Function of deubiquitylating enzymes in the selective degradation of plasma membrane proteins in *Arabidopsis thaliana*

Doctoral thesis for obtaining the academic degree Doctor of Natural Sciences (Dr. rer. nat.)

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Universität Konstanz
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Konstanz, 2022
date of the oral examination: June 03, 2022

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Zusammenfassung


Abstract

Plants are sessile organisms that need to tightly regulate the abundance of plasma membrane (PM) proteins to react to different environmental stimuli. As ubiquitylation is the key signal for selective protein degradation, the stability of PM proteins is regulated by ubiquitylating as well as deubiquitylating enzymes (DUBs). In *Arabidopsis thaliana*, the metalloprotease DUBs AMSH1 and AMSH3 influence the stability of PM proteins. The exact nature of the proteins accumulating in the *amsh1* and *amsh3* mutants is, however, unknown. To expand the knowledge about AMSH function, potential AMSH1 targets were identified with an affinity purification and subsequent MS analysis. As we assumed that AMSH1 targets carry a K63-linked ubiquitin chain, proteins were purified with an ubiquitin-binding construct from *amsh1* and wild-type seedlings. To identify potential AMSH1 targets, *amsh1* and wild-type ubiquitylomes were compared. The observation that potential target proteins were enriched both in the wild-type and *amsh1* background indicates that AMSH1 could stabilize some proteins whereas it destabilizes others. AMSH1 seems to be versatile DUB with different regulatory functions in the Arabidopsis cell.

Selective protein degradation pathways consist of various steps that need to be tightly regulated by ubiquitylation and deubiquitylation. So it is likely that there are multiple Arabidopsis DUBs which can influence the stability of PM proteins. The DUBs OTU11 and OTU12 are localized at the PM in Arabidopsis root cells and could therefore influence the selective degradation of PM proteins. OTU11 and OTU12 bind directly to PM-localized phosphatidylinositol phosphates (PIPs) *in vitro*. As the weak *in vitro* activity of OTU11 against K63-linked ubiquitin was enhanced by binding to liposomes, the catalytic activity of OTU11 seems to be tightly connected to its membrane localization. Furthermore, OTU11 and OTU12 had an influence on the root length of Arabidopsis seedlings, stabilized the artificial endocytosis substrate PMA-GFP-UB at the PM, and had an effect on the endocytic degradation of the PM protein PIN2-GFP. Altogether, the experimental data shows that OTU11 and OTU12 are PM localized DUBs which could have fine-tuning effect on the abundance of PM proteins in Arabidopsis and identified a novel DUB function in the selective protein degradation pathways of PM proteins in Arabidopsis.
1. Introduction

Plants are sessile organisms whose survival depends on their ability to sense and adapt to different environmental factors, such as nutrient availability, biotic and abiotic stress factors or light availability. This process is mediated by plasma membrane (PM)-localized receptor- and transporter proteins (Figure 1). Phytohormones and various developmental processes also influence the abundance of PM proteins. In order to effectively coordinate these processes, plants need to control the abundance of plasma membrane proteins by selective protein degradation (Vierstra, 2012; Isono and Kalinowska, 2017).

1.1. The abundance of PM proteins is regulated by selective protein degradation

Selective protein degradation is mediated by three main pathways, namely the ubiquitin proteasome system, endocytic-, and autophagic degradation (Vierstra, 2012). PM proteins are mostly degraded by endocytosis (Isono and Kalinowska, 2017) whereas Cytosolic proteins are degraded by the proteasome or by autophagy, and organelles are degraded by autophagy (Marshall and Vierstra, 2018).

1.1.1. Selective protein degradation is regulated by ubiquitylation

The key signal for selective protein degradation is ubiquitylation. Ubiquitin binds covalently to a lysine (K) in the target protein with its C-terminus. It can also be conjugated to other ubiquitin molecules at seven different lysines (K48, K63, K29, K33, K27, K11 and K6). Ubiquitin can also form linear (M1) chains with a connection between the N-terminal methionine of the first ubiquitin and the C-terminal glycine of the second ubiquitin molecule. Ubiquitin can form longer chains with different linkage patterns including mixed- and branched chains. Alternatively, proteins can be mono-ubiquitylated in that they are modified with one or more single ubiquitin molecules (Komander and Rape, 2012).

Different ubiquitin chain types have different functions in the cell (Figure 2). In yeast, about 29 % of the ubiquitin chains are K48-linked, 17 % are K63-linked and 28 % are K11-linked ubiquitin chains. All other chain types are less abundant (K6: 11 %, K27: 9 %, K29: 3 % and K33: 3 %) (Komander, 2009; Xu et al., 2009). K48- and K63-linked ubiquitin chains have been extensively studied in various model organisms.
The mono-ubiquitylation of histones is important for the transcription control (Uckelmann and Sixma, 2017). K6 and K27 are involved in DNA damage response in human cells. K33 is implicated in post-Golgi trafficking. The function of K11-linked ubiquitin chains is not well studied. However, it is known that they are important for cell cycle control in human cells. K6 and K27 are involved in DNA damage response in human cells. K33 is implicated in post-Golgi trafficking (Akutsu et al., 2016). Both K33 and K29-linked ubiquitin might be involved in the regulation of autophagy in human cells (Feng et al., 2019). K48-linked chains, especially long K48-linked chains, are the main signal for proteasomal degradation. K63-linked chains are best known to be the signal for endocytosis. Both K63- and K48-linked chains are also involved in autophagy (Zaffagnini et al., 2018). K48- and K63-linked chains are involved in regulatory pathways, e.g. DNA damage response (Fernández-Majada et al., 2016). Whereas K48-linked chains influence the stability of regulatory pathway components, K63-linked chains can also have different functions, e.g. facilitating the binding of interactors (Romero-Barrios and Vert, 2018). Linear chains are important for the NF-kB mediated inflammatory response (Elliott and Komander 2016).

Since protein stability is directly influenced by ubiquitylation, the ubiquitylation status of the proteins must be tightly controlled. The ubiquitylation of target proteins is catalyzed by the Ubiquitin activating enzyme (E1), Ubiquitin-conjugating enzyme (E2) and the Ubiquitin ligase (E3). E1 activates the ubiquitin by the ATP-dependent formation a thioester bond with the C-terminal glycine of ubiquitin. The ubiquitin is then transferred to a cysteine residue in E2, and E2 binds to E3 which mediates the ligation of ubiquitin to the target protein.
Figure 2: Ubiquitylation is a key signal for many processes in eukaryotic cells. K48-linked ubiquitin chains are involved in proteasomal degradation and autophagy. K63-linked ubiquitin chains are involved in endocytic degradation processes, autophagy, regulatory processes and DNA damage response. Mono-ubiquitylation is involved in transcription control. Linear (M1) and K11-ubiquitin chains are involved in regulatory processes, K6 and K27-linked ubiquitin in DNA damage response, K6- and K29-linked chains in autophagy, and K33-linked chains in autophagy and Golgi-trafficking processes.

The interplay between E2, E3, and the target proteins leads to a huge specificity in the ubiquitylation process. Especially important for the specificity is the large number of E3 ligases. In Arabidopsis, there are two E1s, 37 E2s whereas there are about 1500 E3s (Vierstra, 2012). Another layer of specificity is added by the reversibility of ubiquitylation. Ubiquitin chains can be removed or shortened by deubiquitylating enzymes (DUBs) (Clague et al., 2015; Mevissen and Komander, 2017).

1.1.2. PM proteins are degraded by endocytic degradation

PM proteins are degraded by the Endosomal Sorting Complexes Required for Transport (ESCRT)-mediated endocytic degradation pathway. Endocytosed PM proteins are guided by ESCRT-I, ESCRT-II, and ESCRT-III via endosomes to the vacuole where they are degraded. Proteins targeted for degradation are recognized by ESCRTs through their ubiquitin modifications. In order to rescue ubiquitin from degradation, the ubiquitin residues are removed prior to the internalization into the multivesicular bodies (MVBs). In mammals,
ubiquitylated cargo proteins are recognized first by ESCRT-0. In plants, this step is mediated by other adaptor proteins such as the TOM1-like (TOL) proteins (Isono and Kalinowska, 2017; Mosesso et al., 2019). Prior to their endocytic degradation, PM proteins are internalized by clathrin-mediated endocytosis. In clathrin-mediated endocytosis, clathrin-coated vesicles containing the cargo proteins are invaginated and cleaved off from the PM. Dynamin and the Adaptor protein complex (AP-2) are important for this step (Kaksonen and Roux, 2018). The cargo proteins are then bound by ESCRT-I which binds to the ubiquitin chain with its subunit VPS23. ESCRT-I recruits ESCRT-II which binds to the ubiquitin chain by an UEV domain and to the endosomal membrane by a PI(3)P-binding GLUE domain (Vietri et al., 2020). ESCRT-III is recruited by ESCRT-II and sequentially forms polymers with its subunits. The membrane invagination and the scission of intraluminal vesicles is induced by polymer disassembly and rearrangement processes mediated by the ATPase Protein Vacuolar protein sorting 4/SUPPRESSOR OF K(+) TRANSPORT GROWTH DEFECT 1 Vps4/SKD1 (Pfitzner et al., 2020). This process leads to the formation of MVBs. The MVBs transfer the cargo proteins to the vacuole in plants or the lysosome in mammals (Isono and Kalinowska, 2017; Vietri et al., 2020).

1.1.3. Protein aggregates are degraded by autophagy

Macromolecular complexes and organelles that are marked with ubiquitin can be degraded by autophagy. In microautophagy, cytoplasmic material aggregates at the tonoplast from where they are directly internalized to the vacuole. In macroautophagy, cytoplasmic material is internalized first in a double membrane autophagosome. Autophagosomes are formed from Endoplasmic reticulum (ER)-derived phagophores that engulf the cytoplasmic material. The autophagosome then fuses with the vacuole. The inner vesicle is released into the vacuole, where it is degraded. There are multiple AUTOPHAGY-RELATED PROTEINS (ATGs) involved in macroautophagy. The ATG proteins form a cascade with multiple steps (Marshall and Vierstra, 2018). The ubiquitin-like protein ATG8/ Microtubule-associated protein 1A/1B-light chain 3 (LC3) is linked with the lipid-anchor polyethanolamine (PE) to the phagophore. Autophagic cargo proteins bind via their ATG8-interacting motifs (AIM) or Ubiquitin-interacting motif (UIM)-like (UIM) motifs to ATG8, or they are tethered by ATG8-binding adaptor-proteins to the phagophore membrane (Marshall et al., 2019). ATG7 is an E1-like, ATG3 is an E2-like and the ATG12-5–ATG16-like 1 (ATG16L1) complex an E3-like protein for the lipidation of ATG8. To enable the lipidation, ATG8 is processed by the DUB-like enzyme ATG4 (Marshall and Vierstra, 2018). The most prominent function of macroautophagy is the degradation of organelles.
Individual proteins and protein aggregates are also degraded by autophagy (Marshall and Vierstra, 2018).

1.2. DUBs influence the ubiquitylation status of proteins

As ubiquitylation is a key signal for selective degradation of PM proteins, the ubiquitylation status of target proteins must be tightly controlled by ubiquitylating enzymes and DUBs. DUBs can either bind directly to target proteins or to the ubiquitin chain. Exo-DUBs cleave off ubiquitin residues at the N-terminus of the ubiquitin chain and Endo-DUBs cleave at positions between the N-terminal and the C-terminal ubiquitin molecule (Figure 3 A). DUBs can have different chain-type and length preferences. The specificity of DUBs is determined by their ubiquitin binding sites. The ubiquitin-site that binds to the N-terminal ubiquitin or the distal ubiquitin molecule is called the S1 site. The proximal C-terminal ubiquitin is bound to the S1’ site. The binding of the ubiquitin to the S1 and S1’ sites positions the iso-peptide bond in close proximity to the active site to enable the cleavage reaction (Figure 3 B, (Mevissen et al., 2016)). DUBs can have additional ubiquitin binding sites in or outside of the catalytic domain which can contribute to the specificity of the DUBs. Additional ubiquitin binding sites can be also work in trans when they are provided by another protein (Mevissen and Komander, 2017). The ubiquitin binding sites determine the specificity and activity of DUBs. An example for an enzyme whose specificity depends on ubiquitin binding sites in the active domain is shown for human OTUD2. OTUD2 has three ubiquitin binding sites, a S1, S1’, and S2 site, within the active OTU domain that lead to a preference for K11-linked tri-ubiquitin chains. An example for a DUB with an S1’ site outside of the active domain is human OTUD1. The UBA domain of OTUD1 is important for its specificity for K63-linked ubiquitin (Mevissen et al., 2013).

