The role of membrane-trafficking small GTPases in the regulation of autophagy

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Journal of Cell Science 126, 1059–1069

doi: 10.1242/jcs.123075

Summary

Macroautophagy is a bulk degradation process characterised by the formation of double-membrane vesicles, called autophagosomes, which deliver cytoplasmic substrates for degradation in the lysosome. It has become increasingly clear that autophagy intersects with multiple steps of the endocytic and exocytic pathways, sharing many molecular players. A number of Rab and Arf GTPases that are involved in the regulation of the secretory and the endocytic membrane trafficking pathways, have been shown to play key roles in autophagy, adding a new level of complexity to its regulation. Studying the regulation of autophagy by small GTPases that are known to be involved in membrane trafficking is becoming a scientific hotspot and may provide answers to various crucial questions currently debated in the autophagy field, such as the origins of the autophagosomal membrane. Thus, this Commentary highlights the recent advances on the regulation of autophagy by membrane-trafficking small GTPases (Rab, Arf and RalB GTPases) and discusses their putative roles in the regulation of autophagosome formation, autophagosome-dependent exocytosis and autophagosome-lysosome fusion.

Key words: Autophagy, Endocytosis, Exocytosis, GTPase, Rab, Arf, RalB

Introduction

Macroautophagy (hereafter referred to as autophagy) is a bulk degradation process that is characterised by the formation of an isolation membrane (the phagophore or pre-autophagosomal structure) that elongates and fuses in order to sequester a small portion of the cytoplasm, including organelles, soluble material and aggregates (Box 1), thereby forming a double-membrane vesicle, the autophagosome. Autophagosomes then fuse with lysosomes, forming autolysosomes that allow the degradation of the autophagic cargo (Fig. 1). Endosomes also fuse with autophagosomes before they fuse with lysosomes, forming amphisomes (reviewed by Kraft and Martens, 2012; Mizushima and Komatsu, 2011). Autophagy is crucial for several cellular functions, and its regulation has many physiological and pathophysiological implications (Box 1).

A major breakthrough in the autophagy field came from yeast-based genetic analyses, culminating in the identification of 35 autophagy-related genes (ATGs) and elucidation of the basic molecular machinery that regulates the pathway. Most of these genes were found to have mammalian orthologues (Kraft and Martens, 2012; Ravikumar et al., 2010b).

The phagophore assembly sites (PASs) are proposed to be the starting points for autophagosome formation. Unlike in yeast, where autophagosomes are formed at a single PAS next to the vacuole, autophagosomes in mammals are formed randomly throughout the cytoplasm. The formation of the phagophores or pre-autophagosomal structures requires the activity of the class III phosphoinositide 3-kinase Vps34 in order to generate phosphatidylinositol (3)-phosphate [PtdIns(3)*P*]. Vsp34 acts in a large complex together with Atg6 (also known as Beclin 1), Atg14 (also known as Barkor) and Vps15 (also known as p150). The function of PtdIns(3)*P* in autophagy is still unclear, but it appears to aid the recruitment of WD-repeat-domain phosphoinositide-interacting proteins (WIPIs; the mammalian orthologue of Atg18) to the phagophore membrane. The activity of Vps34 is enhanced by its interaction with Beclin 1, which is further regulated by other binding partners, such as Ambra1, the protein product of the UV radiation resistance-associated gene (UVRAG), Bif1 (also known as Zbtb24), the inositol (1,4,5)-trisphosphate receptor (IP3R) and the anti-apoptotic proteins Bcl2 and Bcl-X_L. The binding of Bcl-2 or Bcl-X_L to Beclin 1 inhibits autophagy and this interaction is inhibited by Jun N-terminal kinase 1 (JNK1)-dependent phosphorylation of Bcl-2 that occurs under starvation conditions. The activity of the Vps34 complex is further controlled by upstream autophagy regulators, including the mammalian Atg1 orthologues ULK1 and ULK2, Atg13 and the focal adhesion kinase-family interacting protein of 200 kDa (FIP200). Atg13 binds to ULK1 or ULK2 and mediates their interaction with FIP200 (also known as RB1CC1, the mammalian orthologue of Atg17). Under starvation conditions, Atg13, ULK1 and ULK2 are dephosphorylated, leading to the activation of ULK1 and ULK2, which, in turn, phosphorylate FIP200 to induce the formation of autophagosomes (reviewed by Kraft and Martens, 2012; Ravikumar et al., 2009). These last steps are finely controlled by the classic negative regulator of autophagy, the mechanistic target of rapamycin (mTOR) (Box 2).

After initiation and formation, elongation of the pre-autophagosomal membrane takes place, which requires two ubiquitylation-like reactions. In the first reaction, the ubiquitin-like molecule Atg12 is conjugated to Atg5 in a reaction that involves Atg7 – which is similar to an E1 ubiquitin-activating enzyme (E1-like) – and Atg10, which acts like an E2-ubiquitin-conjugating

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Box 1. Physiological and pathophysiological functions of autophagy

Although autophagy was considered in the past as a nonselective process, several cargo-specific autophagic processes have been recently described, including xenophagy (degradation of intracellular pathogens), aggrephagy (clearance of certain protein aggregates), pexophagy (elimination of peroxisomes), mitophagy (removal of damaged mitochondria) and ribophagy (elimination of ribosomes), which assist the quality control of essential cellular components (reviewed by Mizushima, 2011). Autophagy occurs under basal conditions and can be induced by certain environmental stresses, such as nutrient deprivation, some infections, oxidative stress and treatment with certain drugs (e.g. rapamycin). Under starvation conditions, autophagy is induced and increases the availability of nutrients (e.g. amino acids) by releasing them from proteins and other macromolecules that are targeted for degradation. Indeed, autophagy has roles in both health and disease conditions. It regulates early embryonic development, neonatal starvation, clearance of pathogenic bacteria during infectious processes, cancer-associated mechanisms and degradation of misfolded and aggregation-prone proteins (i.e. tau, mutant α-synuclein, polyglutamine-expanded huntingtin) that are involved in neurodegeneration disorders, such as Alzheimer, Parkinson and Huntington diseases (reviewed by Harris and Rubinsztein, 2012; Mizushima and Komatsu, 2011).

enzyme (E2-like). Subsequently, the Atg12–Atg5 complex interacts noncovalently with Atg16L1, and the Atg12–Atg5–Atg16L1 complex associates with the nascent phagophore. This complex is essential for the elongation of the pre-autophagosomal membrane, but it dissociates from fully formed autophagosomes. In the second ubiquitylation-like reaction, ubiquitin-like molecules of the Atg8 family (LC3, GABARAP or GATE-16) are conjugated to the lipid phosphatidylethanolamine (PtdEtn). Microtubule-associated protein 1 light chain 3β (MAPLC3B or LC3), the best characterised member of the Atg8 family, is synthesised as a precursor form and is cleaved at the C-terminus by Atg4B, resulting in the cytosolic form LC3-I. LC3-I is then conjugated to PtdEtn by Atg7 (E1-like) and Atg3 (E2-like) to form LC3-II, the autophagosome-associated form of LC3. The Atg12–Atg5–Atg16L1 complex may bring LC3 to the site of lipidation and enhance LC3-PtdEtn conjugation, acting like an E3-ubiquitin ligase. In contrast to the Atg12–Atg5–Atg16L1 complex, LC3-II remains associated with the completed autophagosome. After autophagosomal fusion with the lysosome, LC3-II inside the autolysosome is degraded, whereas LC3-II at the cytoplasmic face is recycled in an Atg4-dependent delipidation process (reviewed by Geng and Klionsky, 2008; Shpilka et al., 2011).

