The peroxisomal exportomer directly inhibits phosphoactivation of the pexophagy receptor Atg36 to suppress pexophagy in yeast

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ABSTRACT

Autophagy receptor (or adaptor) proteins facilitate lysosomal destruction of various organelles in response to cellular stress, including nutrient deprivation. To what extent membrane-resident autophagy receptors also respond to organelle-restricted cues to induce selective autophagy remains poorly understood. We find that latent activation of the yeast pexophagy receptor Atg36 by the casein kinase Hrr25 in rich media is repressed by the ATPase activity of Pex1/6, the catalytic subunits of the exportomer AAA+ transmembrane complex enabling protein import into peroxisomes. Quantitative proteomics of purified Pex3, an obligate Atg36 co-receptor, support a model in which exportomer represses Atg36 without assistance from additional membrane factors. Indeed, we reconstitute inhibition of Atg36 phosphorylation *in vitro* using soluble Pex1/6 and define an N-terminal unstructured region of Atg36 that enables regulation by binding to Pex1. Our findings uncover a mechanism by which a compartment-specific AAA+ complex mediating organelle biogenesis and protein quality control staves off induction of selective autophagy.

Keywords: selective autophagy; pexophagy; receptor; Atg36; Hrr25; Pex1/6

INTRODUCTION

The accumulation of damaged organelles is a hallmark of aging and neurodegenerative disease (Mizushima and Levine, 2020). In healthy cells, damaged organelles are detected and eliminated by selective autophagy, a process that involves the capture of organelles in transport vesicles called autophagosomes that deliver organelles to the lysosome for degradation (Gatica et al., 2018). A class of proteins called autophagy receptors targets a diverse range of organelles and other targets for autophagic degradation (Kirkin and Rogov, 2019). Recent work in yeast and mammals has established that autophagy receptors function not only by tethering autophagy targets to autophagosomes, but by initiating *de novo* autophagosome formation via activation of the master autophagy kinase, Atg1/ULK1 (Kamber et al., 2015; Ravenhill et al., 2019; Torggler et al., 2016; Vargas et al., 2019).

To perform effective quality control, autophagy receptors must initiate degradation of damaged organelles while sparing functional organelles (Green and Levine, 2014). Several regulatory mechanisms that enable such selectivity by autophagy receptors have been identified. First, the localization of multiple autophagy receptors is regulated by organelle damage, such that receptors localize only to damaged organelles (Rogov et al., 2014). Second, phosphorylation by cytosolic kinases is critical for the ability of several autophagy receptors to drive organelle degradation (Farré et al., 2013; Tanaka et al., 2014; Wild et al., 2011). Studies of mitochondrial autophagy in mammalian cells have shown that such receptor phosphorylation events can be regulated to enable effective organelle quality control: the kinase TBK1 is recruited to depolarized mitochondria, where it phosphoactivates the autophagy receptor OPTN, enabling selective elimination of damaged mitochondria (Moore and Holzbaur, 2016). For many other

autophagy receptors that are known to be phosphorylated, however, it remains unclear whether their phosphorylation is regulated by signals of organelle damage in order to enable effective organelle quality control.

Recently, multiple groups working in yeast and mammalian cells reported intriguing observations that peroxisomes are rapidly degraded by autophagy in cells that lack Pex1, Pex6, or Pex15 (Law et al., 2017; Nuttall et al., 2014), which form a complex required for peroxisome matrix protein import (Platta et al., 2012). The Pex1/6 subunits form a hexameric AAA-ATPase that is recruited by Pex15 to peroxisomes, where it extracts ubiquitinated Pex5 (a receptor for peroxisome matrix proteins) from the peroxisome membrane to enable additional rounds of protein import (Platta et al., 2004). The accumulation of ubiquitinated proteins on the surface of peroxisomes has been shown to drive selective peroxisome autophagy (also known as pexophagy) (Deosaran et al., 2013; Kim et al., 2008), and it was thus proposed that the accumulation of ubiquitinated Pex5 in exportomer-deficient cells explains why pexophagy is induced in those cells (Law et al., 2017; Nazarko, 2017). However, it remains to be determined why pexophagy is induced by exportomer mutations in yeast, as Pex5 is not required for pexophagy in exportomer-deficient cells and ubiquitination does not appear to be a pexophagy-inducing signal in yeast (Nuttall et al., 2014). All forms of budding yeast pexophagy, including pexophagy driven by exportomer loss, are dependent on the autophagy receptor Atg36 (Motley et al., 2012; Nuttall et al., 2014), whose ability to drive pexophagy depends on its phosphoactivation (Farré et al., 2013; Tanaka et al., 2014). Thus, to gain insight into the mechanism by which exportomer mutations induce peroxisome turnover by selective autophagy, we investigated the molecular basis of Atg36 regulation upon loss of exportomer subunits.

RESULTS

Exportomer mutations enhance Atg36 phosphoactivation by Hrr25

We confirmed that deletions of *pex* genes encoding subunits of the exportomer promote pexophagy during post-logarithmic growth in rich media (Nuttall et al., 2014) (Figure S1A). Furthermore, we confirmed that this process is dependent on the pexophagy receptor Atg36 and Atg11, a scaffold protein that connects autophagy receptors to the Atg1 complex involved in all forms of autophagy (Figure S1A), as well as associated with a modified form of Atg36 that could be resolved by SDS-PAGE (Nuttall et al., 2014) (Figure S1B). Exportomer mutations have been previously shown to enhance removal of peroxisomes but not mitochondria or bulk cytoplasm, thus allowing the autophagy system to effectively exert a form of organelle quality control (Nuttall et al., 2014). Despite being selective, this pexophagy process resembles the mechanism of pexophagy induction by nitrogen starvation, which concomitantly elevates bulk autophagy and cytoplasm-to-vacuole (CVT) transport. In this latter, non-selective context, Atg36 is activated by Hrr25-mediated phosphorylation resulting in the enhanced ability of the modified receptor product to interact with Atg11 (Tanaka et al., 2014). We have obtained three lines of experimental evidence for an analogous mechanism by which exportomer mutants lead to Atg36 activation. First, lambda phosphatase treatment of Atg36-MYC isolated by affinity purification from a *pex1Δ* cell extract converted modified Atg36 to its unmodified form, thus rendering its migration indistinguishable from the wild-type control (Figure 1A). Second, Atg36 was no longer modified in the presence of a Pex3 mutation (*Pex3-177*) (Figure 1B) that abolishes Atg36 phosphorylation by Hrr25 during nitrogen starvation (Meguro et al., 2020; Motley et al., 2012). Third, we took advantage of the previous observation that auxin-inducible degradation (AID) of Pex1 can also enhance pexophagy (Nuttall et al., 2014) (Figure 1C). We were then able to

effectively suppress this conditional *pex1* phenotype by simultaneous AID of Hrr25 (Figure 1C). Lastly, we found that pexophagy caused by AID of Pex1 was abolished by inhibiting the kinase activity of a Shokat mutant version of Hrr25 with the ATP analog 1NM-PP1 (Figure S1A). In sum, these data show that exportomer mutants enable Atg36 phosphoactivation by Hrr25, thus leading to selective pexophagy in the absence of nutritional stress.

The exportomer enables the recycling of several receptors for targeting and translocation of matrix proteins with peroxisome targeting signals (PTSs). This mechanoenzymatic process has not been fully reconstituted with purified components but appears to be driven by the Pex1/6 ATPase. To test if the latter activity also drives Atg36 repression, we used a mutant version of Pex6 that was recently shown to completely abrogate ATP hydrolysis by the Pex1/6 complex *in vitro*, despite its ability to hexamerize with wild-type Pex1 subunits (Ciniawsky et al., 2015) and bind Pex15 (Birschmann et al., 2003). In cells expressing this point mutant version of Pex6 (E832A, Pex6^{WB}), Atg36 phosphoactivation and pexophagy were induced to a similar degree as in *pex6Δ* cells (Figure 1D). These findings demonstrate that exportomer ATPase activity, either directly or indirectly, inhibits Atg36.