There are seven families of DUBs in humans. The ubiquitin-specific proteases (USPs or UBPs), ovarian tumor proteases (OTUs), ubiquitin C-terminal hydrolases (UCHs), the Machado-Joseph domain (MJD) domain proteases, the JAB1/MPN/MOV34 (JAMM) proteases, the motif interacting with Ub-containing (MINDY) proteases, and the zinc finger containing ubiquitin peptidase 1 (ZUP1). All groups, except for the metalloprotease JAMM proteases, are cysteine proteases (Clague et al., 2019). The active center of the cysteine DUBs contains a cysteine residue which acts as nucleophile and attacks the carbon of the iso-peptide bond.
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Figure 3: Cysteine- and Metalloprotease DUBs cleave the iso-peptide bond between two ubiquitin molecules.
A) DUBs cleave ubiquitin chains in the middle (Endo-DUB) or at the end (Exo-DUBs) or they remove ubiquitin molecules (blue) from target proteins (gray).
B) Complex of the OTU domain of the human DUB OTUD7B in complex with its substrate K11-linked di-ubiquitin (Mevissen et al., 2016). PDB ID: 5LRV. Red: active site, turquoise: S1 site, pink: S1’ site, light blue: distal ubiquitin (N-terminal), blue: proximal ubiquitin (C-terminal).
C) The active center of a cysteine DUB (adapted from (Mevissen and Komander, 2017).
D) The active center of a metalloprotease DUB (adapted from (Mevissen and Komander, 2017).

Furthermore, it contains a histidine residue which stabilizes the cysteine in a de-protonated form and an aspartate residue which stabilizes the positively charged histidine (Figure 3 C) (Mevissen and Komander, 2017). Instead of an aspartate, there can also be another hydrogen-bond forming residue, e.g. a serine (Abdul Rehman et al., 2021). The JAMM proteases are zinc dependent metalloproteases. The active site of the zinc-metalloproteases consists of a Zn$^{2+}$-ion forming a complex with conserved residues of the protein and a water molecule. The water molecule acts as a nucleophile and attacks the iso-peptide bond (Figure 3 D) (Mevissen and Komander, 2017).

1.2.1. DUBs have various functions in Arabidopsis

In Arabidopsis there are about 50 different DUBs. The largest groups in Arabidopsis are the UBP and OTU DUBs (Isono and Nagel, 2014). In addition to UBPs and OTUs, there are JAMM, MJD, and UCH DUBs in Arabidopsis. Potential homologs of MINDY proteases
and ZUP1 can be found in the Arabidopsis genome, though it is not clear if they are functional significant (Isono and Kalinowska, 2017).

Arabidopsis UBPs are involved in various cellular processes such as histone deubiquitylation (UBP26/12/13), gametogenesis (UBP3/4), plant immunity (UBP12/13), embryo development (UBP14), cell cycle regulation (UBP15/16), flowering time regulation (UBP26/12/13/15, OTLD1), proteasomal degradation (RPN10), mitochondrial morphogenesis (UBP27), abscisic acid signaling (UBP24), salt tolerance (UBP16), organ size regulation (UBP14/15), embryogenesis (UBP9/12/13/14/19/26), and mitochondria morphology (UBP27) (Isono and Nagel, 2014; March and Farrona, 2017; Zhou et al., 2017; Wu et al., 2019). UBP14 has also been shown to be important for the auxin response (Majumdar et al., 2020). UBP12 and UBP13 have been shown to influence the ubiquitylation status of the transcription factor PIF7 that is involved in shade avoidance response (Zhou et al., 2021). Recently, it was also shown that UBP12 and UBP13 have a function outside of the nucleus and that they regulate the ubiquitylation status and the degradation of the PM-localized brassinosteroid receptor BR insensitive 1 (BR1) (Luo et al., 2022).

The Arabidopsis OTU family is described in section 1.2.2.

1.2.2. The OTU family is conserved among eukaryotes

OTUs are cysteine protease DUBs that were first identified in an ethyl-methanesulfonate (EMS) screen with *Drosophila* as the cause for the tumor-like growth of ovulates (King and Storto, 1988). OTU domains are conserved among eukaryotes and are also present in viruses (Makarova et al., 2000; Clague et al., 2019). Furthermore, OTU domain were found in human pathogenic bacteria (Schubert et al., 2020). The domain composition of OTU domain containing proteins is very diverse and seems to be only conserved among closely related species (Figure 4 A).
Figure 4: OTU DUBs are found in eukaryotes and prokaryotic pathogens. 
A) Scheme of the domain structure of HsOTUD5, ScOTU1 and E. albertii OTU. Domain (gray) and active site (red) positions are taken from Uniprot, the phosphorylation sites (violet) from PhosPhAt 4.0. 
B) Experimentally determined structures of the HsOTUD5 domain (3TMP), the ScOTU1 OTU domain (3BY4) and the EschOTU OTU domain (6W9S).

The structure of the OTU domains is also diverse. This is especially obvious when the OTU domain of HsOTUD5 consisting of six α-helices and four β-sheets is compared with that of ScOTU1 consisting of six α-helices and eight β-sheets (Figure 4 B). Interestingly, the OTU domain of *Escherichia albertii*, a human pathogen, is more similar to the human OTU than to the yeast OTU domain, as it was probably copied from its human host. The diversity in the sequences and the structures might lead to a broad functional diversity as it has been shown for human OTUs (Mevissen et al., 2013).

1.2.2.1. OTU activity and function is influenced by many factors

The activation mechanisms of human DUBs have been extensively studied due to the therapeutic target potential of human OTUs (Mevissen and Komander, 2017). As described before, ubiquitin binding sites within and outside of the active OTU domain influence the chain type specificity of the OTUs. Ubiquitin binding domains can be also provided by another protein (Mevissen et al., 2013) (Figure 5 A).

Another well-known factor influencing the activity of OTUs are post-translational modifications such as phosphorylation and acetylation. Human OTUD5 is activated by
phosphorylation (Figure 5 B). (Huang et al., 2012; Mevissen et al., 2013). Another example is the human OTU7C / Putative DNA-binding protein A20 whose activity against the Ubiquitylated target protein Nuclear factor (NF)-κB essential modulator (NEMO) in the NF-κB pathway is enhanced by phosphorylation (Hutti et al., 2007). The phosphorylation also changes the chain type specificity of A20. Without phosphorylation A20 cleaves K48-linked tetra-ubiquitin, with phosphorylation K63-linked tetra-ubiquitin (Wertz et al., 2015). A similar mechanism has been described for OTUD4 which cleaves K48-linked ubiquitin chains in the un-phosphorylated state and K63-linked chains in the phosphorylated state (Zhao et al., 2018). Tumor necrosis factor receptor-associated factor-binding domain-containing protein (TRABID), a member of the A20-family, is also activated by phosphorylation (Feng et al., 2019; Zhu et al., 2019).

Human OTUD3 is activated by acetylation. The acetylation of a lysine residue enhances the activity of OTUD3 against K63-linked ubiquitin chains (Figure 5 C) (Zhang et al., 2020b). The interaction with activating proteins can also influence the OTU activity (Figure 5 D). The binding of E2 enzymes enhances, for example, the activity of human OTUB1 against K48-linked di-ubiquitin (Wiener et al., 2013). Another factor that could potentially influence the DUB activity of OTU proteins is differential splicing (Figure 5 E).

Figure 5: The activity of human OTU DUBs is influenced by different factors.
A) OTU DUB activity and chain type specificity is influenced by ubiquitin binding sites in the OTU domain, in different parts of the same protein (cis) or other proteins (trans). Adapted from (Mevissen and Komander, 2017).
B) +C) OTU activity can be influenced by different kinds of post-translational modifications: phosphorylation (B) or acetylation (C).
C) OTU activity can be influenced by the interaction with other proteins.
D) OTU activity might be influenced by differential splicing.
E) OTU-DUBS can have an additional, non-catalytic function in the nucleus.
For UBPs, however, there are several examples from different organisms, where differential splicing of the transcript influences the localization of the protein. The changes in the localization pattern have a direct influence on the cellular function. The impact on the catalytic function was, however, not analyzed (Schmitz et al., 2005; Leznicki et al., 2018). It is likely, that a similar mechanism exists for OTUs. A possible candidate might be human OTUD6B. OTUD6B-1 represses protein synthesis in cancer cells and OTUD6B-2 promotes it. The observation that catalytic site mutants could not complement the OTUD6B phenotype suggests that the catalytic activity is important for OTUB6 function. However, the direct connection between DUB activity and the different isoforms has not been analyzed (Sobol et al., 2017). Furthermore, there are some OTU DUBs which have a non-catalytic function in addition to their catalytic function in DNA stress responses (Figure 5 F). Human OTUB1 represses the function of the E3-Ligase RNF168 in response to DNA double strand breaks independently of its catalytic function (Nakada et al., 2010), and human OTUD4 enhances the DNA alkylation damage response independent of its catalytic function (Zhao et al., 2015). As the catalytic activity and the cellular function of OTUs is influenced by many different factors, OTUs are able to fulfill various tasks in the cell.

1.2.2.2. Arabidopsis OTU1, OTU5 and OTLD1 function in the nucleus

In *Arabidopsis thaliana*, there are twelve OTU DUBs with different preferences for the most abundant ubiquitin chain types (K48/K63/linear) *in vitro*. For example, OTU1 preferentially cleaves linear and K48-linked tetra-ubiquitin, and OTU4c cleaves linear, K48- , and K63-linked tetra-ubiquitin. OTU9 and OTU10 cleave both K48- and K63-linked, yet not linear tetra-ubiquitin. OTU3, OTU4c, OTU7a, OTU9, and OTU10 were also able to cleave off a single ubiquitin from GST. There was, however, no visible activity against Small ubiquitin like modifier (SUMO)-GST. OTU12 and OTU11.1 had no activity *in vitro*, although they were expressed on the mRNA level in roots, stems, rosette leaves, cauline leaves, flower clusters, and green siliques (Radjacommare et al., 2014).

Although the three so far characterized Arabidopsis OTUs have a different domain structure, they were all implicated in transcriptional control (Figure 6). OTLD1/OTU6 is a histone deubiquitylase and binds to chromatin, where it promotes the removal of acetylation, ubiquitylation and methylation marks. This leads to the induction of the transcription of major plant growth and development regulator genes (Krichevsky et al., 2011; Keren and Citovsky, 2016).
OTU1 represses the transcription of the *DA1/DA2* genes by the deubiquitylation of *DA1/DA2* chromatin (Keren et al., 2020), and OTU5 influences the methylation of genes in phosphate starvation conditions (Yen et al., 2017; Suen et al., 2018). OTU1 is also reported to be involved in the ER-associated protein degradation pathway (Zang et al., 2020). As both OTU1 and OTLD1 influence plant growth and development, they are essential components of the plant cell. *otu1* knock-down mutants have a smaller leaf, rosette, stem and seed size and the seeds germinated slower than the wild-type seeds (Keren et al., 2020). The phenotype connected to OTLD1 was less severe. The knockout of *OTLD1* led to no visible phenotype. However, the *OTLD1* overexpression led to enhanced plant growth and cell size (Krichevsky et al., 2011; Keren and Citovsky, 2016). OTU5 had no influence on Arabidopsis growth under normal conditions. However, it is important for the phosphate starvation response (Yen et al., 2017; Suen et al., 2018). Although the characterized OTUs influence plant growth and development, none of the characterized Arabidopsis OTUs is essential for plant growth and development. This might indicate that OTUs have a fine-tuning function that helps the plant to adapt to different environmental conditions.

### 1.2.2.3. OTUs in other organisms have functions inside and outside of the nucleus

Many pathogenic bacteria and viruses contain OTU DUBs which disturb cellular defense mechanisms of the host-system by inhibiting the ubiquitin-mediated degradation of proteins in the host cells (van Kasteren et al., 2012). There are also OTUs in fungi. In *S. cerevisiae* there are two OTU DUBs. ScOtu1 which cleaves K48-linked yet not K63-linked multi-ubiquitin chains *in vitro*, protects multi-ubiquitylated target proteins from Cell division cycle protein 48 (CDC48) mediated proteasomal degradation (Rumpf and Jentsch, 2006).
The function of ScOtu2 has not been analyzed. The two OTU DUBs of the fission yeast *Schizosaccharomyces pombe* which are homologs of the *S. cerevisiae* OTUs, localize to the nucleus and cytosol (SpOtu1) and the cytosol (SpOtu2). SpOtu1 and SpOtu2 were not active *in vitro* against the fluorogenic DUB substrate Ubiquitin-7-amino-4-methylcoumarin (Ub-AMC), yet in a proteomic approach, SpOTU1 co-purified with CDC48. So they are probably involved in the proteasomal degradation of target proteins (Kouranti et al., 2010).

In humans, there are 16 OTU DUBs comprising four phylogenetic groups: the A20-like OTUs, the Otubains (OTUB), the OTUDs and the Otulin-like OTUs (Komander et al., 2009; Mevissen et al., 2013). Human OTU DUBs have various functions. There are OTUs that function in the nucleus and are involved in transcription control. OTUB1 (Stanišić et al., 2009) and OTUD1 (Piao et al., 2017) stabilize transcription factors. OTUD1 also reduces the non-proteolytic K63-linked poly-ubiquitination of the transcriptional co-activator Yes-associated protein (YAP) (Yao et al., 2018). OTUD1 is also able to cleave the viral infection induced K6-linked ubiquitination of the IFN regulatory factor 3 (IRF3), which leads to the removal of IRF3 from the promoter region and the reduction of the inflammatory response (Zhang et al., 2020a).

