss reduced migration and proliferation in cultured Neuro-2a and epithelial cells (publication II), and Rab24 overexpression has been linked to increased motility and invasion in hepatocellular carcinoma models (Chen, et al., 2017b). Together, these observations raise the possibility that Rab24-dependent trafficking pathways could influence cell migration not only through impaired cargo delivery to lysosomes but also by altering the turnover of cell surface receptors including integrins. At present, no direct mechanistic links between the migration phenotypes and Rab24-HOPS function have been established. The effect on migration may involve HOPS-dependent or HOPS-independent Rab24 activities. In either case, the direction and magnitude of such effects are likely to be cell-type and disease-context dependent.

A second emerging mechanism in our study is the interplay between Rab24 and Rab7a. Rab7a is a key late endosomal GTPase implicated in lysosome-directed trafficking and it has been linked to HOPS-dependent late endosomal trafficking (McEwan, et al., 2015). However, as discussed in the literature review (2.3.2 *Recruitment of HOPS in metazoan cells*), metazoan HOPS recruitment appears to rely on multiple small GTPases and Rab effector interactions rather than a single Rab7a-driven mechanism. Several Rab7a effectors (e.g., Plekhm1 and Rilp) have been reported to connect Rab7a-defined membranes to HOPS, and additional cues such as Rab2 and Arl8 position HOPS at late endosomes and lysosomes, respectively. In the present work, Rab7a co-precipitated with Rab24-WT, and both Rab24-Q38P and Rab24-S67L mutations reduced Rab7a co-precipitation, indicating that Rab24's association with Rab7a is sensitive to mutations in both the switch I region (Q38P) and the atypical nucleotide-binding region (S67L). Functionally, Rab24 supported Rab7a–HOPS association: significantly less Vps41 was recovered with GFP–Rab7a in Rab24-KO cells compared with WT cells, while silencing Rab7a increased the amount of Vps41 and Vps18 co-precipitating with Rab24. Together, these results argue that Rab24 and Rab7a do not engage HOPS independently, but instead influence each other's ability to associate with HOPS components.

Notably, the proximity-proteomics dataset provides additional context for interpreting the relationship of Rab24, Rab7 and HOPS. In the Rab24-WT versus APEX-NES comparison, a broad set of proteins was identified in the Rab24-proximal fraction. Yet canonical Rab7a effectors that have been linked to HOPS recruitment (such as Rilp and Plekhm-family proteins) were not detected. While this does not exclude their involvement, because proximity labeling can miss low-abundance, transient, or spatially restricted interactions, it suggests that Rab24 is not stably embedded in the same Rab7a–effector assemblies that have been proposed to recruit HOPS in other settings. Similarly, small GTPases that have been implicated in metazoan HOPS positioning in other contexts (including Rab2 and Rab39) were not detected in our dataset. In contrast, Arl8b was identified among the Rab24-proximal proteins, although without statistical enrichment under the applied thresholds. Taken together with the Rab7a co-IP, these observations are compatible with a model in which Rab24 helps establish or stabilize a HOPS interaction state that allows engagement of Rab7a. Rab7a's role may be indirect, transient, or effector-mediated rather than reflecting a stable Rab7a–HOPS recruitment complex. One possible interpretation is a sequential or handover-like mechanism, conceptually related to Rab5-Rab7a conversion during endosome maturation (Rink, et al., 2005), although the timing and regulatory factors controlling any Rab24-to-Rab7a transition remain to be identified.

In addition, the APEX2 dataset did not detect Fis1, a mitochondrial outer membrane protein previously reported to interact with Rab24 in liver tissue extracts (Seitz, et al., 2019). While non-detection in proximity proteomics cannot exclude an interaction, the absence of Fis1 even from the broader set of proteins detected in the Rab24-WT versus APEX-NES comparison is consistent with the possibility that Rab24's interaction network is shaped by cellular context. In this view, Rab24 may engage distinct partner proteins depending on cell type and the contribution of mitochondrial, secretory, or endolysosomal trafficking pathways. This would be compatible with the tissue- and cell-type specificity of Rab24 protein expression observed in publication II.

