Golgi membrane-associated degradation pathway in yeast and mammals

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Abstract

Autophagy is a cellular process that degrades subcellular constituents, and is conserved from yeast to mammals. Although autophagy is believed to be essential for living cells, cells lacking Atg5 or Atg7 are healthy, suggesting that a non-canonical degradation pathway exists to compensate for the lack of autophagy. In this study, we show that the budding yeast *Saccharomyces cerevisiae*, which lacks Atg5, undergoes bulk protein degradation using Golgi-mediated structures to compensate for autophagy when treated with amphotericin B1, a polyene antifungal drug. We named this mechanism Golgi membrane-associated degradation (GOMED) pathway. This process is driven by the disruption of PI(4)P-dependent anterograde trafficking from the Golgi, and it also exists in Atg5-deficient mammalian cells. Biologically, when an Atg5-deficient β -cell line and Atg7-deficient β -cells were cultured in glucose-deprived medium, a disruption in the secretion of insulin granules from the Golgi occurred, and GOMED was induced to digest these (pro)insulin granules. In conclusion, GOMED is activated by the disruption of PI(4)P-dependent anterograde trafficking in autophagy-deficient yeast and mammalian cells.

Keywords GOMED; Golgi membrane; autophagy; anterograde trafficking

Subject Categories Autophagy & Cell Death; Membrane & Intracellular Transport; Metabolism

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Introduction

The intracellular degradation of protein serves a number of functions, including the regulation of protein expression levels and the removal of damaged proteins. Protein degradation is achieved by two mechanisms: the ubiquitin-proteasome system (UPS) and the autophagy–lysosome pathway. It is generally known that UPS degrades individual ubiquitinated proteins in a regulated manner. In contrast, the autophagy–lysosome pathway is utilized for bulk

degradation, although recent studies demonstrated cases in which specific cargos are degraded, namely selective autophagy. The degradation of a large number of proteins and even entire organelles at once is only capable by the autophagy–lysosome pathway, and hence, this process is essential for living cells (Xie & Klionsky, 2007; Mizushima *et al*, 2008, 2011).

The autophagy–lysosome pathway includes two phylogenetically conserved pathways: macroautophagy and microautophagy. Macroautophagy is characterized by the formation of specific double-membrane autophagosomes that sequester cargo material. Autophagosomes fuse with lytic organelles (vacuoles in yeast and lysosomes in mammals) and degrade cargo material (Nakatogawa *et al*, 2009). In microautophagy, cargos are directly incorporated into vacuoles (or lysosomes) via the invagination of their membrane (Müller *et al*, 2000). In addition to these two types of autophagy, mammalian cells have a third autophagy–lysosome pathway, namely chaperone-mediated autophagy, in which cytosolic proteins that are specifically selected by the chaperones are degraded in lysosomes (Arias & Cuervo, 2011). Among these autophagy–lysosome pathways, macroautophagy is believed to be the most efficient bulk degradation pathway.

The molecular mechanisms of macroautophagy (hereafter described as “autophagy” unless otherwise mentioned) has been studied in yeast and mice, and is currently considered to be driven by more than 30 autophagy-related proteins (Atgs) that are well conserved from yeast to mammals (Mizushima *et al*, 2011). Among them, Atg5 and Atg7 are recognized as the molecules most crucial for autophagy, and the lipidation of Atg8 (in yeast) and microtubule-associated protein light chain 3 (LC3) (in mammals) is believed to be the most accurate marker of autophagy induction (Mizushima *et al*, 2011). A large amount of evidence indicates that Atg5-dependent autophagy plays crucial roles in various physiological events in yeast and mammals, and hence, this process is considered to be indispensable for most organisms.

Nonetheless, yeast and mammalian cells lacking Atg5 or Atg7 remain healthy. Furthermore, even embryos lacking Atg5 or Atg7 do not have any abnormalities (Kuma *et al*, 2004; Komatsu *et al*, 2005). Therefore, we considered the possible existence of another bulk degradation pathway that compensate for the lack of Atg5/

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Atg7-dependent autophagy. In mammals, we previously discovered a mechanism we named “the alternative autophagy” as such a pathway (Nishida *et al*, 2009). The role of alternative autophagy is similar to Atg5/Atg7-dependent autophagy, whereby cellular components and organelles are digested within autolysosomes. But the signaling pathway involves Unc-51-like kinase 1 (Ulk1), Beclin1, or Rab9, and not Atg5, Atg7, or LC3.

In this study, we aimed to identify and characterize a novel bulk degradation pathway that compensates for Atg5/Atg7-dependent autophagy in yeast cells. We also examined the phylogenetic conservation of this pathway in mammals. Our results indicated that a novel degradation pathway is activated when Golgi-to-plasma membrane (PM) trafficking is disturbed in *atg5Δ* and *atg7Δ* yeast cells, in which Golgi membranes are stacked, elongated, curved, and generate double-membrane compartments enclosing the cytoplasm and various organelles. These compartments are then fused with vacuoles to degrade cargo molecules. This degradation pathway also functions in Atg5-deficient mammalian cells when Golgi-to-PM trafficking is disrupted. In Atg5-deficient β-cell line and Atg7-deficient β-cells, this pathway functions to suppress insulin secretion by degrading unused insulin granules, when there is a reduction in the glucose concentration of the culture medium (similar situation to the failure of Golgi-to-PM trafficking). Taken together, we here identified and characterized a novel degradation pathway that is generated from the Golgi membrane. We named this pathway the Golgi membrane-associated degradation (GOMED). This pathway is activated when Golgi-to-PM trafficking is disrupted.

Results

Amphotericin B1 induces Atg5-independent bulk degradation in yeast cells

To study the Atg5-independent bulk degradation pathway in yeast, an alkaline phosphatase protein lacking the N-terminal transmembrane domain (pho8Δ60) fused with green fluorescent protein (GFP; GFP-pho8Δ60) was expressed in *atg5Δ* yeast cells. Pho8Δ60 is known to localize in the cytosol and to be delivered to vacuoles, primarily through autophagy (Noda *et al*, 1995). Once GFP-pho8Δ60 is transported to the vacuoles, GFP is released from the fusion protein by Pep4, a vacuolar protease (Epple *et al*, 2001). We observed this cleavage when wild-type (WT) yeast cells, but not *atg5Δ* cells, were starved (Fig 1A), indicating that GFP-pho8Δ60

processing is a good marker of autophagy and that no compensatory pathway exists for starvation-induced autophagy. We then treated GFP-pho8Δ60-transfected *atg5Δ* cells with various stress-inducing drugs to search for stimuli that lead to the degradation of GFP-pho8Δ60 (Appendix Fig S1). Among these drugs, amphotericin B1 (AmphoB) treatment resulted in the efficient processing of GFP-pho8Δ60 in a time- and concentration-dependent manner (Fig 1B and C). Consistent results were obtained when *atg7Δ* cells were treated with AmphoB (Fig 1D). The processing of GFP-pho8Δ60 was concluded to occur in vacuoles in a Pep4-dependent manner, as this processing was suppressed in cells lacking Pep4 (Fig 1E) and as following AmphoB treatment, some GFP-pho8Δ60 was delivered from the cytosol to the vacuoles where Pep4 is localized (Fig 1F). Similarly to GFP-pho8Δ60, the Adh1-monomeric red fluorescent protein (mRFP), another cytosolic protein, was also delivered to vacuoles following AmphoB treatment (Appendix Fig S2). Interestingly, several organelle-specific GFP fusion proteins were also processed following AmphoB treatment (Appendix Fig S3). We observed several bands other than GFP in some AmphoB-treated cells. This might be due to a delay in the maturation process of pep4 (Appendix Fig S4A). All of these findings indicated that Atg5-independent bulk degradation occurred within vacuoles after AmphoB treatment.