Isolation of a multi-subunit Pex3 complex containing Atg36 and the exportomer

PTS receptors have already been excluded as potential mediators of Atg36 activation in *pex1Δ* cells (Nuttall et al., 2014), but a systematic dissection of this mechanism *in vivo* had not been carried out. Thus, we devised an unbiased proteomics approach for detecting Pex1-dependent changes within the Atg36 interactome relevant to pexophagy. Atg36 has relatively low cell abundance and appears to be both inherently unstable and resistant to Hrr25 phosphorylation

when not associated with Pex3, its peroxisomal anchor (Meguro et al., 2020). Thus, we chose Pex3-FLAG as our bait and subjected digitonin-solubilized extracts from post-logarithmic cells to affinity purification. Comparative mass spectrometry with extracts of control cells expressing untagged Pex3 revealed the presence of several previously identified Pex3 interaction partners, including Atg36, Inp1, Pex19, and Pex5, as well as several peroxisome membrane proteins (PMPs) whose insertion is known to be Pex19- and Pex3-dependent (Fang et al., 2004; Hettema et al., 2000; Jones et al., 2001; Sacksteder et al., 2000). Intriguingly, we also identified all three components of the exportomer, which to our knowledge had not been previously linked to Pex3. SDS-PAGE analysis followed by protein staining revealed two prominent high molecular weight species (>100 kDa) as likely candidates for Pex1 (117 kDa) and Pex6 (115 kDa) (Figure 2A). Indeed, we confirmed that Pex1 was a relatively abundant component of the complex by appending a bulky tag (3xV5-AID; ~30 kDa) to its C-terminus and detecting it by Western blotting, as well as by the expected band shift following SDS-PAGE analysis (Figure 2B). We found that the interaction between Pex3 and Pex1 was not affected by the expression of Pex6^{WB} (Figure 2C), indicating that the effect of this mutation on Atg36 phosphorylation and pexophagy induction (Figure 1D) is not simply caused by loss of binding between Pex1 and Pex3.

Next, we addressed the possibility that the exportomer inhibits Atg36 by remodeling the Pex3 interactome. Quantitative mass spectrometry analysis of immunopurified Pex3 from *pex1Δ* cells revealed the expected depletion of Pex6 (11-fold) and a lesser depletion of Pex15 (1.9-fold) (Figure 2D). By contrast, we observed no significant depletion or enrichment of Pex5, Pex19 or known Pex19 clients, suggesting that Pex1 loss does not alter the interaction between Pex3 and any of its other binding partners detectable by this method (Figure 2D). In sum, our quantitative

interactome analysis revealed a direct physical link between the exportomer and Atg36 bound to Pex3 but found no evidence of exportomer-dependent interactions that mediate Atg36 activation by Hrr25.

Defining the mechanism by which the exportomer inhibits Atg36 *in vivo*

Our proteomics analysis raised the formal possibility Atg36 phosphoactivation in *pex1Δ* cells was enabled by Pex19 and its clients. However, we found that the absence of Pex19 was actually by itself sufficient to induce Atg36 phosphorylation comparably to exportomer *pex* mutations (Figure 3A and 1B). Analysis of Pex3 immunopurified from *pex19Δ* cells argues that this effect was indirectly caused by disruption of the exportomer's proximity to Atg36 (Figure 3B). To test this idea further, we devised an engineered system that bypasses Pex19's broad role in the assembly of peroxisomal membrane protein complexes. Our strategy was to first release Atg36 into the cytosol by deleting the transmembrane domain TMD of Pex3 (cytoPex3). A previous study focused on pexophagy regulation by nutritional stress showed that expressing a cytosolic form of Pex3 promotes Atg36 phosphorylation (Meguro et al., 2020). Similarly, we found that our cytoPex3 mutant also enabled Atg36 phosphorylation in the cytosol of post-logarithmic cells (Figure 3C).

Next, we tested if recruitment of Pex1/6 was sufficient to inhibit Atg36 in this cytosolic context by constructing a chimeric protein scaffold comprising the cytosolic domain of Pex15 and the cytosolic domain of Pex3 fused with a short linker (cytoPex15-cytoPex3) (Figure 3C). Strikingly, in cells expressing cytoPex15-cytoPex3, compared to cells expressing cytoPex3, we observed repression of Atg36 phosphorylation, but not when we caused AID of Pex1 (Figure

3C). Importantly, in this engineered context, repression was no longer dependent on Pex19 but remained driven by the exportomer's ATPase activity (Figure S2A and S2B). These data demonstrate that the exportomer's proximity to Atg36 is critical for inhibition of pexophagy but that Pex19 and peroxisomal membrane proteins don't contribute to this mechanism.

To further define the core mechanism of Atg36 inhibition embedded within this stripped-down, membrane-free system, we examined closely the effect of the *pex6^{WB}* mutation on the composition of the cytoPex15-cytoPex3 complex. Quantitative proteomics analysis revealed that the amounts of Pex1 and Pex6 present in the cytoPex15-cytoPex3 complex were not affected, nor was the amount of Inp1, a soluble protein involved in peroxisome inheritance, which, like Atg36, binds Pex3 independently of Pex19 (Figure 3D) (Fagarasanu et al., 2005). Notably, the abundance of Atg36 was increased ~2.2-fold in cytoPex15-cytoPex3 complexes isolated from cells expressing Pex6^{WB} (Figure 3D). In sum, our interactome analysis supports the model that the exportomer directly blocks activation of Pex3-bound Atg36 by Hrr25.

A previous study focused on the exportomer's role in PTS receptor recycling noted the ability of Pex15's soluble domain to dynamically control the ATPase activity of Pex1/6 (Gardner et al., 2018). To determine if this is a general feature of the exportomer mechanism, we tested whether the requirement for Pex15 in Atg36 repression could be altogether bypassed by artificially tethering Pex6 to Pex3 using the rapamycin-dependent FRB/FKBP heterodimerization system (Figure 3E). In cells co-expressing Pex3-FRB and FKBP-Pex6, but not cells expressing Pex3-FRB and FKBP-Pex6^{WB}, addition of rapamycin repressed Atg36 phosphorylation (Figure 3E and

Figure S2C). Thus, Pex15 promotes Pex1/6 association with Pex3 on the surface of peroxisomes but is not strictly required for Atg36 inhibition.

Pex1/6 ATPase inhibits Hrr25 phosphorylation of Atg36 *in vitro*

To further test whether Pex1/6 can directly suppress Atg36 phosphorylation by Hrr25 in the absence of additional factors, we first biochemically reconstituted the latter process using yeast-purified components (Figure S3A) and radiolabeled ATP. Incubation with wild-type but not kinase-dead (K38A) Hrr25 resulted in the time-dependent appearance of ³²P-labeled Atg36 species with progressively reduced gel mobility following SDS-PAGE and autoradiography (Figure S3B). Strikingly, the addition of Pex1/6 purified from *E. coli* (Figure S3A) inhibited overall Atg36 phosphorylation by ~70%, whereas control incubations with Pex1/6^{WB} were indistinguishable from those lacking Pex1/6 altogether (Figure 3F and S3C).

We explored the specificity of Pex1/6 and Atg36 as a AAA-substrate pair in this reconstituted system by analyzing additional reactions with factor substitutions. First, we replaced Pex1/6 with recombinant Cdc48 (and a control ATPase-defective mutant E558Q, Cdc48^{D2}; Figure S3A), a homomeric hexamer with a similar AAA+ architecture, and found only a marginal, ATPase-dependent effect on Atg36 phosphorylation (~10%). Notably, the wild-type preparations of Pex1/6 and Cdc48 had comparable basal ATPase activities (Figure S3D). Second, we substituted Atg19 for Atg36 as the substrate because this CVT pathway receptor also becomes phosphoactivated by Hrr25 during nutrient starvation (Pfaffenwimmer et al., 2014; Tanaka et al., 2014). This control reaction revealed that Pex1/6 inhibition was specific to Atg36 (Figure S3E). Taken together with our previous *in vivo* evidence, these biochemical data provide

complementary support for the model in which the exportomer directly inhibits Atg36 phosphoactivation by Hrr25.

