E3 ubiquitin ligase SP1 regulates peroxisome biogenesis in *Arabidopsis*

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Peroxisomes are ubiquitous eukaryotic organelles that play pivotal roles in a suite of metabolic processes and often act coordinately with other organelles, such as chloroplasts and mitochondria. Peroxisomes import proteins to the peroxisome matrix by peroxins (PEX proteins), but how the function of the PEX proteins is regulated is poorly understood. In this study, we identified the *Arabidopsis* RING (really interesting new gene) type E3 ubiquitin ligase SP1 [suppressor of plastid protein import locus 1 (*ppi1*) 1] as a peroxisome membrane protein with a regulatory role in peroxisome protein import. SP1 interacts physically with the two components of the peroxisome protein docking complex PEX13–PEX14 and the (RING)-finger peroxin PEX2. Loss of SP1 function suppresses defects of the *pex14-2* and *pex13-1* mutants, and SP1 is involved in the degradation of PEX13 and possibly PEX14 and all three RING peroxins. An *in vivo* ubiquitination assay showed that SP1 has the ability to promote PEX13 ubiquitination. Our study has revealed that, in addition to its previously reported function in chloroplast biogenesis, SP1 plays a role in peroxisome biogenesis. The same E3 ubiquitin ligase promotes the destabilization of components of two distinct protein-import machineries, indicating that degradation of organelle biogenesis factors by the ubiquitin–proteasome system may constitute an important regulatory mechanism in coordinating the biogenesis of metabolically linked organelles in eukaryotes.

peroxisome biogenesis | E3 ubiquitin ligase | protein import | peroxin | SP1

Peroxisomes are single-membrane organelles that are present in virtually all eukaryotic cells and host critical metabolic reactions including fatty acid β -oxidation and H_2O_2 degradation (1). In plants, peroxisomes are essential to many metabolic processes such as lipid mobilization, the glyoxylate cycle, photorespiration, detoxification, biosynthesis, and metabolism of plant hormones (2, 3). Some of these metabolic processes are coordinated by peroxisomes and other organelles, e.g., mitochondria and chloroplasts for photorespiration, lipid bodies and mitochondria for lipid mobilization, and chloroplasts for jasmonic acid biosynthesis (3–6). The enzymatic composition of plant peroxisomes is dynamic, depending on developmental and environmental cues. For example, in young seedlings of oilseed plants such as *Arabidopsis*, the major enzymatic content of peroxisomes shifts from the glyoxylate cycle enzymes to photorespiratory enzymes within a few days after germination. This process is induced by light, is achieved through proteome remodeling, and occurs simultaneously with chloroplast development (3, 7–10). The turnover of the peroxisome proteome is correlated with the spatial distribution of peroxisomes, because they are found next to lipid bodies during seed germination but become physically associated with chloroplasts when plants reach photoautotrophic growth in the light (11–13). One can speculate that the development of peroxisomes and chloroplasts may be coregulated to accommodate the functional association of these two organelles in photoautotrophic growth.

The peroxisome proteome is encoded entirely in the nucleus. Peroxisome matrix proteins are imported from the cytosol by the evolutionarily conserved peroxins (PEX proteins) (3, 14). In *Arabidopsis*, PEX5 and PEX7 are cytosolic receptors for peroxisome proteins that contain C-terminal PTS1 (peroxisome targeting

signal type 1) and N-terminal PTS2 sequences, respectively (15, 16). In *Arabidopsis*, PEX5 is also required for PTS2 protein import (16). Two membrane proteins, PEX13 and PEX14, form the docking site for PEX5 and PEX7 (17, 18). After receptor docking, cargo proteins translocate into the matrix before receptors are recycled to the cytosol (19–21). These processes require the RING (really interesting new gene)-finger peroxins PEX2, PEX10, and PEX12 (22–25), the ATPases PEX1 and PEX6 and their membrane tether APEM9 (aberrant peroxisome morphology 9) and the ubiquitin-conjugating enzyme PEX4 and its membrane anchor PEX22 (26–29). Studies from yeast reveal that PEX12-mediated PEX5 monoubiquitination precedes PEX5 recycling (30). Although there is no direct evidence so far for PEX5 ubiquitination in plants, the RING domain of *Arabidopsis* PEX2, PEX10, and PEX12 was shown to possess E3 activity *in vitro*; the *in vivo* targets for their activities remain unclear (31). Disruption of the function of plant PEX proteins causes embryonic lethality or compromises peroxisome function such as β -oxidation (3, 32, 33). Moreover, maintaining the balance between cargo translocation into the peroxisome/receptor docking and receptor recycling back to the cytosol appears to be important for the functional integrity of peroxisomes in plants. For example, as is consistent with both PEX13 and PEX14 being involved in receptor docking at the peroxisome membrane, the peroxisomal defects of *pex14-2* are enhanced by *pex13-1*, a weak allele with mildly reduced PEX13 mRNA levels (17, 18, 34). However, the same *pex13-1* allele partially suppressed the peroxisomal phenotypes of the late-acting peroxin mutant *pex4-1*, which is deficient in the translocation of PEX5 out of the peroxisome. This observation led to the conclusion that the inefficiency in both cargo

Significance

Peroxisomes are eukaryotic organelles crucial for development. Peroxisomal matrix proteins are imported by the peroxisome import machinery composed of peroxins (PEX proteins), but how the function of these PEX proteins is regulated is largely unknown. We discovered in *Arabidopsis* that the ubiquitin–proteasome system regulates peroxisome protein import via an E3 ubiquitin ligase, SP1 (suppressor of *ppi1* locus1), which targets PEX13 and possibly several other components of the peroxisome matrix protein import machinery for degradation. Our data demonstrate that the same E3 ubiquitin ligase can be shared by metabolically linked peroxisomes and chloroplasts to promote the destabilization of distinct components of the two import machineries, suggesting that the ubiquitin–proteasome system may represent an important regulatory mechanism coordinating the biogenesis of functionally associated organelles.