Beyond late lysosomal fusion factors, the Rab24-WT proximity proteome contained multiple proteins linked to ER–Golgi trafficking and Golgi organization, consistent with early results showing that Rab24 localizes to ER/Golgi-associated compartments (Olkkonen, et al., 1993). In the WT versus S67L comparison, highlighted candidates included COPII coat components, the ER–Golgi SNARE Stx5, Golgi matrix proteins, and the Golgi tether Uso1, suggesting that Rab24 functions in proximity to the early secretory pathway machinery (Fig. 12). Additionally, Arf pathway regulators (Arfgef2 and Arfgap2) and several Osbpl family proteins were detected among Rab24-WT proximal proteins, raising the possibility that Rab24 is positioned near protein networks of the ER–Golgi contact

sites. While these associations do not allow to draw conclusions on direct interactions or specific functions, they are in line with the qualitative Sec31a redistribution observed in Rab24 knockout cells (Fig. 13).

Because Q38 lies in the switch I region, impaired GTP loading was suspected and could have explained reduced effector recruitment by the Rab24-Q38P mutant. However, both the GTP-agarose assay and molecular dynamics-based free-energy calculations indicated that Q38P exerts only a mild destabilizing effect on the Rab24–GTP complex, in contrast to the stronger impairment caused by S67L. Hence, the Rab24-Q38P-dependent loss of HOPS and Rab7a interaction is unlikely to be driven primarily by an inability to bind GTP.

Instead, the data are more consistent with Q38P disrupting Rab24's ability to bind its effectors and/or its subcellular targeting. In principle, introduction of a proline residue can lead to local conformational constraints and contribute to loss of function in some proteins. Proline is often discussed as an 'α-helix breaker' because it cannot form the backbone hydrogen-bonding pattern required for α-helix stability (Chakrabarti and Chakrabarti, 1998, Cordes, et al., 2002, Yun, et al., 1991). However, Q38 is not predicted to lie within an α-helix in AlphaFold-based structural models of Rab24 (Fig. 10). Therefore, a helix-disruption mechanism is unlikely to explain the observed phenotype. More importantly, several experimental observations argue against a model in which Q38P causes a globally misfolded, non-functional Rab24 protein. In the proximity-proteomics comparison, Q38P produced a selective reduction in proximity to HOPS subunits (with Vps18 and Vps41 significantly decreased) rather than a broad loss of Rab24-proximal proteins. By contrast, the dominant-negative S67L variant showed a much wider loss of proximity signals, with WT Rab24 retaining proximity to over 70 proteins that were not recovered with S67L under the applied thresholds (Fig. 12). Further, Rab24-Q38P displayed increased colocalization with the cis-Golgi marker Golga2/GM130 compared with Rab24-WT, consistent with altered subcellular targeting that could reduce access of Rab24 to membranes where HOPS-dependent fusion events occur. Alternatively, the increase cis-Golgi localization could be caused by the decreased binding of Rab24-Q38P to the HOPS components. Together, these results support the interpretation that Q38P causes a partial functional defect, which likely affects the conformation or correct targeting required for HOPS/Rab7a engagement rather than eliminating Rab24 function through generalized misfolding. This is in line with the similar levels of Rab24-Q38P and Rab24-WT protein in the stably expressing Neuro-2a cells. Rab24-Q38P was also expressed at protein level in the affected canine Purkinje cells (Agler, et al., 2014a).

