A pex1 Missense Mutation Improves Peroxisome Function in a Subset of Arabidopsis pex6 Mutants Without Restoring pex5 Recycling

Bethany Zolman
University of Missouri-St. Louis, zolmanb@umsl.edu

Kim Gonzalez

Sarah Ratzel

Kendall Burks

Charles Danan

See next page for additional authors

Follow this and additional works at: https://irl.umsl.edu/biology-faculty

Part of the Biology Commons

Recommended Citation

Zolman, Bethany; Gonzalez, Kim; Ratzel, Sarah; Burks, Kendall; Danan, Charles; Wages, Jeanne; and Bartel, Bonnie, "A pex1 Missense Mutation Improves Peroxisome Function in a Subset of Arabidopsis pex6 Mutants Without Restoring pex5 Recycling" (2018). Biology Department Faculty Works. 62. DOI: https://doi.org/10.1073/pnas.1721279115
Available at: https://irl.umsl.edu/biology-faculty/62

This Article is brought to you for free and open access by the Biology at IRL @ UMSL. It has been accepted for inclusion in Biology Department Faculty Works by an authorized administrator of IRL @ UMSL. For more information, please contact marvinh@umsl.edu.
A pex1 missense mutation improves peroxisome function in a subset of Arabidopsis pex6 mutants without restoring PEX5 recycling

Kim L. Gonzalez,a,1, Sarah E. Ratzeb, Kendall H. Burks,a,2, Charles H. Danan,a,3, Jeanne M. Wages,a,4, Bethany K. Zolman,a,b, and Bonnie Bartela,b,5

aDepartment of Biosciences, Rice University, Houston, TX 77005; and bDepartment of Biology, University of Missouri–St. Louis, St. Louis, MO 63121

Contributed by Bonnie Bartel, February 20, 2018 (sent for review December 11, 2017; reviewed by Alison Baker and Jianping Hu)

Plants and animals can store fixed carbon as triacylglycerol (TAG), which can then be mobilized when energy is required. Arabidopsis seedling germination and early growth are fueled by breakdown of TAG stored in oil bodies via fatty acid β-oxidation in peroxisomes, single membrane-bounded organelles. During germination, fatty acids hydrolyzed from TAG are activated with CoA before import through the peroxisomal ABC transporter Pxa1 (1). In the peroxisome, fatty acids undergo β-oxidation to acetyl-CoA, which ultimately can be converted to acetyl-CoA (2). Peroxisomal enzymes are posttranslationally imported into the peroxisome matrix. Peroxisomal enzymes catalyze fatty acid β-oxidation, plant peroxisomes host various other enzymes from membranes to maintain organellar function. The PEX1–PEX6 heterohexameric ATPases are thought to retrotranslocate PEX5 from the peroxisomal membrane, and PEX1–PEX6 dysfunction impairs peroxisome biogenesis in humans and plants. We implemented a pex6 suppressor screen in Arabidopsis and recovered a compensatory pex1 allele that rescues several pex6 defects. Preventing autophagy also improved pex6 peroxisome function, and combining the pex1 and autophagy lesions delivered synergistic benefits. Surprisingly, these different alterations ameliorated pex6 symptoms without notably restoring the sole known function of PEX6, suggesting that PEX1–PEX6 has unexplored functions. Because the pex6 mutations ameliorated by pex1 are analogous to those in human pex6 patients, this study informs research on peroxisome dysfunction in other eukaryotes.

Significance

ATPases have diverse cellular roles, including extracting proteins from membranes to maintain organellar function. The PEX1–PEX6 heterohexameric ATPases are thought to retrotranslocate PEX5 from the peroxisomal membrane, and PEX1–PEX6 dysfunction impairs peroxisome biogenesis in humans and plants. We implemented a pex6 suppressor screen in Arabidopsis and recovered a compensatory pex1 allele that rescues several pex6 defects. Preventing autophagy also improved pex6 peroxisome function, and combining the pex1 and autophagy lesions delivered synergistic benefits. Surprisingly, these different alterations ameliorated pex6 symptoms without notably restoring the sole known function of PEX6, suggesting that PEX1–PEX6 has unexplored functions. Because the pex6 mutations ameliorated by pex1 are analogous to those in human pex6 patients, this study informs research on peroxisome dysfunction in other eukaryotes.


Reviewers: A.B., University of Leeds; and J.H., Michigan State University.

The authors declare no conflict of interest.

Published under the PNAS license.

1Present address: Biology Department, Stanford University, Stanford, CA 94305.
2Present address: Washington University School of Medicine in St. Louis, St. Louis, MO 63130.
3Present address: Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104.
4Present address: Biotechnology Program, Bluegrass Community and Technical College, Lexington, KY 40506.
5To whom correspondence should be addressed. Email: bartel@rice.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1721279115/-/DCSupplemental.

Published online March 19, 2018.
but the PEX6 AAA1 domain lacks a canonical Walker B motif and is unable to hydrolyze ATP (reviewed in ref. 21). *Saccharomyces cerevisiae* PEX1 and PEX6 form a heterohexamer (22 of alternating subunits (23–25). Biochemical experiments (26) indicate that PEX1–PEX6 can thread client proteins through a central pore revealed in structural studies (23–25, 27). Intriguingly, PEX15 is not only a tether (15), but PEX15 lacking its C-terminal transmembrane domain is an in vitro client of the ATPase (26). Human PEX1 also forms a PEX5-interacting homotrimer in the cytosol (28) that may modulate cytosolic PEX5 homooligomerization (29). Moreover, overexpressing PEX6 in yeast suppresses the import defects of a mitochondrial ATPase missense mutation (30), suggesting that PEX6 may also function beyond peroxisomes.

