Graduate Programme in Molecular Biology and Biomedicine

MSc thesis

Targeting of plasma membrane proteins via secretory autophagy

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Abstract

Autophagy has classically been described as a process that delivers cytoplasmic proteins and organelles to lysosomes for degradation. However, autophagosomes (AVs) have also been shown to fuse with the plasma membrane, thereby allowing the secretion of soluble proteins to the extracellular space and the targeting of transmembrane proteins to the plasma membrane. In concert with the notion that degradative and secretory AVs constitute distinct populations, confocal microscopy experiments demonstrated that Stx17 and Sec22b (the degradation- and secretion-specific AV SNAREs, respectively) have distinct patterns. Sequential centrifugation analyses also revealed Sec22b to be enriched in smaller cellular structures compared to Stx17, which could allude to its recruitment on the outer autophagosomal membrane early on in the autophagosomal biogenesis process. Genetic studies in mice have demonstrated the requirement of basal autophagic levels for neuronal function, while it is becoming apparent that autophagy also exerts functions in the pre- and post-synapse. The contribution of secretory autophagy, however, has not been examined as of yet. Preliminary data demonstrate that the outer membrane of murine forebrain-derived autophagosomes is rich in integral proteins. It is thus hypothesized that secretory autophagy could mediate neuron-specific targeting of plasma membrane proteins, and to that end, the process is also examined in non-neuronal cell types.

Περίληψη

Η αυτοφαγία έχει κλασσικά χαρακτηριστεί ως μια διαδικασία που παραδίδει κυτταροπλασματικές πρωτεΐνες και οργανίδια στα λυσοσώματα για αποικοδόμηση. Ωστόσο, τα αυτοφαγοσώματα έχουν επίσης δειχθεί να συντήκονται με την πλασματική μεμβράνη, επιτρέποντας έτσι την έκκριση διαλυτών πρωτεϊνών στον εξωκυττάριο χώρο και τη στόχευση διαμεμβρανικών πρωτεϊνών στην πλασματική μεμβράνη. Σε συμφωνία με την αντίληψη ότι τα αποικοδομητικά και εκκριτικά αυτοφαγοσώματα αποτελούν διακριτούς πληθυσμούς, πειράματα συνεστιακής μικροσκοπίας έδειξαν ότι το Stx17 και το Sec22b (τα αυτοφαγοσωμικά SNAREs ειδικά για αποικοδόμηση και έκκριση, αντίστοιχα) έχουν ξεχωριστά πρότυπα. Αναλύσεις διαδοχικής φυγοκέντρησης επίσης αποκάλυψαν ότι το Sec22b είναι εμπλουτισμένο σε μικρότερες κυτταρικές δομές συγκριτικά με το Stx17, γεγονός που θα μπορούσε να υποδεικνύει τη στρατολόγηση του στην εξωτερική μεμβράνη των αυτοφαγοσωμάτων νωρίς στη διαδικασία βιογένεσής τους. Γενετικές μελέτες σε ποντίκια έχουν δείξει την αναγκαιότητα βασικών επιπέδων αυταφαγίας για τη νευρωνική λειτουργία, ενώ γίνεται φανερό ότι υπάρχουν επίσης ειδικοί ρόλοι της στο προ- και μετα- συναπτικό άκρο. Ωστόσο, η συμβολή της εκκριτικής αυτοφαγίας δεν έχει εξεταστεί ακόμη. Προκαταρκτικά δεδομένα δείχνουν ότι η εξωτερική μεμβράνη αυτοφαγοσωμάτων που προέρχονται από πρόσθιο εγκέφαλο ποντικών είναι πλούσια σε διαμεμβρανικές πρωτεΐνες. Υποθέτουμε ότι η εκκριτική αυτοφαγία θα μπορούσε ειδικά στους νευρώνες να διαμεσολαβήσει τη στόχευση μεμβρανικών πρωτεϊνών, και για το σκοπό αυτό η διαδικασία εξετάζεται επίσης σε μη νευρωνικούς κυτταρικούς τύπους.

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A. INTRODUCTION

A.1 Autophagy and its subtypes

Autophagy is a catabolic pathway that is evolutionarily conserved from yeast to mammals. In contrast to proteasome-mediated degradation, which is limited to the removal of ubiquitinated proteins with a short half-life, autophagy extends its degradative capacity to other macromolecules, such as lipids and nucleic acids, as well as to superfluous or damaged organelles (Mizushima and Komatsu, 2011).

Autophagy is vital for the maintenance of homeostasis. Constitutive, basal autophagic levels have been shown to be critical for quality control; autophagy-mediated removal of defective cellular components and organelles prevents their accumulation (Komatsu and Ichimura, 2010). In addition, autophagy becomes upregulated under various conditions of cellular stress (e.g. nutrient starvation, hypoxia, pathogen infection, radiation) as a cytoprotective response, so as to provide the cell with structural units and energy (Mizushima and Komatsu, 2011). Defective autophagic function has been associated with the pathogenesis of a variety of neurodegenerative, metabolic and immune diseases (Yang and Klionsky, 2010; Mizushima and Komatsu, 2011).

We distinguish three types of autophagy: microautophagy, chaperone-mediated autophagy and macroautophagy (Nikoletopoulou, Papandreou and Tavernarakis, 2015). In the case of microautophagy, small molecules are degraded by direct invagination of the lysosomal membrane. In chaperone-mediated autophagy, no cargo-delimiting vesicles are formed. The KFERQ pentapeptide of misfolded cytoplasmic proteins is instead recognized by heat-shock cognate 70 (HSC70) and co-chaperones, thus facilitating their transport directly to lysosomes via the lysosomal transmembrane receptor LAMP-2A (Mizushima and Komatsu, 2011). Finally, in the case of macroautophagy, hereby referred to as “autophagy”, cytoplasmic fragments and organelles are encapsulated by double-membrane organelles called “autophagosomes”. Autophagosomes then fuse with endosomes or lysosomes (Yang and Klionsky, 2010), where the autophagic cargo, as well as the internal autophagosomal membrane, get degraded by lysosomal hydrolases. The degradation products return to the cytoplasm via lysosomal permeases (Glick, Barth and Macleod, 2010; Rabinowitz and White, 2010).

A.2 The molecular mechanism of autophagy

The steps of autophagy, which are evolutionarily conserved, are the following:

1. Initiation

2. Vesicle nucleation

3. Vesicle elongation

4. Lysosomal fusion

5. Degradation

Each step is strictly regulated and requires ATG (autophagy-related) proteins (Rabinowitz and White, 2010). 35 ATG proteins have been characterized thus far in yeast (Mizushima and Komatsu, 2011), with many of their respective genes having orthologues in other eukaryotic organisms. A subset of *atg* genes is necessary for the autophagosomal formation, with their corresponding gene products being referred to as the core autophagic machinery (Xie and Klionsky, 2007).

Below is a brief overview of the aforementioned steps in mammals (Image 1).

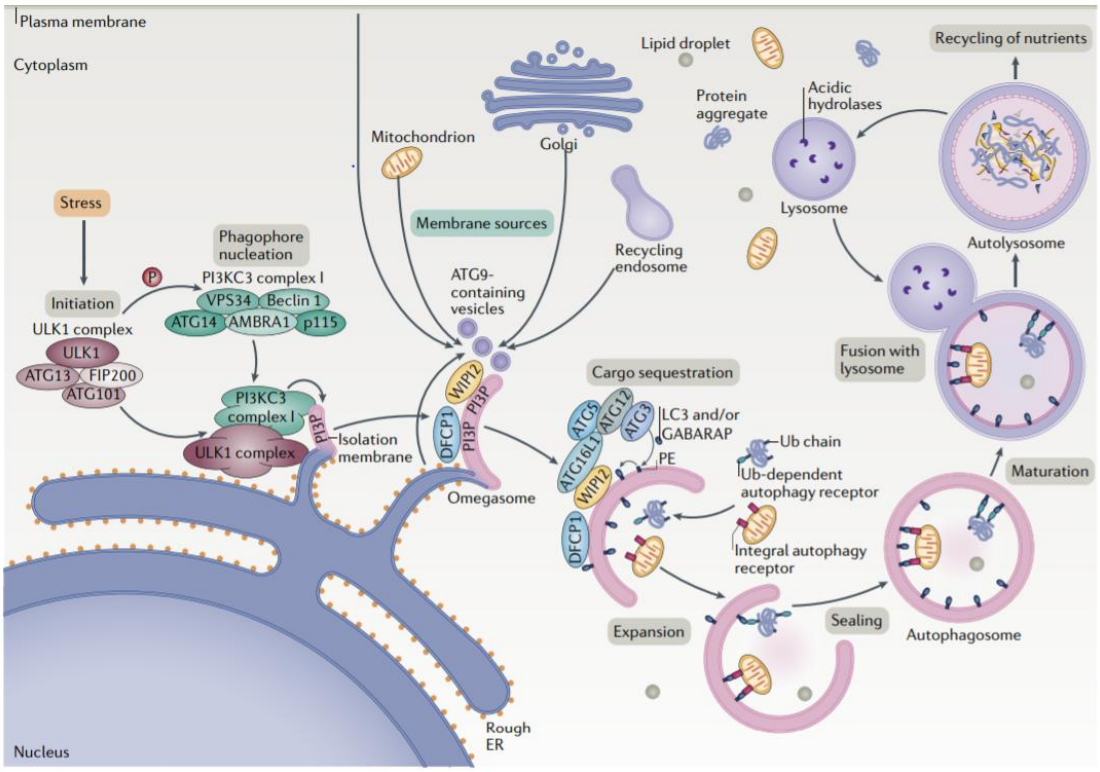

Image 1. Summary of the molecular mechanism of mammalian autophagy.
(Dikic and Elazar, 2018)

A.2.1 Initiation

The mammalian target of rapamycin (mTOR) signaling is vital for autophagic regulation. In the presence of appropriate environmental signals (e.g. nutrient deprivation), the mTOR kinase is inhibited, in which case proteins ULK1 and ATG13 are dephosphorylated and autophagy initiation is rendered feasible. The ULK1 complex, consisting of proteins ULK1, ATG13, ATG101 and FIP200 (also known as

RB1CC1) (Füllgrabe, Klionsky and Joseph, 2014), recruits the BECLIN1-VPS34 complex at the autophagosome formation site (Kim and Lee, 2014).

A.2.2 Vesicle nucleation

The PtdIns3K (class III phosphatidylinositol 3-kinase) complex comprises proteins VPS34 (also known as PIK3C3), VPS15 (also known as PIK3R4), BECLIN1, AMBRA1, ATG14 or alternatively UVRAG, and BIF1 (also known as SH3GLB1) (Füllgrabe, Klionsky and Joseph, 2014). Endoplasmic reticulum (ER)-resident VPS34 converts phosphatidylinositol (PI) to phosphatidylinositol 3-phosphate (PtdIns3P, or PI3P), which then acts as a local signal and employs effector molecules at the lipid membrane and cytoplasmic interface (Dall'Armi, Devereaux and Di Paolo, 2013). Specifically, PI3P recruits proteins DFCP1 and WIPI to initiate nucleation of the double-membrane vesicle (Kim and Lee, 2014).

The membrane origin of autophagosomes remains unclear. However, the contribution of multiple membrane sources, including the ER, mitochondria and the plasma membrane, is widely recognized as a potential scenario (Ravikumar *et al.*, 2010).

A.2.3 Vesicle elongation

At this stage the membrane structure elongates, eventually enveloping the cargo and forming double membrane autophagosomes.

Two systems are instrumental for the process. The first comprises ATG7 (an E1-like enzyme) and ATG10 (an E2-like enzyme), which enable the covalent attachment of ubiquitin-like ATG12 to ATG5 without a need for an E3 enzyme (Nakatogawa, 2013; Füllgrabe, Klionsky and Joseph, 2014). Then, the covalently bound ATG12-ATG5 complex interacts non-covalently with ATG16L, thereby forming the ATG12-ATG5-ATG16L complex (Kim and Lee, 2014).

The second system contributes to the lipidation of LC3 (a ubiquitin-like protein, orthologue of ATG8 in yeast) and its integration to the expanding phagophore. This molecule is synthesized as proLC3 (precursor of LC3), which is then cleaved by the ATG4 protease and yields cytosolic LC3-I. LC3-I proceeds to be coupled to phosphatidylethanolamine (PE) with the help of ATG7 (an E1-like enzyme), ATG3 (an E2-like enzyme) and ATG12-ATG5-ATG16L (acts as an E3 enzyme), thereby forming LC3-II (Kim and Lee, 2014). LC3-II is incorporated in both the inner and outer membrane of the autophagosome (Nikoletopoulou, Papandreou and Tavernarakis, 2015) and contributes to autophagosomal elongation and cargo recognition.

LC3-II is used as an autophagic marker (Mizushima *et al.*, 2004). However, elevated LC3-II levels do not necessarily indicate induction of autophagic activity. In fact, LC3-

II also accumulates when its degradation is inhibited. To that end, autophagic flux is a more reliable indicator of autophagic activity. Its assessment is achieved with LC3 turnover assays, during which cells are treated with lysosomotropic agents, such as chloroquine (an agent preventing lysosomal acidification) or bafilomycin A1 (a factor that inhibits autophagosomal-lysosomal fusion). The differences in the amount of LC3-II in the presence and absence of these reagents represent the amount of LC3 delivered to the lysosomes for degradation (Mizushima, Yoshimori and Levine, 2010). Autophagic flux monitoring can also be achieved by the degradation of selected substrates. p62 interacts directly with LC3 and is thus selectively integrated within autophagosomes. Since p62 is efficiently degraded by autophagy, cellular levels of p62 are inversely related to autophagic activity (Mizushima, Yoshimori and Levine, 2010).