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translocation into the peroxisome (*pex13-1*) and PEX5's recycling back to the cytosol (*pex4-1*) restored the balance between the import and export of PEX5 in the *pex13-1 pex4-1* double mutant (34).

How the function of the peroxins is regulated remains poorly understood. We have been investigating the role of the ubiquitin-proteasome system (UPS) in the biogenesis of peroxisomes and mitochondria (25, 31, 35). The UPS is a key regulatory mechanism that controls various cellular pathways in eukaryotic cells, in which polyubiquitinated proteins are degraded by the 26S proteasome (36, 37). Proteins involved in the UPS pathway were estimated to make up ~5–6% of the *Arabidopsis* proteome; the majority (~1,400) of these proteins are, or are predicted to be, E3 ubiquitin ligases (38). The triad cascade of protein ubiquitination consists of the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) (36). E3s associate with both E2s and substrate proteins to promote substrate-specific ubiquitination; therefore the tremendous diversity of E3 ligases in plant genomes is consistent with the key roles of this class of proteins in defining substrate specificity. The UPS is involved in organelle biogenesis and/or morphogenesis in plants. *Arabidopsis* SP1 [suppressor of *ppi1* (plastid protein import locus 1) 1] is a RING-type ubiquitin ligase that binds to and promotes the degradation of a few components of the TOC (translocon at the outer envelope of chloroplasts) complex (39, 40). We also identified an *Arabidopsis* mitochondrial outer membrane-associated ubiquitin-specific protease, UBP27, which is involved in mitochondrial morphogenesis, possibly through division (35).

This study is part of our continuing effort to identify proteins involved in UPS-mediated regulation of organelle biogenesis. We show that SP1, a RING-type E3 ubiquitin ligase known to regulate chloroplast protein import, is also associated with the peroxisome membrane and targets PEX13 and possibly several other components of the peroxisome protein import machinery for degradation. Thus, the same E3 ligase targets distinct components of the peroxisome and chloroplast protein import apparatuses, indicating that UPS-mediated protein degradation may constitute an important regulatory mechanism by which eukaryotic cells coordinate the biogenesis of metabolically linked organelles.

Results

The *Arabidopsis* E3 Ubiquitin Ligase SP1 Is also a Peroxisome Membrane Protein. While searching the *Arabidopsis* genome for putative peroxisome membrane-localized E3 ubiquitin ligases, we identified a small family of proteins comprising SP1, SPL1 (SP1-like 1), and SPL2 as candidates because of the presence of transmembrane domains (TMDs) in addition to the RING domain on these proteins (Fig. 1*A* and Fig. S1*A*). In a recent study, all three members were found to be associated with chloroplasts, and SP1 was shown to regulate chloroplast protein import (40).

To test the possibility that these E3s are also associated with peroxisomes, translational fusions of the coding region of each protein and YFP were generated. *Arabidopsis* transgenic lines expressing the 35S constitutive promoter-driven fusion proteins were generated in a wild-type Col-0 background that already contained the peroxisomal marker CFP-PTS1 (SKL) (25, 41). Confocal microscopic analysis of T2 plants revealed that, in agreement with the previous report (40), SP1, SPL1, and SPL2 are associated with chloroplasts (Fig. S1*B* and *C*). Interestingly, all three seemed to be concentrated on small (potentially developing) chloroplasts (Fig. S1*B* and *C*); this localization may be related to their proven (SP1) or potential (SPL1 and SPL2) roles in chloroplast protein import. Among the three fusion proteins, only SP1 showed clear peroxisomal localization in different cell types, such as epidermal cells (Fig. 1*B*) and mesophyll cells (Fig. S1*B*). In contrast, SPL1 and SPL2 showed very weak and possibly partial (SPL1) or no (SPL2) peroxisomal localization (Fig. S1*D*). To confirm SP1's localization to peroxisomes, we expressed SP1-YFP by *SP1*'s native promoter (*SP1pro*:SP1-YFP) in leaves of

Fig. 1. Localization of the SP1 protein. (A) Domain structure of the SP1 protein. TM, transmembrane domain. RING, RING domain. C-term, C terminus. Amino acid numbers are indicated. T-DNA insertion sites of in the two *sp1* mutants are also indicated. (B) Peroxisome localization of SP1-YFP. Confocal images were taken in leaf epidermal cells of 2-wk-old *Arabidopsis* T2 plants coexpressing SP1-YFP and CFP-PTS1. (Scale bar, 10 μ m.) (C) Assessment of the purity of peroxisomes isolated from the leaf tissue of transgenic *Arabidopsis* plants coexpressing SP1-YFP and CFP-PTS1. Organelle-specific antibodies used were against *Arabidopsis* VDAC (Voltage-dependent anion channel) (mitochondrial), FtsZ1 (Filamenting temperature-sensitive mutant Z) (chloroplast), and PEX11d (peroxisomal) proteins. CE, crude extract. (D) Peroxisomal membrane association of SP1-YFP. Purified peroxisomes were treated with TE, NaCl, or Na_2CO_3 (pH 11.0) and were separated into soluble (S) and pellet (P) fractions by centrifugation. SP1-YFP and CFP-PTS1 were detected by α -GFP. CFP-PTS1 and PEX11d are controls for matrix and integral membrane proteins, respectively. The matrix protein CFP-PTS1 is often detected in the pellet as well as in the soluble fraction, as has been observed in previous studies.

