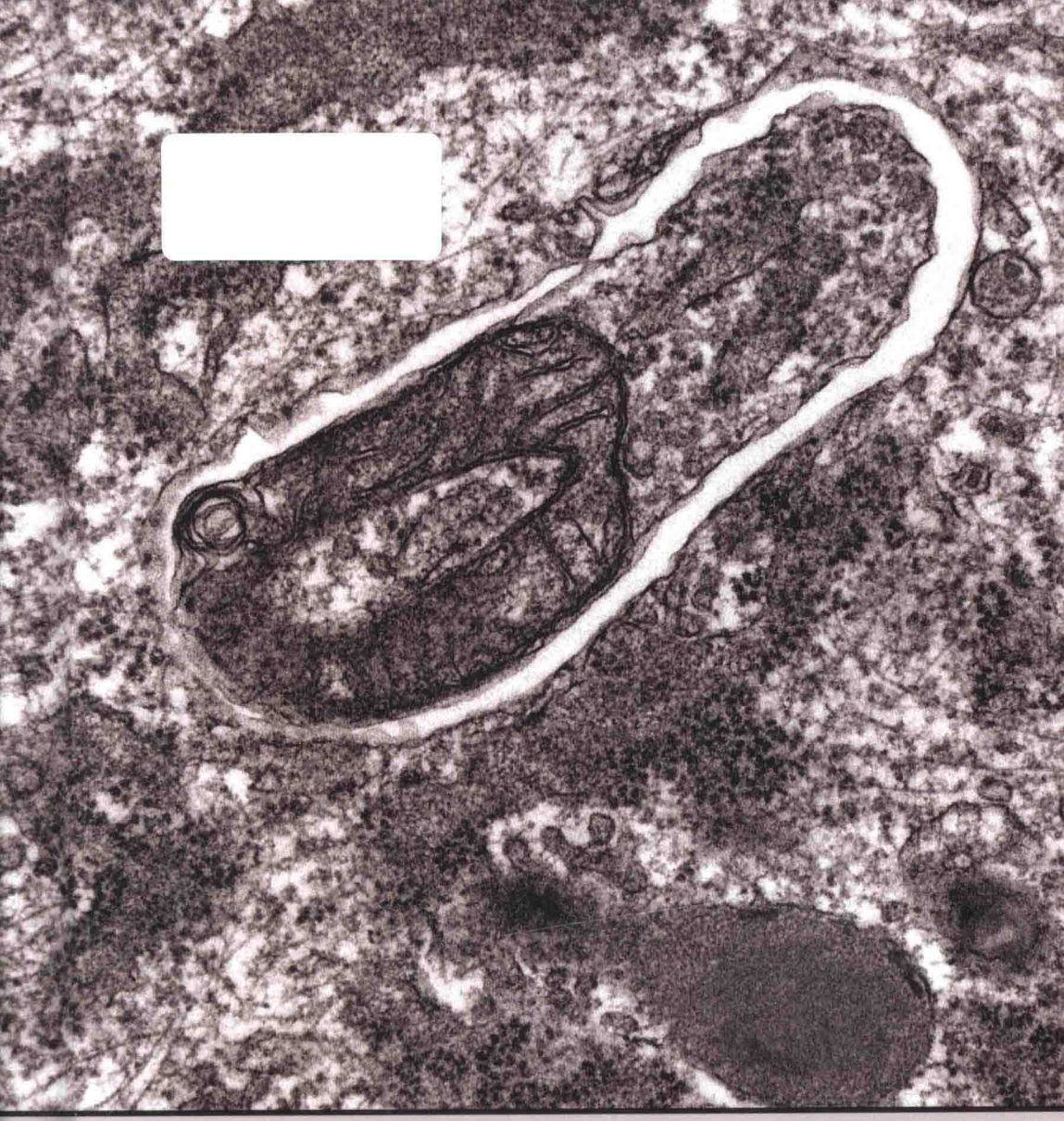

Current Research in AUTOPHAGY

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Preface

It is often said that books are a boon to mankind. They document every progress and pass on the knowledge from one generation to the other. They play a crucial role in our lives. Thus I was both excited and nervous while editing this book. I was pleased by the thought of being able to make a mark but I was also nervous to do it right because the future of students depends upon it. Hence, I took a few months to research further into the discipline, revise my knowledge and also explore some more aspects. Post this process, I begun with the editing of this book.

