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Exploring selective autophagy cargo and machinery using proximity proteomics

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Abbreviations

Aa	Amino acid
AD	Alzheimer's disease
ALFY	Autophagy-linked FYVE protein / WD repeat and FYVE domain-containing protein 3
ALS	Amyotrophic lateral sclerosis
AMPK	5'-AMP-activated protein kinase / Acetyl-CoA carboxylase kinase
APEX2	Ascorbate peroxidase 2
APOE	Apolipoprotein E
ATG	Autophagy-related protein
ATL3	Atlastin 3
ATP / ADP	Adenosine triphosphate / Adenosine diphosphate
BafA1	Bafilomycin A1
BECN1	Beclin-1
BNIP3	BCL2 interacting protein 3
C2	Conserved 2 domain
CC	Coiled-coil domain
CCPG1	Cell cycle progression protein 1
CD63	CD63 antigen
CHMP4B	Charged multivesicular body protein 4b
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
C-terminal	Carboxy-terminal
CUE	Coupling of ubiquitin to ER degradation
DAB	3,3`-Diaminobenzidine
DFCP1	Double FYVE-containing protein 1
DNA	Deoxyribonucleic acid
DUB	Deubiquitination enzyme
e.g.	Exempli gratia (latin for *for example*)
EE	Early endosome
EM	Electron microscopy
ERAD	ER-asoociated degradation
ESCRT	Endosomal sorting complexes required for transport
FAM134B / RETREG1	Reticulophagy regulator 1
FIP200 / RB1CC1	FAK family kinase-interacting protein of 200 kDa / RB1-inducible coiled-coil protein 1
FKBP8	FK506-binding protein 8
FUNDC1	FUN14 domain-containing protein 1
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GAP	GTPase-activating protein
GEF	Guanine-nucleotide exchange factor

GFP	Green fluorescent protein
GTP / GDP	Guanosine triphosphate / Guanosine diphosphate
H_2O_2	Hydrogen peroxide
hATG8	Human ATG8
HD	Huntington's disease
HGS	Hepatocyte growth factor-regulated tyrosine kinase substrate
HP	Hydrophobic pocket
HSC70	Heat shock cognate 71 kDa protein
IMM	Inner mitochondrial membrane
KEAP1	Kelch-like ECH-associated protein 1
KO	Knock out
LC-MS / MS	Liquid chromatography followed by tandem mass spectrometry
LDS	LIR-docking site
LE	Late endosome
LFQ	Label-free quantification
LIR	LC3-interacting region
MAP1LC3	Microbutubule-associated proteins 1 A/AB light chain 3
MS	Mass spectrometry
mTORC1	Mammalian target of Rapamycin complex
NBR1	Next to BRCA1 gene 1 protein
NDP52 / CALCOCO2	Nuclear dot protein 52 / Calcium-binding and coiled-coil domain-containing protein 2
NIX	NIP3-like protein X
NPLOC4	Nuclear protein localization protein 4 homolog
N-terminal	Amino-terminal
OMM	Outer mitochondrial membrane
OPTN	Optineurin
ORF	Open reading frame
PARKIN	E3 ubiquitin-protein ligase parkin
PB1	Phox and Bem1 domain
PD	Parkinson's disease
PE	Phosphatidylethanolamine
PI3KC3	Phosphatidylinositol 3-kinase catalytic subunit type 3 / class III phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PINK1	PTEN-induced putative kinase protein 1
PLEKHM1	Pleckstrin homology domain-containing family member 1
PM	Plasma membrane
polyQ	Polyglutamine expansion
RAB5 / 7	Ras-related protein Rab-5 / -7

RNA	Ribonucleic acid
RTN3	Reticulon 3
SEC62	Translocation protein 62
SILAC	Stable isotope labeling by amino acids in cell culture
SKICH	SKIP carboxyl homology domain
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment receptors
SQSTM1 / p62	Sequestosome-1
STAM	Signal transducing adapter molecule 1
TAX1BP1	Tax1-binding protein 1
TBD	TOM1-binding domain
TBK1	Tank binding kinase 1
TEX264	Testis-expressed protein 264
TMT	Tandem mass tag
TOLLIP	Toll-interacting protein
TOM1	Target of Myb protein 1
TRIM13	Tripartite motif containing 13
Ub	Ubiquitin
UBA	Ubiquitin associated
UBA1	Ubiquitin-like modifier-activating enzyme 1
UBAN	Ubiquitin binding in ABIN and NEMO domain
Ubl	Ubiquitin-like
UBQLN2	Ubiquilin-2
UBZ	Ub-binding zinc fingers
UDS	UIM docking site
UFD1	Ub fusion degradation protein 1
UIM	Ubiquitin interaction motif
ULK1	Unc-51-like kinase 1 / Serine/threonine-protein kinase ULK1
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system
VAPA	Vesicle-associated membrane protein-associated protein A
VAPB	Vesicle-associated membrane protein-associated protein B/C
VCP	Transitional endoplasmic reticulum ATPase / Valosin-containing protein
VPS4 / 15 / 34	Vacuolar protein sorting 4 / 15 / 34
WIPI2	WD repeat domain phosphoinositide-interacting protein 2
z.B.	zum Beispiel
ZF	Zinc finger domain
ZZ	ZZ-type zinc-finger domain

Publications of this thesis

I. Systematically defining selective autophagy receptor-specific cargo using autophagosome content profiling.

Zellner, S., Schifferer, M. & Behrends, C.

Molecular Cell, 2021. Volume: 81(6), 1337-1354 e1338.
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II. Atg4 family proteins drive phagophore growth independently of the LC3/GABARAP lipidation system.

Nguyen, T. N., Padman, B. S., **Zellner, S.**, Khuu, G., Uoselis, L., Lam, W.K., Skulsuppaisarn, M., Lindblom, R. S. J., Watts, E., Behrends, C. & Lazarou, M.

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Zusammenfassung

Autophagie ist ein wichtiger Prozess zur Erhaltung der zellulären Integrität durch die Entsorgung von toxischen Materialien wie zum Beispiel eindringenden Pathogenen, defekten Organellen und fehlgefalteten oder aggregierten Proteinen. Diese Diversität erfordert eine spezielle zelluläre Maschinerie zur korrekten Erkennung der unterschiedlichen Cargos. Autophagie ist in den meisten Zellen bereits unter basalen Bedingungen aktiv, kann aber durch intrinsische oder extrinsische zelluläre Faktoren, z.B. Aminosäurenmangel, deutlich angeregt werden. Die Bildung von Autophagosomen ist ein stark regulierter Prozess, welcher die Aktivierung einer Ubiquitin-ähnlichen Konjugationsmaschinerie beinhaltet. Dies führt zur kovalenten Verknüpfung von Mitgliedern der hATG8 Proteinfamilie, die sich in GABARAPs und MAP1LC3s aufteilt, mit PE (Phosphatidylethanolamin) in der Membran sich bildendender Autophagosomen. Dieser Lipidierung vorausgehend wird die Familie der ATG4 Proteasen (ATG4A-D) benötigt, welche die C-terminalen Glycin Reste der hATG8s freilegt. Lipidierte hATG8s vermitteln die Umschließung des Cargos durch Bindung von selektiven Autophagie-Rezeptoren und fördern die Reifung der Autophagosomen, sowie deren Fusion mit Lysosomen. Letztendlich wird der Inhalt der so entstandenen Autolysosomen durch lysosomale Hydrolasen abgebaut.

Selektive Autophagie-Rezeptoren sind vielfältig und ihr Einsatz hängt von der Art des jeweiligen Cargos ab. Eine Gruppe strukturell ähnlicher Rezeptoren umfasst die sogenannten SQSTM1-ähnlichen Rezeptoren (SLRs), die neben dem Gründungsmitglied SQSTM1 (auch bekannt als p62), auch NBR1, NDP52, OPTN, TAX1BP1 und TOLLIP beinhalten. Obwohl fast alle SLRs bereits mit dem Abbau von aggregierten Proteinen in Verbindung gebracht wurden, ist nur wenig über ihre spezifischen Funktionen und Autophagie-Substrate bekannt. Im Rahmen dieser Arbeit habe ich APEX2-induzierte Nachbarschaftsmarkierung gekoppelt mit limitierter Proteolyse und quantitativer proteomischer Analyse (*proximity proteomics*) angewendet. Damit konnte ich den SLR-spezifischen Inhalt von Autophagosomen in verschiedenen experimentellen Bedingungen bestimmen. Mit Hilfe dieses Ansatzes wurden über 250 verschiedene potenzielle Cargo-Proteine identifiziert, welche unter basalen Bedingungen in Autophagosomen eingefangen wurden und sich deutlich von Proteinen in der Nachbarschaft von zytosolischen SLRs unterscheiden. Obwohl dieser Datensatz eine Vielzahl von Rezeptor-spezifischen Substraten beinhaltet, wurde der Großteil der Cargo-Kandidaten bei mindestens zwei Rezeptoren identifiziert, was auf eine potenzielle Redundanz der Rezeptoren hinweist. Faszinierenderweise konnten einige Cargo-Kandidaten funktionellen Kategorien zugeordnet werden, bei welchen bisher nicht

bekannt war, dass sie durch Autophagie abgebaut werden (z.B. GTPasen). Durch komplementäre biochemische, bildgebende und organellspezifische proteomische Ansätze konnte ich eine Vielzahl dieser Cargo-Kandidaten als *bona fide* Autophagie-Substrate validieren. In einer Reihe von Anschlussexperimenten konnte ich zeigen, dass TOLLIP und dessen Cargo Proteine durch endosomale Membranen umschlossen und anschließend zu Lysosomen transportiert werden. Dieser Prozess wurde zwar bereits als endosomale Mikroautophagie beschrieben, jedoch repräsentiert TOLLIP den ersten Rezeptor dieser alternativen lysosomalen Anlieferungsroute. Mit Hilfe der proteomischen Analyse von Autophagosomen Inhalten konnte ich herausfinden, dass die akute Hemmung von Ubiquitinierung und hATG8-Lipidierung keinen Einfluss auf die meisten basalen Cargo-Kandidaten hatte. Das war sehr überraschend, da diese beiden post-translationalen Modifikationen bisher als wesentliche Merkmale von Rezeptoren gelten, um Cargos zu erkennen und an entstehende Autophagosomen zu binden. Dieses Resultat wirft einige Fragen auf und rüttelt an unserem aktuellen mechanistischen Verständnis der selektiven Autophagie. Zuletzt habe ich unsere proteomische Methode angewendet, um Veränderungen im autophagischen Degradom als Antwort auf drei verschiedene Bedingungen, welche jeweils zur Bildung von zytosolischen Aggregaten führen, zu detektieren. Diese umfangreiche Analyse hat unerwarteterweise gezeigt, dass der Großteil des identifizierten basalen Cargos nicht verändert war. Stattdessen wurden unter diesen Bedingungen zusätzliche Proteine in Autophagosomen eingefangen, welche SLR-spezifische Unterschiede zeigten (**Publikation I**).

In einem kollaborativen Ansatz haben wir die Rolle von ATG4-Proteasen bei der Prozessierung von hATG8s im Kontext von selektiver Autophagie geschädigter Mitochondrien (Mitophagie) untersucht. Durch die Proben-Prozessierung und Analyse von proteomischen Daten aus APEX2-ATG4A-D exprimierenden, Mitophagie induzierten und biotinylierten Zellen konnte ich ATG4 Familienmitglied-spezifische Nachbarschaftsproteine identifizieren. Mit Ausnahme von ATG4C konnten bei allen Familienmitgliedern sowohl überschneidende, als auch spezifische Kandidaten gefunden werden. Bei näherer Betrachtung konnten Proteine, die mit der Bildung von Autophagosomen-Vorläuferstrukturen assoziiert sind, identifiziert werden. Unter diesen befanden sich zwei Regulatoren des Transports von ATG9A, welches in Transportvesikeln zur Bildungsstelle von Autophagosomen rekrutiert wird. Dadurch konnte eine wichtige direkte Interaktion zwischen ATG4A und ATG9A-Vesikeln gezeigt werden, wodurch Letztere als Reaktion auf die Induktion von Mitophagie zu geschädigten Mitochondrien rekrutiert wurden. Außerdem wurde durch kombinatorische Deletion von ATG4- und ATG8-Familienmitgliedern eine neue Funktion von ATG4 Proteinen in der frühen Autophagosomen-Entstehung entdeckt. Interessanterweise war

diese unabhängig von deren proteolytischen Aktivität gegenüber hATG8s (**Publikation II**).