Arabidopsis seedlings by the Fast Agro-mediated Seedling Transformation (FAST) method (42) and observed the same pattern of localization to peroxisomes and chloroplasts (Fig. S1*E* and *F*). Based on these observations, we decided to focus on SP1 to investigate its possible role in peroxisomes.

To assess SP1's peroxisome localization further and to determine the membrane association of SP1 on peroxisomes, we isolated peroxisomes from the leaf tissue of *Arabidopsis* plants stably expressing SP1-YFP and CFP-PTS1. The purity of the isolated peroxisomes was assessed by immunoblot analysis using organelle-specific protein antibodies (Fig. 1*C*). To separate integral membrane proteins from total proteins, the isolated organelles were treated with Tris-EDTA (TE), a high-concentration salt solution (1 M NaCl), and alkaline carbonate (Na_2CO_3 , pH 11.0), respectively. SP1-YFP, along with the peroxisomal membrane protein PEX11d, was detected only in the pellet after each treatment (Fig. 1*D*), suggesting that SP1 is an integral membrane protein of the peroxisome.

SP1 Represses Peroxisome Function. To analyze the function of SP1 in peroxisomes, we obtained from the *Arabidopsis* Biological Resource Center two transferred DNA (T-DNA) insertion mutants that previously had been designated *sp1-2* and *sp1-3* (Fig. 2A) (40). Homozygous mutants were obtained after PCR genotyping of genomic DNA, and *SP1* transcript levels were analyzed using RT-PCR, which showed the absence of full-length *SP1* transcripts in both mutant alleles (Fig. S2A and B). As previously reported (40), the growth and development of these mutants were similar to that in wild-type plants under normal growth conditions (Fig. S2C).

To assess peroxisome functions in the mutants, we used the 2,4-DB (2,4-dichlorophenoxybutyric acid) response assay, which is often used to identify mutants in β -oxidation enzymes or peroxisome biogenesis. 2,4-DB undergoes β -oxidation in peroxisomes to form the active auxin-like derivative 2,4-D, resulting in the inhibition of primary root and hypocotyl elongation when applied to seedlings (32). Both *sp1* mutant alleles were more sensitive than wild type to 2,4-DB (Fig. 2B and Fig. S2D). We also generated transgenic lines stably expressing SP1 under the 35S promoter (Fig. S2E), which were more resistant to 2,4-DB's inhibitory effect on hypocotyl and root elongation (Fig. 2B and Fig. S2D). Hence, peroxisomal β -oxidation activities appeared to be elevated in *sp1* mutants and deficient when *SP1* was overexpressed, suggesting that SP1 plays a repressive role in peroxisomal function, given that peroxisomes are the sole sites of β -oxidation in plants.

Physical and Genetic Interaction Between SP1 and Members of the Peroxisome Protein Import Machinery. To determine whether SP1 affects peroxisome morphology, we introduced the peroxisome marker YFP-PTS1 into *sp1* mutants through transformation. Confocal microscopic analysis of the mutants expressing YFP-PTS1 did not reveal obvious abnormalities in peroxisome morphology (Fig. S2F), suggesting that SP1 does not play a major role in peroxisome morphogenesis. Because SP1 was shown to regulate chloroplast biogenesis by targeting components of the TOC protein import complex for degradation (40), we tested the idea that SP1 may target the peroxisomal protein import machinery. First, we performed coimmunoprecipitation (co-IP) assays using FLAG-tagged SP1 and several YFP-tagged PEX proteins. Similar to the chloroplast TOC33 positive control, PEX13, PEX14, and PEX2 clearly coprecipitated with SP1-FLAG (Fig. 2C). Because of protein instability, we were unable to test the other two RING peroxins, PEX10 and PEX12, in this assay. Based on the co-IP results, we considered PEX13, PEX14, and possibly the RING peroxins as potential substrates for SP1.

Next we examined genetic interactions between SP1 and the PEX proteins. We crossed *sp1* with *pex14-2*, a null allele that carries a T-DNA insertion 41 bp downstream of PEX14's start codon (17). The *sp1 pex14-2* double mutants exhibited marked rescue of the small stature of *pex14-2* (Fig. 3A), although the chlorophyll content was not significantly different from that of *pex14-2* (Fig. 3B). Because peroxisomes are the sole site of fatty acid β -oxidation in plants, mutants defective in β -oxidation, such as *pex14-2*, are often dependent upon exogenous fixed carbon sources, such as sucrose, for seedling establishment (43). On medium without sucrose, *pex14-2 sp1* seedlings overall had longer roots and hypocotyls than *pex14-2* seedlings (Fig. 3C and D and Fig. S3A and B). Although all *pex14-2* seedlings died at 10 d after germination because of their dependence on sucrose, 70–80% of the double mutant seedlings continued to grow (Fig. 3E and F). Compared with *pex14-2* seedlings, the *pex14-2 sp1* seedlings were less resistant to 2,4-DB's inhibitory effect on root and hypocotyl elongation (Fig. 3G and Fig. S3C). Together these data demonstrated that the mutant phenotype of *pex14-2* was significantly rescued by the *sp1* mutation.