The exportomer inhibits pexophagy by binding to Atg36

Our *in vitro* evidence suggests that Atg36 is a novel exportomer substrate. AAA+ proteins can interact with their substrates using a variety of mechanisms but how the exportomer dislocates receptors for matrix proteins from the peroxisome membrane remains poorly understood (Gardner et al., 2018). Unlike these putative substrates, Atg36 is predicted to be a largely unstructured protein (Figures 4A and S4A) and presented an opportunity to define the sequence features of this arguably simpler exportomer substrate. Thus, we took a yeast-two-hybrid (Y2H) approach and found that Atg36 selectively interacted with Pex1 but not Pex6, despite the latter's ability to interact with Pex1 (Figure 4B). This interaction was fully abolished by N-terminal truncation of the first 30 amino acids of Atg36 (Figure 4B). This region is necessary but not sufficient for Atg36 interaction with Pex1, as a fragment corresponding to the N-terminal 59 amino acids of Atg36, but not a fragment corresponding to the N-terminal 30 amino acids, was able to interact with Pex1 (Figure 4B). Importantly, cell microscopy analysis revealed that even more severe N-terminal truncations of Atg36 still efficiently localized to peroxisomes (Figure S4B), thus pointing us to the existence of a C-terminal sequence determinant for peroxisome localization residing between amino acids 150-219 (Figure S4B-D).

A key prediction of the mechanistic model in which Pex1/6 represses pexophagy by binding to Atg36 is that mutations that selectively disrupt this interaction should lead to de-repression. We found support for this idea by analyzing cells expressing N-terminal truncations of Atg36

following post-logarithmic growth (Figure 4C and S4E). Specifically, Atg36 phosphorylation was enhanced by the $\Delta N30$ *atg36* mutation, which was also associated with an Atg11-dependent increase in Pex11-GFP processing (Figure 4C). More severe truncations that encompassed the Atg11-binding site resulted in the expected loss of this constitutive pexophagy phenotype (Figure S4E).

As the final test of the mechanism by which the exportomer inhibits Atg36, we used protein engineering to rescue the $\Delta N30$ *atg36* mutant. Our strategy was to replace the missing Pex1-interacting region with a Pex6-interacting region derived from Pex15 (amino acids 1-30). As predicted, cells expressing Pex15N- $\Delta N30$ Atg36 had a relatively lower level of Atg36 phosphorylation and Pex11-GFP processing (Figure 4C) and both of these suppressor phenotypes were abolished by a mutation in Pex15 that disrupts the Pex6-Pex15 interaction (L22S) (Birschmann et al., 2003). In sum, these data further support a model in which the exportomer acts on Atg36 as a *bona fide* substrate to generate a product resistant to phosphoactivation by Hrr25.

DISCUSSION

Yeast peroxisomes are constitutively degraded by selective autophagy in the absence of exportomer function (Nuttall et al., 2014), but the mechanistic basis for this phenomenon has remained unclear. In this work, we have presented evidence that the exportomer prevents pexophagy in unstarved cells by directly repressing phosphoactivation of the yeast pexophagy receptor, Atg36, by the casein kinase Hrr25. The ability of the exportomer to repress Atg36 phosphorylation is independent of its known role in membrane extraction of peroxisome proteins, as we showed that the exportomer is able to inhibit Atg36 phosphorylation away from the peroxisome or when reconstituted *in vitro* with purified soluble components. Repression of Atg36 phosphorylation by the exportomer requires its AAA-ATPase activity, but does not strictly require Pex15, whose function was bypassed by artificial tethering of Pex1/6 to Pex3.

We found that Pex1/6 physically interacts with Atg36 and mapped the binding site on Atg36 to its N-terminal region. Deletion of this region of Atg36 rendered it constitutively active and induced pexophagy, suggesting that the interaction between Atg36 and Pex1/6 is critical for repression of phosphorylation and pexophagy. However, the molecular mechanism by which Pex1/6 binding to Atg36 decreases phosphorylation of Atg36 by Hrr25 remains unclear. One possibility is that the N-terminal region of Atg36, which is near the critical Hrr25 S97 phosphosite for Atg11 binding (Farré et al., 2013), could provide a docking site for Hrr25, and that Pex1/6 binding to Atg36 disrupts the interaction between Hrr25 and Atg36. Future structural studies, for instance using hydrogen-deuterium exchange mass spectrometry to probe differences in Atg36 structure in the presence or absence of Pex1/6, should help resolve these issues.

Pex3 is required for Atg36 phosphorylation, and it was recently shown that Pex3 promotes Atg36 phosphoactivation by enhancing interaction of the Pex3-Atg36 complex with Hrr25 (Meguro et al., 2020). Due to technical difficulties in purifying a functional form of the soluble domain of Pex3, however, we have been unable to conduct quantitative enzymatic analyses of Pex3's role in promoting Hrr25 phosphorylation of Atg36, leaving uncertain whether the mechanism of stimulation involves substrate-induced conformational changes or a more passive tethering mechanism. It additionally remains unclear whether Pex3 regulates inhibition of Atg36 phosphorylation by Pex1/6, but our work suggests that Pex3 is not absolutely essential for this process, as Pex1/6 was capable of inhibiting Hrr25-mediated phosphorylation of Atg36 in *in vitro* assays conducted in the absence of Pex3. However, we cannot exclude that possible additional disruption of Atg36-Pex3 or Hrr25-Pex3 interactions by Pex1/6 may contribute to inhibition of Atg36 phosphorylation by the exportomer *in vivo*.

Atg36 is known to become phosphorylated by Hrr25 upon starvation, but future studies are required to determine how Atg36 phosphorylation is regulated by changes in nutrient status, and how starvation causes Atg36 to be released from, or overcome, inhibition by Pex1/6. It is known that starvation also increases Hrr25-mediated phosphorylation of two other autophagy receptors, Atg19 and Atg34 (Mochida et al., 2014; Pfaffenwimmer et al., 2014; Tanaka et al., 2014), so one possibility is that Hrr25 is globally activated by starvation signals, which may be sufficient to overcome Pex1/6 suppression. Alternatively, it is possible that exportomer activity (either towards all substrates, or towards Atg36 specifically) decreases during starvation. In this vein, future studies should examine whether the sites at which Atg36 is phosphorylated by Hrr25 differ upon exportomer loss and upon starvation, and whether exportomer loss synergizes with

starvation to increase Atg36 phosphorylation and pexophagy.

Within the context of quality control, our findings raise the intriguing possibility that autophagy is capable of selective organelle clearance using a similar mechanistic logic to two protein homeostasis pathways: the heat shock response and the unfolded protein response (Hetz et al., 2015, 2020; Pincus, 2020; Richter et al., 2010). A key feature of both is the dual ability of compartment-specific chaperones (cytosolic or endoplasmic reticulum (ER)-resident, respectively) to enable negative feedback by functioning as both effectors of protein folding and degradation, as well as repressors of two conserved transcription factors (in yeast, Hsf1 and Ire1, respectively) that restore proteostasis by inducing gene expression of various proteostasis factors, including the chaperones themselves. By analogy, Pex1/6 are known to be mechanonenzymes for both membrane recycling and degradation of Pex5/7, the receptors for targeting soluble proteins to the peroxisome matrix (Cui et al., 2013; Hagstrom et al., 2014; Kragt et al., 2005; Miyata and Fujiki, 2005; Miyata et al., 2009, 2012; Platta et al., 2004, 2007). We demonstrate that Pex1/6 also bind to Atg36 to directly repress wholesale peroxisome destruction but further work is needed to establish if physiological changes in the capacity of this AAA+ system are a readout of peroxisome dysfunction and damage. In this broad view of how different tiers of quality control can be logically connected, our work also raises the attractive possibility that emerging connections between ER protein folding stress and ER-phagy (Molinari, 2021) are the products of direct ER-phagy receptor regulation by ER-associated degradation factors.

Finally, even though the exportomer also appears to function as a repressor of selective pexophagy in mammalian cells (Law et al., 2017), it remains unclear whether the mechanism we

have uncovered in yeast is relevant to the regulation of pexophagy in mammalian cells. Several important differences in the regulation of pexophagy between yeast and mammals – including the use of different autophagy receptor proteins and the apparently unique role of ubiquitin in mammalian pexophagy (Deosaran et al., 2013; Kim et al., 2008; Nordgren et al., 2015; Zhang et al., 2015) – warrant caution in seeking for convergence between these mechanisms. Systematic screening for potential kinase-mediated mechanisms involved in mammalian pexophagy should help resolve this issue. Regardless of these specifics, our work paves the way for future efforts to define a broadly conserved regulatory mechanism of selective autophagy by exploring the ability of organelle-localized enzymes to directly inhibit phosphoactivation of autophagy receptors.