Morphological analysis of the Atg5-independent degradation pathway in AmphoB-treated yeast cells

Because GFP-pho8Δ60 processing was found to occur in vacuoles, we next examined the morphology of the vacuoles in AmphoB-treated yeast cells using phase-contrast microscopy. Given that the absence of Pep4 enables the preservation of structures within vacuoles (Epple *et al*, 2001), we used *atg5Δpep4Δ* cells. Under conditions of vegetative growth and starvation, no visible structures were observed within the vacuoles of *atg5Δpep4Δ* cells, whereas several spherical bodies were found to have accumulated in the vacuoles after AmphoB treatment (Fig 1G and H, and Movies EV1–EV6). These structures moved actively by Brownian motion, similar to autophagic bodies (ABs) (Kirisako *et al*, 1999) (Fig 1G and H, and Movies EV1–EV6). Consistently, we observed AB-like structures enclosing lipid particles, ribosomes, mitochondria, and various other organelles within a single-membrane structure (300–900 nm in size) in the vacuolar lumen of cells exposed to AmphoB, on quick-frozen replicas and thin sections of the frozen-substituted material (Fig 1I and J, and Appendix Fig S4B). The proportion of

Figure 1. Induction of proteolysis in *atg5Δ* yeast cells by AmphoB.

- A–E GFP-pho8Δ60-expressing wild-type (WT), *atg5Δ*, *atg7Δ*, and *atg5Δpep4Δ* cells were starved (A) or treated with AmphoB (B–E) and subjected to Western blotting. Cells were treated with 2.5 μg/ml AmphoB for the indicated time (B) or with the indicated doses for 24 h (C–E). Generation of free GFP was a marker of proteolysis.
- F GFP-pho8Δ60- and Pep4-mRFP-expressing *atg5Δ* cells were cultured with (AmphoB) or without (NT) AmphoB (2.5 μg/ml, 24 h), and localization of GFP-pho8Δ60 and Pep4-mRFP was observed by confocal microscopy. Arrowheads indicate the cells in which GFP-pho8Δ60 was delivered to the vacuoles. Scale bars = 2 μm.
- G, H Accumulation of autophagic body (AB)-like structures by AmphoB in *atg5Δpep4Δ* cells. *pep4Δ* and *atg5Δpep4Δ* cells were starved for 3 h or treated with AmphoB (2.5 μg/ml, 24 h). Scale bars = 2 μm. Cells containing AB-like structures were counted under phase-contrast microscopy. Representative images (G) and the percentage of cells with AB-like structures (H) are shown. **P* < 0.01 versus the value of *pep4Δ* cells.
- I–K Induction of AB-like structures in *atg5Δpep4Δ* cells treated with AmphoB (2.5 μg/ml, 24 h). Representative images of quick-frozen replicas (I) and thin section of the frozen-substituted material (J) are shown. (I) AB-like structures accumulated in the vacuole. Scale bar = 0.5 μm. (J) AB-like structures containing lipid particles and mitochondria accumulated in the cross-section of the vacuole. An autophagosome (AP)-like double-membrane compartment containing a ribosome and Golgi granule is present in the cytosol. Scale bar = 0.5 μm. A magnified photograph is available in Appendix Fig S4B. (K) The cells containing AB-like structures were counted under electron microscopy [no treatment (NT): *n* = 20, AmphoB: *n* = 192]. **P* < 0.01 versus the value of NT.

Figure 1.

cells containing AB-like structures was greater than 70% at 24 h after AmphoB treatment (Fig 1K). Detailed analysis demonstrated that the membranes of AB-like structures contained a few intramembrane particles on both the protoplasmic (*P*-face) and exoplasmic (*E*-face) half-membranes (Fig EV1A), which is completely different from the property of vacuolar membranes as they contain many intramembrane particles (Fig EV1B). This suggests that the AB-like structures did not arise from the vacuolar membranes (microautophagy), but originated outside of the vacuoles. Furthermore, when we deleted *Vtc4*, a molecule crucial for microautophagy (Uttenweiler *et al*, 2007), from *atg5Δpep4Δ* cells and then treated the cells with AmphoB, microautophagic structures were completely absent (Fig EV1C) but AB-like structures were not affected (Fig EV1D). GFP-pho8Δ60 processing also occurred in AmphoB-treated *vtc4Δatg5Δ* cells (Fig EV1E). Taken together, AmphoB treatment results in a novel Atg5-independent bulk degradation pathway accompanied by the formation of AB-like structures.

Golgi involvement in AmphoB-induced Atg5-independent degradation pathway

Ultrastructural analysis of AmphoB-treated *atg5Δpep4Δ* cells exhibited another characteristic feature, namely dramatic morphological changes of the Golgi apparatus. It is widely known that Golgi cisternae are not stacked in *S. cerevisiae* but occur as a single, small layer scattered throughout the cytoplasm (Rossanese *et al*, 1999). However, AmphoB treatment induced the elongation of Golgi cisternae and the formation of Golgi stacks, as in animal and plant cells (Fig 2A–D). Correlative light and electron microscopy (CLEM) showed stacked membranes which merged with Sec7-GFP signals (a *trans*-Golgi marker; Fig EV1F, arrows). Many single membranes were observed surrounding the stacked membranes, which were merged with the mRFP-Sed5 signals (a *cis*-Golgi marker; Fig EV1F, arrowheads). Therefore, these stacked membranes were concluded to have originated from Golgi membranes. Interestingly, we further observed that the distal Golgi cisternae were curved and formed spherical bodies containing cytoplasmic component and organelles

(Fig 2E and F). The spherical bodies were double-membrane compartments, similar to autophagosomes (AP) (Fig EV1G).

Importantly, several of our experimental results indicated that the Golgi-derived spherical bodies are the source of AB-like structures in the vacuoles. Firstly, we observed images of the spherical body separating away from Golgi stacks (Fig 2G) as well as the outer membrane of the spherical body fusing with the vacuolar membrane (Fig 2H and I). The inner membrane compartment appeared to be incorporated into the vacuoles to become AB-like structures (Fig 2H and I). Second, quantitative EM analysis demonstrated that many elongated and stacked Golgi structures and a few AB-like structures were present in the *atg5Δpep4Δ* cells during the initial 6–12 h of AmphoB treatment, but the situation was reversed at 24 h (Fig 2J). Third, GFP-fused Sft2, another *trans*-Golgi marker, exhibited a dot-like pattern in the cytoplasm without AmphoB treatment, whereas it was translocated to the vacuolar membranes (probably derived from the outer membranes of spherical bodies) (Fig 2K “AmphoB”: right cell) and inside of the vacuoles (probably derived from the inner membrane) (Fig 2K “AmphoB”: left cell). Consistent results were obtained from the analysis of Sec7-GFP (Appendix Fig S5A), but not Om45-GFP and Sec71-GFP, a mitochondria and an ER marker, respectively (Appendix Fig S5B and C). Fourth, biochemical fractionation showed that the *trans*-Golgi marker was translocated to the vacuolar membrane by AmphoB treatment (Appendix Fig S5D). Fifth, lack of the Golgi membrane proteins Grh1 and Gvp36 blocked the generation of spherical bodies and AB-like structures as assessed by EM (Fig 2L and M) and phase-contrast microscopy (Fig 2N). AmphoB-induced GFP-pho8Δ60 processing was also inhibited in *grh1Δatg5Δ* and *gvp36Δatg5Δ* cells (Fig 2O). Taken together, these data indicate that Golgi membranes were required for Atg5-independent bulk degradation and for the generation of AB-like structures after AmphoB treatment. Therefore, we named this novel degradation pathway the Golgi membrane-associated degradation (GOMED) pathway.

The morphological features of GOMED were quite similar to autophagy. Furthermore, the deletion of Atg1 (yeast homolog of Ulk1) suppressed GOMED, as assessed by GFP-pho8Δ60 processing

Figure 2. Golgi involvement in AmphoB-induced proteolysis.

