Lipid-mediated activation of plasma membrane-localized deubiquitylating enzymes modulate endosomal trafficking

Karin Vogel¹, Tobias Bläske¹, Marie-Kristin Nagel¹, Christoph Globisch², Shane Maguire³, Lorenz Mattes³, Christian Gude⁴, Michael Kovermann⁵, Karin Hauser³, Christine Peter² & Erika Isono¹

The abundance of plasma membrane-resident receptors and transporters has to be tightly regulated by ubiquitin-mediated endosomal degradation for the proper coordination of environmental stimuli and intracellular signaling. *Arabidopsis* OVARIAN TUMOR PROTEASE (OTU) 11 and OTU12 are plasma membrane-localized deubiquitylating enzymes (DUBs) that bind to phospholipids through a polybasic motif in the OTU domain. Here we show that the DUB activity of OTU11 and OTU12 towards K63-linked ubiquitin is stimulated by binding to lipid membranes containing anionic lipids. In addition, we show that the DUB activity of OTU11 against K6- and K11-linkages is also stimulated by anionic lipids, and that OTU11 and OTU12 can modulate the endosomal degradation of a model cargo and the auxin efflux transporter PIN2-GFP in vivo. Our results suggest that the catalytic activity of OTU11 and OTU12 is tightly connected to their ability to bind membranes and that OTU11 and OTU12 are involved in the fine-tuning of plasma membrane proteins in Arabidopsis.

The survival of plants depends on their ability to sense and adapt to environmental stimuli that are perceived and translated into intracellular processes by plasma membrane (PM)-localized receptors and transporters. As the abundance of PM receptor- and transporter proteins is one of the key parameters determining signal perception and transduction, the amount of PM proteins has to be tightly regulated. This can be achieved by transcriptional regulation, protein transport, posttranslational modifications, or protein degradation. Selective protein degradation of PM proteins is mediated by the protein ubiquitylation and the endosomal sorting complex required for transport (ESCRT) machinery. The ESCRT machinery contains subunits with ubiquitin (UB)-binding motifs in order to retain ubiquitylated PM proteins on endosomal membranes until they are sorted to intraluminal vesicles of multi-vesicular endosomes. Upon fusion of the MVE with the vacuole, the ILVs are released into the vacuolar lumen and degraded by vacuolar enzymes.

Protein ubiquitylation, the covalent modification of proteins at the lysine (K) residue with UB, is achieved by the concerted actions of mainly three enzymes: The E1 ubiquitin activation enzyme, E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin ligase. Any of the seven lysine (K)s in UB (K6, K11, K27, K29, K33, K48, and K63), as well as the N-terminal methionine (M), can be used for oligo- and poly-ubiquitylation, enabling the generation of diverse linkage patterns described as the ubiquitin code. For endosomal degradation, K63-linked UB chains play a predominant role.
In the model plant Arabidopsis thaliana, around 1500 E3s were identified in silico. Besides the specificity conferred by the large number of E3s, there are around 50 deubiquitylating enzymes (DUBs) in Arabidopsis which can modulate protein ubiquitylation. Although DUBs are smaller in number compared to E3s, studies have shown that the timely removal of the UB by DUBs can have consequences in various cellular signaling processes. Arabidopsis DUBs belong to the family of ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (UBPs), ovarian tumor proteases (OTUs), and the Machado–Joseph domain (MJD) domain proteases, the JAB1/MPN/MOV34 (JAMM) proteases and the Zn-finger and USP domain protein (ZUFSP) family. Potential orthologs of motif interacting with UB-containing (MINDY) proteases are present in the Arabidopsis genome, however, their biochemical activities and molecular functions have yet to be determined.

With 27 members, the UBPs are the largest DUB family in Arabidopsis. Depending on their catalytic activity, localization, and interacting proteins, they are involved in broad aspects of plant biology from developmental processes to biotic and abiotic stress responses. In Arabidopsis, there are 12 OTU domain-containing proteins. Among them, OTL1/OT6, OTU1, and OTU5 were reported to be involved in the regulation of gene expression by histone deubiquitylation. OTU1 and OTU5 was also implicated in the ERAD pathway in Arabidopsis. Though recent studies have implicated regulatory roles for DUBs, the molecular and physiological functions of many of the DUBs still remain to be elucidated.

To date, the conserved metallocarboxypeptidase DUB ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM (AMSH) 3, and two UBP DUBs UBP12 and UBP13 are the only DUBs shown to be involved in the endosomal degradation of PM proteins in Arabidopsis. AMSH3 localizes to endosomes and functions together with the ESCRT machinery. In human cells, in addition to AMSH proteins, USP22, USP6, USP8/UBPY, USP9X, USP32, and JosD1 have been shown to modulate the degradation of PM proteins. Except for AMSH, sequence homologs of these DUBs either do not exist in Arabidopsis or have not been characterized. It can thus be assumed that there are further yet uncharacterized DUBs that are regulating endosomal trafficking and PM protein degradation in Arabidopsis.

In this study, we identify two PM-localized Arabidopsis DUBs, OTU11 and OTU12, and show that they are important for the regulation of the endosomal transport of PM proteins. Both OTU11 and OTU12 possess poly-basic motifs which are important for the interaction with phosphoinositides, including PI(4)P and PI(4,5)P2, and for the localization to the PM. One of the poly-basic motifs is located in close proximity to the modeled UB interaction site in the catalytic OTU domain of OTU11 and OTU12. Our results show that the DUB activity of OTU11 and OTU12 is stimulated when liposomes containing anionic lipids are added to the reaction, revealing an activation mechanism for OTU11 and OTU12 upon membrane binding.

Results

OTU11 and OTU12 are localized to the plasma membrane

With the goal to identify Arabidopsis DUBs that are localized to the PM or to endosomal compartments, we conducted a localization study of 18 uncharacterized Arabidopsis DUBs that were selected from the two largest DUB families, namely the UBP- and OTU families. In addition to uncharacterized DUBs, we included UBP27 which was reported to localize on mitochondria, UBP3 and UBP4 which were detected in nuclear extracts, and UBP12 which was reported to localize to the cytosol and nucleus in Arabidopsis seedlings. The selected DUBs were expressed as YFP-fusion proteins in Arabidopsis cell culture-derived protoplasts, and the localization was analyzed under a confocal microscope (Fig. 1a and Supplementary Fig. 1a).

YFP-fused DUBs showed a variety of intracellular localization, which were summarized in Table 1: UBP20, and OTU10 localized to the cytosol, UBP3, UBP4, UBP6, UBP7, UBP9, UBP10, UBP12, and UBP24 to the cytosol and nucleus, UBP25 to the nucleus, UBP22 to the nucleolus, UBP23 to the nucleolus, UBP18 to the endoplasmic reticulum (ER), and OTU11 and OTU12 to the cytosol, nucleus and the PM. UBP27 was localized to rod-like structures that most probably represent mitochondria, as previously reported. UBP22, which was localized in the nucleoplasm in our experiment, was previously shown to localize at euchromatin, a nucleoplasmic structure. These results confirm that DUBs can localize to specific cellular compartments in cell culture-derived protoplasts.

Among the DUBs examined in this study, OTU11 and OTU12 were the only DUBs found at the PM. The PM localization of YFP-OTU11 and YFP-OTU12 was verified with colocalization with the PM-protein FP-SVPI2 (Fig. 1b). Both N-terminally and C-terminally tagged OTU12 localized to the PM (Supplementary Fig. 1b). Since the two splicing forms of OTU11 showed similar localization patterns (Supplementary Fig. 1c, d), we used OTU11.2 for further studies, and unless otherwise specified, OTU11 refers to OTU11.2. To examine the localization of OTU11 and OTU12 in planta, GFP-OTU11 and GFP-OTU12-GFP were expressed under their native promotor in stable transgenic plants. Whereas OTU11pro:GFP-OTU11 was expressed in the meristematic zone, OTU12pro:GFP-OTU12-GFP was expressed in the elongation zone of the seedling root. As in protoplasts, GFP-OTU11 and OTU12-GFP were found at the PM, and colocalyzed with the lipophilic dye FM4-64 (Fig. 1c). The PM in Arabidopsis is enriched in PI(4)P and PI(4,5)P2. When analyzed under a confocal microscope, overexpressed GFP-OTU11 and GFP-OTU12 driven by a 35S promoter both colocalized with the PI(4)P-marker P5R (RFP-1xPHFAPP) at the PM (Supplementary Fig. 1e). For further confirmation, PM protein enrichment was performed using GFP-OTU11 overexpressing seedlings. From the total microsomal fraction (100,000 × g pellet (P100)), the PM and PM-bound proteins were enriched using the nonionic detergent Brij-S8. In the enriched PM fraction, GFP-OTU11 could be detected (Fig. 1d), showing that GFP-OTU11 is indeed associated with the PM in planta. Despite repeated attempts, we could not detect GFP-fused OTU11 and OTU12 expressed under their native promotor and overexpressed GFP-OTU12 by immunoblotting in total extracts, immunoprecipitants, and in membrane-enriched fractions and thus only the results for the 35Spro:GFP-OTU11 line are shown. To examine whether OTU11 and OTU12 are also associated with other endomembrane compartments, the localization of GFP-OTU11 and GFP-OTU12 upon treatment with endosomal transport inhibitors brefeldin A (BFA, ARF-GEF inhibitor) and Wortmannin (WM, PI3K/PI4K inhibitor) was analyzed. BFA bodies and WM-induced aberrant endosomal compartments were stained with the lipophilic dye FM4-64. Accumulation of GFP-OTU11 and GFP-OTU12 to BFA compartments could not be observed after 60 min of BFA incubation (Fig. 1e) nor to WM-induced compartments after 90 min of WM treatment (Fig. 1f). GFP-OTU11 and GFP-OTU12 were at the PM in cells treated with the PI4K-inhibitor phenylarsine oxide (PAO) for 40 min, suggesting that the abundance of PI(4)P is not decisive for the localization of OTU11 and OTU12 to the PM this time period (Fig. 1g).