A.2.4 Lysosomal fusion

Following autophagosomal completion, proteins involved in its formation are released into the cytoplasm to be used anew (Füllgrabe, Klionsky and Joseph, 2014). Mature autophagosomes subsequently fuse with lysosomes, thus leading to the formation of autolysosomes.

Autolysosomal fusion is mediated by SNARE proteins, as is the case for all membrane fusion events. Specifically, after Rab GTPase proteins confer specificity to the imminent fusion event by facilitating the approximation of donor and acceptor membranes in a spatiotemporally regulated manner (Zhao and Zhang, 2019), SNARE proteins form a trans-complex comprised of four a-helical coiled-coil domains arranged in a bundle. From a structural point of view, and depending on whether each SNARE motif contains an arginine (R) or a glutamine (Q) as a central residue, each tetrahelical bundle is formed so that a 3Q:1R stoichiometry is achieved (Hong, 2005). The energetically favorable zippering up of the SNARE-complex leads to a trans- to cis- conformational change, which drives membrane fusion. Eventually, individual SNAREs of the cis-complex are released by the NSF factor for further use (Dingjan *et al.*, 2018).

The autolysosomal fusion event has been shown to be mediated by two sets of SNARE complexes, which function in parallel and independently of one another (Zhao and Zhang, 2019). The firstly identified complex was shown to implicate the autophagosomal protein Syntaxin17 (STX17). More specifically, STX17 is hypothesized to be acquired directly from the cytosolic pool and was shown to be recruited on the external membrane of complete autophagosomal structures (Itakura, Kishi-Itakura and Mizushima, 2012). ATG8 proteins and the IRGM GTPase are instrumental for its recruitment (Kumar *et al.*, 2018), while membrane embedding is rendered feasible by the hairpin-like structure formation of its two

transmembrane domains (Itakura, Kishi-Itakura and Mizushima, 2012). STX17 binding to SNAP29 and lysosomal VAMP8 drives the fusion event and leads to autolysosome formation (Itakura, Kishi-Itakura and Mizushima, 2012).

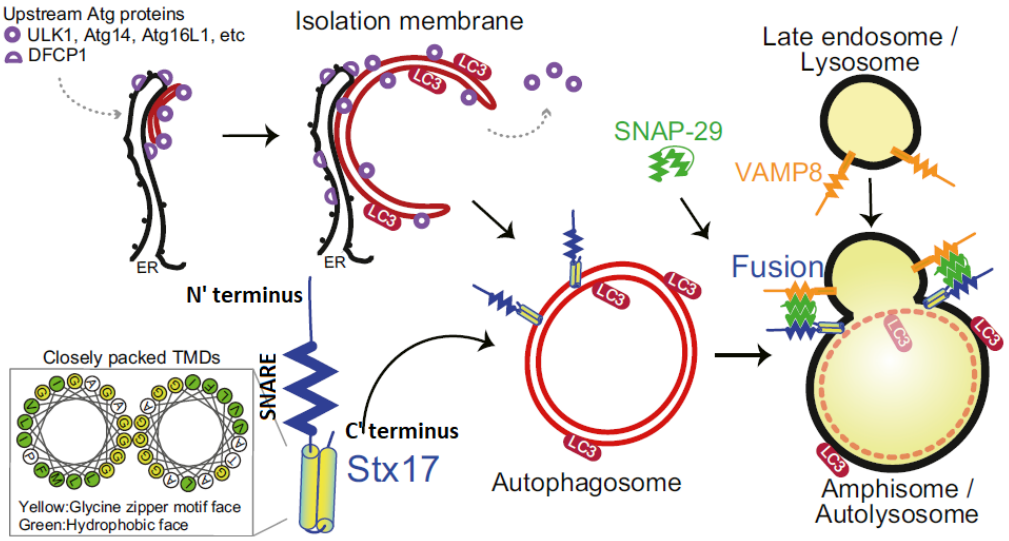

Image 2. The Qa SNARE syntaxin17 in AV-lysosome fusion (Itakura, Kishi-Itakura and Mizushima, 2012)

Recently, however, the aforementioned step was also revealed to be mediated by a second, independently acting SNARE complex, comprising the autophagosomal YKT6, SNAP29 and the lysosomal STX7 (Matsui *et al.*, 2018). Similarly to STX17, YKT6 is considered to be delivered to the autophagosomal membrane directly from the cytosol, while its membrane incorporation is mediated by farnesylation and palmitoylation modifications of two C-terminal cysteine residues (Daste, Galli and Tareste, 2015).

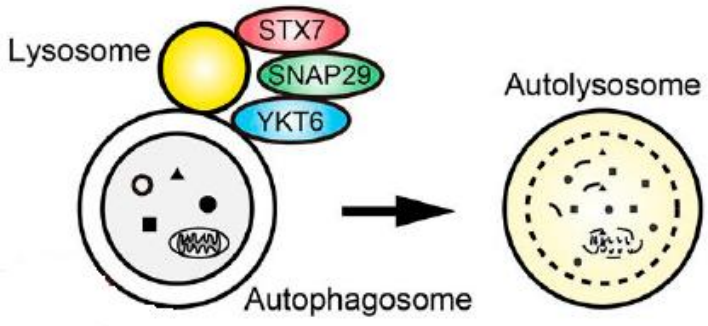

Image 3. The R-SNARE YKT6 in AV-lysosome fusion (Matsui *et al.*, 2018)

According to a recent study in human cardiomyocytes, LAMP-2B was also shown to interact with lysosomal VAMP8 and to mediate AV-lysosomal fusion independently of STX17 (Chi *et al.*, 2019), thereby inviting the inference of further complexity yet to unraveled. The existence of multiple SNARE complexes has been hypothesized to promote fusion efficiency or to enable AV fusion with different lysosomal populations, depending of their VAMP8 and STX7 levels (Zhao and Zhang, 2019).

A.2.5 Degradation

Upon fusion with lysosomes, the cargo and inner membrane of autophagosomes get degraded by lysosomal enzymes (proteases, lipases, nucleases, glucosidases), thus giving rise to an organelle delimited by a single membrane, the autolysosome (Glick, Barth and Macleod, 2010). In contrast to the LC3 of the inner membrane, which is degraded along with the cargo, LC3 of the outer membrane is cleaved off by ATG4 (Yu *et al.*, 2012).

A.3 Molecular identity of lysosomes

Lysosomal proteolysis constitutes one of the two major cellular degradative pathways (the other being the ubiquitin-proteasome pathway). Lysosomes constitute the degradation endpoint for both extracellular and intracellular material, which reach these terminal compartments via the endocytic pathway and by all three autophagic subtypes, respectively. Lysosomal degradative function is mediated by approximately 60 hydrolases, which reside within the lumen and whose enzymatic activity is optimal in acidic conditions (pH 4.5-5.0). Proteins such as LAMP glycoproteins ensure organellar integrity by coating the single membrane and rendering it resistant to the internal acidic environment (Settembre *et al.*, 2013).

Of note, the existence of lysosomes is inextricably linked to endosomal maturation. During this process, sorting functions of early endosomes are gradually restricted; while foundations are laid to accommodate the impending fusion of late endosomes with the degradative compartment (Huotari and Helenius, 2011). Such radical functional changes are reflected by mechanistic complexity, as clearly illustrated by the undermentioned events. The Rab5 to Rab7 switch and the concomitant acquisition of distinct SNARE proteins is vital for altered fusion specificity: late endosomes fuse in a homotypic fashion, as well as with autophagosomes and lysosomes, while recycling with the plasma membrane and fusion with early endosomes is obstructed. In addition, endosomes grow in size and undergo conversions in terms of lipid composition, something which allows the recruitment of different lipid-binding effectors. Intra-luminal vesicle (ILV) formation is indispensable for the presentation of membrane-bound proteins and lipids in an easily digestible form, while vesicles undergo a centripetal movement from the peripheral cytoplasm towards the perinuclear space. Two events are particularly decisive for the biogenesis of lysosomes: a) the acidification due to V-ATPase proton pumps, which results in a luminal pH drop from above 6 to values 6.0-4.9, and b) the gain of lysosomal hydrolases and membrane proteins (Huotari and Helenius, 2011).

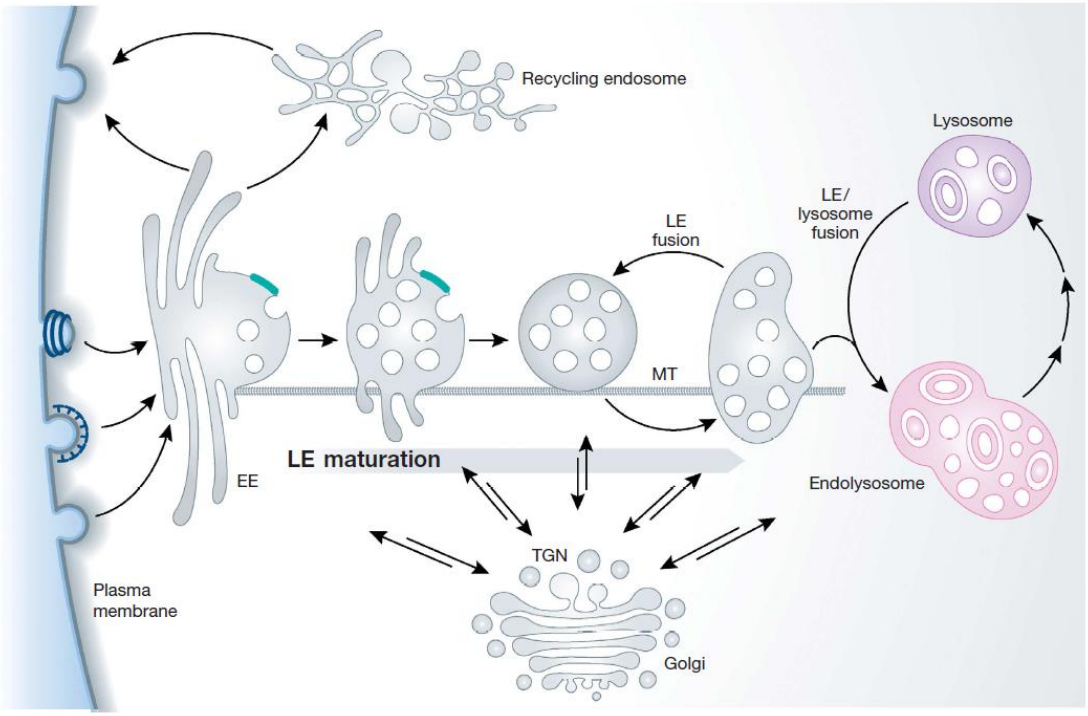

Image 4. The endosomal maturation process (Huotari and Helenius, 2011)

Hydrolase delivery to lysosomes is mediated primarily by mannose 6-phosphate (M6P)-dependent mechanisms (Ghosh, Dahms and Kornfeld, 2003), although M6P-independent mechanisms have also been described (Reczek *et al.*, 2007; Zhao *et al.*, 2014). Briefly, hydrolases acquire M6P modifications and bind to M6P-recognition moieties of transmembrane M6P receptors (MPRs) of the trans-Golgi. Sorting of the resulting complexes to distinct organelles is achieved by the interaction of various proteins with sorting signals of the MPR cytoplasmic tails. MPR-hydrolase dissociation is facilitated by the pH drop along the endosomal pathway: MRPs are thereby recycled back to the TGN and evade lysosomal degradation (Griffiths *et al.*, 1988), while hydrolases reach their destination and exert their function (Ghosh, Dahms and Kornfeld, 2003).

