RAB2 regulates the formation of autophagosome and autolysosome in mammalian cells

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ABSTRACT

Multiple sources contribute membrane and protein machineries to construct functional macroautophagic/autophagic structures. However, the underlying molecular mechanisms remain elusive. Here, we show that RAB2 connects the Golgi network to autophagy pathway by delivering membrane and by sequentially engaging distinct autophagy machineries. In unstressed cells, RAB2 resides primarily in the Golgi apparatus, as evidenced by its interaction and colocalization with GOLGA2/GM130. Importantly, autophagy stimuli dissociate RAB2 from GOLGA2 to interact with ULK1 complex, which facilitates the recruitment of ULK1 complex to form phagophores. Intriguingly, RAB2 appears to modulate ULK1 kinase activity to propagate signals for autophagosome formation. Subsequently, RAB2 switches to interact with autophagosomal RUBCNL/PACER and STX17 to further specify the recruitment of HOPS complex for autolysosome formation. Together, our study reveals a multivalent pathway in bulk autophagy regulation, and provides mechanistic insights into how the Golgi apparatus contributes to the formation of different autophagic structures.

Abbreviations: ACTB: actin beta; ATG9: autophagy related 9A; ATG14: autophagy related 14; ATG16L1: autophagy related 16 like 1; BCPAP31: B cell receptor associated protein 31; BECN1: beclin 1; Ctrl: control; CQ: chloroquine; CTD: cathepsin D; DMSO: dimethyl sulfoxide; EEA1: early endosome antigen 1; GDI: guanine nucleotide dissociation inhibitor; GFP: green fluorescent protein; GOLGA2: golgin A2; HOPS: homotypic fusion and protein sorting complex; IP: immunoprecipitation; KD: knockdown; KO: knockout; LAMP1: lysosomal associated membrane protein 1; LC3: microtubule-associated protein 1 light chain 3; OE: overexpression; PtdIns3K: class III phosphatidylinositol 3-kinase; SQSTM1/p62: sequestosome 1; RAB2: RAB2A, member RAS oncogene family; RAB7: RAB7A, member RAS oncogene family; RAB11A, member RAS oncogene family; RUBCNL/PACER: rubicon like autophagy enhancer; STX17: syntaxin 17; TBC1D14: TBC1 domain family member 14; TFRC: transferrin receptor; TGOLN2: trans-golgi network protein 2; TUBB: tubulin beta class I; ULK1: unc-51 like autophagy activating kinase 1; VPS41: VPS41, HOPS complex subunit; WB: western blot; WT: wild type; YPT1: GTP-binding protein YPT1.

Introduction

Macroautophagy (hereafter referred to as autophagy) is a lysosomal degradative pathway, which is essential to the development and homeostasis [1–3]. The deregulation of autophagy is tightly associated with a variety of human diseases [4,5]. Morphologically, autophagy is initiated from phagophores in mammalian cells. After nucleation, the phagophore membrane expands and ultimately seals to generate an autophagosome, which then fuses with a lysosome or vacuole leading to the degradation of autophagy cargoes [6–9]. How autophagosome forms and matures into autolysosome remain to be the fundamental unresolved questions in autophagy field.

Independent studies have shown that the biogenesis of autophagosomes needs membranes from multiple sources, including ER [10,11], Golgi network [12–15], mitochondria [16], the plasma membrane [8], the endosomes [17–19] and ER-Golgi intermediate [20–23]. It is unresolved that how the membranes from different resources are directionally delivered for the formation and expansion of a phagophore, which eventually seals to form an autophagosome. Due to its widespread distribution and characteristic transmembrane
structure, ATG9A (hereafter referred to as ATG9) is considered the good candidate to deliver membranes from different sources [7,8,17,24–37], but it remains unclear how Golgi-derived ATG9-positive (ATG9⁺) vesicles are transported and integrated into early autophagic structures in mammalian cells.

RAB small GTPases are critical molecular switches in trafficking pathways [38,39]. Several RAB GTPases in autophagy regulation have been identified [40,41], but how these RAB GTPases relay signals sequentially to fulfill the entire autophagy process remains poorly understood. It has been proposed that endocytosis or phagocytosis involves a RAB-conversion mechanism in which signals are seamlessly transduced to promote the maturation of endosomes or phagosomes [42,43], but it is not known whether an analogous regulation also exists in the autophagy pathway.

In previous study, we identified RUBCNL as a vertebrate-specific autophagy regulator [44], and we showed that RUBCNL antagonizes RUBCN/Rubicon to activate the class III phosphatidylinositol 3-kinase (PtdIns3K) during late steps of autophagy. In addition, RUBCNL interacts with STX17 on autophagosomes to promote autolysosome formation. Here, we reported that RAB2A (hereafter referred to as RAB2), another RUBCNL-interactor, unexpectedly regulated both autophagy initiation and termination in mammalian cells. We observed that the Golgi apparatus contributed to autophagy initiation by donating RAB2, which participated in the formation of phagophores by further recruiting and activatingULK1. Next, RAB2 switched to interact with RUBCNL and STX17 to become an autophagosomal GTPase, which further specified the recruitment of HOPS (homotypic fusion and protein sorting) complex to autophagosome to facilitate the fusion with lysosomes. Our study provides mechanistic insights into the regulatory mechanisms underlying the roles of the Golgi apparatus in autophagosome biogenesis and maturation.

