In macroautophagy (hereafter autophagy), cytoplasmic molecules and organelles are randomly or selectively sequestered within double-membrane vesicles called autophagosomes and delivered to lysosomes or vacuoles for degradation. In selective autophagy, the specificity of degradation targets is determined by autophagy receptors. In the budding yeast *Saccharomyces cerevisiae*, autophagy receptors interact with specific targets and Atg11, resulting in the recruitment of a protein complex that initiates autophagosome formation. Previous studies have revealed that autophagy receptors are regulated by posttranslational modifications. In selective autophagy of peroxisomes (pexophagy), the receptor Atg36 localizes to peroxisomes by binding to the peroxisomal membrane protein Pex3. We previously reported that Atg36 is phosphorylated by Hrr25 (casein kinase 1), increasing the Atg36–Atg11 interaction and thereby stimulating pexophagy initiation. However, the regulatory mechanisms underlying Atg36 phosphorylation are unknown. Here, we show that Atg36 phosphorylation is abolished in cells lacking Pex3 or expressing a Pex3 mutant defective in the interaction with Atg36, suggesting that the interaction with Pex3 is essential for the Hrr25-mediated phosphorylation of Atg36. Using recombinant proteins, we further demonstrated that Pex3 directly promotes Atg36 phosphorylation by Hrr25. A co-immunoprecipitation analysis revealed that the interaction of Atg36 with Hrr25 depends on Pex3. These results suggest that Pex3 increases the Atg36–Hrr25 interaction and thereby stimulates Atg36 phosphorylation on the peroxisomal membrane. In addition, we found that Pex3 binding protects Atg36 from proteasomal degradation. Thus, Pex3 confines Atg36 activity to the peroxisome by enhancing its phosphorylation and stability on this organelle.

Macroautophagy (hereafter autophagy) is a highly conserved lysosomal or vacuolar degradation system in eukaryotes (1, 2). In autophagy, cytoplasmic molecules and organelles are sequestered into double-membrane vesicles called autophagosomes, delivered to lysosomes or vacuoles, and degraded by hydrolases within these organelles. Autophagy nonselectively degrades these cellular components to promote their turnover and to supply degradation products as nutrients under starvation conditions (3, 4). In addition, autophagy selectively eliminates proteins or organelles that have become unnecessary or detrimental for cells (5). Recent studies have suggested that degradation of aberrant proteins, mitochondria, and the endoplasmic reticulum by selective autophagy is closely related to neuronal diseases in humans (6).

Previous studies have identified over 40 autophagy-related (Atg) proteins, including core Atg proteins involved in autophagosome biogenesis (7) and autophagy receptors involved in the recognition of degradation targets during selective autophagy (5, 8, 9). Autophagy receptors in yeast bind to degradation targets and interact with Atg11, which recruits core Atg proteins to initiate autophagosome formation in the vicinity of the targets (10). Similarly, some mammalian autophagy receptors, such as p62/SQSTM1 and CCPG1, interact with FIP200, a component of the autophagy-initiation complex (11, 12). In the budding yeast *Saccharomyces cerevisiae*, Atg19, Atg32, Atg34, Atg36, Atg39, Atg40, and Cue5 act as autophagy receptors, which target vacuolar enzymes, mitochondria, α-mannosidase (Ams1), peroxisomes, the nucleus, the endoplasmic reticulum, and polyQ proteins, respectively (13–21).

Selective autophagy should be tightly regulated to avoid undesirable degradation, and most autophagy receptors are regulated by posttranslational modifications (5). In yeast, the mitophagy receptor Atg32 is phosphorylated by casein kinase 2, resulting in increased Atg32–Atg11 interactions and thereby the stimulation of mitophagy initiation (22, 23). Moreover, we and another group have reported that the cytoplasm-to-vacuole targeting pathway (vacuolar enzyme transport via the autophagy machinery), the Ams1 pathway, and pexophagy (selective autophagy of peroxisomes) are regulated in a similar manner (24–26). The receptors Atg19, Atg34, and Atg36 are phosphorylated by the casein kinase 18 Hrr25, and their increased interactions with Atg11 promote the corresponding pathways.