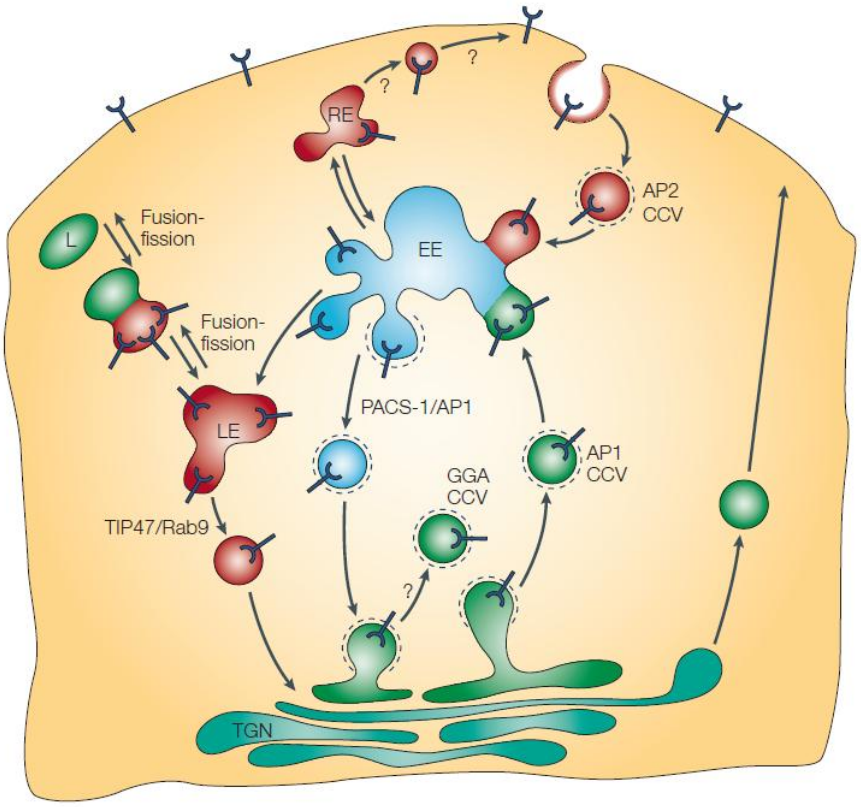

Image 5. Itinerary of lysosomal enzymes from their synthesis to their final destination via M6P-dependent mechanisms (Ghosh, Dahms and Kornfeld, 2003)

As becomes evident from this brief description, the distinction of late endosomes and lysosomes at the molecular level is blurred. Previously considered lysosomal markers (e.g. LAMP1/2) are promiscuously localized throughout the endo-lysosomal system (Andrei *et al.*, 1999; Cheng *et al.*, 2018), and in fact, there is no lysosomal protein that is not also present in late endosomes (Scott, Vacca and Gruenberg, 2014). In any case, assays monitoring hydrolases for lysosomal detection appear to be more accurate than LAMP1/2-labeling methods, since the enzymes are not detectable in the majority of LAMP-positive organelles (Cheng *et al.*, 2018). A more robust method to distinguish lysosomes relies on their physical properties and ultrastructure, seeing as they are spherical and electron-dense. For example, their density was shown to be employed in fractionation experiments to allow their distinction from endosomes (Scott, Vacca and Gruenberg, 2014). Other useful methods for the detection of mature lysosomes include the evaluation of β-hexosaminidase activity (Andrei *et al.*, 1999), or the use of BODIPY-pepstatin A, which specifically binds active Cathepsin D in acidic environments (Cheng *et al.*, 2018).

A.4 Autophagy in neurons

As previously mentioned, functional autophagy is vital for the maintenance of cellular homeostasis, and autophagic defects have been associated with various diseases (Yang and Klionsky, 2010; Mizushima and Komatsu, 2011). However, autophagic integrity is all the more essential in the case of neuronal cells due to their unique properties and functions. Neurons are post-mitotic cells, thus more susceptible to the accumulation of toxic proteins and damaged organelles compared to proliferating cells whose cellular content is diluted through cell division (Son *et al.*, 2012).

Genetic studies in mice indicate that basal autophagic levels are essential for the maintenance of neuronal homeostasis and prevention of neurodegeneration. Mice of *nestin cre; atg5*$^{flox/flox}$ genotype, in which *atg5* is selectively ablated in cells of the neural lineage, manifest motor function deficits and late onset neurodegeneration (Hara *et al.*, 2006). Similarly, mice in which *atg7* is selectively ablated in Purkinje cells, exhibit neurodegeneration and behavioral deficits (Komatsu *et al.*, 2007).

Importantly, neuronal communications take place at synapses, structures with high energetic requirements and rates of protein turnover. As a result, timely clearance of synaptic components is crucial for the maintenance of synaptic function. Inadequate protein removal through autophagy or other degradation pathways results in the accumulation of abnormal or incorrectly folded proteins and the formation of aggregates (Shen *et al.*, 2015). In cases of defective autophagy, aggregates associated with neurodegenerative diseases such as Alzheimer's Disease (AD) and Parkinson's disease (Parkinson's disease, PD) have been observed (Son *et al.*, 2012).

Moreover, we are becoming increasingly aware of selective autophagic roles in pre- and post-synaptic regions. When it comes to the pre-synaptic site, autophagy has been implicated in the degradation of synaptic vesicles and therefore in the regulation of neurotransmitter release from the presynaptic terminal. In fact, mice with chronic autophagic defects in dopaminergic neurons demonstrate increased neurotransmitter release in response to stimulation (Hernandez *et al.*, 2012), thus inviting the inference for a role of autophagy as a brake on presynaptic activity (Shen *et al.*, 2015). Several presynaptic molecules (e.g. Rab26-Plekhg5 (Binotti *et al.*, 2014; Lüningschrör *et al.*, 2017)), EndophilinA/Synaptojanin (Soukup *et al.*, 2016; Vanhauwaert *et al.*, 2017), Bassoon (Okerlund *et al.*, 2018) have also been implicated in the modulation of synaptic vesicle autophagy-mediated degradation.

In terms of the post-synapse, autophagy appears to regulate membrane receptor turnover and to contribute to synapse remodeling (Shen *et al.*, 2015). In *C. elegans*, cell surface $GABA_AR$ receptors have been shown to be degraded by autophagy

(Rowland *et al.*, 2006), while experiments in rat hippocampal cultures have demonstrated the stimulation-induced autophagy-dependent degradation of the GluR1 AMPA receptor subunit (Shehata *et al.*, 2012). In addition, postsynaptic density scaffolds PSD-95, PICK1 and SHANK3 were found to be included within murine brain-derived autophagosomes (Nikoletopoulou *et al.*, 2017).

Moreover, autophagic activity at the post-synapse has been shown to regulate different forms of synaptic plasticity. BDNF, a key regulator of long-term potentiation (LTP), was found to suppress autophagy in the adult brain, and that suppression was shown to be required for BDNF-induced plasticity (Nikoletopoulou *et al.*, 2017). Unpublished data of our lab also demonstrate that autophagic upregulation and concomitant degradation of dendritic spines is required for LTD induction in the hippocampus (Kallergi, Daskalaki *et al.*, in revision). In agreement with the latter, the removal of unnecessary or inappropriate synaptic connections, which is vital for the refinement of neuronal connectivity (Shen *et al.*, 2015), was shown to be insufficient in cases of defective autophagy (Tang *et al.*, 2014; Shen *et al.*, 2015). The resulting increased density of dendritic spines has been associated with neurodevelopmental disorders, such as Autism Spectrum (Autism) Spectrum Disorders, ASD) (Son *et al.*, 2012; Tang *et al.*, 2014).

A.5 Conventional secretory pathway

Most proteins destined towards secretion contain a leader peptide at their N-terminus, which enables them to reach their final destination via the classical ER-to-Golgi pathway. More specifically, the leader sequence interacts with cytoplasmic factors and ER-resident machinery, thereby allowing their translocation to the ER lumen (Claude-Taupin *et al.*, 2017). It is at this stage that transmembrane domains of integral proteins are incorporated within the ER lipid bilayer and that the assumption of the overall protein conformation is achieved (Shao and Hegde, 2011). Upon enclosure within COP-II vesicles, ER proteins bud from specific ER exit sites (ERES). They then pass through the mammalian-specific ER-Golgi intermediate compartment (ERGIC) and move across the Golgi cisternae in a cis- to trans-orientation (*cisternal migration*) (Zahoor and Farhan, 2018). The protein glycan profile is, simultaneously, sculpted: sugar residues are added and modified by about 700 mammalian proteins with a discreet distribution across the ER lumen and Golgi apparatus. As a direct consequence, glycosylations of integral proteins do not face towards the cytosol, but rather towards the interior of the implicated vesicular structures (Stanley, 2011). At the trans-Golgi sorting station, proteins awaiting secretion are segregated from ones targeted towards endosomal/lytic compartments and are incorporated in distinct transport vesicles. Upon fusion with the plasma membrane, soluble proteins are

released to the extracellular milieu, while integral proteins are delivered to the plasma membrane (Zahoor and Farhan, 2018) with glycosylations extruding towards the extracellular space (Stanley, 2011).

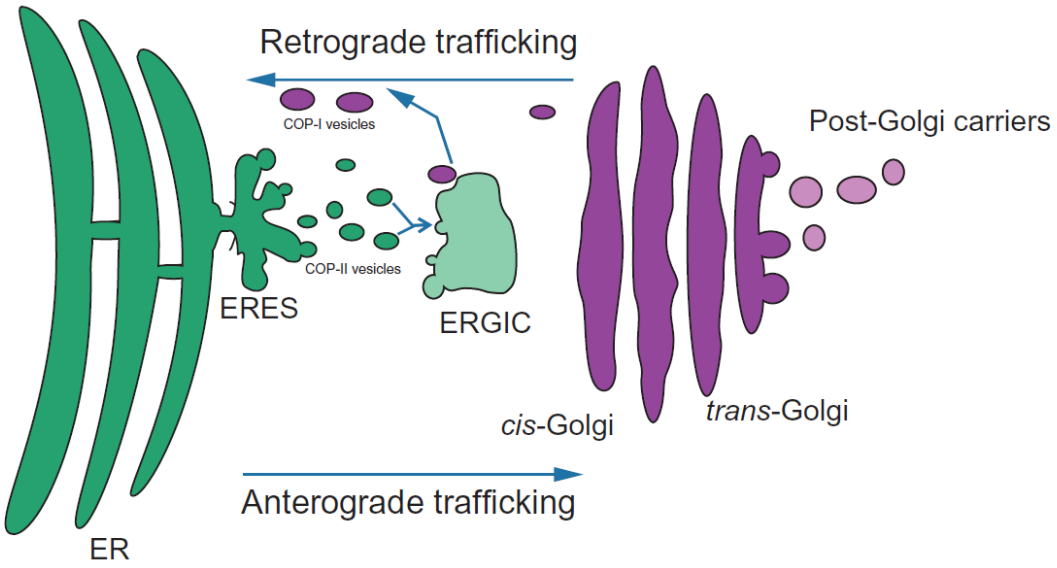

Image 6. Conventional secretion of leader-peptide-containing proteins via the ER-to-Golgi pathway
(Zahoor and Farhan, 2018)

A.6 Unconventional protein secretion (UPS) pathways

While the majority of proteins employ the conventional ER-Golgi pathway, evidence of proteins being secreted independently of the classical route has come to the forefront. The umbrella term of "unconventional secretion" encompasses various processes where cytosolic proteins en route to the extracellular environment, as well as integral ones trafficked to the plasma membrane, do not pass through the Golgi (Davis *et al.*, 2016).

Leaderless cytoplasmic proteins have been shown to directly translocate across pores of the plasma membrane (**Type I** of unconventional secretion). Such proteins (e.g. FGF1, FGF2, annexin A2) are initially recruited by acidic membrane lipids of the inner leaflet and subsequently phosphorylated in a tyrosine residue. Finally, they undergo an oligomerization-induced membrane insertion before being expelled to the extracellular space.

Type II applies to leaderless, lipidated proteins (e.g. a-factor in yeast, m-factor in *Schizosaccharomyces pombe*, germ cell attractant in *Drosophila melanogaster*), which are secreted via ATP-binding cassette (ABC) transporters.

Cytoplasmic leaderless proteins can also undergo secretion through vesicular intermediates (**Type III**) (e.g. Acb1 in yeast, AcbA in *Dictyostelium*, IL1-b in immune system cells), with multiple vesicles having been implicated in the process (e.g.

secretory lysosomes, multi-vesicular bodies, autophagosomes). There appear to be distinct routes for collection of cytoplasmic proteins within secretory vesicles, which can either include translocation across a membrane, or not. As an illustration of the former, IL1b has been shown to translocate from the cytoplasm across the autophagosomal membrane early on during its biogenesis and thus to reside within the intramembrane space (Zhang *et al.*, 2015). In the latter case, a condition-dependent induction of partial unfolding allows the exposure of secretory motifs on a small subset of cytoplasmic proteins (e.g. exposure of diacidic residues on SOD1 and Acb1 upon starvation (Cruz-Garcia *et al.*, 2017), thereby allowing their subsequent recruitment to sorting stations and eventual secretion (Cruz-Garcia, Malhotra and Curwin, 2018).

Finally, a number of transmembrane proteins (e.g. aPS1 and aPS2 in *Drosophila*, CD45, Pendrin, CTFR in humans) have been shown to reach their destination by bypassing the Golgi compartment (**Type IV**) (Rabouille, Malhotra and Nickel, 2012). Although the Type IV vesicular machinery remains largely uncharacterized, it would theoretically entail the formation of vesicular carriers emanating from the ER, followed by carrier fusion with the plasma membrane (either directly or via intermediate compartments) (Gee, Kim and Lee, 2018).

Of note, given the commonalities between UPS types III and IV (stress-dependent activation, implication of GRASP, ATG and heat-shock proteins), it is reasonable that the same vesicles could mediate both the secretion of cytoplasmic proteins and the targeting of transmembrane ones (Rabouille, Malhotra and Nickel, 2012).