Zusammengefasst ist meine Arbeit eine reichhaltige Quelle neuer Autophagie-Cargos und regulatorischer Faktoren und stellt eine große Bereicherung für das Autophagie-Forschungsfeld dar. Die Entdeckung, dass Cargo-Ubiquitinierung und hATG8-Lipidierung nur einen geringen Einfluss haben erfordert die weitere Untersuchung von alternativen Mechanismen und Signalen die zum Abbau führen. Außerdem hat meine Studie zur Identifizierung des ersten Rezeptors für endosomale Mikroautophagie, TOLLIP, geführt, welcher dessen Cargo-Kandidaten entlang des endosomalen Systems an die Lysosomen liefert. Schließlich haben meine nachbarschafts-proteomischen Daten dazu beigetragen, neue Funktionen der ATG4s zu etablieren, wodurch diese bereits mit der frühen Autophagosomen-Bildung assoziiert werden können.

Summary

Autophagy is an important process for maintaining cellular integrity by disposing toxic materials including invading pathogens, defective organelles such as mitochondria and misfolded or aggregated proteins. This diversity requires a specific cellular machinery for proper recognition of the different cargo. Autophagy is active under basal conditions in most cells but can be dramatically increased by cell-intrinsic or -extrinsic cues e.g. by amino acid deprivation (starvation). Autophagosome formation is tightly regulated and involves a ubiquitin-like conjugation machinery whose activation leads to the covalent attachment of hATG8 protein family members (GABARAPs and MAP1LC3s) to PE (phosphatidylethanolamine) which is incorporated into both sides of the forming autophagosomes. As a prerequisite of this lipidation, the family of ATG4 proteases (ATG4A-D) are required to expose the C-terminal glycine residues of the hATG8s. Lipidated hATG8s mediate cargo engulfment by binding to selective autophagy receptors and promote maturation of autophagosomes and their fusion with lysosomes. Ultimately, autophagy cargo is degraded by lysosomal hydrolases.

Selective autophagy receptors are manifold and their involvement is highly dependent on the cargo type. One group of structurally similar receptors encompasses the SQSTM1-like receptors (SLRs) which include the founding member SQSTM1 (also known as p62) as well as NBR1, NDP52, OPTN, TAX1BP1 and TOLLIP. With the exception of NDP52, all of them have been implicated in the disposal of aggregated proteins but understanding of their specific functional roles remained limited. In this study, I employed APEX2 driven proximity proteomics with differentially limited proteolysis to elucidate autophagosomal content in a SLR-specific manner in a number of different experimental settings. This approach led to the identification of more than 250 potential cargo proteins that are engulfed in autophagosomes under basal housekeeping conditions and which differed considerably from those proteins found in the neighborhood of cytosolic SLRs. While this data set contained a number of receptor-specific substrates, the majority of cargo candidates were identified by at least two receptors, indicating potential redundancy. Intriguingly, numerous cargo candidates fell into functional categories (e.g. GTPases) that have not been described to be degraded by autophagy before. Through complementary biochemical, image based and organellar proteomic approaches I exhaustively validated a large number of these cargo candidates as *bona fide* autophagy substrates. In a series of mechanistic experiments, I demonstrated that TOLLIP and its cargo proteins are engulfed by endosomal membranes and subsequently delivered to lysosomes. While this process is known as endosomal microautophagy, TOLLIP is the first receptor of this alternative lysosomal

delivery route. Using autophagosome content profiling, I found that inhibiting ubiquitination and hATG8 lipidation did not affect most of the cargo candidates found under basal conditions. This was very surprising since these two post-translational modifications are thought to be crucial for recognition and tethering of cargo to nascent autophagosomes by receptors. This finding raises a number of questions and challenges our current mechanistic view of selective autophagy. Lastly, I employed our proximity proteomics approach to monitor alterations in the autophagy degradome in response to a build-up of cytosolic aggregates by three different means. This large-scale analysis unexpectedly revealed that the majority of cargo candidates identified under basal, fed conditions was not changed upon aggregate induction. Instead, a number of additional proteins were captured in autophagosomes in these conditions which showed SLR-specific differences (**Publication I**).

In a collaborative effort, we sought to elucidate the role of the ATG4 proteases in hATG8 processing in the context of selective autophagy of damaged mitochondria (mitophagy). By processing and analyzing proximity proteomics samples of APEX2-ATG4A-D expressing cells subjected to mitophagy induction and biotinylation I identified ATG4 family member specific proximity partners. With the exception of ATG4C, the family members showed overlapping but also distinct candidate neighborhood proteins. Closer inspection led to the identification of proteins associated with early autophagosome formation. Among these were two regulators of trafficking of ATG9A, which is a transmembrane protein residing on vesicles that are recruited to the autophagosome formation site. This led to the identification of a direct and crucial interaction between ATG4A and ATG9A vesicles, whereby the latter were recruited to damaged mitochondria upon mitophagy stimulation. Moreover, using combinatorial knockouts of ATG4 and ATG8 family members, a novel role of ATG4 proteins in promoting the formation of early autophagosomes was discovered. Interestingly, this was independent of their proteolytic activity towards hATG8s (**Publication II**).

Collectively, my work provides a comprehensive resource of new autophagy cargo and regulatory factors and represents a major contribution to the autophagy field. The overall surprisingly small impact of cargo ubiquitination or hATG8 lipidation calls for closer inspection of other targeting mechanisms and signals. In addition, my study led to the identification of the first endosomal microautophagy receptor, TOLLIP, which delivers its cargo candidates to lysosomes via the endosomal system. Finally, my neighborhood proteomics data help to establish novel functions of the ATG4 proteins, placing them at the early stage of autophagosome formation.

1. Introduction

1.1. Cellular degradation pathways

The maintenance of cellular homeostasis upon stress conditions such as infection, nutrient starvation or proteotoxic stressors is pivotal to the cell's health and survival. The cell has two major solutions to degrade unwanted cytosolic material, namely proteasomal degradation and autophagosomal-lysosomal degradation (**Figure 1**). Degradation by the proteasome is mainly driven by (poly-) Ub (Ubiquitin) conjugation to a substrate and its subsequent degradation in the 26S proteasome and is referred to as the UPS (Ub-proteasome system) (Thrower et al., 2000). It represents the main degradation pathway for the clearance of short-lived and regulatory proteins as well as damaged and misfolded proteins. However, also ubiquitin-independent proteasomal degradation has been reported (Erales and Coffino, 2014).

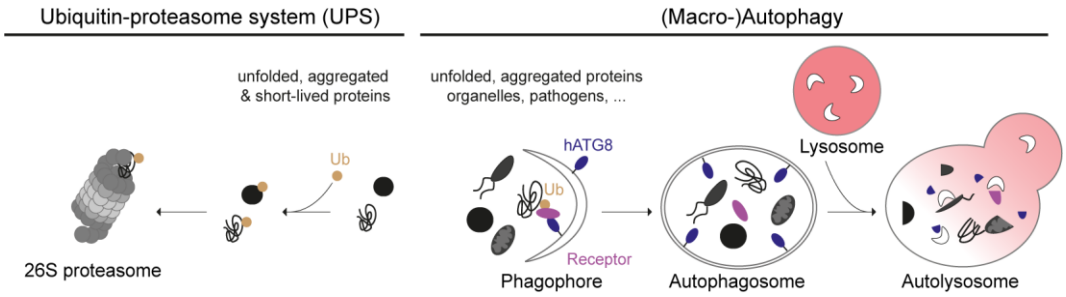

Figure 1: Overview of degradation pathways.
Defective cellular material can be degraded via two major pathways. Either by ubiquitin (Ub)-mediated degradation in the proteasome (left) or by autophagy (right).

The alternative is autophagy, which was long considered a non-selective bulk degradation pathway that enclosed cytoplasm for nutrient recycling in autophagosomes. In the past decade, autophagy has also been shown to occur in a selective manner wherein specific receptors mediate cargo delivery to lysosomes. Similarly to the UPS, autophagy targets damaged or aggregated proteins. In addition, autophagy is also involved in the clearance of whole organelles such as mitochondria, other cytosolic constituents including ribosomes or invading pathogens. There are at least three different autophagy pathways - macroautophagy, microautophagy and chaperone-mediated autophagy - that all lead to cargo degradation by the lysosome. Of these pathways, macroautophagy is the most prominent and best characterized one. The fact that the proteasome itself can be targeted to lysosomal degradation (Cuervo et al., 1995) and that autophagy is induced upon proteasomal inhibition (Iwata et al., 2005; Pandey et al., 2007; Rideout et al., 2004) imply compensatory mechanisms between both processes and underscore the importance of a functional degradation system for the cell's survival.

1.1.1. The ubiquitin-proteasome system (UPS)

Ub is a 76 aa (amino acids) long abundant protein prevalent in the nucleus and cytoplasm. It is tightly packed and has an extremely conserved structure differing only in three amino acids between yeast and human forms (Jentsch et al., 1991; Ozkaynak et al., 1984). Ub either exists in a free form or can get covalently attached as post-translational modification to lysine residues of proteins, a process referred to as ubiquitination, which represents a versatile signal that can govern the fate of proteins in several different ways (Jentsch, 1992). One example is the targeting of ubiquitinated proteins such as cell-cycle proteins like the cyclins or other regulatory proteins for proteasomal degradation. Ub conjugation involves a multi-step enzymatic cascade. First, an Ub-activating E1 enzyme catalyzes the (ATP-dependent) binding of Ub to a cysteine residue of the E1 enzyme. Ub is then transferred to a Ub-conjugating E2 enzyme before it gets covalently attached to a target protein with the help of a Ub E3 ligase (Jentsch, 1992) (**Figure 2**).

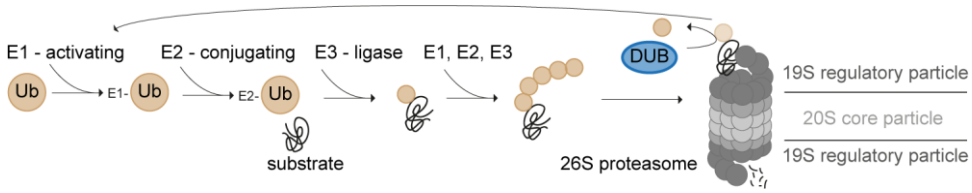

Figure 2: Ubiquitin conjugation to substrates leads to proteasomal degradation.
Ubiquitin is attached to a substrate by the consecutive actions of three enzymes leading to poly-ubiquitinated substrates that are attached to the proteasome. Deubiquitination enzymes (DUBs) remove Ub and the cargo gets proteolysed.

Humans have two E1 enzymes, UBA1 and UBA6 (Ub-like modifier-activating enzyme), around 40 E2 enzymes and several hundred E3 enzymes, thereby achieving selectivity in mediating Ub conjugations (van Wijk et al., 2009). Attachment of one (or several) individual Ub molecule(s) to a substrate is called (multi)mono-ubiquitination. However, each Ub moiety can in principle be subjected to ubiquitination on one or several of its seven lysine and N-terminal methionine residues. This leads to the formation of homo- and heterotypic polyUb chains. Lysine (K) 48 and K63 Ub chains represent the best characterized linkages. The targeting of proteins for hydrolysis in the proteasome is mostly mediated by ubiquitination of K48 but the ubiquitination of other Ub lysine residues (e.g. K11) also contributes to proteasomal degradation (Finley, 2009). Ubiquitination can also drive other cellular responses such as signal transduction, endomembrane trafficking or the DNA damage response (Husnjak and Dikic, 2012). However, here K63 linked ubiquitin chains play a prominent role.