The strong suppression of the *pex14* phenotype by *sp1* prompted us to check the effect of *sp1* mutation on peroxisomal matrix protein import in the *pex14-2* background. We crossed *pex14-2*

Fig. 2. SP1 affects peroxisome function and interacts with several peroxins. (A) *SP1* gene structure. The open box represents the UTR; the black boxes represent exons; the solid line represents the intron. T-DNA insertion sites in the two *sp1* mutants are indicated. Arrows indicate the primers used to amplify full-length cDNA. (B) 2,4-DB response assays. Seedlings were grown on Murashige and Skoog (MS) medium supplemented with 0.5% sucrose and various concentrations of 2,4-DB. The average hypocotyl length of 7-d-old dark-grown seedlings is shown. All data on 2,4-DB are normalized to the data obtained from plants grown on 0.5% sucrose medium with no 2,4-DB. Data are shown as mean $\pm$ SEM; $n = 3$ (>30 seedlings were used for each biological replicate). ** $P < 0.01$; * $P < 0.05$ from wild type. (C) Co-IP of PEX proteins with SP1. YFP-tagged candidate substrate proteins were coexpressed in tobacco leaves with either SP1-FLAG or empty vector (v). The top panel is a representative blot showing the level of SP1-FLAG in co-IP experiments performed for different YFP-tagged proteins.

Fig. 3. The loss of *SP1* function suppresses *pex14-2* mutant phenotypes. (**A**) Four-week-old plants that had been grown for 1 wk in sucrose-containing Linsmaier and Skoog (LS) medium and for 3 wk in soil. (**B**) Leaf chlorophyll contents of the plants shown in **A**. Data are shown as mean $\pm$ SD; $n = 3$ (>30 seedlings were used for each biological replicate). *** $P < 0.001$ from *pex14-2*. (**C**) Ten-day-old seedlings grown on MS medium without or supplemented with 1% sucrose. (**D**) Root length measurement of 10-d-old seedlings grown on MS medium without or supplemented with 1% sucrose. Data are shown as mean $\pm$ SD; $n = 3$ (>30 seedlings were used for each biological replicate). *** $P < 0.001$ from *pex14-2*. (**E**) Ten-day-old seedlings grown on LS medium with no sucrose. (**F**) Quantitative analysis of postgerminative growth of the seedlings shown in **E**. Error bars indicate the SD; $n = 3$ (>100 seedlings for each biological replicate). *** $P < 0.001$ from *pex14-2*. (**G**) Root length measurement of 7-d-old light-grown seedlings grown on MS medium supplemented with 0.5% sucrose and various concentrations of 2,4-DB. The average root length normalized to the data obtained from plants grown on 0.5% sucrose medium without 2,4-DB is shown. Data are shown as mean $\pm$ SEM; $n = 3$ (>30 seedlings were measured for each biological replicate). *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ from *pex14-2*. (**H**) Confocal microscopic images of leaf epidermal cells from 2-wk-old light-grown plants stably expressing 35Spro:YFP-PTS1. Images were acquired with identical microscopic settings. (Scale bar, 20 µm.) (**I**) Immunoblots of total protein extracts from seedlings 3 and 10 days after germination using the anti-thiolase antibody.

plants to transgenic lines stably expressing 35Spro:YFP-PTS1 (44, 45). In contrast to wild-type leaf cells, in which YFP-PTS1 was partially cytosolic in *pex14-2* plants (Fig. 3H), similar to observations in a previous report (17), indicating deficient import of PTS1-containing proteins in the mutant (17). In the *pex14-2 sp1* double mutant, the cytosolic localization of YFP-PTS1 was largely diminished (Fig. 3H), suggesting that PTS1 protein import in

pex14-2 plants was significantly restored as a result of the *sp1* mutation. To assess the impact of *sp1* on peroxisome protein import in *pex14-2* plants further, we also performed immunoblot analysis of a PTS2-containing protein, 3-ketoacyl-CoA thiolase (thiolase), whose precursors are retained for a longer time in mutants defective in PTS2 protein import than in wild-type plants (17). After PTS2-containing proteins are delivered into the matrix, the N-terminal PTS2 peptides are removed by proteases

Fig. 4. Loss of SP1 function enhances *pex4* mutant phenotypes. (A) Ten-day-old seedlings grown on LS medium without sucrose. (B) Quantitative analysis of postgerminative growth of the seedlings in A. Error bars indicate SD; $n = 3$ (>100 seedlings for each biological replicate). $^{**}P < 0.01$; $^{*}P < 0.05$ from *pex4-1*. (C) Ten-day-old seedlings grown on MS medium without or supplemented with 1% sucrose. (D) Average root length of 10-d-old light-grown seedlings. Data are shown as mean $\pm$ SD; $n = 3$ (>30 seedlings for each biological replicate). $^{*}P < 0.05$ from *pex4-1*. (E) 2,4-DB response assays. Seedlings were grown on MS medium supplemented with 0.5% sucrose and

such as DEG15 (46, 47). By evaluating PTS2 protein processing, we could indirectly assess the import of PTS2-containing proteins or the import of PTS1-containing proteases such as DEG15 that are required for PTS2 processing. In 3-d-old seedlings there was a strong accumulation of the thiolase precursor in *pex14-2* plants, but this accumulation was greatly reduced in the *pex14-2 sp1* double mutant (Fig. 3I). In summary, these data provided evidence that the amelioration of the physiological defects of *pex14-2* plants by the *sp1* mutation correlated with improved matrix protein import, further supporting the idea that SP1 plays a negative role in peroxisome protein import.