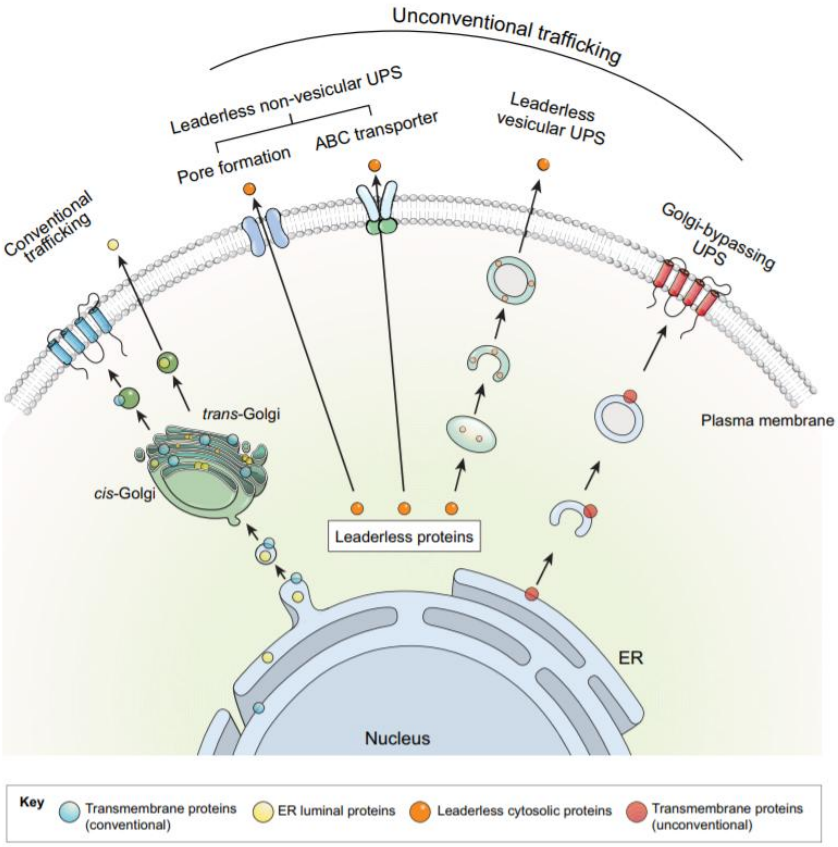

Image 7. All protein secretion pathways with a focus on unconventional ones (Kim, Gee and Lee, 2018)

From an evolutionary perspective, various reasons may underlie the development of unconventional secretion pathways. In terms of proteins that can only be secreted via UPS routes, avoidance of the conditions encountered within the conventional route seems instrumental to their structural integrity and function. Certain proteins demonstrate sensitivity to Golgi-mediated post-translational modifications (PTMs), as was shown by the introduction of a leader peptide to IL1b (normally secreted by UPS Types I & III) and FGF2 (secreted by UPS Type I) and their concomitant defective function due to deleterious PTM acquisition (Lee and Ye, 2018). Moreover, proteins susceptible to the oxidizing milieu of the conventional pathway are able to maintain their activity by avoiding it (Zeitler *et al.*, 2015). Last but not least, the transport of receptors and their ligands via distinct routes offers compartmentalization and prevents unwelcome internal activation and autocrine stimulation (Popa, Stewart and Moreau, 2018). There are also proteins that can be secreted by both conventional and unconventional pathways: for example, upon stress-induced disruption of the ER-Golgi route, the classically secreted lysozyme is rerouted towards autophagy-mediated secretion (Bel *et al.*, 2017). It is therefore possible that UPS is preferred upon stress conditions thanks to its faster secretion speed (Giuliani, Grieve and Rabouille, 2011; Grieve and Rabouille, 2011). It is also within the realms of possibility that UPS is more prominent in cellular compartments where Golgi is absent or scarce (e.g. neuronal dendrites (Hanus *et al.*, 2016; Bowen *et al.*, 2017).

UPS can act as an additional Protein Quality Control (PQC) mechanism, a function particularly valuable in cases when stress-relief is necessary and lysosomal capacity is overwhelmed (Ye, 2018). UPS provides an energy-conserving manner of eliminating aggregates, aggregation-prone species and large cytoplasmic objects (e.g. organelles), thus conferring short-term benefits to the organism (Zahoor and Farhan, 2018). However, should UPS become deregulated, it could contribute to the accumulation of misfolded proteins in the extracellular milieu or to their trans-cellular transmission, thereby proving to be detrimental in the long run (Lee and Ye, 2018; Ye, 2018). UPS also runs the risk of being exploited by pathogens (e.g. bacteria and viruses): their secretion and lack of clearance could result in an infection of neighboring cells and in an increase of their overall population (Claude-Taupin *et al.*, 2017).

A.7 Autophagy-mediated UPS

Apart from *sensu stricto* autophagy, where double membrane, LC3-decorated vesicles fuse with lysosomes to degrade their contents, the process has also been implicated in the unconventional secretion of cytosolic and integral proteins (autosecretion). This finding reinforces the autophagic relevance in degradation-independent functions, while expanding the list of mechanisms which bypass the Golgi or evade ER insertion altogether en route to secretion (Cadwell and Debnath, 2018).

Most autosecreted proteins were identified individually. Their secretion was revealed to be increased with autophagy-inducing stimuli, and to be respectively diminished upon attenuated autophagic function (Ponpuak *et al.*, 2015). However, proteomic analyses have lately been employed to expand our knowledge of autosecreted proteins in a more comprehensive manner (Kraya *et al.*, 2015; Kimura *et al.*, 2017).

Several leaderless cytosolic proteins (e.g. Acb1 in yeast (Duran *et al.*, 2010) and mammalian proteins IL-1β, IL-18 (Dupont *et al.*, 2011a)), which inherently cannot employ the ER-Golgi secretion pathway, were shown to be autosecreted. Autophagy was also found to be implicated in the trafficking of the integral proteins to the plasma membrane: namely, the route is employed by intestinal Paneth cells in order to secrete lysozyme upon ER stress (Bel *et al.*, 2017), whereas autophagy components are implicated in CFTR secretion upon the same conditions (Noh *et al.*, 2018).

Autophagic contribution was additionally illustrated for the export of large cytoplasmic objects, such as organelles (e.g. mitochondria) and microbes, and of aggregate-forming proteins (e.g. the Parkinson's-associated a-synuclein and the Alzheimer's-implicated amyloid beta) (Ponpuak *et al.*, 2015).

A.8 The mechanism of secretory autophagy

Although steps have been taken towards the elucidation of the secretory autophagy mechanism, many elements remain obscure. Below we will describe what is known, starting from the vesicle biogenesis and culminating to their fusion with the plasma membrane, while outlining the emerging challenges.

Firstly, we will examine the **temporal specification of secretory autophagic vesicles**, considering the point of divergence between degradative and secretory autophagic functions. To wit: do secretory vesicles constitute a dedicated subpopulation, or are promiscuous vesicles redirected to the appropriate target depending on the cellular conditions?

The first scenario would entail the marking of secretory autophagosomes from the inception of their biogenesis. In support of this theory, studies in yeast demonstrated dedicated sources for secretory vesicles: their biogenesis seems to occur in the compartment for unconventional protein secretion (CUPS), next to Sec13-positive ER exit sites (Bruns *et al.*, 2011; Cruz-Garcia *et al.*, 2014). CUPS structures are positive for the yeast GRASP Grh1, PI3P, Vps23 of the ESCRT-I complex, as well as autophagic proteins Atg8 and Atg9 (Bruns *et al.*, 2011). Interestingly, Sec12 activity, which is necessary for degradative AV formation in yeast (Ishihara *et al.*, 2001), was found to be dispensable for CUPS formation (Bruns *et al.*, 2011), whereas CUPS-resident Grh1 and Vps23 and not necessary for degradative autophagy. In addition, whereas classical COPII components are indispensable for the formation of classical autophagy nucleation sites (Ishihara *et al.*, 2001), CUPS biogenesis is COPII-independent: the membranes arise, instead, from the fusion of Golgi- and endosomal-derived membranes (Cruz-Garcia *et al.*, 2014). CUPS structures have yet to be identified in mammalian cells, however they exhibit several similarities with mammalian omegasomes: both are associated with the ER, have a characteristic shape and are enriched for PI3P and autophagic proteins. However, although both structures are induced by starvation (Axe *et al.*, 2008; Bruns *et al.*, 2011), CUPS formation was found not to be affected by rapamycin treatment, a classical autophagy-inducing stimulus (Bruns *et al.*, 2011). These subtle differences need to be examined assiduously, but could potentially allude to a differentiation in their functions. Furthermore, the point at which recruitment of SEC22b, the protein of the outer autophagosomal membrane currently serving as a secretory AV marker, occurs is unclear. Albeit no studies have been conducted pertaining specifically to this matter, it is possible that SEC22b is recruited to incomplete autophagic structures. Fractionation studies in human cancer cell lines indicate that SEC22b is detected both in heavier and lighter cellular structures, contrary to STX17 which only fractionates with heavier structures (Kimura *et al.*, 2017; Kumar *et al.*, 2018).

Should, however, biogenesis precede specification, vesicles would have to be marked as secretory en route to their final destination. In agreement with the notion of a divergence point at a later point in the process, cells appear to switch between degradative and secretory autophagy for the same cargo: proteins IL-1b and a-synuclein have been shown to act as substrates of both processes, depending on the circumstances. It, therefore, seems plausible that vesicles arise from common precursors and later on acquire their fate with the acquisition of different fusion proteins (Ponpuak *et al.*, 2015). The vesicular machinery attributed to the secretory route will be further examined below.

The **recognition of secretory cargo** seems to be achieved by different strategies, which in turn affect their place of storage. Members of the tripartite motif (TRIM) family were recently reported as secretory autophagy receptors: IL1b was shown to interact with TRIM16, and to thereby be engulfed within the lumen of the autophagic structure. Although TRIM16 is the sole secretory autohagy receptor detected thus far, allusions have been made to a potential similar function for TRIM10 (Kimura *et al.*, 2017). However, IL1b was shown to translocate within the two autophagosomal membranes early on in the biogenesis process, thereby residing in the intermembrane space and assuming a distinct localization compared to the autophagosomal lumen. The latter accounts for an elegant model, where cargo is released to the extracellular space in a lipid-free form upon fusion with the plasma membrane (Zhang *et al.*, 2015). Whether cargo proteins of secretory autophagy are marked by post-translational modifications (e.g. ubiquitinations) is yet to be discovered (Ponpuak *et al.*, 2015).

The resulting vesicles eventually **fuse with the plasma membrane, thereby bypassing the maturation process** and evading cargo degradation. The exocytosis regulator Rab8a was initially demonstrated to be necessary for the secretion of the proinflammatory cytokine IL1-b (Dupont *et al.*, 2011b). The secretory route was later shown to employ dedicated fusion machinery: SEC22b-positive autophagosomes were revealed to mediate fusion by interacting with Stx3/4 of the plasma membrane, as well as with SNAP23/29 (Kimura *et al.*, 2017).

It should be noted that, apart from cases of direct fusion with the plasma membrane, secretory AVs have also been shown to follow indirect routes and to fuse with other compartments (e.g. lysosomes or multi-vesicular bodies) along the way (Malhotra, 2013; Ponpuak *et al.*, 2015).

Furthermore, our knowledge currently stems from studies focusing on the secretion of selected proteins under different conditions. Such an approach further complicates the landscape and does not allow the inference of a unified picture. The

preferred route and implicated machinery has been proposed to be cargo-, stimulus- and cell line-specific.

A.9 Lysosomes as a secretory compartment

While transport to lysosomes was considered unidirectional, several proteins have been revealed to be released in the extracellular milieu upon lysosomal fusion with the plasma membrane. This emerging secretory function impugns the notion which required proteins en route to secretion to bypass lysosomes (Lee and Ye, 2018).

The mechanism of lysosomal exocytosis was initially believed to be employed specifically by professional secretory cells, such as cells of the immune system (e.g. monocytes) (Andrei *et al.*, 1999). However, it is increasingly appreciated that such a function may be realized by any cell type, such as osteoclasts (Vaes, 1968; Zhao *et al.*, 2008), adipocytes (Villeneuve *et al.*, 2018), fibroblasts and epithelial cells (Rodríguez *et al.*, 1997; Martinez *et al.*, 2000; Blott *et al.*, 2001; Jaiswal, Andrews and Simon, 2002).

Despite growing evidence, whether the secretory function is mediated by degradative lysosomes or by a specialized subpopulation thereof is yet to be established. In addition, due to the previously analyzed conundrum of endosomal-lysosomal distinction, the possibility that secretion originates from late endosomes rather than lysosomes still remains (Lee and Ye, 2018).

B. QUESTIONS AND RESULTS

B.1 Do degradative and secretory autophagosomes constitute discreet populations?

B.1.1 Background

Whether degradative and secretory autophagosomes diverge during their biogenesis or en route to their targets, they are theorized to constitute discreet vesicular populations. To our knowledge, however, this hypothesis has not been definitively proven.

B.1.2 Results

In order to address the question, we set out to explore the effect of nutrient starvation on degradative and secretory autophagosomal populations of hepatic Huh7 cancer cells.

We first needed to establish a starvation protocol. In order to ascertain the appropriate conditions, Huh7 cell cultures were incubated for various lengths of time either in a serum-free medium, or PBS. PBS is an equivalent of Hank's Balanced Salt Solution (HBSS), which is commonly employed for starvation-induced autophagic upregulation in cancer cell lines (Lichtenstein *et al.*, 2011; Hu *et al.*, 2018; Mejlvang *et al.*, 2018).