The eukaryotic 26S proteasome consists of a 20S core particle with 28 subdomains and one or two 19S regulatory particles that mediate unfolding and ATP-dependent translocation of substrates into the catalytic chamber of the 20S core. Ubiquitinated substrates are recognized by Ub receptors residing on the regulatory particle and mediate binding of the substrate before Ub is removed by DUBs (deubiquitination enzymes) (Rackova and Csekes, 2020). Furthermore, shuttling factors that harbor UBA (Ub-associated) or UBL (Ub-like) domains facilitate the targeting of substrates to the proteasome. As such, UBQLN2 (Ubiquilin-2) reversibly binds aggregated proteins via the UBA domain and directly attaches them to the proteasome through interaction with its UBL domain (Hjerpe et al., 2016). Other polyubiquitinated and membrane embedded substrates might require the action of VCP (Valosin-containing protein) in complex with UFD1 (Ub fusion degradation protein 1) and NPLOC4 (Nuclear protein localization protein 4 homolog) before they can get processed by the proteasome (Sato et al., 2019). Following proteolysis, short peptides are released into the cytosol.

1.1.2. Macroautophagy

Macroautophagy, hereafter referred to as autophagy for simplicity, is an evolutionarily conserved process that is tightly regulated by multiple ATG (autophagy)-related proteins through Ubl (Ub-like) conjugation and phosphorylation events. The basic understanding of this pathway stems from research performed in yeast but are conserved in higher eukaryotes (Mizushima et al., 2011). Upon autophagy initiation in mammalian cells, a phagophore is nucleated from pre-existing endomembrane structures such as the ER (endoplasmic reticulum). Expansion of the double-membrane structure around the cargo leads to the formation of an autophagosome which is transported to the endolysosomal compartment for subsequent fusion. Captured autophagic content is processed by various acidic hydrolases before the degraded products are eventually transported back into the cytosol for reuse (Li et al., 2021).

1.1.2.1. Induction of Autophagy

The rate of housekeeping autophagy can be very low under basal conditions with differences across distinct cell types. While autophagy induction tends to be better understood for bulk than selective autophagy, the majority of principles might hold true for both pathways. However, a number of additional mechanisms are in place for selective autophagy. Autophagy initiation is triggered by activation of the ULK1 (unc-51-like kinase 1) complex (Mizushima, 2010). This complex consists of the serine/-threonine kinase ULK1 which assembles with the scaffold protein FIP200 (FAK family kinase-

interacting protein of 200 kDa; also known as RB1CC1), ATG13 and ATG101. Nutrient or energy starvation leads to the upregulation of AMPK (AMP-activated protein kinase) or the dissociation of the inhibitory kinase mTORC1 (mammalian target of Rapamycin complex) from the ULK1 complex thereby enhancing autophagy (Hurley and Young, 2017) (**Figure 3**). Besides ULK1, another member of the ULK1 family, namely ULK2 also seems to play a role in autophagy initiation but its function is less well understood. Activation leads to phosphorylation of ULK1 and its regulatory subunits ATG13 and FIP200 (Ganley et al., 2009; Jung et al., 2009). The formation and nucleation of the phagophore, the early membrane building the autophagosome, relies on two downstream ULK1-mediated phosphorylation events. One is the relocation of vesicles containing the transmembrane protein ATG9A from the Golgi to the phagophore assembly site to potentially provide membrane material (Yamamoto et al., 2012; Young et al., 2006). The other is the generation of PI3P (phosphatidylinositol 3-phosphate) by the PI3KC3 (class III phosphatidylinositol 3-kinase)-C1 complex (Volinia et al., 1995). The latter consists of VPS34 (vacuolar protein sorting), VPS15, BECN1 (Beclin-1) and ATG14L subunits which have catalytic, scaffolding, regulatory and phagophore-targeting functions, respectively (Ma et al., 2017).

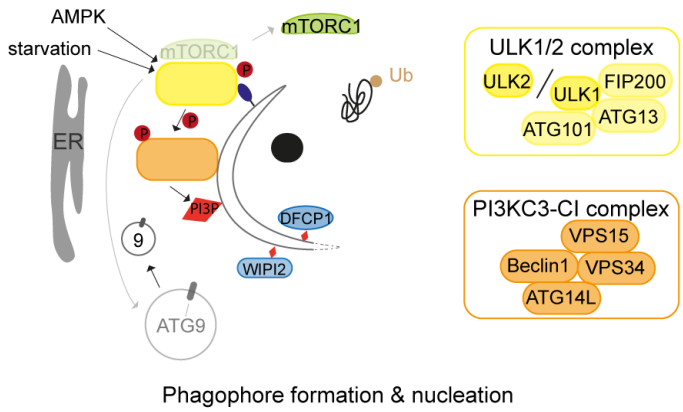

Figure 3: Initiation of autophagy.
Starvation or AMPK regulation induce autophagy. This leads to the formation of a phagophore involving multiple phosphorylation events in order to activate ULK1 and PI3KC3-CI complexes. ER, Endoplasmic reticulum; Ub, Ubiquitin.

This leads to the formation of the omegasome, a platform for the assembly of the phagophore at the ER or ER-mitochondria contact sites, and to the recruitment of downstream factors for autophagosome formation (Axe et al., 2008; Hamasaki et al., 2013). These include additional ATG proteins as well as the PI3P-binding proteins DFCP1 (double FYVE-containing protein 1) and WIPI2 (WD repeat domain phosphoinositide-interacting protein 2) which are drivers for phagophore nucleation and expansion (Axe et al., 2008; Polson et al., 2010).

1.1.2.2. Autophagosome membrane expansion and completion

Phagophore membrane expansion involves two Ubl protein conjugation systems (**Figure 4**). The first Ubl is part of the ATG12-ATG5-ATG16L1 complex which is recruited by WIPI2 binding to ATG16L1 and mediates lipidation of the second Ubl which comprises the human orthologs of the yeast protein Atg8 (hATG8) (Dooley et al., 2014). hATG8 proteins consist of two subfamilies, the GABARAPs (ɣ-aminobutyric acid receptor-associated proteins; including GABARAP, GABARAPL1, GABARAPL2) and the MAP1LC3s (microtubule-associated protein 1 light chain 3 proteins; including -LC3A, -LC3B, -LC3C) which display high sequence similarities. For LC3B, a second splice variant with only one amino acid difference (LC3B2) has been described but so far its functions remain elusive, opposed to LC3B which is the best characterized hATG8 protein to date (Shpilka et al., 2011). Therefore, it is often referred to a total of (at least) 6 hATG8 proteins. The function of a hATG8 family member depends on its post-translational modification. It exists in a free, unbound form in the cytosol (hATG8-I) but can be conjugated to the phospholipid PE (phosphatidylethanolamine) on the forming autophagosome to form hATG8-II (Slobodkin and Elazar, 2013).

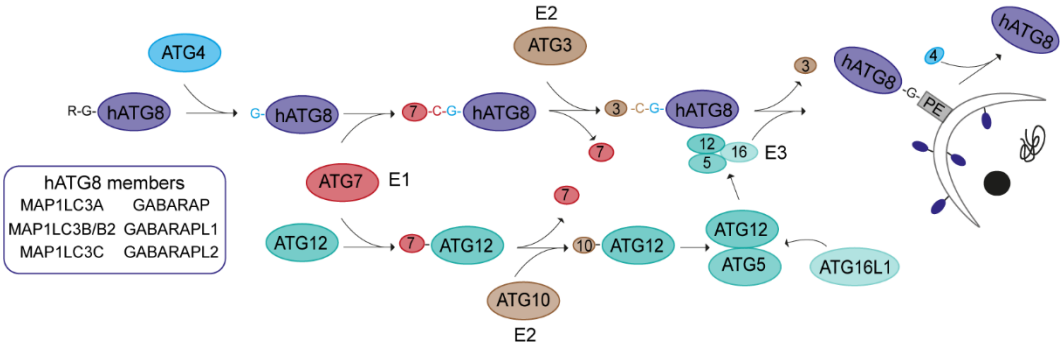

Figure 4: Two ubiquitin-like (Ubl) conjugation systems.
Higher eukaryotes have at least 6 hATG8 family members that can be bound to the forming autophagosome involving ubiquitin-like conjugation events in order to serve a variety of important functions for cargo engulfment, autophagosome maturation and fusion. PE, phosphatidylethanolamine.

Upstream of this process, the covalent binding of ATG12 to ATG5 is facilitated by the E1- and E2-like enzymes ATG7 and ATG10, respectively. Binding of this dimer to ATG16L1 mediates the covalent attachment of hATG8s to PE in a similar Ubl conjugation process. Thereto, the C-terminal glycine residue of hATG8 has to be exposed via cleavage by a member of the ATG4 cysteine protease family (Kirisako et al., 2000). hATG8-I is then activated by the formation of a thioester bond with the catalytic cysteine of ATG7 (E1), transferred to ATG3 (E2) and eventually conjugated to PE on the nascent autophagosomal membrane by the ATG5-ATG12-ATG16L1 complex (E3). The

formation of hATG8-PE conjugates is reversible by ATG4 (Ichimura et al., 2000; Kirisako et al., 2000; Mizushima et al., 2003). ATG4 is the only protease among the ATG proteins. In contrast to a single Atg4 protein in yeast, the ATG4 family in mammals comprises four members: ATG4A, ATG4B, ATG4C and ATG4D (Maruyama and Noda, 2017). Since they contribute to lipidation as well as delipidation of hATG8s, the activity of ATG4 proteins has to be tightly regulated to ensure functional autophagy. Consistent with this notion, post-translational modifications like phosphorylation or ubiquitination were shown to regulate the activity or degradation of ATG4B, respectively (Kuang et al., 2012; Pengo et al., 2017; Yang et al., 2015).

hATG8 lipidation has been used extensively for monitoring autophagosomal flux by assessing the levels of hATG8-II by immunoblotting or using it as a marker for autophagosomes by immunofluorescence microscopy. Once lipidated to either layers of the forming autophagosome (**Figure 5**), hATG8s serve multiple functions with different preferences of the LC3 and GABARAP subfamilies. On the one hand, they bind to LIRs (LC3-interacting regions) in autophagy receptors like p62 (also called SQSTM1 (Sequestosome-1)) to tether the latter to the growing autophagosome (Stolz et al., 2014) while on the other hand, hATG8s contribute to autophagic membrane elongation and closure as well as to the fusion of autophagosomes with lysosomes (Nguyen et al., 2016). Whereas proteins of the LC3 subfamily seem to be more important for phagophore elongation, the GABARAP subfamily is rather important for closure of the autophagic vesicle (Nguyen et al., 2016; Weidberg et al., 2010).

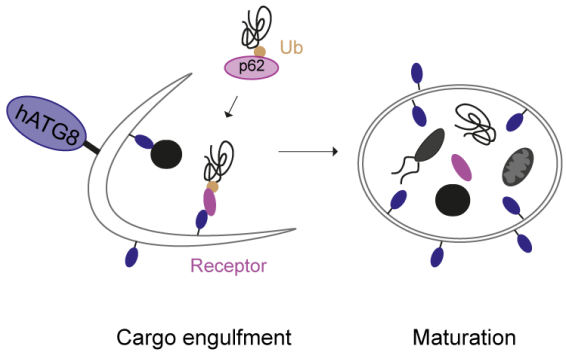

Figure 5: Autophagic vesicles form around the cargo.
Maturation and cargo engulfment require binding of hATG8 proteins to the forming autophagosomes involving two ubiquitin-like cascades (**Figure 4**). Cargo engulfment is mediated by direct binding to the hATG8 protein or mediated by a selective receptor. Ub, ubiquitin.