Human OTUs can have functions both inside and outside of the nucleus. In the nucleus, OTUD4 enhances the DNA alkylation damage response independent of its catalytic function (Zhao et al., 2015). In the cytosol, it is found in RNA-stress granules (Das et al., 2019) and stabilizes the NF-kB pathway component Myeloid differentiation primary response 88 (MyD88) by its K63-directed deubiquitylase activity (Zhao et al., 2018).

Outside of the nucleus human OTUs are involved in various processes. OTUs are known to influence function of components of the ubiquitylation machinery, and thereby the stability of regulatory complexes. OTUD5, also called Deubiquitinating enzyme A (DUBA), inhibits the production of the immune defense factor type I interferon by counteracting the K63-ubiquitin chain assembling E3 protein Tumor necrosis factor receptor-associated factor 3 (TRAF3) (Kayagaki et al., 2007). OTU7B/Cezanne, a K11-specific DUB, influences the stability of TRAF3 (Mevissen et al., 2016), and OTUB1 influences the stability of the Ubiquitin-conjugating enzyme E2 E1 (UBE2E1) (Pasupala et al., 2018). Human OTUs are also involved in selective protein degradation processes. The TRABID activity against K29 and K33-linked ubiquitin chains might, for example, stabilize the autophagy regulator UV radiation resistance associated (UVRAG) (Feng et al., 2019). The human homologue of ScOtu1, YOD1/OTUD2, is part of complex with the Valosin-containing protein (VCP), and has been implicated in the VCP-mediated dislocation of misfolded proteins from the ER to the proteasome (Ernst et al., 2009). Furthermore, human
OTUs are involved in mitosis and the rearrangement of the cytoskeleton. During mitosis, the A20-like OTU DUB VCP/p47-complex-interacting protein (VCPIP) influences the VCP-mediated reassembly of Golgi membranes during mitosis (Wang et al., 2004). OTUD6A influences the stability or the activity of the mitotic Aurora Kinase A (Kim and Kim, 2021) and the stability of Dynamin related protein 1 (DRP1) which affects the mitochondrial fission in cancer cells (Shi et al., 2020).

1.3. DUB influence on membrane protein abundance

The ubiquitylation status of a protein is the key signal for its degradation. Therefore, various DUBs are involved in the endocytic degradation of PM proteins and the degradation of proteins in intracellular membranes by autophagy. Golgi- and ER resident DUBs might regulate the transport and maturation of PM proteins (Figure 7).

1.3.1. AMSH influences endocytic degradation processes

The Arabidopsis metalloprotease DUBs AMSH1 and AMSH3 (ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM) are involved in endocytic- and autophagic protein degradation (Isono et al., 2010a; Katsiarimpa et al., 2013; Katsiarimpa et al., 2014). The amsh3 knockout mutants are seedling lethal, have defects in vacuole morphology, and protein trafficking (Isono et al., 2010a). The knock-down of AMSH1 is less severe and under normal growth conditions amsh1 grows as wild type. amsh1 mutants are defective in autophagy, pathogen defense, and amsh1/amsh1 AMSH3/amsh3 are dwarfish (Katsiarimpa et al., 2013). Both mutants accumulate ubiquitylated proteins. In amsh3 mutants, the ubiquitylated proteins accumulate in the membrane fraction. AMSH1 and AMSH3 function is necessary for the pathogen induced endocytosis of the membrane receptor protein CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) (Katsiarimpa et al., 2014). Furthermore, the active MPN+ domain of AMSH1 preferentially cleaves K63-linked poly-ubiquitin in vitro (Katsiarimpa et al., 2013). We therefore assume that AMSH1 and AMSH3 influence the stability of membrane proteins. However, the exact nature of the proteins that accumulate in the amsh1 and amsh3 mutants is not known.
Figure 7: Various eukaryotic DUBs are involved in membrane-associated processes. Eukaryotic DUBs are involved in the endocytic degradation of PM proteins, autophagy or Golgi-ER-Trafficking processes.

Human AMSH and AMSH-LP have similar structure (Davies et al., 2011), localize to endosomes (Nakamura et al., 2006), and influence the level of the transcription factor gene, the Myc proto-oncogene protein (Kikuchi et al., 2003). Furthermore, AMSH preferentially cleaves K63-linked ubiquitin chains in vitro and stabilizes the endocytosis substrate EGFR at the PM (McCullough et al., 2004). The function of AMSH homologue in S. pombe Suppressor of ste12 deletion protein 2 (Sst2) has not been analyzed in detail. However, it is known to be localized at endosomes (Kouranti et al., 2010) and it has an influence on membrane transport (Beckley et al., 2015). In contrast to AtAMSH1, HsAMSH has not been implicated in autophagy (Jacomin et al., 2018).

1.3.2. Multiple cysteine protease DUBs influence the endocytic degradation processes

Plants need to control the abundance of PM proteins in order to adapt to different conditions in their environment. Ubiquitylation is a key signal for degradation of PM proteins and both ubiquitylating enzymes and DUBs are important to regulate the
ubiquitylation status of PM proteins. The AMSH proteins and UBP12 and UBP13 are reported to be involved in endocytic and autophagic degradation pathways in Arabidopsis (Isono et al., 2010a; Katsiarimpa et al., 2014; Luo et al., 2022). In other eukaryotic organisms there are multiple cysteine protease DUBs known to be important for the regulation of membrane trafficking processes. In *S. cerevisiae* which does not have an AMSH homolog, the endosomal DUB Doa4/UBP4 binds to the ESCRT-III subunits Sucrose non-fermenting protein 7 (Snf7) a and Vacuolar protein sorting (Vps20) and regulates membrane scission events at the endosomes (Johnson et al., 2017; Buysse et al., 2020). The human orthologue of Doa4, USP8/USPY binds to and deubiquitylates the ESCRT-III subunit Charged multivesicular body protein 1b (CHMP1B) (Crespo-Yañez et al., 2018). HsUSP6 localizes at endosomes and influences the abundance of PM proteins via clathrin-independent endocytosis (Martinu et al., 2004; Funakoshi et al., 2014). HsUSP32 localizes to the TGN and regulates late endosomal transport processes (Akhavantabasi et al., 2010; Sapmaz et al., 2019). The membrane binding mechanism of both enzymes has not been analyzed. Other enzymes known to be involved in endocytosis in humans are USP2 (Liu et al., 2013), USP9X (Savio et al., 2016). The *C. elegans* DUB USP46 regulates together with WD40 repeat proteins the abundance of glutamate receptors on the cell surface (Hodul et al., 2021). HsUSP46 is activated *in vitro* by binding to a WD40 repeat protein (Faesen et al., 2011). Human Josephin -1 (JosD1) which consists only of its Josephin domain (except of 23 aa at the N-terminus) and has an influence on endocytosis localizes to the PM in HEK-293 cells. The membrane binding mechanism is unclear. However, there must in membrane binding site or protein-protein-interaction site in the DUB domain. There are potential polybasic motifs in the sequence (BH-Search Tool). Both the PM localization and the *in vitro* activity against K63-linked ubiquitin chains are enhanced by ubiquitination (Seki et al., 2013).

It is likely, that there are more DUBs which influence the endocytosis of PM proteins. In a localization study, 13 human DUBs were found to be localized at the PM (UBP6, UBP9X, UBP13, UBP38, UBP53, Ataxin-3-like protein (ATXN3L), 26S proteasome non-ATPase regulatory subunit 7 (PSDM7), YOD1, TRABID, Cezanne, VCP/IP, JosD1, JosD2). All of these DUBs could potentially influence the abundance of proteins at the PM. The same is true for the five *S. pombe* DUBs that influence membrane transport processes (UBP4, UBP5, UBP9, UBP15, and Sst2) (Beckley et al., 2015). All in all, the abundance of PM is influenced by multiple DUBs in different organism. Therefore, it is likely, that there are, in addition to AMSH1 and AMSH3, further cysteine protease DUBs involved in the endocytic degradation of PM proteins.
1.3.3. DUBs are involved in autophagy

Since ubiquitylation is also a signal for autophagic degradation, DUBs are also involved in autophagy (Jacomin et al., 2018). USP11 restricts autophagy in *C. elegans* (Basic et al., 2021) and cancer cells (Qiao et al., 2021). Human USP13 is reported to regulate autophagy by removing K48-linked ubiquitin chains from VPS34, a core protein of autophagy initiation (Xie et al., 2020). HsUSP8 is also reported to suppress autophagy (Peng et al., 2020). The human OTU DUB OTULIN counteracts the E3-Ligase LUBAC and stabilizes Atg13 by removing linear ubiquitin chains. Thus OTULIN promotes autophagy maturation while blocking autophagy initiation (Chu et al., 2021). The interaction of OTULIN with the plasma membrane bound Tumor necrosis factor receptor (TNFR) upon TNFα treatment is mediated by SNX27, a member of the sorting nexin family protein. This process enables OTULIN to cleave off linear ubiquitin chains from target proteins (Shi et al., 2021). The human OTU DUBs YOD1/OTUD2 (Papadopoulos et al., 2017), Cezanne/OTUD7B (Tian et al., 2021), and TRABID (Feng et al., 2019) have also been implicated in autophagy. As ubiquitylation is a key signal for autophagy, multiple DUBs are involved in the autophagic degradation of proteins in other organisms. In Arabidopsis, only AMSH1 has been described in autophagy.

1.4. Aim of the project

The aim of my project was to identify DUBs that are regulating the stability of PM proteins in *A. thaliana*. First, potential AMSH1 targets were identified with an affinity purification of ubiquitylated proteins from plant extract and subsequent Mass spectrometric (MS) analysis. As ubiquitylated target proteins are enriched in the plant extract of *amsh3* and *amsh1* mutants (Isono et al., 2010a; Katsiarimpa et al., 2013), we decided to purify the ubiquitylated proteins from wild-type and *amsh1* mutant seedlings and to compare the ubiquitylomes. To purify the ubiquitylated proteins from the plant extract, we used tandem affinity constructs with ubiquitin binding domains which have been successfully used to purify ubiquitylated proteins from Arabidopsis seedlings (Kim et al., 2013; Johnson and Vert, 2016) and have the additional advantage that they protect ubiquitin-protein-conjugates from degradation (Hjerpe et al., 2009). As the aim was to specifically enrich K63-linked protein-conjugates that are degraded by endocytosis, the GAT-domain of the ubiquitin adaptor protein TOL6 was used to generate an ubiquitin binding 2xGAT construct. After a single-step affinity purification with three technical replicates for the
amsh1 and Ler genotypes, the ubiquitylome was compared and potential targets were identified.

In the second part, I systematically analyzed the localization of DUBs and identified and characterized DUBs that contribute to the regulation of PM proteins stability. To find potential candidates, we did a localization study in Arabidopsis protoplasts and found OTU11 and OTU12 localized to the PM. The PM localization is a strong indication that the proteins are involved in the selective degradation of PM proteins. To elucidate the function of OTU11 and OTU12, their localization, the membrane binding mechanism, their catalytic activity, and their physiological function were analyzed. Potential interactors that might modulate the function of OTU11 and OTU12 were identified with a yeast two-hybrid screen and a GFP-IP with subsequent MS analysis. The weak in vitro activity of OTU11 and OTU12 was enhanced by liposome binding. *otu11otu12* mutants had slightly longer and OTU11 and OTU12 o/e lines had shorter primary roots than wild-type seedlings. The subtle root length phenotype might be caused by the influence of OTU11 and OTU12 on the endocytosis of the auxin transport PIN2 proteins. Altogether, the experiments conducted in this work added new insights to the existing knowledge about the function of DUBs in the selective protein degradation of plasma membrane proteins in *A. thaliana*. 
2. Results

2.1. Potential AMSH1 targets were identified by the purification of ubiquitylated proteins from amsh1 seedlings

The AMSH proteins AMSH1 and AMSH3 influence the stability of PM proteins, however, the nature of their target proteins is unknown. To find potential target proteins of AMSH1 in Arabidopsis, ubiquitylated proteins were purified from Ler and amsh1 seedlings and subjected to mass spectrometry analysis was performed.

2.1.1. GST-2xGAT binds to tetra-ubiquitin and ubiquitylated proteins

Tandem ubiquitin binding constructs were shown to efficiently enrich ubiquitylated proteins from plant extract. For the purification of ubiquitylated proteins a method was adapted from a previously published proteomics study using His-tagged ubiquitin and GST-TUBE (tandem repeated Ub binding entity, (Kim et al., 2013)).

As K63-linked ubiquitin chains have been shown to be a signal for endocytic degradation, AMSH targets are probably modified with a K63-linked ubiquitin chain. Therefore, the identification of AMSH substrates could be facilitated by a tandem ubiquitin binding construct that specifically binds to K63-linked ubiquitin. To achieve this goal, the ubiquitin binding GAT domain of the plant endocytosis protein TOL6 was cloned in tandem in an E. coli expression vector with a N-terminal GST-Tag (Steffi Klein and Marie-Kristin Nagel) (Figure 8 A+B). Surprisingly, the GST-2xGAT protein did not have any binding specificity in vitro. It bound to free linear, K48-, and K63-linked tetra-ubiquitin (Figure 8 C). It is also able to bind ubiquitylated proteins from the plant extract (Figure 8 D). The strong enrichment of ubiquitylated proteins with the GST-2xGAT construct makes it a useful tool for the purification of ubiquitylated proteins from the plant extract, even though it is not specific for K63-linked ubiquitin chains.
Figure 8: GST-2xGAT binds to free linear-, K48- and K63-linked tetra-ubiquitin chains in vitro and to ubiquitylated proteins in vivo.