In *S. cerevisiae*, peroxisomes proliferate during growth in oleate medium, in which peroxisomal functions are important for cells. A previous study has shown that Atg36-dependent pexophagy is induced by long-term culture in oleate media or by exchanging the medium to nitrogen starvation medium containing glucose (19). In addition, Atg36 is expressed during cell culture in oleate medium and localizes to peroxisomes via the interaction with the peroxisomal membrane protein Pex3. Moreover, Atg36 is phosphorylated by Hrr25, and this phosphorylation promotes the Atg36–Atg11 interaction and triggers pexophagy (24). However, the mechanisms underlying pexophagy regulation are still poorly understood.

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In this study, we reveal two distinct mechanisms by which Atg36 activity is confined to peroxisomes: Proteasomal degradation of cytoplasmic Atg36 and Hrr25-mediated activation of peroxisomal Atg36. In both mechanisms, Pex3 acts as a molecular switch.

Results

Pex3 is involved in the phosphorylation of Atg36

A previous study has shown that the peroxisomal membrane protein Pex3 recruits Atg36 to peroxisomes during pexophagy (19). GFP-tagged Atg36 was detected as multiple bands, mostly representing the protein phosphorylated by Hrr25, under pexophagy-inducing conditions (nitrogen starvation following cell growth in oleate medium) (24) (Fig. 1A). To analyze the phosphorylation of Atg36-GFP, the ATG1 gene, which encodes a protein kinase responsible for autophagosome formation, was knocked out to block degradation of Atg36-GFP via pexophagy. We found that Atg36 phosphorylation was almost completely lost when PEX3 was disrupted (Fig. 1A). Pex3 is a peroxin responsible for the biogenesis of peroxisomes (27). To examine whether peroxisome biogenesis is important for Atg36 phosphorylation, we knocked out other peroxin genes, \( \text{PEX11, PEX14, and PEX19} \). Unlike the results for the knockout of PEX3, the phosphorylation of Atg36-GFP was not substantially impaired by the knockout of these PEX genes (Fig. 1B). These results suggest that Atg36 phosphorylation by Hrr25 requires Pex3, independent of its function in peroxisome biogenesis.

Atg36 phosphorylation requires the interaction with Pex3

Next, we investigated whether the interaction of Atg36 with Pex3 is required for Atg36 phosphorylation by Hrr25. WT Pex3 and the Pex3-177 mutant, which is functional for peroxisome biogenesis but is defective in Atg36 binding (19), were fused with mCherry and expressed from single-copy plasmids in PEX3 knockout cells. The phosphorylation of Atg36-GFP was observed in cells expressing WT Pex3-mCherry but was decreased in cells expressing Pex3-177-mCherry (Fig. 1C), suggesting that the binding of Atg36 to Pex3 is important for Atg36 phosphorylation by Hrr25.

We investigated whether the Atg36–Pex3 interaction on the peroxisome is required for Atg36 phosphorylation. Pex3, which consists of 441 amino acid residues, is anchored to the peroxisomal membrane by its N-terminal transmembrane domain (28). Chromosomal PEX3 was engineered to express Pex3-mCherry lacking the transmembrane domain (Pex3\(^{340-441}\)-mCherry). In addition, because the protein level of Pex3\(^{340-441}\)-mCherry was lower than that of WT Pex3-mCherry, the promoter was replaced with the ADH1 promoter to increase the protein level to that of WT Pex3-mCherry expressed under the original promoter (Fig. 1D). We also confirmed that whereas WT Pex3-mCherry and Atg36-GFP colocalized at peroxisomes (cells expressing Atg36-GFP contain a few clusters of peroxisomes) (19), Pex3\(^{340-441}\)-mCherry and Atg36-GFP were both dispersed throughout the cytoplasm (Fig. 1E). Pex3\(^{340-441}\)-mCherry and WT Pex3-mCherry resulted in similar levels of Atg36-GFP phosphorylation (Fig. 1D). These results suggest that the Atg36–Pex3 interaction per se is important for Atg36 phosphorylation by Hrr25 and does not need to occur on the peroxisome.

