The Cell's Recycling Center: Unveiling the Secrets of Autophagy

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CHAPTER 1. AUTOPHAGY: A PROCESS ESSENTIAL FOR CELLULAR HOMEOSTASIS AND SURVIVAL

Macroautophagy (here referred to as “autophagy”) is a highly conserved cellular process used to degrade misfolded proteins and damaged organelles. It also serves to recycle amino acids and other metabolites during nutrient starvation, and to destroy invading pathogens (Cecconi & Levine, 2008; Dooley et al., 2014; Susmita Kaushik & Ana Maria Cuervo, 2018; Mizushima, 2018; Richter et al., 2016). The molecular machinery, the ATG family of proteins, involved in this process was first identified in 1990s in yeast (Ohsumi, 2014), and several conserved mammalian homologues of these core autophagy proteins have since been identified in higher organisms. Basic steps in the autophagy pathway – ‘formation’ i.e. biogenesis of the phagophore, known previously as an isolation membrane, followed by phagophore membrane ‘extension’ and ‘closure’ to form a nascent autophagosome, which subsequently matures through fusion with an endosome to form an amphisome and finally, the lysosome to form an autolysosome, have been conserved across eukaryotic organisms. Interestingly, in yeast, autophagosomes form at a specific site, called the PAS (Phagophore Assembly Site), and no active transport of these structures towards the vacuole (lysosome in mammals) is required. Thus, microtubule- based molecular motors are dispensable for autophagy in yeast. However, in mammalian cells, *de novo* formed autophagosomes need to be actively transported to lysosomal compartment usually located near the center of the cell by microtubule-based motors, particularly, the minus-end directed motor, dynein. Thus, mammalian autophagy also requires contribution of microtubule motor proteins for maturation and completion of the autophagy pathway. Recent studies have shown that post-mitotic mammalian neurons exhibit high levels of basal autophagy. Several neurodevelopmental and degenerative disorders have been shown to arise from defects in the autophagy pathway (Bordi et al., 2016; S. Gowrishankar et al., 2015; Narendra, Tanaka,

Suen, & Youle, 2008). Given the highly polarized morphology of neurons, and sequestration of neuronal lysosomes mostly in the soma, microtubule-based transport of autophagosomes formed distally along the neurites or at growth cones becomes critical for delivery to and degradation of autophagosomes by the lysosomal compartment. However, the molecular machinery involved in autophagosome transport in neuronal and non-neuronal cells, as well as the molecular mechanisms involved in motor recruitment to autophagosomes are only beginning to be understood (X.-T. Cheng, B. Zhou, M.-Y. Lin, Q. Cai, & Z.-H. Sheng, 2015; Fu, Nirschl, & Holzbaur, 2014).

Autophagy has been classified into several sub-types depending on the molecular mechanism and specialized machinery involved, or the type of cellular cargoes being engulfed by the autophagosomes. *Macroautophagy* was first identified as a non-selective process used for bulk degradation of cytoplasmic contents to recycle basic building blocks in cells. This process involves formation of a double-membraned autophagosome around cytoplasmic organelles, cytosolic proteins, or invading microbes, and the subsequent degradation of these autophagic contents by lysosomes, whereupon the amino acids and the breakdown products from the lysosomal lumen are recycled for synthesizing new building blocks for cells to prolong survival during stress or starvation (Yuchen Feng, He, Yao, & Klionsky, 2014). This is the most prevalent form of autophagy, and it is usually triggered by starvation or nutrient deprivation. My work focuses on this branch of autophagy, and molecular details of this process are discussed later in this chapter.

Microautophagy is a non-selective degradative process that involves direct engulfment of soluble autophagic cargoes into the lytic compartment. Microautophagy is employed by cells to maintain membrane homeostasis, organelle size, and to promote survival under nitrogen restriction. During starvation induced microautophagy, extended membranes on the surface of the lysosome rapidly invaginate to form structures called ‘autophagic tubes’. These autophagic tubes form a constriction at the neck and eventually undergo scission to capture autophagic cargo