A key player in the final maturation of autophagosomes is the small GTPase RAB7 (Ras-related protein Rab7) which is also present on late endosomal membranes (Jäger et al., 2004). Rab GTPases cycle between two states, a cytosolic GDP-bound form (inactive) and a membrane-associated GTP-bound (active) form, with the help of GEFs (guanine-nucleotide exchange factors) and GAPs (GTPase-activating proteins). Thereby, Rab

proteins exert functions such as recruitment of downstream effector proteins, vesicle transport or membrane fusion (Wandinger-Ness and Zerial, 2014). In line with this, RAB7 and LC3 have been reported to interact with FYCO1 resulting in the transport of autophagic vesicles (Pankiv et al., 2010). Once the cargo is enclosed, an autophagosome matures into an autolysosome upon fusion with a LE (late endosome) or lysosome followed by the degradation of its content (**Figure 5**).

1.1.2.3. Autophagosome fusion with the lysosome

The fusion of an autophagosome and a lysosome requires a variety of factors like Rab GTPases, SNAREs (soluble N-ethylmaleimide-sensitive factor attachment receptors) and tethering factors (Kriegenburg et al., 2018) (**Figure 6**). Once the autophagosome is close to the LE or lysosomal membrane, tethering factors build a bridge between these two opposing membranes. One such factor is TECPR1 (tectonin beta-propeller repeat containing protein 1) which promotes fusion through its association with the previously described ATG5-ATG12 dimer (Chen et al., 2012). Another component of the fusion machinery is the HOPS (homotypic fusion and protein sorting) complex which interacts with RAB7 and PI3P. Once the HOPS complex is recruited, it enhances the SNARE protein assembly and also promotes membrane curvature (Kriegenburg et al., 2018).

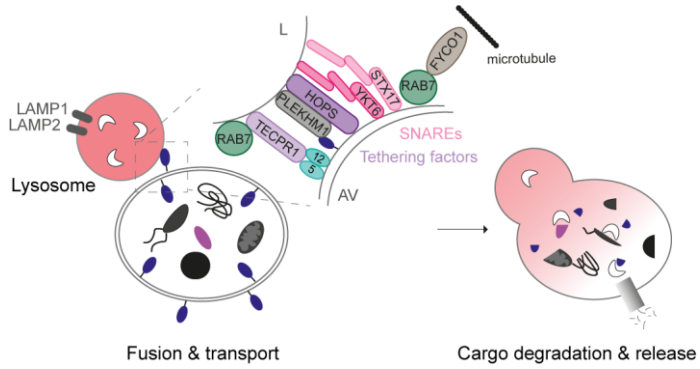

Figure 6: Proteins involved in autolysosome formation.
The mature autophagosome fuses eventually with a lysosome or late endosome in order to degrade and release its content into the cytosol. This fusion requires the RAB GTPase RAB7, as well as SNAREs and tethering factors. L, Lysosome; AV, Autophagic vesicle; 5: ATG5; 12: ATG12.

A direct interactor of the HOPS complex is PLEKHM1 (Pleckstrin homology domain-containing family member 1) that is bound to hATG8-PE proteins on the autophagosomal membrane via its LIR domain and promotes fusion (McEwan et al., 2015). The two SNAREs STX17 (syntaxin 17) and YKT6 have been shown to localize to the mature autophagosome independent of each other and form two separate SNARE complexes by interaction with two other SNAREs to mediate autophagosome-lysosome fusion (Matsui et al., 2018).

Once both membranes have fused, the content of the established autolysosome is degraded by acid hydrolases, among which the cathepsins mediate cleavage (Schulze et al., 2009). A variety of other enzymes take care of digesting other substances with the goal of releasing basic building blocks back to the cytosol for recycling. In order to protect the autolysosomal membrane itself from degradation, a group of proteins containing the lysosomal associated membrane proteins, LAMP1 and LAMP2, form a glycocalyx (Schulze et al., 2009). BafA1 (Bafilomycin A1) has been widely used as a tool to study autophagy due to its inhibitory function on lysosomal acidification which puts a halt on degradation (Mousavi et al., 2001; Yamamoto et al., 1998).

1.1.2.4. Canonical autophagy variations and non-canonical autophagy pathways

Recently, a number of variations of the described, so-called canonical autophagy pathway have been reported that operate without core parts of the autophagy machinery. An example is ATG7- and ATG5-independent autophagy, for which hATG8 lipidation is dispensable but that still requires the activities of ULK1 and BECN1 (Nishida et al., 2009). Similarly, ATG3- or ATG7-independent autophagy was observed in the fruit fly. Here, the Ub E1 enzyme UBA1 was required instead (Chang et al., 2013). Moreover, other pathways have been described in which autophagy initiation occurred independent of ULK1, ULK2 (Cheong et al., 2011) or BECN1 (Scarlatti et al., 2008), showing altogether that multiple steps of autophagy can get bypassed without compromising the functioning of autophagy.

Besides, two non-canonical autophagy pathways were identified that employ parts of the macroautophagy machinery, including LC3, but whose activities do not involve capturing of cytosolic constituents. The first pathway involves phagocytosis which is defined as the digestion of extracellular material through invagination of the plasma membrane and formation of phagosomes. LAP (LC3-associated phagocytosis) is triggered by receptor-mediated responses to pathogens or antigens and has mostly been described for macrophages (Martinez et al., 2015). During LAP, LC3 is conjugated to PE present in single-membrane phagosomes. This process requires the activity of ATG5 and ATG7 as well as the PI3KC3-CII complex, which is similar to PI3KC3-CI but harbors UVRAG instead of ATG14, for its induction. Similar to autophagosomes, LC3-decorated phagosomes are destined for fusion with lysosomes and degradation of their extracellular contents (Martinez et al., 2015). The second pathway is similar to endocytosis which describes a ubiquitous process for the uptake of extracellular or plasma membrane components to the cytoplasm. In LANDO (LC3-associated endocytosis), LC3 is conjugated to single-membrane endosomes that are positive for

RAB5 and clathrin. So far, the proposed function of LANDO seems to be the recycling of receptors. This pathway is also dependent on ATG5, ATG7 as well as the PI3KC3-CII complex but similar to LAP does not require ULK1 complex components (Heckmann et al., 2019; Jülg et al., 2020; Martinez et al., 2015).

1.1.3. Microautophagy

While multiple forms of microautophagy have been described (Oku and Sakai, 2018), the best understood one involves the internalization of cytosolic cargo by invagination of the lysosomal membrane. Thereby, soluble or membrane-bound material is enclosed in intralysosomal vesicles which are degraded by acidic hydrolases (Marzella et al., 1981) (**Figure 7**). This process is conserved from yeast to mammals and occurs either in a non-selective (bulk) or in a selective fashion (Tekirdag and Cuervo, 2018). While in yeast this process has been observed as invagination of the vacuole, in mammals it occurs in LEs or MVBs (multivesicular bodies) (Sahu et al., 2011). Enclosure of cytosolic cargo requires components of the ESCRT (endosomal sorting complexes required for transport) machinery which is also necessary for MVB formation. The selective internalization requires the interaction of the chaperone HSC70 (heat shock cognate of 71 kDa) and its co-chaperones with the limiting endosomal membrane. In this scenario, HSC70 specifically targets proteins harboring a KFERQ-like motif. The process of cargo degradation into LEs or MVBs was termed endosomal microautophagy, underlining that its molecular features overlap with endocytic and autophagic pathways (Sahu et al., 2011; Tekirdag and Cuervo, 2018).

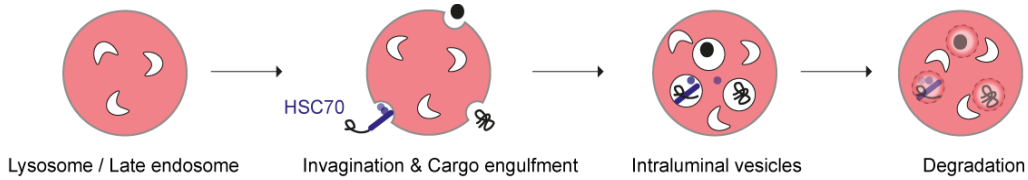

Figure 7: Overview of (endosomal) microautophagy.
During microautophagy cytosolic proteins are delivered to the vacuole (yeast) or LEs / MVBs (mammals) by invagination of their membranes. Intraluminal vesicles are formed and degraded. In a selective form of endosomal microautophagy the chaperone HSC70 can mediate cargo delivery and is thereby degraded alongside its cargo.

Only very recently, selective autophagy receptors have been described to play a role in endosomal microautophagy as well (Mejlvang et al., 2018). In their study, one hour of starvation induced rapid degradation of multiple selective autophagy receptors as well as the two hATG8 members LC3B and GABARAPL2 after their incorporation in LEs or MVBs. Similar to endosomal microautophagy, sorting into intraluminal vesicles required the two ESCRT machinery proteins CHMP4B (Charged multivesicular body protein 4b)

and VPS4 but contrastingly HSC70 was not necessary. Instead functional cholesterol trafficking was essential. Moreover, the authors found that lipidation of hATG8 members presumably onto endolysosomal membranes, was required for the degradation of selective autophagy receptors such as p62 (Mejlvang et al., 2018). Despite this observation, selective autophagy receptors are not known to mediate cargo delivery in this process, which is in contrast to their main function in selective autophagy. Another recent study described hATG8-conjugation dependent cargo loading into intraluminal vesicles of MVBs which ultimately enabled the unconventional secretion of these proteins (Leidal et al., 2020). This process however was exclusively dependent on CHMP4B but not on other ESCRT-machinery proteins. This and other findings led the authors to question whether this process is similar to endosomal microautophagy or other LC3-associated processes (e.g. LANDO) (Leidal et al., 2020). Together, these examples show that a number of endosomal and autophagosomal processes involve similar proteins while they retain some distinguishable mechanisms. However, we are only beginning to understand the interplay of these different degradation and sorting pathways.

1.1.4. Chaperone-mediated autophagy (CMA)

Similar to microautophagy, CMA is driven by the HSC70 chaperone system which recognizes the conserved KFERQ targeting motif in cytosolic substrate proteins (**Figure 8**). Upon binding of HSC70, proteins are unfolded and targeted to the lysosomal membrane protein LAMP2A which mediates their transport into the lysosomal lumen upon its multimerization (Kaushik and Cuervo, 2012).

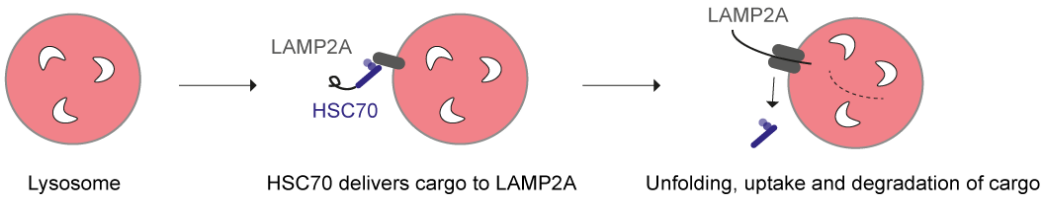

Figure 8: Overview of chaperone-mediated autophagy.
In CMA the chaperone HSC70 recognizes a KFERQ-motif in cargo proteins leading to their transport into the lysosome for degradation. HSC70 and co-chaperones mediate the binding to LAMP2A. Thereby the cargo is internalized in the lysosome while HSC70 dissociates.

CMA occurs under basal conditions but is similar to other autophagy pathways sensitive to cellular stressors such as proteostasis imbalance or starvation (Tekirdag and Cuervo, 2018). The targeting motif can vary but must contain at least one positively charged residue (K, R), hydrophobic residue (F, I, L, V), negatively charged residue (D, E) and one glutamine at either end of the motif (Dice, 1990). Besides, phosphorylation or acetylation can generate KFERQ-like motifs from putative motifs. In order to get detected

for degradation, the motif must be exposed to be accessible for HSC70 recognition. For some proteins, the KFERQ-like motif is hidden but becomes exposed upon cellular stress conditions, which enables a selective degradation of the protein (Kirchner et al., 2019).