We also crossed *sp1* with *pex13-1*, a weak allele with mildly reduced *PEX13* mRNA levels (34). The weak sucrose-dependent and 2,4-DB-resistant phenotypes in *pex13-1* plants were both suppressed in the *sp1 pex13-1* double mutant (Fig. S3 D and E). Hence, the peroxisomal β -oxidation deficiencies in *pex14-2* and *pex13-1* plants were at least partially recovered by the lack of SP1 function, as is consistent with the hypothesis that SP1 negatively regulates the function of these two components of the peroxisome protein import machinery. Because the PEX14 protein is absent in *pex14-2* plants, the observed rescue of the mutant phenotype in *sp1 pex14-2* plants is unlikely to be caused by the stabilization or enhancement of the function of PEX14. Instead, the stabilization/enhancement of other proteins, such as PEX13, may compensate for the loss of PEX14.

SP1's Negative Role in Peroxisome Protein Import Is Restricted to Certain Steps of the Process. PEX13 and PEX14 mediate receptor docking and cargo translocation, which are early steps of peroxisome protein import (3, 17, 18, 34). To determine whether SP1 also impacts peroxins that act in other steps of matrix protein import, we generated additional double mutants, namely *sp1 pex5-10* and *sp1 lon2-2*. PEX5 and LON2 (LON protease-like 2) are involved in different phases of peroxisome protein import: PEX5 is the receptor for matrix proteins, and LON2 is a peroxisomal protease involved in sustained protein import in older seedlings (48, 49). The *pex5-10* mutant allele produces a PEX5 protein that has a deletion in the middle region, has a germination defect, and is dependent on exogenous sucrose for early seedling establishment (48). The *lon2-2* mutant has matrix protein import defects in older seedlings and is partially dependent on exogenous sucrose for seedling development (49). Neither *pex5-10 sp1* nor *lon2-2 sp1* mutants exhibited significant differences from *pex5-10* and *lon2-2* mutants in growth (Fig. S4 A and B) or sucrose dependence (Fig. S4 C–F). Thus, the suppression of peroxisome-related physiological defects by *sp1* is restricted to specific peroxisomal biogenesis mutants.

The *sp1* mutant was also crossed with *pex4-1*, a mutant of the E2 ubiquitin-conjugating enzyme involved in the recycling of PEX5 back to the cytosol, a late step in protein import (29). Lack of SP1 function did not significantly alter the growth of the *pex4-1* mutant (Fig. S4 G and H). However, on medium without sucrose, more *pex4-1 sp1* seedlings than *pex4-1* seedlings exhibited inhibited postgerminative growth (Fig. 4 A and B), and, in the seedlings that were established, the double mutants had shorter roots than *pex4-1* seedlings (Fig. 4 C and D) and were more resistant than *pex4-1* seedlings to 2,4-DB's inhibition of hypocotyl and root elongation (Fig. 4 E and Fig. S4I). Thus, *sp1* appeared to enhance the physiological defects in *pex4-1*; this enhancement is the opposite of its effect on *pex14* and *pex13*. Because the removal of PEX5 from the peroxisome is inefficient in *pex4-1* plants, we reasoned that the lack of SP1 function in the

various concentrations of 2,4-DB. Shown is the average hypocotyl length of 7-d-old dark-grown seedlings normalized to that of seedlings grown on 0.5% sucrose medium with no 2,4-DB. Data are shown as mean $\pm$ SEM; $n = 3$ (>30 seedlings for each biological replicate). $^{*}P < 0.05$ from *pex4-1*.

Fig. 5. SP1's function depends on its RING domain. (A) Sequence of SP1's RING domain. The conserved active Cys and His residues are in red. The underlined residues were replaced in SP1m by the residues shown below in green. Amino acid numbers are indicated. (B) Four-week-old plants that had grown for 1 wk on LS medium and 3 wk on soil. (C and D) Sucrose-dependence assays. Seedlings were grown on MS medium supplemented with different concentrations of sucrose. Average root lengths of 10-d-old light-grown seedlings (C) and average hypocotyl length of 10-d-old dark-grown seedlings (D) are shown. Data are shown as mean $\pm$ SD; $n = 3$ (>30 seedlings for each biological replicate). *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ from *pex14-2*. (E) 2,4-DB response assays. Seedlings were grown on MS medium supplemented with 0.5% sucrose with or without 1.6 μ M 2,4-DB. The average root length of 7-d-old light-grown seedlings is shown. Data are shown as mean $\pm$ SD; $n = 3$ (>30 seedlings for each biological replicate).

pex4-1 sp1 double mutant may lead to the stabilization of peroxins such as PEX13 and PEX14, causing more translocation of PEX5 into the peroxisome and thus increasing the imbalance between the translocation of PEX5 into and out of the peroxisome. Based on these findings, we concluded that SP1's repressive role in peroxisome protein import is restricted mainly to the earlier steps.

