5

At the Intersection of the Pathways for Exocytosis and Autophagy

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1. Introduction

Recent studies have suggested that there are molecular links between the two critical biological processes of exocytosis and autophagy. Exocytosis involves the transport of intracellular vesicles to the plasma membrane of the cell, where vesicular fusion results in the delivery of membrane and protein to the cell surface, and secretion of the vesicular contents. Exocytosis is utilized in, for example, hormone or antimicrobial peptide secretion, the delivery of proteoglycans to the cell surface, cell-cell communication and neurotransmission (Brennwald & Rossi, 2007; He & Guo, 2009). Autophagy is a mechanism for the recycling and degradation of cytoplasmic content, which involves surrounding an area of cytoplasm with a double membrane structure, which then interacts with degradative endosome-lysosome compartments (He & Klionsky, 2009). Autophagy has important functions in a range of cell processes including the maintenance of cellular homeostasis, starvation adaption, energy balance, organelle clearance, immunity and cell death. In human diseases, such as cancers, neurodegenerative disorders (e.g. Huntington's disease), and chronic inflammatory diseases (e.g. Crohn's disease), there have been reports of functional disparity in both of these important membrane-related cellular pathways. There is now increasing evidence that exocytosis and autophagy share molecular machinery and there are a number of reasons why this would be beneficial in terms of cellular function.

Exocytosis and autophagy may be competitively, cooperatively or independently regulated, depending upon the nature of the intracellular and/or extracellular environment. In response to conditions of low or high energy demand, there would be an advantage to the cell in reducing the energy consuming process of secretion, where the membrane from exocytic vesicles could be utilized to enable rapid expansion of the autophagic compartment (i.e. competitive regulation). During an immune response there may be concomitant stimulation of autophagy to degrade an intracellular bacterial pathogen, and exocytosis to release second messengers and antimicrobial effectors. Alternatively, it may be necessary to only upregulate an individual process, which is the case for increased autophagy during organelle and cytoplasm turnover under restricted nutrient supply (e.g. in the bone growth plate) (He & Klionsky, 2009), or increased exocytosis during proteoglycan delivery to the

cell surface (Franken *et al*., 2003). Finally, during the removal of dysfunctional exocytic vesicles or the rapid cessation of secretion (e.g. during neurotransmission), the cell requires organelle-specific molecular machinery, for the nucleation of autophagy (Geng *et al*., 2010).

Despite defects in multiple human syndromes that demonstrate changes in both exocytosis and autophagy, the mutual dependence of these processes on common molecular machinery has only recently been investigated. Evidence indicates that the exocyst complex and its regulator Ral (Ras like GTPase), both of which are known to have a critical function in exocytosis, also appear to be essential for the initiation of autophagy (Bodemann *et al*., 2011). Similarly, the small GTPase Rab11 has a critical role in exocytosis at the recycling endosome and in exocytic vesicle function (van Ijzendoorn, 2006), although in times of starvation where autophagy is induced, Rab11 containing vesicular compartments have been shown to asscociate with autophagosomes (Rab11 positive amphisomes; (Fader *et al*., 2008)). Finally, disruption of the exocytic Rab GTPase Sec4 and its guanine nucleotide exchange factor Sec2, can have significant effects on the anterograde movement of the integral autophagosome membrane protein Atg9 (Autophagy related protein 9), thereby influencing the recruitment of Atg8 to the phagophore assembly site (PAS) (Bodemann *et al*., 2011). The movements of Atg9 and Atg8 are of particular interest as they are both important during the initiation of autophagy (Geng *et al*., 2010; Wang *et al*., 2009). Atg9 has, in turn, been reported to reside on exocytic vesicles that can be converted into a phagophore assembly site (Mari *et al*., 2010; Mari & Reggiori, 2010). The aim of this chapter is to provide an overview of the exocytic and autophagic processes with a focus on the common molecular machinery acting at critical control points. It is this machinery that may facilitate communication between these functionally distinct vesicular compartments and may act as potential sites for regulation.

2. Exocytosis

The exocytic pathway delivers cargo carrying vesicles from either the *trans*-Golgi network (TGN) or recycling endosomes to the plasma membrane, where membrane fusion occurs to release the vesicular content (Figure 1). This vesicular content may be either vesicle membrane proteins directed to the cell surface, or lumenal contents for secretion into the extracellular milieu. This anterograde trafficking route may vary depending upon the cargo and cell type involved (Wurster *et al*., 1990), such as in melanocytes for melanin exocytosis and in neurons for neurotransmission. Exocytosis is also involved in numerous other cellular functions, including immune responses, cell-cell communication, cell growth, cell polarity and neurotransmission.

There are two main exocytic routes from the *trans*-Golgi network to the plasma membrane: the constitutive and the regulated routes (Stow *et al*., 2009). The constitutive route continuously delivers membrane and cargo from the *trans*-Golgi network to the cell surface, and is thought to be utilized for housekeeping functions. Although this process can be up-regulated in response to environmental stress, it is generally representative of a basal level of cell activity and secretion. However, a number of pro-inflammatory cytokines, including TNFα and IL-6, are released from macrophages via the constitutive route, in response to pro-inflammatory stimuli (Shurety *et al*., 2000). The regulated route involves the redirection of newly synthesized cargo to compartments where these molecules are stored until their release is triggered by a specific stimulus (mediated by calcium ion mobilization). In this