In humans, the generally fatal peroxisomal biogenesis disorders (PBDs) are caused by mutations in various PEX genes, most often *PEX1* and *PEX6* (reviewed in ref. 31). *Arabidopsis* PEX1 and PEX6 are 28% and 33% identical at the amino acid level with human PEX1 and PEX6, respectively, and appear to function similarly to their human counterparts (32–34). For example, expressing a human PEX6 cDNA rescues an *Arabidopsis* pex6 mutant (35), and like human PBD patients with dysfunctional PEX6 (36), several *Arabidopsis* pex6 mutants have low PEX5 levels (35, 37). Moreover, three of the six reported *Arabidopsis* pex1 (38) or pex26 (37) missense alleles are equivalent to causal mutations in patients with PBD (www.dbpex.org/home.php) (39–41).

The peroxisomal ATPase complex is also vital for plants. *Arabidopsis* PEX1–I (42) and PEX6–X6 (17, 43) null alleles confer embryo lethality, and the pex1–I missense allele is lethal when homozygous (38), while pex1–I and pex1–2 alter conserved AAA2 residues, and PEX1/pex6–1 and pex7–2 plants display β-oxidation and matrix protein import defects (38). Moreover, pex1–I has decreased PEX1 and PEX6 levels, suggesting that this allele confers instability to the complex. The pex6–1, pex6–3, and pex6–4 missense mutations also lie in or near the AAA2 domain (Fig. 1B and Fig. S1) and result in small pale green plants with β-oxidation defects and low PEX5 levels (35, 37). In contrast, the mild pex6–2 allele lies N-terminal to the AAA1 domain (Fig. 1B), lacks notable β-oxidation defects, complements pex6–1 (44), and displays elevated PEX5 levels (37). The two viable pex6 mutants, pex6–1 (37) and aberrant peroxisome morphology9 (apem9) (17), display distinct phenotypes as well. *apem9* is a mild missense allele that lacks notable β-oxidation defects and has matrix protein import defects only in a subset of tissues (17), whereas the pex6–1 splice-site mutation confers severe β-oxidation defects and clustered peroxisomes (37). Both droplets accumulate in PEX5<sup>−/−</sup> mouse hepatocytes (45), and oil bodies persist during pex6–1, pex6–3, pex6–4, and pex6–1 seedling development after oil bodies have been consumed in WT (37), indicating that the peroxisome retrotranslocation machinery directly or indirectly promotes oil body utilization. Finally, increasing PEX5 levels in pex6–1, pex6–3, pex6–4, and pex6–1 has disparate effects. Excess PEX5 restores growth and oil body utilization in pex6–1 and pex6–3 but not in pex6–4 or pex6–1 (37), hinting that PEX5 recycling is not the sole function of the peroxisomal ATPase.

To learn more about peroxisomal ATPase complex functions, we employed a forward-genetic suppressor screen of *Arabidopsis* pex6–1 and recovered a missense mutation in PEX1 (pex1–1) that restored pex6–1 growth without an exogenous fixed carbon source. Moreover, we found that preventing autophagy also partially ameliorated pex6–1 defects. Rescue of pex6–1 by pex1–1 was more effective than preventing autophagy or reducing PEX13 expression, which also improves pex6–1 peroxisomal function (46). To explore the specificity of pex6–1 restoration, we characterized a suite of pex1–I double mutants, and found that pex1–I improved several physiological and molecular defects in a subset of pex6 mutants while worsening a pex6 mutant. Surprisingly, pex1–I amelioration of pex6 physiological defects was not accompanied by restored PEX5 levels, supporting the hypothesis that PEX1 and PEX6 have roles beyond PEX5 recycling.

**Results**

**The pex1–1 Missense Mutation Improves pex6–1 Growth.** We sought to elucidate functions of the peroxisomal ATPase complex by identifying genes that genetically interact with *PEX6*. Given previous demonstrations that elevating PEX5 or decreasing PEX3 expression ameliorates some physiological defects of the pex1–1 missense allele (35, 46), we screened for pex6–1 suppressors. Like other *Arabidopsis* pex mutants (47–53), *pex6–1* displays impaired growth on medium lacking sucrose (35), providing a facile screen for suppressors with increased growth. We mutagenized *pex6–1* seeds with ethyl methanesulfonate (EMS) and selected dark-grown M<sub>2</sub> seedlings with longer hypocotyls than *pex6–1* on medium lacking sucrose. One suppressor that restored *pex6–1* growth and partially restored IBA responsiveness was retained for further analysis.

After backcrossing to the *pex1–1* parent, we sequenced genomic DNA from the suppressor (Fig. S2) and found a mutation in *PEX1* (Fig. 1A). This G4127-to-A transition in exon 10 causes a glutamic acid to lysine (E748K) change (Fig. 1A) in the region between the two AAA domains (Fig. 1B). We named this allele *pex1–1*. Unlike *pex6–1*, the *pex1–1* single mutant grew similarly to
largely restored the ability of growth. (Recued cauliflower did not similarly suppress all alleles. We compared peroxisome function of with mutants. We found detri-
was WT. Moreover, PEX1 and PEX6 levels were similar in WT levels in the single and double mutants (Fig. 2C), indicating that pex1-1 did not affect stability of the ATPase hexamer components. Suppression was linked to PEX1; growth of suppressor backcross progeny on medium containing IBA or lacking sucrose correlated with pex1-1 inheritance (Fig. 1C). Moreover, pex1-1+/? pex6-1/? seedlings displayed intermediate suppression between homozygous PEX1+/+ pex6-1−/+ and pex1-1−/+ pex6-1−/+ phenotypes (Fig. 1C), indicating semidominance. To determine whether pex1-1 was the causal lesion suppressing pex6-1, we expressed HA-PEX1 from the constitutive 35S cauliflower mosaic virus promoter in pex1-1 pex6-1. We found that dark-grown pex1-1 pex6-1 seedlings expressing HA-PEX1 displayed reduced hypocotyl elongation without sucrose and increased IBA resistance, similar to pex6-1 (Fig. 2A).