SP1's Function in Regulating Peroxisome Biogenesis Depends on Its RING Domain. SP1 was shown to possess E3 ubiquitin ligase activities both in vivo and in vitro (40). The RING domain, which confers ubiquitin ligase activity for this E3, contains well-conserved Cys and His residues (Fig. 5A) (39, 40). To determine the importance of the RING domain for SP1's function, we made five

amino acid substitutions (Cys $\rightarrow$ Ser and His $\rightarrow$ Tyr) in the RING domain to disrupt SP1's E3 activity (Fig. 5A). The mutant SP1m and wild-type SP1 proteins, both driven by the 35S constitutive promoter, were stably overexpressed in the *pex14-2* mutant background. Transgenic lines that showed high expression of SP1m or SP1 were selected for further analysis (Fig. S5A). Overexpression of SP1m, but not of SP1, significantly rescued the growth defect (Fig. 5B), sucrose dependence (Fig. 5C and D), and 2,4-DB resistance (Fig. 5E) in *pex14-2* plants, similar to observations in the *pex14-2 sp1* double mutant (Fig. 3). We also noticed that *pex14-2* 35Spro:SP1 plants were generally slightly smaller (Fig. 5C–E) and were even more sucrose dependent (Fig. 5C and D) than *pex14-2* plants, as is consistent with our earlier conclusion that SP1 plays a

Fig. 6. SP1 promotes the destabilization of several peroxins, and this function depends on the RING domain. (A) Immunoblot analysis of protein extracts from tobacco leaves co-overexpressing SP1 or SP1m and a YFP-tagged candidate substrate protein. YFP-tagged proteins and actin were detected by anti-GFP and anti-actin antibodies, respectively. The actin blot is a representative loading control (for the PEX13 blot). (B) Quantification of the levels of the YFP-tagged proteins shown in A. YFP and actin protein bands were quantified using ImageJ. The amount of each candidate protein coexpressed with SP1 was normalized to the data obtained from the sample coexpressed with SP1m. Data are shown as mean $\pm$ SD; $n = 3$. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ from actin. (C) Immunoblot analysis of total leaf protein from 3-wk-old *Arabidopsis* plants. Endogenous PEX13 and PEX14 proteins were detected by α -PEX13 and α -PEX14 antibodies, respectively, and α -actin was used as the loading control. (D) Immunoblot analysis of total protein from 9-d-old *Arabidopsis* seedlings. Lanes 3 and 4 contain samples from two independent lines overexpressing SP1. (E) Co-IP of YFP-tagged PEX13 or PEX14 with FLAG-tagged ubiquitin. Each YFP-tagged PEX protein, FLAG-UBQ10, and SP1 (or SP1m) were co-overexpressed in tobacco leaves.

repressive role in peroxisome function. Thus, overexpression of SP1m can cause a dominant negative effect on SP1, and the RING domain is indeed required for SP1's function in regulating peroxisome biogenesis.

SP1 Targets PEX13 and Possibly Several Other PEX Proteins for Degradation. The findings in co-IP assays that SP1 interacts with PEX13, PEX14, and PEX2 and that *sp1* suppresses the mutant phenotypes of *pex14* and *pex13* led us to propose that these PEX proteins are the potential substrates for SP1. To test this hypothesis, we first used the tobacco (*Nicotiana tabacum*) transient protein expression system to examine whether SP1 has the ability to affect the stability of the interacting PEX proteins. To this end, various YFP-tagged PEX proteins and wild-type SP1 or the dominant negative SP1m protein were coexpressed (Fig. 6A and Fig. S5B). When coexpressed with wild-type SP1, not only PEX13, PEX14, and PEX2 but also PEX10 and PEX12 were destabilized (Fig. 6A and B). In a similar experiment, YFP-tagged SP1 also promoted the destabilization of PEX13 and PEX14 (Fig. S5C and D). These results were consistent with our co-IP data on PEX13, PEX14, and PEX2 (PEX10 and PEX12 could not be tested in co-IP because of protein instability), suggesting that PEX13, PEX14, and the RING peroxins are possible targets of SP1.

We reasoned that if SP1 promotes the destabilization of its targets, we would see an increased level of the target proteins in *sp1* mutant background and a decrease in the target protein level in SP1 overexpressors. To test this prediction, we performed immunoblot analysis of proteins from various genetic backgrounds using the available PEX13 and PEX14 antibodies. As predicted, the endogenous PEX13 protein level was negatively correlated with the level of SP1 expression (Fig. 6C and D and Fig. S5E). However, the endogenous PEX14 protein level was not very sensitive to changes in SP1 dosage; we observed only mild decreases in the level of PEX14 protein in lines overexpressing SP1 (Fig. 6D). In addition, we found that PEX13-FLAG was generally more susceptible to SP1 overexpression than PEX13-YFP in tobacco leaves (Fig. S5F), possibly because the FLAG tag is much smaller than the YFP tag, making PEX13-FLAG more efficient or more accurate in peroxisome targeting than PEX13-YFP. In summary, SP1 can promote the degradation of PEX13 in tobacco and *Arabidopsis*, and it also can promote the degradation of PEX14 and the RING peroxins, at least in the tobacco transient expression system. Although we did not observe more accumulation of endogenous PEX14 in the SP1 loss-of-function background, PEX14 still could be a substrate of SP1. It is possible that in the SP1 loss-of-function mutant background, the PEX14 level may be subjected to feedback regulatory mechanisms, such