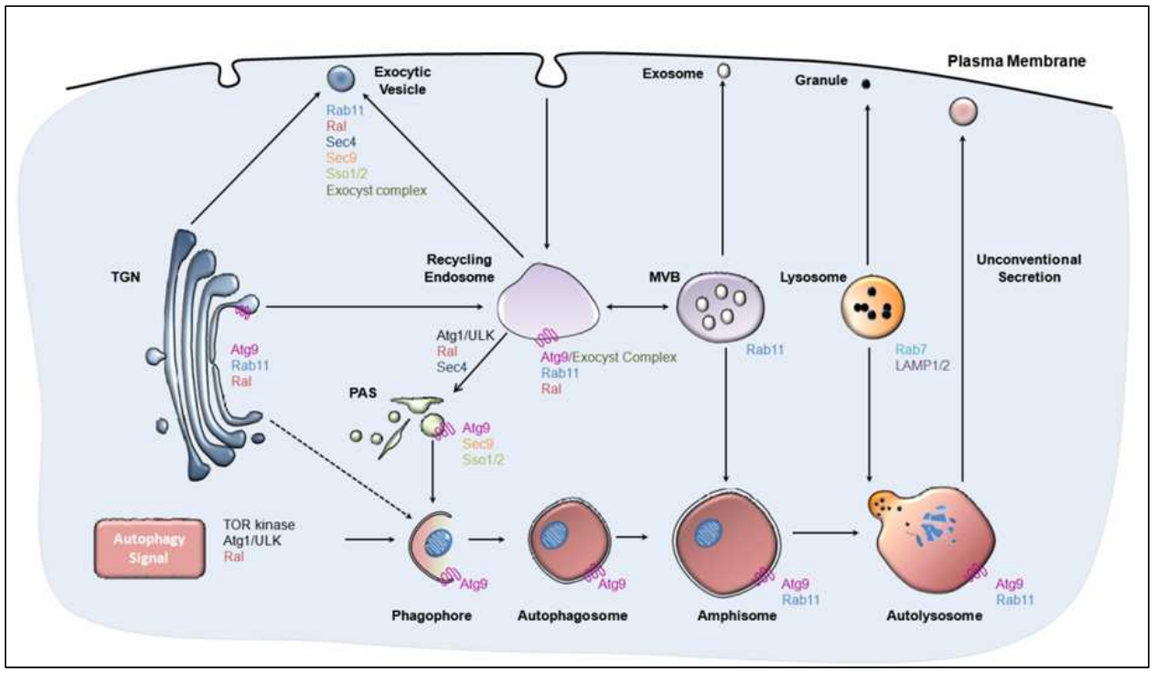

Fig. 1. Proposed model for crosstalk between autophagy and exocytosis.

route, cargo can be trafficked via a number of compartments including recycling endosomes, early endosomes, multivesicular bodies, secretory granules and secretory lysosomes. This pathway is utilized for the immune related secretion of cytokines and antimicrobial peptides, following exposure to pathogens or inflammatory stimuli. Similarly, for neurotransmitter release, exocytosis is stimulated by an increase in the intracellular calcium ion concentration in neurons; which allows the propagation of neuronal function. These different pathways are in dynamic balance with the endocytic pathway, which, apart from facilitating uptake into the cell, allows the recovery of membrane from the plasma membrane following exocytosis, enabling the cell to control its surface area (Khandelwal *et al*., 2010). The molecular machinery that drives exocytosis therefore operates in conjunction with the endocytic machinery, and in some cases may involve common elements that have dual function.

2.1 The molecular machinery for exocytosis

The molecular machinery that facilitates the process of exocytosis can vary with respect to the specific cell type and specialist cargo being transported, although two key molecular complexes are conserved for most membrane associated exocytic events; the exocyst and the soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex (Liu & Guo, 2011; Nair *et al*., 2011). Through interaction with a number of effector molecules, these complexes mediate the tethering, docking and fusion of vesicles with the plasma membrane.

The exocyst is an octomeric complex that is required for the efficient delivery of exocytic vesicles to the plasma membrane (TerBush *et al*., 1996). The components of the exocyst complex were first identified for yeast in the early 1990's, with mammalian orthologues

subsequently being identified (Lipschutz & Mostov, 2002). In yeast, this complex consists of six secretion related proteins (Sec), Sec3, Sec5, Sec6, Sec8, Sec10 and Sec15, with an additional two subunits known as exocyst related proteins (Exo), Exo70 and Exo84. Tethering to the plasma membrane is mediated by GTPase proteins, such as the yeast proteins, Rho3 and Cdc42 (He *et al*., 2007; He & Guo, 2009), or by TC10 in mammalian cells (Dupraz *et al*., 2009; Inoue *et al*., 2006; Pommereit & Wouters, 2007). Recognition of the exocytic vesicle by the exocyst is mediated by the Rab GTPase proteins, Sec4 in yeast (Guo *et al*., 1999; Zajac *et al*., 2005) or Rab11 in metazoans (Novick & Guo, 2002; Novick *et al*., 2006). In mammalian cells, assembly of this complex is controlled by RalA and RalB (Chen *et al*., 2011a; Chen *et al*., 2007; Chen *et al*., 2011b).

Assembly of the exocyst serves to tether exocytic vesicles to a specific plasma membrane site, demarcated by phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), Sec3 and Exo70 (He *et al*., 2007; He & Guo, 2009). The Sec3 and Exo70 membrane associated components of the exocyst act to target vesicles to the site of exocytosis, via the direct association of positively charged residues in the D domain at the C-terminus with PI(4,5)P2, in the plasma membrane (He *et al*., 2007; He & Guo, 2009). Multiple GTPases then regulate the assembly interface of a full octameric exocyst complex. The GTPase proteins Rho3 and Cdc42 also interact with Exo70 and Sec3 to facilitate the assembly of the exocyst complex at the plasma membrane (He *et al*., 2007; Moskalenko *et al*., 2002). In mammalian cells, Exo70 facilitates exocyst association with the plasma membrane through its interaction with TC10 (the orthologue of Cdc42) (Inoue *et al*., 2003; Liu *et al*., 2007). In yeast, secretory vesicles acquire the Rab GTPase protein Sec4, which directly interacts with the exocyst, via the Sec15 subunit, thus allowing the secretory vesicle to be recognised by the exocyst plasma membrane complex. Unlike in yeast, the vesicular targeting of the exocyst in metazoans is thought to occur through interactions between Sec15 and Rab11 (Langevin *et al*., 2005; Wu *et al*., 2005; Zhang *et al*., 2004), and the tethering of the secretory vesicles to the plasma membrane is regulated by Sec5 and Ral (Brymora *et al*., 2001; Chen *et al*., 2011a; Li *et al*., 2007). Active RalA (GTP bound form) interacts with Sec5, and upon delivery of the vesicles to the plasma membrane, the interaction between RalA-Sec5 is broken through the phosphorylation of Sec5 by protein kinase C (PKC) (Chen *et al*., 2011a). Detachment of Sec5 from RalA allows the release of the exocyst complex once the vesicle is delivered to the plasma membrane. The emerging model for the assembly of the exocyst suggests that the components are present as distinct sub-complexes on vesicles and the plasma membrane. In this manner, the assembly of the exocyst may integrate various cellular signalling pathways to ensure that exocytosis is tightly controlled (Sugihara *et al*., 2002).