for degradation (W.-w. Li, Li, & Bao, 2012). Recent studies have revealed that in steady state, homeostatic cells growing in nutrient-rich conditions, autophagy can be used for selective sequestration of specific subcellular cargoes such as aggregated proteins, pathogens, excess or damaged organelles such as peroxisomes (pexophagy), ER (ER-phagy), mitochondria (mitophagy), Lipid droplets (lipophagy), ribosomes (ribophagy) and a variety of pathogens (xenophagy) (Ding et al., 2010) (Kudchodkar & Levine, 2009) (Singh et al., 2009) (MacIntosh & Bassham, 2011) (Reef et al., 2006) (Khaminets, Behl, & Dikic, 2016; Kraft, Reggiori, & Peter, 2009; Rogov, Dotsch, Johansen, & Kirkin, 2014). These selective autophagy processes occur in nutrient-rich growth conditions, where homeostatic cells get rid of their damaged or excess organelles and invading pathogens. Several studies have now shown that defects in these processes might be causal in many neurodegenerative diseases including Parkinson's disease, Alzheimer's disease and Huntington's disease; cancers, heart and liver diseases and aging (Hübner & Dikic, 2020; Kounakis, Chaniotakis, Markaki, & Tavernarakis, 2019; Um & Yun, 2017).

Chaperone-mediated Autophagy (CMA) is an atypical form of autophagy, where proteins marked for lysosomal degradation contain a degradation tag - the KFERQ motif. These proteins are imported into the lysosomal lumen through a sophisticated mechanism involving chaperones and lysosomal transmembrane channel proteins (S. Kaushik & A. M. Cuervo, 2018). During CMA, the KFERQ-like motif containing proteins are selectively delivered to the lysosome by the HSC70 chaperone complex that includes co-chaperones such as HSC40, HSP90, HSP70-HSP90 organizing protein (HOP) and HSP70-interacting protein (HIP). The HSC70 chaperone complex unfolds the degradative protein substrates before delivering them for lysosomal membrane internalization. The degradative cargoes are internalized into the lysosome via the transmembrane, tetrameric channel LAMP2A (Lysosome-associated membrane protein type 2A), whose C-terminal 12aa (amino acid) cytoplasmic tail is required for the docking of HSC70-substrate complex on the lysosomal membrane. Upon tetramerization of the LAMP2A channel, the substrate is released from the LAMP2A tail and translocated into the lysosomal lumen, where

it is degraded (S. Kaushik & A. M. Cuervo, 2018). For the purpose of this study, I have focused on Macroautophagy, hereon referred to as 'Autophagy'.

1.1. Basic Steps in the Autophagy Pathway

Autophagy involves *de novo* in situ formation of a double membraned autophagosome (AP) that encloses cytoplasmic 'cargo' typically marked for degradation through ubiquitination. The formation of this double-membraned autophagosome involves distinct transitional stages namely initiation, elongation and closure, each of which require the contribution of complex multiprotein assemblies.

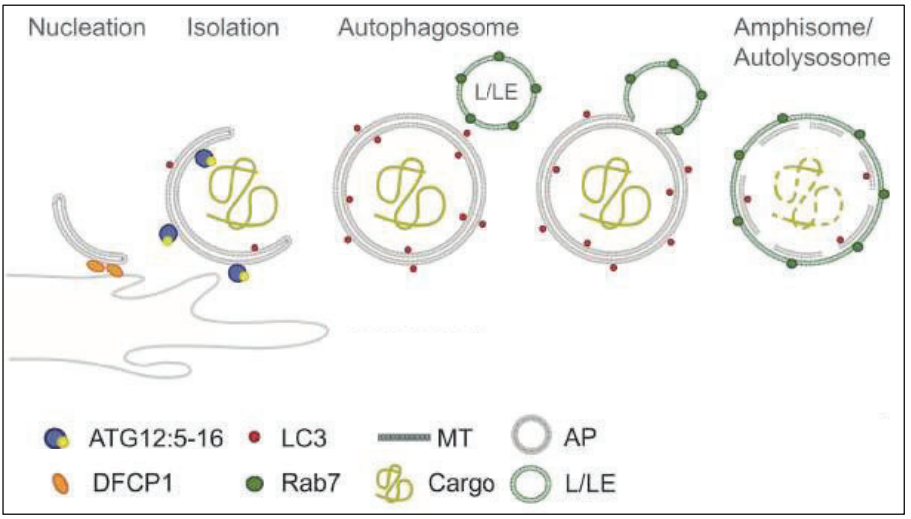

Figure 1. Basic Steps in the Autophagy Pathway.
Autophagosome formation begins at ER nucleation sites decorated with early autophagy proteins such as DFCP1. The isolation membrane (phagophore), labeled with the ATG12:5-16L1 tripartite complex, extends to enclose the autophagic cargo into a fully-formed double-membraned autophagosome, decorated with lipidated LC3 molecules. This nascent autophagosome then fuses with endosomes and lysosomes, to form amphisomes and autophagolysosomes, respectively, where the autophagosomal luminal cargo is degraded by lysosomal proteases.
(*key:* DFCP1: Double FYVE domain containing protein-1, AP: Autophagosome, L/LE: Lysosome/Late Endosome, MT: Microtubule)