The origin of the autophagosomal membrane is still a question that is under debate. However, several sources have been suggested, including the endoplasmic reticulum (ER), the Golgi complex, the mitochondria and the plasma membrane. Autophagosomes have been reported to form in the vicinity of the ER, and electron microscopy studies have shown that pre-autophagosomal isolation membranes (Atg16L-positive membranes) are connected by a small neck-like continuity to the ER (Axe et al., 2008; Hayashi-Nishino et al., 2009; Matsunaga et al., 2010; Ylä-Anttila et al., 2009). Starvation conditions might also favour the growth of the isolation membrane from the outer membrane of the mitochondria (Hailey et al., 2010). The Golgi and *trans*-Golgi network (TGN) have also been suggested as potential autophagosomal membrane sources, as post-Golgi tubulo-vesicular compartments (Atg9-positive structures) undergo homotypic fusion and remodelling to form an isolation membrane (Geng et al., 2010; Mari et al., 2010; Ohashi and Munro, 2010; van der Vaart et al., 2010; Young et al., 2006). Finally, the plasma membrane contributes to early autophagosomal precursor structures, both under basal and starvation conditions, and it is plausible that this source is important in periods of increased autophagic demands, as the large surface area of the plasma membrane can serve as an extensive membrane reservoir (Moreau et al., 2012; Moreau et al., 2011; Ravikumar et al., 2010a; Rubinsztein et al., 2012).

Despite the multiplicity of possible membrane sources for the autophagosome formation and elongation processes, which might not be mutually exclusive, it remains vague how the lipid bilayers are recruited to PASs. Some recent studies have suggested that Atg9 is a putative factor that recruits membranes to the PAS. Atg9 is the only known transmembrane protein among the core Atg proteins that is required for autophagosome formation, and it is, indeed, a key regulator of autophagy induction. It has been postulated to be involved in the supply of lipid bilayers (and, eventually, autophagy regulators found on those bilayers) to the formation of autophagosomes. Atg9-positive vesicles are, indeed, among the first components of the PAS and necessary for the localisation of other Atg proteins in close proximity to the vacuole in yeast, which suggests a key role for Atg9 in the assembly of the core autophagic machinery (Kraft and Martens, 2012; Suzuki et al., 2007). More recently, in yeast, it has been proposed that a pool of mobile cytoplasmic Atg9 vesicles, which mainly derive from the Golgi complex in a process requiring Atg23 and Atg27, dynamically associates with autophagosomal precursors and might undergo continuous cycling, being retrieved to the Golgi, cytoplasmic 'Atg9 reservoirs' and endosomes (Reggiori and Tooze, 2012; Yamamoto et al., 2012). The trafficking of Atg9 is regulated by Atg1, Atg2, Atg18 and Atg17. In mammals, loss of the Atg1 orthologues ULK1 and ULK2 causes Atg9 to remain in the Golgi complex, whereas loss of ATG2A or ATG2B and WIPI2 affects its retrograde movement (reviewed by Reggiori and Tooze, 2012). Further clarification of the roles of Atg9 in the regulation of autophagy might provide some additional clues with regard to the origins of the autophagosome membranes.

After autophagosomes are randomly formed in the cytoplasm, they are transported along microtubules towards the microtubule-organisation centre (MTOC) in a dynein-dependent manner, where the fusion with lysosomes occurs (Kimura et al., 2008). The fusion step is regulated by a number of proteins, including Rab7, the class C Vps protein complex (C-Vps), endosomal sorting complex required for transport (ESCRTs) and soluble NSF attachment protein receptors (SNAREs) (i.e. VAMP8 and VtiB). In addition to this fusion machinery, correct lysosomal function is also required for efficient fusion. For instance, inhibition of the lysosomal proton pump inhibits the fusion of autophagosomes with lysosomes, most probably because of changes in the acidification status of the lysosome (reviewed by Mizushima and Komatsu, 2011; Rubinsztein et al., 2011).

Small GTP-binding proteins or small GTPases are a superfamily of monomeric G proteins (guanine-nucleotide-binding proteins), which is generally classified into five families: the Ras, Rho, Rab (Ypt in yeast), ADP-ribosylation factor (Arf) and Ran families.