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AUTHOR CONTRIBUTIONS

H.Y., R.A.K., V.D. designed and performed the experiments. H.Y., R.A.K., V.D. wrote the paper. V.D. supervised the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. Exportomer mutations enhance Atg36 phosphoactivation by Hrr25.

(A) The indicated extracts were subjected to immunoprecipitation (IP) with anti-MYC magnetic beads. After extensive washing, bound material was treated with lambda phosphatase in the presence or absence of phosphatase (phos.) inhibitors, and then resolved by SDS-PAGE followed by immunoblotting with anti-MYC antibodies. 36-MYC, Atg36-MYC. In previous studies, two Atg36 species were detected in the absence of nutrient stress or exportomer mutations (Meguro et al., 2020, Nuttall et al., 2014); the lower-mobility species might correspond to a basally phosphorylated form of Atg36. In our experiments with MYC-tagged Atg36, we were unable to detect more than one major Atg36 species in the absence of oleate stress or exportomer mutations. The higher- and lower-mobility species we detect under oleate stress or in the absence of exportomer function might correspond to basally phosphorylated and hyperphosphorylated species of Atg36, respectively. See Materials and Methods for additional details.

(B) Extracts derived from cells with indicated genotypes were resolved by SDS-PAGE followed by immunoblotting with anti-MYC, anti-FLAG, and anti-Hsc82 antibodies. Pex3-177 indicates mutant version of Pex3 unable to bind Atg36 (Motley et al., 2012).

(C) Extracts were prepared from cells with indicated genotypes that were treated for 3 h with either 500 μ M auxin or vehicle (DMSO) and resolved by SDS-PAGE, followed by immunoblotting with anti-MYC, anti-FLAG, and anti-HXK antibodies.

(D) Cells expressing Pex11-GFP were grown in oleate medium for 22 hours and then transferred to nitrogen starvation medium. Extracts derived from cells with indicated genotypes were resolved by SDS-PAGE followed by immunoblotting with anti-MYC, anti-GFP, and anti-HXK

antibodies. wt, wild-type allele of *PEX6*; Δ , genomic deletion of *PEX6*; WB, *PEX6* D2 Walker B motif mutant allele (E832A) at the endogenous *PEX6* locus. GFP*, GFP fragments produced upon vacuolar degradation of Pex11-GFP. HKX, hexokinase. Data points represent the mean values determined from three independent experiments. Error bars indicate standard errors.

Figure 2. Isolation of a multi-subunit Pex3 complex containing Atg36 and the exportomer.

(A and B) Detergent-solubilized extracts derived from cells with indicated genotypes were first cleared at $100,000 \times g$ and then immunoprecipitated (IP) with anti-FLAG magnetic beads. (A) Eluates were resolved by SDS-PAGE followed by either silver staining or immunoblotting with anti-FLAG antibodies. Selected proteins identified in the Pex3-FLAG eluate by mass spectrometry are indicated based on predicted molecular weight. (B) FLAG peptide eluates and extract (input) samples were resolved by SDS-PAGE followed by either SYPRO Ruby gel staining or immunoblotting with anti-V5, anti-FLAG and anti-Hsc82 antibodies. Dotted line indicates splicing of identically processed gel-image data from the same gel.

(C) The indicated extracts were subjected to immunoprecipitation (IP) with anti-FLAG magnetic beads. FLAG peptide eluates and extract (input) samples were resolved by SDS-PAGE followed by immunoblotting with anti-V5, anti-MYC, anti-FLAG, and anti-Hsc82 antibodies. wt, wild-type allele of *PEX6*; Δ , genomic deletion of *PEX6*; WB, *PEX6* D2 Walker B motif mutant allele (E832A) at the endogenous *PEX6* locus.

(D) Quantification of protein abundance in Pex3-FLAG eluates for selected proteins identified by mass spectrometry. Plotted is the mean ratio of protein abundance in wt- versus *pex11*-derived Pex3-FLAG eluates. Error bars represent standard deviation.

Figure 3. The exportomer inhibits Atg36 both *in vivo* and *in vitro*.

(A) Extracts derived from cells with indicated genotypes were resolved by SDS-PAGE followed by immunoblotting with anti-MYC and anti-Hsc82 antibodies. wt, wild type.

(B) The indicated extracts were subjected to immunoprecipitation (IP) with anti-FLAG magnetic beads. FLAG peptide eluates and extract (input) samples were resolved by SDS-PAGE followed by immunoblotting with anti-FLAG and anti-V5 antibodies.

(C) Left, Schematic showing predicted pattern of Atg36 phosphorylation if exportomer is sufficient to inhibit Atg36. Right, extracts derived from cells with indicated genotypes that were treated for 3 h with either 500 μ M auxin or vehicle (DMSO) were resolved by SDS-PAGE followed by immunoblotting with anti-MYC, anti-FLAG and anti-V5 antibodies, with lane numbers corresponding to numbered schematics. *, unspecific bands.

(D) The abundance ratios of proteins in cytoPex15-cytoPex3-FLAG eluates derived from cells expressing *PEX6* D2 Walker B motif mutant allele at the endogenous *PEX6* locus (WB) versus eluates from cells expressing *PEX6* wild-type (wt). Values were normalized to the average ratio of quantified Pex3 and Pex15 peptides in each sample to control for variations in total amounts of material immunoprecipitated. Error bars represent standard deviation.

(E) Left, Schematics showing the predicted pattern of Atg36 phosphorylation if bypassing Pex15 by an artificial tethering system (FRB-Rap-FKBP) is sufficient to inhibit Atg36 phosphorylation. Right, extracts derived from cells with indicated genotypes that were treated with 1 μ M rapamycin (Rap) or vehicle (90% ethanol, 10% Tween-20) for 3 h were resolved by SDS-PAGE followed by immunoblotting with anti-MYC, anti-FLAG, and anti-V5 antibodies, corresponding

to schematics. WB, *PEX6* D2 Walker B motif mutant allele (E832A); HXK, hexokinase; Rap, rapamycin. See also Figure S2C.

(F) Purified Atg36 was incubated with ³²P-labeled ATP, Hrr25, and indicated AAA-ATPases, and samples at each time point were resolved by SDS-PAGE followed by autoradiography to measure Atg36 phosphorylation. WB, Pex6 D2 Walker B motif mutant (E832A). D2, Cdc48 D2 ring mutant (E588Q). Data points represent the mean values determined from four repeat experiments. Error bars indicate standard errors.

See Materials and Methods for details on protein chimeras and strain genotypes.

Figure 4. The exportomer inhibits pexophagy by binding to Atg36.

(A) Schematic of Atg36 with predicted secondary structural features indicated (PSIPRED 4.0) (Jones, 1999). Red region, predicted α -helix; green region, predicted β -sheet. AIM, Atg8-family interacting motif, amino acid 33-36. A11BR, Atg11 binding region, amino acids 94-102.

(B) Interaction between exportomer and Atg36 mutants in yeast-two-hybrid assay. Pex1 or Pex6 were fused with the Gal4-activation domain (AD) and Atg36 mutants were fused with Gal4 DNA binding domain (BD). Cells expressing both AD-exportomer and BD-Atg36 mutants were grown on synthetic defined (SD) media plates as indicated.

(C) Cells with indicated genotypes were grown in oleate medium for 22 hours. The extracts were resolved by SDS-PAGE followed by immunoblotting with anti-MYC, anti-GFP, and anti-HXK antibodies. GFP*, GFP fragments produced upon vacuolar degradation of Pex11-GFP. HXK,

430 hexokinase. Data points represent the mean values determined from three independent
431 experiments. Error bars indicate standard errors.

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MATERIALS AND METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Vladimir Denic (vdenic@mcb.harvard.edu).