The inclusion of Rab24-S67L in both APEX2 proximity proteomics and co-immunoprecipitation assays revealed an apparent discrepancy: S67L showed broadly reduced proximity to organelle-associated proteins (including reduced

proximity of HOPS subunits in the APEX2 dataset), whereas co-immunoprecipitation from cell lysates did not show a significant reduction in binding of S67L to Vps41/Vps18/Vps16/Vps33a compared with Rab24-WT. A plausible explanation is that the two approaches capture different aspects of Rab24 biology. APEX2 labeling is performed in intact cells and therefore retains spatial information; because Rab24-S67L shows diffuse cytosolic localization and reduced membrane association, it may biotinylate fewer membrane-bound HOPS components and other organelle-associated proteins. In contrast, co-immunoprecipitation is performed in cell lysates, where organelle localization is disrupted and proteins can diffuse and reassociate, potentially allowing Rab24-S67L to encounter HOPS subunits even if it fails to reach the relevant membrane compartments in living cells. In this view, S67L may retain biochemical binding competence in cell extracts but lack effective compartmental targeting in cells.

Finally, while the core mechanistic findings position Rab24 at lysosome-proximal fusion machinery, the proximity biotinylation data points to the possibility that Rab24's function is broader than HOPS association alone. The APEX2 dataset contained a substantial fraction of proteins linked to Golgi membranes and ER-to-Golgi vesicle trafficking (Fig. 13). Further, Rab24 knockout was associated with a redistribution of the COPII coat marker Sec31a from a perinuclear clustered pattern to a dispersed distribution (Fig. 14). These observations are consistent with Rab24's reported localization to ER/Golgi compartments (Olkkonen, et al., 1993) and suggest that Rab24 may influence the organization of early secretory pathway components in addition to its role at lysosome-directed fusion steps. This interpretation is supported by the Rab24 protein patterns mapped in publication II, which showed prominent Rab24 staining in several epithelial cell types with high membrane traffic demands, including bronchiolar epithelium, kidney proximal tubule epithelium, ependymal cells, and pancreatic endocrine islet cells. These cell types are strongly secretory and/or endocytically active, consistent with an increased requirement for tightly organized ER–Golgi vesicular transport. At the same time, Rab24 expression was not uniformly high across all secretory epithelial populations, for example, pancreatic acinar cells showed much lower staining than the endocrine islet cells, arguing that Rab24 abundance is not a universal marker of a secretory phenotype but may reflect more specific, cell-type-dependent trafficking requirements. How these putative functions relate – whether they represent parallel Rab24 pools acting at different compartments, or a linked pathway in which early secretory trafficking affects lysosome-directed membrane supply – remains an open question. Nonetheless, combined with the robust biochemical evidence for Rab24–HOPS/Rab7a coupling, these findings support a model in which Rab24 contributes to autophagic and endolysosomal homeostasis by coordinating the recruitment or

stability of lysosome-directed fusion machinery rather than by controlling lysosomal degradative capacity.

6.4 Limitations and future directions

Limitations of this thesis should be considered when interpreting the findings and planning follow-up work. First, interpretation of LC3-based pharmacology requires particular caution. As discussed in Section 6.1 *Evaluation of pharmacological modulation of autophagy*, LC3 puncta and even flux estimates depend on experimental conditions and can be influenced by non-canonical LC3-associated pathways; therefore, future compound work should continue to rely on multiple measures of autophagy and clearly defined assay conditions rather than single-marker classification.

Second, the Rab24 tissue- and cancer-profiling work is descriptive and therefore cannot by itself establish causal roles for Rab24 in development, homeostasis or tumorigenesis. In the mouse tissue analyses, Rab24 abundance was quantified by western blotting using total protein normalization and complemented by IHC to resolve cell-type distributions. While this combined approach strengthens anatomical interpretation, protein-level measurements remain sensitive to antibody specificity, fixation/epitope retrieval conditions and differences in tissue composition across ages. Thus, changes in Rab24 levels could in part reflect shifts in cellular makeup (e.g., relative neuron/glia proportions) rather than altered expression within a given cell type. Likewise, although total protein normalization is well suited for comparing tissues with very different housekeeping protein expression profiles, it cannot fully correct for age-dependent changes in overall proteome composition within a tissue. For example, if the total protein content of a tissue increases disproportionately due to accumulation of other proteins, Rab24 could appear to decrease after normalization even if its absolute abundance was unchanged (and vice versa). In the human TMA analyses, Rab24 staining was quantified using H-scores derived from automated classification, which provides a standardized semi-quantitative comparison but does not fully capture intratumoral heterogeneity, differences in sampling between the cores in the TMAs, or clinical variables (e.g., stage, treatment, survival) that were not available for the multicancer cohorts. Moreover, protein and mRNA levels do not necessarily correlate, and the TCGA mRNA comparisons were performed on separate cohorts from the TMAs, limiting conclusion about direct protein–transcript relationship. Finally, although the CRISPR knockout, RNA-seq and migration/proliferation assays provide functional support for Rab24-dependent cellular programs, these experiments were performed in established cell lines and cannot substitute for tissue-matched in vivo studies. In particular, the direction and magnitude of Rab24's effects on cell proliferation and