More specifically, Huh7 cells were exposed to either starvation medium for 1-6 hours prior to fixation, and the induced autophagic effect was evaluated by confocal microscopy **(Fig. 1A)**. LC3 is visualized either as a diffuse signal, corresponding to the cytoplasmic LC3-I pool, or punctuate, representing LC3-II-containing autophagosomal structures (Mizushima *et al.*, 2004). PBS proved to be more effective in inducing autophagy, as indicated by the increase in the number of LC3 puncta at all examined time-points, whereas the serum-free medium elicited a significant autophagic upregulation only upon 4 and 6 hours of incubation **(Fig. 1B)**. It was observed, however, that exposure to PBS also brought about a significant shrinkage of the nucleus at all examined time points **(Fig. 1C)**. Consequently, a 6-hour incubation with serum-free medium was chosen as the optimal protocol, seeing as it significantly induces autophagy without affecting the nuclear size and overall morphology.

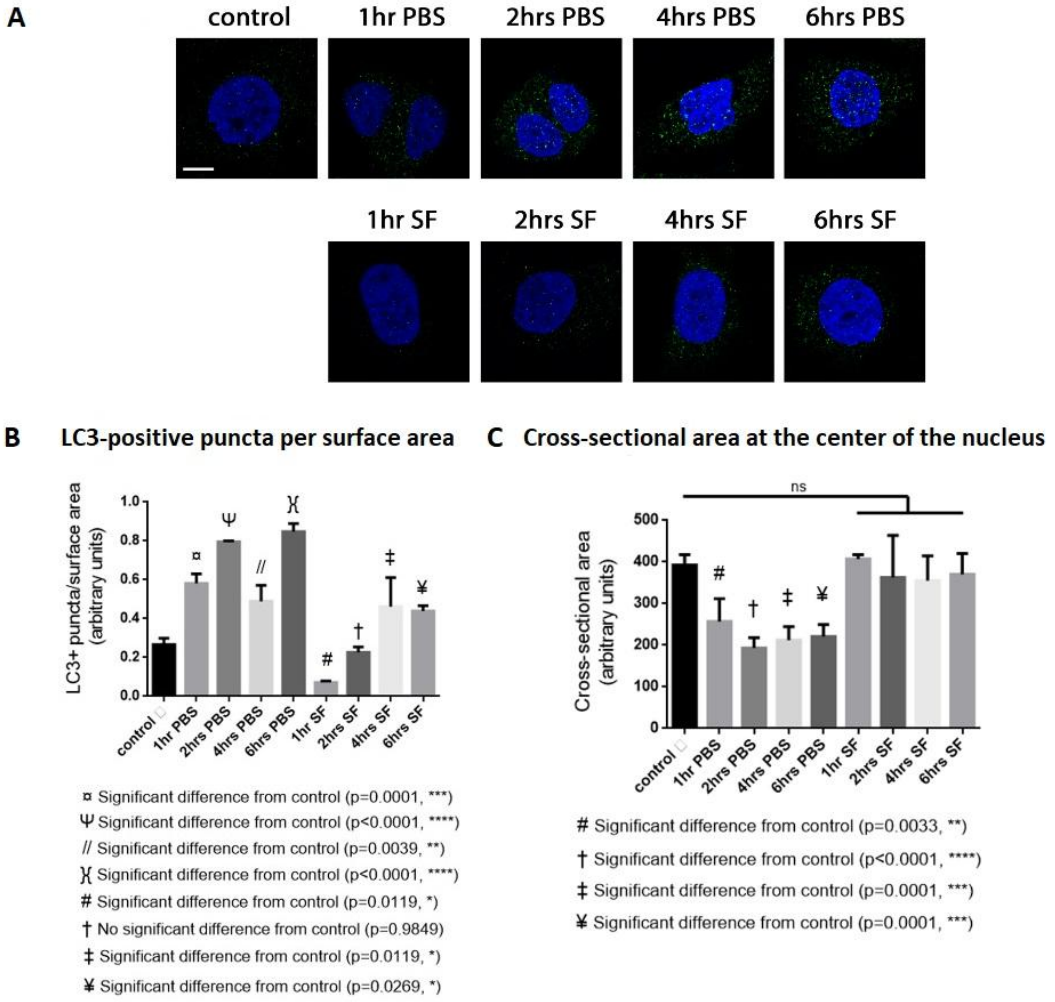

Figure 1. Evaluation of starvation protocols for autophagy induction in Huh7 cells. A) Confocal images of Huh7 cells immunolabeled with an antibody against LC3. Immunolabeling was performed in control cultures or upon incubation with PBS or serum-free (SF) medium for the indicated durations. Scale bar: 10um. **B)** Graph showing the normalized number of LC3-positive puncta in control cultures and upon the indicated protocols for autophagic induction. Statistical analyses were performed using ANOVA. Bars represent mean values +/- SD (N= 3-5 cells in each condition). **C)** Graph showing the cross-sectional area at the center of the nucleus in control conditions and upon the indicated protocols for autophagic induction. Statistical analyses were performed using ANOVA. Bars represent mean values +/- SD (N= 3-5 cells in each condition).

We then wished to examine the effect of starvation on the secretory subpopulation of autophagosomes, as denoted by the surface marker SEC22b (Kimura *et al.*, 2017). Consistently with previous findings (Kimura *et al.*, 2017), both LC3-II and SEC22b were shown to be induced upon starvation of Huh7 cells **(Fig. 2A)**, while an increased fraction of each marker was illustrated to colocalize with the second one **(Fig 2B,C)**. These findings suggest a starvation-induced upregulation of secretory autophagy.

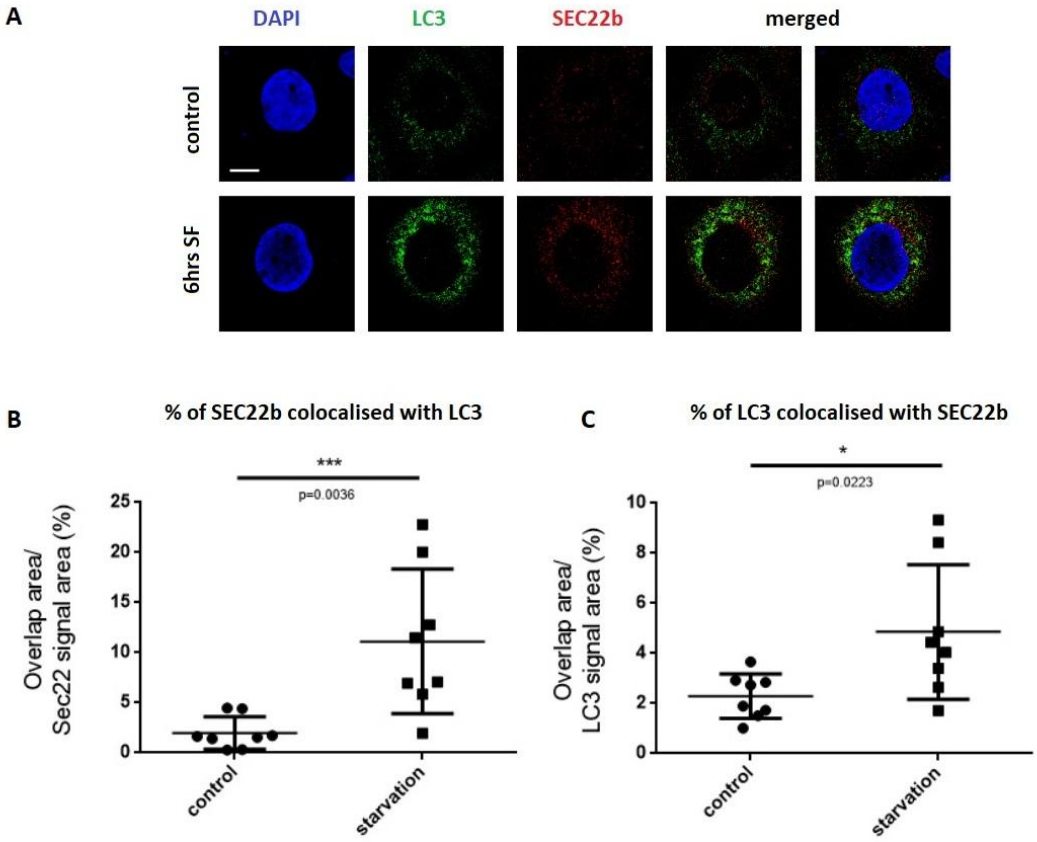

Figure 2. Secretory autophagy is induced upon starvation in Huh7 cells. A) Confocal images of Huh7 cells immunolabeled with antibodies against LC3 and SEC22b. Immunolabeling was performed in control conditions or upon a 6-hour incubation with serum-free medium for autophagy induction. Scale bars: 10um. **B, C)** Graphs showing colocalisation of SEC22b with LC3 in the indicated conditions. Statistical analyses were performed using student's t-test. Bars represent mean values +/- SD (N=8 cells in each condition).

Finally, we wanted to examine the effect of starvation on secretory and degradative autophagosomal populations, as denoted by markers SEC22b and STX17, respectively (**Fig. 3A**). Both at control and experimental conditions, there appears to be a low colocalization percentage, which does not significantly increase upon starvation (**Fig 3B,C**). One would argue that the aforementioned colocalization could stem from resolution limitations; signal overlap does not necessarily indicate colocalization in a cellular structure. Therefore, it appears that degradative and secretory autophagosomes constitute discreet populations.

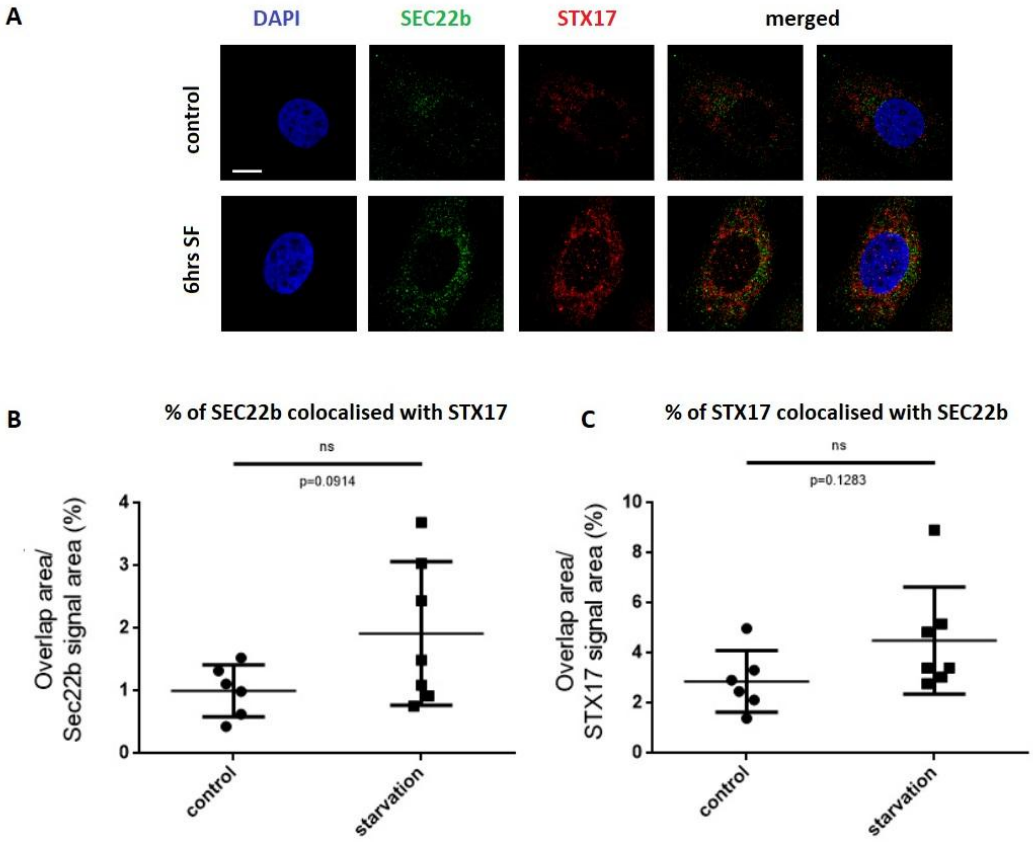

Figure 3. Degradative and secretory autophagosomes constitute discreet populations in Huh7 cells. A) Confocal images of Huh7 cells immunolabeled with antibodies against SEC22b and STX17. Immunolabeling was performed in control conditions or upon a 6-hour incubation with serum-free medium for autophagy induction. Scale bars: 10um. **B, C)** Graphs showing colocalisation of SEC22b with STX17 in the indicated conditions. Statistical analyses were performed using student's t-test. Bars represent mean values +/- SD (N= 6 cells in control conditions, 7 cells in starvation conditions).

B.2 Does secretory autophagy mediate the targeting of plasma membrane proteins?

B.2.1 Background

Genetic studies have revealed the importance of baseline autophagy for the maintenance of neuronal integrity and functionality; autophagic ablation in the totality or in distinct populations of the neural lineage elicits the manifestation of motor and behavioral deficits (Hara *et al.*, 2006; Komatsu *et al.*, 2007). Given the dependency of neuronal cells on robust quality control pathways, the beneficial effects of autophagy have thus far been interpreted through the lens of its degradative functions. Autophagy has been implicated in the removal of protein aggregates (Hara *et al.*, 2006; Komatsu *et al.*, 2007) and damaged organelles (Anding and Baehrecke, 2017), as well as in the degradation of compartment-specific

proteins (Rowland *et al.*, 2006; Shehata *et al.*, 2012; Nikoletopoulou *et al.*, 2017; Kallergi, Daskalaki *et al*., in revision). However, the recently emerging autophagic contributions to unconventional secretion have not been taken into consideration.