The contradictory role of autophagy i.e. cell survival versus cell death; draws the focus on the importance of keeping in mind this double-edged nature in future developments of the currently promising autophagy-modulating therapies. This book highlights some of the demanding research topics related to autophagy and also analyzes the recent developments in its molecular mechanisms. The emphasis is on the role this basic cell defense mechanism plays in studying various diseases which include autophagy in infectious diseases, in neurodegenerative diseases, and cell death.

I thank my publisher with all my heart for considering me worthy of this unparalleled opportunity and for showing unwavering faith in my skills. I would also like to thank the editorial team who worked closely with me at every step and contributed immensely towards the successful completion of this book. Last but not the least, I wish to thank my friends and colleagues for their support.

Editor

Contents

Section 1

New Insights into Mechanisms of Autophagy

Rab GTPases in Autophagy

Yuko Hirota, Keiko Fujimoto and
Yoshitaka Tanaka

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1. Introduction

Rab proteins constitute a subfamily of small GTPases that play important roles in the spatio-temporal regulation of intracellular vesicle transport [1-3]. Rab GTPases represent a large family of small guanosine triphosphate (GTP)-binding proteins that comprise more than 60 known members. In mammalian cells, it is well established that different Rab proteins localize on distinct membrane-bound compartments, where they regulate multiple steps in membrane traffic, including vesicle budding, fusion, and movement, through cycling between an inactive guanosine diphosphate (GDP)-bound form and an active GTP-bound form [3]. Guanine nucleotide exchange factor (GEF) shifts GTPase from its inactive GDP-bound form to its active GTP-bound form, while GTPase activating domain protein (GAP) inactivates GTPase [Figure 1]. Many structural and biological studies have shown that specific amino acid mutation can make possible to keep Rab GTPase in its GDP-bound form or GTP-bound form; therefore, expression of GDP-bound form or GTP-bound form could imitate its function [3].

Autophagy is a degradation pathway that delivers cytoplasmic components and intracellular organelles at random and/or in a selective manner to lysosomes via doubled-membrane organelles called autophagosomes [4]. Although autophagy is induced by exposure of cells to nutrient- or growth factor-deprived medium, it also occurs at basal levels in most tissues and contributes to the routine turnover of cytoplasmic materials. So far, in the process of the formation of autophagosomes, many mammalian homologues of yeast *ATG* (autophagy-related) genes have been identified and extensively characterized, demonstrating that the molecular machinery of autophagy has been conserved from yeasts to mammals [5]. Analyses of the Atg proteins have identified two ubiquitin-like conjugation systems that are required for autophagosome formation [6]. Among these proteins, Atg5 and LC3 (a mammalian homolog of Atg8) have been analyzed in more detail. An Atg12-Atg5 conjugate is necessary