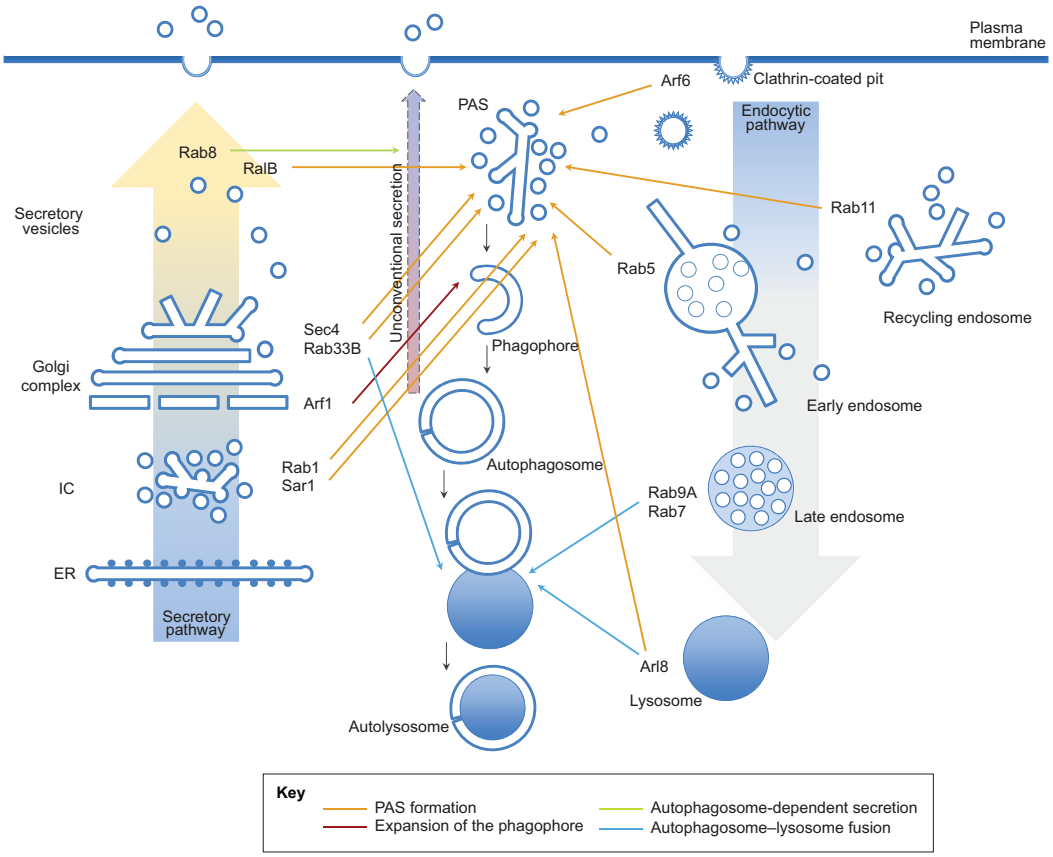

Fig. 1. Schematic illustration of the main functions of Rab, Arf and RalB small GTPases in autophagy and their intersections between the secretory, exocytic and autophagic pathways. Arf6 and Rab33B are involved in the formation of Atg16L1-autophagosome precursors, whereas Rab1, Rab11 and Sec4 are likely to regulate Atg9-mediated autophagosome formation. Autophagosome formation and expansion is also controlled by Arf1, Rab5 and Sar1. RalB takes part in the isolation of the pre-autophagosomal membrane and maturation of autophagosomes through a mechanism dependent on the exocyst complex, whereas Rab8 is a critical factor involved in a new, unconventional form of secretion that relies on autophagy. The autophagosome-lysosome fusion step is mainly dependent on Rab7, but also requires Arl8 and Rab33B. Rab7 has also been implicated in the maturation of GcAvs during bacterial infection, whereas Rab9A appears to regulate the enlargement of GcAvs and their fusion with lysosomes. IC, intermediate compartment; ER, endoplasmic reticulum; PAS, phagophore assembly site.

These proteins are only present in eukaryotes from yeast to human and are essential for multiple cellular processes. The Ras proteins mainly regulate cell proliferation and gene expression. Rho, Rac and Cdc42 proteins of the Rho family regulate both cytoskeleton reorganisation and gene expression. Ran proteins control nucleo-cytoplasmic transport during the G1, S and G2 phases of the cell cycle and microtubule organisation during the M phase, and Rab and Arf proteins regulate intracellular membrane trafficking by controlling membrane identity and vesicle budding, uncoating, motility, tethering and fusion (reviewed by Takai et al., 2001). All small GTPases have consensus amino acid sequences that are able to specifically bind GDP and GTP, being inactive when bound to GDP (cytosolic location) and active when bound to GTP (membrane location). They also have an intrinsic GTPase activity to hydrolyse bound GTP to GDP and phosphate (Pi) (Fig. 2) (reviewed by Takai et al., 2001). Generally, intracellular vesicle transport involves a series of steps: (1) budding of a vesicle from the donor membrane, (2) targeting of the vesicle to the acceptor membrane, (3) docking of the vesicle to the target membrane and, (4) fusion of the vesicle with the target membrane. Arf and Rab proteins, the small GTPases that we focus in this Commentary, are essential for the regulation of these steps. Arf proteins mainly control the budding process, whereas Rab proteins control the targeting, docking and fusion processes (reviewed by Kahn et al., 2005; Stenmark, 2009; Takai et al., 2001).

It is becoming clear that the Rab and Arf GTPases (as well as their molecular partners), which thus far have been associated with endocytic and exocytic vesicular transport, are also required for the regulation of autophagy. For example, several Rab GAPs have been identified to interact with LC3 and might act as molecular switches between the different membrane-trafficking pathways (Popovic et al., 2012). Although not the focus of this Commentary, small GTPases of the Rho and Ras families have also been implicated in the regulation of autophagy. For instance, RhoA positively regulates starvation-induced autophagy through a mechanism that is likely to depend on actin, whereas Rac1 has the opposite effect (Aguilera et al., 2012). In addition, Rag GTPases together with the Ragulator complex mediate amino-acid-dependent recruitment of mTOR to the lysosomal surface where it becomes active in a mechanism that involves the small GTPase Ras homolog enriched in brain (Rheb) (Sancak et al., 2010; Sancak et al., 2008).

In the following sections, we focus on the main findings that have implicated Rab and Arf GTPases in autophagy. We first

Box 2. mTOR-dependent and mTOR–independent signals regulating autophagosome formation

An extensive array of signals regulates the formation of autophagosomes. Generally, they can be categorised into mTOR-dependent or mTOR-independent stimuli. The mTOR pathway is a classic negative regulator of autophagy that is conserved from yeast to mammals. mTOR activity is inhibited under starvation conditions and rapamycin treatment, which results in the partial dephosphorylation of its targets Atg13, ULK1 and ULK2; this activates ULK1 and ULK2 to phosphorylate FIP200 and, thereby, induces autophagy (Hosokawa et al., 2009; Jung et al., 2009). In addition, mTOR is positively and negatively regulated by a plethora of other stimuli. For example, depending on the oncogenic or genotoxic stress, p53 can activate AMP-activated protein kinase (AMPK), which directly activates ULK1 and also inhibits mTOR, or upregulate phosphatase and tensin homologue (PTEN), which inhibits mTOR through inhibition of the Akt kinase (reviewed by Ravikumar et al., 2009; Rubinsztein et al., 2011). In addition, AMPK can also inhibit mTOR activity through the tuberous sclerosis complex 1 (Tsc1), Tsc2 and Ras homology enriched in brain (Rheb) (reviewed by Ravikumar et al., 2009; Rubinsztein et al., 2011). mTOR can also be regulated by GTPases that influence its lysosomal localisation and activity during starvation conditions (Saci et al., 2011; Sancak et al., 2010; Sancak et al., 2008). Recent work has also described that the G-protein-coupled taste receptor complex T1R1–T1R3 acts as a sensor for amino acids, which then regulates mTOR activity and autophagy (Wauson et al., 2012). AMPK can also regulate autophagy independently of mTOR. Under certain circumstances, such as less Ca^{2+}-transfer from the ER to the mitochondria, AMPK activates autophagy without any overt impact on mTOR (Cárdenas et al., 2010). Another well-characterised mTOR-independent signal that regulates autophagy includes the inhibition of inositol monophosphatase (IMPase), which reduces free inositol and inositol (1,4,5)-trisphosphate [Ins(1,4,5)*P*3] levels, resulting in an upregulation of autophagy (Sarkar et al., 2005). Further studies have shown that this pathway is regulated by cyclic AMP (cAMP) and intracellular Ca^{2+}. Increased intracellular levels of cAMP activate the exchange protein directly activated by cAMP (Epac, also known as RAPGEF), which in turn leads to activation of phospholipase ε (PLCε), increased production of Ins(1,4,5)*P*3 and release of Ca^{2+} from the ER. Increased cytosolic Ca^{2+} then leads to the activation of calpains that cleave and activate guanine-nucleotide-binding protein G-protein α-subunit (Gsα), which is responsible for the production of cAMP, forming a regulatory feedback loop (reviewed by Ravikumar et al., 2009; Ravikumar et al., 2010b; Renna et al., 2010). A change in Ca^{2+} levels can also regulate autophagy through an mTOR-dependent mechanism via a signalling pathway that involves Ca^{2+}/calmodulin-dependent protein kinase kinase-β (CAMKK2) and AMPK (Høyer-Hansen et al., 2007).

review small GTPases that affect autophagosome formation – with particular attention on Atg16- and Atg9-precursors – before discussing how small GTPases regulate autophagy-dependent secretion and autophagosome delivery to lysosomes. Rab and Arf GTPases involved in the regulation of autophagy are summarised in Fig. 1 and Table 1.