Results

Golgi-resident RAB2 relocates to autophagic membrane structures through microtubule-based vesicular transport

In our previous study, Mass spectrometry (MS) analysis of the proteins showed that co-immunoprecipitated (co-IP) with RUBCNL resulted in the identification of RAB2 as another potential RUBCNL-binding partner [44]. Therefore, RAB2 might be an autophagy regulator. To test this notion, we first investigated its subcellular localization. Because the reliable antibodies for imaging of endogenous RAB2 were not available, we established stable cell lines expressing FLAG-RAB2 at a level that was close to its endogenous counterpart (Figure 1(a)). Next, we employed this stable cell line to optimize the imaging conditions, which enabled transiently expressed GFP-RAB2 or mCherry-RAB2 to behave similarly to FLAG-RAB2 (Figure 1(b,c)). Subsequently, we applied these conditions for following confocal microscopy analysis. In unstressed cells, RAB2 predominantly resided on the Golgi apparatus, as exhibited by extensive colocalization with GOLGA2 or TGOLN2/TGN46 (Figure 1(d,e) and S1). In addition, the localization of RAB2 on ER, late endosomal and autophagic membrane structures was apparent, albeit at relatively lower levels. In contrast, the localization of RAB2 on early endosomes and mitochondria was limited. Interestingly, autophagy stimulation by Torin1 treatment significantly enhanced the colocalization of RAB2 with autophagic markers, except for ATG9 (Figure 1(d,e) and S1). Meanwhile, the overlay percentage of RAB2 with Golgi markers was reduced. These results showed that RAB2 is a Golgi-resident small GTPase, and implied that RAB2, upon autophagy induction, may relocate from the Golgi network to autophagic structures. To test this hypothesis, we used nocodazole, a microtubule polymerization inhibitor in cell culture and performed confocal microscopy analysis, knowing that intracellular vesicular trafficking depends on microtubules. Nocodazole treatment largely abolished the colocalization of RAB2 with autophagic markers, except for ATG9, while the overlay between RAB2 and Golgi markers remained largely unaltered (Figure 1(d,e) and S1). These results demonstrated that RAB2 largely co-existed with a portion of ATG9 on Golgi apparatus-related membrane structures, and that Golgi-resided RAB2 relocated to autophagic structures through microtubule-based vesicle trafficking. These observations allowed us to outline the RAB2 trafficking routes in mammalian cells, as shown in Figure 1(f).

RAB2 regulates autophagy initiation

The colocalization pattern of RAB2 suggested a potential function of RAB2 in autophagy. To test this idea, we generated RAB2-knockout (KO) U2OS cell lines (Figure 2(a)), and we found that the levels of LC3-II were significantly reduced in the absence of RAB2 in both autophagy-stimulated and -unstimulated cells, indicating that RAB2 KO resulted in a defect in LC3 lipidation. Consistently, RAB2 depletion significantly diminished cytosolic LC3 puncta (Figure 2(b,c)), and this defect could be rescued by the re-expression of wild-type (WT) RAB2 (Fig. S2A and S2B). LC3 lipidation is mainly catalyzed by ATG12–ATG5–ATG16L1 on the elongating phagophore membrane [45]. Indeed, membrane recruitment of endogenous ATG16L1 was abolished in RAB2 KO cells (Figure 2(d,e)). In addition, RAB2 knockdown (KD) in mouse liver led to SQSTM1/p62 accumulation and the defects in the biogenesis of autophagic membrane structures in vivo (Fig. S2C, S2D and S2E). More importantly, RAB2 KO eliminated the formation of the earliest autophagic structures labelled by endogenous ULK1 or GFP-ATG13 (Figure 2(f,g), S2F and S2G). Collectively, these data indicated that Golgi-derived RAB2⁺ vesicles participated in autophagy initiation. The observation that autophagy stimuli decreased the colocalization of GOLGA2/GM130 and RAB2 (Figure 1(e) and S1) led us to hypothesize that there might be functional correlation between GOLGA2 and RAB2 in autophagy initiation. Indeed, RAB2 was able to co-IP with GOLGA2, which was consistent with previous study [46], and their interaction was decreased in autophagy-stimulated cells indicating that autophagy stimuli dissociated RAB2 from GOLGA2 (Figure 2(h)). Consistently, GOLGA2 depletion by either shRNA knockdown (KD) (Fig. S2H, S2I and S2J) or Crispr-Cas9-mediated knockout (Figure 2(i-j)) was able to elevate LC3 lipidation levels and to increase the colocalization of RAB2 and LC3. Together, these data suggested
Figure 1. Golgi-derived RAB2+ vesicles fuse into autophagic membrane structures by vesicular trafficking. (a) Western-blot (WB) analysis of FLAG-RAB2 stable cell lines using anti-RAB2 antibody. (b) WB analysis of GFP-RAB2 and endogenous RAB2 level using anti-RAB2 antibody. (c) The subcellular localization of mCherry-RAB2 was similar to the stably expressed FLAG-RAB2. (d) Confocal microscopy analysis of GFP-RAB2 with TGOLN2 and LC3 as indicated under untreated, Torin1- or Torin1 plus nocodazole-treated conditions. Scale bars: 10 µm. (e) Quantification of colocalization presented in Figure 1 (d) and S1. Data were shown as mean ± SD, *p < 0.05, **p < 0.01; 'ns' indicates no statistical significance. (f) Schematic representation of the trafficking routes of RAB2. Red arrow heads indicated the routes uncovered in this work.
that autophagy stimuli liberate a population of RAB2⁺ vesicles from the Golgi network for autophagy initiation.