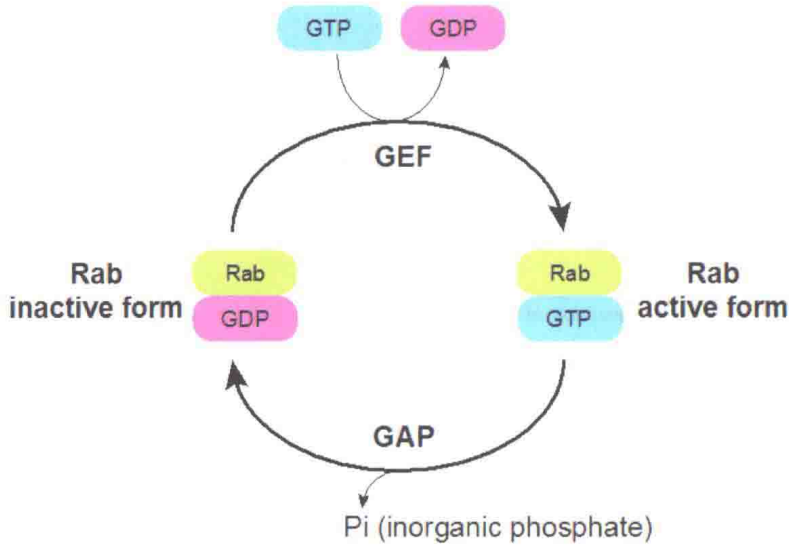

Figure 1. The Rab switch

for elongation of the isolation membrane on its outer side [7]. ProLC3 is processed by Atg4, a cystein protease, to a cytosolic form, LC3-I, exposing a carboxyl terminal glycine [8]. LC3-I is subsequently activated by the E1-like protein Atg7, and conjugates phosphatidylethanolamine to its C-terminal glycine via the E2-like enzyme Atg3, producing a membrane-bound form, LC3-II [9]. LC3-II localizes on the isolation membrane and the autophagosome membrane [10,11]. Because the amount of LC3-II correlates with the number of autophagosomes, detection of LC3-II by Western blotting can be used to measure autophagic activity. In addition, dot-like or ring-like staining of LC3 in immunofluorescence is widely utilized as a specific marker of the formation of autophagosomes.

Akin to the involvement of Rab proteins in vesicle transport processes, there is a growing body of evidence that many Rab proteins, such as Rab7, Rab9, Rab11, Rab24, Rab32, and Rab33, function in the formation and/or maturation of autophagic vacuoles. Each of these Rab proteins localizes to distinct intracellular compartments and thereby appears to be involved in a distinct step of autophagic flux. In this chapter, we focus on the roles of these Rab proteins in the regulation of the autophagic process.

2. Rab7 GTPase and autophagy

2.1. Rab7 GTPase and autophagosome maturation

Rab7 is a member of the Rab family, which is involved in vesicle transport from early endosomes to late endosomes/lysosomes as well as lysosome biogenesis [12, 13]. Rab7 is also

implicated in the fusion between autophagosomes and lysosomes, i.e., autophagosome maturation [14-16]. Rab7 wild-type (WT) and active-forms of Rab7 (Rab7Q67L) are associated with ring-shaped vesicles labeled with the autofluorescent compound monodansylcadaverine (MDC), which is preferentially incorporated into mature autophagosomes and autolysosomes, and with LC3, which preferentially labels immature autophagosomes, indicating the association of Rab7 with autophagic vesicles [14]. On the other hand, overexpression of the inactive form of Rab7 (Rab7T22N) causes a marked increase in the size of MDC- and LC3-positive vesicles and the number of LC3-positive vesicles, but reduces the number of MDC-positive vesicles, indicating that the inactive-form of Rab7 impaired the fusion between autophagic vacuoles and lysosomes. Similar results were also obtained in cells depleted of Rab7 by RNAi [15]. Collectively, these results suggest that Rab7 is not essential for the initial step of autophagosome maturation, but is involved in the final step of the maturation of late autophagic vacuoles, possibly in the fusion with lysosomes [14, 15]. Interestingly, Rab7T22N that was diffusely localized in the cytoplasm under nutrient-rich conditions was redistributed to the membrane of MDC-positive vacuoles by amino acid starvation or by rapamycin treatment [14]. Thus, Rab7 is targeted to the autophagosomal membranes by a TOR (target of rapamycin)-kinase signal transduction mechanism in response to starvation.

SNARE proteins and the class C Vps (C-Vps) complex as well as Rab7 have been implicated in mammalian autophagy. In *Saccharomyces cerevisiae*, the fusion of autophagosomes and vacuoles is assumed to proceed in an identical manner to that of endocytic fusion, depending on SNARE proteins, Rab GTPase Ypt7, the yeast ortholog of mammalian Rab7, and its GEF, C-Vps tethering complex, all of which are known as regulators of the endocytic pathway [17-19]. Interestingly, while Rab7 and the C-Vps complex component Vps16 are essential for endocytic fusion with lysosomes, Rab7 but not Vps16 is required for complete autophagy flux in an autophagy induced by thapsigargin, an inhibitor of the sarco/ER Ca^{2+} ATPase [20]; therefore, autophagosomal-lysosomal fusion might be controlled by a molecular mechanism distinct from general endocytic fusion.