OTU11 and OTU12 do not have transmembrane domains. To understand the molecular basis of the PM localization of OTU11 and OTU12, we analyzed the primary amino acid sequences of OTU11 and OTU12. OTU12 has a glycine at position 2 after the methionine and OTU11 and OTU12 do not have transmembrane domains. To understand the molecular basis of the PM localization of OTU11 and OTU12, we analyzed the primary amino acid sequences of OTU11 and OTU12. OTU12 has a glycine at position 2 after the methionine and OTU11 and OTU12 do not have transmembrane domains.
We next examined the hydrophobicity of the amino acid sequences of OTU11 and OTU12 with the basic and hydrophobic (BH)-search tool and found two regions with BH-scores above 0.6. These regions correspond to amino acids 153–163 (polybasic motif [PBM1]) and 236–242 (PBM2) in OTU11 and amino acids 131–141 (PBM1) and 210–216 (PBM2) in OTU12 (Fig. 2a–d and Supplementary Fig. 2a). To establish whether the basic amino acids in PBM1 and PBM2 are responsible for the PM-localization of OTU11 and OTU12, we mutated six basic amino acids [lysine (K) and arginine (R)] in each region to alanine to generate mutants 6A1 and 6A2 (Fig. 2a, b).
mutations reduce the BH-score in the PBMI and PBMB, respectively (Fig. 2c, d).

To investigate the importance of PBMs for PM localization, we analyzed the localization of XFP-fused wild-type OTU11 (OTU11(WT)), OTU11(6A1), and OTU11(6A2) in Arabidopsis root cell culture-derived protoplasts. RFP-OTU11(WT) localized in 64% of the cells to the PM (n = 22), whereas the 6A1 mutation reduced the number of cells with YFP signals at the PM to 31% (n = 32), and the 6A2 mutation abolished the PM localization completely (n = 20) (Fig. 2e and Supplementary Fig. 2b). Similarly, whereas wild-type RFP-OTU12 localized 100% to the PM (n = 77), YFP-OTU12(6A1) localized in 20% of the cells to the PM (n = 36), and YFP-OTU12(6A2) localized in none of the observed cells to the PM (n = 15) (Fig. 2f and Supplementary Fig. 2b). These results indicate that both PBMI and PBMB in OTU11 and OTU12 are necessary for their PM localization. The importance of PBMI was confirmed by the observation that the 6A1 mutation in GFP-OTU11 and GFP-OTU12 leads to the complete loss of PM localization in planta (Fig. 2g–i). PBMB, but not PBMI, is conserved in closely related OTU9 and OTU10 (Supplementary Fig. 2c). OTU9 and OTU10 do not localize to the PM in protoplasts (Fig. 1a) despite the presence of PBMB, suggesting that the PBMB alone is not sufficient for the PM localization of OTU11 and OTU12.

OTU11 and OTU12 modulate protein stability at the PM

PM-localized receptors and transporters translate extracellular stimuli to intracellular signaling pathways and are thus tightly controlled both in abundance and activity. To regulate their abundance, UB-dependent endocytic protein degradation plays a pivotal role. For the regulation of protein stability, ubiquitylating enzymes and DUBs both are important. To test whether OTU11 and OTU12 are involved in the regulation of PM proteins, we investigated T-DNA insertion knock-out lines for OTU11 and OTU12 (Supplementary Fig. 3a–c). The double mutant otu11otu12 did not show changes in the accumulation of ubiquitylated proteins (Supplementary Fig. 3d), nor showed obvious developmental defects under continuous light, long-day, and short-day conditions (Supplementary Fig. 3e). However, the single mutant otu12 and the double mutant otu11otu12 both showed a slight but significant increase in the primary root length at the seedling stage (Fig. 3a, b). The otu11otu12 phenotype was complemented by the introduction of OTU2pro:OTU12-GFP, indicating that the GFP-fusion construct is functional, and the phenotype is caused by the T-DNA insertion in OTU12 (Fig. 3c, d and Supplementary Fig. 3f). The overexpression of OTU11 and OTU12 led to the reduction of the primary root length (Fig. 3e, f and Supplementary Fig. 3g). However, with expression at similar levels, the root length of the OTU11(6A1) overexpressing protoplasts was significantly longer than the OTU11(WT) overexpressing seedlings (Fig. 3g and Supplementary Fig. 3h). Thus, the 6A1 mutation could impact the physiological function of OTU11, although we cannot rule out that the slightly different expression levels of the WT and 6A1 variants contribute to this difference.

Primary root growth is regulated by numerous factors, including PM-residing proteins such as phytohormone receptors, ion transporters, and protein transport machineries. To examine whether OTU11 and OTU12 affect the endosomal transport of PM-proteins, we first used the model cargo PMA-GFP-UB which is transported to the vacuole for degradation in a ubiquitin- and ESCRT-dependent manner. When expressed in Arabidopsis root cell-derived protoplasts, PMA-GFP-UB localizes on membrane compartments along the endosomal degradation pathway from the PM to the vacuole (Fig. 4a). We hypothesized that if OTU12 is deubiquitylating poly-ubiquitylated PMA-GFP-UB, the loss of OTU12 will lead to an enhanced transport of PMA-GFP-UB to the vacuole. In contrast, overexpression of OTU12 would reduce the population of polyubiquitylated PMA-GFP-UB, and stabilize
To analyze this, we generated a CRISPR-Cas9 construct with a target sequence in the first exon of OTU12 (Fig. 4b). The effect of CRISPROTU12 and the non-binding CRISPR m control on the expression of RFP-OTU12 was verified on an immunoblot before the experiment (Fig. 4c).

When co-expressed with a CRISPR-Cas9 construct, PMA-GFP-UB localized to the vacuole in 48% of the cells, whereas it did so in 19% and 27% of cells transformed with PMA-GFP-UB alone or PMA-GFP-UB together with CRISPR m-Cas9, respectively (Fig. 4d, e). When PMA-GFP-UB was co-transformed with CRISPR-Cas9, the number of cells with GFP signals at the PM was reduced to 14%, whereas it was 29% and 24% for cells expressing PMA-GFP-UB alone or PMA-GFP-UB with CRISPR m-Cas9, respectively. This result indicates that the transport of PMA-GFP-UB is more efficient when OTU12 is depleted from the cell.

When OTU12 was overexpressed, PMA-GFP-UB was associated to the PM in 48% of the analyzed cells whereas 17% of the cells showed PMA-GFP-UB in the vacuole. When expressed alone, PMA-GFP-UB showed localization to the PM and to the vacuole in 10% and 30% of the cells.
transferred to dark, vacuolar signals were observed in more cells in the wild-type (Fig. 5a, b, d, e). When PIN2-GFP-expressing seedlings were grown on 0.0723 (ns: not significant), wild-type/OTU12 P = 0.0417 (**0.001 < P < 0.01), wild-type/OTU12 P = 3.28 × 10^{-2} (**P < 0.001), two-tailed t test, no equal variance. The experiment was conducted three times, and one representative result is shown. c Photographs of 7-day-old wild-type, OTU12pro:OTU12-GFP. Scale bars: 1 cm. d Box plot of primary root length of 7-day-old wild-type (n = 280 seedlings), OTU12pro:OTU12-GFP (n = 256 seedlings). Center line, median; box limits, first and third quartiles; whiskers, 1.5x interquartile range; points, outliers. Wild-type/OTU12 P = 0.158 (ns: not significant), wild-type/OTU12 P = 8.0 × 10^{-4} (**P < 0.001), wild-type/OTU12 P = 3.07 × 10^{-17} (**P < 0.001), wild-type/GFP-OTU12 #2 P = 5.46 × 10^{-16} (**P < 0.001), two-tailed t test, no equal variance. The experiments were conducted three times and one representative result is shown. e Photographs of 7-day-old wild-type and OTU12pro:GFP-OTU11 (OTU12pro:OTU11 overexpressor o/e) and OTU11pro:GFP-OTU12 (GFP-OTU12 o/e #2) seedlings. Scale bars: 1 cm. f The primary root length of 7-day-old wild-type (n = 196 seedlings), GFP-OTU11 o/e (n = 171 seedlings), GFP-OTU12 o/e #1 (n = 197 seedlings), and GFP-OTU12 o/e #2 (n = 213 seedlings) is shown as a box plot. Center line, median; box limits, first and third quartiles; whiskers, 1.5x interquartile range; points, outliers. Wild-type/GFP-OTU11 o/e P = 8.08 × 10^{-4} (**P < 0.001), wild-type/GFP-OTU12 o/e #1 P = 3.07 × 10^{-17} (**P < 0.001), wild-type/GFP-OTU12 o/e #2 P = 5.46 × 10^{-16} (**P < 0.001), two-tailed t test, no equal variance. The experiments were repeated twice and one representative result is shown. Source data are provided as a Source Data file.

respectively (Fig. 4f, g). Overexpression of the 6A1 variant of OTU12 increased the PM localization of PMA-GFP-UB to 23%, though the effect was weaker than the wild-type OTU12 (Fig. 4f, g), indicating that PBM1 is important for the function of OTU12. These results indicate that OTU12 regulates the transport of PMA-GFP-UB from the PM to the vacuole.

To investigate whether OTU11 and OTU12 also regulate the endosomal transport of PM proteins in planta, we analyzed the auxin efflux carrier PIN2-GFP in the roots of wild-type and otu11otu12 seedlings. PIN2 is a transmembrane protein shown to be degraded in a K63-linked polyubiquitylation-dependent manner. The localization of PIN2-GFP was indistinguishable between the wild-type and otu11otu12 (Fig. 5a–c). However, when treated with the endosomal transport inhibitors BFA and WM, PIN2-GFP accumulated into BFA bodies and WM compartments earlier in the otu11otu12 mutant compared with the wild-type (Fig. 5a, b, d, e). When PIN2-GFP-expressing seedlings were transferred to dark, vacuolar signals were observed in more cells in the otu11otu12 seedlings than in the wild-type (Fig. 5c, f). This result shows that the absence of OTU11 and OTU12 affects the rate of endosomal transport of PIN2-GFP and probably also on the subsequent degradation of PIN2-GFP in the vacuole. In contrast, otu11otu12 did not show a significant difference in the accumulation of FM4-64 to the BFA bodies (Supplementary Fig. 4a, b) nor a root gravitropism phenotype (Supplementary Fig. 4c), as in the ubiquitylation-deficient PIN2-33K mutation. Thus, OTU11 and OTU12 could be involved in fine-tuning the ubiquitylation status and degradation rate of PIN2. When DUB activity of OTU11 and OTU12 are missing at the PM, a larger population of PIN2-GFP can remain polyubiquitylated and sent to the endosomal degradation route, which could result in their faster accumulation in BFA and WM compartments as well as in the vacuole in otu11otu12.