Physiological defects of pex mutants are often ascribed to matrix protein import defects (48, 50–53), and the proteolytic processing of PTS2 proteins that occurs after import (54) can be used as an import proxy. We found that the pex1-1 mutant fully processed PTS2 proteins and partially restored PTS2 processing of thiolase and PMDH in pex6-1 (Fig. 2B). Moreover, expressing HA-PEX1 decreased thiolase and PMDH processing in pex1-1 pex6-1 (Fig. 2B). Because expressing HA-PEX1 in pex1-1 pex6-1 counteracted the beneficial effects of pex1-1 (Fig. 2A and B), we concluded that the identified pex1-1 mutation was the causal pex6-1 suppressor.

### pex1-1 Effects Are Specific to the PEX1-PEX6-PEX26 Complex

To determine if the pex1-1 lesion improved peroxisome function in general, we crossed pex1-1 to pxa1-1, which is impaired in the peroxisomal transporter that moves β-oxidation substrates into the peroxisome (55). pex1-1 did not significantly restore pxa1-1 growth on medium without sucrose (Fig. 2D), indicating that pex1-1 did not improve pex6-1 seedling growth by bypassing the requirement for peroxisomal fatty acid β-oxidation.

To examine the specificity of the pex1-1 suppression among pex6 alleles, we compared peroxisome function of pex1-1 pex6-1 to double mutants of pex1-1 with pex6-2 (44), pex6-3 (37), and pex6-4 (37). Intriguingly, pex1-1 did not similarly suppress all pex6 mutants. In addition to improving growth without sucrose, pex1-1 increased sensitivity of pex6-1 dark-grown hypocotyls to IBA (Fig. 3A) and slightly increased pex6-1 hypocotyl sensitivity to the IBA analog 2,4-dichlorophenoxybutyric acid (2,4-DB) (Fig. 3B). Similarly, pex1-1 rescued pex6-2 physiological defects, largely restoring IBA and 2,4-DB responsiveness and the ability to grow without sucrose in the dark (Fig. 3A and B). Although pex1-1 did not significantly improve IBA-induced lateral rooting in pex6-1, pex1-1 largely restored the ability of pex6-3 to respond to IBA in this assay (Fig. 3C). Aside from a partial restoration of growth without sucrose in pex1-1 pex6-4 (Fig. 3A), pex1-1 did not significantly alter hypocotyl IBA (Fig. 3A) or 2,4-DB (Fig. 3B) responsiveness or IBA-responsive lateral rooting (Fig. 3C) in the weak pex6-2 allele or the strong pex6-4 allele.

We also examined the impact of combining pex1-1 with pex26-1 (37), which is defective in the tether that recruits the PEX1-PEX6 hexamer to the peroxisome (17). In contrast to the restorative or neutral effects on pex6 mutants, we found detrimental effects when combining pex1-1 with pex26-1 (Fig. 3). The pex1-1 pex26-1 double mutant scarcely grew in the absence of sucrose.

---

**Fig. 2.** Overexpressing PEX1 in pex1-1 pex6-1 phenocopies pex1-1 defects: pex1-1 does not restore pxa1-1 growth. (A) Expressing HA-PEX1 increases resistance to the inhibitory effects of IBA and hypocotyl elongation dependence on exogenous sucrose of dark-grown pex1-1 pex6-1 seedlings. Two independent pex1-1 pex6-1 35S:HA-PEX1 lines (A and B) are shown. Seedlings were grown as described in the legend to Fig. 1C. Bars indicate mean hypocotyl lengths (n ≥ 13), and error bars indicate SD. For thiolase and PMDH, precursor (p) and mature (m) proteins are indicated. (C) PEX1 and PEX6 levels resemble WT levels in pex1-1. An immunoblot of 8-d-old light-grown seedling extracts was serially probed with antibodies to the indicated proteins. For thiolase and PMDH, precursor (p) and mature (m) proteins are indicated. (C) PEX1 and
(Fig. 3A) and remained fully resistant to IBA (Fig. 3A and C) and 2,4-DB (Fig. 3B), demonstrating that the pex1-1 mutation was not beneficial to all peroxisomal ATPase complex mutants.

Monitoring overall growth of the suite of single and double mutants revealed that pex1-1 resembled WT throughout development (Fig. 4). As previously documented (37), pex6-2 displayed minimal growth defects, whereas pex6-1, pex6-3, pex6-4, and pex26-1 seedlings were small and pale (Fig. 4A and B). We found that pex1-1 generally increased root growth (Fig. 4A and B) and rosette size (Fig. 4B) of pex6 seedlings. Unlike the worsened seedling growth without sucrose (Fig. 3A), pex1-1 did not notably alter pex26-1 growth when sucrose-supplemented (Fig. 4A and B) or following transfer to soil (Fig. 4C). Whereas mature pex6-2, pex6-4, and pex26-1 plants resembled WT with or without pex1-1, the pex1-1 mutation appeared to alleviate the smaller adult plant size of pex6-1 and pex6-3 mutants (Fig. 4C).

To examine the molecular consequences of the pex1-1 lesion, we compared PTS2 processing in seedlings. As with pex6-1, pex1-1 slightly improved PTS2 processing of PMDH in pex6-3 (Fig. 3D). In contrast, pex1-1 did not notably alter PTS2 processing in pex6-2 or pex6-4 (Fig. 3D), and PTS2 processing of thiolase and PMDH in pex26-1 was worsened by pex1-1 (Fig. 3D). Thus, the effects of pex1-1 on PTS2 processing mirrored the suppression seen in β-oxidation phenotypes.

We also monitored levels of several peroxisomal matrix proteins in the double mutants. Catalase levels are elevated in certain peroxisome-defective mutants (56), perhaps because catalase undergoes less oxidative damage and degradation when β-oxidation is impaired. Consistent with the changes in β-oxidation phenotypes (Fig. 3A–C), pex1-1 lessened catalase accumulation in the pex6-3 mutant and further elevated catalase levels in pex26-1 (Fig. 3D). pex1-1 did not dramatically alter catalase levels in pex6-1, pex6-2,
or pex6-4 (Fig. 3D), adding to the evidence that pex1-1 does not uniformly restore all pex6 mutants’ defects.