Following cell surface membrane tethering by the exocyst complex, SNARE proteins facilitate the final step of exocytosis by bringing together the vesicular and plasma membranes for fusion. There are two groups of SNARE proteins; t-SNAREs, such as syntaxin1 and Sec9, which are found on the inner leaflet of the plasma membrane of cells and denote the target membrane; and v-SNAREs, which are found on a range of membrane compartments and denotes the vesicular membrane (Shorer *et al*., 2005; Stow *et al*., 2006). These proteins work by cognate pairing of t-SNAREs with their opposing v-SNAREs to form a four helix bundle, which allows the two membranes to be brought into close proximity, and this facilitates membrane fusion (Stow *et al*., 2006). A number of studies have

provided evidence of interactions between SNARE proteins and components of the exocyst complex (Bao *et al*., 2008; Hattendorf *et al*., 2007; Wiederkehr *et al*., 2004; Zhang *et al*., 2005). In yeast, this interaction is orchestrated though WD-40 adaptor proteins Sro7p and Sro77p (Zhang *et al*., 2005), which are homologues of lethal giant larvae (Lgl); first identified as a tumor suppressor in *Drosophila* (Gateff, 1978) and since demonstrated to play a role in cell polarity (Bilder *et al*., 2000). Sro7p and Sro77p interact with the exocyst components Sec6 and Exo84 as well as t-SNARE Sec9, thus providing a link between these two complexes to mediate the final steps of membrane fusion and exocytosis (Zhang *et al*., 2005).

3. Autophagy

Autophagy is responsible for a number of routine housekeeping functions, including the elimination of defective proteins, the prevention of abnormal protein aggregate accumulation, the turnover of glycogen, the removal of intracellular pathogens and the recycling of aged or dysfunctional organelles. These functions are likely to be critical for autophagy-mediated protection against aging, cancer, neurodegenerative disease and infection (Levine & Kroemer, 2008). Autophagy involves the engulfing of cytoplasmic content into a double membrane vesicle, which is used to mediate the degradation of the internalised contents following interaction with endosome and lysosome compartments (Figure 1). Autophagy normally occurs at a basal level, but stimuli such as starvation, hormonal and developmental signals, accumulation of unfolded proteins or invasion of microorganisms, can each modulate the rate of autophagic activity (Meijer & Codogno, 2004).

3.1 The induction and sequence of the autophagic process

The process of autophagy is mediated by the recruitment of autophagy related proteins to the limiting membranes of the forming phagosome, where they assemble the so-called pre-autophagosomal structure. This nucleation step is known to occur at sites adjacent to mitochondria in yeast (Mari & Reggiori, 2010), while other eukaryotes are thought to have multiple nucleation sites that may include the endoplasmic reticulum, Golgi, mitochondria and secretory vesicles (Hailey *et al*., 2010; Hamasaki & Yoshimori, 2010; Militello & Colombo, 2011; Tooze & Yoshimori, 2010; Weidberg *et al*., 2011). Autophagosomes are formed via the expansion of the isolation membrane to completely surround an area of cytoplasm. Maturation of the autophagosome involves fusion with a multivesicular body to form an amphisome, which subsequently fuses with a lysosome to become a fully functional autolysosome. Through the action of lysosomal enzymes, the degradation process then recycles molecular constituents back into the cytoplasm.

3.2 The molecular machinery involved in autophagosome formation and maturation

The induction and nucleation of autophagy is dependent on the successive assembly of a number of complexes within the cytoplasm, such as the Atg1-Atg13 (or mammalian unc-51-like kinase 1(Ulk1)–Atg13) kinase complex and the Atg5-Atg12 ubiquitin-like conjugation system. Up-stream signalling pathways lead to the activation of the Atg1/Ulk1 complex, which in turn recruits other members of the autophagic machinery to the site of nucleation. The exact mode for this recruitment is as yet unknown, however the individual step-specific complexes are well described for yeast and higher eukaryote systems.

One of the upstream regulators, targets of rapamycin (TOR) acts as an inhibitor of autophagy. Inactivation of TOR leads to the assembly of an active Atg1 complex; Atg1:Atg13:Atg17 in yeast (Kamada *et al*., 2000; Nakatogawa *et al*., 2009), and Ulk1:Atg13:FIP200 in higher eukaryotes (Chang & Neufeld, 2010; Mehrpour *et al*., 2010). In the latter case, activation is thought to occur via a change in Atg13-mediated phosphorylation of Ulk1 (Chang & Neufeld, 2010), although the exact site and the induction signal for this initiation step remains unclear (Chang & Neufeld, 2009, 2010; Mehrpour *et al*., 2010).