Small GTPases involved in the formation of autophagosomes

Formation of Atg16L1-positive autophagosome precursors

The formation of phagophore precursor vesicles that are derived from the plasma membrane is dependent on clathrin-mediated endocytosis and on the interaction between Atg16L1 and clathrin heavy chain (Ravikumar et al., 2010a). Arf6, a small GTPase that is typically localised at the plasma membrane and the endocytic compartment, was shown to have a role in the formation of autophagosomes in an endocytosis-dependent manner. For instance, Arf6 promotes autophagosome formation through the activation of phosphatidylinositol (4)-phosphate 5-kinase (PIP5K), which produces phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2]. Both Arf6 and PtdIns(4,5)P_2 are present on autophagic precursors, but not on autophagosomes (Moreau et al., 2012). Arf6 knockdown or strategies that inhibit the formation of PtdIns(4,5)P_2 reduce the delivery of membranes to Atg5–Atg12–Atg16L1-positive and LC3-negative phagophore precursors, thereby inhibiting autophagy (Moreau et al., 2012). It is important to note that Arf6 can regulate autophagy not only by modulating endocytosis, but also by influencing the membrane flow from compartments other than the plasma membrane, or by modulating phospholipase D activity, whose activity has been shown to regulate autophagy (Dall'Armi et al., 2010), because an Arf6 mutant that is unable to activate phospholipase D reduces the formation of autophagosomes compared with Arf6 wild type (Moreau et al., 2012).

Recently, Rab33B, a Golgi-resident small GTPase that is involved in Golgi-to-ER transport (Jiang and Storrie, 2005; Valsdottir et al., 2001), has also been identified as an Atg16L1 interactor (Itoh et al., 2008). Rab33B interacts with Atg16L1 in a GTP-dependent manner through its coiled-coil domain, which is different from the domain that is involved in the Atg5–Atg12–Atg16L1 interaction. Overexpression of mutant Rab33B strongly suppresses autophagy, whereas Rab33B knockdown has no effect on autophagosome formation. This result suggests that Rab33B acts indirectly on autophagy through the sequestration of a yet unidentified Rab33B binding partner that is required for autophagy. This hypothesis is not without precedent, as OATL1 was identified as a molecular partner of Rab33B that is involved in the maturation of autophagosomes (see below) (Itoh et al., 2011).

Regulation of autophagosome formation through small GTPases associated with the Golgi complex or recycling endosomes

Atg9 is mostly found on recycling endosomes and on cytoplasmic mobile vesicles that are derived from the Golgi complex, and colocalises with TGN46, cation-independent mannose-6-phosphate receptor (CIMPR), Rab7 and Rab9 (Longatti et al., 2012; Yamamoto et al., 2012; Young et al., 2006). Depletion of Atg9 inhibits autophagosomal synthesis (Young et al., 2006), and upregulation of autophagy results in a redistribution of Atg9 from the TGN to endosomal membranes that are also LC3-positive (Young et al., 2006).

Sar1 and Rab1 GTPases control the membrane traffic from the ER to the Golgi through an intermediate compartment (Lippincott-Schwartz et al., 2000; Plutner et al., 1990; Saraste and Kuismanen, 1992), and were found to participate in the early stages of autophagy (Zoppino et al., 2010). Interestingly, yeast cells that are deficient in the guanine nucleotide exchange factor (GEF) Sec12 (also known as Preb) – which activates Sar1 – are characterised by an impairment of autophagosome formation, whereas Sar1 overexpression can rescue this phenotype (Ishihara et al., 2001). This suggests that Sar1 induces autophagy through a mechanism that is dependent on Sec12 (Zoppino et al., 2010).

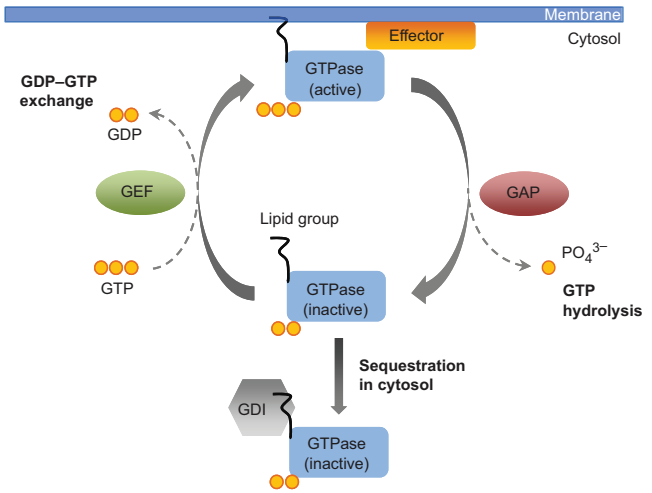

Fig. 2. GDP-GTP exchange cycle of small GTPases. Small GTPases cycle between two interconvertible forms, the GDP-bound inactive and GTP-bound active forms, and have intrinsic GTPase activity that hydrolyses bound GTP to GDP and Pi. Cyclical activation, inactivation and translocation of small GTPases are regulated by guanine nucleotide exchange factors (GEFs) that catalyse the exchange of GDP to GTP, guanosine-nucleotide dissociation inhibitors (GDIs) that prevent the exchange of GDP to GTP, maintaining a pool of small GTPases in an inactive state in the cytosol, and GTPase-activating proteins (GAPs) that increase the rate of GTP hydrolysis to GDP. GTPases interact with various downstream effectors that modulate their activity and/or localisation. For example, in the case of Rab proteins, their hypervariable C-terminal domains specifically interact with coat components, motors and SNAREs, which confer to them a high specificity for target membranes. Most of small GTPases also contain sequences at their C-termini that are targets of proteolysis or post-translational modifications by methylation or lipid groups, such as geranylgeranyl, palmitoyl, farnesyl and myristoyl moieties. Those lipid modifications are generally necessary for the binding of small GTPases to membranes and regulators, and for the activation of downstream effectors.

Moreover, depletion of one of the components of the TRAPPI complex (a Rab1 GEF) also causes a defect in the organisation of the pre-autophagosomal structure (Meiling-Wesse et al., 2005), supporting a role for Rab1 in this pathway (Zoppino et al., 2010). Remarkably, Atg11 has been recently described as an effector of the Rab1 orthologue in yeast (Ypt1), and the Atg11–Ypt1 complex is necessary for the assembly of the PAS during normal growth conditions. The Atg11–Ypt1 complex was found to colocalise with Trs85, a Ypt1 activator subunit, which is also important for the regulation of autophagy. In addition, Ypt1 and Trs85 interact on Atg9-positive membrane structures that can serve as a source for the PAS membrane (Lipatova et al., 2012). Trs85 also directs TRAPPIII (a Ypt1 GEF) to the PAS, reinforcing the role of Ypt1 and Rab1 in the regulation of autophagy (Lynch-Day et al., 2010).