A) Domain structure of the GST-2xGAT construct with the GAT domain of TOL6 (At2g38410). GST=Glutathione-S-transferase, GAT=GGA (Golgi-localized, gamma-ear containing, ADP-ribosylation-factor-binding protein) and TOM (target of Myb), TOL=TOM like protein.

B) Homology model of the TOL6 GAT domain with ubiquitin (template: PDB ID 1yd8, complex of the human GGA3 GAT domain with ubiquitin (Prag et al., 2005).

C) The ubiquitin binding domain construct (GST-2xGAT) was incubated with free linear, K48 and K63 tetra-ubiquitin chains. GST was used as a negative control. The GST-2xGAT construct binds to linear, K48 and K63 tetra-ubiquitin chains in vitro. Black arrowhead: GST-2xGAT, white arrowhead: GST.

D) The ubiquitin binding domain construct (GST-2xGAT) was incubated with extract from amsh1 seedlings. GST was used as a negative control. The GST-2xGAT construct binds to ubiquitylated proteins in the plant extract. Black arrowhead: GST-2xGAT, white arrowhead: GST.

2.1.2. 6His-UBQ/amsh1 lines were identified

To enable a His-tag purification of ubiquitin-protein conjugates from amsh1 and the dominant negative AMSH3-AXA line, both lines were crossed with a Col-0 line expressing a poly-His-Ubiquitin transgene (6His-UBQ) (Saracco et al., 2009), Figure 9 A) by Marie-Kristin Nagel. Homozygous amsh1 and AMSH3-AXA lines were identified by genotyping (Figure 9 B). amsh3 mutants were not used. They are unsuitable for large scale proteomic experiment due to their embryo lethality. The presence of the 6His-UBQ transgene was verified by genotyping (Figure 9 C) and an anti-Ubiquitin blot (Figure 9 D). However, the induction in the AMSH-AXA did not work reliably (Figure 9 E). Both amsh1 and 6His-UBQ
/ amsh1 lines accumulated ubiquitylated proteins in the plant extract (Figure 9 F) in comparison to wild-type seedlings. As there was a stable accumulation of ubiquitin-protein-conjugates in the 6His-UBQ / amsh1 seedlings and it is easy to obtain enough material from amsh1 seedlings, I decided to use them for the proteomic experiments.

Figure 9: amsh1 and 6His-UBQ / amsh1 seedlings accumulate ubiquitylated proteins.
A) The 6His-UBQ transgene consists of 6 tandem repeats of the ubiquitin coding region expressed under a 35S promoter. Each of the ubiquitin genes is N-terminally linked to a 6xHis-tag (adapted from (Saracco et al., 2009)).
B) Exemplary genotyping result of the 6His-UBQ / amsh1 and 6His-UBQ / AMSH3 AXA, showing the existence of the amsh1 TDNA insertion and the Dex-inducible AMSH3-AXA construct.
C) Exemplary genotyping result of the 6His-UBQ / amsh1 and 6His-UBQ / AMSH3 AXA, showing the existence of the His-UB transgene.
D) The ubiquitin western blot of Col-0 and 6His-UBQ / AMSH3 AXA seedling extract shows a second His-tagged Mono-ubiquitin in the extract of 6His-UBQ / AMSH3 AXA seedlings.
E) Ubiquitin- and AMSH3-blot of the total plant extract of 6His-UBQ / AMSH3 AXA seedlings treated with dexamethasone.
F) Ubiquitin and AMSH1 western blot of the total plant extract of wild-type, amsh1, and 6His-UBQ / amsh1 seedlings. Ubiquitylated proteins were accumulated in both amsh1 and 6His-UBQ / amsh1 seedlings.
2.1.3. Both the in gel as well as the in solution digest are suitable for the MS-analysis of affinity purified proteins

In the first large scale experiment I wanted to clarify if a two-step purification with an affinity purification and a Ni-NTA-purification was a suitable method for the identification of AMSH1 targets. The second goal of this experiment was to compare the in the gel and the in the solution method. For this, the total cell extract of 40 g of 6His-UBQ / amsh1 seedlings was mixed with GST-2xGAT. The eluate was then subjected to Ni-NTA-purification. To compare both methods, the eluates were treated with trypsin in the gel or in the solution and analyzed by mass spectrometry (Julia Mergner / Küster Lab, TUM) (Figure 10 A). Col-0 and 6His-UBQ / Col-0 was used as a negative control. Ubiquitylated proteins were successfully isolated from the plant extract (Figure 10 B). To analyze the proteomics data protein intensities were normalized with the iBAQ (intensity-based absolute quantification) method. For this, protein intensities are summed up by the MS software (Maxquant) and normalized by the protein size. This balances the effect that larger proteins are detected more often than smaller proteins. The normal distribution of the iBAQ intensities in the samples indicates that the MS data had a broad detection range (Figure 11 C).

![Diagram](image_url)

**Figure 10:** Ubiquitylated proteins were enriched from 6His-UBQ / amsh1 seedlings with a 2-step purification method.

A) Ubiquitylated proteins from whole cell extract of 10-day old 6His-UBQ / amsh1, 6His-UBQ / Col-0, and Col-0 seedlings were purified with a combination of an affinity purification step with the GST-2xGAT constructs and a His-purification step. The proteins in eluate 2 were digested in gel and in solution and analyzed by mass spectrometry (MS) to compare both methods by Julia Mergner in the Küster Lab (TUM).

B) Ubiquitin blot and silver staining gel of the tandem affinity purification. Ubiquitylated proteins are enriched in the eluates after the affinity purification step (eluate 1) and the Ni-NTA-step (eluate 2).
To analyze the proteomics data, protein intensities were normalized with the iBAQ (intensity-based absolute quantification) method. For this, protein intensities are summed up by the MS software (Maxquant) and normalized by the protein size. This balances the effect that larger proteins are detected more often than smaller proteins. The normal distribution of the iBAQ intensities in the samples indicates that the MS data had a broad detection range (Figure 11 C).

Figure 11: There were only small differences between the 6His-UBQ / amsh1 sample and the controls.
A) The iBAQ intensities of the proteins were distributed according to a normal distribution in both in gel and in solution samples.
B) The pairwise correlation of the proteins found in all samples showed a correlation of $R^2 > 0.850$ with both methods, indicating a high similarity between the samples.
C) In the in solution experiment, the two 6His-UBQ samples clustered together. In the in gel experiment, the two Col-0 controls clustered together.
D) The Venn diagrams of all proteins found in the samples showed that nearly all proteins were found in all three samples. In general, more proteins were found with the in solution approach (1327) than with the in gel approach (835).
As the protein intensities in all samples were very similar, there was a high correlation between the samples (Figure 11 B). The same trend is visible in the clustering (Figure 11 C). In the in solution dataset, the samples with the 6His-UBQ-transgene clustered together, in the in gel dataset, the Col-0 samples clustered together. As Col-0 is the absolute negative control, the in solution clustering is more plausible. As already indicated by the correlation analysis, most of the identified proteins were found in all samples (Figure 11 D). The in solution analysis was more sensitive than the in gel analysis. In the in solution experiment, 1327 proteins were identified in comparison to 835 the in gel experiment. This is comparable to the numbers from the TUBE ubiquitylome where 1031 proteins were detected (Kim et al., 2013).

In both the in solution and the in gel experiment, there were proteins that were at least two times enriched in the 6His-UBQ / amsh1 sample in comparison with the controls. We found 84 in the in solution dataset and 194 in the in gel dataset (Figure 12 A+ B). These proteins could be potential AMSH1 targets. In both the in solution and the in gel dataset there were non-specifically bound proteins that were depleted in the 6His-UBQ samples in comparison to the Col-0 control (Figure 12 C +D).

As there was only one replicate for each genotype and method, a statistical analysis could not be performed. Thus it is not clear whether the proteins enriched 6His-UBQ / amsh1 sample are AMSH1 targets. There was only a small overlap between the enriched proteins from the in solution and in gel dataset (Figure 12 E). Among the 12 proteins in the overlapping region were no promising candidates for AMSH1 targets. Three proteins in the list were GST variants, which are probably contaminants from the affinity purification matrix. A subcellular localization analysis conducted with the internet tool SUBA4 showed, that most of the proteins enriched in the 6His-UBQ / amsh1 sample were not localized at the PM or in endosomes (Figure 12 F), suggesting that the proteins enriched in the 6His-UBQ / amsh1 sample are probably no AMSH1 targets.

The experiment highlighted several major problems. Unspecific binding during the His-purification step probably complicated the identification of AMSH1 targets. To avoid this problem, further experiments were performed without the His-purification step. Additionally, at least three replicates would be necessary to identify AMSH1 targets. The data analysis did not show clear advantage for the in gel or the in solution digestion. The detection of proteins was more sensitive with the in solution method. This, however, did not lead to identification of more selectively enriched proteins in the amsh1 sample. In further experiments the in gel method was used as the GST-band can be removed from the gel which reduces unspecific signals in the MS analysis.
Figure 12: There was nearly no overlap between potential AMSH1 targets in the in gel and the in solution analysis.
A) + B) Venn diagrams of proteins that were at least 2x (log2=1) enriched in the 6His-UBQ/amsh1 (aH) sample compared to the 6His-UBQ/Col-0 (CH) and Col-0 (C) controls in the in solution (A) and in gel experiment (B). Proteins that were at least 2x (log2=1) enriched in the CH control in comparison to the C control are also shown.
C) + D) Venn diagram of proteins that were at least 2x (log2=1) depleted in the aH and CH dataset in comparison to the respective controls for the in gel (C) and in solution (D) experiment.
E) There was nearly no overlap between the selectively enriched proteins in the aH samples of the in gel and in solution experiment. Only 12 proteins from 84 proteins (in solution) and 194 proteins (in gel) were enriched in both samples.
F) The subcellular localization analysis with the internet tool SUBA4 showed that most of the selectively enriched proteins were localized in the cytosol.

2.1.4. Both GST-TUBE and GST-2xGAT selectively enriched ubiquitylated proteins

The goal of the second large scale experiment was to compare the efficiency of the affinity purification of ubiquitylated proteins with the GST-2xGAT and the GST-TUBE construct from amsh1 seedlings. For this, the total plant extract of 40 g of amsh1 and Ler seedlings was incubated for 6 hours at 4°C with GST-, GST-2xGAT and GST-TUBE affinity beads.
GST-beads were used as a negative control. The bound proteins were eluted with 1xSDS. The samples were digested in the gel and analyzed by MS by Julia Mergner from the Küster Lab (TUM) (Figure 13 A). Ubiquitylated proteins were detected in the eluate of both GST-2xGAT and GST-TUBE samples (Figure 13 B). As in the first experiment, the protein intensities in all samples were distributed normally (Figure 14 A) and there was a correlation for all samples (Figure 14 B+C). The high correlation was unexpected as the ubiquitin blot implicated a bigger difference between the samples and the negative control. A reason for the high similarity between the genotypes could be that AMSH1 only targets a small part of the proteins in the cell and the abundance of most ubiquitylated proteins are not changed by the knockout of AMSH1.

GST, GST-2xGAT, and GST-TUBE samples clustered together. This implies that the protein intensities differed more between the samples than between the genotypes (Figure 14 D). In the Venn diagrams of the identified proteins, there was a higher overlap between the GST controls and the GST-2xGAT samples than with the GST-TUBE samples (Figure 14 E). Overall, less proteins were identified in the GST-TUBE sample than in the GST-2xGAT sample. Over 50% of the detected proteins were common in all samples, probably representing unspecific binding.

**Figure 13: Ubiquitylated proteins were enriched with GST-2xGAT and GST-TUBE from amsh1 and Ler seedlings.**

A) Whole cell extract of 10 day-old wild-type (WT) and amsh1 seedlings was mixed with the ubiquitin binding domain constructs GST-2xGAT and GST-TUBE (provided by the Vierstra Lab) and GST as a negative control. The extract and the ubiquitin binding constructs were incubated for 6h at 4°C, eluted and analyzed by LC-MS with in gel digestion. All mass spectrometric analyses for this experiment were performed by Julia Mergner of the Küster Lab at the Technical University of Munich.

B) Ubiquitin blot showing the presence of ubiquitylated proteins in the GST-2xGAT and GST-TUBE samples and less ubiquitylated proteins in the GST controls.
Figure 14: The identification of AMSH1 targets is complicated by unspecific binding.

C) The protein intensities were distributed according to a normal distribution in all samples.
D) The pairwise correlation of the protein intensities found in the *amsh1* and wild-type (WT) GST, GST-2xGAT and GST-TUBE samples showed a correlation of $R^2 > 0.80$.
E) The pairwise correlation of the protein intensities of the *amsh1* and wild-type samples was over $R^2 > 0.80$.
F) The protein intensities of the GST, GST-2xGAT and GST-TUBE samples clustered together.
G) The Venn diagrams of all proteins found in the samples showed that 54% of all proteins were found in all three *amsh1* samples and 62% in all three wild-type samples. In total, 4101 proteins detected in the wild-type samples and 4276 in the *amsh1* mutant samples with at least one peptide.