The solution to the mystery of the origin of autophagic compartment may lay in the biology of Atg9, the only trans-membrane autophagic protein that is present throughout autophagosome maturation. In yeast, Atg9 has been observed to form clusters near the mitochondria, suggesting the possibility of a membrane pool for autophagy (Mari & Reggiori, 2010). However, an equivalent structure has yet to be identified in other eukaryotes, and there may be multiple nucleation sites, including the endoplasmic reticulum, Golgi, mitochondria (Hailey *et al*., 2010; Hamasaki & Yoshimori, 2010; Tooze & Yoshimori, 2010) and plasma membrane (Ravikumar *et al*., 2010). Recent studies in mammalian cells showed that Atg9 initially resides at the Golgi and is trafficked to recycling endosomes (Wang *et al*., 2011; Webber *et al*., 2007; Webber & Tooze, 2010). This suggests the involvement of the Golgi complex in the autophagic pathway. Starvation dependent trafficking of mammalian Atg9 to the pre-autophagosomal structure requires the Atg1/Ulk1 kinase, Atg13, as well as p38 MAPK interaction protein, p38IP (Webber & Tooze, 2010). Following Atg9 recruitment, nucleation of the pre-autophagosomal structure limiting membrane is controlled by a protein complex containing a member of the vacuolar protein sorting family, Vps34, and Atg6/Beclin1. Atg6 is crucial for the recruitment of other autophagic proteins to the pre-autophagosomal structure, while Vps34 kinase phosphorylates phosphatidylinositol (PI_3P) in order to recruit Atg8 and Atg18 (Kundu & Thompson, 2008; Polson *et al*., 2010).

Two ubiquitin-like conjugation systems are required for the expansion and closure of the autophagosome, Atg5-Atg12 and the Atg8-phosphotidylethanolamine complex (Ichimura *et al*., 2000; Mizushima *et al*., 1998). In the first of these systems the conjugated form of Atg5-Atg12 associates with Atg16 dimers to become a multimeric Atg5-Atg12-Atg16 complex. It is believed that this Atg5-Atg12-Atg16 complex is required for the formation of pre-autophagosomal structures, and allows association with the second Atg8 related conjugation system. In the second ubiquitin-like conjugation system, cytosolic Atg8, or LC3 (microtubule-associated protein 1 light chain 3) in mammals, is modified by the attachment of the phospholipid anchor phosphatidylethanolamine, or PE. This step results in the localisation of Atg8/LC3-PE to the isolation membrane of the phagophore and may contribute to the expansion of autophagic membranes (McPhee & Baehrecke, 2009; Nakatogawa *et al*., 2007).

Once the autophagosome is closed by fusion of the expanding edges of the phagophore, its maturation proceeds through fusion with multi-vesicular bodies, late endosomes and lysosomes (Razi *et al*., 2009). It has been suggested that fusion of the autophagosome with endocytic compartments is facilitated by endosome membrane fusion machinery (Eskelinen, 2005) including the membrane targeting proteins Rab11 and Rab7 GTPases and membrane fusion protein complexes, such as SNAREs, ESCRT proteins, Vps28, Vps25, Vps32, Deep

Orange (Dor)/Vps18 and Carnation (Car)/Vps33a (Fader *et al*., 2008; Gutierrez *et al*., 2004a; Gutierrez *et al*., 2004b; Simonsen & Tooze, 2009), as well as the lysosomal membrane proteins, Lamp-1 and Lamp-2 (Tanaka *et al*., 2000). After fusion with the lysosome, degradation of protein, lipid, glycogen, RNA, DNA and other contents is dependent upon the action of lysosomal acid hydrolases (Koike *et al*., 2005; Tanaka *et al*., 2000; Tanida *et al*., 2005). The small molecules (e.g. amino acids and sugars) are then transported back to the cytosol for protein synthesis and the maintenance of other cellular functions (He & Klionsky, 2009).

4. Diseases that show links between exocytosis and autophagy

Altered regulation of exocytosis and autophagy has been shown in a number of debilitating diseases including cancer (Gozuacik & Kimchi, 2006; Levine, 2007; Miracco *et al*., 2007; Pattingre *et al*., 2005; Tayeb *et al*., 2005), neurodegenerative diseases (Gao & Hong, 2008; Keating, 2008; Yu *et al*., 2005), and chronic inflammatory diseases (Barbier, 2003; Barrett, 2008; Cadwell *et al*., 2010; Fujita *et al*., 2008; Homer *et al*., 2010; Rioux, 2007; Saitoh *et al*., 2008).

In cancer, the uncontrolled cell proliferation that results in tumor outgrowth is associated with increased secretion of pro-oncogenic proteins and lysosomal enzymes. Thus, lysosomal cathepsins, acid phosphatase and various glycosidases have been used as diagnostic markers and to define metastatic potential in a range of cancers (Tappel, 2005). The underlying reason for this increase in lysosomal enzyme secretion may be linked to the increase in endosome-lysosome membrane recycling that is required to maintain plasma membrane area during rapid cell division (Boucrot & Kirchhausen, 2007). Increased lysosomal enzyme secretion has also been associated with extracellular matrix degradation and this can facilitate metastasis (Tayeb *et al*., 2005). The migration of metastatic cancer cells also involves upregulated exocytosis, as a means of membrane delivery to the leading edge of the migrating cell. This allows the formation of lamellipodia and filopodia, and thereby cellular movement. Exocytosis and cell division are both high energy demand cellular processes, and so it is not too surprising that autophagy has also been implicated in the carcinogenic process, as a means of energy supply.