Poly-basic motifs in OTU11 and OTU12 bind to phospholipids PBMs can interact with anionic lipids such as phosphatidylinositol phosphates (PIPs) which are minor membrane phospholipids contributing to membrane compartmentalization and signaling. To investigate whether the PBMs in the OTU domain serve as the
shows that the N-terminal half of OTU11 that contains neither of the anionic lipids, we tested GST-OTU11(N) and GST-OTU11(OTU), which also interacted with PI(3)P, PI(4)P, PI(5)P, PI(3,5)P2, PI(4,5)P2, PI(3,4,5)P3, and also with PA (Fig. 6b, c). To identify the region interacting with anionic lipids, we tested GST-OTU11(N) and GST-OTU11(OTU12m), which did (Fig. 6b), and when 6A1 and 6A2 mutations were combined, lipid binding of OTU11 was further reduced (Fig. 6b), suggesting that both PBMs act synergistically. A similar result was obtained when GST-OTU11(OTU-6A1) was used for the assay (Fig. 6d). In contrast, for GST-OTU12, both the 6A1- and 6A2 mutations affected the binding of GST-OTU12 to anionic lipids (Fig. 6c).

To analyze the interaction of OTU11 with PIPs in membranes, we next prepared liposomes generated with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) containing no PIPs or 5% of one of PI(3)P, PI(4)P, or PI(4,5)P2. OTU11 showed interaction with all PIP-containing liposomes, whereas it bound liposomes without PIPs only weakly (Fig. 6e, f). OTU12 also bound all PIP-containing liposomes and also to liposomes with no equal variance. The expression of 3xFLAG-Cas9 in (d) was verified with an anti-FLAG immunoblot. An anti-ACTIN antibody was used as loading control on the same membrane. Source data are provided as a Source Data file.
Lipid binding could lead to conformational changes of OTU11
The alpha fold model of the OTU-domain of OTU11 and OTU12 shows that PBM1 is located in the proximity of residues comprising the active site of both OTU11 and OTU12 (Fig. 7a–c). Interaction of the PBM1 with the membrane could thus influence the conformation of the catalytic site and modify the accessibility of UB molecules to the catalytic center. To test whether binding to liposomes changes the conformation of OTU11, we performed circular dichroism spectroscopy analysis (Supplementary Fig. 6a). Using a secondary structure analysis algorithm, the overall estimated helical content of the protein changed from 57 to 44% upon binding to PI(4,5)P2-containing liposomes, which shows that OTU11 indeed binds via the PBM1 patch to PI(4,5)P2 (Fig. 7d, e and Supplementary Movie 1). For the 6A1 mutants, we observed a generally reduced binding to the membrane compared with the wild-type variants, with a lower number of hydrogen bonds for the missing basic amino acids in PBM1 in OTU11(Δ21-6A1) (Supplementary Fig. 6b–d). As these unspecific binding were not observed in simulations using OTU11(OTU-WT) (Supplementary Fig. 6e–g), residues outside of the OTU domain must contribute to the unspecific binding. Residue-specific analysis of the interactions in OTU11(Δ21-WT) and OTU11(Δ21-6A1) illustrates that interactions in the N-terminal helix and PBM2 are partly compensating for the missing basic amino acids in PBM1 in OTU11(Δ21-6A1) (Fig. 7f, g and Supplementary Fig. 6h–m). As a result, PBM1 in OTU11(Δ21-6A1) does not participate in the binding to lipids and is oriented away from the membrane (Supplementary Fig. 6i and Supplementary Movie 2). Next, we analyzed the influence of membrane binding of OTU11 to the catalytic site. Upon membrane binding, wild-type and 6A1 OTU11(Δ21) showed differences in the distance pattern of the catalytic triad (Fig. 7h, i). These results suggest that binding of OTU11 to the membrane through PBM1 could have a stabilizing effect on the active site.
Fig. 6 | PBM in OTU11 and OTU12 are required for their interaction with anionic lipids in vitro. a Lipid species spotted on the membrane for the lipid overlay assay. The solid dark gray and light gray spots indicate lipids that showed interactions in the lipid overlay assay. b-d Lipid overlay assays with GST, GST-OTU11(WT), GST-OTU11(6A1), and GST-OTU11(6A2) (b), GST, GST-OTU12(WT), GST-OTU12(6A1), and GST-OTU12(6A2) (c) and GST, GST-OTU11(OTU), GST-OTU11(OTU-6A1), and GST-OTU11(N) (d). Bound proteins were detected with an anti-GST antibody. e Representative gel images of liposome sedimentation assays using GST-OTU11(WT) and GST-OTU12(WT). GST fusion proteins were incubated with the liposome buffer alone or with liposomes (PC/PE) containing 5% of PI(3)P, PI(4)P, or PI(4,5)P2. SDS-PAGE gels were stained with TCE. M molecular mass marker. f Quantification of the result in (e). Box plot shows the results of the quantification of three independent experiments. Center line, median; box limits, first and third quartiles; whiskers, 1.5× interquartile range; points, outliers, n = 3 experiments. g Representative gel images of liposome sedimentation assays of GST-OTU11(WT) and GST-OTU11(6A1). GST fusion proteins were incubated with the liposome buffer alone or with liposomes (PC/PE) containing 5% of PI(3)P, PI(4)P, or PI(4,5)P2. M molecular mass marker. h Quantification of the result in (g). Box plot shows the results of the quantification of three independent experiments. Center line, median; box limits, first and third quartiles; whiskers, 1.5× interquartile range; points, outliers, n = 3 experiments. i A representative gel image of liposome sedimentation assays of GST-OTU11(WT) or GST-OTU11(N). GST fusion proteins were incubated with liposomes (PC/PE) containing 5% of PI(4,5)P2 or with the liposome buffer alone. M molecular mass marker. j Quantification of the result in (i). Box plot shows the results of the quantification of three independent experiments. Center line, median; box limits, first and third quartiles; whiskers, 1.5× interquartile range; points, outliers, n = 3 experiments. Source data are provided as a Source Data file.
The DUB activity of OTU11 and OTU12 is stimulated by anionic lipids
We then analyzed whether the binding to lipids could affect the DUB activity of OTU11 and OTU12 and first investigated the DUB activity of OTU11 and OTU12 in vitro. At an equimolar substrate to enzyme ratio (DUB: substrate = 7.5 pmol:7.5 pmol), recombinant GST-OTU11 and GST-OTU12 both showed only weak activity (Fig. 8a, b) in accordance with a previously published result\(^1\). When the enzyme was added in excess, the DUB activity could be observed clearly (Fig. 8a, b). The DUB activity towards K63-linked tetra-UB was not dependent on the isoform of OTU11, as both isoforms OTU11.1 and OTU11.2 showed comparable activity (Supplementary Fig. 7a). The DUB activity was
dependent on an intact OTU domain since a mutation in the conserved cysteine residue (C112A) abolished the activity of GST-OTU11 (Supplementary Fig. 7b). Recombinantly purified OTU11 could lack post-translational modifications which could be important for the DUB activity. We immunoprecipitated GFP-OTU11 from Arabidopsis total extracts and found the majority of the isolated GFP-OTU11 to be phosphorylated (Supplementary Fig. 7c). This is in accordance with information about potential phosphorylation sites from the PhosPhAt 4.0 database and a recent proteomics study40 (Supplementary Fig. 7d). However, the phosphorylation status of GFP-OTU11 did not affect its DUB activity (Fig. 8e).

To examine whether the DUB activity of OTU11 and OTU12 is stimulated by binding to the membrane, we prepared liposomes and analyzed first the DUB activity of GST-OTU11 against K63-linked tetra-UB in the presence or absence of liposomes. Liposomes (PC/PE) without or with 5% of PI(4,5)P2 were incubated with either 25 pmol of GST-OTU11 (WT) or GST-OTU11(6A1) that binds less efficiently to liposomes. We then added 25 pmol Ubc to the liposome-OTU11 mixture and analyzed the DUB activity of OTU11. When pre-incubated for 15 min with liposomes containing PI(4,5)P2, GST-OTU11(WT) showed a stronger DUB activity compared to preincubation with only neutral lipids. The activation was weaker when GST-OTU11(6A1) was used for the DUB assay (Fig. 8d), indicating that the binding of GST-OTU11 to lipids could be one of the mechanisms for the activation of OTU11. To test whether the binding of OTU11 to lipids modulates the DUB activity towards other UB linkage types, we conducted DUB assays with linear, K6, K11, K29, K33, and K48 tetra-UB as substrates. GST-OTU11 was pre-incubated with liposomes with or without PI(4,5)P2 before the addition of the UB oligomers. The DUB activity of OTU11 towards K6 and K11-linked tetra-UB was also stimulated in the presence of liposomes containing PI(4,5)P2 (Fig. 8e).

To analyze the DUB activity in a more quantitative manner, we next used di-UB FRET TAMRA as a substrate for the DUB assay (Fig. 8f–k), which emits fluorescence upon cleavage of the di-UB. Prior to the addition of di-UB FRET TAMRA, wild-type or 6A1 variants of OTU11 and OTU12 were pre-incubated with liposomes (PC/PE) with or without 5% PI(4,5)P2 for 15 min. The DUB activity of OTU11(WT) and OTU12(WT), but not of OTU11(6A1) and OTU12(6A1), was stimulated by liposomes containing PI(4,5)P2. The activation of OTU11(WT) was dependent on the presence of PI(4,5)P2 in the liposomes as liposomes with only PC/PE could not activate OTU11 and OTU12. The 6A1-mutant could not be activated by the addition of liposomes, indicating that the interaction between OTU11 and the PI4Ps in the membrane is necessary for the activation. The activation was observed when K63-linked di-UB FRET TAMRA was used as a substrate but not for K48-linked di-UB FRET TAMRA (Fig. 8h–k). The stimulation of DUB activity was observed for both OTU11 and OTU12 isoforms (Supplementary Fig. 7f). The activation did not depend on specific anionic lipids, as preincubation with both PA (Supplementary Fig. 7f) and PI(3)P-containing liposomes (Supplementary Fig. 7g) also stimulated the DUB activity of OTU11.