Rab1a has also been involved in the clearance of protein aggregates, peroxisomes and *Salmonella* (Huang et al., 2011), as well as in the homeostasis of α-synuclein, the most abundant component in Lewy bodies (the histological marker for Parkinson disease). Overexpression of α-synuclein inhibits autophagosome formation by disrupting Rab1a homeostasis and secretion. Consistent with this observation, siRNA knockdown of Rab1a inhibits autophagosome formation and increases the accumulation of autophagic substrates (similar to α-synuclein overexpression). Interestingly, Rab1a knockdown or α-synuclein overexpression cause Atg9 mislocalisation and decrease its colocalisation with LC3-positive vesicles (Winslow et al., 2010), suggesting impairment of Atg9-dependent autophagosome formation.

Arf1 and the Arf GEF Sec7 also localise and act in the Golgi complex (Franzusoff and Schekman, 1989; Novick et al., 1980). In yeast, as in mammalian cells, the Golgi complex plays an important role in membrane sorting and protein secretion and blockage of Golgi transport in yeast severely impairs autophagy (van der Vaart et al., 2010). In particular, Arf1 and Sec7 appear to be essential for the expansion of the phagophore, rather than for the correct assembly of the autophagic machinery at the pre-autophagosomal structure (van der Vaart et al., 2010). Thus, in yeast, membranes or proteins that are provided by the Golgi complex are necessary for the formation of the autophagosome (van der Vaart et al., 2010). Sec4 (a member of Rab family) and its GEF Sec2 have similar functions in autophagy; both are localised at the Golgi and provide membrane for autophagosome formation. In Sec2- and Sec4-mutant cells, Atg9 anterograde movement is impaired and Atg8 is inefficiently recruited to the phagophore membrane, resulting in reduced autophagosomes formation (Geng et al., 2010).

It is important to note that, in addition to its role in the expansion of the phagophore, Arf1 also regulates autophagy through the mTOR signalling pathway. A screen of *Drosophila melanogaster* small GTPases found that mTORC1 activity is regulated by members of Arf and Rab GTPases, such as Arf1 and Rab5 (Li et al., 2010). Upregulation of Arf1 or Rab5 strongly inhibits mTOR activity in response to amino-acid-starvation, whereas glucose-induced activation of mTOR is not affected by Arf1 and Rab5. Moreover, Rab5, in *Drosophila*, selectively inhibits mTOR activation (Li et al., 2010).

As mentioned before, Atg9 trafficking occurs not only through the Golgi and the TGN but also through recycling endosomes. Atg9 localisation and trafficking in this compartment is essential for the initiation and progression of autophagy (Orsi et al., 2012). Rab11 is a small GTPase that localises in the pericentriolar endosomal recycling compartment and is responsible for the recycling of the transferrin receptor through that compartment (Ren et al., 1998). Rab11 was initially implicated in the regulation of autophagy (Huttenhower et al., 2009) at the level of fusion between multivesicular bodies (MVBs) and autophagosomes (Fader et al., 2008; Fader et al., 2009). Autophagy inducers, such as rapamycin or starvation, cause indeed enlargement of Rab11-positive vacuoles and a remarkable colocalisation of Rab11 with LC3 (Fader et al., 2008). Subsequent studies have suggested additional roles for Rab11. An important functional correlation between Rab11 and the regulation of autophagy was obtained from a genetic screen of Rab GTPase activating proteins (GAPs), in which several negative regulators of starvation-induced autophagy were identified (Longatti et al., 2012). The Rab GAP TBC1D14 was shown to colocalise and interact with the autophagy kinase ULK1, which, in turn, localises to recycling endosomes. Loss of Rab11 prevents TBC1D14-induced tubulation of recycling endosomes by inhibiting autophagosome formation. Atg9 also localises in recycling endosomes together with ULK1. Taken together, these data show that Rab11-dependent vesicular transport from recycling endosomes contributes to and regulates autophagy (Longatti et al., 2012).

Table 1. Rab and Arf GTPases involved the regulation of autophagy

Small GTPase	Primary subcellular localisation	Role in autophagy	References
Arf1	Golgi complex	Expansion of the phagophore and inhibition of mTORC1 activity.	van der Vaart et al., 2010; Li et al., 2010
Arf6	Plasma membrane and endocytic compartment	Atg16L1-precursor formation by regulating endocytosis, PIP5K and phospholipase D.	Moreau et al., 2012
Arl8	Lysosomes	Lysosomal redistribution in a pH-dependent manner, affecting autophagosome formation and autophagosome-lysosome fusion.	Korolchuk et al., 2011
Rab1	Intermediate compartment (ER to Golgi transport)	Autophagosome formation and PAS assembly by controling the correct localisation of Atg9.	Meiling-Wesse et al., 2005; Zoppino et al., 2010; Winslow et al., 2010; Lynch-Day et al., 2010; Huang et al., 2011; Lipatova et al., 2012
Rab5	Early endosomes	Regulation of Vps34 activity and inhibition of mTOR-dependent signalling.	Ravikumar et al., 2008; Li et al., 2010; Su et al., 2011
Rab7	Late endosomes	Autophagosome-lysosome fusion, autophagic lysosomal restoration, transport of autophagosomes and GcAV formation, most likely by recruiting Atg5-positive membranes to GAS.	Kirisako et al., 1999; Gutierrez et al., 2004; Jager et al., 2004; Yamaguchi et al., 2009; Yu et al., 2010; Pankiv et al., 2010; Sakurai et al., 2010; Ganley et al., 2011
Rab8	Secretory vesicles (TGN to plasma membrane transport)	Regulation of unconventional secretion by a mechanism dependent on autophagosome exocytosis.	Dupont et al., 2011; Pilli et al., 2012
Rab9	Golgi and late endosomes	Enlargement of GcAVs and fusion of GcAVs with lysosomes in Group A *Streptococcus*-induced autophagy.	Nozawa et al., 2012
Rab11	Recycling endosomes	Autophagosome formation in a mechanism dependent on ULK1 and the Rab-GAP TBC1D14.	Longatti et al., 2012
Rab33B	Golgi complex	Atg12–Atg5–Atg16L1 complex recruitment to pre-autophagosomal structures, inducing conjugation of LC3 to PtdEtn; also interferes with the fusion of autophagosomes with lysosomes.	Itoh et al., 2008; Itoh et al., 2011
RalB*	Secretory vesicles (Exocyst)	Assembly of active ULK1 and Beclin-1–Vps34 complexes on the exocyst, which is required for the isolation of the pre-autophagosomal membrane and maturation of autophagosomes.	Bodemann et al., 2011
Sar1	Intermediate compartment (ER to Golgi transport)	Autophagosome formation by a mechanism most likely dependent on the Sec12 GEF.	Ishihara et al., 2001; Zoppino et al., 2010
Sec4	Golgi complex and TGN-derived vesicles	Provides membrane for autophagosomes formation and is involved in the anterograde movement of Atg9 and recruitment of Atg8 to the phagophore membrane.	Geng et al., 2010

*It should be noted that RalB is a Ras-related protein and does not belong to the Rab or Arf families. Although this Commentary mainly focuses on the role of Rab and Arf small GTPases on autophagy, we have included RalB, as part of the exocyst, since this complex is also involved in vesicle trafficking, such as the Rab and Arf proteins.