During autophagy, autophagosomes fuse with lysosomes to degrade materials within them by lysosomal hydrolases. So far, little is known about the fate of autolysosomes. Recently, it has been shown that mTOR regulates the termination of autophagy and reformation of lysosomes [21]. When cells are exposed to starvation, mTOR is inhibited, leading to the autophagy induction; however, prolonged starvation causes reactivation of mTOR and this reactivation generates proto-lysosomal tubules and vesicles from autolysosomal membranes to reform into functional lysosomes. Interestingly, the dissociation of Rab7 from autolysosomes is required for the reformation of lysosomes, and overexpression of the active form of Rab7 results in the accumulation of enlarged autolysosomes [21]; therefore, mTOR might regulate the reformation of lysosomes from autolysosomes via Rab7.

2.2. Rab7 GTPase and pathogen-containing autophagosome

Many pathogens are sequestered in phagosomes and fated to be degraded, since these phagosomes undergo a process of maturation, fusing with lysosomes [22, 23]; however, some pathogens reside in vacuoles that interact with other organelles, such as mitochondria, ER and

Golgi, while others escape from phagosomes or remain in vacuoles that neither acidify nor fuse with lysosomes [24]. In contrast, *Coxiella burnetii* bacteria, the agent of Q fever in humans and of coxiellosis in other animals, live and replicate in acidified compartments with phagolysosomal characteristics. Lysosomal membrane proteins and enzymes are found in vacuoles containing *C. burnetii* [25]. In HeLa cells infected with *C. burnetii*, vacuoles containing these parasites and labeled with acidotropic probe LysoTracker were also labeled with MDC and LC3. Moreover, 3-methyladenine and wortmannin, known as reagents to inhibit the early stage of autophagosome formation, blocked the development of *Coxiella*-containing vacuoles [26]. These results suggest that *Coxiella*-containing vacuoles interact with the autophagic degradation pathway. Interestingly, exogenously expressed wild-type Rab7 and the active form of Rab7 colocalize with *Coxiella*-containing vacuoles, whereas the inactive form of Rab7 does not. This indicates that Rab7 associates with the biogenesis of *Coxiella*-containing vacuoles [26]. Also, the initial formation of Group A *streptococcus*-containing autophagosome-like vacuoles is prevented by expression of the inactive form of Rab7 or downregulation of Rab7 expression with RNAi, suggesting that Rab7 is required for the early stage of the formation of Group A *streptococcus*-containing autophagosome-like vacuoles [27].

2.3. Rab7 GTPase and interaction molecules

Rubicon (Run domain protein as Beclin 1 interacting and cysteine-rich containing) is a component of the class III phosphatidylinositol 3-kinase (PI3KC3) complex. PI3KC3 forms two protein subcomplexes that localize to autophagosomes or early endosomes and perform distinct functions. The autophagosomal subcomplex consists of the PI3KC3 core complex (hVps34, p150/Vps15, and Beclin 1) and Atg14L [28]. Atg14L is the targeting factor for this complex to the early stage of autophagosomes. The endosomal complex is composed of the PI3KC3 core complex, UV irradiation resistance-associated gene (UVRAG) and Rubicon. UVRAG activates PI3KC3 and is needed to mature autophagosomes and endosomes through its direct interaction [29]. UVRAG also interacts with C-Vps, and this interaction accelerates autophagosome recruitment and activation of Rab7, which facilitates autophagosome maturation [30]. On the other hand, Rubicon specifically interacts with Rab7 through the common C-terminal domain, called a regulator of G-protein signaling homology (RH) domain but not RUN domain (for RPIP8, UNC-14, and NESCA) to inhibit autophagosome maturation [31, 32]. The overexpressed active form of Rab7 competed with UVRAG for Rubicon binding much more efficiently than the inactive form of Rab7 [31]. Thus, Rubicon is a negative regulator of autophagosome maturation. Interestingly, Rubicon homologue, PLEKHM1, which contains an RH domain, specifically interacted with Rab7, and this interaction is important for their function [32]. In contrast to Rubicon, PLEKHM1 does not directly suppress autophagosome maturation. Rubicon, but not PLEKHM1, also interacted with the Beclin 1-PI3-kinase complex [32]. Rubicon functions to regulate the endocytic and autophagic pathways under the control of the association with Beclin 1-PI3-kinase complex or Rab7.