Sites of Autophagosome Formation

Initiation

Autophagy machinery is recruited to Phagophore Assembly site (PAS) in yeast or omegasomes in mammals to initiate nucleation of the phagophore (Dikic & Elazar, 2018). Class III PI-3 Kinases play critical roles in regulating membrane trafficking in mammalian cells, including in autophagy **(Lindmo & Stenmark, 2006)**. VPS34, a PI-3 kinase and its accessory protein Beclin-1 (VPS30), are essential for induction of autophagy (Akio Kihara, Takeshi Noda, Naotada Ishihara, & Yoshinori Ohsumi, 2001) (Koyama-Honda, Itakura, Fujiwara, & Mizushima, 2013). Inhibition of PI 3-kinases using small molecule inhibitors such as LY294002, 3-Methyladenine and Wortmannin is sufficient to inhibit autophagy (Blommaart, Krause, Schellens, Vreeling-Sindelarova, & Meijer, 1997). Induction of autophagy results in the activation of the PI3KC3 (Phosphatidylinositol 3-kinase catalytic subunit type 3), which is the catalytic subunit of the PI3K complex. The PI3K complex consists of VPS34, VPS15, Beclin1 and ATG14, which through VPS34-mediated phosphorylation of phosphatidylinositols on the ER membrane, generates PI3P (Phosphatidylinositol 3-phosphate). PI3P in turn serves to accumulate autophagic machinery at that ER site (A. Kihara, T. Noda, N. Ishihara, & Y. Ohsumi, 2001). On mammalian autophagosomes, this lipid is predominantly present in the outer leaflet of the AP membrane, while in yeast it is present on the inner leaflet, suggesting evolutionary differences in the mechanism of AP formation from yeast to mammals (Cheng et al., 2014). The formation of PI3P-rich domains within the ER membrane results in the enrichment of FYVE zinc finger domain containing protein (DFCP1) (Hayashi-Nishino et al., 2009; Ylä-Anttila, Vihinen, Jokitalo, & Eskelinen, 2009). The translocation of DFCP1 from the cytoplasm to the PI3P-rich ER membrane subdomains can also be triggered in response to Beclin1 and VPS34 activity and can be enhanced in the presence of extracellular cues such as nutrient starvation. The domain within DFCP1 spanning amino acids 416-543 is sufficient for its localization to ER, independent of its two FYVE domains (Axe et al., 2008; Cheung, Trinkle-Mulcahy, Cohen, & Lucocq, 2001). In

addition to ER membranes, recent studies have shown that APs can be formed from other membranous compartments in a cell including mitochondria, Golgi, ERGIC, plasma membrane, recycling endosomes as well as ER exit sites **(Abada & Elazar, 2014; Lindmo & Stenmark, 2006)**.

Machinery involved in building the autophagosome

As a first step in mammalian autophagosome nucleation, Ulk1 complex: UNC-51 like kinases along with FIP200, ATG13, ATG101, is recruited to the site of phagophore formation (Chan, Longatti, McKnight, & Tooze, 2009). Secondly, PI3K complex (which contains VPS34) is recruited to the phagophore formation site. Following mTOR inhibition or amino acid starvation, Ulk1 is dephosphorylated, which results in its activation. Ulk1 in turn phosphorylates Beclin1 on Serine 14, which activates the PI3K complex (Russell et al., 2013). Finally, the ATG5:12-16L1 complex is recruited to this site to enable elongation of the autophagic membrane. The FIP200 subunit of Ulk1 complex is able to interact with ATG5, which may help in concurrent recruitment of the Ulk1 and ATG5:12-16 complexes to the phagophore (Gammoh, Florey, Overholtzer, & Jiang, 2013) (Nishimura et al., 2013). Mammalian WD repeat domain Phosphoinositide-interacting proteins (WIPIs) have been shown to recruit ATG16L1 to the expanding phagophore. The interaction site within ATG16L1 that binds WIPI2 is distinct from its binding sites for ATG5:12 complex. The ATG5:12-16L1 complex is essential for lipidation of the ATG8 homologues LC3 and GABARAPs onto the growing isolation membrane. The nascent pro-ATG8 proteins (pro-LC3 in mammals) are cleaved by a cysteine protease, ATG4, at their C-terminal domains to expose a glycine residue that is essential for conjugation to PE (Slobodkin & Elazar, 2013). Four different ATG4 homologues exist: ATG4B has been shown to possess widest spectrum of substrates including LC3, ATG4A is more specific to GABARAPs and ATG4C and D show minimal activities (M. Li et al., 2011). The ATG4-processed ATG8s are then activated by an E1- Ubiquitin ligase like enzyme ATG7, and conjugated to PE on the isolation membrane through the activity of another Ub-like enzyme ATG3 (Hamasaki et al., 2013). This process converts nascent free-