There is, however, controversy in the literature regarding the pro-survival and pro-death functions of autophagy (Hippert *et al*., 2006; Kundu & Thompson, 2008; Levine, 2007; Levine & Kroemer, 2008). The cyto-protective role that autophagy has under conditions of starvation or low energy supply, prevents apoptosis (Boya *et al*., 2005), and is therefore thought to promote cancer cell growth and survival within solid tumors prior to vascularization. In stark contrast, the suppression of autophagy via a number of regulatory pathways can lead to tumorigenesis (Gozuacik & Kimchi, 2006; Levine, 2007; Miracco *et al*., 2007; Pattingre *et al*., 2005). The increased tumorigenesis observed in *beclin1/Atg6* and *Atg5* murine mutants, and the high number of mono-allelic deletion mutations in these genes observed in different types of human cancer, indicate a direct tumor suppressor role for autophagy (Aita *et al*., 1999; Hippert *et al*., 2006; Kundu & Thompson, 2008; Levine, 2007). In addition, p53 and PTEN, which are frequently mutated in cancer patients, can stimulate autophagy (Bae *et al*., 2007; Lindmo *et al*., 2006; Shin *et al*., 2011; Wang *et al*., 2011); while PI3K, p38 MAPK and Akt, which are often activated in cancer, can suppress autophagy (Webber & Tooze, 2010). The apparent disparate roles of autophagy in cancer make it difficult to ascertain its exact function, and it also remains unclear whether exocytosis and autophagy are acting independently or as inter-linked

processes in this disease. However, in one study, the trafficking of lysosomes in cancer cells was found to be linked to autophagosome formation through the common molecular machinery of the microtubule –dependent motor protein KIF5B (kinesin heavy chain protein 5B; Cardoso *et al*., 2009) a protein previously demonstrated to be involved in exocytosis (Varadi *et al*., 2002).

Neurodegenerative disorders including Parkinson's, Huntington's and Alzheimer's disease are progressive disorders, which have in common the loss of function of neurons in discrete areas of the central nervous system. This loss of function is thought to be a result of aggregation of misfolded proteins. Autophagy has a role in the degradation of misfolded protein (Yu *et al*., 2005), and the functional loss of *Atg5* or *Atg7* results in the accumulation of ubiquitinated protein aggregates, and a neurodegenerative phenotype (Hara, 2006; Komatsu *et al*., 2006). Furthermore, altered autophagy has been shown to be linked with altered exocytosis in a number of neurodegenerative disorders, leading to impaired release of neurotransmitters and increased inflammation (Gao & Hong, 2008; Keating, 2008). This highlights a direct link between autophagy and the recycling of the specialist secretory vesicles that control neurotransmission at the synaptic terminals of neurons.

Some inflammatory diseases, such as Crohn's disease, are thought to be caused by a breakdown in the regulation of exocytosis leading to increased secretion of pro-inflammatory factors (Barbier, 2003; Cadwell *et al*., 2010). In addition, a number of genetic screens of patients suffering from Crohn's disease have identified mutations in autophagy related genes (Barrett, 2008; Rioux, 2007). The autophagy related protein Atg16L is thought to function as a scaffold for LC3 lipidation, by dynamically localizing to the source of membrane involved in autophagosome formation (Fujita *et al*., 2008). A genetic defect in *Atg16L* may decrease the efficiency by which pathogens can be cleared from cells via autophagy, evoking an increased inflammatory response (Fujita *et al*., 2008; Homer *et al*., 2010; Saitoh *et al*., 2008). In addition, there is mounting evidence that defects in this autophagy gene can also lead to defects in exocytosis, causing a build-up of secretory granules in specific cell types (Cadwell *et al*., 2009). These concurrent defects in both exocytosis and autophagy may be one more piece of evidence for co-regulation and a shared molecular link between these two cellular processes, and raises the important question: is there common molecular machinery for exocytosis and autophagy?

5. Exocytosis and autophagy: Common cellular functions and molecular machinery

Exocytosis and autophagy are essential for a number of common biological processes, including; the immune response (Govind, 2008; Minty *et al*., 1983; Murray *et al*., 1998; Ostenson *et al*., 2006), cell growth (Brennwald & Rossi, 2007; Orlando & Guo, 2009; Wei & Zheng, 2011; Zhang *et al*., 2005), cell proliferation and apoptosis (Kundu, 2011; Shin *et al*., 2011; Zeng *et al*., 2012), and multicellular organism development (Gutnick *et al*., 2011; Hu *et al*., 2011; Sato & Sato, 2011; Tra *et al*., 2011). Autophagy and exocytosis both involve membrane trafficking and fusion events and so similar groups of molecular machinery may be required for both processes: such as GTPase proteins, that facilitate membrane tethering and SNARE proteins which are involved in membrane fusion. There is increasing evidence indicating shared molecular machinery between these processes, which provokes questions concerning possible dual regulation as a means of balance for these pathways.

Autophagy and exocytosis can have opposing or synergistic roles in cell function. For example, during times of reduced nutrient availability autophagy is stimulated, allowing cells to recycle cytoplasmic components, and exocytosis is reduced to conserve cellular constituents and energy (Shorer *et al*., 2005). In response to stimulation, specialised secretory cells (e.g. chromaffin neuroendicrine cells) divert energy utilization towards the exocytic pathway (Malacombe *et al*., 2006). Conversely, when a cell is presented with an immune challenge, both exocytosis and autophagy can be upregulated; exocytosis for the release of immune response factors and autophagy to clear invading pathogens from cells (Stow *et al*., 2009). Given these findings, it would appear to be of advantage to cells to have a mechanism coordinating the activity of these two processes.

5.1 The endosomal network is involved in both exocytosis and autophagy

The dynamic flow of membrane and membrane proteins within a cell is mediated through the endosomal network (Figure 1). For example, lipids and proteins from the plasma membrane are recovered by the cell for cytosolic recycling via compensatory endocytosis, which also allows for the maintenance of membrane homeostasis at the sites of active exocytosis; directing endocytosed membrane back into the endosomal network or Golgi for degradation or recycling (Sramkova *et al*., 2009). This type of endocytosis is of particular importance in specialized secretory cells, such as, bladder umbrella cells (Khandelwal *et al*., 2008; Khandelwal *et al*., 2010), neurons (Kim & von Gersdorff, 2009; Llobet *et al*., 2011; Logiudice *et al*., 2009) and neuroendocrine cells (Engisch & Nowycky, 1998; Barg & Machado, 2008). This allows for the rapid recycling of secretory vesicles back into the reserve pool. Endosomes are at the nexus of the exocytic and autophagic pathways allowing for the sorting and directing of membrane. Thus, in yeast, Atg9 clusters are connected with both the endocytic and exocytic systems, and delivered to the phagophore assembly site via recycling endosomes (Geng *et al*., 2010; Mari *et al*., 2010). The recycling endosome's exocytic function is involved in the maintenance of cell polarity through the sorting of membrane proteins such as clathrin and cadherin (Farr *et al*., 2009). The recycling endosome machinery also plays a role in the fusion of multivesicular bodies with autophagosomes, which is an essential step in phagosome maturation (Fader & Colombo, 2009; Razi *et al*., 2009; Tooze & Razi, 2009). Recent studies have suggested a significant overlap of the molecular machinery used in these two biological processes (Bodemann *et al*., 2011; Geng *et al*., 2010). This involves the exocyst complex and its regulators (e.g. small GTPases), as well as membrane fusion machinery (e.g. SNAREs; Table 1).