Fig. 7. A working model for SP1's role in peroxisome protein import in *Arabidopsis*. PEX5 and PEX7 are cytosolic receptors for peroxisome matrix proteins. The cargo-receptor complexes dock to the docking site formed by two membrane proteins, PEX13 and PEX14. The exact function of the E3 ubiquitin ligases PEX2, PEX10, and PEX12 in *Arabidopsis* is still unclear. The export of PEX5 to the cytosol is dependent on the ubiquitin-conjugating enzyme PEX4, which needs PEX22 as its membrane anchor, and the AAA ATPases PEX6 and PEX1, which are tethered to the membrane by APEM9. The balance between cargo translocation and receptor recycling is also important for the function of the machinery. SP1 interacts with PEX13, PEX14, and PEX2 in co-IP. SP1 promotes the ubiquitination and destabilization of PEX13 and promotes the destabilization of PEX14, PEX2, PEX10, and PEX12, at least in tobacco. Therefore, SP1 regulates peroxisome biogenesis by associating with and destabilizing PEX13 and possibly several other peroxins in peroxisome matrix protein import. Double-headed arrows indicate a physical interaction in co-IP. Dashed arrows indicate hypothesized SP1-mediated ubiquitination. PEX13 is shown in bold because it is the most likely target of SP1.

as increased activity of proteins functionally redundant with SP1, increased degradation of overly accumulated PEX14, and/or decreased *PEX14* transcription. Also, our immunoblot method may not be sensitive enough to detect subtle changes in the PEX14 protein level in the plant. However, in the tobacco transient protein expression system, the capacity of such feedback mechanisms may be exceeded by the overexpression of SP1m (or SP1) and substrate proteins, thus demonstrating a much stronger SP1-mediated destabilization of the substrates.

To test whether SP1 promotes the association of ubiquitin with its potential substrate proteins, we performed *in vivo* ubiquitination assays. Because PEX2, PEX10, and PEX12 are ubiquitin E3 ligases, which often auto-ubiquitinate themselves, we tested only PEX13 and PEX14. FLAG-tagged ubiquitin, YFP-tagged PEX13 (or PEX14), and SP1 (or SP1m) were coexpressed in tobacco leaves, and the proteins pulled down by the α -FLAG antibody were analyzed by immunoblot analysis. In total protein input, PEX13 and PEX14 were much less stable when they were coexpressed with wild-type SP1 than when they were coexpressed with SP1m (Fig. 6E, Input and Fig. S5G). However, PEX13 and PEX14 apparently were more concentrated in the pull down when they were coexpressed with SP1 rather than with SP1m (Fig. 6E, IP). Hence, SP1 appears to be able to increase the association of ubiquitin with PEX13 and PEX14. More importantly, modified forms of PEX13, which likely represent ubiquitinated PEX13 because FLAG-UBQ10 was the bait for the co-IP, were strongly intensified in the presence of overexpressed SP1 (Fig. 6E). Therefore, our data show that SP1 is able to promote the ubiquitination of PEX13, at least when both proteins are overexpressed in tobacco. Given that SP1 interacts with PEX13 in co-IP, *sp1* suppresses the mutant phenotype of *pex13* and *pex14*, and SP1 promotes the destabilization of PEX13 in both the tobacco transient protein expression system and *Arabidopsis pex14-2* plants, we conclude that PEX13 is most likely a

direct target of SP1 for ubiquitination and destabilization in *Arabidopsis*.

Discussion

The molecular mechanisms that regulate the function of components of the peroxisome import machinery are largely unknown. In this study, we identified the *Arabidopsis* RING-type E3 ubiquitin ligase SP1 as a peroxisome membrane protein that regulates peroxisome biogenesis. A previous study on SP1 by Ling et al. (40) used transient expression of 35Spro:SP1-YFP in protoplast to show SP1's association with chloroplasts. In our study, we used both 35Spro:SP1-YFP and SP1pro:SP1-YFP and the peroxisomal marker protein to demonstrate the association of SP1 with peroxisomes and chloroplast in both tobacco and *Arabidopsis*. Results from our co-IP, genetic, cell biological, pull-down, and *in vivo* ubiquitination analyses led us to a working model in which SP1 plays a negative regulatory role in peroxisome import and function by promoting the destabilization of components of the peroxisome import machinery. Specifically, SP1 interacts with PEX13 and prompts PEX13's destabilization through the UPS and likely targets PEX14 and the RING peroxins PEX2, PEX10, and PEX12 as well (Fig. 7). Lack of functional SP1 may stabilize its target PEX proteins, such as PEX13, thus partially compensating for the loss of PEX14 in *pex14-2* plants and the reduction of PEX13 in *pex13-1* plants. Lack of SP1 function is expected to increase PEX5 translocation into peroxisomes and enhance *pex4-1* defects that stem from the retention of PEX5 in the peroxisome membrane.

The *sp1* single mutant was never identified from forward genetic screens because it does not display obvious growth- or organelle-specific phenotypes. This absence of obvious phenotypes in the single mutant is consistent with SP1 being a negative regulator instead of a major component of peroxisome and chloroplast protein import. SP1 was identified in a suppressor screen of *ppi1* (or *toc33*) by its role in chloroplast protein import (40) and from our *in silico* study followed by *in vivo* targeting and functional analyses of its role in peroxisome protein import. The role of SP1 in protein import in both chloroplasts and peroxisomes could be revealed clearly only in the background of an import mutant, such as *toc33* and *pex14*. Consistent with this result and with our findings of the partial suppression of *pex14* in the *pex14 sp1* double mutant, Ling et al. (40) showed direct evidence of the increased chloroplast protein import caused by *sp1* mutation only in *toc33* mutants but not in the wild-type background.