To identify potential AMSH1 targets, the list of proteins that were at least twice enriched in all *amsh1* samples were compared. The Venn diagram shows that 111 proteins were selectively enriched in both the GST-2xGAT and the GST-TUBE sample, yet not in the GST-control (Figure 15 A). The Venn diagram also shows that the proteins enriched in the GST-2xGAT and the GST-TUBE samples. As the proteins enriched in the GST-2xGAT
and the GST-TUBE sample might be ubiquitylated, they were compared with published ubiquitylomes (Figure 15 B +C). The TUBE ubiquitylome from the Vierstra Lab (Kim et al., 2013) was chosen due to the similarity of the method. As potential AMSH1 targets carry a K63-linked ubiquitin chain, the K63 sensor ubiquitylome (Johnson and Vert, 2016). There was only a small overlap between our dataset and the ubiquitylomes. Around 52 (10 %) of the proteins enriched in the GST-2xGAT sample and 17 (3.8 %) of the proteins enriched in the GST-TUBE sample were found in the TUBE dataset from the Vierstra Lab.

Figure 15: 2xGAT and TUBE enriched different proteins in amsh1.
A) Venn diagram of proteins that were at least 2x (log2=1) enriched in the amsh1 (A1) GST, GST-2xGAT (GAT) and GST-TUBE (TUBE) samples compared to the wild-type samples (WT). Potential AMSH1 targets are 111 proteins, that were selectively enriched in the GST-2xGAT and GST-TUBE samples, yet not in the GST control.
B) Venn diagram comparing proteins that were at least 2x enriched in the GAT amsh1 sample and the TUBE amsh1 sample to the Vierstra TUBE ubiquitylome (Kim et al., 2013).
C) Venn diagram comparing proteins that were at least 2x enriched in the GAT amsh1 sample and the TUBE amsh1 sample to the K63-Sensor ubiquitylome (Johnson and Vert, 2016).
D) Venn diagram of the 111 proteins selectively enriched in the 2xGAT and the TUBE dataset, the potential AMSH1 targets, the K63 and the TUBE ubiquitylome.
Only 17 proteins were found in all three datasets. There was nearly no overlap with the K63-ubiquitylome, 2 (0.4 %) of the proteins enriched in the GST-2xGAT sample and 6 (1.3 %) of the proteins enriched in the GST-TUBE sample were found in the K63 ubiquitylome. Only two proteins were found in all three datasets. Finally, the potential AMSH1 targets, the proteins that were selectively enriched both in the GST-2xGAT and GST-TUBE sample and not in the GST sample, were compared to the published ubiquitylomes (Figure 15 D).

One of the proteins was found in the K63-, twelve were found in the TUBE ubiquitylome. Among the potential targets that were also found in the published ubiquitylomes there were five PM localized, three Golgi-localized and one vacuolar protein (Table 1). In an analysis of the subcellular localization of the proteins enriched in the GST-2xGAT and the GST-TUBE sample with the internet tool SUBA4, most proteins were more or less evenly distributed between cytosol, plastid, nucleus, Golgi+ ER, PM, and the extracellular fraction (Figure 16). However, in the GST-2xGAT sample there were about 50 % more proteins (16 %) enriched that localize to the Golgi+ ER than in the GST-TUBE sample (9 %). As the overall number of enriched proteins was higher in the GST-2xGAT sample than in the GST-TUBE sample, I used the GST-2xGAT construct for the final experiment.

Table 1: Proteins that were at least 2x enriched with both 2xGAT and TUBE in the amsh1 mutant samples and were also found in the K63 or the TUBE ubiquitylome.
At2g36380 was found in the K63 ubiquitylome (Johnson and Vert, 2016), the other proteins in the TUBE ubiquitylome (Kim et al., 2013). The subcellular localization was assigned with the SUBA4 internet tool.

<table>
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<th>Gene</th>
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<td>Golgi</td>
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<tr>
<td>AT2G45540</td>
<td>WD-40 REPEAT FAMILY PROTEIN</td>
<td>Golgi</td>
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<td>METAL ION-BINDING PROTEIN</td>
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2.1.5. Potential AMSH1 targets were isolated with GST-2xGAT

To identify potential AMSH1 targets, a final large scale affinity purification experiment was conducted with the GST-2xGAT construct and amsh1 and wild-type (Ler) seedlings. To enable a statistical interpretation of the results the experiment was performed with three biological replicates and two separate measurements per replicate. The plant extract of 3 x 40 g of amsh1 and Ler (WT) seedlings was incubated for 6 h at 4°C with GST-2xGAT affinity beads. The bound proteins were eluted with 1xSDS. The samples were digested in the gel and analyzed by MS (Eva Höllmüller, Stengel Lab, University of Konstanz, Figure 17 A). Ubiquitylated proteins were detected in the eluate of all replicates (Figure 17 B).

For the analysis of the proteomic data, the protein intensities were normalized with the LFQ method (data label-free quantification) by the MS software (MaxQuant). In the LFQ method, a median ratio is calculated from peptides from the same protein in all samples, and the protein intensity is normalized to this median ratio. The LFQ method is recommended for the comparison of sample with a very similar composition as it is the case in this experiment. The protein intensities (LFQ intensities) in all samples were distributed normally (Figure 17 A). The correlation analysis showed that the differences between the biological replicates are in the same range as the differences between wild-type and amsh1 samples (Figure 17 B). Due to the small differences between the samples the clustering of the protein intensities was only partly plausible. The protein intensities of all technical replicates, yet not all biological replicates clustered together (Figure 17 C).
Figure 17: Ubiquitylated proteins were enriched with the GST-2xGAT construct from amsh1 and wild-type (Ler) seedlings.
A) Whole cell extract of 10-day-old Ler and amsh1 seedlings was mixed with the GST-2xGAT, incubated for 6h at 4°C, eluted with 5x Laemmli, and analyzed by LC-MS with in gel digestion. The experiment was performed with three biological replicate for each genotype and all replicates were measured twice. All mass spectrometric analyses and the in gel digestion for this experiment were performed by Eva Höllmüller of the Stengel Lab at the University of Konstanz.
B) Ubiquitin-blot showing the presence of ubiquitylated proteins in the eluate of the three Ler and amsh1 replicates. For the in gel digestion, 100 µL of the eluate was loaded on a CBB gel. The GST-2xGAT protein (50 kDa) band was not used for the MS analysis.

In total, 1966 proteins were identified with at least one peptide. 978 proteins were found in all three wild-type samples whereas 876 proteins were found in the amsh1 samples (Figure 19 A). 774 proteins were found in all three wild-type and amsh1 replicates (Figure 19 B). The Venn diagrams and the correlation analysis show that as in the two previous experiments there were only subtle differences between the wild-type and amsh1 dataset. Surprisingly, in this experiment there were more proteins in the wild-type control than in the amsh1 samples. As amsh1 seedlings accumulate ubiquitylated proteins, this result was not expected. A reason for this result could be that AMSH1 regulates the abundance of its target proteins differently. The presence of AMSH1 might stabilize some of its target proteins, and de-stabilize other proteins.
Figure 18: There were only subtle differences between *amsh1* and *Ler* samples.

A) The LFQ intensities of the proteins were distributed according to a normal distribution in all measurement.

B) Exemplary pairwise correlation plots of the protein intensities (LFQ) found in the *amsh1* and *Ler* (WT) samples. Technical replicates (1, 2) had correlation coefficients $R^2 > 0.95$, biological replicates (A, B, C) had correlation coefficients $R^2 > 0.75$. The pairwise correlations between *Ler* and *amsh1* samples were in the same range as the correlations between the biological replicates, e.g. $R^2 = 0.7820$ for wild type A1 and *amsh1* A1.

C) The clustering of the LFQ intensities shows a high similarity of the technical replicates (1, 2), which all clustered together. The clustering of the biological replicates (A, B, C) was less consistent, *amsh1* (Mut) A had a higher similarity with wild type B and C than with *amsh1* B and C.
Figure 19: More proteins were in the Ler than in amsh1 samples.
A) The Venn diagrams of all proteins found in the samples showed that more proteins, 50% (978) of all proteins, were found in all three wild-type samples than in all three amsh1 samples (45% (876) of all proteins). In total, 1966 proteins were detected with at least one peptide.
B) Venn diagram showing the overlap between the proteins identified in all wild-type samples (978) and amsh1 (876) samples. Most of the proteins (774) were identified in both genotypes.

To find potential AMSH1 targets, the dataset of the amsh1 sample and the wild-type control were compared with a T-Test (s0=0, fdr=0.05) by Eva Höllmüller (Figure 20 A). 51 proteins were significantly enriched in amsh1 whereas 42 proteins were enriched in the wild type. 45% of the proteins enriched in amsh1 were cytosolic proteins (Figure 20 B). 30% localized to the plastid or the mitochondrion. 30%, 16 out of 51, proteins enriched in the amsh1 mutant were ribosomal proteins. In the wild-type sample, the number of cytosolic proteins was smaller, only 15% of the proteins were cytosolic. About 50% of the enriched proteins localized to the plastid, the PM, the extracellular space or the Golgi + ER fraction which includes also endosomal proteins (Figure 20 C). As the potential AMSH1 targets were expected to be membrane-associated, they could be among the proteins enriched in the wild-type sample. In the amsh1 dataset, 15 proteins (30%) were found in the TUBE ubiquitylome and 2 proteins (4%) in the K63-ubiquitylome (Figure 21 A). In the wild-type dataset, two proteins (5%) were found in both ubiquitylomes, five (12%) in the TUBE ubiquitylome and three (7%) in the K63-ubiquitylome. The 30% overlap of the proteins enriched in the amsh1 sample and the 24% overlap of proteins in the wild-type sample with the published ubiquitylomes could suggest that these proteins are ubiquitylated. The proteomic analysis gave a first impression about the nature of potential AMSH1 targets. However, if the identified proteins are targeted by AMSH1 must be elucidated in further experiments.
Figure 20: Less cytosolic proteins were significantly enriched in the Ler control than in the amsh1 sample.

A) Visualization of the statistical analysis in a Volcano plot by Eva Höllmüller. The statistical analysis was performed with a T- Test in Perseus (s0=0, fdr (false discovery rate) =0.05). Potential AMSH1 targets were significantly enriched in both the wild-type (WT) control and the amsh1 samples.

B+C) Subcellular analysis with SUBA4 of the significantly enriched proteins in the amsh1 sample (B) and the wild-type (WT) control (C). In amsh1, most of the 51 enriched proteins localized to the cytosol, plastid, or mitochondrion. In the wild type, most of the 42 enriched proteins localized to plastid, the PM, the cytosol, or the Golgi+ER.

Figure 21: More than 25 % of the selectively enriched proteins were found in published ubiquitylomes.

Venn diagram of the significantly enriched proteins in the amsh1 (A) and the Ler (WT) sample with the K63- and the TUBE ubiquitylome.
2.2. OTU11 and OTU12 might be involved in the degradation of PM proteins

2.2.1. OTU11 and OTU12 localize to the PM in Arabidopsis protoplasts

The second aim of my studies was to identify and analyze the function of additional DUBs which might influence the degradation of membrane proteins in Arabidopsis thaliana. In previous studies it was shown that AMSH1 and AMSH3 are involved in the regulation of PM protein abundance in Arabidopsis. As the degradation of PM proteins depends on many ubiquitylation-regulated steps, there are probably further DUBs that influence the abundance of PM proteins in Arabidopsis. To identify DUBs that are important for the endocytic degradation of PM proteins, we performed a systematic localization study of fluorophore-tagged UBPs and OTUs in Arabidopsis root cell derived protoplasts.

Localization studies have shown that UBP20 and OTU10 localized to the cytosol, UBP3, UBP4, UBP6, UBP7, UBP10, UBP12, and OTU9 to the cytosol and nucleus, UBP25 to the nucleus, UBP22 to the nucleolus, UBP23 to the nucleolus, and UBP18 to the endoplasmic reticulum (ER). OTU11 and OTU12 localized to the cytosol, nucleus and the PM (Figure 22 A). UBP27 was localized at rod-like structures which are probably mitochondria (Pan et al., 2014) (Figure 22 B).

The nucleolus localized UBP22 was reported to be involved in histone deubiquitylation and to be localized to euchromatin, a nucleoplasmic structure (Nassrallah et al., 2018). UBP12 was shown to localize to the cytosol and nucleolus in Arabidopsis seedlings and was detected both in cytosolic and nuclear extracts in previous reports (Cui et al., 2013). UBP3 and UBP4 were shown to be in nuclear extracts and probably also in cytosolic extracts of Arabidopsis seedlings (Chandler et al., 1997), suggesting that transient overexpression of YFP-fusion proteins in protoplasts was a suitable system for the localization analysis of DUBs in Arabidopsis. As the PM localization is a strong indication that both OTU11 and OTU12 are important for the regulation of membrane-associated processes, OTU11 and OTU12 were chosen for further studies.
Figure 22: Arabidopsis UBP and OTU DUBs are localized to different compartments in root cell derived protoplasts.