Phosphatidylinositol-3-phosphate (PI3P) is essential for autophagosome formation. Although the PI3P function in autophagy is unknown, it is already considered that effector proteins containing the FYVE (Fab1/YOTB/Vac1/EEA1) domain or PX (phox) domain are recruited to

and activated on PI3P-enriched membranes. Recently, FYCO1 was identified as a novel protein interacting with LC3, Rab7, and PI3P [33]. FYCO1 interacts with Rab7 and PI3P via part of the coiled-coil domain and FYVE domain, respectively. Overexpression of FYCO1 redistributes LC3, Rab7, and ORP1L, a Rab7 effector protein, to the cell periphery in a microtubule-dependent manner [33]. In contrast, FYCO1 depletion leads to the accumulation of perinuclear clustering autophagosomes, indicating that FYCO1 binds to PI3P via its FYVE domain and functions as a Rab7 and LC3 effector molecule with microtubules plus end-directed transport.

3. Rab9 GTPase and autophagy

Rab9 GTPase resides in late endosomes, in which Rab7 localizes in a distinct microdomain, and plays a role in vesicle transport from late endosomes to the TGN [34]. Rab9 depletion using siRNA decreased the size of late endosomes and reduced the number of late endosomes/lysosomes, which were clustered in the perinuclear region [35], implying that Rab9 is associated with the maintenance of late endosomes/lysosomes.

Generally, it has been believed that Atg5 and Atg7 are essential for mammalian autophagy [36, 37]. In contrast, mouse embryonic fibroblasts deficient in Atg5 or Atg7 can still form autophagosomes and autophagic flux can function when exposed to autophagy-inducible stress conditions, and the lipidation of LC3 (autophagosome membrane-bound form) is also dispensable for this Atg5-/Atg7-independent autophagy [38]. Interestingly, in this alternative process of autophagy, but not in Atg5/Atg7-dependent conventional autophagy, the formation of autophagosomes seemed to be regulated in a Rab9-dependent manner by the fusion of isolation membranes with the TGN- and late endosome-derived vesicles [38]. In fact, the localization of Rab9 to autolysosomes was slightly increased with the active form of Rab9 (Rab9Q66L), but decreased with the dominant-negative form of Rab9 (Rab9S21N). Additionally, Rab9 silencing by siRNA decreased the number of autophagosomes but induced the accumulation of isolation membranes [38]. Thus, Rab9 plays a significant role in Atg5-/Atg7-independent autophagy.

4. Rab11 GTPase and autophagy

Rab11 has been shown to associate with perinuclear recycling endosomes and regulate transferrin recycling in CHO or BHK cells [39]; however, in K562, an erythroleukemic cell line, Rab11 localizes at MVBs, which are equivalent to late endosomes and are released into the extracellular space as so-called exosomes [40]. Overexpression of wild-type Rab11 and its active-form mutant produced large MVBs. Induction of autophagy by starvation or mTOR inhibitor rapamycin significantly increased the fusion between MVBs and autophagosomes [41]. This fusion was disturbed by the Ca^{2+} chelator BAPTA-AM and by the expression of the inactive form of Rab11 [41], indicating that the fusion of MVB with autophagosomes is a calcium- and Rab11 activity-dependent event.

Rab GTPase activity is controlled by GEF and GAP. Thirty-eight putative RabGAPs with a Tre-2/Bub2/Cdc16 (TBC) domain have been identified [43]. Recently, it was thought that RabGAP might be associated not only with the cellular endomembrane system but also with autophagy. In fact, TBC1D5 is identified as an interacting partner of LC3 and retromer complex and regulates the autophagy pathway and retrograde transport of cation-independent mannose 6-phosphate receptor from endosomes to the TGN [44]. Another RabGAP, TBC1D14, can bind a mammalian homologue of Atg1p ULK1, as can Rab11, and disrupts recycling endosome traffic [45]. Furthermore, under starvation conditions, TBC1D14 and Rab11 modulate the membrane transport from recycling endosomes to generate autophagosomes. TBC1D14 overexpression caused the tubulation of ULK1- and Rab11-positive recycling endosomes irrespective of nutrition conditions, impairing their function and preventing autophagosome formation [45]. However, the tubulation of recycling endosomes induced by the expression of TBC1D14 was dependent on Rab11 expression, since Rab11 depletion using siRNA gave rise to a loss of tubules and a diffuse distribution of TBC1D14 throughout the cytosol [45]. Amino acid-deprived starvation caused TBC1D14 relocation from recycling endosomes to Golgi, while the ULK1- and LC3-positive recycling endosome membrane was incorporated into the forming autophagosomes [45]. Thus, TBC1D14- and Rab11-dependent membrane transport from recycling endosomes participates in and controls starvation-induced autophagy.