**Autophagy stimuli trigger RAB2-mediated ULK1 acquisition and activation to facilitate the formation of a phagophore**

The fact that RAB2 KO abolished the formation of the earliest autophagic structures marked by ULK1 implied that the Golgi-derived RAB2⁺ATG9⁺ vesicles participate in the formation of phagophores. In yeast, both ATG9 and ATG1 are required for the formation of phagophore assembly site [47,48]. Therefore, we envisioned that RAB2 might regulate ULK1 and ATG9 to facilitate the formation of the phagophore in mammalian cells. We optimized the conditions by intentionally expressing GFP-ATG9 at very low levels which enabled faithful resembling of endogenous ATG9 (Fig. S3A). We observed that in unstimulated cells majority of RAB2⁺ATG9⁺ vesicles were positive for GOLGA2 but not ULK1, however, this pattern was largely reversed when

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**Figure 2.** RAB2 is required for autophagy initiation in mammalian cells. (a) Measurement of LC3 lipidation. Control or clonal RAB2 KO U2OS cell line were untreated and treated with EBSS and/or bafilomycin A₁ (Baf A₁) for 2 h, and then analyzed by WB. (b) Control and clonal RAB2 KO U2OS (A3 and A40) were treated with Torin1 for 2 h, which was followed by fixation, anti-LC3 immunostaining and confocal microscopy analysis. Scale bars: 10 µm. (c) Quantification of LC3 puncta described in (B). Data are shown as mean ± SD, ***p < 0.001. (d) Measurement of early autophagic membrane structures positive for endogenous ATG16L1, which was quantified in (e). Scale bars: 10 µm. Data are shown as mean ± SD, **p < 0.01. (f) Measurement of early autophagic membrane structures positive for endogenous ULK1, which was quantified in (g). Scale bars: 10 µm. Data are shown as mean ± SD, **p < 0.01. (h) Co-IP of HA-RAB2 and FLAG-GOLGA2 under uninduced and autophagy-induced conditions. (i) Immunostaining analysis of endogenous GOLGA2 in GOLGA2 knockout (KO) and control HEK293 cells (j) Measurement of LC3 puncta and RAB2-LC3 colocalization in GOLGA2 KO U2OS cells, which was quantified in (k and l). Scale bars: 10 µm. Data are shown as mean ± SD, ***p < 0.01.
autophagy was stimulated (Figure 3(a,b) and S3B). Because reliable antibodies for the immunoprecipitation of endogenous RAB2 were lacking, we used the cell line that stably expressed FLAG-RAB2 at a level close to its endogenous counterpart (Figure 1(a,c)). Consistently, autophagy-stimulated dissociation of RAB2 from GOLGA2 was accompanied by the increased interaction of RAB2 with ULK1 (Figure 3(c)), and the colocalization of RAB2 and ULK1 were enriched by wormtin, which is capable of accumulating early autophagic structures (Fig. S3C and S3D). In addition, we did not detect the interaction between GOLGA2 and ULK1 (data not shown), which indicated that they may bind to RAB2 in a sequential manner. Furthermore, RAB2 interacted with ATG9 and facilitated the colocalization of ATG9 and ULK1 (Figure 3(d-f)). In contrast, RAB2 KO did not affect the colocalization of ATG9 and TFRC (transferrin receptor), and ATG9 depletion decreased the colocalization of RAB2 and TFRC (Fig. S3E, S3F, S3G and S3H). Importantly, RAB2 appeared to regulate ULK1 activation (Figure 3(g,h)), which was further confirmed by the alteration of ULK1-mediated phosphorylation of ATG14 and ATG9 in the RAB2 KO, OE and RAB2 WT -rescued KO cells. Biochemical dissection showed that RAB2 directly interacted with the HORMA domain of ATG13 and the RIR (417–827aa, RAB2-Interaction Region, which was named in this study) of ULK1 (Figure 3(i-k), S3I and S3J), and RAB2 was required for the integrity ULK1-ATG13 complex (Figure 3(k,l)). Since the phosphorylation of various of autophagy machineries including ATG9 and ATG14 by ATG1/ULK1 is critical for autophagy initiation [25,49–51], these results indicated that RAB2 regulates ULK1 recruitment and activation to further propagate signals for autophagy initiation.