A) Selected confocal pictures of UBP and OTU DUBs, that were transiently expressed Arabidopsis root cell derived protoplasts with a 35S CaMV promotor and an N-terminal YFP-tag. The DUBs localized to the cytosol (OTU10, UBP20), cytosol and nucleus (UBP6, UBP7, UBP10, UBP12, OTU9), nucleus (UBP25), nucleolus (UBP22), nucleolus (UBP23), mitochondria (UBP27) endoplasmic reticulum (ER) (UBP18) and PM, cytosol and nucleus (OTU11, OTU12), bar=10 µm.

B) Summary of the localization patterns observed in the protoplast experiment.
2.2.2. OTU11 and OTU12 are two closely related OTU DUBs

OTU11 and OTU12 contain a C-terminal OTU domain with a cysteine protease active site (Figure 23 A). Both OTU11 and OTU12 have a potential membrane binding motif in the OTU domain, the polybasic motif 1 (PBM1), and at the C-terminus, the polybasic motif 2 (PBM2), that were identified with the BH-search tool. The polybasic motifs consist of basic amino acids that are positively charged in the cell and can thus bind to the negatively charged head groups of phospholipids in cellular membranes (Simon et al., 2014). PBM1 is conserved in OTU11 and OTU12, yet it is not found in OTU8, OTU9, and OTU10 (Figure 24). PBM2, however, is found in OTU9, OTU10, OTU11, and OTU12. In the N-terminus of OTU11 there are six phosphorylation sites, in the N-terminus of OTU12 there is one phosphorylation site (Wang et al., 2013b; Lin et al., 2015; Roitinger et al., 2015; Mergner et al., 2020b). The OTU domain of OTU11 and OTU12 could be modelled with a high confidentiality with AlphaFold (Uniprot, (Jumper et al., 2021)). The N-terminus of OTU11 an OTU12 could not be modelled with a high confidentiality, it seems to have no rigid structure (Figure 23 B+C).

![Figure 23: OTU11 and OTU12 are two closely related OTU DUBs.](image)

A) Scheme of the domain structure of OTU11 and OTU12. Phosphorylation sites are marked in violet, PBM1 in yellow and PBM2 in brown, the active site amino acids in red.
B+C AlphaFold-Models (Jumper et al., 2021) of OTU11 (B) and OTU12 (C). The OTU domain is marked in blue, the active site in red, PBM1 in yellow and PBM2 in brown.
2.2.3. OTU11 and OTU12 are expressed in different tissues

The expression profile of OTU11 and OTU12 could give some information about the biological function of the enzymes. In a large scale expression analysis, OTU11 and OTU12 transcripts were expressed in similar amounts in most Arabidopsis tissues (Mergner et al., 2020b, Figure 25 A). In contrast to OTU11, OTU12 could not be detected on the protein level in the majority of Arabidopsis tissues (Figure 25 B). As GFP-OTU12 could not be detected on western blots in this study (compare Figure 28 A), the detection of OTU12 may be technically not feasible. Neither OTU11 nor OTU12 were enriched in a specific tissue. OTU11 exists in two splicing isoforms, OTU11.1 and OTU11.2, whereby OTU11.2 has additional five amino acids in the C-terminus (Figure 26 A). When a fragment containing the splicing site of OTU11 was amplified from the cDNA of wild-type Arabidopsis seedlings, there were two peaks, showing both isoforms are transcribed in Arabidopsis seedlings (Figure 26 B).

The different transcripts could encode versions of OTU11 with different properties. On the protein level, the isoforms could have different localization pattern. To test this, 35S:GFP-OTU11.1 and 35S:GFP-OTU11.2 was expressed in Arabidopsis seedlings (Figure 26 C). The isoforms did not have a different localization pattern, both GFP-OTU11.1 and GFP-OTU11.2 localized to the cytosol, the nucleus and the PM.
The isoforms might also have differences in their activity. To analyze the catalytic activity of the two isoforms, an *in vitro* DUB Assay with both OTU11.1 and OTU11.2 was performed. The GST-tag was cleaved off with PreScission-protease. In this assay both isoforms had an equally weak activity against K63-linked di- or tetra-ubiquitin. So the isoforms generated by differential splicing had no influence on the *in vitro* DUB activity of OTU11 (D). The functional significance of the two splicing of OTU11 has to be elucidated by further studies.
Figure 26: The differential splicing of OTU11 has no influence on the localization and the \textit{in vitro} DUB activity.

A) Alignment of the differential splicing site in OTU11.1 and OTU11.2. 
B) RT-PCR showing that OTU11.1 and OTU11.2 are expressed in the cDNA of Arabidopsis seedlings. Constructs with OTU11.1 (KV118) and OTU11.2 (KV119) were used as a control, water as a negative control. Peaks are marked with black arrowheads. The double peak in the cDNA sample shows that both isoforms are expressed.
C) Selected confocal pictures of 35S: GFP-OTU11.1 and OTU11.2 roots. Both isoforms localize to the cytosol, nucleus and PM. bar=10 µm.
D) Anti-OTU11 and anti-ubiquitin blot of an \textit{in vitro} DUB assay with recombinant OTU11.1 and OTU11.2 (28 kDa) and K63-linked di/tetra-ubiquitin. There was no difference in the cleavage efficiency of the between the two isoforms.

2.2.4. OTU11 and OTU12 localize to the PM in Arabidopsis root cells

To analyze the PM localization of OTU11 and OTU12, 35S:GFP-OTU11 and 35S:GFP-OTU12 Arabidopsis lines were crossed with a red PM-marker line, P5R (UBQ10prom:2xCherry-1x PH(FAPP1)) (Simon et al., 2014). The Pleckstrin homology (PH) domain of the human Pleckstrin homology domain-containing family A member 3 (FAPP1) is a phospholipid binding domain that binds to the PM-resident phosphatidylinositol-phosphate PI(4)P. GFP-OTU11 and GFP-OTU12 localized to the
cytosol and the PM and co-localized with P5R at the PM (Figure 27 A). The co-localization is also visible in the intensity profiles of the GFP-OTU11, GFP-OTU12 and P5R (Figure 27 B). As in the protoplasts, in the 35S:GFP-OTU12 seedlings there was also a GFP-signal in the nucleus. It is, however, unclear if the nuclear signal is caused by full length GFP-OTU12 or truncations products containing GFP. To verify that GFP-OTU11 and GFP-OTU12 localize to the PM and not to the cell walls, 35S:GFP-OTU11 and 35S:GFP-OTU12 seedlings were treated with a hyperosmotic mannitol solution which led to the shrinkage of the cells, whereas the cell walls stayed in the same place. Both GFP-OTU11 and GFP-OTU12 stayed in the cell during plasmolysis, so both GFP-OTU11 and GFP-OTU12 are localized at the PM and not at the cell wall (Figure 27 C).

Figure 27: 35S:GFP-OTU11 and OTU12 localize to the PM in Arabidopsis root cells.  
A) Confocal pictures showing, that GFP-OTU11 and GFP-OTU12 co-localize with P5R at the PM in root epidermis cells of Arabidopsis seedlings when expressed with a 35S-promotor.  
B) Intensity profiles of an exemplary epidermis cell from the pictures in (A) showing that GFP-OTU11 and GFP-OTU12 co-localize with P5R.  
C) Confocal pictures of 35S:GFP-OTU11 and 35S:GFP-OTU12 seedling root cells treated for 2 h with 1 M Mannitol. The GFP signal stayed in the cell during the plasmolysis.
Figure 28: GFP-OTU11 is found in the S100, P100 and ePM fraction.
A) Anti-GFP, anti-H⁺-ATPase, anti-UGPase and anti-PDR8 blot of whole cell extract of 7- to 10-day old 35S: GFP-OTU11 expressing Arabidopsis seedlings (S8), that was separated in a soluble (S100) and an insoluble, membrane protein containing fraction (P100). GFP-OTU11 was detected both in the soluble S100 fraction and the insoluble P100 pellet. GFP-OTU12 could not be detected. B) Anti-GFP, anti-H⁺-ATPase, anti-UGPase and anti-Sec21 blot of a simplified enrichment of the PM fraction with Brij58 of 35S: GFP-OTU11 seedlings. GFP-OTU11 was detected in the S100 fraction, in the P100 and in the enriched PM fraction (ePM). UGPase=UTP--glucose-1-phosphate uridylyltransferase 1, Sec21= Gamma subunit, COP vesicles, PDR8= ABC transporter G family member 36.

The PM localization of GFP-OTU11 was further examined with fractionation experiments. The clarified whole cell extract (S8) of 7- to 10-day-old 35S:GFP-OTU11 Arabidopsis seedlings was separated in a soluble (S100) and an membrane containing fraction (P100) with ultracentrifugation at 100,000 x g. Full-length GFP-OTU11 was detected both in the soluble (S100) and the membrane fraction (P100) (Figure 28 A) whereas the GFP-tag alone was mainly found in the S100 fraction. The experiment did not work for OTU12, as full-length GFP-OTU12 could not be detected on the western blot. In contrast, the PM proteins PDR8 and H⁺-ATPase are only found in the P100 fraction and the cytosolic protein UGPase only in the S100 fraction. As the P100 pellet contains membranes from all cellular compartments, we next performed an enrichment of PM proteins (Collins et al., 2017). To enrich the PM-proteins, the microsomal P100-pellet was treated with the detergent Brij-58. After the treatment, the microsomal proteins were removed by ultracentrifugation, whereas the PM proteins stayed in the enriched PM pellet (ePM). The enrichment of PM proteins was verified with the PM-resident H⁺-ATPase. In contrast, the amount Golgi-localized peripheral membrane protein Sec21 was not enriched in the ePM fraction. Full length GFP-OTU11 was found in the S100, P100 and ePM fraction which shows that GFP-OTU11 is associated with the PM (Figure 28 B).
Figure 29: OTU11p:GFP-OTU11 and OTU12p:GFP-OTU12 localize the PM in elongated root cells and root hairs.


In the meristematic zone GFP-OTU11 localizes to the cytosol and rod-like structures, GFP-OTU12 to rod-like structures (A). In the transition zone between the meristem and the elongation zone GFP-OTU11 localized to the cytosol and the PM, GFP-OTU12 to rod like structures (B). In elongated cells and root hairs GFP-OTU11 localized to the PM (C, pictures by Marie-Kristin Nagel) and GFP-OTU12 localized to the PM and the nucleus (D).
To examine the localization of OTU11 and OTU12 in under their own promoters, native promoter-driven GFP-OTU11 and GFP-OTU12 lines were generated and analyzed (Tobias Bläske, Marie-Kristin Nagel). In the meristematic zone, GFP-OTU11 localized to the cytosol and rod-like structures, and GFP-OTU12 localized to rod-like structures (Figure 29 A). In the transition zone between the meristematic zone and the elongation zone, GFP-OTU11 could be detected at the PM and in the cytosol, whereas GFP-OTU12 still localized to rod-like structures (Figure 29 B). In elongated cells and in root hairs both GFP-OTU11 and GFP-OTU12 localized to the PM (Figure 29 C +D). GFP-OTU12 also localized to the nucleus in elongated cells. The localization analysis of the own promoter lines shows that the PM localization of GFP-OTU11 and GFP-OTU12 depends on the cell type and that there are differences in the localization pattern of GFP-OTU11 and GFP-OTU12. GFP-OTU11 and GFP-OTU12 were both visible on the PM of elongated cells. In addition to this, GFP-OTU11 was localized to the PM in cells of the elongation zone and in meristematic cells it was cytosolic. GFP-OTU12 was not localized at the PM in the meristematic zone or the elongation zone. Probably it was not expressed in the meristematic or the elongation zone.

2.2.5. The OTU domains of OTU11 and OTU12 bind to phospholipids in vitro

To identify the sequence motifs required for membrane binding, the localization of the N-terminus (N), the OTU domain (OTU), and the OTU domain with the C-terminus of YFP-OTU12 was analyzed in protoplasts. Furthermore, the localization of the N-terminus (N) and the OTU domain (OTU) of GFP-OTU11 was analyzed in Arabidopsis seedlings (Figure 30 A). Full-length OTU12 localized to the PM in all analyzed protoplasts. The OTU domain and the N-terminus of OTU12 lost the PM localization completely, the addition of the C-terminus to OTU domain rescued the PM localization in 40 % of the protoplasts (Figure 30 B). In contrast to the full length version, the N-terminus and the OTU domain of OTU11 did not localize to PM in Arabidopsis seedlings (Figure 30 D). The localization experiment with YFP-OTU12 show that the C-terminus is required for PM localization, yet it is not sufficient to rescue the PM localization completely. Only full-length YFP-OTU12 is localized at the PM in all cells. As the OTU domain and the N-terminus of GFP-OTU11 did not localize to the PM in Arabidopsis seedlings, it is probable that the same is true for GFP-OTU11.
Figure 30: The N-terminus and the OTU domain of OTU11 and OTU12 alone do not localize to the PM.

A) Scheme showing the truncation constructs used for protoplast transformation and Pip Strips.