- A–D Generation of Golgi stacks by AmphoB. Stacked Golgi cisternae are observed in quick-frozen replicas (A–C) and substituted thin section (D) of *atg5Δpep4Δ* cells treated with AmphoB (2.5 μg/ml) for the indicated time. (B) The magnified image shows the area indicated by the square in (A). (C, D) A representative Golgi stack with four cisternae is shown. Scale bars = 1 μm (A) and 0.5 μm (B–D).
- E–G Generation of AP-like double-membrane compartment from the stacked Golgi. A quick-frozen replica (E) and a substituted thin section (F, G) are shown. Scale bars = 0.5 μm. The distal stacks are extended, curved, and form AP-like structure (E, F). Mitochondria are encircled by the Golgi membrane (F). (G) The AP-like structure was separated away from Golgi stacks.
- H, I The scene that AP-like structure was fused with vacuole. A magnified image of (H) is shown in (I). In *atg5Δpep4Δ* cells treated with AmphoB (2.5 μg/ml, 24 h), outer membrane of AP-like structure (red arrows) was fused with vacuolar membrane (blue arrows). Inner membranes of AP-like structure (yellow arrowheads) were entered into the vacuoles to become AB-like structure. Green arrows indicate the membrane fusion site. Scale bars = 0.5 μm (H) and 0.2 μm (I).
- J The number of elongated single cisternae (green bars), Golgi stacks (red bars), and AB-like structures (blue bars) was counted over time using electron microscopy images. Error bars indicate s.e.m. (3 h: *n* = 21, 6 h: *n* = 20, 12 h: *n* = 193, 24 h: *n* = 192).
- K Translocation of the *trans*-Golgi protein to the vacuolar membrane induced by AmphoB treatment. GFP-Sft2-expressing *atg5Δ* cells (*trans*-Golgi marker protein) were incubated with or without AmphoB (2.5 μg/ml, 24 h), and localization of GFP-Sft2 was assessed by confocal microscopy. Arrowhead indicates the GFP signal on the vacuolar membrane. Scale bars = 2 μm.
- L–O Grh1 and Gvp36 involvement in AmphoB-induced autophagy-like proteolysis. (L, M) Representative electron microscopy images of *grh1Δatg5Δpep4Δ* (L) and *gvp36Δatg5Δpep4Δ* (M) treated with AmphoB (2.5 μg/ml, 24 h). AP-like and AB-like structures were absent. (L) A unilamellar Golgi cisterna was observed in the cytoplasm (dotted square). Scale bar = 1 μm. (M) Formation of a Golgi stack, but not Golgi curvature, was observed (dotted square). Scale bar = 0.5 μm. (N) Indicated cells were treated with AmphoB for 24 h and the number of cells containing AB-like structures was counted by phase-contrast microscopy. **P* < 0.01 versus the value of *atg5Δpep4Δ* cells. (O) GFP-pho8Δ60-expressing *atg5Δ*, *grh1Δatg5Δ*, and *gvp36Δatg5Δ* cells were cultured with or without AmphoB (2.5 μg/ml, 24 h) and subjected to Western blotting for GFP. An unidentified band at 32 kDa (asterisk) is non-specific band because it was present in Pep4-lacking *grh1Δatg5Δ* cells.

Figure 2.

and EM analyses (Appendix Fig S6A–C). However, we did not observe any involvement of other autophagy genes (Appendix Fig S6D–I). Furthermore, we cannot deny the possibility that this proteolysis is performed simply by the consequence of Golgi membrane deformity. Therefore, we did not consider GOMED to be a type of autophagy in this study.

Reduction in Golgi phosphatidylinositol 4-phosphate levels is required for GOMED

The unique morphology of the Golgi membranes during GOMED led us to hypothesize that molecules that regulate Golgi function may be involved in GOMED. Given that AmphoB directly perturbs ergosterol (Mouri *et al*, 2008) and that the amount of ergosterol in the ER regulates phosphatidylinositol 4-phosphate [PI(4)P] levels on the Golgi membrane (Beh & Rine, 2004), AmphoB may reduce Golgi PI(4)P levels and thereby induce GOMED. To test this hypothesis, we first examined PI(4)P localization (Roy & Levine, 2004) using the PI(4)P reporter GFP-2xPH^{Osh2}. PI(4)P localized to the Golgi and PM in healthy *atg5Δ* cells (Fig 3A, “*atg5Δ* 37°C”), as described previously (Stefan *et al*, 2011). Golgi localization of PI(4)P was confirmed by the overlap of its signals with those of Golgi markers (Fig EV2A). After AmphoB treatment, the GFP-2xPH^{Osh2} signals were spread throughout the cytosol and had disappeared from the Golgi (Fig 3A “*atg5Δ* AmphoB”). Similar results were observed when Golgi PI(4)P levels were monitored using the Golgi-specific PI(4)P reporter GFP-GOLPH3 (Mousley *et al*, 2012) (Fig 3B), suggesting that AmphoB treatment, as expected, reduced Golgi PI(4)P levels.

We next investigated whether a reduction in Golgi PI(4)P levels is required for AmphoB-induced GOMED. To this end, we increased Golgi PI(4)P levels by genetically engineering *Sac1*, a PI(4)-phosphatase localized to the Golgi and the ER. As expected, when analyzing *sac1-23/atg5Δ* cells (Foti *et al*, 2001), a temperature-sensitive mutant that loses PI(4)-phosphatase activity at 37°C, we observed Golgi-localized PI(4)P signals even after AmphoB treatment (Fig 3A and B “*sac1-23 atg5Δ* AmphoB”). Importantly, in these cells,

AmphoB-induced GOMED was markedly inhibited, as assessed by GFP-pho8Δ60 processing (Fig 3C), EM observations (Figs 3D and EV2B and C), and phase-contrast microscopy (Fig EV2D). Although the suppression of GOMED was partial, this was due to the presence of other Golgi PI(4)-phosphatases, namely *Inp52* and *Inp53* (Guo *et al*, 1999). In fact, when we deleted *Inp52* and *Inp53* from *atg5Δ* cells, we also observed marked suppression of AmphoB-induced GOMED (Fig EV2E and F). These results indicated that a reduction in Golgi PI(4)P levels is crucial for the induction of GOMED.

We then investigated whether a direct reduction in Golgi PI(4)P levels is sufficient to induce GOMED. *pik1-83* is a temperature-sensitive mutant that loses its PI(4)-kinase activity in the Golgi at or above 37°C (Audhya *et al*, 2000). Localization analysis confirmed the loss of PI(4)P at the Golgi in *pik1-83/atg5Δ* cells at 37°C (Fig 3A and B “*pik1-83 atg5Δ*”). In *pik1-83/atg5Δpep4Δ* cells, AB-like structures were induced after changes in temperature as judged from phase-contrast microscopy (Fig 3E) and EM observations (Fig 3F and G). Importantly, freeze replica immunolabeling demonstrated the presence of HA-tagged *Sec7* (a *trans*-Golgi marker) on the membrane of AP-like (Fig 3H) and AB-like structures (Fig EV2G), indicating the Golgi-mediated generation of GOMED. Note that Golgi membranes were also immunolabeled by *Sec7*-HA (Fig 3H). These data indicated that a direct reduction in Golgi PI(4)P levels is sufficient to induce GOMED.

Golgi PI(4)P regulates several Golgi functions (Mayering, 2012). Therefore, an important question is which of these functions is needed for GOMED. Defects in lipid sorting induced by *Osh1* or *Osh4* deletion and defects in retrograde trafficking induced by *Vps74* deletion did not activate GOMED (Appendix Fig S7A). In contrast, the temperature-sensitive deletion of *Gga1* and *Gga2*, proteins responsible for anterograde trafficking in conjunction with PI(4)P (Demmel *et al*, 2008), induced GOMED (Fig 3I and J, and Appendix Fig S7A), recapitulating the loss of PI(4) kinase. To further identify the type of anterograde trafficking machinery required for GOMED, we generated *atg5Δ* cells lacking each of the components of the membrane trafficking machinery. As a result, we

Figure 3. Reduction in Golgi PI(4)P levels is required for GOMED.