**RAB2 interacts with RUBCNL and STX17 to become an autophagosomal GTPase**

RAB2 also colocalized with autophagosomal markers, including RUBCNL and STX17 (Figure 1(e)), implying that RAB2 was probably retained on autophagosomes after the closure step of autophagosome formation. Therefore, we further dissected RAB2 subcellular localization after the disruption of autophagosome maturation by silencing VPS4I, the essential functional subunit of HOPS complex, and we observed the colocalization of LC3 and LAMP1 was significantly reduced (Figure 4(a,b) and Fig. S4A), confirming that the loss of the tethering function of HOPS led to a defect in autolysosome formation. Similarly, RAB2 showed impaired colocalization with RAB7 or LAMP1 in the absence of functional HOPS. In contrast, HOPS inactivation did not affect colocalization of RAB2 with STX17 and RUBCNL (Figure 4(a,b) and S4A), indicating that RAB2 colocalized with RUBCNL and STX17 on autophagosomes rather than with LAMP1 or RAB7 on lysosomes or late endosomes. Consistent with our previous Mass spectrometry (MS) analysis, RUBCNL selectively interacted with RAB2 (Figure 4(c)), and their interaction was mapped to both the N- and C-termini of RUBCNL, which partially overlapped with the HOPS-binding regions of RUBCNL (Figure 4(d,f)) [44]. Further co-IP assays and in vitro pulldown assay using purified recombinant proteins showed that RUBCNL and STX17 directly interacted with three forms of RAB2, with a preference for the GDP-bound form (Figure 4(f-i)). It is well-established that membrane-associated RAB GTPases are recycled constantly to the cytoplasm via GDI-mediated membrane extraction [38] and that GDI2 is responsible for detachment of RAB2 from membrane structures [52]. Therefore, we hypothesized that RUBCNL or STX17 may inhibit GDI2-mediated membrane extraction of RAB2. Indeed, the interaction between RAB2 and GDI2 was largely disrupted in RUBCNL OE cells (Figure 4(j)). Moreover, RUBCNL KD significantly reduced the colocalization of RAB2 with STX17 and LC3 (Fig. S4B). Because autophagosome-targeting of RUBCNL is partially dependent on STX17 [44], we concluded that RUBCNL and STX17 prevent GDI2-mediated membrane extraction to maintain RAB2 on autophagosomes.

**The autophagosomal trimeric complex containing RAB2, RUBCNL and STX17 recruits HOPS complex for autophagosome maturation**

We then postulated that the autophagosomal trimeric complex containing RAB2, STX17 and RUBCNL further specifies the recruitment of HOPS complex to facilitate autophagosome maturation. Indeed, RAB2 but not RAB1 interacted and colocalized with HOPS complex (Figure 5(a,b) and data not shown). Next, we performed autophagosome maturation assays using the tandem mCherry-GFP-LC3 construct [53,54] (Figure 5(c-e)), and we observed that RAB2 KO significantly reduced autophagosome maturation, as measured by the percentage of mCherry-GFP puncta in U2OS cells. Furthermore, the colocalization of LC3 and LAMP1 puncta, the indicator for autophagosome-lysosome fusion, was reduced in RAB2 KO cells but increased in RAB2 OE cells (Figure 5(f,g)). Notably, large vesicles positive for both LAMP1 and LC3 only appeared in RAB2 OE cells, which further demonstrated the accelerated autolysosome formation driven by RAB2 OE (Figure 5(f,h)). Overexpression of RAB2 mutants was able to inhibits autophagy flux (Fig. S5A). Furthermore, we monitored the delivery of autophagosomal membranes to lysosomes by GFP-LC3 processing assays [54,55]. The appearance of free GFP, the indicator of the lysis of the inner autophagosomal membrane was blocked in RAB2 KO cells (Fig. S5B and S5C). RAB2 appeared to regulate early autophagic structures (Fig. S3C and S3D), which was consistent with the observations by other groups [56,57]. However, its dual role in both endocytosis and autophagy was not due to a direct effect on lysosome biogenesis or maturation, because cathepsin D maturation was not altered by RAB2 KO (Fig. S5E). Overall, our data showed that the autophagosomal tripartite complex containing RAB2, STX17 and RUBCNL recruited HOPS for autophagosome maturation (Fig. S5F).