floating pro-ATG8 homologues (LC3-I) to their membrane-tethered lipidated LC3-II form. ATG7 activates ATG12 and covalently conjugates it to ATG5 through the activity of an E2 -Ub ligase like enzyme ATG10. This ATG5:12 complex then acts like an E3 Ub-ligase complex that serves to facilitate the process of lipidation *in vivo*.

Autophagosome Membrane Elongation and Membrane Sources

The size of the fully-formed double membraned autophagosome is variable, and chiefly dependent on the size of the cargo that is engulfed by the autophagosome. The AP cargoes range from small precursor aminopeptidases (~150nm) to cytoplasmic organelles such as mitochondria and bacteria which can be several micrometers in size (Stolz, Ernst, & Dikic, 2014). After the initiation of autophagosome formation, the phagophore membrane is further expanded by membranes originating from the Golgi, plasma membrane, and recycling endosomes (Lamb, Yoshimori, & Tooze, 2013). The AP membrane expansion can occur through three distinct molecular pathways involving Class III PI3 Kinases, ATG9 and RAB proteins, each of which are located on the Golgi apparatus.

Recent studies have also implicated the ER-Golgi Intermediate compartment (ERGIC) and the COP-II coated vesicular trafficking from Golgi in AP membrane expansion. In particular, ERGIC recruits an early AP nucleation component ATG14, and is both necessary and sufficient for AP formation *in vitro* as well as *in vivo* (Ge, Melville, Zhang, & Schekman, 2013).

ATG9, the only multi-spanning transmembrane ATG family protein, is also critical for AP membrane expansion. ATG9A is enriched in the trans golgi network (TGN) and is exported from the TGN to the periphery of the cell by a multi-subunit adaptor AP-4 (Mattera, Park, De Pace, Guardia, & Bonifacino, 2017). In the absence of AP-4, ATG9 accumulates in the TGN, which in turn leads to a decrease in LC3-I to LC3-II conversion and defects in AP formation, as well as accumulation of autophagic substrates in the cell (De Pace et al., 2018). ATG9 also localizes to the recycling endosome compartment. Syntaxin-18 (SNX18), a membrane remodeling protein, is involved in the membrane tubulation from recycling endosomes, to which ATG9 is recruited and

eventually trafficked out of the recycling endosome back to TGN (Soreng et al., 2018). SNX-18 interacts with dynamin-2 for the eventual scission of ATG9-containing tubules which are also positive for ATG16L1. These proteins are then transported to the sites of AP formation which have been shown to be WIPI-2 positive. However, the motors and/or adaptors involved in the tubulation of ATG9-ATG16L1 - positive membranes from REs and their subsequent transport are yet unknown. Under nutrient-rich, steady state conditions, ATG9 is localized to trans golgi network, recycling endosomes and late endosomes, and shows extensive colocalization with TGN-46, Rab7 and Rab9 (Young et al., 2006). Upon nutrient starvation, ATG9 gets redistributed from the TGN onto the late endosomes and vesicular/ tubular compartments near Golgi through the action of BAR protein BAX-interacting Factor-1 (BIF1) (Y. Takahashi et al., 2011). The ATG9-rich vesiculotubular structures are recruited to the phagophore membrane independent of the early ATG proteins such as ULK1 and WIPI2. However, they show a dynamic interaction with phagophore membranes rather than stable structural incorporation into the growing phagophore membrane. Thus, ATG9 compartments may predominantly provide the membrane required for the expansion of the phagophore through transient interactions (Orsi et al., 2012).