motility are likely to vary depending on cell type, consistent with the cancer-type-specific expression patterns observed (Chen, et al., 2017b, Deng, et al., 2022). Future work using animal models, larger clinically annotated patient cohorts, and validation of antibody-based staining (e.g., independent antibodies or proteomic measurements) will be required to distinguish whether altered Rab24 levels primarily reflect the tissue from which a tumor originated, the tumor differentiation state, adaptive responses of tumor cells, or direct functional contributions to tumor progression.

Third, the Rab24–HOPS/Rab7a mechanism proposed in Section 6.3 *Rab24 in autophagy and endolysosomal trafficking* is supported by convergent proximity labeling, co-immunoprecipitation and cellular phenotyping, but key mechanistic details remain unresolved. Priority follow-up work should include:

1. Defining if Rab24 binds directly to Vps41/Vps18 (and mapping interaction interfaces and nucleotide-state dependence).
2. Testing Rab24 function in more physiologically relevant neuronal systems (e.g., Purkinje-neuron models).
3. Testing of specialized lysosomal functions, given the emerging HOPS-subunit roles in lysosome-directed biosynthetic trafficking. Lysosomal membrane protein composition and lipid export pathways should be investigated.
4. Testing of functional consequences of defects in Rab24-mediated membrane fusion, e.g. via measuring long-lived protein degradation in Rab24-KO vs WT cells.

In summary, while this thesis provides a framework for pharmacological evaluation of basal autophagy phenotypes and identifies a mechanistic connection between Rab24 and HOPS/Rab7a-associated lysosome-directed trafficking, future studies combining additional functional assays with in vivo-relevant models will be necessary to establish when Rab24 acts as an active regulator of lysosome-directed fusion, and when its abundance mainly reflects cell identity and/or differentiation state.

7 Summary/Conclusions

Based on the results of this PhD thesis, the following conclusions can be made:

1. Candidate compounds identified in a high-content microscopy screening showed distinct, condition-dependent autophagy-modulating profiles: famciclovir and partly aciclovir primarily reduced LC3 flux under basal conditions, whereas anisomycin and vioprolides reduced LC3 flux more broadly. In Atg4D-mutant dog fibroblasts (NVSD model), anisomycin and famciclovir reduced the elevated basal LC3-vesicle phenotype toward wild-type levels, but did not correct autophagy flux to the level of wild-type cells.

2. Rab24 protein abundance is strongly dependent on tissue and developmental stage in mice, with prominent enrichment in adult brain and kidney. Rab24 abundance also shows marked cell-type specificity, it is particularly enriched in neurons and selected epithelial populations. In human tissue microarrays, Rab24 protein levels differed across tumor entities, indicating cancer-type-specific expression rather than a uniform direction of change.

3. Mechanistically, Rab24 supports lysosome-directed autophagic trafficking steps most likely by associating with the HOPS tethering complex and Rab7a. The ataxia-associated Rab24-Q38P mutation selectively weakens Rab24 interactions with HOPS subunits and Rab7a without causing a major defect in GTP binding. These results support a model in which Rab24 promotes HOPS-dependent autophagosome/amphisome–lysosome fusion.

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