**Discussion**

In this study, we suggest a model in which RAB2 links the Golgi apparatus to autophagosome formation and maturation. It should be noted that RAB2 interacts with several Golgins, including GOLGA2, to maintain the homeostasis of the Golgi
Figure 3. RAB2 regulates ULK1 recruitment and activation for autophagy initiation. (a) Confocal microscopy analysis of the colocalization of GFP-ATG9, mCherry-RAB2 and ULK1 or GOLGA2, and quantification was shown in (b). Scale bars: 10 µm. Data are shown as mean ± SD; **p < 0.01; 'ns' indicates no significance. (c) Co-IP of RAB2 and GOLGA2 or ULK1 under unstressed and starved conditions. (d) Co-IP of HA-RAB2 and FLAG-ATG9 using HA-GFP as a negative control. (e) Confocal microscopy analysis of the colocalization of GFP-ATG9 and ULK1 in RAB2 WT and KO U2OS cells under Torin1 treatment, and quantification was shown in (f). Scale bars: 10 µm. Data are shown as mean ± SD; **p < 0.01. (g) WB analysis of ULK1 phosphorylation at Serine555, ATG14 phosphorylation at Serine29 and ATG9 phosphorylation at Serine14 in RAB2 WT, KO or OE U2OS cells cultured in complete medium. Long exposure (LE), short exposure (SE). (h) WB analysis of phosphorylation of ULK1 and ATG14 in rescued RAB2 KO cells. (i) Dissection of RAB2 and ATG13 interaction by co-IP assay. (j) Dissection of RAB2 and ULK1 interaction by co-IP assay. (k) Co-IP of FLAG-ATG13 and ULK1 in RAB2 WT or KO cells. (l) Schematic representation of RAB2 interaction with the HORMA domain of ATG13 and the RIR (RAB2-Interaction Region, named in this study) domain of ULK1.
apparatus [58–61]. Whether other Golgins also have a similar role in autophagy initiation requires further investigation. In addition, it seems that GOLGA2 may inhibit autophagy through other mechanisms [62]. Furthermore, it is not known what are the upstream signals in dissociating RAB2 from GOLGA2 upon autophagy stimulation. We recently noticed that starvation altered the phosphorylation pattern of GOLGA2 (unpublished data), and the characterization of the putative kinases involved is currently undertaken in the lab.

**Figure 4.** RAB2 directly interacts with RUBCNL and STX17 to become an autophagosomal GTPase. (a) Confocal microscopy analysis of the colocalization of the organelle markers as indicated in VPS41 KD and control cells, and quantification was shown in (b). Scale bars: 10 µm. Data are shown as mean ± SD, **p < 0.01, *p < 0.05. (c) Co-IP of FLAG-RUBCNL with GFP-tagged RAB GTPases, and co-IP of FLAG-RUBCN with GFP-WT RAB7 or GFP-RAB7(71L) were applied as positive controls. (d) Co-IP of HA-RUBCNL or mutants with HA-RAB2. (e) Summary of the interaction of RUBCNL with different binding partners. (f) Co-IP of HA-RUBCNL with FLAG-WT RAB2, FLAG-RAB2Q65L or FLAG-RAB2N119I. (g) In vitro GST-pulldown assay using purified recombinant proteins for FLAG-RUBCNL and GST-WT RAB2, RAB2Q65L or RAB2N119I as indicated. (h) Co-IP of FLAG-STX17 with HA-RAB1, HA-RAB2 or HA-RAB7. (i) Schematic representation of the trimeric complex of RUBCNL, STX17 and RAB2 on autophagosome. (j) Co-IP of FLAG-GDI2 and HA-RAB2 in RUBCNL OE and control cells.

RAB2 may regulate autophagy initiation through three different mechanisms. First, RAB2 transports Golgi-derived ATG9 vesicles to the phagophore assembly sites. Previous works have shown that plasma membrane- and recycling endosome-derived ATG9 vesicles contribute to autophagosome biogenesis [17,18,28]. Our work indicates that the Golgi-derived ATG9 vesicles are delivered by RAB2 for the construction of early autophagic structures, because the colocalization of ULK1 and ATG9, but not that of ATG9 and TFRC, was significantly reduced in RAB2 KO cells, and the
translocation of RAB2, but not that of ATG9, to recycling endosomes was abolished by nocodazole treatment. Second, RAB2 recruits ULK1 to phagophore assembly sites, as ULK1 appears to be soluble and forms a diffused cytosolic pattern in the absence of RAB2. Previous studies have demonstrated that ULK1 may have a tethering function, which is independent of its kinase activity [29,63–65]. It has been indicated that ATG9 is self-interacted [66], and it requires at least three ATG9 vesicles to mark a PAS in yeast [7]. Therefore, it is conceivable that RAB2-ULK1 interactions may directly contribute to the initial tethering of ATG9 vesicles prior to fusion, which enables the small donating vesicles to mature into a phagophore. Third, RAB2 facilitates ULK1 activation to propagate signals for autophagy initiation. The acquisition of ULK1 to RAB2 vesicles may result in clustering of the ULK1 complex, which is essential for ULK1 activation [47,65,67,68]. Next, activated ULK1 phosphorylates ATG9 to enable the ATG9 vesicles to fuse into phagophores [25,49,50,69], and
phosphorylates ATG14 and BECN1 to activate PtdIns3K for nucleation [51,70].