Pex3 directly promotes Atg36 phosphorylation by Hrr25

Our previous in vitro kinase assay using recombinant Hrr25 and GST-tagged Atg36 demonstrated that Hrr25 directly phosphorylates Atg36 (24). The cytoplasmic region of Pex3 (Pex3\(^{340-441}\)) was purified and added to this in vitro reaction. When GST–Atg36 was incubated with Hrr25 and ATP in the absence of Pex3\(^{340-441}\), the band of GST–Atg36 was slightly upshifted (Fig. 2A). The addition of Pex3\(^{340-441}\) to the reaction caused upshifted bands of most GST–Atg36. The upshifted band of GST–Atg36 disappeared by treatment with a protein phosphatase, and the disappearance was impeded by phosphatase inhibitors (Fig. 2B). Moreover, Pex3–177\(^{340-441}\), which is defective in the interaction with Atg36, had no effect on the band shift of GST–Atg36 by Hrr25 (Fig. 2A). These results demonstrated that Pex3 interacts with Atg36 and directly promotes its phosphorylation by Hrr25.

Pex3 enhances the interaction of Atg36 with Hrr25

Previous studies have shown that Atg36 interacts with Pex3 (19) and that Hrr25 is dispensable for the interaction (24). Consistent with the fact that Atg36 is a substrate of Hrr25, immunoprecipitation of Hrr25-GFP coprecipitated Atg36-myc (Fig. 3A). This coprecipitation depended on Pex3, suggesting that Pex3 increases the interaction of Atg36 with Hrr25. Furthermore, Pex3-myc was co-immunoprecipitated with Hrr25-GFP, and Atg36 knockout abolished this coprecipitation (Fig. 3B). These results suggest that Pex3 directly interacts with Hrr25 in association with Atg36 or indirectly interacts with Hrr25 via Atg36.

Pex3 protects Atg36 from proteasomal degradation

We noticed that, in addition to the decrease in phosphorylation, the protein levels of Atg36 were substantially lower in cells lacking Pex3 (Fig. 1A and B) or expressing the Pex3-177 mutant deficient for the interaction with Atg36 than in cells with WT Pex3 (Fig. 1C). In addition, Atg36 levels were almost normal in the presence of cytoplasmically expressed Pex3 (Pex3\(^{340-441}\)) (Fig. 1D). These results suggested that Pex3 binding is important for not only the Hrr25-mediated phosphorylation of Atg36 but also the protein stability of Atg36. We found that the treatment of PEX3 knockout cells with the proteasome inhibitor MG132 substantially increased Atg36-GFP in both oleate and nitrogen starvation media, suggesting that Atg36 is susceptible to degradation by the proteasome in the absence of Pex3 (Fig. 4A). MG132 also increased Atg36-GFP in pex3-177 cells (Fig. 4B), and it had a weaker effect on Atg36-GFP levels in cells expressing WT Pex3 (Fig. 4, A and B). These results suggest that Pex3 binding to Atg36 blocks proteasomal degradation of Atg36.

Discussion

In this study, we investigated the mechanisms underlying pexophagy regulation, with a focus on the pexophagy receptor.
Atg36. We first showed that Pex3 is involved in the Hrr25-mediated phosphorylation of Atg36, independent of peroxisome biogenesis and the localization of these proteins to peroxisomes (Fig. 1).

In vitro analysis using recombinant proteins revealed that Pex3 directly promotes Atg36 phosphorylation by Hrr25 (Fig. 2). Moreover, co-immunoprecipitation analysis suggested that the interaction of Atg36 with Hrr25 is enhanced by Pex3 and that Hrr25 directly or indirectly interacts with Pex3 depending on Atg36 (Fig. 3). These results allow us to propose a model for the spatial regulation of Atg36 phosphorylation by Hrr25 (Fig. 5). Although cytoplasmic Atg36 is not a good substrate for Hrr25, it becomes efficiently phosphorylated by Hrr25 when bound to Pex3 in the peroxisomal membrane. Pex3 and Atg36 may both interact with Hrr25 to retain the kinase at the complex, facilitating Atg36 phosphorylation by Hrr25 (Fig. 5i). Alternatively, Pex3 binding may induce a conformational change in Atg36, increasing Atg36 affinity to Hrr25 (Fig. 5ii). The conformational change may also increase the accessibility of Hrr25 to phosphorylation sites in Atg36. Pex3-dependent phosphorylation clearly enables the specific activation of Atg36 that has been correctly localized to the peroxisome as a degradation target.