Contrary to candidate-based approaches, an unbiased approach was employed for the elucidation of the autophagic cargo (Emmanouela Kallergi, unpublished results). Specifically, purified autophagosomes were isolated from murine forebrains, and were then subjected (or not) to a Proteinase K (PK) treatment. This serine protease has a broad cleavage specificity and thus "shaves off" the exposed proteins of the outer autophagosomal membrane. Following carbonate extraction, a process which allows discrimination between soluble and membrane sample fractions, the resulting material underwent quantitative proteomic analysis (10-plex TMT-labelling MS) (Mikhail Savitski, EMBL PCF). Upon comparison of the PK-treated pellet fraction with the untreated one, it emerged that many proteins (315) were PK-sensitive and thus localized to the outer autophagosomal membrane. Of these, 142 had one or multiple transmembrane domains. This finding was in stark contrast to ultrastructural analyses of hepatocyte-derived autophagosomes, which demonstrated their outer membrane to be protein-poor (Fengsrud *et al.*, 2000; Fengsrud *et al.*, 2000).

We therefore hypothesized that secretory autophagy could mediate cell surface protein targeting in neurons.

B.2.2 Results

In order to ascertain whether targeting of plasma membrane proteins via secretory autophagy is a neuron-specific process, we set out to examine it in a different cell type. Due to technical issues, murine livers could not be employed as starting material, and thus Huh7 cells were used instead.

Previously established protocols for autophagosomal isolation from tissues (Strømhaug *et al.*, 1998; Nikoletopoulou *et al.*, 2017) were adapted, and eventually enabled us to isolate autophagic vesicles from Huh7 cell cultures, albeit at low quantities (**Fig. 4**).

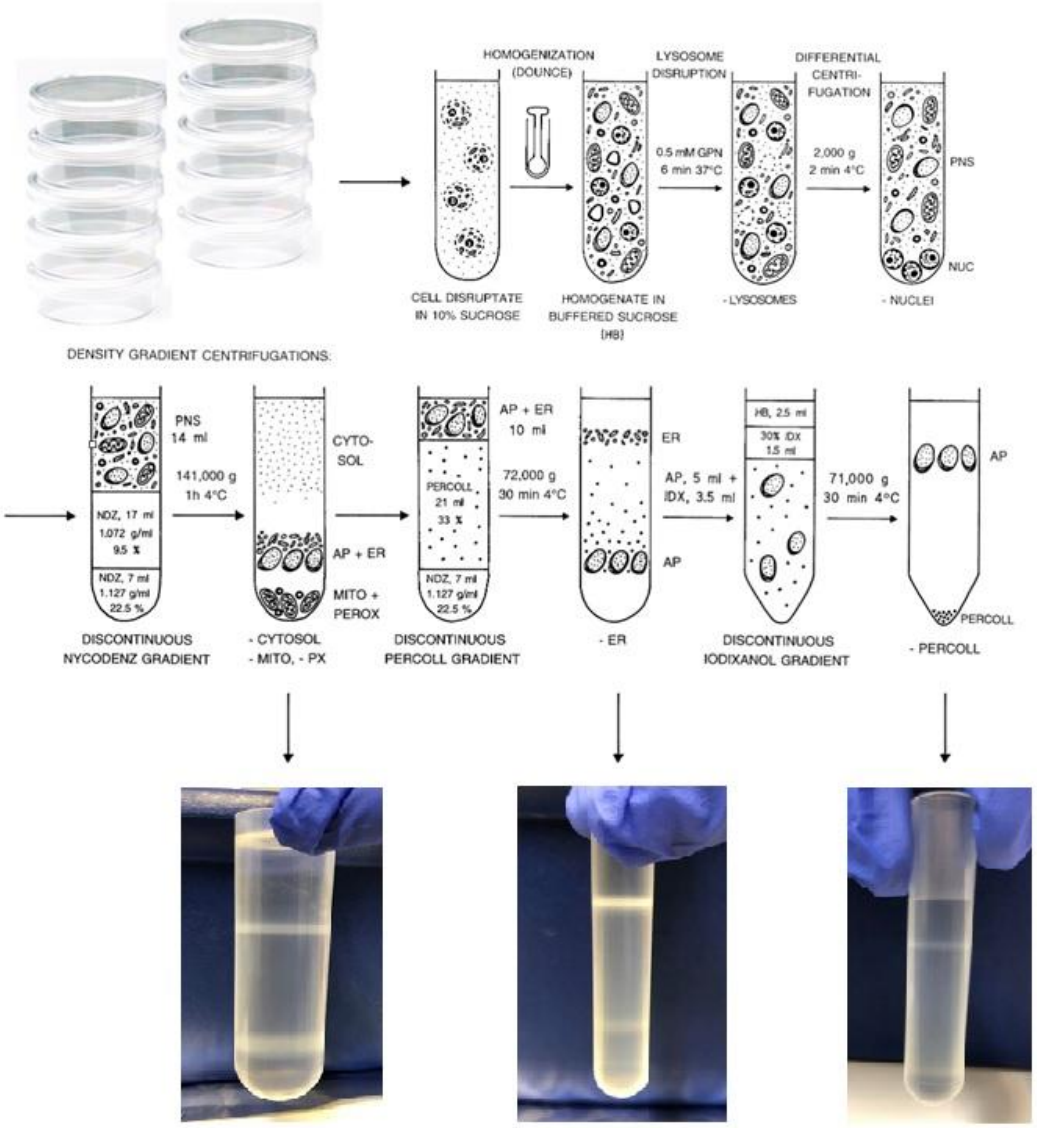

Figure 4. Autophagosomal isolation from Huh7 cells (adapted from Strømhaug *et al.*, 1998)

Samples were collected throughout the process for purity evaluation, and were assessed alongside a forebrain-derived autophagosomal sample as a reference.

Huh7-derived autophagosomes appeared enriched for the LC3-II autophagosomal marker, while they were primarily devoid of the nuclear TATA-binding protein (TBP). Protein disulfide isomerase (PDI), an ER-resident enzyme, was present to some extent in the autophagosomal fraction, which is however not surprising considering its previous identification as autophagic cargo (unpublished data of our lab). Since the autophagosomal fraction was largely free of nuclear and ER contaminants, we considered the vesicles to be purified. Both examined autophagosomal fractions were positive for ATG16L1, a protein that associates with phagophores and

dissociates prior to autophagosomal membrane closure (Rubinsztein, Shpilka and Elazar, 2012), and thus contained immature and mature vesicles alike (**Fig. 5A**).

Carbonate extraction experiments were performed in parallel on Huh7- and murine forebrain-derived autophagosomes, while its efficacy was assessed by the localization of soluble and membrane-bound proteins after the fact. In the case of forebrain-derived autophagic vesicles, soluble proteins were successfully separated from membrane ones, as indicated by the segregation of the soluble P62 in the supernatant and the localization of the membrane-bound LC3-II in the pellet fraction. However, when it comes to Huh7-derived autophagosomes the results were not explicit: while LC3-I and LC3-II localized to the supernatant and pellet fractions respectively, thereby alluding to the success of the protocol, P62 appeared to stick in part to the pellet fraction (**Fig. 5B**).

Due to the low yield of the isolation protocol, the sample was not sufficient to undergo treatments and proteomic analysis. It is thus to be repeated in liver tissue.

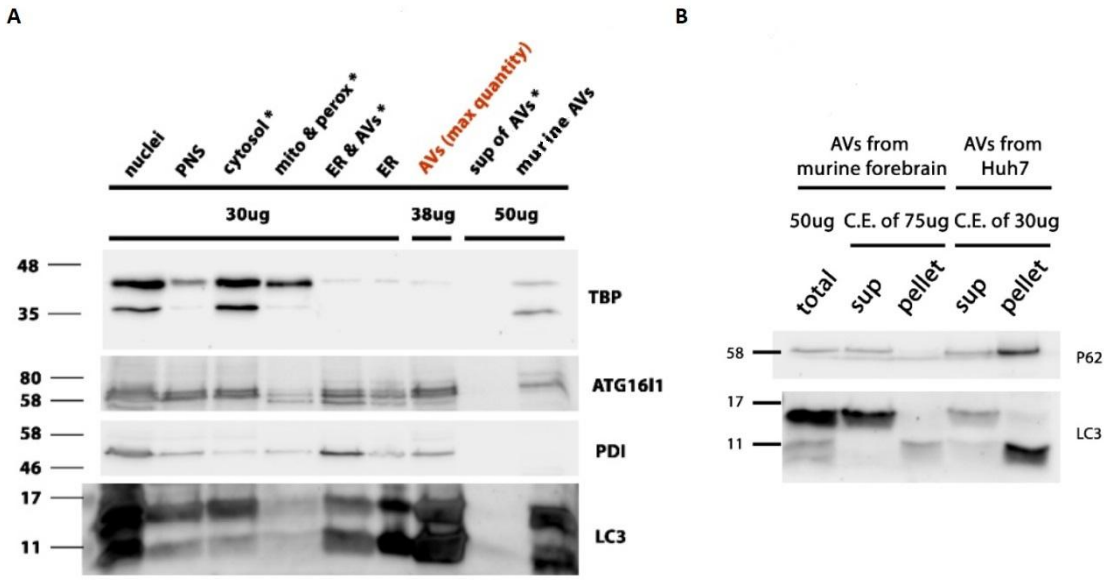

Figure 5. Evaluation of Huh7-derived autophagosomes. A) Western blot analysis for TBP, ATG16L1, PDI, LC3 in Huh7 lysates acquired throughout the autophagosomal isolation process. A murine forebrain-derived autophagosomal sample was used as a reference, while samples denoted with an asterisk underwent TCA precipitation prior to gel separation. **B)** Western blot analysis for P62 and LC3 in lysates acquired upon carbonate extraction of murine forebrain- and Huh7-derived autophagosomes.

The protein glycosylation profile is sculpted across the conventional secretory pathway, with core molecules being added in the ER and further modifications occuring in the Golgi (Breitling and Aebi, 2013). Given that autophagosomes are

thought to arise primarily from the ER and could therefore theoretically incorporate core-glycosylated proteins, in the event that secretory autophagy does indeed serve as a neuron-specific mechanism of cell surface protein targeting, neuronal plasma membranes would need to be enriched in core-glycosylated proteins. That has indeed been shown to be the case, while trafficking of these proteins was demonstrated to occur in a Golgi-independent fashion (Hanus *et al.*, 2016).

Again in the spirit of juxtaposition with a non-neuronal cell type, evaluation of the Huh7 plasma membrane N-glycosylation profile was achieved via labeling with lectin biotin-conjugates under non-permeabilizing conditions (**Fig. 6**). Huh7 cells displayed high levels of core- and mixed- glycosylated proteins, as indicted by strong Concanavalin A (ConA) and ricin agglutinin (RCA) labeling respectively, while complex glycosylation-indicative wheat germ agglutinin (WGA) staining appeared fainter.

In the future, changes in the cell surface N-glycosylation profile will be evaluated upon disruption of the secretory autophagy pathway.

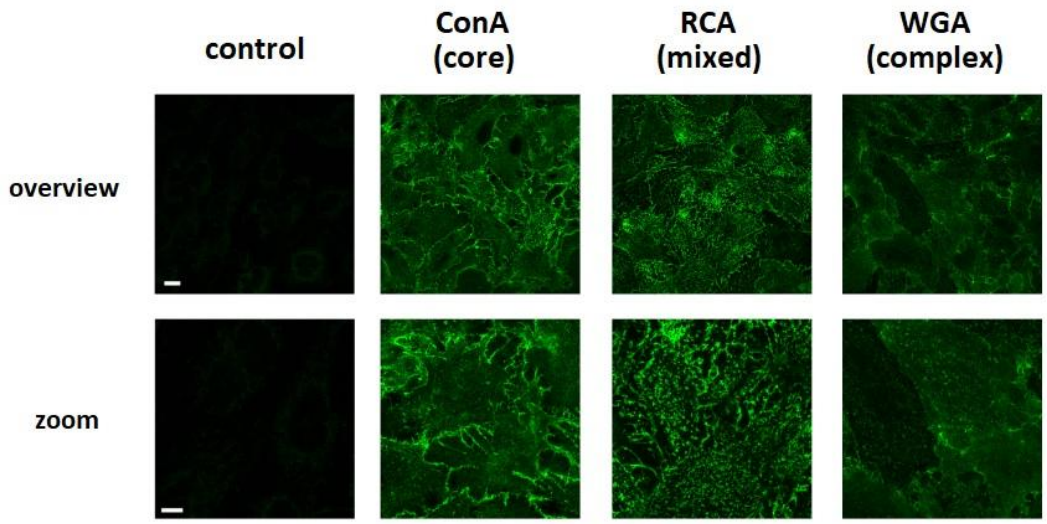

Figure 6. The cell surface of Huh7 cells is rich in core- and mixed-N-glycosylated proteins. Confocal images of Huh7 cells labeled with lectin biotin-conjugates under non-permeabilizing conditions**.** Scale bar for overview: 20um, scale bar for zoom, 10um.