Studies in yeast have shown that YPT1 is recruited and activated by its GEF, TRAPPIII, at PAs, which is followed by YPT1-mediated ATG1 recruitment [35,71]. RAB1, the mammalian orthologue of YPT1, also regulates autophagy [28,72–77]. In addition, RAB1, together with TRAPPIII and TBC1D14, maintains an ATG9 pool on the Golgi apparatus by acquiring the vesicles from recycling endosomes [28]. Moreover, a circuit involving TBC1D14, RAB11 and ULK1 on the recycling endosome also contributes to autophagy initiation [18]. Although RAB2 homolog has not been detected in yeast [78], RAB2 appears to be conserved in model organisms ranging from C. elegans and Drosophila to humans. We demonstrated here that the Golgi network contributes membranes and protein machineries including RAB2 and ATG9 to autophagy, which is different from RAB1- or RAB11-mediated pathways linking recycling endosomes to autophagy in mammalian cells.

A series of studies have shown that RAB2 is essential for endocytic pathways [79–81] in which C. elegans GOP1 and its mammalian homolog CLEC16A activates RAB2 as GDF (GDI displacement factor) [38,81]. RUBCNL appears to have a similar GDF function, because RUBCNL preferentially interacts with the GDP-bound form of RAB2, and RUBCNL inhibits RAB2-GD2 interactions. In addition, because both STX17 and RAB2 localize on different compartments [82,83], to avoid disadvantageous mistargeting of HOPS complex, it might be necessary to form a tripartite complex with RUBCNL on autophagosomes to further specify the recruitment of HOPS complex for autophagosome maturation. Whether the complex containing RUBCNL, STX17 and RAB2 also functions in endocytic pathway is currently not known. During the preparation of this manuscript, two groups reported that the Drosophila RAB2 regulates autophagosome maturation by recruiting HOPS complex [57,84], which is in accordance with second part of our findings in this work. However, this work provides further insights into the mechanisms of how RAB2 recruits HOPS to promote autophagosome maturation by introducing RUBCNL and STX17 as the cofactors. In addition, a possible function of RAB2 in autophagy initiation in Drosophila was not addressed, which may worth further investigation.

In conclusion, this study not only identifies RAB2 as a unique GTPase participating in the formation of both autophagosome and autolysosome in mammalian cells, but also provides further mechanistic insights into the mechanisms of how the Golgi apparatus contributes to bulk autophagy pathway.

Materials and methods

Antibodies

Anti-SQSTM1 (MBL, PM045), anti-ATG16L1 (MBL, PM040), anti-GOLGA2 (MBL, PM179-3), anti-LC3 (MBL, PM036), anti-AIFM2 (Cell Signaling Technology, 5318), anti-CTSD (Santa Cruz Biotechnology, sc-6486), anti-EGRF (Santa Cruz Biotechnology, sc-120), anti-RAB2 (BBI Life Sciences, D122959-0200), anti-RAB1A (Protein tech, 11671–1-AP), anti-VPS41 (Santa Cruz Biotechnology, sc-377118), anti-ULK1 (Santa Cruz Biotechnology, sc-390904), anti-LAMP1 (Santa Cruz Biotechnology, sc-20011), anti-TFRC (Santa Cruz Biotechnology, sc-32272), anti-BCAP3 (Santa Cruz Biotechnology, sc-393810), anti-TOMM20 (Santa Cruz Biotechnology, sc-17764), anti-SQSTM1 (pSer403) (Genetex, GTX128171), anti-ATG14 (Cell Signaling Technology, 5504), anti-LAMP1 (Cell Signaling Technology, D2D11), anti-EEA1 (Cell Signaling Technology, C45B10), anti-p-ATG14 (529) (Cell Signaling Technology, 13155S), anti-TC (Sigma, L8918), anti-RAB2 (Abcam, GR188995-4), anti-HA-Tag-HRP (MBL, M180-7), anti-FLAG-Tag-HRP (MBL, M185-7), anti-HA (Biomedig, 16B12), anti-GFP (Santa Cruz Biotechnology, M048-3), anti-MYC (Santa Cruz Biotechnology, 9E10), anti-ubiquitin (Cell Signaling Technology, 3936T), anti-LAMP1 (D2D11) (Cell Signaling Technology, 9091S) anti-ULK1 (D8H5) (Cell Signaling Technology, 8054S), anti-p-ULK1 (S555) (Cell Signaling Technology, 5869S), anti-ATG9 (Cell Signaling Technology, 13509S), anti-p-ATG9 (S14) (Donated by Prof. Chen Quan), Alexa Fluor 488 (Abcam, GR238847-1), Alexa Fluor 546 (Thermo Fisher Scientific, A11003), Alexa Fluor 546 (Thermo Fisher Scientific, A11010), Alexa Fluor 405 (Thermo Fisher Scientific, A31556), Alexa Fluor 405 (Thermo Fisher Scientific, A81553), Alexa Fluor 488 (Thermo Fisher Scientific, A11008), Alexa Fluor 488 (Thermo Fisher Scientific, A11001).