5.2 Small GTPases at the cross road of exocytosis and autophagy

5.2.1 Ral small GTPase

Ras-like proteins (Ral) are small GTPases that function as an essential component of the cellular machinery regulating the post-Golgi targeting of exocytic vesicles to the plasma membrane (Balasubramanian *et al*., 2010; Chen *et al*., 2007; Kawato *et al*., 2008; Kim *et al*., 2010; Ljubicic *et al*., 2009; Lopez *et al*., 2008; Rondaij *et al*., 2008; Rosse *et al*., 2006; Shipitsin & Feig, 2004; Spiczka & Yeaman, 2008). Ral function is directly mediated by its interaction with the exocyst complex (Feig, 2003; Kawato *et al*., 2008; Mark *et al*., 1996; Mott *et al*., 2003), in particular Sec5 which has been shown to be essential for Ral-exocyst dependent exocytosis (Fukai *et al*., 2003; Moskalenko *et al*., 2002).

Protein	Role in Exocytosis	References	Role in Autophagy	References
Ral	Interacts with exocyst via Sec5 to facilitate the tethering of vesicles to the plasma membrane.	(Balasubramanian *et al*., 2010; Brymora *et al*., 2001; Chen *et al*., 2011a; Chen *et al*., 2007; Fukai *et al*., 2003; Kawato *et al*., 2008; Li *et al*., 2007; Ljubicic *et al*., 2009; Lopez *et al*., 2008; Mark *et al*., 1996; Moskalenko *et al*., 2002; Mott *et al*., 2003; Shipitsin & Feig, 2004)	RalB but not RalA involved in initation of autophagy in mammalian cell lines. Over expression of active RalB enhances autophagy while depletion decreases autophagy.	(Bodemann *et al*., 2011)
Rab11	Bound to exocytic vesicles and is involved in the anterograde trafficking of vesicles from recycling endosomes to the plasma membrane. Interacts with the exocyst component Sec15 to assist tethering of vesicles to the plasma membrane.	(Langevin *et al*., 2005; Oztan *et al*., 2007; Shandala *et al*., 2011; Ward *et al*., 2005; Wu *et al*., 2005; Zhang *et al*., 2004)	Facilitates fusion of the autophagosome with endocytic compartments.	(Fader *et al*., 2008)
Sec4	Allows the interaction of the secretory vesicle with the exocyst complex via Sec15 to facilitate tethering to the plasma membrane.	(Guo *et al*., 1999)	Involved in the recruitment of Atg9 to the PAS.	(Geng *et al*., 2010)
Exocyst Complex	Octomeric complex required for tethering of exocytic vesicles to the plasma membrane in a site specific manner	(He *et al*., 2007; He & Guo, 2009; Jin *et al*., 2011; Langevin *et al*., 2005; Morgera *et al*., 2012)	Proposed as a scaffold for the initiation of autophagy complexes.	(Bodemann *et al*., 2011; Farré & Subramani, 2011)
Sso1/2-Sec9	t-SNARE that denotes the site of exocytosis on the plasma membrane, possibly through interactions with the exocyst complex and its effectors	(Aalto *et al*., 1993; Brennwald *et al*., 1994)	Involved in the formation of Atg9 associated tubule-vesicular clusters emanating from the PAS	(Nair *et al*., 2011)

Protein	Role in Exocytosis	References	Role in Autophagy	References
VAMP7	Involved in constitutive exocytosis in a number of cell types	(Galli *et al*., 1998; Oishi *et al*., 2006)	Involved in lysosome fusion during autophagosome maturation	(Fader *et al*., 2009)
Atg9	Unknown role but has been found on secretory vesicles. May have a role in unconventional secretion	(Bruns *et al*., 2011; Mari *et al*., 2010)	Transmembrane protein required for the transport and assembly of membrane during autophagosome formation	(He *et al*., 2009)
Atg16L	Involved in secretion from secretory granules in intestinal Paneth cells	(Cadwell *et al*., 2008; Cadwell *et al*., 2009)	Functions as a scaffold for LC3 lipidation, required during autophagosome formation	(Fujita *et al*., 2008)

Table 1. Proteins involved in both autophagy and exocytosis

In addition to its well documented role in exocytosis, recent evidence from mammalian cell cultures indicates that RalB is involved in the formation of autophagosomes (Bodemann *et al*., 2011). The crucial role for RalB as an upstream activator of autophagy is illustrated by the fact that the over-expression of its active GTP-bound form was sufficient to induce autophagy, even in the absence of autophagy-specific stimuli (Bodemann *et al*., 2011). RalB is present on sites of nascent autophagosome formation, together with Beclin1 and Atg5, and its depletion, similar to the depletion of Atg5 and Beclin1, significantly impaired the formation of starvation-induced LC3/Atg8 punctae and the turnover of LC3/Agt8. Interestingly, depletion of RalB also impaired the digestion of autophagocytosed *Salmonella typhimurium*. The autophagy-related function of RalB appears to be mediated by its effector Exo84, a component of the exocyst complex (Bodemann *et al*., 2011). Activated by starvation, RalB triggers Exo84 interaction with the autophagy initiation component Beclin1. Intriguingly, the alternative RalB roles in exocytosis and autophagy appear to be driven by environmental signal/s, as nutrient availability determines the RalB coupling preferences to a down-stream effector; endogenous RalB preferentially associates with Exo84 in nutrient poor conditions and with Sec5 under nutrient rich conditions (Bodemann *et al*., 2011). This model has not been investigated in higher eukaryotes, but these findings in yeast suggest a role for the exocyst complex as a scaffold for the assembly of a number of important autophagy initiators.