The glyoxylate cycle enzyme isocitrate lyase (ICL) is degraded during early seedling development (57) as the glyoxylate cycle becomes obsolete, and pex6 mutants degrade ICL more slowly than WT (44, 57). We found that changes in ICL levels in 5-d-old seedlings mirrored catalase changes; pex1-1 decreased ICL levels in pex6-3 and further increased ICL levels in pex26-1 but did not decrease ICL levels in pex6-1, pex6-2, or pex6-4 (Fig. 3D).

**pex1-1 Improves Oil Body Utilization in pex6-1 and pex6-3.** Because pex6 and pex26 mutants inefficiently utilize cotyledon oil bodies (37), we examined oil bodies by staining neutral lipids with Nile red. Confocal microscopy revealed persisting oil bodies in 5-d-old pex1-1, pex6-3, pex6-4, and pex26-1 pavement and hypocotyl cells that were not observed in WT or pex1-1 (Fig. 5). pex1-1 alleviated oil body persistence in pex6-1 and pex6-3 and partially reduced oil body persistence in pex6-4, but failed to alleviate oil body persistence in pex26-1 (Fig. 5). The improvement of oil body utilization conferred by pex1-1 mirrored and presumably explains the improved growth of these lines without supplemental sucrose (Fig. 3A).

**pex1-1 Slightly Improves GFP-PTS1 Import in pex6-1 and pex6-3.** The minor improvement of PTS2 processing observed when pex1-1 was combined with pex6-1 or pex6-3 (Fig. 3D) suggested that pex1-1 might be improving matrix protein import. To directly assess import, we examined the localization of GFP-PTS1 in the pex1-1 double mutants by using confocal microscopy. WT, pex1-1, pex6-2, and pex1-1 pex6-2 displayed similar GFP-PTS1 puncta, and GFP-PTS1 remained largely cytosolic in the pex1-1 pex6-4 double mutant (Fig. 5). In contrast, the predominantly cytosolic GFP-PTS1 in pex6-1 and pex6-3 mutants was slightly improved by pex1-1, as evidenced by the appearance of some GFP-PTS1 puncta in the pex1-1 pex6-1 and pex1-1 pex6-3 double mutants (Fig. 5).

**pex1-1 Does Not Restore PEX5 Levels in pex6 Mutants.** Most Arabidopsis pex6 mutants (35, 37) and human pex6 patients (36) have low PEX5 levels, presumably because PEX5 is polyubiquitinated and degraded in the absence of efficient recycling. In contrast, immunoblotting revealed that PEX5 levels were not decreased in pex1-1 (Figs. 2B and 3D). Surprisingly, PEX5 levels remained low in seedlings when pex1-1 was combined with pex6-1, pex6-3, pex6-4, or pex26-1 (Fig. 3D). Moreover, PEX5 levels remained elevated when pex1-1 was combined with pex6-2 (Fig. 3D). As expected, PEX5 levels remained low in pex1-1 pex6-1 35S:HA-PEX1 (Fig. 2B).

Because pex1-1 did not restore PEX5 levels in pex6 mutants, we interrogated PEX5 localization by using centrifugation to separate cytosolic and membrane-associated proteins. PEX5 is excessively membrane associated in pex6-1 (46) and pex6-3 (37). We found that PEX5 distribution between the supernatant and pellet in pex1-1 resembled WT and that pex1-1 did not dramatically alter the excessive association of PEX5 with the membrane fraction in pex6-1 (Fig. 6) or pex6-3 (Fig. S3).

We also examined PEX1 and PEX6 localization in these fractionation experiments. Like PEX5, PEX1 and PEX6 were approximately evenly distributed between the supernatant and pellet fractions in WT (Fig. 6 and Fig. S3). This distribution did not markedly change in the pex1-1 or pex6 single or double mutants (Fig. 6 and Fig. S3), suggesting that the pex6-1 and pex6-3 mutations do not impair peroxisome association of the pex6 protein and that altering pex6 localization is not the basis of pex1-1 suppression.

**Overexpressing PEX5 in pex1-1 pex6-1 Further Rescues pex6-1 Deficiencies.** Because PEX5 levels remained low in pex1-1 pex6-1 (Fig. 3D), we tested whether overexpressing PEX5 could further restore peroxisome function. We found no discernible effect of excess PEX5 on pex1-1 or WT growth, IBA sensitivity, or PTS2 processing (Fig. 7). As previously reported (35, 44), excess PEX5 improved pex6-1 growth without sucrose (Fig. 7A) and partially restored PTS2 processing (Fig. 7C). We found that overexpressing PEX5 in pex1-1 pex6-1 further improved growth without sucrose (Fig. 7A) and IBA responsiveness (Fig. 7A and B), although a slight defect in PTS2 processing remained (Fig. 7C). This additive pex6-1 suppression by pex1-1 and 35S:PEX5 suggests that pex1-1 pex6-1 remains limited by decreased PEX5 recycling.

**Preventing Autophagy Also Restores Peroxisome Function in pex6-1.** To illuminate the mechanism of pex1-1 suppression, we compared the suppression effects of pex1-1 vs. a previously characterized pex6 suppressor, pex13-1, a partial loss-of-function allele that may ameliorate pex6-1 defects by decreasing PEX5 docking at the
was more effective than and and pex1-1 (Fig. 8A). loss-of-function mutants atg7-3 improved C pex1- and heterozygote. Indeed, the semidominant suppression (Fig. 1) and pex6-1 (Fig. 8) suppressors and characterized the pex26-1 mutation, suggesting that PEX1 and did not similarly rescue all and pex6 pex6-3 IBAGenesis (Fig. 3) pex1-1 B improved PTS2 processing in pex6-1 atg7-3 pex6-1. Like WT, 5-d-old Arabidopsis pex1-3 missense allele that emerged from this screen (Fig. 1). B oil body utilization (Fig. 5) and growth in the absence of (Fig. 8) to slightly improve (35, 37, 44), and www.pnas.org/cgi/doi/10.1073/pnas.1721279115 pex1-1 in restoring growth without su suppressors, and characterized the pex6-1 allele that emerged from this screen (Fig. 1). B oil body utilization (Fig. 5) and growth in the absence of (Fig. 8) to slightly improve (35, 37, 44), and www.pnas.org/cgi/doi/10.1073/pnas.1721279115