- A Localization of cellular PI(4)P. GFP-2xPH^{Osh2}-expressing *atg5Δ*, *sac1-23/atg5Δ*, and *pik1-83/atg5Δ* cells were incubated with or without AmphoB (2.5 μg/ml) for 6 h at 37°C, and their localization was observed by confocal microscopy. Scale bars = 2 μm.
- B The same experiments were performed using GFP-GOLPH3 (a Golgi PI(4)P-monitoring protein) instead of GFP-2xPH^{Osh2}. Scale bars = 2 μm.
- C, D Reduction of AmphoB-induced GOMED by the deletion of Golgi PI(4) phosphatases. (C) Indicated cells expressing GFP-pho8Δ60 were cultured with or without AmphoB (2.5 μg/ml, 24 h) and subjected to Western blotting for GFP. (D) The number of AP-like and AB-like structures was counted using EM images (Fig EV2B and C). Error bars indicate s.e.m. (*atg5Δpep4Δ* cells: *n* = 22 and *sac1-23/atg5Δpep4Δ* cells: *n* = 32. **P* < 0.01 vs the value of *atg5Δpep4Δ* cells.)
- E–H Induction of GOMED by the deletion of Golgi PI(4) kinase. (E) Indicated cells were incubated at 37°C (temperature shift) for 6 h, and cells containing AB-like structures were counted by phase-contrast microscopy. **P* < 0.01 versus the value of *atg5Δpep4Δ* cells. (F) Representative electron microscopy image of *pik1-83/atg5Δpep4Δ* cells at 3 h after temperature shift. AP-like structure (arrow) and AB-like structures were generated. Scale bar = 0.2 μm. (G) The number of AP-like and AB-like structures was counted using EM images. Error bars indicate s.e.m. (*atg5Δpep4Δ* cells at 37°C: *n* = 54 and *pik1-83/atg5Δpep4Δ* cells at 37°C: *n* = 63). **P* < 0.01 versus the value of *atg5Δpep4Δ* cells. (H) *Sec7*-HA-expressing *pik1-83/atg5Δ* cells were incubated at 37°C (temperature shift) for 3 h, and freeze replica immunolabeling was performed as described in Materials and Methods. *Sec7*-HA-positive signals (labeled with 15-nm gold particle) were observed on the surface membrane of AP-like structure and Golgi membrane. Scale bar = 0.1 μm.
- I–L Genetic (I, J) and pharmacological (K, L) alterations in anterograde trafficking from the Golgi are required for GOMED. (I) Indicated cells were incubated at 37°C (temperature switch) for 3 h, and the cells containing AB-like structures were counted by phase-contrast microscopy. **P* < 0.01 versus the value of *atg5Δpep4Δ* cells. (J) Indicated cells were also observed using EM. Arrow indicates AP-like structure. Scale bar = 0.5 μm. (K, L) *atg5Δpep4Δ* cells were treated with (CBM) or without (NT) CBM (1 mM, 3 h), and cells containing AB-like structures were counted by phase-contrast microscopy (K). **P* < 0.01 versus the value of NT. Cells were also observed using EM (L). AB-like structures were observed. Scale bar = 0.5 μm.
- M–O Disturbance of anterograde trafficking from the Golgi to the PM in the cells with GOMED. Indicated cells expressing Hsp150-HA(3×) were treated as follows: NT, no treatment; starve, starvation for 6 h; AmphoB, 2.5 μg/ml for 24 h (M); CBM, 1 mM for 24 h (N); temperature shift, from 25 to 37°C for 3 h (O). The cells were then lysed and examined for Hsp150-HA(3×) expression by Western blotting. Accumulation of O-glycosylated Hsp150-HA(3×) indicated the trafficking failure from the Golgi to the PM.

Figure 3.

observed GOMED in *ypt31Δypt32^{A141D}/atg5Δpep4Δ* cells (Ortiz et al, 2002) (Appendix Fig S7B), which lack the components for protein export from the Golgi to the PM, whereas other trafficking mutants (Kaiser & Schekman, 1990; Becherer et al, 1996; Luo & Gallwitz, 2003) (*sec23-1*; ER to Golgi, *pep12Δ*; Golgi to vacuole, *ypt6Δ*; endosome to Golgi) and endocytosis mutants (Singer-Krüger et al, 1994; Smaczynska-de Rooij et al, 2010) (*vps1Δ* and *ypt51Δypt52Δypt53Δ*) did not induce GOMED (Appendix Fig S7B). Pharmacologically, the addition of 1, 3-cyclohexanebis(methylamine) (CBM), a compound that interferes with coatomer binding to Golgi membranes and thereby inhibits anterograde trafficking (Hu et al, 1999), also induced GOMED in *atg5Δpep4Δ* cells (Fig 3K and L). The failure of trafficking from the Golgi to the PM can be identified by the accumulation of O-glycosylated Hsp150 (see Materials and Methods section). Importantly, cells with GOMED [induced by treatment with AmphoB (Fig 3M) or CBM (Fig 3N), and GGA1/GGA2 deficiency (Fig 3O)] exhibited a disruption of Golgi trafficking. When Golgi-to-PM trafficking is disrupted and GOMED is activated, the undelivered secretory proteins may be degraded. This hypothesis was verified by the accumulation of invertase, a secreted protein, in the vacuoles in *pik1-83/atg5Δpep4Δ* cells after a temperature shift (Appendix Fig S7C). Taking these findings together, we concluded that a disturbance of anterograde trafficking from the Golgi to the PM is crucial for the induction of GOMED in yeast cells.

To examine whether GOMED is induced in WT cells, we expressed GFP-Atg8, a known autophagy marker, and Sec7-mRFP in *pik1-83* cells. The inhibition of anterograde trafficking by the temperature shift generated AB and AB-like structures, as assessed by the formation of GFP-Atg8 puncta and Sec7-mRFP puncta in the vacuoles (Fig EV3A). Freeze replica immunolabeling confirmed these findings because there were two types of structures in the vacuoles: GFP-Atg8-positive AB and Sec7-HA-positive AB-like structure (Fig EV3B), indicating the induction of autophagy and GOMED. Most structures had either one of these signals and only a few of the structures contained both signals (Fig EV3C). Note that the membrane of AB-like structure was similar to that of AB (Fig EV3B).

We also examined the extent of GFP-Sft2 processing during AmphoB treatment and found that the processing in *atg5Δ* cells and *grh1Δ* cells (GOMED-deficient cells) was less than that of WT cells, and deletion of both genes completely abolished GFP-Sft2 processing (Fig EV3D). These data indicated that AmphoB induced GOMED even in WT yeast cells.

Disruption of Golgi trafficking induces GOMED in mammals

GOMED is characterized by the generation of Golgi membrane-associated structures accompanied by proteolysis. To address whether a disruption in anterograde trafficking from the Golgi induces GOMED in mammals, we added CBM into the culture medium of Atg5-deficient mouse embryonic fibroblasts [Atg5 knock-out (KO) MEFs]. EM analysis showed the generation of autophagosome (AP)-like and autolysosome (AL)-like structures (Fig 4A and B, and Appendix Fig S8A). The AL-like structures were well merged with the large puncta of mCherry-syntaxin 6 (Golgi marker) by CLEM analysis (Fig 4C). Representative AL-like structures are identified by the enlarged ring-like immunofluorescence signals of lysosomal membrane proteins, such as Lamp1 and Lamp2, which appear due to the fusion of lysosomes and autophagosome-like structures. Such ring-like structures were increased in a time-dependent manner after CBM treatment (Fig 4D and E). Furthermore, consistent with CLEM analysis, large ring-like structures of Lamp2 fluorescence were merged with GFP-syntaxin 6 (Fig 4F), suggesting the involvement of the Golgi membrane in the generation of AL-like structures. Silencing of Grasp65 blocked the induction of CBM-induced large Lamp2 puncta formation (Fig 4E), indicating that CBM-induced AL-like structures were generated from the Golgi membrane. We further examined whether these AL-like structures are capable of degrading proteins. To this end, we used a red fluorescent protein (RFP)-GFP tandem protein, which can detect degradation compartments because GFP fluorescence, but not RFP fluorescence, becomes weak within acidic compartments (Kimura et al, 2007). As shown in Fig 4G, only a few degradation

Figure 4. Pharmacological and genetic induction of GOMED in Atg5-deficient MEFs.