Yeast strain construction and PCRs

Yeast strains are listed in Table S1. Deletion strains were constructed in the BY4741 or W303 backgrounds (mating type **a**) by standard PCR-mediated gene knockout. $3\times FLAG$, $13\times MYC$, *GFP*, *mCHERRY*, *emCitrine*, *Turquoise*, *NeonGreen*, $3\times V5-AID$, *FRB-3\times HA*, and $3\times FLAG-FKBP12$ (Euroscarf) cassettes were used to modify gene loci using standard PCR-mediated gene tagging. To introduce *OsTIR1* into the genome, a plasmid containing *OsTIR1* (Nishimura et al., 2009) provided by A. Amon was digested with *PmeI* and transformed into yeast for integration at the *leu2* or *his3* locus. The *as-HRR25* yeast strain bearing a gatekeeper mutation (I82G) in *HRR25* (Petronczki et al., 2006) at its endogenous locus was provided by C. Shoemaker. *cytoPEX3-FLAG* was constructed by replacing the endogenous *PEX3* ORF with an allele of *PEX3* lacking codons 2-45. *cytoPEX15-cytoPEX3-FLAG* was constructed by replacing the endogenous *PEX3* ORF with the product of an overlap extension PCR that fused, in frame, PCR-generated fragments corresponding to codons 1-330 of *PEX15*, a glycine-serine linker, and codons 46-441 of *PEX3*. The N-terminal truncated mutants of *ATG36* were constructed by replacing the endogenous *ATG36* ORF with the alleles of *ATG36* lacking the N-terminal region as indicated. The product of an overlap extension PCR that fused, in frame, two DNA fragments encoding 1-30 of *PEX15* and 31-294 of *ATG36* was used to generate *Pex15N-Atg36ΔN30* mutant.

Mutagenesis was performed using QuikChange (Stratagene) mutagenesis. Unless otherwise indicated, genomic allelic exchanges were performed using standard *URA3* replacement and 5-FOA counter-selection. Primer sequences for all strain constructions are available upon request.

Yeast cell growth and whole cell extract preparation for immunoblotting

Saturated, overnight cultures were diluted to 0.2 OD₆₀₀ units and grown for 5 h in YPD media to mid-log phase unless indicated otherwise. Cells were collected by centrifugation at 2,800 × *g*, stored at -80 °C, resuspended in 100 µl SDS-PAGE sample loading buffer per OD₆₀₀ unit, and boiled for 5 min. Samples were cooled and cleared for 1 min at 15,000 × *g* before loading. In Figure S1A, cells were grown in SD media until saturation, diluted to 0.2 OD₆₀₀ units in SD media and grown until reaching 2-4 OD₆₀₀ units. Cells were cultured at 30 °C.

To induce Pex1 and Hrr25 degradation, cells were treated with 500 µM 3-indoleacetic acid (auxin) (Sigma) in DMSO or mock-treated with DMSO alone. To inhibit Hrr25(as), cells were treated with 20 µM 1NM-PP1 (a gift from A. Hansen) for 20 h prior to sample collection. To induce the overexpression of target genes under a ZD promoter, cells were treated with 1 µM b-estradiol in ethanol or mock-treated with ethanol alone. In Figures 3E and S2C, cells were treated with 1 µM rapamycin (LC Laboratories) in 90% ethanol/10% Tween-20 or mock-treated with 90% ethanol/10% Tween-20 alone.

Yeast cell growth and lysate preparation for immunoprecipitations

Saturated, overnight cultures were diluted 1:100 and grown for 8 h in YPD media (1% yeast extract, 2% peptone, 2% dextrose) to mid-log phase. YP5D (1% yeast extract, 2% peptone, 5% dextrose) media was seeded with logarithmically growing cells to achieve a final OD₆₀₀ of ~2.0-3.0 after 9-11 doublings. Cells were pelleted at 3,000 × *g* for 20 min, washed with distilled water

and pelleted in 50 ml Falcon tubes ($3,000 \times g$ for 1 min). Washed cell pellets were weighed and resuspended in 1 ml lysis buffer (50 mM HEPES-KOH pH 6.8, 150 mM KOAc, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 M sorbitol) per 6 g pellet. Lysis buffer cell suspensions were frozen, drop-wise, in liquid nitrogen and the resulting frozen material was ground in the presence of cOmplete protease inhibitor cocktail (Roche) using a Retsch PM100 ball mill (large scale, 1 liter cultures) or Retsch MM400 ball mill (small scale, 25 OD₆₀₀ units maximum). Frozen lysate powder was stored at -80 °C.

Atg36-MYC immunoprecipitation and phosphatase treatment

After thawing 50 OD units frozen lysate powder in 0.5 ml HNP buffer (50 mM HEPES-KOH pH 6.8, 150 mM KOAc, 2 mM MgOAc, 1 mM $CaCl_2$, 15% glycerol, 1% NP-40, 1 $\times$ phosphatase inhibitors (5 mM sodium fluoride, 62.5 mM beta-glycerophosphate, 10 mM sodium vanadate, 50mM sodium pyrophosphate)), lysates were cleared twice at $3,000 \times g$ at 4 °C. Protein G Dynabeads (Invitrogen) were pre-equilibrated with mouse anti-MYC (9E10) antibody (Sigma) and added to clarified extract for 2 h at 4 °C. Beads were collected, washed four times in HN buffer (HNP buffer without phosphatase inhibitors), and resuspended in 40 μ l lambda phosphatase buffer (NEB). Two separate 8- μ l aliquots of beads derived from each strain were treated with 1 μ l lambda phosphatase (NEB) in 10 μ l reactions containing, additionally, either 1 μ l 10 $\times$ phosphatase inhibitors or 1 μ l water for 30 min at room temperature, before the reaction was terminated with equal volume 2 $\times$ SDS-PAGE sample loading buffer.

Immunoprecipitation of Pex3-FLAG complexes

Frozen lysate powder (0.4 g) was prepared as described above. After thawing in 1.6 ml HNP buffer, lysates were cleared twice at $3,000 \times g$ for 5 min, then at $100,000 \times g$ for 30 min. Protein

G Dynabeads were pre-equilibrated with mouse anti-FLAG antibody (Sigma) and added to clarified extract for 3 h at 4 °C. After extensive washing with HNP buffer, proteins were eluted via two sequential 30-min incubations with 20 µl 1 mg/ml 3 × FLAG peptide in HNP, at room temperature. The two eluates were pooled and analyzed by immunoblotting and SYPRO Ruby staining. For cytoPex15-cytoPex3-FLAG and cytoPex3-FLAG purifications, immunoprecipitations were performed similarly, but HP buffer (i.e. lacking detergent) (50 mM HEPES-KOH pH 6.8, 150 mM KOAc, 2 mM MgOAc, 1 mM CaCl₂, 15% glycerol, 1 × phosphatase inhibitors) was used in place of HNP.

Mass spectrometry

Affinity-purified complexes were prepared in triplicate (biological replicates), as described above, and analyzed by mass spectrometry at the Thermo Fisher Scientific Center for Multiplexed Proteomics (Harvard Medical School). Sample processing steps included SDS-PAGE purification of proteins followed by alkylation of cysteine and in-gel trypsin digestion. Peptides were extracted from the gel and desalted followed by peptide labeling with Tandem Mass Tag 10-plex reagents (Cargnello et al., 2012). Multiplexed quantitative mass spectrometry data were collected on an Orbitrap Fusion mass spectrometer operating in a MS3 mode using synchronous precursor selection for the MS2 to MS3 fragmentation. MS/MS data were searched against a Uniprot yeast database with both the forward and reverse sequences using the SEQUEST algorithm. Further data processing steps included controlling peptide and protein level false discovery rates, assembling proteins from peptides, and protein quantification from peptides (Weekes et al., 2014). Statistical analysis was performed using a one-way ANOVA and Benjamin Hochberg corrected *p*-values were calculated for each protein. For analysis of Pex3-FLAG and cytoPex15-cytoPex3-FLAG complexes, only those proteins with >2 quantified

peptides were plotted. Abundance ratios for each protein in Figure 3D were first normalized to the mean ratio (i.e., from Pex6^{WB}- versus Pex6-wt-derived cells) of Pex3 and Pex15 peptides to account for variations in the amount of immunoprecipitated cytoPex15-cytoPex3-FLAG complexes.