5. Rab24 GTPase and autophagy

Rab24 is localized to perinuclear reticular structures that partially colocalize with marker proteins for ER, cis-Golgi, and ER-Golgi intermediate compartments [46]. Under starvation conditions, Rab24 relocated to large vesicles, where LC3 was localized. The appearance of these vesicles was enhanced in the presence of vinblastin, an agent that disrupts microtubules and prevents fusion of autophagosomes with lysosomes [46]. Interestingly, since no such distribution change was observed in cells expressing the mutant Rab24S67L that introduced the mutation into the GTP-binding motif, normally functioning Rab24 protein appears to be required for the formation of autophagosomes in response to starvation. *Coxiella burnetii* survives and replicates in MDC- and LC3-positive phagolysosomal compartments and Rab7 participates in the formation of *Coxiella*-containing vacuoles [26]. Overexpression of Rab24 or LC3 also accelerated the occurrence of *Coxiella*-containing vacuoles early after infection [47]. The expression of the Rab24 mutant (Rab24S67L), which does not localize to autophagosomes, significantly reduced the number and size of the phagolysosomal structures, although the inhibitory effect was not enduring but mutant expression delayed the generation of phagolysosomes [47]. Taken together, these results suggest that overexpression of proteins involved in the autophagic pathway, such as Rab24, increases the development of phagolysosomes for *Coxiella* replication.

Rab24 is also supposed to contribute to the degradation of aggregated proteins in rat cardiac myocytes. Glucose deprivation induced the formation of aggregates and aggresomes of polyubiquitinated proteins, and then they colocalized with exogenously expressing green

fluorescent protein (GFP) tagged-LC3 and endogenous Rab24 [48]. Autophagy induced by glucose deprivation seemed to depend on the reactive oxygen species, because the treatment with N-acetylcysteine prevented aggresome formation and autophagy [48]. These results might imply that glucose deprivation induces oxidative stress, which is involved in aggresome formation and autophagy via Rab24 in cardiac myocytes.

6. Rab32 GTPase and autophagy

Mouse Rab32 and Rab38 operate in a functionally redundant manner in regulating skin melanocyte pigmentation and regulate post-Golgi trafficking of tyrosinase and tyrosinase-related protein 1, thereby suggesting their critical roles in melanosome maturation [49]. In *Xenopus* melanophores, Rab32 is involved in the regulation of melanosome transport by cAMP-dependent protein kinase A [50]. Although Rab32 is expressed in most human tissues [51], little is known about the physiological roles of Rab32 in tissues and cells other than melanocytes.

6.1. Rab32 GTPase and constitutive autophagy

Rab32 is supposed to localize to ER [52] and ER and mitochondria [53]. We showed previously that Rab32 participates in constitutive autophagy in HeLa cells derived from cervical cancer [52]. The expressed wild-type or GTP-bound active form of human Rab32 was primarily localized to the ER. Interestingly, overexpression of the wild-type or active form of Rab32 induced the formation of autophagic vacuoles containing LC3, the ER-resident protein calnexin and late endosomal/lysosomal membrane protein LAMP-2 even under nutrient-rich conditions. Moreover, the localization of Rab32 to ER was necessary for the formation of autophagosomes [52], because the expression of a mutant Rab32 deleted two cysteine residues that are essential for association with the membrane, impaired autophagy vacuole formation [50]. There is a long-standing debate concerning from where the autophagosomal membrane is derived. So far, two possibilities have been proposed: it arises from pre-existing organelles, such as the ER or Golgi, or from de novo formation [54]. Our findings mentioned above postulate, therefore, that Rab32 facilitates the formation of autophagic vacuoles whose membranes are derived from the ER. In addition, expression of the inactive form of Rab32 or depletion of Rab32 expression by siRNA caused the formation of p62 and ubiquitinated protein-accumulating aggresomes and prevented constitutive autophagy [52]. Thus, these results imply the physiological importance of Rab32 in the cellular clearance of aggregated proteins by basal constitutive autophagy.