- A–I Induction of GOMED in Atg5 KO MEFs by CBM treatment. (A, B) EM analysis revealed the formation of AP-like and AL-like structures. Atg5 KO MEFs were treated with CBM (2 mM) for 3 h. A representative low-magnification image (A) and high-magnification images (B) are shown. Scale bar = 1 μm (A) and 0.2 μm (B). The arrowheads and arrows indicate AL-like and AP-like structures, respectively. (C–F) Involvement of Golgi membranes in the generation of AL-like structures. (C) CLEM analysis of mCherry-syntaxin 6-expressing Atg5 KO MEFs. Cells were treated with CBM (2 mM) for 24 h and observed using fluorescence and electron microscopy. mCherry-syntaxin 6 puncta merged with the AL-like structures. Scale bar = 5 μm. "N" indicates nucleus. A magnified image of the dashed square is shown in the inset (scale bars = 2 μm). (D, E) Requirement of Grasp65 in the generation of AL-like structures. Atg5 KO and Grasp65-silenced Atg5 KO MEFs were treated without (NT) or with CBM (2 mM) for the indicated times followed by immunostaining with an anti-Lamp2 antibody. Representative images (at 24 h) are shown in (D). Scale bars = 5 μm. A magnified image of the dashed square is shown in the inset (scale bars = 1 μm). CBM induced large ring-like Lamp2 fluorescence. (E) Percentages of cells with large ring-like Lamp2 immunofluorescence (mean ± s.e.m., *n* = 4). **P* < 0.01 versus the value of Atg5 KO MEF. (F) Colocalization of Lamp2 and GFP-syntaxin 6 in CBM-treated Atg5 KO MEFs. GFP-syntaxin 6-expressing Atg5 KO MEFs were treated with (CBM) or without (NT) CBM (2 mM) for 6 h and immunostained with an anti-Lamp2 antibody. The ring-like Lamp2 fluorescence merged with the signal for syntaxin 6. Scale bar = 5 μm. A magnified image of the dashed square is shown in the inset (scale bars = 2 μm). (G) The monomeric red fluorescent protein (mRFP)-green fluorescent protein (GFP) tandem protein assay revealed the induction of GOMED. Atg5 KO MEFs stably expressing tandem mRFP-GFP were incubated without (no treatment) or with CBM (2 mM) for 24 h. Red signals indicate acidic compartments. Lysosomes were counterstained with an anti-Lamp2 antibody (cyan). Scale bars = 5 μm. Regions of interest (ROI) are indicated by dashed squares; arrowheads indicate GOMED structure (scale bars = 2 μm). (H, I) Degradation of GFP-fused proteins indicated the induction of GOMED. Atg5 KO MEFs stably expressing VSVG-GFP (H) or M6PR-GFP (I) were incubated with CBM (5 mM) for the indicated times. Cell lysates were subjected to immunoblotting with the anti-GFP antibody.
- J–M Genetic induction of GOMED in Atg5 KO MEFs. Atg5 KO MEFs were treated with the indicated small interfering (si)RNAs for 24 h, and GOMED was assessed by EM (J–L) and the mRFP-GFP tandem protein assay (M). Representative images are shown in (J, K). Arrows indicate AL-like structures. Scale bars = 2 μm. (L) The number of AP-like and AL-like structures was calculated from the EM images of cells treated with the indicated siRNAs. Error bars indicate s.e.m. (siControl: *n* = 23, siPi4k2α + 3β: *n* = 20, siArfaptin1: *n* = 20). **P* < 0.01 versus the value of siControl. (M) The same experiments as in (G) were performed using cells treated with the indicated siRNAs. Representative images are shown. Arrowheads indicate GOMED structure. Scale bars = 5 μm.

Figure 4.

compartments (containing red fluorescence) were observed in healthy Atg5 KO MEFs, whereas CBM-treated Atg5 KO MEFs displayed many large degrading structures (Fig 4G arrowheads). Importantly, all the red fluorescent compartments were merged with the large ring-like structure of Lamp2 fluorescence. These results indicated that the addition of CBM induced GOMED in Atg5 KO MEFs, as in *atg5Δ* yeast cells.

Because CBM inhibits Golgi-to-PM trafficking (Appendix Fig S8B), leading to the induction of GOMED, we hypothesized that the cargo molecules of Golgi trafficking as well as some Golgi membranes are digested by GOMED. To evaluate this hypothesis, we expressed the vesicular stomatitis virus ts045G protein fused to GFP (VSVG-GFP) in Atg5 KO MEFs and then treated the cells with CBM. The VSVG-GFP protein is normally transported from the Golgi to the cell surface (Presley *et al*, 1997). As shown in Fig 4H, VSVG-GFP was degraded upon CBM treatment in a time-dependent manner. Consistently, immunofluorescence analysis revealed that a large proportion of the VSVG-GFP signals merged with the large ring-like structure of Lamp2 fluorescence in the presence of E64d (Appendix Fig S8C). E64d is a lysosomal protease inhibitor that enables the detection of intralysosomal constituents by inhibiting lysosomal enzymes. The GFP-fused mannose-6-phosphate receptor, which is a Golgi membrane-localized protein, was also degraded by CBM (Fig 4I). Collectively, these findings suggested that the cargo molecules of Golgi trafficking might be target substrates of GOMED.

In WT MEFs, CBM induced conventional autophagy, but to a lesser extent than that induced upon starvation treatment, as assessed by LC3 modifications and p62 degradation (Appendix Fig S9A). When wild-type MEFs expressing GFP-syntaxin 6 and mCherry-LC3 were treated with CBM and immunostained for Lamp2, some Lamp2 puncta were colocalized with mCherry-LC3 and others were colocalized with GFP-syntaxin 6 (Appendix Fig S9B), suggesting that CBM-induced GOMED even in WT MEFs.

The induction of GOMED by the inhibition of Golgi trafficking was further confirmed by genetic manipulation. EM confirmed the presence of representative GOMED structures in the Atg5 KO MEFs in which PI4K2 α and PI4K3 β were silenced, both of which are Golgi-localized PI(4)kinases (Fig 4J and L), similar to those in yeast cells (Wang *et al*, 2003). The RFP-GFP tandem protein assay also confirmed the induction of GOMED (Fig 4M). Similar results were observed when arfaptin 1, which inhibits trafficking from the Golgi to the PM (Gehart *et al*, 2012), was silenced in Atg5 KO MEFs (Fig 4K–M). These results indicated that defects in Golgi-localized PI(4)kinases and disruption of the Golgi-to-PM pathway induce GOMED in mammalian cells.

Induction of GOMED in glucose-deprived Atg5/Atg7-deficient pancreatic β -cells

Finally, we addressed the biological roles of GOMED. Based on the observation that the disruption of Golgi trafficking induced GOMED and the knowledge that (pro)insulin granules are synthesized in the Golgi and then secreted in a similar way to Golgi-to-PM trafficking, we hypothesized that if insulin secretion was disrupted, GOMED would be activated to digest the unused insulin granules. To test this hypothesis, we deleted Atg5 from MIN6 cells (Fig EV4A and B), an insulin-secreting mouse β -cell line (Miyazaki *et al*, 1990). The addition of CBM inhibited Golgi trafficking, so that insulin secretion

was reduced (Fig EV4C). In these cells, we observed AP-like and AL-like structures enclosing insulin granules by EM (Fig EV4D–F), as observed in Atg5 KO MEFs.