**Discussion**

The peroxins in the ATPase complex (PEX1, PEX6, and PEX26) are implicated in removing PEX5 from the peroxisomal membrane after cargo delivery (14), allowing recycled PEX5 to promote another round of matrix protein import. Analyses of Arabidopsis pex1 (38), pex6 (35, 37, 44), and pex26 (17, 37) loss-of-function mutants have begun to elucidate the roles of the ATPase complex peroxins in PEX5 management (reviewed in ref. 3). For example, four Arabidopsis pex6 missense mutations confer disparate phenotypes that suggest impairment of different aspects of PEX6 function (35, 37, 44). In an effort to expand our understanding of PEX6, we conducted a screen for pex6-1 suppressors and characterized the pex1-1 missense allele that emerged from this screen (Fig. 1).

Because PEX1 and PEX6 are present in a 1:1 ratio in a heterohexamer, the pex1-1 semidominant suppression (Fig. 1C) likely reflects the multiple possible combinations of pex1-1 and PEX1 with pex6-1. For example, only 12.5% of heterohexamers would be expected to carry three WT PEX1 subunits in a PEX1/ pex1-1 heterozygote. Indeed, the Arabidopsis pex1-3 missense allele confers peroxisome-related defects when heterozygous and lethal when homozygous (38), highlighting the sensitivity of this hexamer to perturbation even when some WT PEX1 remains.

**pex1-1 Ameliorates Defects in a Subset of pex6 Alleles Without Restoring PEX5 Levels.** pex1-1 did not similarly rescue all pex6 mutants (Table S1). pex1-1 had little or no effect on pex6-2 physiological and molecular defects (Fig. 3), pex1-1 slightly improved pex6-4 oil body utilization (Fig. 5) and growth in the absence of sucrose (Fig. 3A) without notably improving IBA responsiveness or PTS2 processing (Fig. 3A, C, and D). In contrast, pex1-1 improved pex6-1 and pex6-3 IBA responsiveness (Fig. 3A and C), growth on medium lacking sucrose (Fig. 3A), PTS2 processing (Fig. 3D), GFP-PTS1 import (Fig. 5), and oil body utilization (Fig. 5). Interestingly, pex1-1 improved pex6-3 more fully than the pex6-1 allele used in our

---

**Fig. 5.** pex1-1 slightly improves pex6-1 and pex6-3 GFP-PTS1 import and improves oil body utilization in pex6 mutants but does not improve oil body utilization in pex26-1. Confocal images of cotyledon epidermal cells (A) and hypocotyl cells (B) of 5-d-old seedlings carrying 35S:GFP-PTS1 (green) stained with Nile red (magenta). Emissions were collected at 490–519 nm for GFP and 587–643 nm for Nile red. Seedlings were screened for germination on day 2 after plating, and individuals that had germinated by day 2 were imaged on day 5. (Scale bar: 20 μm.)
We initially expected that pex1-1 impairment of pex6 physiology and matrix protein import might be caused by improved PEX5 degradation. Consistent with this notion, the pex1-1 mutants would be useful to test this hypothesis. Adding to the evidence of PEX1 rescue specificity is the observation that the other two reported PEX1 missense alleles, the mild pex1-2 allele and the more severe pex1-3 allele, confer lethality in combination with pex6-1 (38), suggesting that simply decreasing PEX1 function does not improve activity of complexes carrying mutated PEX6. Moreover, pex1-1 worsened rather than improved pex26-1 growth defects (Fig. S3A), PTS2 processing (Fig. 3D), catalase and ICL levels (Fig. 3D), and cytosolic, and mitochondrial (mito) ATP synthase subunit α and PEX14 localization in the organelle fraction. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

Fig. 6. PEX5 remains excessively membrane-associated in pex1-1 pex6-1. Homogenates (H) prepared from 6-d-old dark-grown WT, pex1-1, pex6-1, and pex1-1 pex6-1 seedlings were separated by centrifugation to isolate cytosolic supernatant (S) and an organelar pellet, which was resuspended and recentrifuged to provide a final organelar pellet (P) fraction. Fractions were subjected to immunoblotting with the indicated antibodies. HSC70 is cytosolic, and mitochondrial (mito) ATP synthase subunit α and PEX14 localize in the organelle fraction. The positions of molecular mass markers (in kilodaltons) are indicated on the left.

Fig. 7. pex1-1 and overexpressing PEX5 additively restore pex6-1 defects. (A and B) Overexpressing PEX5 improves pex1-1 pex6-1 dark-grown (A) and light-grown (B) physiology. Plant lines with (+) and without (−) 2SSS:PEX5 were grown on the indicated media for 1 d under yellow-filtered light before moving to darkness for 5 d (A) or were grown for 8 d under continuous yellow-filtered light (B). Bars indicate mean hypocotyl lengths (A; n ≥ 14) or root lengths (B; n ≥ 13), and error bars indicate SD. Means not sharing a letter above the bar are significantly different as determined by one-way ANOVA (P < 0.001). (C) Overexpressing PEX5 improves PTS2 processing in pex1-1 pex6-1 seedlings. An immunoblot of 8-d-old light-grown seedling extracts was serially probed with antibodies to the indicated proteins. Pre-cursor (p) and mature (m) PMDH are indicated. HSC70 is a loading control.