Protein Purification

Pex1/6. The coding sequence of *S. cerevisiae* Pex1 wild-type was fused with a C-terminal streptavidin-binding peptide (SBP) tag and inserted into pCOLA-Duet vector. The expression plasmids of His-tagged Pex6 wild-type and mutants were gifts from A. Martin (Gardner et al., 2018). For both the wild-type and mutant complexes, Pex1 and Pex6 subunits were co-expressed in BL21-CodonPlus (DE3)-*RIPL* cells. The expression strain was grown in 2xYT media (16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter) with ampicillin and kanamycin at 37 °C and induced at an OD₆₀₀ of 0.6–0.9 with 0.4 mM IPTG for a further 16 h growth at 18 °C. The *E. coli* cells were harvested at 5,000 × g for 10 min at 4 °C and resuspended in Buffer A (25 mM HEPES pH 7.6, 100 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 0.5 mM EDTA, 20 mM imidazole, 0.5 mM ATP, 10% glycerol and cOmplete protease inhibitor cocktail (Roche)) and were frozen at –80 °C or lysed by homogenization (Avestin Emulsiflex C3) at 15,000 psi at 4 °C. Cell debris and unlysed cells were pelleted at 48,000 × g and the supernatant was incubated with Ni-NTA resin (Qiagen) for 2 h at 4 °C. The resin was poured into a gravity flow column and washed with 100 mL Buffer A and 50 mL Buffer B (Buffer A plus 30 mM imidazole) sequentially. Pex1/6 complexes were eluted by 25 mL Buffer C (Buffer A plus 480 mM imidazole) and the eluate was incubated with 1 mL High Capacity Streptavidin Agarose resin (Pierce) for 2 h at 4 °C. The agarose resin was poured into a gravity flow column and washed with 50 mL Buffer A. Pex1/6 complexes were eluted by Buffer D (Buffer A plus 4 mM biotin). Purified Pex1/6 was dialyzed

into Buffer A for storage and *in vitro* assays.

Atg19. *S. cerevisiae* Atg19 was cloned into pGEX6P-1 resulting in a N-terminal GST fusion protein. The protein was purified from BL21-CodonPlus (DE3)-*RIPL* cells as described in the literature (Sawa-Makarska et al., 2014). Cells were grown at 37 °C in LB media (10 g tryptone, 5 g yeast extract and 10 g NaCl per liter) with ampicillin to an OD₆₀₀ of 0.6, induced with 0.4 mM IPTG for a further 16 h growth at 18 °C. Harvested cells were resuspended in Buffer E (50 mM HEPES pH 7.5, 300 mM NaCl, 2 mM MgCl₂, 2 mM mercaptoethanol, and cOmplete protease inhibitor cocktail (Roche)) and were frozen at –80 °C or lysed by homogenization (Avestin Emulsiflex C3) at 15,000 psi at 4 °C. Cell debris and unlysed cells were pelleted at 48,000 × g and the supernatant was incubated with glutathione beads (GE Healthcare) for 1 h at 4 °C. The beads were poured into a gravity flow column and washed with 100 mL Buffer E. The protein was cleaved from the GST tag by incubation with PreScission protease (GE Healthcare) overnight at 4 °C. The supernatant containing the protein was concentrated and applied to a Superdex™ 200 10/30 GL column (GE Healthcare) and eluted with Buffer F (25 mM HEPES pH 7.5, 150 mM NaCl and 1 mM DTT). Fractions containing pure protein were pooled, concentrated and stored at –80 °C.

Hrr25. Wild-type and kinase dead mutant (K38A) versions of Hrr25 were purified as described in the literature (Peng et al., 2015). Cell strains carrying leucine-deficient plasmids of *P_{GAL}-HRR25-Strep-6×His* and *P_{GAL}-hrr25(K38A)-Strep-6×His* were gifts from D. Drubin (Peng et al., 2015). Cells were grown in 25 ml leucine-dropout medium containing 2% glucose at 30 °C overnight. The culture were inoculated into 700 ml leucine-dropout medium containing 2% raffinose to OD₆₀₀ of ~2 at 30 °C. 100 ml 20% galactose and 200 ml 5x YP (50 g yeast extract and 100 g peptone per liter) were added to the final concentration of 2% and 1x YP, respectively.

After additional 6-7 h growth at 30°C, cells were harvested, washed twice with ice-cold water, and resuspended in ½ v/w ratio of lysis buffer/yeast, and frozen in drops in liquid N₂. The resulting frozen materials were lysed using a Retsch PM100 ball mill. Frozen lysate powder was stored at –80 °C or thawed with 2x w/w ratio of Buffer G (25 mM HEPES pH 7.6, 100 mM NaCl, 100 mM KCl, 10% glycerol and cOmplete protease inhibitor cocktail (Roche)). Lysates were cleared twice at 3,000 × g at 4 °C to remove cell debris and unlysed cells. The supernatant was incubated with Ni-NTA resin (Qiagen) for affinity purification as described above. The eluate from Ni-NTA resin was concentrated, applied to a Superdex™ 200 10/30 GL column (GE Healthcare) and eluted with Buffer H (25 mM HEPES pH 7.6, 400 mM NaCl, 10% glycerol). Fractions containing pure protein were pooled, concentrated and stored at –80 °C.

Atg36. *S. cerevisiae* Atg36 fused with C-terminal myc tag was cloned into the plasmid vector from D. Drubin, resulting in a leucine-deficient plasmid: *P_{GAL}-6×His-Atg36-10×myc*. Atg36 was overexpressed in strain D1074 (*Mata lys2::Pgall-GAL4 pep4::HIS3 bar1::hisG*) (St-Pierre et al., 2009) by galactose induction and cells were collected and lysed using a Retsch PM100 ball mill as described above. The frozen lysate powder was thawed with 2x w/w ratio of Buffer G and cleared twice at 1000 × g at 4 °C to remove unlysed cells, and then at 20,000 × g at 4 °C to collect the pellet. The pellet was dissolved in 10 ml Buffer G containing 6 M guanidinium chloride. The solution was further cleared at 20,000 × g at 4 °C and the supernatant was incubated with Ni-NTA resin (Qiagen) for 2 h at 4 °C. The resin was poured into a gravity flow column and sequentially washed with Buffer G containing 6 M, 4 M, 2 M, 1 M, 0.6 M, 0.4 M, 0.2 M, 0.1 M and 0.05 M guanidinium chloride; 100 mL Buffer G; and 50 mL Buffer G containing 50 mM imidazole. Atg36 was eluted from Ni-NTA resin by Buffer G containing 500 mM imidazole. The eluate was concentrated and applied to a Superdex™ 200 10/30 GL column

(GE Healthcare) and eluted with Buffer G. Fractions containing pure protein were pooled, concentrated and stored at -80 °C.

Protein concentrations were determined by measuring light absorbance at 280 nm and using extinction coefficients predicted from the proteins' sequence (ExPASy's ProtParam). The integrity and purity of proteins were evaluated by SDS-PAGE. ATPase activity was measured as reported previously (Nørby, 1988).

***In vitro* kinase assay**

500 nM Atg36 was incubated at 30 °C with wild-type Hrr25 or Hrr25 K38A mutant (200 nM in Figure 3F and Figure S3C, and 2 µM in Figure S3B), in the absence or presence of 200 nM ATPases (wild-type Pex1/6 and Walker B mutant (E832A), wild-type Cdc48 and D2 mutant (E588Q)) in kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT, 1 mM ATP, 10 mM creatine phosphate, 0.2 mg/mL creatine kinase) supplied with γ-³²P-ATP (Perkin-Elmer) (Figure 3F). 1 µM Atg19 was incubated at 30 °C with 500 nM wild-type Hrr25 in the absence or presence of 200 nM Pex1/6 (wild-type and Walker B mutant (E832A)) in kinase buffer (Figure S3E). The samples at each time points were resolved by SDS-PAGE followed by autoradiography. The ³²P-labeled species of Atg36 or Atg19 were quantified by FIJI.

Pexophagy assay

Pexophagy was induced as described previously (Motley et al., 2012, Tanaka et al., 2014). Yeast cultures grown in synthetic dextrose (SD) + casamino acids (CA) + ATU medium (0.17% yeast nitrogen base (YNB) without amino acids and ammonium sulfate (YNB w/o aa and as), 0.5% ammonium sulfate, 2% glucose, 0.5% CA, 0.002% adenine sulfate, 0.002% tryptophan, and

0.002% uracil)) overnight were diluted 10-fold with SO + CA + ATU medium (0.17% YNB w/o aa and as, 0.5% ammonium sulfate, 0.12% oleate, 0.2% Tween 40, 0.1% glucose, 1% CA, 0.1% yeast extract, 0.002% adenine sulfate, 0.002% tryptophan, and 0.002% uracil) and grown for 20 h. Cells were harvested, washed with autoclaved water twice and inoculated into SD-N medium (0.17% YNB w/o aa and as, 2% glucose). Samples at each time point were precipitated with 10% trichloroacetic acid and resolved by SDS-PAGE followed by Western blot. The abundances of the target proteins were quantified by FIJI.