Taken together, our study has identified OTU11 and OTU12 as PM-localized DUBs in Arabidopsis and suggests that the DUB activity of OTU11 and OTU12 could be regulated through their binding to anionic lipids in the PM (Fig. 9). By modulating the ubiquitylation status of plasma membrane proteins, OTU11 and OTU12 could fine-tune the endosomal degradation of plasma membrane proteins in Arabidopsis.

**Discussion**

The localization of DUBs to cellular membranes can be determined by the transmembrane domain(s) in the DUB, protein modifications, membrane-interacting motifs, by signal peptides, or by the interaction with other membrane-localized proteins. Phosphoinositides show distinct localization patterns across cellular membranes and can regulate cellular processes by recruiting specific effector proteins41. In Arabidopsis, the PM was shown to be enriched in PI(4)P and PI(4,5)P2. OTU11 and OTU12 did not show specific binding towards PI(4)P and PI(4,5)P2 in vitro and were not removed from the PM upon treatment with the PI4K-inhibitors WM or PAO. As the affinity of most lipid-binding domains towards anionic lipids is considered to be too low for stable and efficient recruitment42,43, the recruitment of OTU11 and OTU12 to the PM could be achieved by additional interactions with membrane lipids, PM-localized proteins, or with the ubiquitin on the target proteins.

Although the 6A2 mutation did not affect the binding of OTU11 to anionic lipids in the lipid overlay assay, when introduced into XFP-OTU11, it led to the abolishment of PM localization. One explanation could be that PBMB2 is responsible for the correct targeting of OTU11 by functioning as an interaction surface for PM-localized interactors of OTU11. For OTU12, however, both PBMB1 and PBMB2 seem to function as lipid binding motifs, suggesting a difference in the membrane binding mechanism of OTU11 and OTU12.

Various posttranslational modifications such as phosphorylation, ubiquitylation, and acetylation were shown to influence the activity of human DUBs OTUD5A, JOSD1, and OTUD3, respectively. The activity of human OTUD5 and the OTU-domain-containing DUB A20 were both shown to be stimulated by phosphorylation44,45. Furthermore, enhancement of the DUB activity of A20 was linkage-specific in which only the activity for K63-linked poly-UB was enhanced46. A similar mechanism has been described for OTUD4, which cleaves K48-linked UB chains when not phosphorylated and K63-linked chains when phosphorylated. Although we found OTU11 to be phosphorylated in vivo and OTU12 also has predicted phosphorylation sites, the phosphorylation did not obviously affect the DUB activity. Whether and which regulatory role phosphorylation has on the function of OTU11 and OTU12 will be an interesting topic for further studies.

The DUB activity of OTU11 and OTU12 can be stimulated upon binding to anionic lipids in vitro, which suggests that OTU11 and OTU12 could be activated when they are bound to cellular membranes in vivo. The Legionella effector LotA is a DUB that binds to PI(3)P and a DUB
from the human pathogen *Orientia tsutsugamushi* binds to phosphatidylserine in vitro. However, whether the DUB activity of these DUBs is regulated through binding to membrane lipids has not been investigated.

The molecular dynamics simulation shows that the OTU11 (6A1) could bind eventually to membranes by increased interactions outside the PBM1. This could explain the weak DUB activation of OTU11(6A1) observed in the immunoblot-based DUB assay in which OTU11 was incubated with 7.5 pmol of GST-OTU11 for 1 h at 21 °C. The experiments were repeated at least three times, and one representative image is shown.

![Figure 8](https://doi.org/10.1038/s41467-022-34637-3)

**Fig. 8** The in vitro DUB activity of OTU11 and OTU12 is stimulated by anionic lipids. a, b DUB assays with GST-OTU11 (a) and GST-OTU12 (b), 7.5 pmol of K63-linked tetra-UB was incubated with 7.5 pmol (substrate: enzyme ratio 1:1), 25 pmol (1:3), 50 pmol (1:6), 100 pmol (1:12), or 250 pmol (1:30) of GST-OTU11 or GST-OTU12 for 1 h at 21 °C. The experiments were repeated at least three times, and one representative image is shown.

c DUB assay with phosphatase treated GFP-OTU11 purified from 35Spro:GFP-OTU11 expressing *Arabidopsis* seedlings and 15 pmol of K63-linked di-UB. active: active phosphatase, inact: heat-inactivated phosphatase. The experiment was repeated at least three times; one representative image is shown.

d, e DUB assays with GST-OTU11 (WT) and GST-OTU11(6A1) pre-incubated with liposomes generated with PC and PE (PC/PE) alone (−PIP) or with liposomes (PC/PE) containing 5% PI(4,5)P2 (+PIP). In (d), 25 pmol of GST-OTU11(WT) or GST-OTU11(6A1) was incubated for the indicated time with 25 pmol of K63-linked tetra-UB (negative controls: tetra-UB with +PIP or −PIP liposomes, incubated for 4 h). In (e), 25 pmol of GST-OTU11 pre-incubated with +PIP and −PIP liposomes, was mixed with 25 pmol of linear, K6-, K11-, K27-, K29-, K33-, or K48-linked tetra-UB for 4 h at 21 °C. The experiments were repeated at least three times; one representative image is shown.

f, g, h Effect of liposomes on the DUB activity of OTU11 (f−h) and OTU12 (i−k). 3.75 pmol of Recombinant OTU11(WT), OTU11(6A1), OTU12(WT), and OTU12(6A1) were pre-incubated with either liposome with or without PI(4,5)P2 [lipo(+PIP) and lipo(−PIP), respectively], or the liposome buffer alone before the addition of 3.75 pmol of di-UB FRET TAMRA K48 pos1 (f, i), K48 pos2 (g, j), and K63 pos1 (h, k). The assays were conducted at least three times. The result of one representative measurement is shown. Error bars: standard deviation of a technical quadruplicate, center of the error bars: mean of the quadruplicates. Source data are provided as a Source Data file.
incubated for 4 h with tetra-ubiquitin. In contrast, during the 30 min of incubation for the fluorescence-based DUB assay, the activation of OTU11(6A1) could not be observed. The low lipid binding affinity of OTU11(6A1) and the subsequent activation could be the reason why the expression of OTU11(6A1) is affecting the primary root length growth, albeit to a lesser extent than the overexpression of OTU11(NT). OTU11 and OTU12 are also found in the nucleus, though it is not clear whether they associate with the nuclear envelope. Whether OTU11 and OTU12 require temporal binding to the nuclear membrane for activation, the nuclear function of OTU11 and OTU12, and also the nature of OTU11 and OTU12 targets are open questions that await future studies.

AMSH family proteins and UBPI2/UBPI3 do not localize to the PM. Whether endocytic cargos in plants are already ubiquitylated at the PM is not clear and may differ from protein to protein. A number of endocytic cargos in plants are already ubiquitylated at the PM, the DUB activity of OTU11 and OTU12 can be stimulated, and OTU11 and OTU12 can deubiquitylate ubiquitin-modified proteins at the PM. Deubiquitylation of PM proteins could affect their affinity to ubiquitin-binding domains of ESCRT and ESCRT-accessory proteins. Thus, for the UB-dependent and ESCRT-mediated endosomal degradation, the balance of the activity of ubiquitylating- and deubiquitylating enzymes can determine protein stability. OTU11 and OTU12 can be part of the molecular mechanisms modulating the endosomal transport and subsequent vacuolar degradation of PM proteins.

**Methods**

**Molecular cloning**

All primers used in this study are listed in Supplementary Table 1, and all plasmids used in this study are listed in Supplementary Table 2.

The **CDS** of the cloned genes was amplified with Phusion polymerase (New England Biolabs) from Arabidopsis cDNA or existing constructs. RNA was extracted from Arabidopsis seedlings with the Nucleospin RNA Kit for plants and fungi (Macherey-Nagel), and cDNA was generated with M-MuLV Reverse Transcriptase (New England Biolabs). Mutations were introduced with a two-step Overlap-PCR with Phusion polymerase (New England Biolabs).

To generate the YFP-fusion constructs pKV10 (UBP6), pKV11 (UBP9), pKV12 (UBP12), pKV13 (UBP18), pKV14 (UBP20), and pKV15 (UBP27), Gateway entry clones of the coding sequences of UBPs (Atg51710, UBPI0, Atg510590, UBPI2, Atg439170, UBPI8, Atg43670, UBPI8, Atg439790, UBPI2, Atg439370) were obtained from ABRC, and the ORF was transferred into pExTagYFP (MPI Cologne). The coding sequences of UBPs (Atg39910, UBPI4, Atg222340, UBP7, Atg321280, UBP9, Atg405700, UBP22, Atg5109700, UBP23, Atg579990, UBP24, Atg438900, UBP25, Atg314400, OTU9, Atg404250, TAGYFP (MPI Cologne) to generate 35S-promotor driven YFP-fusion constructs pKV17 (UBP3), pKV9 (UBP4), pKV19 (UBP7), KV5/KV6 (UBP9), KV13/KV14 (UBP22), KV15/KV16 (UBP23), CG3/C4 (UBP24), Kv17/Kv18 (UBP25), CG3/CG4 (OTU9), CG17/CG18 (OTU10), CG17/CG18 (OTU12), and the PCR products were cloned via the Gateway entry vector pDONR 207 (Thermo Fisher Scientific) into the Gateway destination vector pExTagYFP (MPI Cologne) to generate 35S-promotor driven YFP-fusion constructs pKV17 (UBP3), pKV9 (UBP4), pKV19 (UBP7), KV5/KV6 (UBP9), KV13/KV14 (UBP22), KV15/KV16 (UBP23), CG3/C4 (UBP24), Kv17/Kv18 (UBP25), CG17/CG18 (OTU9), CG17/CG18 (OTU10), CG17/CG18 (OTU12), and the PCR products were cloned via the Gateway entry vector pDONR 207 (Thermo Fisher Scientific) into the Gateway destination vector pExTagYFP (MPI Cologne).