Diverse pex6-1 Suppression Mechanisms Suggest Complexity of PEX6 Functions. Decreasing PEX13 expression via the pex13-1 allele partially suppresses pex6-1 physiological defects without notably improving PTS2 processing or PEX5 levels (Fig. 8A and B) (46). These findings suggest that pex6-1 defects stem not only from reduced matrix protein import but also from excessive PEX5 buildup.

Fig. S1, which is separated by a linker from the AAA2 domain where the lesions in pex6-1, pex3-3, and pex6-4 mutants are found (37). The pex1-1 analogous residue in human p97 (Gly376) is at least 25 Å from the residues corresponding to the pex6-1, pex6-3, and pex6-4 mutations in the neighboring subunit (Fig. S1), indicating that suppression is unlikely to arise from direct interaction of the mutated residues. In contrast, the analogous pex1-1 residue is only ∼7 Å from the ATP bound by the AAA1 domain and ∼11 Å from the linker that connects the AAA1 and AAA2 domains, hinting that pex1-1 might influence AAA1 ATP binding and/or communication between the AAA1 and AAA2 domains (Fig. S1).

Adding to the evidence of pex1-1 rescue specificity is the observation that the other two reported PEX1 missense alleles, the mild pex1-2 allele and the more severe pex1-3 allele, confer lethality in combination with pex6-1 (38), suggesting that simply decreasing PEX1 function does not improve activity of complexes carrying mutated PEX6. Moreover, pex1-1 worsened rather than improved pex26-1 growth defects (Fig. 3A), PTS2 processing (Fig. 3D), catalase and ICL levels (Fig. 3D), and oil body utilization (Fig. 5). This exacerbation suggests that pex1-1 further impedes ATPase function in pex6-1 even though pex1-1 does not notably impair peroxisome function as a single mutant.

Interestingly, the pattern of pex1-1 suppression of pex6 and pex26 alleles mirrored the range of restorative or exacerbative effects of overexpressing PEX5 in these mutants. PEX5 overexpression worsens the peroxisome-related defects of pex26-1 (37), fails to rescue pex6-2 (44), slightly rescues a subset of pex6-4 defects (37), substantially rescues pex6-1 (35, 37), and most fully rescues pex6-3 (37). PEX5 accumulation in the peroxisomal membrane is detrimental to peroxisome function (46, 64), and the parallel suppression patterns conferred by PEX5 overexpression or pex1-1 reinforces the hypothesis that these pex6 and pex26-1 mutations differentially disrupt retrotranslocation of polyubiquitinated PEX5 for proteasomal degradation (37). Analysis of PEX5 ubiquitination states in these mutants would be useful to test this hypothesis.

We initially expected that pex1-1 improvement of pex6 physiology and matrix protein import might be caused by improved PEX5 recycling. The pex6-1, pex6-3, pex6-4, and pex6-2 mutants have low PEX5 levels (35, 37) that can be increased by proteasomal inhibition (37, 52), suggesting that PEX5 is ubiquitinated and degraded when the ATPase complex is dysfunctional. Contrary to our expectations, PEX5 levels remained low in all pex1-1 combinations with pex6 and pex26 mutant except pex6-2 (Fig. 3D), which has elevated rather than reduced PEX5 levels (37). Moreover, examination of PEX5 membrane association to determine whether pex1-1 might improve removal of PEX5 from the membrane without decreasing PEX5 degradation revealed that PEX5 remained excessively membrane-associated in pex1-1 pex6-1 and pex1-1 pex6-3 (Fig. 6 and Fig. S3). Although a slight recycling improvement would be difficult to discern in this assay, these results hint that dysregulation of PEX6 functions in addition to PEX5 retrotranslocation may contribute to pex6 phenotypes, as has been suggested in plants (44, 57) and yeast (65).

These hypothetical ubiquitinated PEX1–PEX6 clients might be generated by the PEX2–PEX10–PEX12 complex (66) or other peroxisome-associated ubiquitin–protein ligases (67).

Fig. 8. PEX5 improves PTS2 processing in pex1-1 pex6-1 seedlings. The positions of molecular mass markers (in kilodaltons) are indicated on the left.
relieves this detrimental accu-
pex1-1
phenotypes when auto-
suffers from heightened pexophagy.
Arabidopsis pex6-1
PEX5
pex6

dysfunction.
import
further
(Figs. 3

knockdown cells (20), and the
PEX6 has
mutant. LON2 is a peroxisomal protease that
pex6-1 atg7-3
and 6). Moreover, increasing PEX5 levels via
PEX6 levels that accompany
pex1-1
(green)
further ameliorated defects remaining in the
IBA responsiveness and growth without sucrose, and

PEX5
PEX1
643 nm for Nile red.
pex1-1
0.001). (atg7-3
and
by preventing pexophagy.
synergistically improve GFP-PTS1 im-
526 nm for GFP
benefits. Seedlings were grown as in the legend to

PEX1
–
null allele also
does not primarily suppress
fewer defects in the
(46); neither defect was relieved

PEX1
6
symptoms can be relieved through
pex13-1
in the membrane and that pex13-1 relieves this detrimental accumu-
lation by decreasing PEX5 peroxisomal docking. Our observa-
tion that pex1-1 slightly improves pex6-1 and pex6-3 PPS2 processing
(Fig. 3D) and matrix protein import (Fig. 5A) implies that pex1-1
does not act by reducing PEX5 docking at the peroxisome.

We found that the autophagy-defective atg7-3-null allele also
partially ameliorated physiological and molecular defects of
pex6-1 (Fig. 8), suggesting that decreasing constitutive pexophagy
is beneficial or that pex6-1 suffers from heightened pexophagy.
Similarly, preventing autophagy lessens the physiological defects
of yeast pex1 and pex6 mutants (65), mammalian PEX1 and
PEX26 knockout cells (20), and the Arabidopsis PEX1/pex1-3
heterozygote (38). This suppression implies that, when the ret-
rotranslocation machinery is disabled, polyubiquitinated PEX5
(or other ATPase clients) marooned on the peroxisome mem-
brane triggers not only proteasomal degradation of PEX5 (37, 52),
but also pexophagy of the entire organelle. Alternatively or in
addition, the elevated PEX1–PEX6 levels that accompany
autophagy prevention (Fig. 8B) could provide additional ATPase
function that slightly improves peroxisome function.