At least two Rab proteins associated with the golgi apparatus have been shown to participate in phagophore membrane expansion. GTP-bound active form of Rab1 is essential for the formation of DFCP1-positive nucleation sites on the ER. Another protein, Rab33B has also been shown to directly interact with ATG16L1, and is required for AP membrane expansion, however the exact molecular mechanisms involved remain to be elucidated (Itoh et al., 2008; Mochizuki et al., 2013).

Finally, plasma membrane derived Clathrin-coated vesicles (CCVs) can also provide membrane lipids to the growing phagophore. These newly-formed CCVs become ATG16L1 positive but remain EEA1-negative and grow in size through homotypic fusion facilitated by vesicle membrane associated protein 7 (VAMP-7); and eventually may acquire LC3 (Moreau, Ravikumar, Renna, Puri, & Rubinsztein, 2011). Recycling endosomes may also contribute to AP

membrane expansion through TBC1D14 and Syntaxin18 (Knævelsrud et al., 2013; Longatti et al., 2012).

Before the closure of the phagophore membrane, the ATG family proteins dissociate from it, while LC3 and its homologues persist on the fully-formed AP (Klionsky, 2005). Lipidated LC3 is enriched on the inner membrane of the post-closure AP and remains associated until AP degradation by the lysosomal proteases.

After the closure of the newly formed autophagosome, it has to be severed from the membrane source from which it was formed. The machinery involved in this process is only beginning to be identified. Recent evidence suggests that in the final stages of autophagosome formation, ESCRT-III component CHMP2A localizes to the phagophore membrane and mediates the abscission of this membrane. Inhibition of the AAA-ATPase VPS4 which is required for the assembly of functional ESCRT-III complexes also impedes the formation of a fully-formed autophagosome (Yoshinori Takahashi et al., 2018). Further work reveals that Rab5-dependent recruitment of ATG17- vacuolar-sorting protein Snf7 complex to APs results in ESCRT-mediated abscission of AP membrane and subsequent closure (Zhou et al., 2019).

Autophagosome Maturation and Fusion

AP-Lysosome fusion results in the release of the single-membraned autophagic body into the lumen of the lysosome (Dikic & Elazar, 2018). Fully-formed APs first fuse with late endosomes to form amphisomes. Rab11 decorates multivesicular bodies (MVBs) and is necessary for the fusion between APs and MVBs. The v-SNARE VAMP3 is required for AP-MVB fusion but not for the fusion of the resulting amphisome with the lysosome. This latter fusion event requires another v-SNARE protein VAMP7 (Fader, Sánchez, Mestre, & Colombo, 2009). Depleting the ESCRT complex subunits results in decreased autophagic degradation in cells. Especially, mutations in the ESCRT-III subunit CHMP2B also result in the accumulation of ubiquitin-p62-ALFY positive aggregates in cells, suggesting an impaired AP turnover (Filimonenko et al., 2007). APs show two types of fusions with lysosomes: complete fusions and kiss-and-run fusions, where only

partial contents of the AP lumen are transferred to the lysosome and both AP and lysosome still persist as two independent organelles (Jahreiss, Menzies, & Rubinsztein, 2008).

Lysosomal distribution in the cytoplasm is sensitive to the cytoplasmic pH. Starvation makes the cytoplasm more alkaline and induces the movement of lysosomes towards the MTOC, i.e. the cell center. For example, upon acidification of cytoplasmic pH to 6.5 due to external stimuli such as acetate treatment, the lysosomes move out towards the very periphery of the cell but return to cell center upon removal of the stimulus (Heuser, 1989; Korolchuk et al., 2011). Thus, upon starvation, the lysosomal compartment is sequestered closer to the MTOC, and newly formed autophagosomes have to be actively transported in the retrograde direction by cytoplasmic dynein towards the lysosomal compartment for their turnover. Thus, starvation might induce dynein-mediated retrograde transport of autophagosomes to increase autophagic flux, a possibility not yet tested experimentally.

Regeneration of the Lysosome from Autophagolysosome

During autophagy, autophagosomes fuse with lysosomes to get their luminal content degraded by the lysosomal proteases. Thus, at the peak of autophagy, APs fuse with lysosomes to form autophagolysosomes (APLs). However, it can be discerned that there is a relative decrease in the total cellular lysosomal pool upon APL formation, and lysosomes have to be reformed after the attenuation of autophagy to restore a steady state pool of cellular lysosomes.

Lysosomal efflux permeases are required for reactivating mTOR kinase and restoring homeostasis. One such efflux permease, named *Spinster*, localizes to the mammalian Lamp1-positive lysosomal membrane. Depletion of spinster from cells results in enlargement of lysosomes, which is even more pronounced upon nutrient starvation. Studies show that in the absence of spinster activity, the autophagic lysosomal reformation in cells in inhibited (Rong et al., 2011).