In the methylotrophic yeast Komagataella phapphi (formerly Pichia pastoris), Atg30 functions as a pexophagy receptor...
Despite no sequence similarity between Atg30 and Atg36, these proteins share a number of functional characteristics. Similar to Atg36, Atg30 is recruited to peroxisomes by interacting with Pex3 (30). In addition, Atg30 is also phosphorylated by Hrr25, and this phosphorylation is likely to increase the Atg30 interaction with Atg11 (29–31). However, unlike in S. cerevi-
Experimental Procedures

Yeast strains and media

The yeast strains used in this study are listed in Table S1. Gene disruption and tagging were performed by a PCR-based method (36). Yeast cells were grown in SD medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose, 0.5% casamino acids, 0.002% adenyl sulfate, 0.002% tryptophan, and 0.002% uracil) at 30°C. Cells carrying pRS316-derived plasmids were cultured in SD medium without uracil. To induce pexophagy, cells were grown in these media for 24 h (A600 reached 6), diluted 10-fold with SO medium (0.17% YNB w/o aa and as, 0.5% ammonium sulfate, 0.1% glucose, 0.12% oleate, 0.2% Tween 40, 1% casamino acids, 0.1% yeast extract, 0.002% adenine sulfate, 0.002% tryptophan, and 0.002% uracil) at 30°C. Cells carrying pRS316-derived plasmids were cultured in SD+CA+ATU medium without uracil. To induce pexophagy, cells were grown in these media for 24 h (A600 reached ~6), diluted 10-fold with SO+CA+ATU medium (0.17% YNB w/o aa and as, 0.5% ammonium sulfate, 0.1% glucose, 0.12% oleate, 0.2% Tween 40, 1% casamino acids, 0.1% yeast extract, 0.002% adenine sulfate, 0.002% tryptophan, and 0.002% uracil), and grown for 18 h (A600 reached ~4); cells lacking PEX genes grew normally during this time period probably because SO+CA+ATU medium contained 0.1% glucose), and the medium was replaced with SD-N (0.17% YNB w/o aa and as and 2% glucose). 20 mM MG132 dissolved in DMSO was added to the media to a final concentration of 100 µM for proteasome inhibition.

Plasmids

Oligonucleotides used for plasmid construction are listed in Table S2. The pGEX-6P-Pex330-441 plasmid for GST-Pex330-441 expression in Escherichia coli was constructed as follows. The DNA sequence encoding Pex330-441 was amplified by PCR using genomic DNA from BY4741 (37) and the oligonucleotides Pex3-2mCherry 500 bp up and Pex3-2mCherry-PGK down, and cloned into the single-copy vector pRS316 using BamHI and EcoRI. The pex3-177 mutation was introduced into this plasmid as described above to construct pRS316-pex3-177-2mCherry.

Immunoblotting

Yeast cell pellets (~3–6 A600 units) were suspended in 200 µl of water, mixed with 200 µl of 0.2 M NaOH, stayed at room temperature for 5 min, and centrifuged at 5000 × g for 5 min at 4°C. The pellets were suspended in (5 × A600 units) µl of 2× urea sample buffer (75 mM MOPS-NaOH, pH 6.8, 4% SDS, 8 M urea, and 200 mM DTT) by vortexing at 65°C for 5 min and centrifuged at 15,000 × g for 1 min, and the supernatants were used for immunoblotting. Monoclonal antibodies against GFP (IL-8; Clontech), mCherry (a gift from Dr. Toshiya Endo), GST (B-14; Santa Cruz Biotechnology), and myc (9E10; Santa Cruz Biotechnology) were used for the detection of tagged proteins.
**Fluorescence microscopy**

Fluorescence microscopy was performed as described previously (38) using an inverted fluorescence microscope (IX83; Olympus) equipped with an electron-multiplying charged coupled device camera (ImagEM C9100-13; Hamamatsu Photonics), a 150 objective lens (UAPON 150XOTIRF, NA/1.45; Olympus), a 488 nm blue laser (50 milliwatt; Coherent) for GFP excitation, and a 588 nm yellow laser (50 milliwatt; Coherent) for mCherry excitation. MetaMorph (Molecular Devices) and Fiji (ImageJ) (39, 40) were used for image acquisition and processing, respectively.