1.1.5. The endo-lysosomal system

Many cellular processes such as hormone signaling, nutrient uptake, immune response, cell surface receptor recycling and endomembrane trafficking involve the endocytic pathway and thus its functionality is essential for a homeostatic cell (Gilleron et al., 2019; Gleeson, 2014). The endocytic system involves cycling of vesicles between the endomembrane compartments, which include the ER, the Golgi apparatus, endosomes and lysosomes and also cycling to and from the PM (plasma membrane) (Balderhaar and Ungermann, 2013) (**Figure 9**).

Endocytic vesicles containing internalized receptors and their cargo bud inward from the plasma membrane and fuse with EEs (early endosomes). Subsequently, the vesicles release their cargo in the EE and the receptor can be transported back to the PM (Langemeyer et al., 2018). The EE functions as a sorting platform for vesicles from different membrane compartments such as the PM or the Golgi. An important functional endocytic protein is RAB5 which resides on the membrane of endocytic vesicles and EEs and exerts its function as a small GTPase which includes the recruitment of effector proteins (Balderhaar and Ungermann, 2013). One such effector is the tethering protein EEA1 (early endosome antigen 1) which promotes the fusion between endocytic vesicles and EE (Christoforidis et al., 1999). The EE undergoes a morphology change to become a LE or MVB which is accompanied by a change in the GTPase from RAB5 to RAB7 (Langemeyer et al., 2018). Similar to its described role for autophagosome-lysosome fusion, RAB7 promotes fusion of LE/MVB with lysosomes involving the membrane tethering HOPS complex and the RAB7 interactor PLEKHM1 (McEwan et al., 2015; Vanlandingham and Ceresa, 2009). Owing to the switch between RAB proteins, RAB5 and RAB7 serve as biochemical markers to distinguish EEs from LEs, respectively.

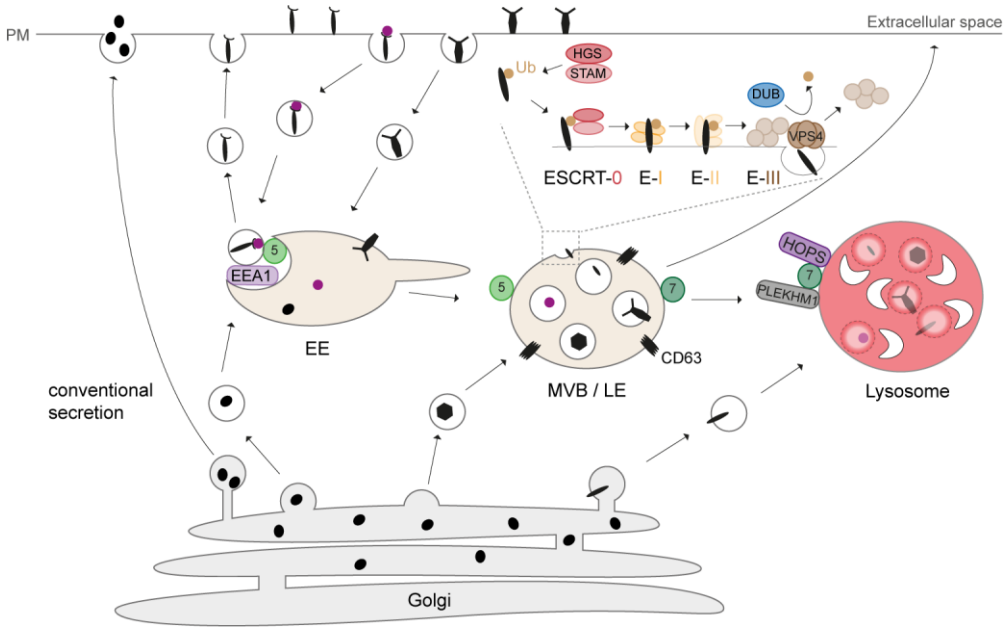

Figure 9: Vesicle transport routes in the endo-lysosomal system.
Proteins are transported through different stages of endosomes in order to be secreted or degraded by lysosomal hydrolases. Endocytic vesicles fuse with EEs that mature into LEs/MVBs. Sorting at MVBs is mediated by ubiquitination and sequential recruitment of the ESCRT machinery. PM, plasma membrane; EE, early endosome; MVB, multivesicular body; LE, late endosome; 5, RAB5; 7, RAB7; DUB, deubiquitination enzyme; Ub, ubiquitin.

The formation of MVBs requires a series of ESCRT complexes (0, I, II and III) that lead to the invagination of the limiting membrane to form the typical morphological structure that was observed already over 60 years ago (Palade, 1955) (**Figure 9**). However, a detailed mechanistic understanding of this process was only possible with the identification of multiple MVB-associated VPSs approximately 45 years later (Babst et al., 2002a; Babst et al., 2002b; Katzmann et al., 2001; Katzmann et al., 2003). Ubiquitinated substrates are recognized by soluble receptors like TOM1 (target of Myb protein 1) and TOLLIP (Toll-interacting protein) or the ESCRT-0 complex consisting of HGS (hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (signal transducing adapter molecule 1). All of these have domains for Ub-binding and interact with clathrin which coats transport vesicles (Katoh et al., 2006; Takahashi et al., 2015). ESCRT-I and ESCRT-II are sequentially recruited while the ubiquitinated cargo, including PM receptors or other cargo proteins, is passed on by the different complexes after being dispatched from the receptors (Hurley, 2010). At last, ESCRT-III is recruited containing the polymeric ATPase VPS4. This results in the disassembly of the complex proteins and invagination of the membrane containing the cargo in order to form intraluminal vesicles. Notably, the substrate is deubiquitinated with the purpose of recycling Ub prior to the inward budding. MVBs can then either deliver their cargo to lysosomes or secrete their content instead (Piper and Katzmann, 2007). Given its

presence on the MVBs as well as on LEs, the tetraspanin CD63 is considered as a marker for these compartments (Kobayashi et al., 2000) (**Figure 9**).

1.2. Selective autophagy

1.2.1. Selective autophagy pathways

Since the identification of the first mammalian selective autophagy receptor, a variety of soluble and transmembrane proteins were discovered that serve a similar purpose. The founding member of this protein category, p62, was reported to bind noncovalently to polyubiquitinated proteins (Vadlamudi et al., 1996) and to interact with hATG8 proteins on forming autophagosomes via its LIR domain to promote the degradation of aggregated proteins (Bjorkoy et al., 2005; Pankiv et al., 2007). Many of the identified selective receptors share these two key molecular features: a Ub-binding entity and an AIM (ATG8 family-interacting motif), also referred to as LIR. Due to their structural and functional similarity with p62 (alias SQSTM1), NBR1 (Next to BRCA1 gene 1 protein), NDP52 (Nuclear dot protein 52, also known as CALCOCO2 (Calcium-binding and coiled-coil domain-containing protein 2)), OPTN (Optineurin), TAX1BP1 (Tax1-binding protein 1) and TOLLIP are referred to as SLRs (SQSTM1-like receptors) (Deretic, 2012). Many of the SLRs are implicated in multiple selective autophagy pathways but their redundancy remains largely unexplored yet.

In contrast to starvation induced bulk autophagy, selective autophagy starts with the recognition of cargo by one or several distinct receptors. The emerging view is that cargo receptors recruit the autophagy machinery to initiate autophagosome formation in proximity of the bound cargo. Consistent with this notion, NDP52 has been shown to recruit the ULK1 complex to ubiquitinated cargo, leading to ULK1 kinase activation (Vargas et al., 2019). Moreover, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) KO (knockout) of five SLRs hindered ULK1 complex and hATG8 recruitment to defective mitochondria (Lazarou et al., 2015). Similarly, TAX1BP1 and p62 were reported to directly interact with the ULK1 complex component FIP200 (Ravenhill et al., 2019; Turco et al., 2019).

Multiple selective autophagy pathways were described and named after the cargo that is targeted for degradation. The most prominently studied ones are the degradation of mitochondria (mitophagy), ER-proteins (ER-phagy or reticulophagy) and aggregated proteins (aggrephagy) which are described in more detail below. Additional selective autophagy pathways include invading pathogens (xenophagy), ribosomes (ribophagy), lysosomes (lysophagy), lipid droplets (lipophagy), glycogen (glycophagy), peroxisomes

(pexophagy), ferritin (ferritinophagy) and parts of the nucleus (nucleophagy). For most of them, at least one receptor has been identified to mediate specific cargo engulfment by autophagosomal membranes (**Figure 10**) (Li et al., 2021).

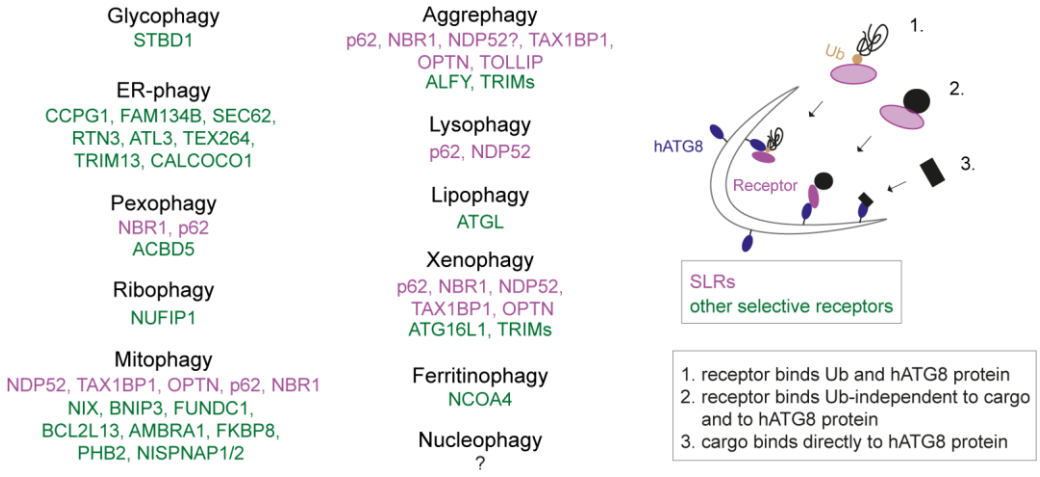

Figure 10: Overview of known receptors in selective autophagy pathways.
SLRs and other receptors are assigned to distinct selective autophagy pathways. Three mechanisms for cargo targeting to the autophagic membrane are depicted. Ub, ubiquitin.

Besides the binding of the receptors through their Ub-binding entities, cargo can also be recognized in an Ub-independent fashion by direct binding of the receptor (Levine and Kroemer, 2019; Padman et al., 2019) or potentially through other degradation signals that have yet to be uncovered. A third possibility of engulfment is the ability of cargo to directly interact with hATG8-II on the membrane of forming autophagosomes (**Figure 10**). How exactly SLRs mediate cargo engulfment is summarized in the following chapters.