For investigation under more physiological conditions, we reduced the glucose concentration of the culture medium to suppress insulin secretion (Fig 5A) and examined the induction of GOMED. EM analysis showed the generation of AP-like and AL-like structures enclosing and digesting insulin granules (Fig 5B–D). These structures were frequently observed close to the Golgi apparatus (Fig EV4G). CLEM analysis showed that Lamp1-GFP and mCherry-syntaxin 6 signals were merged with AL-like structures (Fig 5E), suggesting the Golgi membrane-mediated generation of AL-like structures. We also observed the degradation of (pro)insulin after glucose deprivation (Fig 5F). Consistently, immunofluorescence analysis showed that (pro)insulin signals merged with large Lamp2 dots in glucose-deprived, but not normal, Atg5 KO MIN6 cells in the presence of E64d (Fig 5G). Colocalization of these signals was not observed when lysosomal fusion was blocked by bafilomycin A1 (Fig 5G). Bafilomycin A1 also blocked the reduction of (pro)insulin by glucose deprivation (Fig 5F). The re-addition of glucose into the glucose-deprived medium also suppressed AP-like and AL-like structures (Fig EV4H and I) and (pro)insulin degradation (Fig EV4J). Collectively, these results indicated that (pro)insulin granules are degraded by GOMED after glucose withdrawal in MIN6 cells lacking Atg5.

Similar experiments were performed using primary β -cells isolated from Atg7^{F/F} Rip-cre mice, in which Atg7 is deleted specifically in β -cells (Ebato *et al*, 2008). We found that these cells undergo degeneration and form inclusion bodies, as previously described (Ebato *et al*, 2008) (Fig EV5A and B). When insulin secretion was suppressed by a reduction in the glucose concentration of the medium, we observed the induction of AP-like structures containing (pro)insulin granule (Fig 6A) and degrading the insulin granules (Fig 6B). Multiple Golgi vesicles were also enclosed in AP-like structures (Fig 6C and D). Quantitative analysis of the EM photographs of Atg7 KO β -cells revealed that AL-like structures increased at least 15 min after glucose deprivation, became fully activated at 30 min, and decreased at 60 min (Fig 6E). Bafilomycin A treatment decreased and increased AL-like structures and AP-like structures, respectively (Figs 6F and EV5C). Interestingly, mitochondria and some inclusion bodies were also enclosed and degraded by GOMED structures (Figs 6G and H, and EV5D and E). The degradation of mitochondria may have the effect of rapidly decreasing ATP levels, given that ATP is essential for insulin secretion.

Previous investigators examining the morphology of glucose-deprived WT β -cells showed that the digestion of old and fresh insulin granules was performed via crinophagy (Orci *et al*, 1984; Schnell & Borg, 1985; Schnell *et al*, 1988) and starvation-induced nascent granule degradation (Goginashvili *et al*, 2015) (SINGD), respectively. These pathways degrade (pro)insulin granules via their direct fusion with lysosomes. We also observed extensive crinophagy/SINGD (Fig 6I and J), as well as a small amount of (pro)insulin-containing autophagic vacuoles in WT β -cells (Fig 6K). These findings indicated that crinophagy and SINGD are the main pathways for the degradation of (pro)insulin granules in glucose-deprived WT β -cells as reported previously. In contrast, in Atg7 KO β -cells, crinophagy/SINGD were largely suppressed (Fig 6J)

Figure 5. Induction of GOMED in Atg5-deficient MIN6 cells.

- A** Suppression of insulin secretion in Atg5 knockout (KO) MIN6 cells after glucose deprivation. Atg5 KO MIN6 cells were incubated with normal culture medium (NT) or glucose-depleted medium for 1 h. The amount of secreted insulin is expressed as the percentage of that of the untreated control (NT) (mean $\pm$ s.e.m., $n = 3$). * $P < 0.01$ versus the value of NT.
- B, C** EM analysis revealed the induction of GOMED structures. Atg5 KO MIN6 cells were subjected to glucose deprivation for 1 h. Representative images are shown. The white arrow and black arrows indicate the AP-like structure and insulin vesicles, respectively. Scale bars = 0.2 μ m.
- D** The number of AP-like and AL-like structures was calculated from the EM images of cells left untreated (NT) or subjected to glucose deprivation. Error bars indicate s.e.m. (NT: $n = 17$, glucose deprivation: $n = 84$). * $P < 0.01$ and # $P < 0.05$ versus the value of NT.
- E** Involvement of Golgi membrane in the generation of GOMED. Lamp1-GFP/mCherry-syntaxin 6-expressing Atg5 KO MIN6 cells were subjected to glucose deprivation and analyzed by TEM and confocal microscopy. CLEM analysis revealed that AL-like structures (arrows) merged with Lamp1-GFP fluorescence and syntaxin 6 fluorescence. Scale bars = 5 μ m. A magnified image of the dashed square is shown in the inset (scale bars = 2 μ m).
- F** GOMED-mediated degradation of (pro)insulin by glucose deprivation of Atg5 KO MIN6 cells. Atg5 KO MIN6 cells were incubated in glucose-depleted medium in the absence or presence of bafilomycin A1 (10 nM) for the indicated times. The cell lysates were then subjected to immunoblot analysis using an anti-(pro)insulin antibody.
- G** Immunofluorescence analysis of (pro)insulin and Lamp2. Atg5 KO MIN6 cells were left untreated (NT) or subjected to glucose deprivation for 1 h in the presence of E64d (25 μ M) or bafilomycin A1 (10 nM) and were stained with anti-(pro)insulin and anti-Lamp2 (lysosomal marker) antibodies and observed by fluorescence microscopy. Scale bar = 1 μ m. Arrowheads indicate the colocalization of (pro)insulin with GOMED structures.

Figure 6. Induction of GOMED in Atg7-deficient pancreatic β -cells.

A–D EM analysis demonstrated the induction of GOMED. Primary Atg7 KO β -cells were subjected to glucose deprivation for 1 h. A representative AP-like structure containing insulin granule is shown in (A). An AL-like structure containing insulin granules is shown in (B). Scale bars = 0.2 μ m. (C, D) GOMED structures containing Golgi vesicles. An AP-like structure containing multiple Golgi vesicles (arrows) (C) and an AL-like structure fusing with a lysosome (arrowhead) (D) are shown. Scale bar = 0.5 μ m. A magnified image of the dashed rectangle is shown in the inset (scale bar = 0.2 μ m). AP-like structure contained double membrane.

E The total number of AL-like structures was calculated from the EM images of WT and Atg7 KO β -cells after glucose deprivation. Error bars indicate s.e.m. (n = 46–163). * P < 0.01 and [#] P < 0.05 versus the value of 0 min.

F The total number of GOMED structures was calculated from the EM images of Atg7 KO β -cells after glucose deprivation in the absence or presence of bafilomycin A1 (10 nM). Error bars indicate s.e.m. (n = 109–116). * P < 0.01 versus the value of 60 min.

G–J EM analysis showed the induction of mitophagy and crinophagy/SINGD. WT and Atg7 KO β -cells were subjected to glucose deprivation for 1 h. Representative images showing mitophagy in Atg7 KO β -cells and crinophagy/SINGD in WT β -cells are shown in (G) and (I), respectively. Arrowhead (G) and white arrow (I) indicates mitophagy and crinophagy/SINGD, respectively. Arrows indicate insulin granules (I). Scale bars = 0.5 μ m. (H, J) The number of structures representative of mitophagy (H) and crinophagy/SINGD (J) per cell was calculated from the EM images of β -cells after glucose depletion. Error bars indicate s.e.m. (n = 46–163). * P < 0.01 and [#] P < 0.05 versus the value of 0 min.

K The number of (pro)insulin granule-containing AL-like structure was calculated from the EM images of wild-type (WT) cells and Atg7 KO β -cells after glucose deprivation. Error bars indicate s.e.m. (n = 46–163). * P < 0.01 and [#] P < 0.05 versus the value of 0 min.

(probably owing to the requirement for Atg7 in crinophagy/SINGD), and instead, GOMED was activated (Fig 6K), suggesting that GOMED plays a role in compensating for crinophagy/SINGD.

Because the failure of conventional autophagy is associated with type 2 diabetes (Abe *et al*, 2013), GOMED may play a crucial role in the regulation of insulin secretion in diabetic patients. Taken

together, these findings suggest that glucose deprivation induces GOMED and digests (pro)insulin granules in Atg7 KO β -cells.