Unless otherwise specified, all **OTU11** constructs are based on the splice variant **OTU11.2**. For 35S:Spro:GF-OTU11 (pKV30) and 35S:Spro:GF-OTU12 (pKV31), the coding sequences of **OTU11** and **OTU12** were cloned into pFastBac, and the ORF of **OTU12** was transferred to a pExTagYFP-vector, in which the GFP was replaced with TagRFP. To generate 35S:Spro:OTU12-RFP (pKV214) the ORF of **OTU12** was amplified from cDNA with primer pairs CBG25/CG26 (UBP9), CBG27/CG28 (UBP4), KV1/KV2 (UBP7), KV5/KV6 (UBP9), KV13/KV14 (UBP22), KV15/KV16 (UBP23), CBG3/C4 (UBP24), KV17/KV18 (UBP25), CBG3/CG4 (OTU9), CBG5/CG6 (OTU10), CBG7/CG18 (OTU11), CBG9/CG20 (OTU12). The PCR products were cloned via the Gateway entry vector pDONR 207 (Thermo Fisher Scientific) into the Gateway destination vector pExTagYFP (MPI Cologne) to generate 35S-promotor driven YFP-fusion constructs pKV17 (UBP3), pKV9 (UBP4), pKV19 (UBP7), KV5/KV6 (UBP9), KV13/KV14 (UBP22), KV15/KV16 (UBP23), CG3/C4 (UBP24), KV17/KV18 (UBP25), CBG3/CG4 (OTU9), CBG5/CG6 (OTU10), CBG7/CG18 (OTU11), CBG9/CG20 (OTU12). The ORF of **SYPI21** (Atg318200) was amplified with primers KV274/KV275 and cloned via the Gateway entry vector pDONR 207 (Thermo Fisher Scientific) into the Gateway destination vector pExTagYFP (MPI Cologne).
and cloned between the BamHI/SalI sites of pGEX-6P1 (Sigma Aldrich). To introduce an active site mutation in KV363 and cloned between the EcoRI/SalI sites of pGEX-6P1 (Sigma Aldrich). To generate OTU domain of OTU11 was amplified using primers KV380/KV381 to yield pKV195. To generate the active site cysteine was mutated to alanine (C112A) with primers KV380/KV381 or with primers LBb1.3/FA134. For OTU12m (pKV216), the ORF was amplified with primers CG46/KV104 and after selection, homozygous OTU12m mutant plants with homozygous PIN2-GFP expression were identified. To generate GFP-fusion lines for OTU11 and OTU12, wild-type Arabidopsis plants (Col-0) or OTU12m plants were transformed with plasmids by the Agrobacteria-mediated floral dip method. For the root length assays, seedlings were grown in long day (16 h light/8 h dark) conditions on ½ MS medium for 5–7 days. The length of the primary roots was measured with the freehand tool in Fiji. Significance was tested using t test in Excel (two-tailed, no equal variance), and P values less than 0.05 was considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001).

Microscopy

The localization of the fluorophore fusion proteins was analyzed with a confocal laser microscope LSM 880 with Airyscan (Zeiss). Proto-plastation of Arabidopsis root cell culture and protoplast transformation were conducted as described previously. For confocal microscopy, Arabidopsis seedlings were grown vertically for 5 to 7 days. For drug treatments, seedlings were incubated in ½ MS liquid media supplemented with 50 µM brefeldin A (BFA, Sigma Aldrich) for 60 min, 33 µM Wortmannin (WM, Merck) for 90 min, or 60 µM phe-nylsine oxide (PAO, WVR) for 40 min at room temperature. FM4-64 was diluted to 2 µM in ½ MS liquid media, and seedlings were incubated at room temperature. For the dark treatment, seedlings were incubated for 6 h in the dark at 21 °C. GFP-, YFP-, and RFP-fusion proteins were analyzed using the 63x/1.40 PlanaPOCHromot (Oil) objective and the 488 nm, 514 nm, and 560 nm laser for excitation, respectively. Images were obtained with 4× line averaging. To quantify fluorescence signals, Fiji was used.

Recombinant protein purification and DUB assays

GST-fused proteins were purified using Glutathione Magnetic Agarose Beads (Thermo Fischer Scientific) or Protino™ Glutathione Agarose 4B (Machery-Nagel) following the manufacturer’s instructions. For DUB assays with recombinant DUBs, 7.5 pmol to 250 pmol of OTU11 or OTU12 was mixed with the indicated amounts K63-linked tetra-UB (Boston Biochem) in 15 µl DUB assay buffer [50 mM Tris (pH 7.2), 25 mM KCl, 5 mM MgCl₂, 2 mM DTT] and incubated at 21 °C for 6 h. To test the activation by liposomes, 50 µl of 3.57 pmol/µl GST-OTU11 and GST-OTU11(6A1) in liposome buffer were mixed with 50 µl of liposomes with and without PIP₃, pre-incubated for 15 min at room temperature, and 14 µl of the mixture was incubated with 1 µl of 25 pmol/µl tetra-UB of Ko(5), K1(1), K2(9), K3(3), or K48 linked tetra-UB (Boston Biochem, K48: Enzo Life Sciences) for 4 h at room temperature.

For DUB assays with GFP-OTU11, GFP-OTU11 was immunoprecipitated from 5 g of 10-day-old Arabidopsis seedlings. The seedlings were frozen in liquid nitrogen and ground with a pestle and mortar. The resulting powder was mixed with 500 µl of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 2 mM DTT, 0.5 mM EDTA, and 0.1% Nonidet P-40. The extract was centrifuged at 13,000 × g for 15 min and the supernatant was mixed with 4 µl of a 1:1 mixture of anti-GFP magnetic agarose beads and anti-GST magnetic agarose beads. After washing, the protein-containing beads were treated with active and heat-inactivated A Protein Phosphatase (NEB) for 1 h at 30 °C. After the phosphatase treatment, the beads were washed with 150 µl of K63-linked di-UB (Boston Biochem) for 4 h at 21 °C in 15 µl of DUB assay buffer.

DUB assays were terminated by adding 5 µl of 5xSDS sample buffer [310 mM Tris-HCl (pH 6.8), 50% Glycerol, 10% SDS, 0.5% bromphenol blue, 3.5% B-mercaptoethanol]. Samples were resolved on NuPAGE™ Bis-Tris Mini Protein Gels (4–12%) (Thermo Fischer Scientific) and analyzed by gel staining or immunoblotting with an anti-UBP4(DI)

Plant material and growth conditions

Arabidopsis seedlings were surface sterilized with 1% NaOCl, stratified at 4 °C in the dark for 1–3 days, and grown on ½ MS (2.15 g/L Murashige & Skoog (MS) medium including vitamins (Duchefa), 250 mg/L MES, pH 5.8) or MS with sucrose (pH 5.8) (4.3 g/L Murashige & Skoog medium including vitamins (Duchefa), 250 mg/L MES, 1% Sucrose) under long day (16 h light, 8 h dark) or continuous light conditions at 21 °C for 5–10 days as indicated for each experiment. To generate otu1otu12 double mutants, the T-DNA insertion mutants otu1 (SALK05296) and otu2 (SALK13251) were crossed with each other and double-homozygous lines were identified by genotyping. For genotyping otu1 (SALK05296), the genomic fragment was amplified with primers FA134/135, and the T-DNA insertion was confirmed with primer pairs LBB1.3/FA134. For otu2 (SALK13251), the genomic fragment was amplified with primers FA130/131 and the T-DNA insertion was confirmed with primers LBB1.3/FA131. The PIN2pro:PIN2-GFP line in Col-0 background was described previously. PIN2pro:PIN2-GFP was crossed with otu1otu2 and after selecting, homozygous otu1otu2 mutant plants with homozygous PIN2-GFP expression were identified. To generate GFP-fusion lines for OTU11 and OTU12, wild-type Arabidopsis plants (Col-0) or otu1otu2 plants were transformed with plasmids by the Agrobacteria-mediated floral dip method. For the root length assays, seedlings were grown in long day (16 h light/8 h dark) conditions on ½ MS medium for 5–7 days. The length of the primary roots was measured with the freehand tool in Fiji. Significance was tested using t test in Excel (two-tailed, no equal variance), and P values less than 0.05 was considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001).
antibody (Santa Cruz). For fluorescence-based DUB assays, 3.75 pmol of K48-linked di-UB FRET TAMRA (post and pos2) or K63-linked di-UB FRET TAMRA (pos) (Biotechnne) were mixed with an equimolar amount of recombinant DUBs and incubated in the TAMRA assay buffer [20 mM Tris (pH 7.5), 100 mM NaCl, 2 mM DTT, 0.1 mg/ml BSA] with or without liposomes. The fluorescence was measured in a flat-bottom 384-well plate in an Infinium™-PLEX plate reader (Tecan) using the 540 nm excitation- and 590 nm emission filters. Di-UB FRET TAMRA (5-Carboxytetramethylrhodamin) is a Förster resonance energy transfer (FRET)-based substrate for the monitoring of DUB activity. In the di-UB substrate, one of the ubiquitin moieties carries a donor fluorophore, Rhodamine, and the other one an acceptor fluorophore, TAMRA. The two fluorophores interact with each other by FRET in the uncleaved molecule. The cleavage of di-UB FRET TAMRA releases the TAMRA residue, which leads to an increase in fluorescence at 590 nm upon excitation at 540 nm.