B.3 When is SEC22b recruited on the surface of autophagic structures?

B.3.1 Background

As of yet, it is unknown when SEC22b is recruited on the outer membrane of secretory autophagosomes. However, such an event could take place in incomplete vesicular structures: sequential centrifugation studies in HEK293T (Kumar *et al.*, 2018) and HeLa cells (Kimura *et al.*, 2017) have demonstrated SEC22b precipitation

both at low and high speed centrifugation rounds. On the contrary, STX17 is only present in heavier structures, as indicated by its precipitation solely upon low speed centrifugation rounds (Kumar *et al.*, 2018).

However, information concerning the distribution of SEC22b-positive organelles on the basis of sedimentation rate is not available in murine brain cells.

B.3.2 Results

Isolated forebrains were initially homogenized and then underwent three low-speed centrifugation rounds so as to remove unbroken cells, cellular debris and nuclei. The resulting post-nuclear supernatant (PNS) underwent a high-speed centrifugation round, thereby giving rise to an unsedimentable fraction (supernatant S1) and a precipitate (pellet P1). The S1 fraction then underwent an additional centrifugation round of even higher speed, thus leading to the P2 pellet and S2 supernatant (**Fig.7A**).

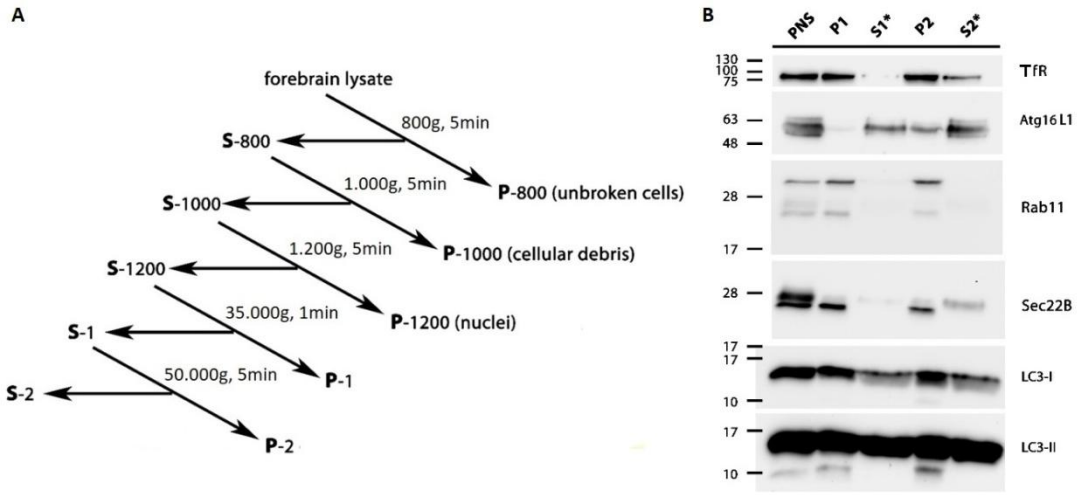

Figure 7. A) Fractionation protocol, **B)** Western blot analysis for TfR, Atg16L1, Rab11, SEC22b and LC3 in samples acquired upon sequential centrifugations of forebrain lysate.

The recycling endosome-specific markers Rab11 and transferrin receptor (TfR) (Kobayashi and Fukuda, 2013), are detected in both pellets, which is expected since these organelles vary in size. Interestingly enough, SEC22b appears to exist in two forms: whereas two bands are detected in the PNS, only the upper one is apparent in soluble fractions (S1, S2), whereas the lower one is the predominant one in membrane fractions (P1, P2) (**Fig. 7B**). We sought to examine whether a phosphorylation modification was accountable for the difference in solubility. Although no effect was noted upon Lambda Protein Phosphatase treatment of the PNS, a molecular weight shift of the control protein (ATG13) was also not apparent,

therefore impeding the inference of conclusions about the irrelevance of the modification (data not shown). It is probable that lipid-conjugation could facilitate SEC22b association with the outer autophagosomal membrane, in which case the lower apparent molecular weight of the lipid-bound form could be attributed to increased hydrophobicity and migration rate. Such a prospect will be addressed in the future.

LC3-II was detected in both pellets, whereas a significant amount of soluble LC3-I was detected in the S2 supernatant. In addition, S2 appeared enriched in ATG16L1, a protein vital for the early phases of autophagosome formation (Xiong *et al.*, 2018) (**Fig. 7B**). The latter two findings lead us to repeat the fractionation experiment, with the addition of an extra centrifugation step of the S2 supernatant, so as to precipitate any remaining autophagosomal structures (**Fig. 8**).

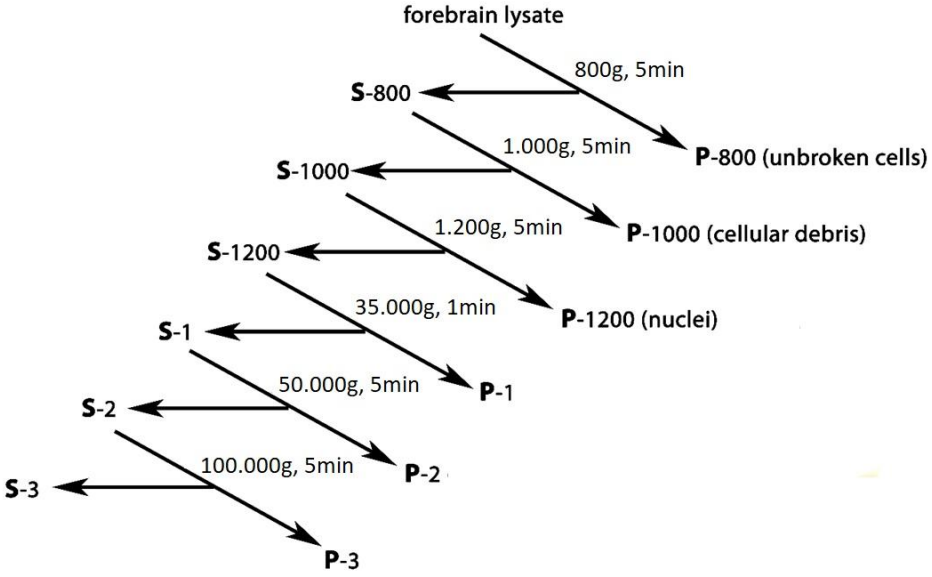

Figure 8. Overview of fractionation protocol

Indeed, more autophagic structures were precipitated, as indicated by LC3-II presence in the P3 fraction (**Fig. 9**). Contrary to studies in HEK293T cells, where SEC22b precipitates upon a 100.000g centrifugation while STX17 does not (Kumar *et al.*, 2018), the picture is less explicit in murine forebrain cells. While the trend persists, with SEC22b appearing enriched in the lighter P3 pellet and STX17 in the heavier P1 pellet, both markers seem to precipitate in all pellet fractions (**Fig. 9**). The SEC22b enrichment in lighter fractions could allude to its recruitment on early, incomplete autophagic structures.

In order to solidify our claim, we examined the fractionation of DFCP1, which is a marker for omegasomes and phagophore nucleation sites (Mercer, Gubas and

Tooze, 2018), as well as that of WIPI2 and ATG16L1. These proteins localize to phagophore structures and are paramount for LC3 lipidation, while they dissociate prior to autophagosomal membrane closure and are absent from mature vesicles (Polson *et al.*, 2010; Li *et al.*, 2017). Contrary to our hypotheses, these markers were not enriched in light fractions along with SEC22b, but rather in heavy ones (**Fig. 9**), possibly due to the fact that growing phagophores are associated with the ER (Ylä-Anttila *et al.*, 2009; Graef *et al.*, 2013; Sanchez-Wandelmer, Ktistakis and Reggiori, 2015).

In order to gain further insight into the colocalization of autophagosomal subpopulation-specific markers (STX17 and SEC22b) with early autophagosomal structures (as denoted by DFCP1, WIPI2 and ATG16L1), double staining experiments are to be conducted.

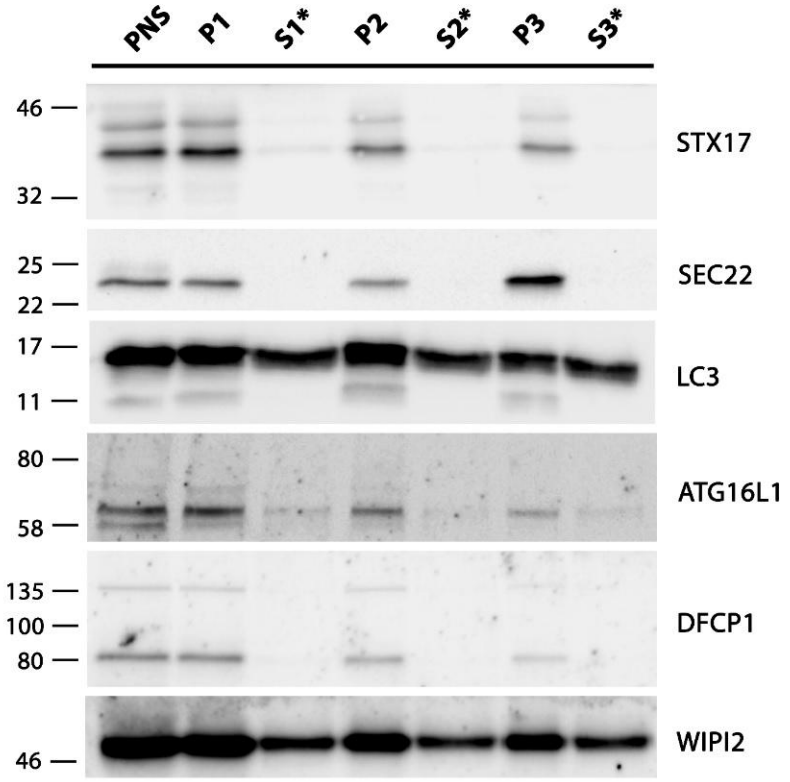

Figure 9. Western blot analysis for STX17, SEC22b, LC3, ATG16L1, DFCP1 and WIPI2 in samples acquired upon sequential centrifugations of forebrain lysate**.**

B.4 Can mature lysosomes of murine forebrain cells be discriminated on the basis of centrigugation speed?

B.4.1 Background

While lysosomes have been shown to promote the secretion of some proteins (Lee and Ye, 2018), it is generally accepted that evasion of lysosomal degradation is paramount for secretion. Andrei and colleagues (Andrei *et al.*, 1999), using human monocytes as starting material and employing the same fractionation protocol as we did (**Fig. 7A**), demonstrated mature lysosomes to be precipitated solely on P2, whereas P1 was devoid of them. In addition, the unconventionally secreted protein pro-IL1b was shown to be excluded from the P2 fraction and to be contained within endolysosomal-related vesicles of the P1 fraction.

In murine forebrain cells, whether a pronounced discrimination of mature lysosomes can be made on the basis of centrifugation speed has yet to be unravelled.

B.4.2 Results

We repeated the previously employed protocol that yields 3 pellet fractions upon increasing centrifugation speeds (**Fig 8**).

Although both LAMP proteins and hydrolase enzymes are delivered to lysosomes via the endosomal pathway, hydrolases were shown to be more useful for lysosomal detection due to their not being detectable in the majority of LAMP-positive organelles (Cheng *et al.*, 2018). To that end, cathepsins B and D were employed to detect lysosomal presence in the different fractions. Cathepsins are synthesized as pre-proforms, with the pre- part being removed co-translationally and the proform undergoing glycosylations within the Golgi. Finally, the mature part is targeted to lysosomes (Katunuma, 2010).

As expected, LAMP1 was present in all pellet fractions. The mature form of Cathepsin B (31kDa) appeared enriched in the P2 pellet, alluding to an enrichment of mature lysosomes in that particular fraction. However, Cathepsin D staining revealed many bands, the pattern of which was identical in all three pellet fractions (**Fig. 10**). In order to ascertain which bands correspond to the mature form, the experiment was repeated upon incubation of the initial lysate with glycyl-L-phenylalanine 2-naphthylamide (GPN). This molecule gets degraded within lysosomes, thus leading to intralysosomal accumulation of hydrolysis products and osmotic degradation of the organelles (Berg *et al.*, 1994). In that context, the mature cathepsin form is expected to be released from lysosomes and would therefore not be precipitated upon centrifugation. However, no change was noted in the cathepsin pattern, either due to the fact that the treatment was not successful or due to non-specific binding of the antibody (data not shown).