5.2.2 Yeast Rab small GTPase Sec4

The yeast Rab GTPase Sec4 and its activator Sec2 have well-established roles in the tethering of secretory vesicles to sites of active exocytosis, in a process mediated by interaction with the exocyst complex component Sec15 (Geng *et al*., 2010) . Recent studies indicate that Sec2 and Sec4 also have a role in anterograde trafficking of the autophagic membrane protein Atg9, as silencing of Sec4 blocked the delivery of Atg9 to the pre-autophagosomal structure

(Geng *et al*., 2010). Furthermore, when the domain of Sec4 that is known to interact with Sec15 was altered, the effect on autophagy was equivalent to the effect of Sec4 silencing. Taking into account that there is no apparent role for Sec15 in autophagy, this suggests that autophagy-specific proteins may compete for this Sec15-binding domain in order to switch the function of activated GTP-bound Sec4 between exocytosis and autophagy.

5.2.3 Metazoan Rab11 small GTPase

Rab11 is a small GTPase, which is most often referred to as a recycling endosome marker. However, it has also been observed on vesicles bound for exocytosis (Shandala *et al*., 2011; Ward *et al*., 2005), and amphisomes; an intermediate compartment that is formed during autophagosome maturation, prior to lysosomal fusion (Fader & Colombo, 2009)

The exocytic role for Rab11 is mediated by its association with the Sec15 exocyst component. This has been shown in MSCK cells (Oztan *et al*., 2007; Zhang *et al*., 2004), and in *Drosophila* photoreceptor and sensory neuron cells (Wu *et al*., 2005). Rab11 is important for the anterograde trafficking of; numerous membrane receptors (Chernyshova *et al*., 2011), the epithelial sodium channel complex of the cortical collecting duct of the kidneys (Butterworth *et al*., 2012), and DE-Cadherin in polarised cells (Langevin *et al*., 2005; Wu *et al*., 2005; Zhang *et al*., 2004), as well as the calcium dependent exocytosis of growth hormones (Ren *et al*., 1998; Takaya *et al*., 2007). A number of intracellular pathogens, such as *Porphyromonas gingivalis*, influenza A and HIV, have been reported to hi-jack Rab11 dependent anterograde trafficking as a means of escape from host cells (Kadiu & Gendelman, 2011; Momose *et al*., 2011; Takeuchi *et al*., 2011).

An example of coordinated exocytosis and autophagy comes from the biology of multivesicular bodies (MVBs). MVBs are specialised late endosomes, a crucial intermediate in the internalization of nutrients, ligands and receptors into small intraluminal vesicles, also known as exosomes (Fader & Colombo, 2009). Rab11 decorates MVBs and is involved in both the biogenesis of MVBs and exosome release (Fader *et al*., 2008). During the maturation of hematopoietic progenitors into reticulocytes and erythrocytes, proteins that are not required at the mature stage are sequestered into exosomes of MVBs. In this scenario Rab11 is involved in the targeting of MVBs to the plasma membrane, where exosomes are released into the extracellular milieu (Fader & Colombo, 2006). Active Rab11 is also required for the interaction of MVBs with autophagosomes, where the resulting calcium-stimulated fusion of these organelles promotes efficient degradation of autophagic contents (Fader *et al*., 2008; Savina *et al*., 2005). Thus, Rab11 may represent a critical regulator of membrane flow between recycling endosomes (as a source of exocytic vesicles) and multivesicular bodies, where it can be engaged in both autophagic maturation and secretion.

5.3 The exocyst and the initiation of autophagy

Components of the exocyst complex involved in regulated and polarized exocytosis have also been shown to associate with a number of essential autophagy proteins (Bodemann *et al*., 2011). Exocyst components Sec3 and Sec8 interact *in vitro* with positive (FIP200, ATG14L) and negative (RUBICON) regulators of autophagy, as well as with the phagophore expansion complex Atg5-Atg12 (Bodemann *et al*., 2011). The functionality of these physical interactions is confirmed by the fact that LC3/Atg8 autophagosome formation was impaired

in cells depleted for core exocyst components. For example, the depletion of Sec8 rendered cells insensitive to starvation stimulation, and impaired autophagy to the same extent as seen for the depletion of Atg5 and Beclin1 (Bodemann *et al*., 2011). Further interrogation of this system showed that the localization of exocyst components, with the autophagy initiator Atg1/Ulk1 and other proteins involved in isolation membrane formation, was altered following the induction of autophagy (Bodemann *et al*., 2011). As these processes are under the control of Ral or Rab, the differential recruitment of the exocyst to the target membrane might depend on the signals upstream of these small GTPases.

5.4 SNARE proteins and membrane fusion during exocytosis and autophagy

Both autophagosome maturation and anterograde vesicle trafficking via the exocytic route involve a series of membrane fusion steps, the execution of which is controlled by SNAREs. Recent studies in yeast have indicated that some exocytic t-SNAREs may also play a role in membrane dynamics during autophagy (Geng *et al*., 2010; Nair *et al*., 2011). The anterograde trafficking of the key autophagic membrane determinant, Atg9, depends on interaction with exocytic Sso1-Sec9, as well as on the endosomal t-SNARE Tlg2 and the v-SNAREs Sec22 and Ykt6. Sso1/2 and Sec9 SNAREs are also responsible for the formation of the Atg9 associated tubular-vesicular clusters emanating from the pre-autophagosomal structure, and their depletion results in Atg9 localization to small vesicular structures, possibly *trans*-Golgi network derived secretory vesicles, that fail to be delivered to the pre-autophagosomal structure (Nair *et al*., 2011). This failure of Atg9 delivery to pre-autophagosomal structure abolishes Atg8 recruitment, and thereby abrogates autophagosome biogenesis (Geng *et al*., 2010; Nair *et al*., 2011) .