Preventing autophagy also improves peroxisome function in the
Arabidopsis lon2 mutant. LON2 is a peroxisomal protease
that promotes sustained matrix protein import (68) by preventing pre-
mature autophagy of peroxisomes (69). Unlike the apparently
complete suppression of multiple lon2-2 phenotypes when auto-
phagy is precluded (69, 70), preventing autophagy by introducing
atg7-3 only slightly ameliorated pex6-1 (Fig. 8) or PEX1/pex1-3 (38)
defects, confirming that the PEX1–PEX6 hexamer contributes to
peroxisome functions beyond the prevention of pexophagy.

Because PEX5 levels remained low in pex1-1 pex6-1 (Fig. 3D), we
hypothesized that pex1-1 might increase removal and proteasomal
degradation of polyubiquitinated PEX5 in the peroxisome mem-
brane, thus reducing PEX5-triggered pexophagy and increasing
pex1-1 peroxisome function without restoring PEX5 levels. How-
ever, we found that combining pex1-1 with pex6-1 atg7-3 further
improved peroxisome function; matrix protein import resembled
WT in pex1-1 pex6-1 atg7-3 (Fig. 8C) despite low PEX5 levels (Fig.
8B). The observation that pex1-1 further improved pex6-1 import
even in the complete absence of autophagy (Fig. 8C) indicates that
pex1-1 does not primarily suppress pex6-1 by preventing pexophagy.
Because preventing autophagy in pex6-1 was insufficient to restore
import, it follows that pex1-1 partially increases pex6-1 function.
Perhaps preventing autophagy allows more time for the pex1-
1 pex6-1 hexamer to extract PEX5 (for recycling or degradation),
resulting in improved peroxisome import and function.

In addition to decreased PEX5 levels (35), PEX5 is excessively
membrane-associated in pex6-1 (46); neither defect was relieved
by pex1-1 (Figs. 3D and 6). Moreover, increasing PEX5 levels via
transgenics (35) or by combination with a ubiquitination ma-
chinery mutant (71) partially suppresses pex6-1 defects. Over-
expressing PEX5 further ameliorated defects remaining in the
pex1-1 pex6-1 double mutant (Fig. 7). This additive rescue sug-
gests that pex1-1 and PEX5 overexpression may restore different
facets of pex6 dysfunction.

Our observations that pex6 symptoms can be relieved through
multiple avenues (Table S1) without restoring PEX5 recycling,
the sole known function of PEX6, suggest that PEX1–PEX6 has
additional functions. For example, our results suggest that
PEX1–PEX6 removes a pexophagy-promoting signal from the
peroxisome membrane. Moreover, several Arabidopsis pex6
mutants retain seedling oil bodies after they have been fully utilized
in WT seedlings (37). Similarly, mice lacking PEX5 accumulate
lipid droplets in liver cells (45). Regulation of TAG mobilization
is complex; overexpressing PEX5 (37), preventing autophagy
(Fig. 8C), or introducing pex1-1 (Figs. 5 and 8C) each restore oil
body utilization in Arabidopsis pex6-1. Our characterization of

were collected after staining 5-d-old seedlings carrying 35S:GFP-PTS1 (green)
with Nile red (magenta). Emissions were collected at 490–526 nm for GFP
and 586–643 nm for Nile red.

Fig. 8. pex1-1 augments benefits provided to pex6-1 by preventing auto-
phagy without increasing PEX5 levels. (A) pex1-1, atg7-3, and pex13-1 im-
prove pex6-1 IBA responsiveness and growth without sucrose, and pex1-1
further increases atg7-3 benefits. Seedlings were grown as in the legend to
Fig. 1C. Bars indicate mean hypocotyl lengths (n = 16), and error bars indi-
cate SD. Means not sharing a letter above the bar are significantly dif-
ferent as determined by one-way ANOVA (P < 0.001). (B) Preventing
autophagy increases PTS2 processing in pex6-1, and this increase is further
improved by pex1-1. An immunoblot of 8-d-old light-grown seedling ex-
tracts was serially probed with antibodies to the indicated proteins. Pre-
cursor (p) and mature (m) PMDH are indicated. HSC70 is a loading control.
(C) Preventing autophagy and pex1-1 synergistically improve GFP-PTS1 im-
port in pex6-1. Confocal images of cotyledon epidermal and hypocotyl cells

Gonzalez et al.
this pex1 allele that selectively restores a subset of pex6 mutant defects expands our understanding of the peroxisomal ATPase complex and provides tools for the future investigation of the roles of this essential ATPase complex in recycling PEX5, preventing pexophagy, and promoting oil body utilization.

Materials and Methods

Plant Materials and Growth Conditions. The Columbia-0 (Col-0) accession of Arabidopsis thaliana was the WT control, and all mutants were in the Col-0 background. atg-7-3 (SAIL_11_H07; 60, 61), pex1-1 (35), pex2-6 (44, pex3-6 (37), pex4-6 (37), pex5-3 (ALK; 006744; 46), pex13-1 (46), pex2-6 (37) and pxa1-1 (55) were previously described. pex-1 and pex-11 pex6-1 mutants were backcrossed at least once before phenotypic assays. Genotype types were determined by using PCR-based markers (Table S2). Seedlings were moved from plates to soil (SunGro MetroMix 366) after 1–2 wk and were grown under constant white light at 22 °C.

We crossed previously described WT 35S:PEX5 and pex1-1 35S:PEX5 expressing a PEKS cDNA (35) to pex1-1 pex6-1 to isolate pex1-1 pex1-1 pex6-1 lines overexpressing PEX5, respectively. Transgenic plants were selected by using Basta resistance and by PCR-amplifying PEX5 using intron-spanning primers (Table S2).