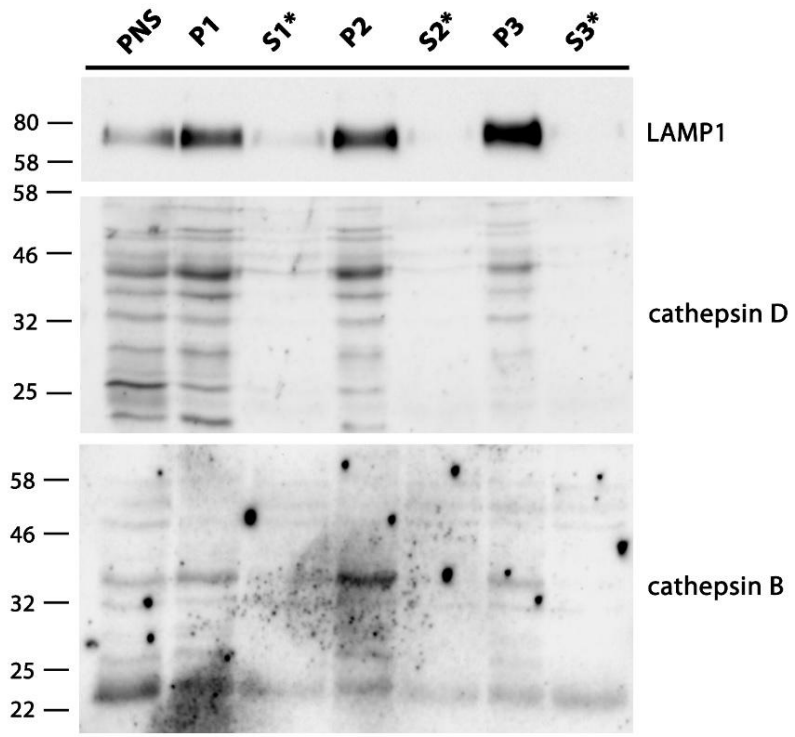

Figure 10. Lysosomal marker distrubution on sequential centrifugation-derived murine brain samples. Western blot analysis for LAMP1, Cathepsin B and Cathepsin D in samples acquired upon sequential centrifugations of forebrain lysate.

C. DISCUSSION

The current study aims to investigate the contribution of secretory autophagy to neuronal function, and specifically to plasma membrane protein targeting.

The point at which SEC22b, the secretory AV marker, is recruited on the outer AV membrane is not known. Our data allude to SEC22b being recruited on incomplete autophagosomal structures, whereas STX17 is known to be recruited to the external membrane of complete autophagosomes (Itakura, Kishi-Itakura and Mizushima, 2012). Although more experiments need to be conducted, these findings allude to a divergence between the two autophagosomal fates early in the biogenesis of the respective vesicles, a notion also corroborated by studies in yeast (Bruns *et al.*, 2011; Cruz-Garcia *et al.*, 2014).

Irrespectively, however, of their biogenesis, secretory vesicles are eventually targeted to the plasma membrane in a SEC22b-dependent fashion (Kimura *et al.*, 2017), whereas degradative ones fuse with lysosomes via STX17-including SNARE complexes (Itakura, Kishi-Itakura and Mizushima, 2012). Consistently, we found

STX17 and SEC22B markers to not colocalize both upon control and autophagy-inducing conditions, thereby corroborating the existence of distinct degradative and secretory vesicular populations.

Contrary to established notions of a protein-poor outer autophagosomal membrane, preliminary data of our lab demonstrate the outer membrane of forebrain-derived autophagosomes to be rich in integral proteins. Furthermore, these proteins localize both to the pre- and post-synapse and have a role in synaptic transmission. Among them, we find ionotropic glutamate receptors (e.g. AMPAR subunits GluR1, GluR2, GluR3 and NMDAR subunits NR1, NR2A, NR2B), metabotropic glutamate receptors (e.g. subunits mGluR2, mGluR3, mGluR5, mGluR7), ionotropic GABA receptors (e.g. $GABA_A$ subunits alpha-1, beta-2, gamma-2) and metabotropic GABA receptors (e.g. $GABA_B$ subunits 1 and 2) (unpublished data of our lab).

Interestingly enough, mRNAs for 75% of the identified proteins have previously been found to localize on dendrites and to be locally translated (Cajigas *et al.*, 2012). Given that transmembrane proteins reach the plasma membrane via vesicular intermediates and that dendrites are primarily devoid of Golgi structures (Hanus *et al.*, 2016; Bowen *et al.*, 2017), it is reasonable that unconventional targeting pathways would be employed in this scenario. Indeed, several synaptic molecules have been shown to bypass the Golgi apparatus en route to secretion (Bowen *et al.*, 2017). In addition, neuronal membrane proteins have been shown to demonstrate an enrichment in core glycosylated proteins; a pattern concordant with early stages of the glycosylation process and in stark contrast with the usually heavily glycosylated membrane proteins. In fact, trafficking of such proteins was shown to occur in a Golgi-independent fashion, although the exact mechanism remains elusive (Hanus *et al.*, 2016). Several findings, therefore, support the probability of a role of secretory autophagy in neuronal protein membrane targeting.

Generally speaking, during targeting of proteins to the plasma membrane, domains that face towards the ER lumen are eventually exposed on the cell surface, whereas the ones that face towards the cytoplasm do as across their journey (Cooper and Hausman, 2007). Consistently with this principle, the peptides of integral proteins that were found to be reduced upon PK treatment of autophagosomes, correspond to those that, according to Uniprot, face towards the cytoplasm once the protein has assumed its position in the plasma membrane (unpublished data of our lab). This finding alludes to positioning of proteins on the outer autophagosomal membrane in such a way that would allow their proper orientation in the target membrane upon fusion.

D. MATERIALS AND METHODS

Mice

For fractionation experiments, wild-type animals with a C57BL/6 genetic background were used. They were 2-6 months of age.

The mice were housed in a room with a 12 hr/12 hr light/dark cycle and free access to food and water. The experiments were conducted in accordance with the guidelines of the FORTH Animal Ethics Committee (FEC).

Western blot

Proteins were separated on a polyacrylamide gel (7.5-15%) and transferred to a nitrocellulose membrane with a 0.2 pore size (Millipore). Membranes were incubated in 5% BSA in PBS-T (100 mM Na_2HPO_4, 100 mM NaH_2PO_4, 0.5 N NaCl, 0.1% Tween-20) for 1 hour at room temperature, and then in 5% BSA in PBS-T with primary antibodies for 14-16 hours (4^{o}C). After three 10-min washes with PBS-T, membranes were incubated in 2% BSA with the corresponding secondary horseradish peroxidase-conjugated antibody (Abcam) for 1 hour at room temperature. After three 10-min washes with PBS-T, the membranes were developed by chemiluminescence (SuperSignal Chemiluminescent Substrate, West Pico and Femto, ThermoFisher Scientific ; ChemiGlow West, ProteinSimple) according to the manufacturer's instructions.

Huh7 cell cultures

Huh7 cells were plated in 12-well plates, which contained 15-mm glass coverslips previously coated overnight with poly-D-lysine (Sigma-Aldrich). The seeding concentration was 10^5cells/well, and they were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 3,5mM L-glutamine, 5 μg/μl penicillin and 12.5 μg/μl streptomycin. After incubation for 14-16 hours at 37^{o}C and 5% CO_2, cells underwent (or not) starvation, in which case they were washed with PBS and then incubated in starvation medium (DMEM without serum, or PBS).

Immunostaining

Huh7 cells cultured on coverslips were initially washed with PBS and then fixed by applying 4% paraformaldehyde (PFA) in PBS for 15-20 minutes (37^{o}C). Cells were, then, rehydrated by three 10-minute washes with PBS. Coverslips underwent incubation with blocking dilution (10% fetal bovine serum (FBS) and 0.2% Triton-X in PBS) for 1 hour at room temperature, followed by incubation with primary antibodies diluted in blocking solution for 14-16 hours (4^{o}C). After three ten-minute

washes with PBS, incubation with secondary antibodies dissolved in PBS ensued for 1 hour at room temperature. The secondary antibodies used were the following: Alexa 488 anti-rabbit (1: 1000), Alexa 594 anti-mouse (1: 1000). Finally, three ten-minute washes were carried out with PBS. Images Photographs of fluorescently labeled proteins were acquired on a confocal laser microscope (SP8 inverted confocal, Leica Microsystems).

Autophagosomal isolation from Huh7 cell line

The following was developed based off of previously described protocols (Strømhaug *et al.*, 1998; Nikoletopoulou *et al.*, 2017).

Huh7 cells were cultured in 10-50 culture dishes of 150mm diameter until they reached 80% confluency. Upon medium aspiration followed by a PBS wash, cells were scraped and collected in PBS in room temperature. After cell pelleting and PBS aspiration, cells were incubated for 10 minutes in Buffer A (10% (w/v) sucrose, 10mM HEPES and 1mM EDTA) in room temperature. Cellular homogenization was achieved in a Dounce homogenizer, by 150 strokes of the tight-fitting pestle. The homogenate was then diluted in homogenization buffer (HB) (250mM sucrose, 10mM HEPES, 1mM EDTA pH 7.3): HB was added in a volume half of the Buffer A and contained 1.5mM glycyl-L-phenylalanine 2-naphthylamide (GPN), so as for the resulting dilution to have a 0.5mM GPN concentration. The sample was incubated at 37°C for 10min for the lysosomes to be osmotically disrupted and then cooled at 4°C. From this point onwards, everything was done at 4°C. The homogenate was centrifuged at 2000g for 2min (4°C) and the supernatant was collected. 4ml of HB were added to the obtained nuclear pellet, and the centrifugation step was repeated. The resulting supernatant was combined with the previous one, to give a single post nuclear supernatant (PNS). Cytosol, mitochondria and peroxisomes were removed with discontinuous Nycodenz gradients: the gradient comprised 7ml of 22.5% Nycodenz (1.127 g/ml) and 17ml of 9.5% Nycodenz (1,072 g/ml), while the 10-11ml of PNS were layered on top. Upon centrifugation at 141.000g for 1 hour at 4°C, the interface containing autophagosomes and endoplasmic reticulum was isolated and diluted with an equal volume of HB buffer. The dilution was centrifuged for 20 minutes at 13.000g (4°C), and the resulting pellets were resuspended in a final volume of 1ml HB. ER removal was achieved with Nycodenz-Percoll gradients: the gradient comprised 0.86ml of 22.5% Nycodenz (1,127 g/ml) at the bottom and 2.57ml of 33%Percoll on top, while 0.98ml of the sample was layered at the top. The material was then centrifuged for 30 minutes at 72.000g (4°C), and the interface was collected. The material was diluted with 0.7V of 60% Optiprep, and removal of Percoll silica particles was realized by placing 2.06ml of the diluted material overlayed with 0.34ml of 30% Optiprep and a top layer of 0.6ml HB. The material was then centrifuged for 30min at 71.000g (4°C), resulting in sedimented Percoll

particles at the bottom of the tube and an autophagosome band floating at the Optiprep/HB interface. Autophagosomes were collected and diluted in three volumes of HB buffer. Upon gentle mixture, the material was centrifuged for 20 minutes at 13.000g (4°C). The supernatant was largely removed, and the pellet was resuspended in the remaining 15-20ul. The resulting samples were frozen in liquid nitrogen and stored for downstream manipulations.

Fractionation experiments

The following was adapted from previously described protocols (Andrei *et al.*, 1999; Cheng *et al.*, 2018).

Adult mice of a C57BL/6 genetic background were employed. Cortices and hippocampi were isolated on ice, and were washed with homogenization buffer (HB) [250mM sucrose, 20mM Tris-HCl (pH 7.4), 1mM EGTA, 1mM EDTA]. They were then resuspended in HB (7.5ml HB per 1g of tissue) and transferred to a Dounce glass homogenizer. Homogenization was achieved by 20 strokes with the tight-fitting pestle, and the sample was centrifuged for 5 minutes at 800g (4^{o}C) to remove unbroken cells. The supernatant underwent an additional centrifugation for 5 minutes at 1000g (4^{o}C) for the removal of cellular debris, and the process was repeated for 5 minutes at 1200g (4^{o}C) so as to achieve removal of nuclei. The resulting supernatant was diluted 10-fold in HB and was then centrifuged for 1 minute at 35.000g (4^{o}C). Samples of the resulting pellet (P1) and supernatant (S1) were retained for analysis, and S1 underwent an additional centrifugation round for 5 minutes at 50.000g (4^{o}C), thereby yielding P2 and S2. S2 was further centrifuged for 5 minutes at 100.000g (4^{o}C), thus producing P3 and S3.

Carbonate extraction

The autophagosomal sample underwent centrifugation for 30 minutes at 13.000g (4^{o}C), so as to pellet autophagic vesicles. Upon supernatant aspiration, the pellet was incubated with freshly prepared, ice-cold Na_2CO_3 (0.1M) (200ul per 100ug of protein) for 30 minutes (4^{o}C). The sample was then centrifuged in an airfuge, at 20psi for 30 minutes (4^{o}C). The resulting pellet contained the membrane fraction of the organelles, whereas the soluble one was found in the supernatant.

Prior to analysis, the supernatant was concentrated with a trichloroacetic acid (TCA) precipitation protocol. Briefly, TCA was added to the sample in a 10% w/v final concentration, and was incubated for 20 minutes at 4^{o}C. After a centrifugation for 20 minutes at 13.000g (4^{o}C), the supernatant was removed and 200ul of 100% acetone were added to the pellet. Upon centrifugation for 20 minutes at 13.000g (4^{o}C), the supernatant was removed and the pellet was left to air-dry for 5 minutes. The pellet was finally resuspended in 2x Laemli buffer.

Statistical analysis

Analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). Data were expressed as means ± S.D., and statistical significance was determined using Student's t-test or ANOVA. Differences with $p<0.05$ were deemed to be statistically significant.

E. LITERATURE

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