Regulation of ULK1-mediated formation of autophagosomes

The exocyst, a protein complex involved in tethering transport vesicles to the plasma membrane, has been identified to act as a scaffolding complex for starvation-induced autopagy (Bodemann et al., 2011). The Ras-like small GTPase RalB, through its direct binding to the exocyst component Exo84, induces the assembly of active ULK1 and Beclin-1–Vps34 complexes at the exocyst, which is required for the isolation of the pre-autophagosomal membrane and maturation of autophagosomes (Bodemann et al., 2011). Interestingly, Vps34 activity has also been found to be regulated by Rab5, a small GTPase that is localised in the early endosomes (Ravikumar et al., 2008; Su et al., 2011). Given the role of the exocyst in the transport of vesicles between the plasma membrane and the Golgi, one can hypothesise that the exocyst mediates the movement of membranes between the plasma membrane and autophagosomes.

Formation of autophagosomes during bacterial infection

Although the most accepted role of Rab7 in autophagy refers to its ability to control the autophagosome-lysosome fusion step (see below), there are a few studies suggesting that Rab7 also participates in autophagosome-like vacuole formation during the invasion of certain microorganisms, such as *Streptococcus pyogenes* (also known as Group A streptococcus and hereafter referred to as GAS) (Sakurai et al., 2010; Yamaguchi et al., 2009). This bacterium infects non-phagocytic cells through endocytosis, but subsequently escapes the endolysosomal pathway. After this, GAS is engulfed by an autophagosome-like vacuole, which eventually acquires lysosomal enzymes that induce its proteolysis. The formation of a GAS-containing autophagosome-like vacuole (GcAV) is triggered by canonical autophagic proteins such as Atg5 (Nakagawa et al., 2004), and these vesicles are decorated with LC3 (Nakagawa et al., 2004; Yamaguchi et al., 2009). Rab7 was found to be involved in GcAV formation and maturation, most probably by assisting in the recruitment of LC3 to GcAVs and inducing homotypic membrane fusion during the enlargement process of GcAVs (Yamaguchi et al., 2009). Rab7, indeed, appears to participate in the coalescence of multiple small Atg5-positive membranes that localise around the GAS bacteria, suggesting it has a role in the early phase of GcAV formation (Sakurai et al., 2010; Yamaguchi et al., 2009).

In addition to Rab7, Rab9A has also been implicated in the enlargement of GcAVs and the fusion of GcAVs with lysosomes

during GAS-induced autophagy. However, starvation-induced autophagy does not appear to be regulated by this small GTPase, suggesting that different types of autophagy require different factors that confer distinct specificities and functions to the different autophagy pathways (Nozawa et al., 2012).

Small GTPases involved in autophagosome-mediated exocytosis

The autophagy machinery is also involved in regulating the secretion of the contents of granules or secondary lysosomes, such as Acyl-CoA-binding protein (Acb1) in yeast, and interleukin 1β and interleukin 18 (IL1B and IL18, respectively) in mammalian cells (Deretic et al., 2012; Dupont et al., 2011; Manjithaya et al., 2010). This newly described form of secretion, which has been named autophagy-based unconventional secretion, is dependent on Rab8a, a small GTPase that is located at the Golgi and canonically involved in the polarised sorting from the Golgi to the plasma membrane. This autophagy-dependent secretory pathway enables cytosolic proteins to exit the cell without entering the conventional ER-to-Golgi secretory pathway. It remains unclear why cells would use one particular secretion system for specific proteins instead of other systems. However, this recently unveiled aspect of autophagy may be relevant in the context of inflammatory diseases, such as cystic fibrosis and Crohn disease (Cadwell et al., 2008; Cadwell et al., 2010; Gee et al., 2011; Saitoh et al., 2008). Autophagy can regulate the unconventional trafficking of the cystic fibrosis transmembrane conductance regulator (CFTR), the protein that is mutated in cystic fibrosis, from the ER to the plasma membrane without passing through the Golgi. The impaired conventional Golgi-mediated secretion and cell surface expression of the CFTRΔ508 mutant protein can be rescued by directing it to an unconventional Golgi reassembly stacking protein (GRASP)-dependent secretion pathway using autophagosomes as a vehicle (Gee et al., 2011). In the context of Crohn disease, autophagy participates in the regulated secretion of lysozyme from Paneth cells (Cadwell et al., 2008), which is an important component of the intestinal immune response. With regard to bacterial infection, Rab8 has been shown to be involved in the autophagic elimination of *Mycobacterium tuberculosis* var. bovis BCG through its interaction with TANK-binding kinase 1 (TBK1), which is involved in the phosphorylation of the autophagic adaptor p62 and assembly of the autophagy machinery (Pilli et al., 2012).

Small GTPases involved in the trafficking of autophagosomes and lysosomes and their fusion

Rab7 as a main regulator of autophagosome-lysosome fusion

The fusion of autophagosomes with the vacuole (the lysosome in yeast) is compromised in *Saccharomyces cerevisiae* mutants that lack the yeast Rab7 orthologue Ypt7, resulting in the accumulation of autophagosomes in the cytoplasm (Kirisako et al., 1999). Many other studies that subsequently emerged aimed to characterise the mechanisms involved in the regulation of the fusion event by Rab7. Rab7 was found to regulate the maturation of late autophagic vacuoles and the formation of the autolysosome both under nutrient replete and starvation conditions through a mechanism that is dependent on the binding and hydrolysis of GTP (Gutierrez et al., 2004; Jäger et al., 2004) (Fig. 3A). More recently, a role for Rab7 has also been reported in a process named autophagic lysosomal restoration (ALR), which involves the termination of autophagy and formation of nascent lysosomes from autolysosomes in a mechanism that depends on the reactivation of mTOR. Treatment of cells with GTPγS, a non-hydrolysable analogue of GTP, completely inhibits ALR, and overexpression of constitutively active Rab7 that is permanently associated with membranes, also abrogates ALR, resulting in the accumulation of enlarged and long-lasting autolysosomes. Inhibition of ALR by rapamycin also inhibits the dissociation of Rab7 from autolysosomes, suggesting that mTOR and Rab7 together participate in the regulation of ALR (Yu et al., 2010).