Protein interaction analysis in yeast-two-hybrid system

The GAL4-based Matchmaker yeast 2-hybrid system (Clontech Laboratories Inc.) was used for yeast-two-hybrid analysis. Full-length open reading frames or truncated forms of the target proteins were inserted into pGADT7 (AD) or pGBKT7 (BD) vectors (Clontech Laboratories). The AD and BD constructs were transformed into *S. cerevisiae* strains Y2H gold (Clontech Laboratories). The representative transformants on SD -Leu, -Trp plates were used for Y2H analysis. The strains were grown in SD -Leu -Trp medium to log phase and plated on the selective plates (SD -Leu, -Trp or SD -Leu -Trp, -His, with 10 mM 3-amino-1,2,4-triazole (3-AT)) to test protein interaction.

Fluorescence Microscopy

Cells were grown in synthetic dropout medium to logarithmic phase, concentrated, and imaged at room temperature on a microscope (Axiovert 200M; Carl Zeiss) equipped with a Yokogawa CSU-10 spinning disk, using an oil-immersion 63 × objective (NA of 1.4). Images were acquired using a Cascade 512B EM-CCD detector (Photometrics) and the Metamorph 7.8.8 acquisition software (Molecular Devices). Images were converted to maximum intensity projections in FIJI,

635 and adjusted for brightness and contrast – equally for all images within each panel – in

636 Photoshop (Adobe) or FIJI.

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A

B

C

D

Figure 4

A

B

C

797 **SUPPLEMENTAL INFORMATION**

798 **Table S1. List of yeast strains, related to Figure 1-4, S1-S4**

Figure Panel	Strain Number	Strain Genotype
1A	BY4741	<i>MATa ura3Δ0 his3Δ1 leu2Δ0 met15Δ0</i>
1A	VDY2603	<i>BY4741 ATG36-13×MYC::HIS</i>
1A	VDY2604	<i>BY4741 ATG36-13×MYC::HIS pex1Δ::KAN</i>
1B	VDY2738	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
1B	VDY2662	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS pex1Δ::KAN</i>
1B	VDY2817	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex6Δ::URA</i>
1B	VDY2754	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex15Δ::URA</i>
1B	VDY2663	<i>BY4741 pex3Δ::PEX3_177-3×FLAG::NAT ATG36-13×MYC::HIS pex1Δ::KAN</i>
1C	VDY2632	<i>BY4741 ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
1C	VDY2633	<i>BY4741 ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN HRR25-3×V5-AID::URA leu2Δ::pGPD-OsTIR1::LEU</i>
1D	VDY5861	<i>BY4741 ATG36-13×MYC::HIS PEX11-GFP::HPH pex6Δ::PEX6(WB)</i>
1D	VDY5860	<i>BY4741 ATG36-13×MYC::HIS PEX11-GFP::HPH pex6Δ::URA</i>
1D	VDY5859	<i>BY4741 ATG36-13×MYC::HIS PEX11-GFP::HPH</i>
2A,D	VDY2604	<i>BY4741 ATG36-13×MYC::HIS pex1Δ::KAN</i>
2A,D	VDY2682	<i>BY4741 ATG36-13×MYC::HIS PEX3-3×FLAG::NAT pex8Δ::LEU</i>
2A,D	VDY2683	<i>BY4741 ATG36-13×MYC::HIS PEX3-3×FLAG::NAT pex8Δ::LEU pex1Δ::KAN</i>
2B	VDY2603	<i>BY4741 ATG36-13×MYC::HIS</i>
2B	VDY2661	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS</i>
2B	VDY2684	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN</i>
2C	VDY2632	<i>BY4741 ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
2C	VDY2738	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
2C	VDY2817	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex6Δ::URA</i>
2C	VDY3670	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex6Δ::PEX6(WB)</i>

3A	VDY2738	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
3A	VDY3656	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex5Δ::URA</i>
3A	VDY3657	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex14Δ::URA</i>
3A	VDY2755	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN pex19Δ::LEU</i>
3B	VDY2632	<i>BY4741 ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
3B	VDY2684	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN</i>
3B	VDY2753	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN pex14Δ::URA leu2Δ::pGPD-OsTIR1::LEU</i>
3B	VDY2754	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN pex15Δ::URA leu2Δ::pGPD-OsTIR1::LEU</i>
3B	VDY2755	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN pex19Δ::LEU</i>
3C	VDY2738	<i>BY4741 PEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
3C	VDY2750	<i>BY4741 pex3Δ::URA ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
3C	VDY2767	<i>BY4741 pex3Δ::cytoPEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
3C	VDY3426	<i>BY4741 pex3Δ::cytoPEX15-cytoPEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
3D	VDY3676	<i>BY4741 ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex15Δ::URA pex19Δ::HPH</i>
3D	VDY3677	<i>BY4741 pex3Δ::cytoPEX15-cytoPEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex15Δ::URA pex19Δ::HPH</i>
3D	VDY3678	<i>BY4741 pex3Δ::cytoPEX15-cytoPEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex15Δ::URA pex19Δ::HPH pex6Δ::PEX6(WB)</i>
3E	VDY3504	<i>W303 MATa fpr1Δ::NAT tor1-1 PEX3-FRB-3×HA::HIS ATG36-13×MYC::KAN pex15Δ::TRP1 pex6Δ::3×FLAG-FKBP-PEX6</i>
3E	VDY3511	<i>W303 MATa fpr1Δ::NAT tor1-1 PEX3-FRB-3×HA::HIS ATG36-13×MYC::KAN pex15Δ::TRP1 pex6Δ::3×FLAG-FKBP-PEX6(WB)</i>
4C	VDY5859	<i>BY4741 PEX11-GFP::HPH ATG36-13×MYC::HIS</i>
4C	VDY5862	<i>BY4741 PEX11-GFP::HPH atg36Δ::URA</i>
4C	VDY5863	<i>BY4741 PEX11-GFP::HPH atg36Δ::Atg36Δ30-13xMYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>

4C	VDY5864	<i>BY4741 PEX11-GFP::HPH atg36Δ::Atg36Δ30-13xMYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU atg11Δ::URA</i>
4C	VDY5865	<i>BY4741 PEX11-GFP::HPH atg36Δ::Pex15N-Atg36Δ30-13xMYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
4C	VDY5866	<i>BY4741 PEX11-GFP::HPH atg36Δ::Pex15N_L22S-Atg36Δ30-13xMYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
S1A	VDY2603	<i>BY4741 ATG36-13×MYC::HIS</i>
S1A	VDY2604	<i>BY4741 ATG36-13×MYC::HIS pex1Δ::KAN</i>
S1A	VDY2757	<i>BY4741 ATG36-13×MYC::HIS pex1Δ::KAN atg11Δ::LEU</i>
S1A	VDY2635	<i>BY4741 atg36Δ::URA pex1Δ::KAN</i>
S1A	VDY2664	<i>BY4741 hrr25Δ::HRR25(as) pex1Δ::KAN</i>
S1B	VDY2603	<i>BY4741 ATG36-13×MYC::HIS</i>
S1B	VDY2604	<i>BY4741 ATG36-13×MYC::HIS pex1Δ::KAN</i>
S1B	VDY2726	<i>BY4741 ATG36-13×MYC::HIS pex6Δ::URA</i>
S1B	VDY2730	<i>BY4741 ATG36-13×MYC::HIS pex15Δ::URA</i>
S2A	VDY3426	<i>BY4741 pex3Δ::cytoPEX15-cytoPEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
S2A	VDY3514	<i>BY4741 pex3Δ::cytoPEX15-cytoPEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex19Δ::HPH</i>
S2B	VDY3426	<i>BY4741 pex3Δ::cytoPEX15-cytoPEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
S2B	VDY3477	<i>BY4741 pex3Δ::cytoPEX15-cytoPEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex6Δ::URA</i>
S2B	VDY3478	<i>BY4741 pex3Δ::cytoPEX15-cytoPEX3-3×FLAG::NAT ATG36-13×MYC::HIS PEX1-3×V5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU pex6Δ::PEX6(WB)</i>
S2C	VDY3499	<i>W303 MATa fpr1Δ::NAT tor1-1 ATG36-13×MYC::KAN</i>
S2C	VDY3501	<i>W303 MATa fpr1Δ::NAT tor1-1 ATG36-13×MYC::KAN PEX3-FRB-3×HA::HIS</i>
S2C	VDY3506	<i>W303 MATa fpr1Δ::NAT tor1-1 ATG36-13×MYC::KAN pex6Δ::3×FLAG-FKBP-PEX6</i>
S2C	VDY3500	<i>W303 MATa fpr1Δ::NAT tor1-1 ATG36-13×MYC::KAN pex15Δ::TRP1</i>
S2C	VDY3512	<i>W303 MATa fpr1Δ::NAT tor1-1 ATG36-13×MYC::KAN PEX3-FRB-3×HA::HIS 3×FLAG-FKBP-PEX6</i>
S2C	VDY3504	<i>W303 MATa fpr1Δ::NAT tor1-1 ATG36-13×MYC::KAN PEX3-FRB-3×HA::HIS pex15Δ::TRP1 pex6Δ::3×FLAG-FKBP-PEX6</i>
S2C	VDY3511	<i>W303 MATa fpr1Δ::NAT tor1-1 ATG36-13×MYC::KAN PEX3-FRB-3×HA::HIS pex15Δ::TRP1 pex6Δ::3×FLAG-FKBP-PEX6(WB)</i>
S4B	VDY5867	<i>BY4741 PEX11-mCHERRY::HPH ATG36-NEONGREEN::HIS</i>
S4B	VDY5868	<i>BY4741 PEX11-mCHERRY::HPH atg36Δ::ATG36ΔN149-</i>