Lysosomal reformation from APL starts with the initiation of tubulation from the APL membrane. This process of tubulation is mediated by kinesin motor Kif5B. Knockdown of Kif5B and introduction of the motility-defective Kif5B mutant both prevent APL tubulation, suggesting that this particular kinesin motor is essential for APL tubulation. Kif5B directly interacts with PIP2 and is enriched on the PIP2 microdomains on the vesicular membrane. Clathrin and PIP5K1B (Phosphatidylinositol-4-Phosphate 5-Kinase Type 1 Beta) mediate formation of PIP2-rich membrane subdomains on the surface of autolysosomes, which in turn can be bound by Kif5b. In this manner, clathrin may promote kinesin recruitment to APLs and induce tubulation to reform lysosomes (Du et al., 2016). PIP5K1B helps convert PI(4)P to PIP2. Knock down of PIP5K1B indeed results in the reduction of clathrin recruitment to autolysosomes. It is also observed that PIP2 is selectively enriched onto the buds coming off of APL membranes, while PI4P is ubiquitously present on APL membranes. Thus, it appears that PIP5KB1 regulates membrane lipid composition to initiate tubulation and eventual lysosomal reformation.

PIP5K1A (Phosphatidylinositol-4-Phosphate 5-Kinase Type 1 Alpha) also shows a striking localization to the membrane buds i.e. tubulation, and has been shown to be required for the pinching-off of the proto-lysosomes from the APL. Consistent of a role for Clathrin-PIP2 interaction for proto-lysosome fission, depletion of AP2 also results in a block in formation of ‘reformation tubules’ (which eventually form lysosomes) (Rong et al., 2012). This process of reforming the full complement of lysosomes post-autophagy induction is mTOR-dependent. Upon prolonged starvation and induction of autophagy, lysosomal degradation and recycling of APL cargoes reactivates mTOR and induces the reformation of lysosomes (L. Yu et al., 2010).

It has also been shown that the large GTPase Dynamin 2/ DNM2 is required for the scission of proto-lysosomes from the reformation tubules. Acute or chronic inhibition of Dynamin 2 results in four- to five-fold increase in the size of APLs. When DNM2 inhibition is combined with nutrient starvation, it results in extensive tubulation from the APL compartment. Thus, lysosomal reformation is increased as autophagic flux increases upon nutrient starvation. The APL tubules

show a striking localization of DNM2 along their length. When the wild type DNM2 is expressed in cells, this protein localizes to tubules and also shows scission of these tubules into small vesicular structures i.e. proto-lysosomes (Schulze et al., 2013). PI4KIIIbeta (Phosphatidylinositol 4-kinase III beta) with an active kinase domain plays an important role in negative regulation of the APL tubulation. Knockdown of PI4KIIIbeta results in extensive APL tubulation, but no proto-lysosome formation, which in turn causes a loss of lysosomal contents, and failure in maintaining a steady lysosomal pool (Sridhar et al., 2013). mTOR kinase also controls APL membrane tubulation through VPS34 kinase. In nutrient-rich conditions, mTOR kinase directly phosphorylates UVRAG at two serine residues. Phosphorylated UVRAG then binds VPS34 kinase, and activates it, which results in inhibition of APL tubulation. However, upon nutrient starvation and mTOR inhibition, UVRAG and VPS34 kinase complex is deactivated, and tubulation is induced. mTOR, in this way, also promotes scission of newly formed lysosomes by activating UVRAG. This in turn increases the pool of PI3P on APL membrane, the lipid that marks the site of tubule scission by dynamin. This lysosome-localized PI(3)P generated by UVRAG-VPS34 kinase complex has also been shown to be important for the scission of proto-lysosomes (Munson et al., 2015).

A separate study shows two new proteins, Spastizin (SPZ15) and Spatacsin (SPZ11), upon depletion, cause an enlargement of LAMP1 positive compartments in cells. In *wt* cells, prolonged starvation and attenuation of autophagy results in restoration of the size of the autophagolysosomes. However, in Spastizin and Spatacsin depleted cells, the APLs remain enlarged and do not get cleared. Finally, depletion of these proteins results in decreased ALR in cells upon short-term starvation and induction of autophagy (Chang, Lee, & Blackstone, 2014). Consistent with this, Spatacsin knockout in mice results in severe neuronal loss in the cerebral cortex and the cerebellum, mimicking spastic paraplegia-like features. In these mice, Purkinje cells accumulate abnormal large APLs, which in turn has been shown to decrease the regeneration of lysosomes and deplete their steady state pool in these cells (Varga et al., 2015).