Unlike PEX13, no modified PEX14 bands representing ubiquitinated PEX14 were detected in our *in vivo* ubiquitination assay (Fig. 6E). This result is similar to that reported in a similar *in vivo* ubiquitination experiment using TOC75, in which the authors speculated that the lack of detectable ubiquitinated TOC75 suggests that TOC75 is less ubiquitinated than TOC33 and TOC159 or is more quickly degraded through UPS when ubiquitinated (40). This scenario may apply to PEX14 as well. However, if PEX14 is indeed much less ubiquitinated by SP1, SP1's promotion of PEX14 degradation in the tobacco protein transient expression system may be caused indirectly by the degradation of PEX13. Because PEX13 and PEX14 form the receptor docking complex, they may stabilize each other, a notion that was supported by the reduction of the level of PEX13 protein in *pex14-2* mutants (17). Therefore, even if PEX14 is not or is less ubiquitinated by SP1, PEX13 ubiquitination mediated by SP1 may be sufficient to facilitate the turnover of both PEX13 and PEX14, because the destabilization of one protein can cause the destabilization of the other component of the same protein complex. Last, the coprecipitation of unmodified TOC159, TOC75, and TOC33 with FLAG-ubiquitin in *in vivo* ubiquitination assays (40) is similar to the coprecipitation of unmodified PEX13 and PEX14 with FLAG-ubiquitin in our study. We thus speculate that ubiquitin and substrate protein may interact physically even before the ubiquitination reaction occurs or that

unmodified PEX14 and PEX13 coprecipitate with modified PEX13 because these two proteins are in the same complex. Nevertheless, whether SP1 can mediate the ubiquitination of PEX14 and the RING peroxins remains to be clarified.

In plants, peroxisomes and chloroplasts function closely in a few metabolic pathways, including photorespiration (3, 50). As a result, the level of enzymes housed in these two organelles may need to be coordinately adjusted during developmental and environmental changes. For example, after germination, or when dark-grown seedlings are exposed to light, chloroplasts develop and actively import photosynthetic proteins, and seed peroxisomes turn into photorespiratory peroxisomes to metabolize phosphoglycolate, a product of the oxygenase activity of the photosynthetic enzyme Rubisco (7, 12). Our study demonstrated that SP1 plays a dual role in protein import for both chloroplasts and peroxisomes. The *sp1 toc33* double-mutant plants are greener than the *toc33* single-mutant plants (40), but the chlorophyll content in *pex14-2 sp1* plants is not significantly different from that of *pex14-2* plants (Fig. 3B), possibly because the pale leaf color in *pex14-2* plants is an indirect consequence of disrupted PEX14 function and thus is not highly responsive to a partial rescue of the peroxisome defects in these plants. E3 often has multiple substrates (37, 51). SP1 was found to interact with and promote the degradation of multiple TOC proteins required for chloroplast protein import (40). Likewise, SP1 may target multiple peroxins at the peroxisome membrane.

The previous study of SP1's role in chloroplasts showed that *sp1* mutants are less efficient in meeting the developmental requirement for chloroplast proteome change, such as when etiolated seedlings are exposed to light or when leaf senescence is induced (40). Another study reported that SP1 (DAL1) and SPL1 (DAL2) are involved in the regulation of reactive oxygen species (ROS) accumulation and programmed cell death (52). A more recent study showed that SP1 promotes stress tolerance by depleting TOC components, leading to diminished ROS produced in photosynthesis (53). We speculate that SP1's function in coping with developmental changes and stress conditions also may be attributed to its regulatory role in peroxisome biogenesis. ROS are key signaling molecules in plant stress response (54), and peroxisomes possess numerous ROS-producing and -decomposing enzymes (3). Hence, developmental or environmental cues that induce changes in the chloroplast proteome may also trigger

proteome adjustment of the peroxisome. This notion is particularly interesting, given that these two organelles also share a major component of the division machinery, the dynamin-like GTPase DRP5B (55). Thus, both the organelle division and the protein import processes of peroxisomes and chloroplasts share key factors that ultimately may contribute to their linked metabolism.

All three members of the *Arabidopsis* SP1 protein family share limited sequence similarities with the human MAPL (mitochondrial-anchored protein ligase) protein, a regulator of mitochondrial fission that targets the dynamin-related protein DRP1 (56). Neither SP1 nor MAPL has obviously similar sequences in yeast or nematode, suggesting that this family of E3 enzymes may be present only in higher eukaryotes. The plant SP1-like E3s seem to be more divergent and can be divided into two subclasses, with SP1 and SPL1 in one subclass and SPL2 in the other (Fig. S6 and Dataset S1). Human MAPL is localized to both mitochondria and peroxisomes, but its function in peroxisomes is unclear (57). It is possible that this family of E3 enzymes targets to and regulates the biogenesis of multiple organelles across diverse species. Whether the animal MAPL-like proteins also govern organelle import and the roles of the *Arabidopsis* SPL1 and SPL2 proteins in organelle biogenesis or morphogenesis remain to be elucidated.

Materials and Methods

Arabidopsis plants were grown at 22 °C with 70% humidity and a 14-h day of white light at 70–80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. *N. tabacum* plants were grown at 24 °C with 70% humidity and a 14-h day of white light treatment at 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The *Arabidopsis* Col-0 ecotype was the wild-type reference. The sources of *Arabidopsis* transgenic lines and detailed methods are described in *SI Materials and Methods*. Primers used in this study are listed in Table S1; vectors used in this study are listed in Table S2.

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