Immunoblotting, protein staining, and PM enrichment

Immunoblotting was conducted using the following primary antibodies: anti-H+-ATPase (5000× diluted, Agrisera, AS07260), anti-UcGase (3000× diluted, Agrisera, AS05066), anti-SEC21 (1000× diluted, Agrisera, AS05327), anti-GFP (1000× diluted, 3H9, Chromobiotic, 3189-100), anti-RFP (1000× diluted, Sigma Aldrich, GT1610), anti-UB (500–1000× diluted, P4D1, Santa Cruz, sc-8017), anti-ACTIN (50× diluted, JLA20, Sigma Aldrich), and anti-FLAG (1000× diluted, M2, Sigma Aldrich, F3594). Polyclonal OTU11- and GST-antibodies were raised in rabbits using full-length OTU11 or GST, as an antigen, respectively (Eurogentec). OTU11- and GST-specific antibodies were purified from the rabbit serum first with Protein A Agarose (Thermo Fisher Scientific) and subsequently using recombinant OTU11- or GST-immobilized NHS-activated agarose (Thermo Fisher Scientific), respectively. Both antibodies were diluted 1000×. The anti-OTU11 antibody did not detect endogenous OTU11 however, could detect overexpressed GFP-OTU11 or purified OTU11 in in vitro experiments. Primary antibodies were verified by comparison with the molecular weight marker and comparison with appropriate negative controls. All antibodies that were not generated for this study were used as recommended by the manufacturer. The anti-GST and anti-OTU11 antibody that were generated in this study were used as specified in “Methods” and the Reporting Summary. As secondary antibodies, anti-rat-HRP (80,000× diluted, Roche, A9037), anti-mouse-HRP (80,000× diluted, Sigma Aldrich, A9044), anti-rabbit-HRP (80,000× diluted, Sigma Aldrich, A0543), anti-mouse IgG DyLight 488 (5000× diluted, Thermo Fisher Scientific, 35503), and anti-rabbit IgG DyLight 650 (5000× diluted, Thermo Fisher Scientific, 45456) were used, and protein bands were detected in an Amersham™ Imager 600 (Cytiva). Protein gels were stained with Coomassie brilliant blue R250, TCE and SYPRO Ruby Protein Gel Stain (Thermo Fisher Scientific) and analyzed in an Amersham™ Imager 600 (Cytiva). Protein enrichment was carried out according to a previously published protocol. To analyze the phosphorilation status of proteins, proteins extracted from total plant extracts by immunoprecipitation were treated with λ Protein Phosphatase (NEB) and analyzed using Pro-Q™ Diamond Phosphoprotein Gel Stain (Thermo Fisher Scientific) according to the manufacturer’s instructions with a Typhoon™ laser-scanner (Cytiva). Protein bands were quantified using the Amersham™ Imager 600 software (Cytiva) or Fiji. Uncropped and unprocessed pictures of the gels and western blots are shown in the Source Data File.

Lipid overlay assays

Lipid overlay assays were conducted using PIP Strips™ Membranes (Thermo-Fisher) following the instructions of the manufacturer. PIP Strips™ Membranes were blocked for 6 h with 3% fatty acid-free BSA at room temperature and incubated with 1 μg/μl GST-fusion proteins for 1 h at room temperature or overnight at 4 °C. The binding of the GST-fused proteins was analyzed using an anti-GST antibody.

Liposome preparation and sedimentation assay

Lipid vesicles were prepared as described previously. For the liposome sedimentation assay, freshly prepared liposomes (80% PC and 20% PE or 75% PC, 20% PE, and 5% PIPs) were mixed with 5 μg of recombinant protein and incubated for 15 min at room temperature in a liposome buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM MgCl₂, 1 mM DTT, 50 mM sucrose]. The mixture was subsequently centrifuged for 25 min in a Beckman TL120.1 rotor at 100,000 × g at 4 °C. The supernatants were collected and mixed with 5xSDS sampling buffer. The pellets were dissolved in liposome buffer and mixed with 5xSDS sampling buffer. The proteins in the supernatant and the pellet were analyzed by SDS-PAGE and TCE staining. The gels were imaged using an Amersham Image 600 (GE Healthcare) and quantified using Fiji.

Genotyping, RT-PCR, and quantitative real-time PCR

The insertion was confirmed with a genotyping PCR using the left border primer LB0L3 and gene-specific primers (OTU11: FA134/FA135, FA130/FA131, FA132/FA131). To examine the expression level of OTU11 and OTU12, total DNA was isolated using the NucleoSpin RNA Plant Kit (Macherey-Nagel) followed by cDNA synthesis using M-MULV Reverse Transcriptase (New England Biolabs). The cDNA was analyzed in a RT-PCR reaction with gene-specific primers TB04/TB05 for OTU11, TB24/ TB25 OTU12 and TB24/εGFP r for OTU12-GFP. SYNTAXIN 121 (SYN121) primers KV274/KV275 were used as a control. The expression levels of 35Spro:GFP-OTU11 and 35Spro:GFP-OTU12 were analyzed by quantitative real-time PCR (qRT-PCR) with primers KV470/KV471 and KV472/ KV473, respectively. ACTIN2 primers ACT2 fw/ACT2 rv were used as a control.

D Dephosphorylation assay

The phosphorylation status of the proteins was analyzed by the addition of Phos-tag™ Acrylamide (Fujifilm/Wako) to an SDS-PAGE separating gel (7.5% acrylamide, 50 μM Phos-tagTM, 36 μM MnCl₂) as described elsewhere.

Circular dichroism spectroscopy

Circular dichroism spectra were recorded with a J815 spectrometer (Jasco) at room temperature using a quartz cell with a path length of 0.1 cm. Six scans were accumulated at a scanning speed of 100 nm/s and a wavelength interval of 0.1 nm. Spectra were recorded from 180 to 300 nm, and the average of 3–6 independent measurements was calculated. Secondary structure analysis was performed with DichroWeb using the CDSSRT analysis with the reference dataset S0P18007. CDSSRT analysis assigns an overall helical content of 57% for the protein with a normalized root-mean-square deviation (NRMSD) of 0.004. For the protein with PI(4,5)P₂-containing liposomes, the secondary structure analysis provides an overall helical content of 44% with a NRMSD of 0.002. A NRMSD value ≤0.01 is rated as suitable for CDSSRT analysis.

Molecular dynamics simulation studies

All molecular dynamics simulations were obtained by GROMACS version 2021.4. We used the CHARMM36 [46,47] forcefield for the OTU11 (alpha fold [48] model) and lipids together with the tip3p water model [49]. The force-field parameters for the system have been obtained from input generator tools in CHARMM-GUI [50] using Membrane Builder [51]. The wild-type OTU11Δ(1-21) protein simulations were preceded with a 500 ns long bulk water equilibration. All variants, two
different initial setups were performed: one where the PBMII motif was already placed close to a membrane patch containing inositol lipids and one with the protein placed more distantly from the membrane in the bulk water. The membrane water system was built as hexagonal box and consisted of a phospholipid bilayer POPE/POPC/SAP24 (SAP24 - PI(4,5)P₂ protonated on P4) with 56/210/14 lipids, respectively, for simulations with OTU11(Δ21), and the distantly placed OTU12(OTU). For the OTU12(OTU) close to the membrane, lipids in the ratio of 45/180/12 were used. The box height was set to 13 nm in order to allow the protein to be able to reside fully in bulk water without contact to the membrane. The box was solvated with TIP3P water and neutralized with sodium chloride together with an additional concentration of 150 mM, resulting in 22,147 up to 29,399 water molecules, 138 to 167 sodium ions and 89 to 109 chloride ions.

The simulation setup followed the protocol from the membrane builder. The following settings have been applied. The leapfrog integrator was utilized together with hydrogen bonds being constrained by the LINCS algorithm in order to enable a time-step of 2 fs. A modified cutoff for short-ranged electrostatic and Lennard Jones interactions of 1.2 nm was used, where a switching function was applied to smoothly approach the cutoff between 1.0 and 1.2 nm. Long-range Coulomb interactions were calculated by particle mesh Ewald method. The neighbor list was updated every 10 steps. Initially, all systems were energy minimized with the steepest-descent algorithm for 5000 steps with position restraints for the protein and position and dihedral restraints for the lipids (protein: 4000 kJ/mol nm² for backbone atoms, 2000 kJ/mol nm² for sidechain atoms, lipids: 1000 kJ/mol nm² and 1000 kJ/mol rad² for lipids). In the next step, six consecutive equilibration simulations followed. First two in a canonical (NVT) and next four in the microcanonical (NPT) ensemble. For the first three with a reduced timestep of 1 ps and 125 ps length and the latter three with a 2 ps timestep and 500 ps length.

The position and dihedral restraints are reduced stepwise from step 1 starting with the restraints applied in the energy minimization and reducing the forces by 50% in every step for the position restraints. Dihedral restraints started from 1000 kJ/mol rad² to 400 kJ/mol rad² and then by 50% until no restraints are applied in the last step of the equilibration. The temperature during the equilibration steps was maintained at 298.15 K by using the Berendsen thermostat with a coupling time of 1 ps. The systems were simulated in a semi-isotropic ensemble. The pressure was set to 1 atm using the Nose-Hoover thermostat and the equilibration. During the production runs, the temperature and pressure was maintained with the Nose-Hoover thermostat and the Parrinello-Rahman barostat using the same coupling times as before. The length of the production runs were 2000 ns for the protein placed closely to the membrane and 1000 ns for the protein placed more distantly from the membrane. The protocol of the bulk simulation consisted of energy minimization with position restraints on the protein (400 kJ/mol nm² for backbone atoms, 40 kJ/mol nm² for sidechain atoms) followed by a 100 ps long NVT and subsequent NPT run with isobaric pressure coupling with position restraints and finally a 500 ns long NPT simulation without position restraints.

Analysis of molecular dynamics simulations
To describe the interaction strength of the OTU11 protein with the membrane, we calculated the following features. The number of hydrogen bonds, contacts, salt bridges, and their strength and residue-wise probability. For the calculation of the number of hydrogen bonds and contacts between OTU11 variants and the lipid membrane, we used the gromacs hbond tool applying the default settings for the number of H-bonds between both groups and for contacts among all atoms within a plain cutoff of 0.35 nm. The salt bridges were calculated with MDAnalysis by counting all contacts within 0.4 nm between nitrogen atoms from the sidechains of lysine and arginine and the charged oxygens of the phosphate groups of the PI(4,5)P₂. Reported are the timelines and the corresponding distributions together with the probability of a residue to be in contact with PI(4,5)P₂ and the average number of contacts per residue. RMSD and RMSF values for the Cα alpha carbons were calculated to describe the structural properties of the protein, and pairwise distances between residues of the catalytic triad was calculated to characterize the catalytic center. Simulation snapshots were generated with VMD. Further data that support the findings of this study and all biological materials used in this study are available from the corresponding author upon reasonable request.