Another group of SNAREs, the vesicle-associated membrane proteins (VAMPs), appear to be involved in membrane fusion events in both autophagy and exocytosis. One the one hand, during autophagosome maturation, it has been shown that VAMP3 and VAMP7 are required for sequential fusion with multivesicular bodies and lysosomes respectively (Fader *et al*., 2009). In HeLa cells, VAMP7 has been shown to be involved in homotypic fusion of Atg16L1 positive vesicles to allow autophagosome biogenesis (Moreau *et al*., 2011). On the other hand, it has been recently demonstrated that VAMP7 is involved in constitutive exocytosis in HSY cells (Oishi *et al*., 2006), and in apical trafficking of exocytic vesicles in polarized epithelial cells, such as MDCK cells and CaCo-2 cells (Galli *et al*., 1998). VAMP3 has been postulated to be a v-SNARE for early and recycling endosomes, with a role in constitutive exocytosis, but its role might be redundant as mice with a null mutation for this gene were normal in most endocytic and exocytic pathways, including constitutive exocytosis (Wang *et al*., 2004; Yang *et al*., 2001). The question remains whether these functions of SNAREs are restricted to specific tissues, or universal for all tissues, and if so, what are the upstream signals that direct these SNAREs to either the exocytic or autophagic membranes?

5.5 The role of autophagy genes in secretion

There is emerging evidence of involvement of autophagy proteins in polarized secretion involving lysosomes. In bone resorptive osteoclasts, Atg5, Atg7, Atg4B, and LC3/Atg8 participate in directing lysosomes to fuse with the plasma membrane and in the release of the lytic enzyme cathepsin K into the extracellular space (DeSelm *et al*., 2011). This type of

lysosome-regulated exocytosis is not restricted to osteoclasts involved in bone remodelling, and has been described for lysosome related organelles in many other specialist cell types, such as melanosomes in melanocytes and lytic granules in neutrophils (Blott & Griffiths, 2002; Chen *et al*., 2012; Luzio *et al*., 2007). In yeast grown under conditions of nitrogen starvation, autophagy genes are required for the secretion of an acyl coenzyme A binding protein (Acb1) (Bruns *et al*., 2011; Duran *et al*., 2010; Manjithaya *et al*., 2010; Skinner, 2010). This unconventional route of secretion is initiated at sites that are positive for the Golgi assembly and stacking protein (GRASP65) homologue 1 (Grh1), which attracts core autophagy-related proteins Atg9 and Atg8 to a novel compartment (Bruns *et al*., 2011). These Acb1-containing autophagosomes then evade fusion with the lytic vacuole, fusing instead with recycling endosomes to form multivesicular body carriers, which then fuse with the plasma membrane in a t-SNARE Sso1 dependent fashion, to release Acb1. It is still not clear how beneficial or economical it is for cells to use these unconventional routes of secretion.

6. Summary

Despite the similarity in requirements for membrane dynamics in the processes of exocytosis and autophagy, correlations between the molecular machinery used for both of these processes are only beginning to be elucidated. The exocytic and autophagic functions of cells are critical for the maintenance of cell homeostasis and the exchange of membrane between intracellular compartments and the cell surface. In addition, the fusion and fission events that remodel the exocytic vesicle and the autophagosome are likely to require much of the same molecular machinery. Therefore, it is likely that there is co-ordinated control of these two processes to ensure that they can be regulated with respect to each other. Members of the exocyst complex, and some autophagy related proteins, have already been shown to have functions in their opposite processes, and the involvement of the Ral small GTPases in the global control of exocytosis and autophagy mirrors the role of Rab small GTPases in the control of endosome trafficking. There are many intriguing questions brought about by recent findings. What is the decision making signal that diverges the components of the shared machinery from one pathway to another? Is there a common upstream signal for both pathways, be it through the insulin receptor/mTOR (Webber & Tooze, 2010), MAPK (Webber & Tooze, 2010), redox (Lee *et al*., 2012), or are there combinations of these signals? Or is there a yet to be defined intrinsic factor of the autophagic or exocytic membrane, with a changing affinity for vesicular compartments? This is a very interesting time to be exploring the intersection of the exocytosis and autophagy pathways, particularly while we are is still looking for the key controllers of cellular homeostasis in cancers, neurodegenerative and immune disorders.

7. References

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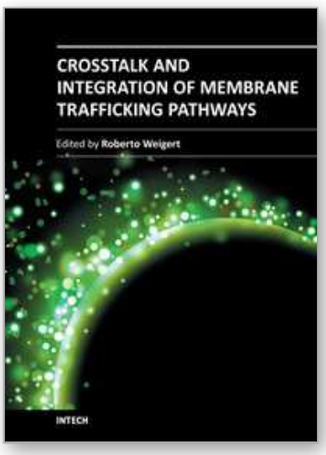

Crosstalk and Integration of Membrane Trafficking Pathways
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Membrane traffic is a broad field that studies the complex exchange of membranes that occurs inside the cell. Protein, lipids and other molecules traffic among intracellular organelles, and are delivered to, or transported from the cell surface by virtue of membranous carriers generally referred as "transport intermediates". These carriers have different shapes and sizes, and their biogenesis, modality of transport, and delivery to the final destination are regulated by a multitude of very complex molecular machineries. A concept that has clearly emerged in the last decade is that each membrane pathway does not represent a close system, but is fully integrated with all the other trafficking pathways. The aim of this book is to provide a general overview of the extent of this crosstalk.

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