1.2.1.1. Mitophagy

Maintaining intact mitochondria is essential for cellular homeostasis as they are important for ATP production and furthermore serve functions in calcium homeostasis, immune signaling and cell death (Chen et al., 2020; Pickles et al., 2018). Mitochondria are highly dynamic as they undergo constant fission and fusion events. Therefore, multiple mechanisms have evolved to turnover old, damaged or surplus mitochondria in order to retain a functional organelle population. In general, mitophagy is divided into two major pathways which are dependent on or independent of the involvement of PINK1 (PTEN-induced putative kinase protein 1) and PARKIN (E3 ubiquitin-protein ligase parkin) (Palikaras et al., 2018). In healthy mitochondria, PINK1 localizes to the IMM (inner mitochondrial membrane). Upon loss of the mitochondrial membrane potential after mitochondrial damage, PINK1 is no longer imported but accumulates on the OMM (outer mitochondrial membrane) where it gets activated by auto-phosphorylation

(Palikaras et al., 2018; Pickles et al., 2018). Subsequently, PINK1 phosphorylates ubiquitin and the cytosolic E3 Ub ligase PARKIN which in turn conjugates phosphorylated ubiquitin to multiple substrates on the OMM (Li et al., 2021). This ubiquitin signal is recognized by selective autophagy receptors which recruit components of the autophagy machinery to initiate the growth of phagophore membranes around the damaged mitochondria. TAX1BP1, NDP52, OPTN and to a lesser extent NBR1 and p62 have been shown to localize to damaged mitochondria (Lazarou et al., 2015; Li et al., 2021). The serine/threonine kinase TBK1 (Tank binding kinase 1) phosphorylates OPTN, NDP52 and p62, thereby increasing their Ub binding affinities, and is essential for the recruitment of these receptors to damaged mitochondria. Conversely, TAX1BP1 recruitment is independent of TBK1 but requires PINK1 (Heo et al., 2015). The IMM protein PHB2 (Prohibitin-2) was also found to function in PARKIN-dependent mitophagy and mediate clearance of OMM damaged mitochondria by binding to LC3 (Wei et al., 2017). Other receptors implicated in mitophagy are the OMM proteins NIX (NIP3-like protein X), BNIP3 (BCL2 interacting protein 3), FUNDC1 (FUN14 domain-containing protein) and FKBP8 (FK506-binding protein 8) (**Figure 10**). They bind directly to hATG8-II on mitochondria-engulfing autophagosomal membranes (Palikaras et al., 2018). Remarkably, mitochondrial matrix proteins were also shown to promote mitophagy. Upon mitochondria depolarization, NIPSNAP1 and NIPSNAP2 accumulate on the OMM and recruit autophagy proteins, including the receptors NDP52, p62, NBR1 and TAX1BP1 as well as the adaptor ALFY (WD repeat and FYVE domain-containing protein 3) and preferentially members of the GABARAP subfamily of hATG8 proteins (Princely Abudu et al., 2019). Interestingly, besides the manifold proteins described to serve as mitophagy receptors, the phospholipid cardiolipin, which primarily resides at the IMM relocates to the OMM of damaged mitochondria and triggers mitophagy through interaction with LC3 (Chu et al., 2013). In summary, various proteins acting as mitophagy receptors ensure that mitochondria are effectively cleared to retain their functionality and prevent the buildup of damaging reactive oxygen species.

1.2.1.2. ER-phagy

The ER is a large heterogenous membrane-bound organelle comprised of the nuclear envelope and a peripheral network of tubules and sheets. It is involved in protein biosynthesis and secretion and plays roles in calcium storage, signal transduction, detoxification as well as lipid and hormone synthesis (Wilkinson, 2020). Due to its manifold functions and morphologically distinct domains, a number of mechanisms exist that tightly control ER integrity. Perturbations of proteostasis due to the accumulation of

misfolded proteins or an excessive amount of proteins in the ER induce ER stress and trigger a signaling cascade and transcriptional program called the UPR (unfolded protein response), which leads to upregulation of a large fraction of the proteostasis machinery, thereby increasing the cellular capacity for protein folding, secretion and degradation (Walter and Ron, 2011). Clearance of misfolded ER proteins is mediated by ERAD (ER-associated degradation) which involves retrotranslocation of proteins out of the ER with the help of SEC61 and the AAA+ ATPase p97 (also known as VCP) and their subsequent targeting to the proteasome for degradation (Smith et al., 2011). Aggregated proteins in the ER are typically not cleared by ERAD but rather by autophagic degradation of parts of the ER (Grumati et al., 2018). So far, nine receptors have been identified to mediate ER-phagy (**Figure 10**). These receptors are either membrane proteins such as RTN3 (Reticulon 3), FAM134B (alias Reticulophagy regulator 1, RETREG1), SEC62 (Translocation protein 62), CCPG1 (Cell cycle progression protein 1), ATL3 (Atlastin 3), TEX264 (Testis-expressed protein 264) and TRIM13 (Tripartite motif containing 13) or soluble, cytosolic proteins such as CALCOCO1 (Nthiga et al., 2020). CALCOCO1 binds to GABARAP proteins via its LIR domain and a second UIM (ubiquitin-interaction motif) and simultaneously interacts with the ER membrane tethers VAPA and VAPB (Vesicle-associated membrane protein-associated protein A / B) (Nthiga et al., 2020). CCPG1 also binds hATG8 proteins in a LIR-dependent manner and can bind to FIP200 in order to mediate ER-phagy for aggregated or misfolded ER luminal proteins (Smith et al., 2018). All of the other ER-phagy receptors, except TRIM13, share the ability to interact with various hATG8s via LIRs in order to recruit the phagophore while ATL3 selectively binds GABARAP subfamily members (Li et al., 2021). Interestingly, compared to other selective autophagy pathways such as mitophagy or xenophagy, ubiquitination is not required for most ER-phagy processes. The only exception so far is the E3 ligase TRIM13 which ubiquitinates itself and other proteins. Ubiquitinated TRIM13 is bound by p62 which in turn binds hATG8 proteins. In parallel, TRIM13 also recruits the VPS34-BECN1 complex to promote phagophore nucleation (Ji et al., 2019).

1.2.1.3. Aggrephagy

The native state of proteins can be compromised by a number of factors including faulty translations, aberrant folding, altered post-translational modifications and oxidative damage. Molecular chaperones (also known as heat shock proteins) can shield and rescue proteins from these conditions but if this is not possible, misfolded proteins are ultimately sent for degradation. Concentrating individual aggregation-prone proteins in a larger protein inclusion, termed aggresome, is thought to be an intermittent defense

mechanism since the latter seems to be less toxic to the cell than multiple smaller aggregates (Johnston et al., 1998; Lamark and Johansen, 2012). However, uncleared aggregates are a major pathological hallmark of many neurodegenerative disorders. In AD (Alzheimer's disease), besides the loss of synapses, hyperphosphorylated tau forms intracellular neurofibrillary tangles and amyloid-β accumulates extracellularly in plaques (Plaza-Zabala et al., 2017). These manifestations of a failed proteostasis are accompanied by the inability to transport and acidify autolysosomes (Lee et al., 2010; Nixon et al., 2005). Mutations in presenilin-1, causative for an early-onset form of AD, also contribute to decreased autophagic flux by impairing lysosomal degradation (Menzies et al., 2017). Up-regulation of autophagy has shown to decrease the deposition of amyloid-β plaques, suggesting that disease pathology and autophagy are closely connected (Spilman et al., 2010; Tian et al., 2011).

PD (Parkinson's disease) is characterized by the loss of dopaminergic neurons and the formation of cytoplasmic α-synuclein inclusions called Lewy bodies which are indicative of defective cellular degradation pathways (Choi et al., 2013). Consistent with this notion, an accumulation of autophagosomes has been observed in brain samples from PD patients (Anglade et al., 1997; Dehay et al., 2010). The fact that mutations in PINK1 and PARKIN are linked to familiar forms of PD (Choi et al., 2013) gave rise to the hypothesis that aberrant mitophagy contributes to the pathophysiology of PD. Similarly, in HD (Huntington's disease) cytoplasmic aggregates are formed. They result from mutant huntingtin carrying an aggregation-prone polyglutamine extension (polyQ) and were shown to negatively interfere with autophagy cargo recognition (Martinez-Vicente et al., 2010). Moreover, polyQ aggregates sequester key autophagy proteins such as BECN1, thereby disrupting autophagy (Shibata et al., 2006). In ALS (amyotrophic lateral sclerosis), mutations in several risk genes encoding VCP, OPTN, *C9orf72*, TBK1, UBQLN2 and p62 as well as the accumulation of cytosolic aggregates and autophagosomes highlight a potential role of defective autophagy in disease onset or progression (Vicencio et al., 2020).

The majority of these diseases share the labeling of protein inclusions with Ub which can be recognized and targeted by selective autophagy receptors. Among them, p62 and NBR1 are the best understood receptors mediating aggregate clearance (Kirkin et al., 2009; Pankiv et al., 2007). More recently OPTN, TOLLIP and TAX1BP1 have also been associated with aggrephagy (Korac et al., 2013; Lu et al., 2014; Maruyama et al., 2010; Sarraf et al., 2020). Thus, almost all SLRs are involved in aggrephagy and detailed targeting mechanisms of each receptor will be introduced in the following chapter.

Besides these neurodegenerative diseases, failure of autophagy is implicated in a variety of other diseases including cancer (Guo and White, 2016; Jacob et al., 2017), inflammatory diseases (Netea-Maier et al., 2016), metabolic diseases and heart dysfunction (Wang et al., 2017). In addition, autophagy plays a role in aging where autophagy processes are reduced due to downregulation of autophagy genes (e.g. ATG5, ATG7 and BECN1) (Lipinski et al., 2010).

1.2.2. Selective autophagy receptors

1.2.2.1. Common characteristics

For tethering cargo to the forming autophagosome, all SLRs employ two important domains. A Ub-binding entity to bind ubiquitinated cargo and a LIR to interact with hATG8 proteins (**Figure 11**).

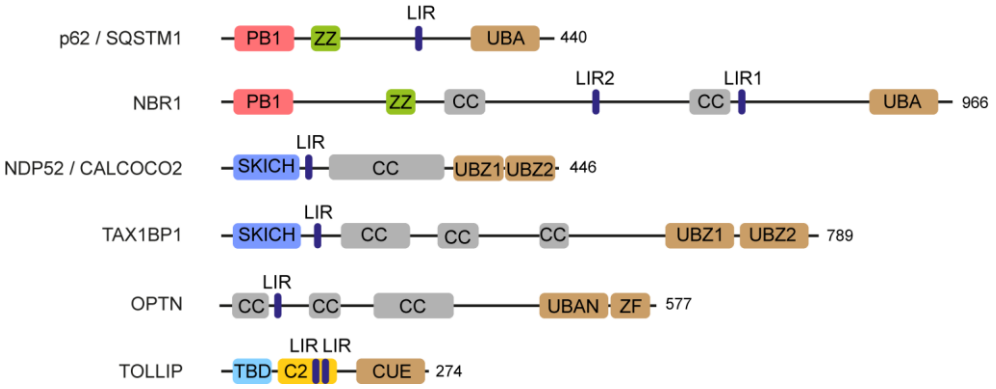

Figure 11: Schematic structures of SLRs.
Topology, length and important domains of selective autophagy receptors are represented. PB1, Phox and Bem1 domain; ZZ, ZZ-type zinc-finger domain; LIR, LC3-interaction region; UBA, Ub associated domain; CC, coiled-coil domain; SKICH, SKIP carboxyl homology domain; UBZ, Ub-binding zinc fingers, ZF, zinc finger domain; UBAN, Ub binding in ABIN and NEMO domain; TBD, TOM1-binding domain; C2, conserved 2 domain; CUE, coupling of ubiquitin to ER degradation.

LIRs have a consensus sequence of [W/F/Y]XX[L/V/I] wherein X stands for any amino acid (Birgisdottir et al., 2013). Amino acids preceding this core (e.g. Q, N or S) also contribute to hATG8 binding. The LDS (LIR-docking site) on hATG8 proteins consists of two hydrophobic pockets (HP1 and HP2) formed by the N-terminal helical extension and the UBL domain that are both important for the LDS-LIR interaction. N- and C-terminal regions flanking these pockets add to the binding specificity and affinity and contain indispensable amino acids for LIR binding in some cases (Johansen and Lamark, 2020). Notably, LIRs are also found in several other autophagy machinery components such as ULK1, ULK2, FIP200 and ATG13, though they do not always serve degradative purposes (Alemu et al., 2012).

Besides the LIR-LDS interaction, it was recently discovered that hATG8 proteins also possess a UDS (UIM docking site) that binds to Ub interacting motifs in cargo receptors (Marshall et al., 2019). Intriguingly, the ER-phagy receptor CALCOCO1 binds to the GABARAP family by employing LIR-LDS and UIM-UDS type of interactions (Nthiga et al., 2020). Another important feature of the SLRs is the ability for dimerization or multimerization which is conferred by several distinct domains that vary between the receptors. Multiple families of structurally diverse Ub-binding entities were described that bind noncovalently to Ub (Husnjak and Dikic, 2012). Different modes of Ub binding allow for preferences of Ub chain types or lengths and optimal conformation for the respective entity to increase binding affinity (Randles and Walters, 2012). The next chapters will give a more detailed overview of the six SLRs.