Chemicals and reagents

Torin1 (Selleck Chemicals, S2287), bafilomycin A1 (Selleck Chemicals, S1413), chloroquine (Sigma, C6628), EGF (PeproTech incorporated, AF–100–15), nocodazole (Sigma, M1404), puromycin (Sigma, P7255), Lipofectamine 3000 (Thermo Fisher Scientific, L3000015), Earle’s basic salt solution (Thermo Fisher Scientific, 1816327), restriction enzymes (Thermo Fisher Scientific), GST agarose (Protegene, PC014), 2× Taq Master Mix (Protegene, ME013), 2× Ultra-Pfu Master Mix (Protegene, ME026), CloneExpress II One Step Cloning Kit (Vazyme Biotech, C112-01), 2× Phanta Master Mix (Vazyme Biotech, P511).

Cell lines

U2OS (ATCC), HEK293T (ATCC), HeLa (ATCC), HEK293 (ATCC), RAB2ΔU2OS (constructed in our lab), FLAG-RAB2 2 U2OS (Constructed in our lab), FLAG-RAB2 HEK293T (constructed in our lab), HA-RUBCNL HEK293T (constructed in our lab), MYC-RUBCNL HEK293T (constructed in our lab).

Oligonucleotides

VPS41 shRNA 1 (GCTTTGACAGTCAGAGGCTTT), GOLGA2 shRNA 1 (CGAGAATGATGAGGTGAAGAT), GOLGA2 shRNA 2 (GCCGAGTTTGGAAAGGTGAGT), ATG9 shRNA (GTGGACTATGACATCCTATTT).

Recombinant DNA

pEGFP-C1 (Clontech, PT32595), pEGFP-N1 (Clontech, PT3027-5), pmCherry-N1 (Clontech, PT3974-5),
**sgRNA vectors** into each mouse. Four weeks later, protein samples were collected in pH 7.4, 150 mM NaCl, 0.5% NP-40 (Sigma, 56741), sgRNA vector by Lipofectamine 3000. Twenty-five μL pLK-cas9-Rab2 vector were co-transfected into the Flp-In™ 293T and Flp-In™ U2OS cells. Cells were passed into 2 to 4 10-cm plates. WB was performed to screen single cell clones with FLAG-RAB2 or anti-GOLGA2 antibodies. Then, WB verified single cell clones were trypsinized and seeded into 96-well plates and grow in presence of hygromycin. WB was performed to verify FLAG-tagged protein expression.

### Stable cell lines construction

FLAG-RAB2/293T and FLAG-RAB2/U2OS were obtained by Flp-In™ System. The pOG44 plasmid and the pCDNA3-FRT-RAB2 vector were co-transfected into the Flp-In™ 293T and Flp-In™ U2OS cells. Cells were passed into 2 to 4 10-cm plates and were diluted and seeded into 96-well plates. WB was performed to screen single cell clones with anti-RAB2 or anti-GOLGA2 antibodies. Then, WB verified KO cell clones were sent for sequencing verification.

### Generation knockout cell lines for GOLGA2 and RAB2

pLKO-cas9-RAB2 and pLKO-cas9-GOLGA2 sgRNA vectors with designed gDNA sequence 5′GCTCGAATGATAAC TGCTGATATTCTCTCAACTG3′ and 5′TGCCTATCTCTTCAAGTACATCTTCAAGAGA were cloned. U2OS or HEK293 cells were seeded in a 6-well plate with 50% confluency one day before transfection. Cells were transfected with 2 μg pLKO-cas9-RAB2 sgRNA or pLKO-cas9-GOLGA2 sgRNA vector by Lipofectamine 3000. Twenty-four h later, regular medium was replaced with medium containing 1 μg/ml puromycin. After 2 days incubation, cells were diluted and seeded into 15cm dishes. Two weeks later, single cell clones were trypsinized and seeded into 96-well plates. WB was performed to screen single cell clones with anti-RAB2 or anti-GOLGA2 antibodies. Then, WB verified KO cell clones were sent for sequencing verification.

### Immunoprecipitation and western blot

Cell pellets were homogenized in TAP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40 [Sigma, 56741], 1 mM NaF, 1 mM Na3VO4, 1 μM EDTA, 10 nM MG132 [Selleck Chemicals, S2619], protease inhibitor cocktail [Bimake, B14001], phosphatase inhibitor cocktail [Bimake, B15001]) and incubated on ice for 30 min. The cell lysate was cleared by centrifugation at 16,873 g for 30 min. The supernatant was incubated with antibody-conjugated beads (Bimake, B23102) and rotated for 2 h at 4°C. After incubation, the beads were washed 3 times with TAP buffer. WB was performed following standard procedures.