**Protein purification**

GST-Atg36, Hrr25, Pex3-40-441, Pex3-177-40-441 were purified as described previously (41). Briefly, *E. coli* BL21 cells carrying pGEX-6P–based plasmids expressing GST-fused Atg36, Hrr25, Pex3-40-441, or Pex3-177-40-441 were grown in LB medium (10 mg/ml tryptone, 5 mg/ml yeast extract, 10 mg/ml NaCl, and 1 mM NaOH) containing 50 μg/ml ampicillin, and treated with 0.1 mM isopropyl-β-D-thiogalactopyranoside at 16°C for 18 h. Cell pellets were suspended in buffer B (20 mM HEPES-KOH, pH 7.2, and 150 mM NaCl) containing 5 mM DTT, 0.5 mM ethylenediaminetetraacetic acid, and 0.1 mM phenylmethylsulfonyl fluoride. The lysates were prepared as described previously (41) and rotated with GSH-Sepharose 4B resin (GE Healthcare) at 4°C for 2 h. The resins were washed with buffer B, and GST-Atg36 was eluted with 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 10 mM reduced GSH. To elute GST-free Hrr25, Pex3-40-441, and Pex3-177-40-441, the resins were treated with the PreScission Protease (GE Healthcare), which cleaves the linker between GST and the fused proteins. The purified proteins were concentrated using Vivaspin 6000–10,000 columns (Sartorius), supplemented with glycerol at a final concentration of 25%, and stored at −80°C.

**In vitro kinase assay**

0.3 μM GST-Atg36 was incubated with 0.05 μM Hrr25 at 30°C in the presence or absence of 3 μM Pex3-40-441 or Pex3-177-40-441 in 20 mM HEPES-KOH, pH 7.2, containing 150 mM NaCl, 1 mM MgCl₂, 0.2 mM DTT, and 1 mM ATP.

**Phosphatase treatment**

After in vitro phosphorylation reactions, samples were previously (42) treated with 0.5% Triton X-100 at 4°C for 30 min on a rotator, and centrifuged at 15,000 × g for 15 min. The supernatants were mixed with GFP-nanobody (GFP-binding protein)–conjugated magnetic beads (42) and rotated at 4°C for 2 h. The beads were washed with buffer A, and the bound proteins were eluted by mixing the beads in SDS sample buffer (50 mM Tris-HCl, pH 7.5, 2% SDS, 8% glycerol, 20 mM DTT) at 65°C for 5 min.

**Data availability**

All of the data are contained within the manuscript.

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**Author contributions**—S. M. and H. N. conceptualization; S. M., X. Z., and H. K. data curation; S. M. formal analysis; S. M. and H. N. validation; S. M. investigation; S. M. and H. N. writing—original draft; X. Z. and H. K. resources; X. Z., H. K., and H. N. writing—review and editing; H. N. supervision; H. N. funding acquisition; H. N. project administration.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviation**—The abbreviations used is: YNB w/o aa and as, yeast nitrogen base without amino acids and ammonium sulfate.

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**Spatial regulation of the pexophagy receptor Atg36**

Yeast cells were disrupted in buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10% sorbitol) containing 2 mM phenylmethylsulfonyl fluoride and 2× protease inhibitor mixture (cOmplete, EDTA-free; Roche) using a Multi-beads Shocker (Yasui Kikai) and 0.5-mm YZB zirconia beads. The lysates were treated with 0.5% Triton X-100 at 4°C for 30 min on a rotator, and centrifuged at 15,000 × g for 15 min. The supernatants were mixed with GFP-nanobody (GFP-binding protein)–conjugated magnetic beads (42) and rotated at 4°C for 2 h. The beads were washed with buffer A, and the bound proteins were eluted by mixing the beads in SDS sample buffer (50 mM Tris-HCl, pH 7.5, 2% SDS, 8% glycerol, 20 mM DTT) at 65°C for 5 min.
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