1.2.2.2. p62 / SQSTM1

The first described selective autophagy receptor was p62 (Bjorkoy et al., 2005; Komatsu et al., 2007; Pankiv et al., 2007), a protein that has previously been shown to bind Ub (Vadlamudi et al., 1996) and whose abundance was found to be sensitive to starvation and blockage of autophagy flux by BafA1 (Bjorkoy et al., 2005). Subsequent studies in ATG7 and p62 KO mice showed that p62 regulates the formation of larger, less toxic aggregates which are degraded by autophagy along with p62 (Komatsu et al., 2007). p62 also plays a role in the response to oxidative stress by activating NRF2, a protein cleared by the UPS under normal conditions but which is stabilized and activated by KEAP1 (Kelch-like ECH-associated protein 1) upon stress. Stabilization leads to transcription of cytoprotective enzymes which can also be triggered by p62, and to toxicity when autophagy is not functional. Moreover, it was shown that p62 also mediates KEAP1 degradation via autophagy also leading to the stabilization of NRF2 (Komatsu et al., 2010). Recent fluorescence correlation spectroscopy studies in unperturbed fed cells revealed that p62 distributes in two populations of either small oligomers or large structures, the latter of which might represent LC3-positive autophagosomes or protein aggregates (Zaffagnini et al., 2018). The PB1 (Phox and Bem1) domain at its N-terminus is necessary for polymerization which greatly enhances LC3 binding and drives autophagosomal engulfment of p62 bound cargo. Binding of ubiquitinated cargo is mediated by the UBA domain located at the C-terminus of p62 while its N-terminal Ub-binding ZZ (ZZ-type zinc finger) might regulate the oligomerization status of p62 (Zaffagnini et al., 2018) (**Figure 11**). The LIR of p62 was identified as a 22 aa long sequence able to bind LC3 and represents the first described LIR (Ichimura et al., 2008; Noda et al., 2008). Moreover, p62 was shown to directly interact with a C-terminal part

of FIP200 via a region surrounding the LIR domain of p62. Thereby, the autophagy initiation machinery is recruited to Ub-positive inclusions. This binding is mutually excluding the binding to LC3B which can outcompete FIP200 binding in order to promote autophagosome engulfment and closure (Turco et al., 2019). p62 is also able to form liquid-liquid phase separated condensates containing mobile ubiquitinated proteins as an alternative to the formation of aggregates containing misfolded proteins (Sun et al., 2018).

Besides its aforementioned roles, p62 is also involved in the degradation of peroxisomes (Kim et al., 2008) and bacteria (Cemma et al., 2011; Mostowy et al., 2011). Interestingly, in the latter, it was shown that clearance of different bacterial species involve distinct requirements of the selective receptors. In *Shigella flexneri* and *Listeria monocytogenes*, p62 and NDP52 have been reported to engage in their clearance dependent or independent of each other, respectively (Mostowy et al., 2011). Moreover, lysosomes that are damaged beyond repair can be degraded by autophagy. However, only NDP52 and p62 have been implicated in lysophagy so far (Koerver et al., 2019; Thurston et al., 2012).

1.2.2.3. NBR1

NBR1 has a similar domain structure as p62 but is more than twice its size (**Figure 11**). The N-terminal PB1 domains of p62 and NBR1 were shown to mediate direct binding between both receptors. NBR1 also binds Ub labeled proteins via its C-terminal UBA domain (Lamark et al., 2003). In contrast to p62, the PB1 domain of NBR1 does not support oligomerization. Instead the first CC (coiled-coil) domain of NBR1 is important for dimer formation. Although the LIRs differ between p62 and NBR1, both receptors bind to all hATG8 family members *in vitro* (Kirkin et al., 2009). NBR1 harbors a second LIR motif which mediates hATG8 binding *in vitro* but on its own is not sufficient for the interaction in yeast. Hence, its function remains hitherto unclear. NBR1 was shown to be recruited to p62 bodies, increased in abundance in cells that lack p62 or ATG7 and colocalized with Ub-positive protein aggregates (Kirkin et al., 2009). NBR1 has also been identified as a receptor for defective ribosomal products which accumulate upon autophagy inhibition whereas p62 was dispensable (Wenger et al., 2012). NBR1 has further been found to serve as an aggrephagy receptor in plants (Jung et al., 2020), highlighting its conserved role in mediating the disposal of aggregated proteins. In addition, NBR1 plays a role in pexophagy. Therein, NBR1 alone is sufficient for the clearing of peroxisomes but the clearing is enhanced by its binding to p62 (Deosaran et al., 2013). The role for NBR1 in mitophagy however is not clear since opposing findings

have been reported for NBR1's function in PARKIN mediated mitophagy (Gao et al., 2015; Shi et al., 2015). Similar to p62 and NDP52, NBR1 is also involved in xenophagy and enhances the recruitment of the other two xenophagy receptors to cytosolic *Shigella* (Mostowy et al., 2011).

1.2.2.4. OPTN

OPTN possesses three CC domains, a LIR domain for binding hATG8 family members and C-terminal UBAN (Ub binding in ABIN and NEMO domain) and ZF (zinc finger) domains that mediate Ub binding (Ryan and Tumbarello, 2018) (**Figure 11**). OPTN shows a preference to bind poly- rather than mono-Ub and can directly bind to the kinase TBK1. TBK1 mediated phosphorylation of the LIR and UBAN domains of OPTN results in increased activation of the receptor by enhancing its binding affinity for hATG8 proteins and Ub. Therefore, OPTN plays an important role in the degradation of cytosol invading bacteria (Wild et al., 2011) and defective mitochondria (Richter et al., 2016). OPTN is functionally redundant with NDP52 in mitophagy (Lazarou et al., 2015) while both receptors non-redundantly mediate xenophagy (Wild et al., 2011).

OPTN was shown to be important for the phagophore recruitment of the ATG5-ATG12-ATG16L1 E3 ligase scaffold required for hATG8 lipidation. Phagophores still formed upon OPTN KO in mice but reduced levels of lipidated hATG8 were observed (Bansal et al., 2018). During PINK1 / PARKIN dependent mitophagy, OPTN and NDP52 both translocate to the phagophore a second time to then recruit ULK1 complex components. Intriguingly, this second wave of OPTN and NDP52 recruitment amplified mitophagosome formation in a Ub-independent but LIR-dependent manner (Padman et al., 2019). Besides mediating the disposal of pathogens and depolarized mitochondria, OPTN also helps to clear protein aggregates. Here, recognition of aggregates is conferred by its C-terminal CC domain and is not dependent on Ub. Nevertheless, OPTN can bind to polyubiquitinated aggregates in p62 bodies through its UBAN domain (Korac et al., 2013). In addition to its role as selective autophagy receptor, OPTN contributes to other cellular functions such as endomembrane trafficking, transcriptional regulation and control of cell division (Korac et al., 2013).

1.2.2.5. NDP52

NDP52, also known as CALCOCO2, is related to TAX1BP1 (CALCOCO3) and CALCOCO1, all of which were identified as selective autophagy receptors. They share an N-terminal SKICH (SKIP carboxyl homology) domain followed by a conserved LIR motif and one or three CC regions. At the C-terminus, they harbor one (CALCOCO1) or

two (TAX1BP1, NDP52) zinc finger motifs for binding Ub (Nthiga et al., 2020) (**Figure 11**). The LIR motif is atypical since it lacks the first, aromatic residue that mediates binding to the HP1 pocket in the LDS of hATG8 family members. While NDP52 was found to bind solely to LC3C (von Muhlinen et al., 2012), it is not known whether this preference is conserved for TAX1BP1 or CALCOCO1. NDP52 has the largest CC region which is necessary for its dimerization. Dimerized NDP52 represents its most prevalent form and is important for LC3C binding (Kim et al., 2013). The SKICH domain seems to serve multiple functions. It is implicated in the disposal of accumulated phosphorylated tau protein associated with AD (Jo et al., 2014). Moreover, recruitment of FIP200 and TBK1 adaptors to bacteria is mediated via a region within the SKICH domain. Thereby, the ULK1 and TBK1 kinase complexes are recruited to nascent autophagosomes to promote xenophagy (Ravenhill et al., 2019). NDP52 protects cells against depolarized mitochondria and invading bacteria like *Salmonella* (Cemma et al., 2011; Lazarou et al., 2015; von Muhlinen et al., 2012). Intriguingly, this latter function is not only mediated by Ub but also involves an interaction with the lectin galectin-8, which senses endomembrane ruptures (Thurston et al., 2012). Consistent with this finding, NDP52 is also recruited to damaged endosomes (Falcon et al., 2018).

Besides its roles in xenophagy and mitophagy, NDP52 might mediate the clearance of protein aggregates due to its homology to the aggrephagy receptor TAX1BP1. However, this remains to be experimentally tested. Notably, NDP52 controls the degradation of the microRNA processor and effector proteins DICER and AGO2 (Gibbings et al., 2012). Possibly, the three homologs, NDP52, TAX1BP1 and CALCOCO1 have evolved to mediate distinct functions. These might also differ among species, since no functional NDP52 is found in mice or *Xenopus* whereas TAX1BP1 is conserved among all vertebrates (Tumbarello et al., 2015).

1.2.2.6. TAX1BP1

Originally, TAX1BP1 was described as a protein involved in immune responses and preventing apoptosis as well as being a transcriptional regulator, highlighting its multifaceted roles in the cell (Verstrepen et al., 2011). TAX1BP1 aides autophagosome maturation through its binding to Myosin VI with both C-terminal UBZ (Ub-binding zinc finger) domains of which only the second one is mediating Ub binding (Hu et al., 2018). Besides the structural features that are shared with NDP52, the SKICH domain of TAX1BP1 additionally mediates TBK1 adaptor binding whereby TBK1 is recruited and phosphorylates TAX1BP1 at multiple sites (Fu et al., 2018). This activation is indispensable for its role as a selective receptor in xenophagy and mitophagy. Compared

to NDP52, however, TAX1BP1 carries three shorter CC regions and does not harbor a galectin-8 binding site (**Figure 11**), indicating that both proteins potentially differ in their ability to detect invading pathogens and defective endosomes (Fu et al., 2018). TAX1PB1 is more abundant in human brain tissue and rat neurons than any of the other SLRs and consistent with a recently proposed role as a neuronal aggrephagy receptor, KO of TAX1BP1 in mice resulted in accumulation of Ub-positive inclusions (Sarraf et al., 2020). In addition, TAX1BP1 serves as adaptor for the turnover of ferritin by chaperoning the ferritinophagy receptor NCOA4 which is necessary for ferritin binding (Goodwin et al., 2017). Unexpectedly, this function of TAX1BP1 is independent of its LIR and hence hATG8 binding but requires ULK1, FIP200, VPS34 and ATG9A. Iron homeostasis is important for a number of cellular functions and iron excess is toxic to the cell. Ferritin can encage iron which can be released upon lysosomal degradation. While loss of FIP200 did not affect ferritin turnover, TBK1 was indispensable for it (Goodwin et al., 2017).

1.2.2.7. TOLLIP

TOLLIP has an N-terminal TBD (TOM1-binding domain) followed by a C2 (conserved 2) domain and a C-terminal CUE (coupling of Ub to ER degradation) domain (**Figure 11**). The C2 domain is responsible for binding to phospholipids such as PI3P but also harbors two LIRs (Lu et al., 2014). In addition, the C2 domain is also able to bind Ub and mediate oligomerization of TOLLIP (Mitra et al., 2013). In contrast to other SLRs, TOLLIP's role in selective autophagy pathways seems to be limited to aggrephagy. TOLLIP was identified to mediate the clearance of polyQ aggregates in a cellular model of HD (Lu et al., 2014; Oguro et al., 2011). Surprisingly, it did so with a higher affinity for polyubiquitin binding and greater clearance efficiency of soluble polyQ than p62. Consistently, the yeast homologue of TOLLIP, Cue5, was also shown to be an aggrephagy receptor targeting defective ubiquitinated proteins (Lu et al., 2014).