Several groups have been focusing their efforts on the understanding of the molecular mechanisms, as well as in the identification of the molecular complexes that are involved in the regulation of autophagy by Rab7. FYVE and coiled-coil-domain-containing protein (FYCO1) has been identified in a recent study as a Rab7 effector protein that is able to bind LC3 and $PtInsP_3$ and mediate microtubule plus-end-directed transport of autophagic vesicles (Pankiv et al., 2010). FYCO1 seems to have a role in the redistribution of Rab7- and LC3-positive vesicles to the cell periphery in a microtubule-dependent manner that might, for instance, interfere with the fusion of autophagosomes with lysosomes. Indeed, the authors identified a potential kinesin-binding site in the central part of the coiled-coil region of FYCO1 (Pankiv et al., 2010). Although the physiological implications of this mechanism were not explored in depth, the authors propose a mechanism whereby FYCO1 preferentially localises at the ER in a conformation that prevents its binding to kinesins under nutrient-rich conditions. However, upon starvation, FYCO1 binds to the microtubule plus-end-directed motors and redistributes pre-autophagosomal membrane compartments to the sites of autophagosome formation throughout the cytosol. It is also proposed that, after the formation of autophagosomes, FYCO1 competes with the dynein recruitment complex for binding to Rab7, providing a regulated bidirectional transport of autophagosomes along microtubule tracks (Pankiv et al., 2010) (Fig. 3B).

Another factor that is involved in the regulation of Rab7 is Rab7-interacting lysosomal protein (RILP), a component of the complex that is responsible for the binding of Rab7 to dynactin–dynein1 (Fig. 3B). Interestingly, the interaction between Rab7 and RILP is positively affected by the activation of Rab7 through the complex of class C-Vps (also known as HOPS complex) – a tethering protein complex that serves multiple membrane fusion events (e.g. autophagosome fusion with late endosomes and lysosomes) – and UVRAG, a protein known to induce autophagy and membrane curvature through a mechanism that is dependent on Beclin 1 and PtdIns 3-kinase class III (Liang et al., 2008). Indeed, the interaction between C-Vps and UVRAG stimulates Rab7 activity and promotes autophagosome maturation and fusion with lysosomes (Liang et al., 2008) (Fig. 3A). The GDP-GTP exchange on Rab7 necessary for its activation is likely to be dependent on the GEF activity of the C-Vps complex (Liang et al., 2008; Rink et al., 2005; Wurmser et al., 2000). The Rab7-RILP interaction is also regulated by the insulin-like growth factor 1 (IGF1)–AKT pathway during neuronal autophagy (Bains et al., 2011), unveiling further complexities of the regulation of autophagy by Rab7. An additional level of Rab7 regulation is also provided by rubicon (Run domain beclin-1-interacting and cysteine-rich-containing protein), another Beclin 1-binding