### Immunofluorescence

Cells grown on coverslips were transfected with different plasmids, then fixed in 4% paraformaldehyde in PBS (Thermo Fisher Scientific, 10010023) for 20 min at room temperature and permeabilized with 0.1% Triton X-100 (Sangon Biotech, T0694) in PBS for 20 min. Following permeabilization, cells were treated with block buffer (1% BSA (BBI Life Sciences, A600332), 0.1% Triton X-100 in PBS) for 1 h at room temperature. Cells were incubated with primary antibodies diluted in block buffer overnight at 4°C. Cells were washed 3 times with PBS, each for 10 min, followed by incubation with Alexa Fluor-conjugated secondary antibody (Life Technologies) in block buffer for 1 h at room temperature. Slides were examined by using a laser scanning confocal microscope (Zeiss LSM 800).

### EGFR degradation assay

Cells cultured in 12-well plates were grown to approximately 80% confluency. Cells were serum-starved overnight (12–18 h). EGFR endocytosis was stimulated by adding of 200ng/ml EGF (peprotech, AF-100–15) in DMEM containing 20 mA HepES and 0.2% BSA. Four different time lapse after EGF stimulation, the cells were boiled in 100 μl x SDS loading buffer. Samples were analyzed by WB of EGFR.

### RAB2 knockdown in mouse liver by AAV-mediated shRNA expression in vivo

All animal experiments were performed under the guidelines of the institutional Animal Care and Use Committee at Zhejiang University. Mice were maintained in a barrier facility, at normal room temperatures, on a regular 12 h light and 12 h dark cycle. For Rab2 KD in mouse liver, eight-week-old male C57BL/6J were applied for studies. shRNA-Rab2 sequence: GCCATATCCTTCAAGATGTACTTGAAGAGATAGGCTTTTTT. pAV-U6-GFP inserted with nonsense sequence were used as control. Male mice were used for the AAV gene transfer studies. The mice in each experiment were randomized. pAV-U6-shRNA-Rab2-GFP and pAV-U6-GFP AAV particles were injected with 5 × 10¹¹ vg into each mouse. Four weeks later, protein samples isolated from mouse liver, which were equalized with BCA kit, were performed with WB using anti-SQSTM1 antibody. The other group of mice, which were injected with pAV-U6-shRNA-Rab2-GFP and pAV-U6-GFP AAV particles, were starved for two days. Livers from normal feeding and starved mice were prepared for transmission electron microscopy (TEM).

### Protein extraction from tissue

Liver samples (200 mg) were homogenized in 1 ml TAP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM NaF, 1 mM Na3VO4, 1 μM EDTA, 10 mM MG132,
supplemented with protease, phosphatase inhibitors and deacetylase inhibitors (Selleck Chemicals, S1045)) using a homogenizer at 4°C for 45 s, and the homogenates were cleared by centrifugation at 16,873 g for 20 min and 200,000 g for 60 min, respectively. The supernatants were used for WB analysis or immunoprecipitation.

**Autophagy analysis**

For LC3-II degradation assay, U2OS cells with RAB2 KO or WT were treated with 250 nM Torin1 or vehicle at different time points, and whole cell lysates were briefly sonicated in 1× SDS loading buffer, and incubated at 100°C for 5 min, then subjected to WB analysis with antibodies against LC3. For autophagosome maturation assays, U2OS with RAB2 KO or WT were transfected with GFP-mCherry-LC3, 16 h post-transfection, the cells were treated with EBS at 37°C for 1 h, and analyzed by fluorescence microscopy. To determine how RAB2 mutants affect autophagic flux, stable cell lines of flagtagged WT RAB2, RAB2Q65L and RAB2N119I were treated with Torin1 treatment for different times. Cell lysates were analyzed by WB using SQSTM1 and LC3 antibodies. For GFP-LC3-RFP-LC3ΔG cleavage assay, RAB2 KO or WT U2OS cells were seeded in 12-well plates. When reaching 70% confluency, cells were transfected with GFP-LC3-RFP-LC3ΔG. Twenty-four h later, cells were treated with Torin1 for inducing autophagy for different times. Cells were boiled in 100 μl 1× SDS loading buffer. Samples were analyzed by WB using GFP, RFP and LC3 antibodies. For Electron microscopy, RAB2 KO and WT cells were treated with 250 nM Torin1 or vehicle (DMSO) for 2 h. The cells were harvested and washed with PBS at room temperature. The cell pellets were fixed in 0.1 M PBS buffer containing 2.5% (w:v) glutaraldehyde at 4°C for 24 h. The cells were washed with 0.1 M PBS three times for 15 min each time. The cells were post-fixed in 0.1 M PBS buffer containing 1% osmium tetroxide for 1 h at 4°C and then washed with water three times for 15 min each time. When processing resumed, the cells were dehydrated in graded alcohols, embedded in Epon 812 (SPI, 660-AB), sectioned with ultramicrotome (Leica, Germany), and then stained with uranyl acetate and lead citrate. The sections were examined with a transmission electron microscope (JEOL-1230, Japan). For each representative figure which was shown, and at least three different experiments, unless otherwise noted. A P value < 0.05 was considered statistically significant.

**Quantification and statistical analysis**

Statistical analyses were performed using the Student’s t test in SPSS 17.0 software. Values are expressed as mean ± SD of at least three independent experiments, unless otherwise noted. A P value < 0.05 was considered statistically significant.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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