### References

1. Paez Valencia, J., Goodman, K. & Otegui, M. S. Endocytosis and endosomal trafficking in plants. Annu. Rev. Plant Biol. 67, 309–335 (2016).
2. Mosesso, N., Nagel, M. K. & Isono, E. Ubiquitin recognition in endocytic trafficking—with or without ESCRT-O-J. Cell Sci. 132, jcs22868 (2019).
3. Komander, D. & Rape, M. The ubiquitin code. Annu. Rev. Biochem. 81, 203–229 (2012).
4. Vierstra, R. D. The expanding universe of ubiquitin and ubiquitin-like modifiers. Plant Physiol. 160, 2–14 (2012).
5. Majumdar, P. & Nath, U. De-ubiquitylases on the move: an emerging field in plant biology. Plant Biol. (Stuttg.) 22, 563–572 (2020).
6. March, E. & Farrona, S. Plant deubiquitinases and their role in the control of gene expression through modification of histones. Front. Plant Sci. 8, 2274 (2017).
7. Mewissen, T. E. T. & Komander, D. Mechanisms of deubiquitinase specificity and regulation. Annu. Rev. Biochem. 86, 159–192 (2017).
8. Clague, M. J., Urbe, S. & Komander, D. Breaking the chains: deubiquitylating enzyme specificity begets function. Nat. Rev. Mol. Cell Biol. 20, 338–352 (2019).
9. Isono, E. & Nagel, M. K. Deubiquitylating enzymes and their emerging role in plant biology. Front. Plant Sci. 5, 56 (2014).
10. Hermanns, T., Pichlo, C., Baumann, U. & Hofmann, K. A structural basis for the diverse linkage specificities within the ZUFSP deubiquitinase family. Nat. Commun. 13, 401 (2022).
11. Yan, N., Doelling, J. H., Falbel, T. G., Durski, A. M. & Vierstra, R. D. The ubiquitin-specific protease family from Arabidopsis. AtUBP1 and 2 are required for the resistance to the amino acid analog canavanine. Plant Physiol. 124, 1828–1843 (2000).
12. Zhou, H., Zhao, J., Cai, J. & Patil, S. B. UBIQUITIN-SPECIFIC PROTEASES function in plant development and stress responses. Plant Mol. Biol. 94, 565–576 (2017).
13. Wu, R. et al. Protein partners of plant ubiquitin-specific proteases (UBPs). Plant Physiol. Biochem. 145, 227–236 (2019).
14. Radjacomarre, R., Usharani, R., Kuo, C. H. & Fu, H. Distinct phylogenetic relationships and biochemical properties of Arabidopsis ovarian tumor-related deubiquitinases support their functional differentiation. Front. Plant Sci. 5, 84 (2014).
16. Keren, I. & Citovsky, V. The histone deubiquitinase OTL1 targets euchromatin to regulate plant growth. *Sci. Signal.* 9, ra125 (2016).

17. Keren, I., Lacroix, B., Kohrman, A. & Citovsky, V. Histone deubiquitinase OTU1 epigenetically regulates DA1 and DA2, which control Arabidopsis seed and organ size. *iScience* 23, 100948 (2020).

18. Yen, M. R. et al. Deubiquitinating enzyme OTU5 contributes to DNA methylation patterns and is critical for phosphate nutrition signals. *Plant Physiol.* 175, 1826–1838 (2017).

19. Suen, D. F. et al. The deubiquitinating enzyme OTU5 regulates root responses to phosphate starvation. *Plant Physiol.* 176, 2441–2455 (2018).

20. Luo, Y. et al. Deubiquitinating enzymes UBPI2 and UBPI3 stabilize the brassinosteroid receptor BRI1. *EMBO Rep.* 23, e53354 (2022).

21. An, Z. et al. Regulation of the stability of RGF1 receptor by the ubiquitin-specific proteases UBPI2/UBPI3 is critical for root meristem maintenance. *Proc. Natl Acad. Sci. USA* 115, 1123–1128 (2018).

22. McCullough, J., Clague, M. J. & Urbe, S. AMSH is an endosome-associated ubiquitin isopeptidase. *J. Cell Biol.* 166, 487–492 (2004).

23. Liu, Z. et al. The ubiquitin-specific protease USP2a prevents endocytosis-mediated EGFR degradation. *Oncogene* 32, 1660–1669 (2013).

24. Funakoshi, Y., Chou, M. M., Kanaho, Y. & Donaldson, J. G. TRE1/USP6 regulates ubiquitylation and trafficking of cargo proteins that enter cells by clathrin-independent endocytosis. *J. Cell Sci.* 127, 4750–4761 (2014).

25. Row, P. E., Prior, I. A., McCullough, J., Clague, M. J. & Urbé, S. The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation. *J. Biol. Chem.* 281, 12618–12624 (2006).

26. Savio, M. G. et al. USP8X controls EGFR fate by deubiquitinating the endocytic adaptor Eps15. *Curr. Biol.* 26, 173–183 (2016).

27. Cui, X. et al. Ubiquitin-specific proteases UBPI2 and UBPI3 act in circadian clock and photoperiodic flowering regulation in Arabidopsis. *Plant Physiol.* 162, 897–906 (2013).

28. Nazareth, A. et al. DET1-mediated degradation of a SAGA-like deubiquitination module controls H2Bub homeostasis. *eLife* 7, e37892 (2018).

29. Noack, L. C. & Jaillais, Y. Precision targeting by phosphoinositides: how PI3 directs endomembrane trafficking in plants. *Curr. Opin. Plant Biol.* 40, 22–33 (2017).

30. Pan, R., Kaur, N. & Hu, J. The Arabidopsis mitochondrial membrane-bound ubiquitin protease UBP27 contributes to mitochondrial morphogenesis. *Plant J.* 78, 1047–1059 (2014).

31. Deubiquitination of BES1 by UBP12/UBP13 promotes brassinosteroid signaling and plant growth. *Plant Commun.* 3, 100348 (2022).

32. Martins, S. et al. Internalization and vacuolar targeting of the brassinosteroid hormone receptor BRI1 are regulated by ubiquitination. *Nat. Commun.* 6, 6151 (2015).

33. Kasai, K., Takano, J., Miwa, K., Toyoda, A. & Fujiwara, T. High boron-induced ubiquitination regulates vacuolar sorting of the BOR1 protein from the human pathogen Orientia tsutsugamushi modulates host membrane trafficking by multiple mechanisms. *Mol. Cell Biol.* 42, e000722 (2022).

34. Katsiarimpa, A. et al. The Arabidopsis deubiquitinating enzyme AMSH3 interacts with ESCRT-III subunits and regulates their localization. *Plant Cell* 23, 3026–3040 (2011).

35. Majeran, W., Le Caer, J. P., Ponnala, L., Meinnel, T. & Giglione, C. Targeted profiling of Arabidopsis thaliana subproteomes illuminates Co- and posttranslationally N-terminal myristoylated proteins. *Plant Cell* 30, 543–562 (2018).

36. Breska, H., Guag, J., Remmert, K., Chacko, S. & Korn, E. D. An experimentally based computer search identifies unstructured membrane-binding sites in proteins: application to class I myosins, PAKs, and CARMIL. *J. Biol. Chem.* 285, 5728–5747 (2010).

37. Herberst, S. et al. Artificial ubiquitylation is sufficient for sorting of a plasma membrane ATPase to the vacuolar lumen of Arabidopsis cells. *Planta* 236, 63–77 (2012).

38. Schink, K. O., Tan, K.-W. & Stemmark, H. Phosphoinositides in control of membrane dynamics. *Annu. Rev. Cell Dev. Biol.* 32, 143–171 (2016).

39. Huang, O. W. et al. Phosphorylation-dependent activity of the deubiquitinating enzyme DUBA. *Nat. Struct. Mol. Biol.* 19, 171–175 (2012).

40. Seki, T. et al. JosD1, a membrane-targeted deubiquitinating enzyme, is activated by ubiquitination and regulates membrane dynamics, cell motility, and endocytosis*. *J. Biol. Chem.* 288, 17145–17155 (2013).

41. Zhang, Z. et al. Acetylation-dependent deubiquitination of OTU3 controls MAVS activation in innate antiviral immunity. *Mol. Cell* 79, 304–319.e307 (2020).

42. Huttin, J. et al. IkappaB kinase beta phosphorylates the K63 ubiquitinase NEMO, PAKS, and CARMIL. *Proc. Natl Acad. Sci. USA* 115, 2027 (2018).

43. Watz, I. E. et al. Phosphorylation and linear ubiquitin direct A20 inhibition of inflammation. *Nature* 528, 370–375 (2015).

44. Zhao, Y. et al. OTUD4 is a phospho-activated K63 deubiquitinase that regulates MyD88-dependent signaling. *Mol. Cell* 69, 505–516.e508 (2018).

45. Küberl, T., Kitao, T., Ando, H. & Nagai, H. LotA, a Legionella deubiquitinase, has dual catalytic activity and contributes to intracellular survival, membrane dynamics, cell motility, and endocytosis*. *Nature* 579, 409–414 (2020).

46. Berk, J. M., Lee, M. J., Zhang, M., Lim, C. & Hochstrasser, M. OtDUB a plasma membrane ATPase to the vacuolar lumen of Arabidopsis thaliana upregulates ER- and Golgi-based protein export*. *Plant Physiol.* 176, 1826–1838 (2017).

47. Simon, M. L. et al. A multi-colour/multi-affinity marker set to visualize phosphoinositide dynamics in Arabidopsis. *Plant J.* 77, 332–347 (2014).

48. Majeran, W., Le Caer, J. P., Ponnala, L., Meinnel, T. & Giglione, C. Targeted profiling of Arabidopsis thaliana subproteomes illuminates Co- and posttranslationally N-terminal myristoylated proteins. *Plant Cell* 30, 543–562 (2018).

49. Breska, H., Guag, J., Remmert, K., Chacko, S. & Korn, E. D. An experimentally based computer search identifies unstructured membrane-binding sites in proteins: application to class I myosins, PAKs, and CARMIL. *J. Biol. Chem.* 285, 5728–5747 (2010).

50. Herberst, S. et al. Artificial ubiquitylation is sufficient for sorting of a plasma membrane ATPase to the vacuolar lumen of Arabidopsis cells. *Planta* 236, 63–77 (2012).

51. Leitner, J. et al. Lysine63-linked ubiquitylation of PIN2 auxin carrier protein governs hormonally controlled adaptation of Arabidopsis root growth. *Proc. Natl Acad. Sci. USA* 109, 8322–8327 (2012).