Thus, a new set of proteins are being discovered that play an important role in lysosomal reformation from the autophagolysosomes. The process of ALR seems to be critical for maintaining an active steady state pool of lysosomes in cells, and for an efficient turnover of the newly forming autophagosomes. Defects in ALR might be causal for a steady depletion of lysosomal pool in aging and diseased cells, a possibility not yet explored.

1.2. Microtubule-based Transport of Autophagosomes

The Phagophore assembly site (PAS) in yeast is located on the vacuole (similar to the lysosome in mammals) and as such, the APs formed in yeast do not rely on active microtubule-based transport by retrograde motors to reach the vacuole. Unlike in yeast, mammalian autophagosomes can form at diverse sites within the cytoplasm, distant from the lysosomal compartment which is usually located close to the center of the cell. Thus, in mammals, autophagosomes have to be actively transported by the MT-minus end directed motor towards the lysosomal compartment for degradation of the autophagic cargo (Hollenbeck, 1993). Directional transport of autophagosomes is even more critical for the autophagy pathway in highly asymmetric, polarized cells such as neurons (Chklovskii, 2004). The Cathepsin-positive degradative compartment is restricted to neuronal cell body, while the autophagosomes can form at any site within the soma or the neurites, which tend to extend for several hundreds of micrometers *in vivo (Swetha Gowrishankar et al., 2015; Yap, Digilio, McMahon, Garcia, & Winckler, 2018b)*. Thus, especially in neurons, dynein-mediated transport of APs in axons and potentially, dynein and kinesin-mediated transport of APs in dendrites (given the mixed polarity of microtubules) is critical for AP turnover (Millecamps & Julien, 2013). Several recent studies in mammalian neurons have shown that cytoplasmic dynein adaptor proteins have the ability to directly bind Rab7 on the late, post-LE fusion autophagolysosomes, as well as to LC3 on nascent autophagosomes. Thus, both APs and APLs can be actively transported along microtubules towards the lysosomal compartment for their degradation. One study has shown that JIP1, a

motor adaptor protein, recruits both the anterograde motor kinesin and the dynein accessory protein, dynactin. JIP1 is required for the retrograde transport of a subset of APs in axons (Fu et al., 2014). Phosphorylation of a serine residue within JIP1 acts as a switch between retrograde and anterograde transport, thus allowing bidirectional transport of autophagosomes in neurons. In addition, LC3 binding to JIP1 through its conserved LIR (LC3-Interacting Region) motif also acts as a regulatory mechanism to prevent kinesin binding to JIP1, and to ensure a robust retrograde transport of APs within the axon.

Another study on a motor adaptor protein SNAPIN proposes a different mechanism for AP transport. SNAPIN associates with the late endosomal membranes and recruits dynein to LEs. Upon LE fusion with APs, the SNAPIN bound motor is in turn acquired by the newly formed autophagolysosome (APL) which then starts moving retrogradely towards the cell body of the neuron (X. T. Cheng, B. Zhou, M. Y. Lin, Q. Cai, & Z. H. Sheng, 2015). Furthermore, in keeping with this model, inhibition of AP-LE fusion by knocking down Syntaxin-17, results in decreased acquisition of dynein by APs, inhibition of their retrograde transport and consequently, accumulation of APs in the distal axon of DRG neurons.

Distally formed neuronal autophagosomes are also involved in the AP-2 mediated retrograde transport of BDNF-activated TrkB receptors to neuronal soma (Kononenko et al., 2017). This function is important for neuronal survival, and for maintaining axonal integrity and preventing axonal degeneration.

There is also a possibility that the components of the core autophagy machinery might be actively transported by microtubule motor- based transport to subcellular sites of AP formation. However, the specific motors and adaptors involved in this process need to be investigated.

1.3. Regulation of Autophagy Pathway in Mammals

The process of autophagy is highly regulated in mammalian cells. During homeostatic conditions, autophagy is suppressed, however basal low levels of autophagy are detected in cells.