		<i>NEONGREEN::HIS</i>
S4B	VDY5869	<i>BY4741 PEX11-mCHERRY::HPH atg36Δ::ATG36ΔN180-NEONGREEN::HIS</i>
S4B	VDY5870	<i>BY4741 PEX11-mCHERRY::HPH atg36Δ::ATG36ΔC219-NEONGREEN::HIS</i>
S4B	VDY5871	<i>BY4741 PEX11-mCHERRY::HPH atg36Δ::ATG36ΔC180-NEONGREEN::HIS</i>
S4B	VDY5872	<i>BY4741 PEX11-mCHERRY::HPH atg36Δ::ATG36_150-219-NEONGREEN::HIS</i>
S4C, D	VDY5873	<i>BY4741 PEX11-mCHERRY::HPH ATG36-NEONGREEN::HIS Pex3-3xFLAG::NAT his3Δ::LEU::pZ4EV</i>
S4E	VDY5859	<i>BY4741 PEX11-GFP::HPH ATG36-13xMYC::HIS</i>
S4E	VDY5862	<i>BY4741 PEX11-GFP::HPH atg36Δ::URA</i>
S4E	VDY5863	<i>BY4741 PEX11-GFP::HPH atg36Δ::Atg36Δ30-13xMYC::HIS PEX1-3xV5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
S4E	VDY5874	<i>BY4741 PEX11-GFP::HPH atg36Δ::Atg36Δ59-13xMYC::HIS PEX1-3xV5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>
S4E	VDY5875	<i>BY4741 PEX11-GFP::HPH atg36Δ::Atg36Δ89-13xMYC::HIS PEX1-3xV5-AID::KAN leu2Δ::pGPD-OsTIR1::LEU</i>

SUPPLEMENTAL FIGURES

Figure S1. The exportomer inhibits pexophagy in yeast, related to Figure 1.

(A) Cells with indicated genotypes and expressing Pex11-GFP on a low-copy vector were cultured for 20 h in synthetic defined media in the presence of 20 μ M 1NM-PP1, where indicated (+), or vehicle (DMSO), to monitor pexophagy. The corresponding cell extracts were resolved by SDS-PAGE followed by immunoblotting with anti-GFP. HRR25(as), analog-sensitive allele of Hrr25. GFP*, GFP fragments produced upon vacuolar degradation of Pex11-GFP. See Materials and Methods for additional details.

(B) Extracts derived from cells with indicated genotypes were resolved by SDS-PAGE followed by immunoblotting with anti-MYC antibodies.

Figure S2. The exportomer inhibits Atg36 phosphorylation *in vivo*, related to Figure 3.

(A) and (B) Extracts derived from cells with indicated genotypes were resolved by SDS-PAGE followed by immunoblotting with anti-MYC and anti-HXK antibodies. (A) wt, wild-type; *pex19Δ*, genomic deletion of *PEX19*. (B) wt, wild-type allele of *PEX6*; Δ , genomic deletion of *PEX6*; WB, *PEX6* D2 Walker B motif mutant allele (E832A) at the endogenous *PEX6* locus.

(C) Extracts derived from cells with indicated genotypes that were treated with 1 μ M rapamycin (Rap) or vehicle (90% ethanol, 10% Tween-20) for 3 h were resolved by SDS-PAGE followed by immunoblotting with anti-MYC, anti-FLAG, anti-HA, and anti-HXK antibodies. WB, *PEX6* D2 Walker B motif mutant allele (E832A); HXK, hexokinase; Rap. rapamycin.

Figure S3. The exportomer inhibits Atg36 phosphorylation *in vitro*, related to Figure 3.

(A) Purified proteins used in *in vitro* kinase assay were resolved by SDS-PAGE followed by Coomassie blue staining. K38A, kinase-dead mutant of Hrr25. WB, Pex1/6 bearing D2 Walker B motif mutant (E832A) in Pex6. D2, Cdc48 D2 ring mutant (E588Q).

(B) Purified Atg36 was incubated with ^{32}P -labeled ATP and Hrr25 or Hrr25^{K38A} *in vitro*, for indicated incubation time. Samples were resolved by SDS-PAGE followed by autoradiography.

830 (C) Purified Atg36 was incubated with ³²P-labeled ATP, Hrr25 and Pex1/6 for 15 min. Hrr25
831 only sample was used as the control. Samples were resolved by SDS-PAGE followed by
832 autoradiography.

833 (D) ATPase activities of AAA-ATPases used in Figure 3F were monitored by measuring the
834 absorbance at NADH 340 nm, using an assay in which regeneration of hydrolyzed ATP was
835 coupled to the oxidation of NADH (see Materials and Methods for details).

836 (E) Purified Atg19 was incubated with ³²P-labeled ATP Hrr25, and indicated Pex1/6 complexes.
837 WB, Pex6 D2 Walker B motif mutant (E832A). Samples at each time point were resolved by
838 SDS-PAGE followed by autoradiography. Data points represent the mean values determined
839 from three repeat experiments. Error bars indicate standard errors.

840

Figure S4. The exportomer inhibits pexophagy by binding to Atg36, related to Figure 4.

(A) Disordered regions in Atg36 predicted by PSIPRED 4.0 (Jones and Cozzetto, 2015).

(B) Cells expressing Pex11-mCherry and NeonGreen-tagged Atg36 mutants were imaged by confocal microscopy (594 nm channel for mCherry and 488 nm channel for NeonGreen). Cells expressing $\Delta N149$ and $\Delta C219$ Atg36 mutants exhibit increased numbers of Pex11-mCherry puncta, suggesting the N- and C-terminal domains of Atg36 may be involved in the regulation of peroxisome size, clustering, or abundance.

849 (C) Cells expressing Pex11-mCherry and Atg36-NeonGreen under control of their endogenous
850 promoters, and a second copy of Atg36-FLAG expressed on a CEN plasmid (full-length or
851 peroxisome localization domain, amino acid 150-219 of Atg36) under control of the pZEV
852 promoter were imaged by confocal microscopy. Where indicated, cells were treated with 1 μ M
853 β -estradiol (b-est) for 3 hours before imaging.

854 (D) Extracts derived from cells imaged in (C) with indicated genotypes were resolved by SDS-
855 PAGE followed by immunoblotting with anti-FLAG and anti-HXK antibodies. HXK,
856 hexokinase.

857 (E) Cells expressing Pex11-GFP were grown in oleate medium for 22 hours and then transferred
858 to nitrogen starvation medium. Extracts derived from cells with indicated genotypes were
859 resolved by SDS-PAGE followed by immunoblotting with anti-MYC, anti-HXK, and anti-GFP
860 antibodies. GFP*, GFP fragments produced upon vacuolar degradation of Pex11-GFP. HXK,
861 hexokinase.