As well as Rab7, Rab32 also seems to participate in phagosome maturation in pathogen-induced autophagic degradation by infection with *Salmonella enterica serovar Typhimurium* [55] or *Mycobacterium tuberculosis* [56], especially in the recruitment of lysosomal enzyme cathepsin D to phagosomes containing *M. tuberculosis* [56]. Rab32 and some other Rabs localized to *M. tuberculosis*-containing phagosomes transiently, and the expression of the inactive form of Rab32 showed the impairment of its recruitment to phagosomes [56], but had no effect on

phagosomal fusion with lysosomes [55]. Therefore, these results imply that Rab32 regulates the recruitment of cathepsin D to the phagosomes.

6.2. Rab32 GTPase and interaction molecules

Recently, it has been reported that, in *Drosophila*, Rab32 colocalized with LysoTracker labeling lysosomes and GFP-Atg8 (LC3 homologue), indicating that Rab32 is localized at lysosomes and/or autophagosomes during programmed autophagy for metamorphosis to differentiate the fat body, salivary gland, and midgut [57]. Previously, Ma et al. reported that the Claret encoded by *claret*, a member of the granule group eye color genes [58], coprecipitated not only with Rab-RP1, a Rab GTPase encoded by *Drosophila lightoid*, but also with its human homologues, Rab32 and Rab38 [59]. Furthermore, the autophagosome formation was impaired in Rab32/*lightoid* mutants and Rab32 GEF/*claret* mutants, suggesting that Rab32 activity is required for the autophagic process of the fat body [57]. Previously, it has been suggested that autophagy impairment reduces lipid accumulation and impairs adipocytes differentiation in mice [60, 61]. In fact, downregulation of autophagy in *Drosophila* led to a decrease in the size of lipid droplets in *atg*-related genes in knocked down *Drosophila* fat body cells [57]; therefore, Rab32 appears to regulate lipid storage by controlling autophagy. Another report showed that Rab32 is upregulated in the epidermis and midgut during metamorphosis in *Helicoverpa armigera* [62], suggesting that Rab32 may participate in organeogenesis in insects.

In addition to GEF, a GAP for Rab32, RUTBC1, was identified [63]. RUTBC1 is a TBC domain-containing protein that binds to Rab9A in a nucleotide binding-state-dependent manner both in vitro and in vivo but has no GAP activity for Rab9A [63]; however, RUTBC1 acts as a GAP for Rab32 and Rab33B, and its TBC domain stimulates GTP hydrolysis [63]. Therefore, RUTBC1 may function in the autophagy process, as both Rab32 and Rab33B are suggested to be regulatory factors of autophagy.

6.3. Rab32 GTPase and disease

Recently, *RAB32* and *IL23R (interleukin receptor 23)* were identified as susceptibility genes for leprosy in a genome-wide association study [64], although *IL23R* was previously reported to be a gene involved in Crohn's disease [65]. Leprosy, also known as Hansen's disease, is a chronic granulomatous infectious disease caused by *Mycobacterium leprae*, which affects both peripheral nerves and mucosa of the upper respiratory tract. As Rab32 participates in regulating the recruitment of cathepsin D to phagosomes containing *M. tuberculosis* [56], referred to above, Rab32 may have function in the pathogenesis of leprosy, such as host defense against *M. leprae* infection.

7. Rab33 GTPase and autophagy

Rab33 has two isoforms, Rab33A and Rab33B. Rab33B is expressed ubiquitously, although Rab33A is expressed exclusively in the brain and cells of the immune system [66]. Rab33B is localized at the Golgi apparatus [67], although Rab33A also targets dense-core vesicles in