B) Representative confocal pictures of the protoplasts transformed with 35S::YFP-OTU12, 35S::YFP-OTU12 (N), 35S::YFP-OTU12 (OTU), and 35S::YFP-OTU12 (OTU). Full length YFP-OTU12 localized to the PM in all analyzed protoplasts. YFP-OTU12 (N) and YFP-OTU12 (OTU) lost the PM localization completely, YFP-OTU12 (C) lost the PM localization in 60% of the analyzed protoplasts.

C) Confocal pictures of 35S::GFP-OTU11 (N) and 35S::GFP-OTU11 (OTU) seedling root cells. Both YFP-OTU11 (N) and YFP-OTU11 (OTU) lost the PM localization.
To test if OTU11 and OTU12 are able to bind directly to membranes, an in vitro lipid overlay assay was performed (Figure 31 A). Both GST-OTU11 and GST-OTU12 were able to bind to phospholipids in vitro (Figure 31 B). They specifically bound to phosphatidylinositolphosphates (PIPs) and phosphatidic acid. PIPs are negatively charged phospholipids which are a minor but key components of bio-membranes. In plant cells, P(3)P is found in endosomes, P(4)P in endosomes and the PM, and P(4,5)P2 is mainly found in the PM (Simon et al., 2014). Thus, the binding to PIPs could contribute to membrane binding in vivo. To identify the motif required for membrane binding, lipid overlay assays with GST-OTU11 (N) and the GST-OTU11 (OTU), and GST-OTU12 (OTU) were conducted. GST-OTU11 (N) lost its ability to bind to PIPs. However, both GST-OTU11 (OTU) and GST-OTU12 (OTU) were able to bind to PIPs as the full length proteins (Figure 31 C+ D).

**Figure 31:** Full length GST-OTU11 and GST-OTU12 and the OTU domains bind to PIPs in vitro.
A) Scheme of the in vitro lipid binding PIP Strip™ membrane showing the position of the individual phospholipid spots on the membrane.
B) PIP Strip Assay of GST, GST-OTU11 and GST-OTU12. Both GST-OTU11 and GST-OTU12 bound to the PIPs on the PIP Strip.
C) PIP Strip Assay of GST, GST-OTU11 (N) and GST-OTU11 (OTU). GST-OTU11 (N) did not bind to PIPs, GST-OTU11 (OTU) bound to the PIPs.
D) PIP Strip Assay of GST and GST-OTU12 (OTU). GST-OTU12 (OTU) bound to PIPs.
Whereas the OTU domains of OTU11 and OTU12 do not localize to the PM \textit{in vivo}, they bind to PIPs \textit{in vitro}. Therefore, there must be at least two sequence motifs which influence the PM localization of OTU11 and OTU12. In the OTU domain there must a phospholipid binding motif. In other parts of the sequence there must be sequence motifs with a different function that are also required for the PM-association.

\section*{2.2.6. PBM1 and PBM2 influence the PM localization in protoplasts and phospholipid binding \textit{in vitro}}

Polybasic motifs consist of a stretch of basic amino acids that can interact with the negatively charged head groups of charged phospholipids such as the PIPs (Figure 32 A).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure32}
\caption{Mutations of PBM1 and PBM2 should abolish the membrane binding ability of OTU11 and OTU12.}
\begin{enumerate}
\item A stretch of basic amino acids, that is positively charged in the cellular environment, a polybasic motif, can mediate the binding of a protein to negatively charged head groups of membrane phospholipids.
\item Scheme showing the position and the amino acid sequence of the potential polybasic motifs that are predicted in the sequence of OTU11 and OTU12 by the BH-Search Tool. PBM1 is in the OTU domain (OTU11: aa 153 to 163, OTU12: aa 131 to 141), PBM2 in the C-terminus (OTU11: aa 236 to 242, OTU12: aa 210 to 216). To analyze the influence of the motif on the PM localization, the basic amino acids in the motifs were mutated to alanine (red).
\item Plots of the predicted BH-Score of OTU11, OTU11 6A1 and 6A2 (C) and of OTU12, OTU12 6A1 and 6A2 (D). According to the prediction, the membrane binding ability of the polybasic motif should be abolished by the mutations. black=wild type; grey, long dashes = 6A1; grey, short dashes=6A2, threshold=0.6, window=10.
\end{enumerate}
\end{figure}
Both OTU11 and OTU12 contain conserved potential polybasic motifs within the OTU domain (PBM1) and at the C-terminus (PBM2). PBM1 is not conserved in the OTU domain of the non-PM-localized homologues of OTU11 and OTU12, OTU9 and OTU10 (Figure 33). Therefore, it could be important for the PM-association of OTU11 and OTU12. The C-terminal PBM2 could explain the partial rescue of PM localization of YFP-OTU12 (C) in protoplasts. As PBM2 is conserved in OTU9 and OTU10, it cannot be the only reason for the PM localization (Figure 33). Probably both PBM1 and PBM2 contribute to the binding of OTU11 and OTU12 in vivo. To analyze the influence of PBM1 and PBM2 on the PM localization and the phospholipid binding of OTU11 and OTU12, the basic amino acids in the motifs were exchanged with the uncharged amino acid alanine. PBM1 was mutated in the 6A1 variant, PBM2 was mutated in the 6A2 variant. In the M3 variant both motifs were mutated (Figure 32 B). According to the prediction, the mutations should abolish the membrane binding ability of OTU11 and OTU12 in the respective regions (Figure 32 C+D).

The influence of the mutations on the PM localization of OTU11 and OTU12 in vivo was analyzed in Arabidopsis protoplasts (Figure 34). Wild-type OTU12 localized to the PM in all protoplasts, wild-type OTU11 in about 50% of the protoplasts. The 6A1 variation partly reduced the PM localization of OTU12, the PM localization was only retained in 15% of the protoplasts. In the experiment with the OTU11 6A1 variant, the number of protoplasts with PM localization was slightly reduced from 60 to 50% compared to wild-type OTU11.

The 6A2 and M3 variants of OTU11 and OTU12 lost the PM localization completely. This confirms the result of the localization analysis with the truncated versions of OTU11 and OTU12 (compare Figure 30). The localization experiment with the mutant variants shows that PBM2 is essential for the PM localization in vivo.

Figure 33: Alignment of the amino acids sequences of AtOTU11, OTU12, OTU9, OTU10. Yellow: PBM1, orange: PBM2.
PBM1 also contributes to the PM localization \textit{in vivo}, yet it is not essential. The influence of PBM1 and PBM2 on the binding to phospholipids \textit{in vitro} was analyzed with the 6A1 and 6A2 variants of OTU11 \textit{in vitro} lipid overlay assay, a PIP Strip Assay (Figure 35 A). GST-OTU11 6A2 had the same binding profile as wild-type GST-OTU11. GST-OTU11 6A1 had a reduced binding affinity for most PIPs, namely PI(4)P, PI(3,4)P_2, PI(4,5)P_2, PI(3,5)P_2, PI(3,4,5)P_2. The binding to PI(3)P and PI(5)P was not affected (Figure 35 B). GST-OTU11 M3 lost its binding affinity for all PIPs, except of PI(3)P. As the nearly complete loss of binding of GST-OTU11 M3 cannot be explained by an additive effect of the 6A1 and 6A2 variations, this result might be an artifact. The lipid overlay assay was repeated with GST-OTU11 (OTU) and GST-OTU11 (OTU-6A1). Compared to GST-OTU11 (OTU), GST-OTU11 (OTU-6A1) had a reduced binding affinity to most PIPs, except of PI(3)P and PI(5)P (Figure 35 C). As PI(4)P and PI(4,5)P_2 are the two PIPs found in the PM, the loss of binding to these PIPs might explain the loss of PM localization \textit{in vivo} observed for the 6A1-variants of OTU11 and OTU12.

In summary, the results of the localization analysis and the PIP Strips show, that both PBM1 and PBM2 influence the PM-association of OTU11 and OTU12. PBM1 seems to be a polybasic motif, which specifically influences the bind of OTU11 and OTU12 to the PM-localized PIPs PI(4)P and PI(4,5)P_2.

![Figure 34: The C-terminal motif is essential for the PM localization of OTU11 and OTU12 in protoplasts.](image)

Representative confocal pictures of the protoplasts transformed with 35S:YFP-OTU12, 35S:RFP-OTU12 6A1, 35S:YFP-OTU12 6A2, 35S:YFP-OTU12 M3, 35S:YFP-OTU11.2, 35S:RFP-OTU11.1 6A1, 35S:YFP-OTU11.2 6A2, and 35S:YFP-OTU11.2 M3 constructs. YFP-OTU12 localized to PM in all protoplasts. YFP-OTU12 6A1 retained the PM localization in 15% of the protoplasts, YFP-OTU12 6A2 and YFP-OTU12 M3 lost the PM localization completely. YFP-OTU11.2 had PM localization in 60% of the protoplasts, RFP-OTU11.1 6A1 in 50% of the protoplasts. YFP-OTU11.2 6A2 and YFP-OTU11.2 M3 lost the PM localization completely.
As only full-length OTU12 shows complete PM localization, there are, presumably, also one or more sequence motifs in the N-terminus of OTU12 which influence the PM localization. As the N-terminally tagged version of OTU12 is localized at the PM, it is improbable that the potential myristoylation motif MGXX (Majeran et al., 2018) at the N-terminus is required for PM localization. It is more likely that the N-terminus contains an additional binding domain for an adaptor protein. All in all, the experiments show that OTU11 and OTU12 have complex membrane binding mechanism that depends on at least two different sequence motifs.

**Figure 35: PBM1 influences the binding of GST-OTU11 to PIPs in vitro.**
A) Scheme of the *in vitro* lipid binding PIP Strip™ membrane showing the position of the individual phospholipid spots on the membrane.
B) PIP Strip Assay of GST, GST-OTU11, GST-OTU11 6A1, GST-OTU11 6A2 and GST-OTU11 M3. GST-OTU11 able to bind to all PIPs, phosphatidic acid and phosphatidylinerine. GST-OTU11 6A1 had a reduced binding affinity to all PIPs, except of PI(3)P and PI(5)P. The binding affinity of GST-OTU11 6A2 was not affected. GST-OTU11 M3 lost the binding affinity to all phospholipids except of PI(3)P.
C) PIP Strip Assay of GST, GST-OTU11(OTU) and GST-OTU11 (OUT-6A1). The mutation abolished the binding of the OTU domain to most PIPs, except of PI(3)P and PI3,5P2.
2.2.7. OTU11 and OTU12 are only weakly active *in vitro*

Human OTU enzymes have distinct chain type or chain length preferences, which directly influence the function in the cell (Mevissen et al., 2013). It is unclear whether this is also the case for Arabidopsis OTUs (Radjacommare et al., 2014).

**Figure 36:** GST-OTU11, GST-OTU12, GST-OTU11(D), and GST-OTU(N) were purified from bacteria.

A) + B) Total protein staining (A) and GST-blot (B) of GST-OTU11 (black arrowhead) and GST-OTU12 (white arrowhead) isolated from bacteria. **CBB** = Coomassie staining.

C) Total protein staining (CBB) of PreScission-cleaved bacterial OTU11.1 and OTU11.2 (black arrowhead).

D) Total protein staining of GST-OTU11 (D) (black arrowhead) isolated from bacteria.

E) Total protein staining of GST-OTU11 (N) (black arrowhead) isolated from bacteria.

F) Chromatogram of a typical Äkta™ run with OTU11 with a cation exchange column (SP HP) and a salt gradient. OTU11 forms broad shoulder between fractions A10 and B8.

G) Protein staining gel of the Äkta™ - fractions from the run in (A). OTU11 (28 kDa, black arrowhead) is distributed between fractions A10 and B8.
As OTU11 and OTU12 is localized at the PM and PM-localized proteins are known to carry a K63-linked ubiquitin chain, we assumed that K63-linked ubiquitin is the preferred substrate of OTU11 and OTU12. Therefore, the \textit{in vitro} DUB activity of OTU11 and OTU12 against K63-linked ubiquitin was analyzed. For this GST-OTU11, GST-OTU12, GST-OTU11 (D) and GST-OTU11(N) were expressed in Rosetta bacteria strains, purified with affinity purification and eluted with glutathione or PreScission protease (Figure 36 A to D). In ion exchange chromatography the bacteria-expressed OTU11 without GST-tag formed broad shoulder, instead of a distinct peak (Figure 36 E) and OTU11 is distributed among many fractions (Figure 36 F).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure37.png}
\caption{OTU11 and OTU12 are weakly active \textit{in vitro} against K63-linked tetra- and di-ubiquitin.}
A) +B) SYPRO Ruby total protein staining and anti-ubiquitin blot of an \textit{in vitro} DUB assay with GST-OTU11.1 (A) and GST-OTU12 (B). 7.5, 25, 50, 100, 250 pmol of enzyme were mixed with 7.8 pmol K63-linked tetra-ubiquitin. Cleavage products were visible in the reactions with more than 50 pmol tetra-ubiquitin.
C) Fluorescence based DUB Assay with 100, 25, 7.5 pmol of GST-OTU11, and 7.5 pmol of TAMRA-K63-linked di-ubiquitin in the reaction with 100 pmol of GST-OTU11 there was a measurable activity.
\end{figure}
For this reason, the proteins were not further polished before use. Firstly, an \textit{in vitro} DUB assays with different amounts of recombinant GST-OTU11 and GST-OTU12 was performed. Both OTU11 and OTU12 were only able to cleave K63-linked tetra-ubiquitin when they were added in more than 5x excess (50 pmol enzyme: 7.5 pmol substrate) (Figure 37 A+B). There was nearly no cleavage in the samples with an enzyme to substrate ratio of 1:1. In comparable DUB assays with OTUs from other organisms, the enzymes were able to cleave their ubiquitin substrate, at 1:1 enzyme/substrate ratio (Mevissen et al., 2013; Dzimianski et al., 2019; Schubert et al., 2020). To test if recombinant OTU11 is more active against shorter ubiquitin chains, a fluorescence-based DUB Assay with K63-linked di-ubiquitin was performed. OTU11 was only able to cleave the substrate when the enzyme was added in excess (about 13x enzyme (100 pmol) than substrate (16 pmol), Figure 37 C). As for the tetra-ubiquitin, there was no cleavage with 1:1 enzyme to substrate ratio. This shows, that OTU11 is equally active against di- and tetra-ubiquitin \textit{in vitro}. Reaction temperature and time do not explain the weak \textit{in vitro} activity.