To isolate peroxisome-targeted GFP in pex1-1 and double/triple mutants, we crossed pex1-1 to WT, pex6-2, pex6-3, pex4-6, or pex2-6 containing 35S: GFP-PST5 (35, 37, 44), crossed pex1-1 pex6-1 to pex6-1 35S:GFP-PST5 (35), and crossed pex1-1 pex6-1 35S:GFP-PST5 to atg-7 33S:GFP-PST1. The transgene was tracked by using PCR amplification with primers annealing to the promoter and GFP (Table S2).

Seedlings were grown on plant nutrient (PN) medium (72) solidified with 0.6% (wt/vol) agar and supplemented with 0.5% (wt/vol) sucrose (PN medium) and IBA from an ethanol-dissolved stock solution as indicated. Physiological assays monitoring seedling growth and IBA responsiveness were conducted as described previously (37) and were repeated at least twice with similar results.

Mutant Isolation. pex1-1 seeds were soaked in 0.12% EMS for 16 h in the dark, washed extensively, and allowed to germinate in liquid 1/6x PN medium supplemented with 0.5% sucrose to promote germination. Seedlings were transferred to soil to allow M2 seed set. pex1-1 was isolated by screening pex1-1 M2 seedlings grown on PN for individuals with elongated dark-grown hypocotyls. Putative suppressors were transferred to PNS in the ability to elongate hypocotyls in the dark without sucrose.

Whole-Genome Sequencing. Approximately 2,000 backcrossed seedlings were grown under continuous white light for 14 d on sterile filter paper on PNS. Seedlings from three lines were pooled to reduce the appearance of homozygous noncausal background mutations, and genomic DNA was prepared as described previously (73). DNA was sequenced at the Genome Technology Access Center at Washington University in St. Louis by using an Illumina HiSeq 2000 sequencer and was analyzed as described previously (69, 74) to identify EMS-consistent lesions in predicted coding sequences, introns, and UTRs that were absent in our laboratory version of Col-0. More than 80% of the genome had 10-fold sequence coverage.

DNA Constructs and Plant Transformation. 35S:HA-PEX6 was previously described (37). A PEX1 cDNA in pENTR/SD-DTOPO was transferred into the pEG201 destination vector (75) using LR clonase II (Invitrogen) to give 35S: HA-PEX1, which was transformed into Agrobacterium tumefaciens GV3101 (pMP90) and used to transform pex1-1 pex6-1 and WT using the floral dip method (77). Transformants were selected on PNS plates containing 8–10 μg/mL Basta; homozygous lines were identified by monitoring Basta resistance in the T1 generation.

Immunoblot Analysis. Seedling tissue was prepared for immunoblotting as described previously (37). Primary antibodies were incubated with membranes overnight as follows: rabbit anti-PEX1 (1:200; ref. 38), anti-PEX5 (1:100; ref. 35), anti-PEX6 (1:800–1,000; ref. 46), anti-PEX14 (1:10,000; Agrisera AS08), anti-catalase (1:20,000; ref. 78), anti-ICL (1:1,000; ref. 79), anti-PMDH2 (1:2,000; ref. 80), anti-thiolese (1:2,500–1,500; ref. 57), mouse anti-mitochondrial ATP synthase subunit α (1:2,000; Mitoscience M5507) and anti-HSC70 (1:5000–1:1000; SPA-817; StressGen Biotechnologies), and rat anti-PA (1:100–1:500; clone 3F10; Roche). Membranes were incubated for 3–6 h with HRP-conjugated secondary antibodies (1:5,000) before washing and imaging using WesternBright ECL substrate (Advansta). Membranes were sequentially probed with various antibodies without stripping. Films were scanned by using a flatbed scanner. Immunoblotting experiments were performed at least twice with similar results.

Confocal Fluorescence Microscopy. Seedlings were grown under continuous light on PNS, and germination was scored 2 or 3 d after plating. Cotyledons and hypocotyls from individual seedlings with emerging hypocotyls were imaged. Cotyledons were stained with 5 μg/mL Nile red and imaged on day 5. A Carl Zeiss 710 confocal microscope with a 63x oil-immersion objective and a Meta detector was used to capture fluorescence. A 488-nm Argon laser was used to image 0.8-μm optical sections. Each image is an average of four images with 12-bit depth. Confocal microscopy experiments were repeated at least four times with similar results.

Cell Fractionation. An approximately equal mass (±0.03 g) of seedlings was collected from each genotype. Tissue was processed as described previously (64). Fractionation experiments were repeated twice with similar results.

Statistical Analysis. One-way ANOVA with Duncan’s test was used to assess statistical significance (SPSS Statistics, version 24; IBM). Different letters above bars in the figures denote significant differences (P < 0.001).

Accession Numbers. Sequence data can be found in the Arabidopsis Genome Initiative under the accession numbers At1g03000 (PEX1) and At1g03000 (PEX6). Other proteins aligned in Fig. 18 were human PEX1 (NP_000457), PEX6 (NP_000278.3), and p97 (NP_000957).

ACKNOWLEDGMENTS. We thank Shin Goto and Miki Nishimura for the plasmid pMA-1.0 and Masayoshi Maeshima, Steven Smith, and Richard Trelease antibodies recognizing ICL, PMDH, and catalase, respectively; and Yun-Ting Kao, Kathryn Smith, and Zachary Wright for critical comments on the manuscript. This research was supported by National Institutes of Health (NIH) Grant R01GM079177, National Science Foundation (NSF) Grant MCB-1516966, Robert A. Welch Foundation C-1329, NSF Graduate Research Fellowship DGE-0940902 (to K.L.G.), and an NSF training program EHR-0966303 (to K.L.G.). Genome sequencing at the Genome Technology Access Center at Washington University School of Medicine was supported by NIH Grants P30CA18142 and UL1RR024992. Confocal microscopy performed in this study used equipment obtained through NIH Shared Instrumentation Grant S10RR026399.