Upon nutrient deprivation or acute stress, autophagy is upregulated which triggers formation of new autophagosomes and degradation of ubiquitinated substrates. The autophagy pathway is controlled by a number of different cellular signaling pathways, which sense and respond to cell stress and nutrient availability, amongst other environmental cues. The major autophagy regulator is mTOR (mechanistic target of rapamycin) kinase, which negatively regulates autophagy during homeostatic conditions.

mechanistic Target of Rapamycin (mTOR)

mTOR kinase serves a critical role in sensing nutrient deprivation and other forms of stress. Inhibition of mTOR activates autophagy and prolongs cell survival. However, how mTOR regulates neuronal autophagy remains a critical, but poorly understood question (J. Kim, M. Kundu, B. Viollet, & K.-L. Guan, 2011; Maday & Holzbaur, 2016; Robert A. Saxton & Sabatini, 2017).

Several studies in non-neuronal cells have addressed the mechanism of mTOR control of autophagy pathway. During steady-state growth conditions, mTOR constitutively inhibits autophagy by phosphorylating several key protein kinases required to initiate autophagosome formation. mTOR phosphorylates ULK1 kinase, which prevents its association with autophagy proteins FIP200, ATG101 and ATG13. This in turn inhibits phagophore initiation and blocks AP formation (J. Kim, M. Kundu, B. Viollet, & K. L. Guan, 2011). In nutrient-rich conditions, an active mTOR kinase also phosphorylates TF-EB, a transcription factor required for the transcription of lysosomal biogenesis and autophagy-related genes (A. Roczniak-Ferguson et al., 2012). During nutrient-rich conditions, mTOR phosphorylation of TF-EB prevents its nuclear translocation, thus blocking the transcription of TF-EB downstream target genes. Inhibition of mTOR kinase or nutrient starvation results in TF-EB translocation to the lysosomal membrane where it co-localizes with mTOR, and finally to the nucleus. In steady state cells, mTOR phosphorylates TF-EB at Serine 211, which induces TF-EB binding to 14-3-3 proteins and its retention in the cytoplasm. Inhibition of mTOR signaling upon starvation results in decreased TF-EB phosphorylation and

loss of 14-3-3 mediated retention of the transcription factor in the cytoplasm, leading to its nuclear translocation (Agnes Roczniak-Ferguson et al., 2012). This is an obligate step to upregulate autophagy-specific genes. Consistent with this finding, depletion of TF-EB prevents the upregulation of lysosome biogenesis and autophagy genes in cells even upon nutrient starvation (Settembre et al., 2012). Upon amino acid feeding, and mTOR kinase reactivation, mTOR phosphorylates nuclear TF-EB on Serine residues 138 and 142 in close proximity of a nuclear export signal, which induces its CRM1-mediated export from the nucleus.

mTOR plays a critical role in nutrient sensing in mammalian cells. Rag GTPases (RagA, B, C and D) are Ras family proteins that control mTOR signaling pathway *via* amino acid sensing. These proteins regulate mTOR localization to the Rab7-positive compartment in cells, which brings mTOR spatially closer to its activator, Rheb (Sancak et al., 2008). Constitutively active RagB expression induces hyperactivation of mTOR kinase and its constitutive localization at the Rab7-positive membranes, irrespective of amino acid levels in cells, thus making mTOR resistant to amino acid signaling (Sancak et al., 2008). Ragulator, which consists of a multi-subunit protein complex, including p14, p18, MP1, C9orf52 and HBXIP, promotes GTP nucleotide loading onto the RagA-B homodimeric complex, thus serving as a GEF for RagA and RagB (L. Bar-Peled, Lawrence D. Schweitzer, R. Zoncu, & David M. Sabatini, 2012). This interaction between Rag GTPases and Ragulator becomes stronger upon amino acid starvation and weakens upon refeeding, where the Ragulator shows preferential binding to nucleotide-free Rag proteins, consistent with its role as a GEF. Ragulator is critical for the recruitment of Rag GTPases to lysosomal membranes, which then recruit mTOR to lysosomes in an amino acid -dependent manner. mTOR kinase undertakes nutrient sensing in conjunction with several multiprotein complexes, such as GATOR1, GATOR2, Sestrins, Folliculin-FNIP and KICSTOR, to regulate the amino acid dependent mTOR signaling pathway (Bar-Peled et al., 2013; L. Bar-Peled, L. D. Schweitzer, R. Zoncu, & D. M. Sabatini, 2012; Chantranupong et al., 2016; Chantranupong et al., 2014; R. A. Saxton, Chantranupong, Knockenhauer, Schwartz, & Sabatini, 2016; Tsun et al.,