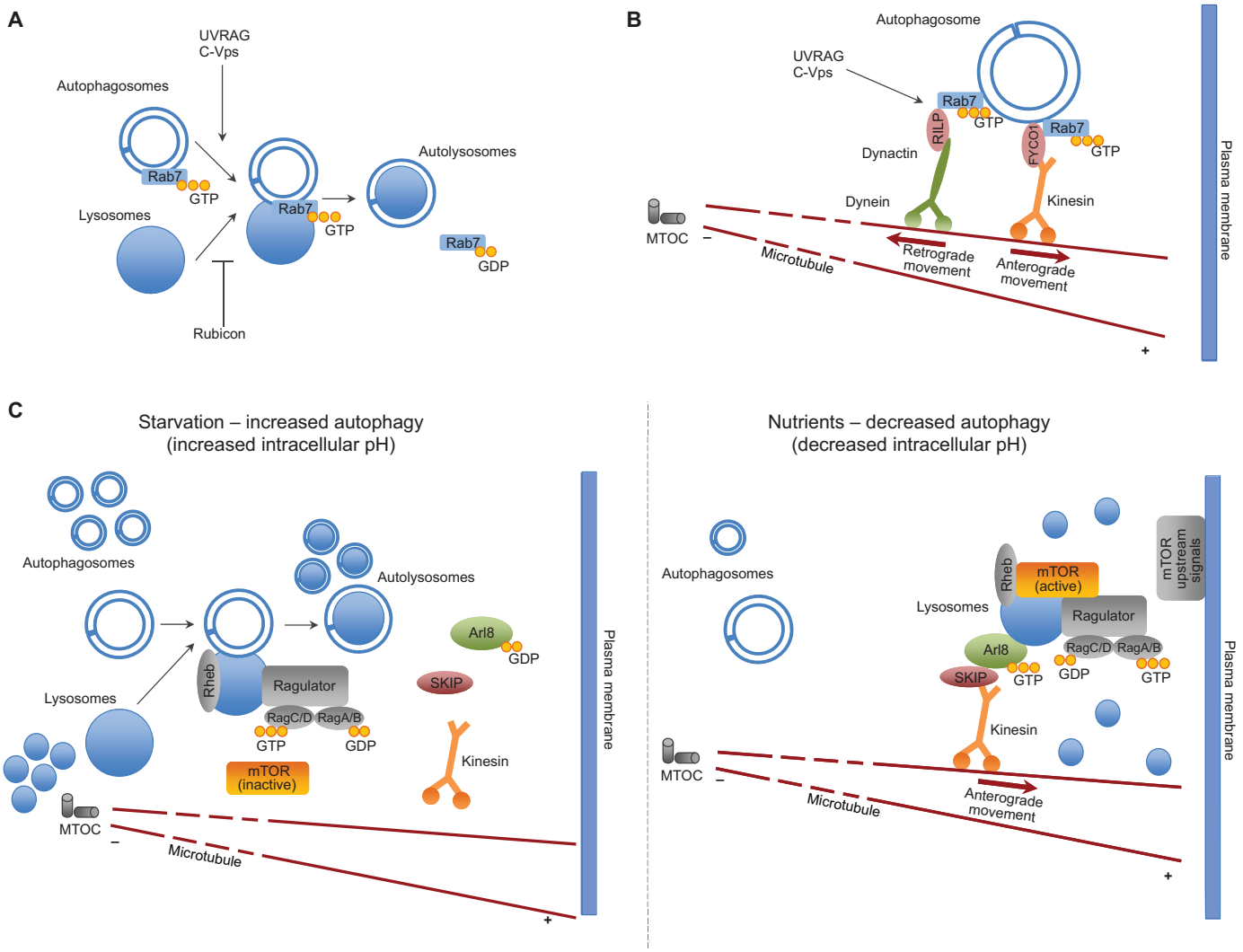

Fig. 3. Rab7- and Arl8-ancillary machinery involved in the positioning of lysosomes and autophagosomes, and their fusion. (**A**) Rab7 has been implicated in the fusion between autophagosomes and lysosomes through a mechanism that is dependent on the binding and hydrolysis of GTP. The UVRAG–C-Vps complex appears to activate Rab7 activity and stimulate autophagosome-lysosome fusion, whereas Rubicon inhibits this step. (**B**) The movement of autophagosomes has been suggested to rely on a precise balance between dynein- and kinesin-dependent mechanisms. Starvation conditions are likely to promote the binding of FYCO1 to kinesin, and to Rab7 and LC3-II in the membrane of autophagosomes, inducing a redistribution of the pre-autophagosomes throughout the cytosol. FYCO1 appears to compete with the RILP-dynactin-dynein complex for the binding to Rab7, providing means to regulate the bidirectional movement of autophagosomes along microtubules. RILP-Rab7 interaction is further controlled by the UVRAG–C-Vps complex. (**C**) Left: starvation conditions, characterised by an increase of the intracellular pH, inhibit the recruitment of Arl8B (which is kept on the cytosol in a GDP-bound form) and of the kinesin KIF2A to the lysosomal membrane; this favours the accumulation of lysosomes and the fusion between lysosomes and autophagosomes in a perinuclear region close to the MTOC. mTOR is also inhibited under starvation conditions, favouring the formation of new autophagosomes. Right: after nutrient replenishment (characterised by a decrease of the intracellular pH), Arl8 bound to GTP is recruited to the lysosomal membrane in a complex with pleckstrin-homology-domain-containing family M member 2 (PLEKHM2; here referred to as SKIP) and kinesin, which binds to microtubules (Korolchuk et al., 2011; Rosa-Ferreira and Munro, 2011). Lysosomes are subsequently transported towards the cell periphery. mTOR is also recruited to the lysosomal membrane, where it becomes activated, by a mechanism that is dependent on Ragulator and the small GTPases RagA/B, RagC/D and Rheb (located at the lysosome) and by upstream mTOR activating-signals that are located at the plasma membrane, resulting in an inhibition of autophagy. The centrifugal movement of lysosomes also diminishes the encounter between lysosomes and autophagosomes, which interferes with their fusion and clearance of autophagic substrates.

protein. Rubicon has been shown to inhibit the maturation and fusion steps during autophagy (Matsunaga et al., 2009; Zhong et al., 2009). Interestingly, rubicon appears to sequester UVRAG from the C-Vps complex and block Rab7 activation (Sun et al., 2010), suggesting that it terminates Rab7-dependent maturation of autophagosomes by modulating UVRAG in the C-Vps complex (Tabata et al., 2010) (Fig. 3A).

Rab7 activity can also be regulated by changes of intracellular Ca^{2+} signalling. For instance, the ER stress inducer thapsigargin, which raises cytosolic Ca^{2+} levels by inhibiting the Ca^{2+} pump of the ER, was shown to block autophagy, both under basal or starvation conditions, by inhibiting recruitment of Rab7 to autophagosomes and the fusion of autophagosomes with lysosomes without affecting endocytosis (Ganley et al., 2011).

This response appears to be independent of a functional ER stress response pathway, because thapsigargin also blocks autophagy in ER-stress inositol requiring enzyme (IRE)-null cells (Ganley et al., 2011; Williams et al., 2008).

The role of other small GTPases in autophagosome–lysosome fusion

In addition to Rab7, other trafficking-associated small GTPases probably also regulate the autophagosome-lysosome fusion step. For instance, Rab33B, a Golgi-resident Rab protein that is involved in retrograde transport, might have an indirect role in this process through the activity of its GAP OATL1 (also known as TBC1D25) (Itoh et al., 2011). It has been proposed that activated Rab33B recruits the Atg12–Atg5–Atg16L1 complex to pre-autophagosomal structures, thereby inducing the subsequent conjugation of LC3 to PtdEtn. Following this, OATL1 recognises LC3 in the autophagosomal membrane in proximity to Rab33B and inactivates Rab33B through its GAP activity, exerting a feedback loop. This mechanism appears to be involved in autophagosomal maturation and conversion of autophagosomes to autolysosomes, because inactivation of Rab33B through overexpression of OATL1 inhibits the encounter of autophagosomes and lysosomes, whereas overexpression of constitutively active Rab33B reduces the rate of fusion between autophagosomes and lysosomes (Itoh et al., 2011).

Another GTPase that indirectly regulates autophagosome–lysosome fusion is the Arf-like GTPase Arl8B. A decrease of the intracellular pH – induced, for example, through nutrient-rich conditions – increases recruitment of Arl8B and the kinesin KIF2A to lysosomes, which promotes their centrifugal movement along microtubules towards the cell periphery (Korolchuk et al., 2011). This occurs concomitantly with the activation of mTORC1, which inhibits autophagosome formation and decreases autophagosome-lysosome fusion because encounters of autophagosomes and lysosomes in the perinuclear region are less likely (Korolchuk et al., 2011). Activation of mTORC1 primarily occurs at the surface of lysosomes through a mechanism that depends on Rheb, ragulator and the Ras-related GTPases RagA or RagB, and RagC or RagD (Sancak et al., 2010; Sancak et al., 2008), as well as on the intracellular positioning of lysosomes – as many signals upstream of mTOR are located at the cell periphery (Korolchuk and Rubinsztein, 2011) (Fig. 3C).

Conclusions

In this Commentary, we have described how a number of small GTPases modulate different steps of autophagy, including autophagosome formation, autophagosome secretion, autophagosome trafficking and fusion with lysosomes. For instance, Arf6 and Rab33B are small GTPases implicated in Atg16L-mediated autophagosome formation, whereas the small GTPases Rab1, Rab11 and Sec4 are likely to be involved in the formation of Atg9-autophagosome precursors. Autophagosome formation and expansion is also controlled by Arf1, Rab5 and Sar1. Although the most-accepted role of Rab7 in autophagy refers to its ability to control the autophagosome–lysosome fusion step, it is also involved in the formation and maturation of autophagosomes during bacterial infection, as is the small GTPase Rab9A. In addition, RalB has been implicated in the regulation of ULK1-mediated formation of autophagosomes, whereas Rab8 controls a new and unconventional form of secretion that relies on autophagy. The trafficking of autophagosomes and lysosomes, as well as their fusion has been shown to mainly depend on Rab7, but also requires Arl8 and Rab33B (Fig. 1 and Table 1).

Despite extensive research efforts, the molecular networks and complexes that support the functions of these small GTPases in autophagy, as well as their spatial and temporal control, are still not completely understood and important questions still need to be answered. In addition, it is still unclear how small GTPases reach the autophagosomal membrane. Understanding this step may shed some light into the mechanisms that underlie the intersections between endocytic, secretory and autophagic pathways. An interesting hypothesis is that a pool of Atg9-positive membranes can cycle dynamically between the endosomal compartment, the secretory pathway and the pre-autophagosomal structures (Reggiori and Tooze, 2012; Young et al., 2006), which might explain how small GTPases are recruited to the surface of autophagosomes in a mechanism that is independent of the direct fusion between different compartments. However, one should not exclude the canonical process for the recruitment of small GTPases to membranes without the need of vesicular transport or fusion-dependent events, which relies on their direct recruitment from the cytoplasm to the membrane by a mechanism that is dependent on the exchange of GDP to GTP.

Future studies on the regulation of autophagy through small GTPases may also help us to understand the origins of the autophagosome membrane, because recent evidence suggested that Atg9-dependent autophagosome formation is regulated by various small GTPases and that it dynamically cycles between the phagophore, Golgi complex, endosomes and cytoplasmic ‘Atg9 reservoirs’. One can, therefore, hypothesise that the primary location of these GTPases provides clues about the membrane sources for the formation of the autophagosome.

Acknowledgements

We are grateful to Fiona Menzies, Mariella Vicinanza and Maurizio Renna for critical reading of the manuscript.

Funding

We thank the Wellcome Trust for a Principal Research Fellowship [to D.C.R.) and a Strategic Grant to the Cambridge Institute for Medical Research.

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