52. Heilmann, I. Phosphoinositide signaling in plant development. *Development* 143, 2044–2055 (2016).

53. Jumper, J. et al. Highly accurate protein structure prediction with AlphaFold. *Nature* 596, 583–589 (2021).

54. Mergner, J. et al. Mass-spectrometry-based draft of the Arabidopsis proteome. *Nature* 579, 409–414 (2020).

55. Schink, K. O., Tan, K.-W. & Stemmark, H. Phosphoinositides in control of membrane dynamics. *Annu. Rev. Cell Dev. Biol.* 32, 143–171 (2016).

56. Wang, Z. et al. Acetylation-dependent deubiquitination of OTU3 controls MAVS activation in innate antiviral immunity. *Mol. Cell* 79, 304–319.e307 (2020).
borate transporter in Arabidopsis thaliana. J. Biol. Chem. 286, 6175–6183 (2011).
57. Barberon, M. et al. Monoubiquitin-dependent endocytosis of the iron-regulated transporter 1 (IRT1) transporter controls iron uptake in plants. Proc. Natl Acad. Sci. USA 108, E450–E458 (2011).
58. Yin, X. J. et al. Ubiquitin lysine 63 chain forming ligases regulate apical dominance in Arabidopsis. Plant Cell 19, 1889–1911 (2007).
59. Konstantinova, N. et al. WAVY GROWTH Arabidopsis E3 ubiquitin ligases affect apical PIN sorting decisions. Nat. Commun. 13, 5147 (2022).
60. Shin, L. J. et al. IRT1 degradation factor1, a ring E3 ubiquitin ligase, regulates the degradation of iron-regulated transporter1 in Arabidopsis. Plant Cell 25, 3039–3051 (2013).
61. Zhou, J. et al. Regulation of Arabidopsis brassinosteroid receptor BRII endocytosis and degradation by plant U-box PUB12/PUB13-mediated ubiquitination. Proc. Natl Acad. Sci. USA 115, E1906–e1915 (2018).
62. Lu, D. et al. Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. Science 332, 1439–1442 (2011).
63. Shimada, T. L., Shimada, T. & Hara-Nishimura, I. A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of Arabidopsis thaliana. Plant J. 61, 519–528 (2010).
64. Binder, A. et al. A modular plasmid assembly kit for multigene expression, gene silencing and silencing rescue in plants. PLoS ONE 9, e86218 (2014).
65. Wang, Z. P. et al. Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. Genome Biol. 16, 144 (2015).
66. Nagel, M. K., Vogel, K. & Isono, E. Transient expression of ESCRT components in Arabidopsis root cell suspension culture-derived protoplasts. Methods Mol. Biol. 1988, 163–174 (2019).
67. Abas, L. et al. Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. Nat. Cell Biol. 8, 249–256 (2006).
68. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).
69. Hameed, D. S., Sapmaz, A. & Ovaa, H. How chemical synthesis of ubiquitin conjugates helps to understand ubiquitin signal transduction. Bioconjugate Chem. 28, 805–815 (2017).
70. Ladner-Keay, C. L., Turner, R. J. & Edwards, R. A. Fluorescent protein visualization immediately after gel electrophoresis using an in-gel trichloroethanol photoreaction with tryptophan. Methods Mol. Biol. 1853, 179–190 (2018).
71. Collins, C. A., Leslie, M. E., Peck, S. C. & Heese, A. Simplified enrichment of plasma membrane proteins from Arabidopsis thaliana seedlings using differential centrifugation and Brii58 treatment. Methods Mol. Biol. 1564, 155–168 (2017).
72. Blume, J. J., Halbach, A., Behrendt, D., Paulsson, M. & Plomann, M. EHD proteins are associated with tubular and vesicular compartments and interact with specific phospholipids. Exp. Cell Res. 313, 219–231 (2007).
73. Vogel, K. & Isono, E. Detection of phosphorylation on immunoprecipitates from total protein extracts of Arabidopsis thaliana seedlings. Methods Mol. Biol. 2177, 169–182 (2020).
74. Miles, A. J., Ramalli, S. G. & Wallace, B. A. DichroWeb, a website for calculating protein secondary structure from circular dichroism spectroscopic data. Protein Sci. 31, 37–46 (2022).
75. Compton, L. A. & Johnson, W. C. Jr. Analysis of protein circular dichroism spectra for secondary structure using a simple matrix multiplication. Anal. Biochem. 155, 155–167 (1986).
76. Manavalan, P. & Johnson, W. C. Jr. Variable selection method improves the prediction of protein secondary structure from circular dichroism spectra. Anal. Biochem. 167, 76–85 (1987).
77. Abdul-Gader, A., Miles, A. J. & Wallace, B. A. A reference dataset for the analyses of membrane protein secondary structures and transmembrane residues using circular dichroism spectroscopy. Bioinformatics 27, 1630–1636 (2011).
78. Abraham, M. J. et al. GROMACS: high performance molecular simulations through multi-level parallelism from laptops to supercomputers. Softw. Pract. Exp. 1–2, 19–25 (2015).
79. Pronk, S. et al. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. Bioinformatics 29, 845–854 (2013).
80. Bjelkmar, P., Larsson, P., Cuenedt, M. A., Hess, B. & Lindahl, E. Implementation of the CHARMM force field in GROMACS: analysis of protein stability effects from correction maps, virtual interaction sites, and water models. J. Chem. Theory Comput. 6, 459–466 (2010).
81. Huang, J. et al. CHARMM36m: an improved force field for folded and intrinsically disordered proteins. Nat. Methods 14, 71–73 (2017).
82. Klauda, J. B. et al. Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. J. Phys. Chem. B 114, 7830–7843 (2010).
83. Venable, R. M. et al. CHARMM all-atom additive force field for sphingomyelin: elucidation of hydrogen bonding and of positive curvature. Biophys. J. 107, 134–145 (2014).
84. Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W. & Klein, M. L. Comparison of simple potential functions for simulating liquid water. J. Chem. Phys. 79, 926–935 (1983).
85. Brooks, B. R. et al. CHARMM: the biomolecular simulation program. J. Comput. Chem. 30, 1545–1614 (2009).
86. Lee, J. et al. CHARMM-GUI input generator for NAMD, GROMACS, AMBER, OpenMM, and CHARMM/OpenMM simulations using the CHARMM36 additive force field. J. Chem. Theory Comput. 12, 405–413 (2016).
87. Wu, E. L. et al. CHARMM-GUI Membrane Builder toward realistic biological membrane simulations. J. Comput. Chem. 35, 1997–2004 (2014).
88. Jo, S., Lim, J. B., Klauda, J. B. & Im, W. CHARMM-GUI Membrane Builder for mixed bilayers and its application to yeast membranes. Biophys. J. 97, 50–58 (2009).
89. Jo, S., Kim, T. & Im, W. Automated builder and database of protein/membrane complexes for molecular dynamics simulations. PLoS ONE 2, e880 (2007).
90. Lee, J. et al. CHARMM-GUI Membrane Builder for complex biological membrane simulations with glycolipids and lipoproteins. J. Chem. Theory Comput. 15, 775–786 (2019).
91. Van Gunsteren, W. F. & Berendsen, H. J. C. A Leap-frog algorithm for stochastic dynamics. Mol. Simul. 1, 173–185 (1988).
92. Hess, B., Bekker, H., Berendsen, H. J. C. & Fraaije, J. G. E. M. LINCS: a linear constraint solver for molecular simulations. J. Comput. Chem. 18, 1463–1472 (1997).
93. Essmann, U. et al. A smooth particle mesh Ewald method. J. Chem. Phys. 103, 8577–8593 (1995).
94. Berendsen, H. J. C., Postma, J. P. M., Gunsteren, W. F. V., DiNola, A. & Haak, J. R. Molecular dynamics with coupling to an external bath. J. Chem. Phys. 81, 3684–3690 (1984).
95. Nosé, S. A molecular dynamics method for simulations in the canonical ensemble. Mol. Phys. 52, 255–268 (1984).
96. Hoover, W. G. Canonical dynamics: equilibrium phase-space distributions. Phys. Rev. A 31, 1695–1697 (1985).
97. Parrinello, M. & Rahman, A. Polymorphic transitions in single crystals: a new molecular dynamics method. J. Appl. Phys. 52, 7182–7190 (1981).
98. Michaud-Agrawal, N., Denning, E. J., Woolf, T. B. & Beckstein, O. MDAnalysis: a toolkit for the analysis of molecular dynamics simulations. J. Comput. Chem. 32, 2319–2327 (2011).
Acknowledgements
The authors would like to thank Franziska Anzenberger (Technical University of Munich) and Lea Held (University of Konstanz) for earlier contributions to the work, Sina Geißler (University of Konstanz) for preliminary MD simulations of OTU11, Swen Schellmann (University of Cologne) for the PMA-GFP-UB construct and Jiří Friml (Institute of Science and Technology Austria) for the PIN2pro:PIN2-GFP line. We thank the BioImaging Center Core facility and the Botanical Garden of the University of Konstanz and the High Performance and Cloud Computing Group at the Zentrum für Datenverarbeitung of the University of Tübingen and the state of Baden-Württemberg (bwHPC). The work in the authors’ laboratories is supported by grants from the German Science Foundation (IS 221/6-1 to E.I., INST 37/935-1 FUGG to C.P.).

Author contributions
K.V. and E.I. designed the study and analyzed the experiments, and K.V. performed most of the experiments. T.B. has carried out cloning and biochemical analyses. M.-K.N. has performed protoplast transformations and M.-K.N. and E.I. performed confocal imaging and analysis together with K.V. C.G. performed the initial localization study of DUBs in protoplasts. M.K. prepared the panel with structural models of OTU11 and OTU12. S.M., L.M., and K.H. performed the CD spectroscopic analysis and C.G. and C.P. conducted the molecular dynamics simulation analysis. K.V. and E.I. wrote the manuscript with the input of all other authors.

Funding
Open Access funding enabled and organized by Projekt DEAL.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-34637-3.

Correspondence and requests for materials should be addressed to Erika Isono.

Peer review information Nature Communications thanks Hongyong Fu, Barbara Korbei and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permissions information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022