Discussion

In this study, we showed the following novel findings: (i) Yeast cells have a novel Golgi membrane-associated degradation (GOMED) of intracellular constituents; (ii) this pathway is phylogenetically conserved from yeast to mammals; (iii) disruption of the trafficking from the Golgi to the PM is a trigger of GOMED, by which undelivered cargo molecules are degraded; and (iv) GOMED functions to digest (pro)insulin granules in glucose-deprived autophagy-deficient β -cells. These findings showed the presence of a novel protein degradation pathway that can compensate for Atg5/Atg7-dependent autophagy and also indicated the molecular mechanisms and biological roles of this pathway. These findings are crucial for fully understanding intracellular protein degradation.

The morphological and functional features of GOMED were quite similar to autophagy, that is, (i) the presence of structures similar to autophagosomes (double-membrane compartments) containing cytosolic and organellar constituents, (ii) the generation of AB-like and AL-like structures (single-membrane compartments), and (iii) bafilomycin-sensitive fusion between lysosomes and AP-like structures. Therefore, it might be possible to categorize GOMED as a type of autophagy, particularly with its resemblance to alternative autophagy, which is an Atg5/Atg7-independent type of bulk degradation involving autophagic structures. The machinery for alternative autophagy is also generated from Golgi membranes together with late endosome membranes and is physiologically utilized for the degradation of mitochondria during erythrocyte maturation (Honda *et al*, 2014). Despite the morphological and functional similarities between GOMED and autophagy, we hesitate to categorize GOMED into autophagy, because we do not have sufficient molecular evidence to conclude that these two machineries are the same. Further studies will be necessary to clarify this point.

Decreased Golgi trafficking is a trigger of GOMED in both yeast and mammals. Although there are several differences between the anterograde trafficking machinery of yeast and mammals, the basic molecules involved are phylogenetically conserved. PI(4)P is one such molecule playing important roles in the biogenesis of Golgi-derived transport vesicles through its effector proteins. In this context, PI(4)P functions as a platform onto which these effector proteins are recruited. Therefore, a decrease in Golgi PI(4)P levels blocks the exit of transport vesicles, resulting in the accumulation of proteins that are delivered from the Golgi. The absence of effector proteins, such as Gga1/Gga2 in yeast and arfaptin 1 in mammals, also blocks trafficking from the Golgi (Demmel *et al*, 2008; Gehart *et al*, 2012). Given that a decrease in anterograde trafficking, but not other Golgi functions, induced GOMED in yeast and mammals, the accumulation of proteins appears to be a phylogenetically conserved trigger of GOMED. This protein accumulation may induce morphological changes of the Golgi apparatus, *that is*, stacking and elongation of Golgi cisternae and the induction of GOMED.

Increasing lines of evidence indicate that unused or damaged cellular constituents are the target substrates of autophagy. For example, unused mitochondria were degraded by autophagy

when culture conditions were shifted from aerobic to anaerobic in yeast (Priault *et al*, 2005). We accordingly hypothesized that GOMED may degrade undelivered proteins from the Golgi to compensate for autophagy. This hypothesis was verified by the accumulation of invertase in the vacuoles of yeast cells and the degradation of VSVG and insulin granules in Atg5 KO MEFs and Atg5 KO MIN6 cells, respectively, supporting the notion that one of the biological roles of GOMED is to avoid the accumulation of proteins in Golgi cisternae and to reduce Golgi stress. This scenario appears similar to the induction of Atg5-dependent autophagy to digest misfolded proteins in response to ER stress (Yorimitsu *et al*, 2006).

GOMED is required in cells lacking conventional autophagy. However, this process also functions in WT cells when the Golgi-to-PM transport machinery is disrupted. When β -cells are subjected to glucose deprivation, crinophagy and SINGD are the main pathways for the degradation of unused (pro)insulin and GOMED plays a subsidiary or compensatory function. In contrast, GOMED may play major roles in CBM-treated yeast cells and mammalian cells. Further studies are needed to elucidate the biological events in which GOMED is predominantly involved.

Materials and Methods

Reagents

The following antibodies were used for immunoblot and immunofluorescence assay: anti-GFP (Santa Cruz, #sc-9996), anti-HA (Santa Cruz, #sc-7392), anti-(pro)insulin (Takara, #M178), anti-insulin (Cell Signaling, #8138), anti-Atg5 (Sigma-Aldrich, #A0731), anti-LC3 (nanoTools, #0231-100), anti-GS28 (BD Bioscience, #611184), and anti-actin (Millipore, #MAB1501). Enzymes used for recombinant DNA techniques were purchased from Takara Bio Inc., TOYOBO, and New England Biolabs. Other chemicals were obtained from Nacalai Tesque (Tokyo, Japan).

DNA construction for yeast cells

Descriptions of the plasmids used in this study are included in Appendix Table S1. pRS-GFP and pRS-mRFP expression vectors were used to create fusion constructs. The pRS vector series has been described previously (Sikorski & Hieter, 1989). Coding sequences were amplified by PCR using PrimeStar (Takara Bio Inc.) or Pfu Turbo (Stratagene). Plasmids were sequenced to ensure that no mutations were introduced due to manipulations. Mutant constructs were generated by site-directed mutagenesis by Pfu Turbo and were confirmed by sequencing.

Yeast strains and media

The yeast strains used in this study are listed in Appendix Table S2. Gene deletions and epitope tags were introduced into the yeast by homologous recombination. Transformation into yeast was performed using the standard lithium acetate method. Unless otherwise mentioned, yeast cells were grown with shaking at 30°C in rich medium (YPD; 1% yeast extract, 2% peptone, 2% dextrose) and synthetic media (0.68% yeast nitrogen

base without amino acids, 0.5% casamino acids) containing 2% dextrose supplemented with necessary amino acids. For starvation, yeast cells were grown in liquid SD(-N) medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% dextrose). For AmphoB and CBM treatments, yeast cells were cultured in rich medium to stationary growth phase, and the cells were then shifted to rich medium containing AmphoB or CBM for 24 h with shaking at 30°C.

GFP processing assay in yeast cells

Yeast cells with GFP fusion proteins were precipitated with trichloroacetic acid for 10 min on ice. Cells were collected by centrifugation at $23,200 \times g$ for 10 min. After washing twice with 1 ml of ice-cold acetone, cells were air-dried and suspended in 100 μ l of sample buffer. After disruption of the cell walls by vortexing with an equal volume of acid-washed glass beads for 3 min, proteins were boiled at 100°C for 3 min. Samples were then loaded on a 15% polyacrylamide gel and electrophoresed. A standard semidry Western blot transfer procedure was performed using a PVDF membrane. After blotting, membranes were probed by incubation with anti-GFP antibody overnight at 4°C. After washing the membrane twice with T-TBS for 10 min, the second antibody was applied for 1 h at room temperature. After washing membranes, the GFP signal was detected using the ECL kit.

Fluorescence and phase-contrast microscopy in yeast cells

For fluorescence microscopy, yeast cells transfected with GFP fusion proteins or mRFP fusion proteins were visualized with a confocal microscope (Zeiss; LSM510 system). Samples were also observed by DIC microscopy (Nikon; TE-2000), and video images were taken at $200\times$ magnification using a CCD camera (Keyence). For phase-contrast microscopy, yeast cells were examined under an Olympus BS2 microscope with a $100\times$ oil-immersion objective for phase-contrast optics. Images were obtained using DP2-BSW.

Measurement of trafficking efficiency from Golgi to plasma membrane

Yeast cells with HA(3 $\times$)-tagged Hsp150 were precipitated with trichloroacetic acid for 10 min on ice. Cells were collected by centrifugation and performed Western blotting using anti-HA antibody as described in GFP processing assay. This protein is transported from the ER through the Golgi apparatus and subsequently secreted away. The ER-localized form is detected at 87 kD, whereas the Golgi-localized form is at 150 kD in SDS-PAGE, due to the glycosylation. To examine the extent of each size of HA-Hsp150, we can estimate the trafficking efficiency from the ER to Golgi and Golgi to PM.