Besides its function in aggrephagy, TOLLIP is also implicated in other cellular pathways. For example, TOLLIP is a regulator of innate immune responses by associating with TOLL-like receptors that act upon binding to certain patterns of intruding pathogens (Capelluto, 2012). Moreover, TOLLIP is involved in endomembrane trafficking and associates with TOM1 to sort endosomal ubiquitinated transmembrane proteins (Katoh et al., 2004; Yamakami et al., 2003). In response to mitochondrial stress, TOLLIP directs mitochondrial-derived vesicles to lysosomes in a manner dependent on PARKIN, Ub and TOM1 (Ryan et al., 2020). KO of TOLLIP disrupted endolysosomal fusions in macrophages (Baker et al., 2015) and resulted in neuron death in mice deficient of the

AD risk factor APOE (Apolipoprotein E) (Chen et al., 2017). These mice showed accumulated autophagosomes, increased p62 and PARKIN levels as well as prominent amyloid-β and α-synuclein deposits (Chen et al., 2017). Furthermore, TOLLIP was found to be decreased in elderly humans and patients suffering from AD (Cribbs et al., 2012).

1.3. Proximity proteomic profiling

Over the past years, several methods have been developed to map the neighborhood of proteins in order to identify potential binding partners in a spatially resolved manner and thus advance our understanding of their biological functions. These techniques can be roughly divided into two classes which take advantage of either an engineered biotin ligase or peroxidase. Fusion of a protein of interest to these enzymes eventually results in the local biotinylation of surrounding proteins which then can be enriched by specific binding matrices and identified by mass spectrometry. This approach is therefore referred to as proximity proteomics. The developed methods differ from each other and both have advantages and disadvantages which will be addressed in the following chapter.

1.3.1. Proximity labeling methods

Proximity labeling using a mutant form of the *Escherichia coli* biotin ligase BirA is often referred to as BioID. It was first applied in mammalian cell culture. The enzymatic reaction requires biotin as substrate which is ATP-dependently catalyzed to an biotinyl-5'-AMP product (Lane et al., 1964) that is attached to proximal lysine residues in neighboring proteins (Roux et al., 2012). Fusion of the 35 kDa sized BirA to a protein of interest targets BirA to the respective localization of this bait and allows to biotinylate its proximity (Roux et al., 2012). The labeling radius has been mapped to approximately 10 nm (Kim et al., 2014).

The alternative approach involves an ascorbate peroxidase (i.e. APEX or its improved version APEX2) which catalyzes the generation of phenoxyl radicals that covalently modify electron-rich side chains of nearby proteins (20 nm radius) (Lam et al., 2015). Here, biotinylation is triggered by a 30 min incubation with the substrate biotin-phenol followed by a 1 min-pulse of H_2O_2 to induce peroxidase activity. The reaction is immediately stopped using radical quenchers. Due to a very short labeling time and short half-life of the radical (< 1 ms) APEX-based approaches provide a snapshot of dynamic protein neighborhoods (Rhee et al., 2013). In comparison, the BirA method, which requires labeling with biotin for at least several hours, is likely to capture more static

proximal proteins. Another advantage of the peroxidases over BirA and its derivatives is their compatibility with EM (electron microscopy). Here, catalyzation of the substrate DAB (3,3'-diaminobenzidine) upon enzymatic activation with H_2O_2 increases the contrast for EM when combined with electron-dense osmium staining (Martell et al., 2012). On the other hand, a disadvantage of the APEX methods is the use of H_2O_2 which can be toxic for certain cell types despite its short incubation time and relatively low dosage (1 mM) (Han et al., 2018). Another important consideration is the size of the enzyme tag which could influence cellular targeting of the fusion partner. The original BirA ligase has a molecular weight of 35 kDa compared to APEX2 with a size of 28 kDa and moreover, BirA fusions were reported to falsely localize. An improved version of BirA was reduced to only 27 kDa in size and showed more efficient labeling (Han et al., 2018; Kim et al., 2016). Further mutagenesis of BirA gave rise to two variants named TurboID and miniTurbo. Both rely on biotin as substrate but their labeling times were drastically decreased to 10 min, therewith making TurboID or miniTurbo mediated proximity labeling useful for capturing more transient protein neighborhood populations (Branon et al., 2018). To increase the spatiotemporal resolution, split versions of both BirA (Schopp et al., 2017) and APEX2 (Han et al., 2019) have been developed recently. They rely on the fusion of the N- or C-terminal fragments of the enzyme with different bait proteins, which only allow proximity labeling when both fragments are brought together due to the binding of both bait proteins.

1.3.2. Mass spectrometry as readout for proximity proteins

The most widely used method for the analysis of complex protein mixtures is LC (liquid chromatography) coupled to MS/MS (tandem mass spectrometry). This provides a high-resolution method for the identification and quantification of proteins. Since protein mixtures are very heterogeneous, it is necessary to reduce their complexity before samples can be processed via LC-MS/MS. Therefore, proteolytic enzymes like trypsin are used to digest proteins into defined peptides. Prior to digestion, proteins need to be denatured (e.g. by urea), their disulfide bonds reduced and the peptide amines alkylated. Following C18 solid-phase extraction purification, peptide mixtures are separated by a gradient of solvents on the LC and converted to a mist of charged droplets. This is facilitated by electrospray ionization as the mixture elutes from fine capillaries (Fenn et al., 1989). Ionized droplets are sucked into and guided through the mass spectrometer through high vacuum and masses of the ionized analytes are sorted by electric and magnetic fields. These separated ions are analyzed due to their mass to charge ratio and the ion current is amplified to be representable as mass spectrum (Pitt, 2009).

Different labeling techniques can be combined with MS. The most common ones include metabolic labeling methods like SILAC (stable isotope labeling by amino acids in cell culture) and chemical labeling methods such as TMT (tandem mass tag). SILAC describes the cultivation of cells in heavy or light amino acids in order to compare different conditions. After harvesting, cells are mixed in an equal ratio in order to process the samples together, thus reducing the overall number of samples. MS intensities of spectra are directly comparable but its main limitation is that only a limited number (3 - 5) of conditions can be compared. TMT labeling allows labeling of more samples (up to 16) and takes place after sample reduction, alkylation and digestion. One alternative to both labeling methods is the so called “label-free” processing of samples. Here, no time and cost intensive labeling is required. Through the implementation of algorithms for the detection of differences in “label-free” samples (label-free quantification (LFQ)), it has become a reliable method to detect robust changes between protein samples (Cox and Mann, 2011; Wong et al., 2017).

1.3.3. Autophagosome profiling

A number of approaches have been developed to monitor the contents of autophagosomes. Early studies used gradient centrifugation approaches to obtain mitochondrial-lysosomal fractions from rat liver that were further fractionated to purify autophagosomes. Ultrastructural analysis of this preparation indeed revealed autophagosomal structures containing cytosol, glycogen and less frequent also mitochondria and ER structures (Marzella et al., 1982). This technique was repeatedly adapted and applied for autophagosome purification from rodent hepatocytes in combination with autophagosome-enriching treatments (Koga et al., 2010; Stromhaug et al., 1998). Combining gradient centrifugation with SILAC proteomics identified the proteasome as autophagy cargo in a LC3-overexpressing breast cancer cell line grown in amino acid starvation and lysosomal degradation conditions (Dengjel et al., 2012). A similar approach performed in pancreatic and breast cancer cell lines compared inhibited autophagosome formation and blocked autophagosome degradation. Among the identified proteins were autophagy receptors, hATG8 family members and proteins involved in autophagosome-lysosome fusion. Importantly, this approach discovered the first and to date only ferritinophagy receptor NCOA4 (Mancias et al., 2014). An alternative approach relied on the overexpression of GFP-LC3 in HEK293 cells and capturing autophagosomes using immune isolation which was analyzed by MS (Gao et al., 2010).

The disadvantages of purifying autophagosomes using differential centrifugation methods lie in the long procedure that is prone to sample loss across multiple centrifugation rounds and the risk of contamination by other cellular compartments. Moreover this technique does not allow the differentiation of proteins residing in- or outside the autophagosome. To overcome these problems, autophagosome content profiling was established which takes advantage of APEX2 driven proximity proteomics. In this approach, APEX2 is directed to autophagosomes through fusions with hATG8 proteins. By combining proximity biotinylation in cells with proteinase K treatment of homogenates, this approach ensures that only proteins inside autophagosomes are captured since all cytosolic biotinylated proteins are digested. SILAC based proteomics of cells grown in the absence and presence of BafA1 which enriches autophagosomes led to the identification of cargo candidates in a hATG8 family specific manner (Le Guerroue et al., 2017).

1.4. Aim of the study

Autophagy is a major cellular degradation process. Due to its function in keeping the cell in balance with nutrient supply and disposal of potentially harmful substances, it plays important roles in many human diseases. While the autophagy machinery is well described at the level of autophagosome formation, maturation and fusion with the lysosome, far less is known about the cargo proteins that are selectively degraded by autophagy. My main PhD thesis project aims to close this challenging gap in our understanding by applying label-free APEX2 driven autophagosomal content profiling to identify cargo candidates in a SLR-specific manner under basal (fed) growth conditions. Moreover, these autophagy substrates are characterized using biochemical, cell biological and proteomics methods. Given that most of the SLRs have known functions in aggrephagy, I furthermore exploit this approach to determine whether the autophagosome content changes upon proteostasis imbalance.

In a tight collaboration with the group of Michael Lazarou (Monash University, Australia), I seek to characterize the group of cysteine proteases that is responsible for the reversible lipidation of hATG8s. So far, only very little is known about family member specific roles of ATG4 proteins during selective autophagy, their redundancy and potential tasks they might have other than hATG8 processing. To address these open questions, I employ APEX2 driven proximity biotinylation upon PINK1 / PARKIN mitophagy induction to identify the protein neighborhood of each ATG4 family member.

2. Publications and contributions

2.1. Publication I

Systematically defining selective autophagy

receptor-specific cargo

using autophagosome content profiling.

Published as:

Zellner, S., Schifferer, M. & Behrends, C. (2021). Systematically defining selective autophagy receptor-specific cargo using autophagosome content profiling. Molecular Cell, 81(6), 1337-1354 e1338. doi: 10.1016/j.molcel.2021.01.009

Contribution:

As first author of this publication, I contributed to the conceptualization of the study. I created all figures, wrote the first draft and edited the manuscript. I conducted, processed and analyzed all experiments with the exception of electron microscopy experiments (Fig. 2A, 5H and S6I). For these I grew and treated the cells.

2.2. Publication II

Atg4 family proteins drive phagophore growth independently of the LC3/GABARAP lipidation system.

Published as:

Nguyen, T. N., Padman, B. S., Zellner, S., Khuu, G., Uoselis, L., Lam, W.K., Skulsuppaisarn, M., Lindblom, R. S. J., Watts, E., Behrends, C. & Lazarou, M. (2021). Atg4 family proteins drive phagophore growth independently of the LC3/GABARAP lipidation system. *Molecular Cell, 2021. Volume: 81(9), 2013-2030.e9 https://doi.org/10.1016/j.molcel.2021.03.001*

Contribution:

I contributed to this publication by performing all mass spectrometry experiments (Fig. 4, S5E). This involved cloning of the ATG4A-D into APEX2 vectors as well as sample processing and data analysis.

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation mit dem Titel „Exploring selective autophagy cargo and machinery using proximity proteomics“ selbstständig und ausschließlich unter Verwendung der angegebenen Hilfsmittel verfasst habe. Ich versichere, dass alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen sind.
Weiterhin versichere ich, dass die hier vorgelegte Dissertation nicht in gleicher oder ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 25.02.2022

Susanne Zellner

Teile dieser Arbeit sind bereits in internationalen Fachzeitschriften veröffentlicht worden.