Yeast subcellular fractionation

Cells were collected and converted to spheroplasts as described previously (Sato *et al*, 1995). To examine the efficiency of Pep4 maturation, spheroplasts were resuspended in 10 ml of ice-chilled lysis buffer (0.2 M sorbitol, 50 mM potassium acetate, 2 mM EDTA,

20 mM HEPES-KOH (pH 6.8), 1 mM DTT, 20 mg/ml PMSF, 5 mg/ml antipain, 1 mg/ml aprotinin, 0.5 mg/ml leupeptin, and 0.7 mg/ml pepstatin) and immediately homogenized with a Potter–Elvehjem homogenizer on ice. After removal of unbroken spheroplasts, the lysates were subjected to centrifugation at $13,000 \times g$ for 15 min at 4°C to yield an intermediate speed supernatant fraction, and further centrifuged at $100,000 \times g$ for 60 min at 4°C to obtain crude membrane fraction.

To isolate vacuole and vacuolar membrane, spheroplasts were suspended in 10 volumes of buffer A (10 mM Mes/Tris (pH 6.9), 0.1 mM $MgCl_2$, 12% Ficoll-400) and homogenized with Dounce homogenizer. The solution was suspended in 10 ml of buffer B (10 mM MES/Tris (pH 6.9), 0.5 $MgCl_2$, 8% Ficoll-400) and centrifuged at $51,900 \times g$ for 30 min at 4°C. The white layer on top was collected and resuspended in buffer B, and then 5 ml buffer B' (10 mM MES/Tris (pH 6.9), 0.5 $MgCl_2$, 4% Ficoll-400) was layered on top. After centrifugation ($51,900 \times g$, 30 min at 4°C), the intact vacuoles were recovered from the top. The intact vacuoles were converted to vacuolar membrane by diluting 10 volumes of buffer C (10 mM Mes/Tris (pH 6.9), 0.5 mM $MgCl_2$, 25 mM KCl) followed by centrifugation ($37,000 \times g$, 20 min at 4°C).

Mammalian cell culture

MEFs were generated from WT, Atg5 KO embryos at E13.5, and were immortalized with SV40 T antigen. MIN6 cells were kindly provided by Prof. J. Miyazaki (Osaka University). Atg5 KO MIN6 cells were generated by the CRISPR/Cas9 system (Cong *et al*, 2013). Briefly, a 20-bp mouse Atg5-targeting sequence (GAGAGTCAGC TATTGACGT) was synthesized (Eurofins) and introduced into px330 (Addgene). MIN6 cells were co-transfected with the plasmid and pcDNA3.1 (Invitrogen), which contains the neomycin gene, and G418 selection (800 μ g/ml) was initiated 24 h later. After 48 h of selection, the MIN6 cells were reseeded to allow single colony formation. The knockout of Atg5 was confirmed by anti-Atg5 immunoblot. MEFs and MIN6 cells were grown in modified Dulbecco's modified Eagle's medium (DMEM).

Pancreatic islets were isolated from WT or Atg5^{F/F} Rip-cre C57/B6J mice by collagenase digestion, as previously described (Ebato *et al*, 2008) and incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum. After the incubation, the medium was exchanged for glucose-deprived medium for immunofluorescence and EM.

Transfection in mammalian cells

For DNA transfection, the retroviral expression plasmids, pMSCV-mCherry-syntaxin 6-Hygro, pMSCV-VSVG-GFP-Hygro, pMSCV-M6PR-GFP-Zeo, pMSCV-mRFP-GFP-Hygro, and pMSCV-Lamp1-GFP-Zeo were constructed and introduced into MEFs and MIN6 cells by retroviral infection.

Briefly, Plat-E, retroviral packaging cells were plated and transfected 24 h later by calcium phosphate transfection method. Virus-containing supernatant was collected at 48, 60, and 72 h post-transfection and Atg5 KO MEFs were infected consecutively three times every 12 h with 4 μ g/ml polybrene. The medium was changed 12 h after the last infection, and hygromycin selection (200 μ g/ml) or zeocin selection (50 μ g/ml) was started 24 h later.

For transient DNA transfection, pcDNA3.1Hygro-GFP-syntaxin 6 and pcDNA3.1Hygro-mCherry-LC3 were constructed and introduced into MEFs with the Neon electroporation system (Invitrogen) according to the manufacturer's instructions.

For small interfering (si)RNA transfection, 1×10^6 cells were transfected with 10 μ g of siRNA using the Amaxa electroporation system (kit V, program U-20) according to the manufacturer's instructions. FlexiTube siRNA (QIAGEN) was used.

Electron microscopy

Yeast cells were placed between gold plates (Balzers) or sapphire glasses (Niko Optical) and frozen quickly by soaking in liquid nitrogen. Frozen samples in the gold plates were fractured in a BAF400D (Balzers). Replicas were obtained as described previously (Arakawa-Kobayashi *et al*, 2004). The glass samples were fractured in liquid nitrogen and fixed by the freeze-substitution method (Arakawa-Kobayashi *et al*, 2004). Replicas and thin sections were observed with a JEM 1010 (JEOL) operating at 100 and 80 kV, respectively.

Mammalian cells were fixed by a conventional method (1.5% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, followed by an aqueous solution of 1% osmium tetroxide). Fixed samples were embedded in Epon 812, and thin sections (70–80 nm) were then cut and stained with uranyl acetate and lead citrate for observation under a Jeol-1010 electron microscope (Jeol) at 80 kV.

Freeze replica immunolabeling

Yeast freeze-fracture replicas were washed by PBS and incubated with 2.5% SDS in 0.1 M Tris-HCl (pH 7.4) at 60°C for 12 h. The samples were subsequently blocked with 3% BSA in PBST and treated with immunogold-labeled anti-GFP and anti-HA antibodies at 4°C overnight. After antibody treatment, replicas were washed with 0.1% BSA in PBST, followed by distilled water, and subjected to EM observation.

Correlative light and electron microscopy (CLEM)

For merge of confocal fluorescence microscopy photograph and TEM photograph, samples were quick-frozen and transferred to 0.0001% OsO₄ and 0.01% glutaraldehyde in acetone (−80°C for 24 h, −30°C for 6 h, −15°C for 1 h, 4°C for 30 min), and washed by acetone. After replacing acetone to PBS(−), the samples were visualized with a confocal microscopy. They were then fixed by Karnovsky solution (1.5% paraformaldehyde and 3% glutaraldehyde in phosphate buffer) for 15 min at room temperature and 1% OsO₄ at 4°C for 5 min. After dehydration, the samples were embedded in Epon, and thin sections were observed with a JEM 1010 (JEOL) operating at 80 kV.

Immunofluorescence analysis

Cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% saponin, and stained with primary antibodies followed by secondary antibodies (Invitrogen). The cells were then mounted in ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole

(DAPI) and examined by confocal microscopy (Zeiss; LSM510 system).

Insulin secretion assay

MIN6 cells were starved for 1 h in Krebs–Ringer buffer containing 2 mM glucose. After starvation, medium was exchanged for normal culture medium (glucose 23 mM) or glucose-deprived medium (glucose 0.2 mM) to measure glucose-stimulated insulin secretion. After 1 h, the medium was collected and the remaining cells were lysed with RIPA buffer to measure total protein content. Secreted insulin amount was measured with an ultra-sensitive mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga). Secreted insulin was normalized to total protein content and presented as a percentage of the non-treated cells.

Statistical analysis

Results are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical evaluation was performed using Prism (GraphPad) software. Comparisons of multiple datasets were made using one-way ANOVA followed by Tukey's *post hoc* test. Statistical significance was established for *P*-values of < 0.05 . Statistical analyses of nonrandom associations between two categorical variables were examined using Fisher's exact test.

Expanded View for this article is available online.

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Author contributions

HY and SA discovered GOMED, HY performed biochemical analyses of yeasts and mammals, SA and TK performed EM analyses, TM, YF, and HW performed a part of experiments using *Atg7* KO β -cells, YT supervised data interpretation, and SS designed the research and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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