\textbf{Figure 38: Temperature and reaction time has no influence on the \textit{in vitro} DUB activity of GST-OTU11.}

A) SYPRO Ruby gel and anti-ubiquitin (P4D1) blot of an \textit{in vitro} DUB assay with GST-OTU11.1 and K63-linked tetra-ubiquitin at 21°C and 30°C. There was no difference in the cleavage efficiency of the enzyme between 21°C and 30°C.

B) CBB gel and anti-P4D1 blot of an \textit{in vitro} DUB assay with GST-OTU11.1 and K63-linked tetra-ubiquitin. In the DUB assay 40 pmol of recombinant GST-OTU11.1 was mixed with 7.8 pmol (250 ng) K63-linked tetra-ubiquitin and incubated at 21°C for 2h, 4h, and overnight (on). The amount of cleavage product in the DUB assay did not increase with the reaction time.
The increase of the reaction temperature from 21°C to 30°C had no influence on the DUB activity (Figure 38 A). An extension of the incubation time also did not enhance the cleavage efficiency (Figure 38 B). To test if the N-terminal sequence of OTU11 shields the ubiquitin binding site in vitro, a DUB assay with only the OTU domain was performed.

GST-OTU11 (OTU) was not active against K63-linked tetra-ubiquitin in vitro (Figure 39 A). Therefore, it is improbable that the N-terminus is obstructing ubiquitin binding in vitro. To analyze if the weak in vitro activity of OTU11 is dependent on its OTU domain, a catalytic site mutant (C112R) was tested. GST-OTU11 (C112R) was not active in vitro (Figure 39 A). The loss of activity in the catalytic site mutant shows that OTU11 is a cysteine protease DUB with a very weak activity against free ubiquitin chains in vitro. As the N-terminus is required for the OTU11 activity in vitro, it could provide an additional substrate binding site.

To test if the N-terminus is involved in the binding of ubiquitin, an in vitro binding assay with GST-OTU11 (N) was performed. In contrast to the positive control, GST-2xGAT, GST-OTU11 (N) was not able to bind to free K63-linked tetra-ubiquitin chains in vitro (Figure 39 B), so it does probably not have an additional ubiquitin-binding site and there must be another reason, why it is required for activity.

**Figure 39:** The N-terminus of OTU11 is necessary for its in vitro DUB activity.

F) SYPRO Ruby gel and anti-P4D1 blot of an in vitro DUB assay GST-OTU11.1, GST-OTU11 D and GST-OTU11 (C112R). Both the removal of the N-terminus and the mutation of the active site cysteine (C112) to arginine abolished the DUB activity.

G) Anti-GST and anti-P4D1 blot of an in vitro binding assay with 100 pmol of K63-linked tetra-ubiquitin and 100 pmol of GST-OTU11(N). GST- was used as a negative, the ubiquitin-binding GST-2xGAT as a positive control. GST-OTU11 (N) did not bind to free K63-linked tetra-ubiquitin chains in vitro.
2.2.8. Plant derived GFP-OTU11 was slightly more active than bacterial GST-OTU11

Another reason for the weak activity of the bacteria-expressed GST-OTU11 could be the lack of post-translational modifications in the *in vitro* system. To test if post-translational modifications are important for the activation of OTU11, GFP-OTU11 was purified from extract of 7- to 10-day-old 35S:GFP-OTU11 Arabidopsis seedlings. The purified GFP-OTU11 was mixed on beads with K63-linked di- and tetra-ubiquitin. GFP-OTU11 was active against both K63-linked di-ubiquitin and tetra-ubiquitin. However, it was less active than the positive control GFP-AMSH3 and there was some background activity in the negative control GFP for K64-linked tetra-ubiquitin (Figure 40 A). In this experiment it was not possible to quantify the amount of enzyme before the DUB assay. To assess the efficiency of the enzyme, substrates and enzymes were quantified in the gel after the DUB Assay. In the DUB assay with GFP-OTU11, the ratio of enzyme to substrate was around 1-2:1, suggesting that plant-derived GFP-OTU11 was slightly more active than the bacterial enzyme. To analyze if GFP-OTU11 extracted from plant extract could bind to ubiquitin, it was mixed with non-hydrolysable K48- and K63-linked di-ubiquitin. Similar to GST-OTU11, GFP-OTU11 also did not bind di-ubiquitin *in vitro* (Figure 40 B). An additional reason for the low activity against K63-linked ubiquitin could be a specific preference for another chain type. To analyze this possibility, GFP-OTU11 on beads was mixed with all chain types of di-ubiquitin. GFP was used as a negative control. In this DUB assay GFP-OTU11 did not show a chain type specificity. Instead, it was weakly active towards K6-, K11-, K29- K63-, and K48-linked di-ubiquitin (Figure 40 C).
Figure 40: GFP-OTU11 extracted from plant extract is weakly active against K6-, K11-, K48- and K63-linked di-ubiquitin and K63-linked tetra-ubiquitin.

A) SYPRO-Ruby gel of an DUB assay with GFP-OTU11 extracted from Arabidopsis seedlings and K63-linked di- and tetra-ubiquitin. GFP was used as a negative control, AMSH3-GFP as a positive control. GFP-OTU11 did not show any preference for di- or tetra-ubiquitin.

B) Anti-GFP and anti- ubiquitin blot of binding assay with GFP-OTU11 and non-hydrolysable K63-linked and K48-linked di-ubiquitin. GFP was used as a negative control. GFP-OTU11 did not bind to the di-ubiquitin. Black arrowhead=GFP-OTU11, white arrowhead=GFP.

C) SYPRO-Ruby gel of an DUB assay with GFP-OTU11 extracted from Arabidopsis seedlings and lin-, K6-, K11-, K29-, K33-, K48- and K63-linked di-ubiquitin. GFP was used as a negative control. GFP-OTU11 was equally active against K6-, K11-, K29-, K48- and K63-linked di-ubiquitin. white arrowhead: mono-ubiquitin.
2.2.9. Phosphorylation does not influence the activity or the PM localization of OTU11

As there are six reported phosphorylation sites in the N-terminus of OTU11, we speculated that OTU11 is phosphorylated in vivo. As OTU12 only has one reported phosphorylation site in its N-terminus, it is not clear if this is also true for OTU12. However, there is an additional potential phosphorylation site in OTU12 (S42) that is conserved among OTU11 and OTU12 (Figure 33). Although proteins expressed in *E. coli* are less frequently phosphorylated than in eukaryotes, it cannot be ruled out completely that recombinant GST-OTU11 is phosphorylated (Macek et al., 2008). To test if bacterial GST-OTU11 is phosphorylated, it was treated with phosphatase and the phosphorylation status of the protein was analyzed by a phosphostain.

**Figure 41: GFP-OTU11 is phosphorylated in the plant extract.**
A) Phosphostain (ProQ Diamond) and total protein stain (SYPRO Ruby) of recombinant GST-OTU11 treated with λ- phosphatase. In the GST-OTU11 sample there was no intensity difference between the sample with the active phosphatase and the controls in the phosphostain (p=phosphatase, ip =inactivated phosphatase).
B) Phosphostain (ProQ Diamond) and total protein stain (SYPRO Ruby) of phosphatase treated GFP-OTU11, GFP-OTU12 and GFP. The intensity difference between the sample with the active phosphatase and the controls in the phosphostain shows that GFP-OTU11 phosphorylated in the plant extract (p=phosphatase, ip =inactivated phosphatase).
C) Total protein stain (SYPRO Ruby) of a Phostag-acrylamide and a standard 10% acrylamide gel of phosphatase treated GFP-OTU11. The shift of the protein band in the sample with the active phosphatase in comparison to the respective bands in the controls shows that GFP-OTU11 is phosphorylated (p=phosphatase, ip =inactivated phosphatase).
There was no difference between the GST-OTU11 samples with and without active phosphatase in the phosphostain. This shows that the recombinant protein is not phosphorylated (Figure 41 A). In contrast, there was a clear difference for GFP-OTU11 between the samples with and without active phosphatase in the phosphostain (Figure 41 B). As this difference was not observed for GFP-tag alone, the phosphorylation sites must be in the OTU11 protein. The phosphorylation status of GFP-OTU12 could not be analyzed, as the protein level in the GFP-IP was too low for detection. The apparent phosphorylation of GFP-OTU11 in the phosphostain was verified with a Phostag-gel. The shift between the samples with and without active phosphatase in the Phostag-gel confirmed that GFP-OTU11 is phosphorylated in planta (Figure 41 C). To analyze whether the DUB activity of GFP-OTU11 is influenced by phosphorylation, the phosphatase treatment was combined with an in vitro DUB Assay. There was no difference in the cleavage efficiency between the samples with and without active phosphatase. This clearly shows that phosphorylation status of GFP-OTU11 does influence its DUB activity (Figure 42). The phosphorylation status of the enzyme could also influence the PM localization of OTU11 and OTU12 in the plant cell. To analyze this, phospho-mimic and phospho-dead versions of OTU11 and OTU12 were expressed in protoplasts. In the phosphor-mimic variants, the potential phosphorylation sites were changed to glutamine, in the phospho-dead variants, to alanine (Figure 43 A). Neither the phospho-mimic nor the phospho-dead version of OTU11 and OTU12 lost the PM localization in the protoplasts (Figure 43 B). In summary, the results showed that though GFP-OTU11 is phosphorylated in planta, though the phosphorylation is not important for the DUB activity of OTU11 and PM localization of OTU11 and OTU12.

![Figure 42: The phosphorylation status of GFP-OTU11 does not influence its catalytic activity. Phosphostain (ProQ Diamond) and total protein stain (SYPRO Ruby) of a DUB assay with phosphatase treated GFP-OTU11. The phosphorylation status of GFP-OTU11 had no influence on the DUB activity (p=phosphatase, ip =inactivated phosphatase).](image)
2.2.10. GST-OTU11 is activated by liposome binding \textit{in vitro}

As OTU11 is localized at the PM and might target PM proteins, it would be plausible that OTU11 needs lipid binding to assume its active conformation. The proximity of PBM1 and PBM2 to the catalytic active site in the structural model of OTU11 (Figure 44 A), could influence the ubiquitin binding and the cleavage activity of OTU11. N-term To rule out that the 6A1 variation disturbs the catalytic activity of GST-OTU11, GST-OTU11, and GST-OTU11 6A1 were incubated in more than 10x excess with K63-linked tetra-ubiquitin. Both versions of OTU11 were able to cleave K63-linked tetra-ubiquitin (Figure 44 B). Although the cleavage efficiency of the 6A1 variant was lower, the mutation did not interfere with the catalytic activity of OTU11.
**Figure 44: GST-OTU11 is activated by liposome binding.**

A) Homology and *ab initio* model (trRosetta, template hsOTUD5 domain, 3tmp) of OTU11 (black) in complex with ubiquitin (blue). The position of the ubiquitin is taken over from the template. The potential polybasic motifs (pink), which are probably important for membrane binding, are in close proximity of the potential ubiquitin binding site.

B) Anti-ubiquitin blot and whole protein staining of an *in vitro* DUB Assay with 100 pmol GST-OTU11.2, GST-OTU11.1 6A1 and 16 pmol K63-linked tetra-ubiquitin. GST-OTU11 was more active than GST-OTU11 6A1.

C) Anti-ubiquitin and anti-OTU11 blot of a DUB Assay with Liposomes. GST-OTU11.2 and GST-OTU11.1 6A1 (25 pmol) was pre-incubated for 15 min RT with PI(4,5)P₂-containing liposomes produced by Tobias Bläske, mixed with 24 pmol (750 ng) of K63-linked tetra-ubiquitin and incubated for 1 and 4 hrs. Liposome buffer was used as negative control. The activity of both GST-OTU11 WT and 6A1 is enhanced by liposome binding. The difference is larger in the wild-type samples.

To test if lipid binding has an influence on the activity, OTU11 WT and 6A1 was pre-incubated with PI(4,5)P₂-containing liposomes produced by Tobias Bläske. PI(4,5)P₂ was chosen as it is the PIP species that is only found in the PM of Arabidopsis cells. The pre-incubated enzymes were mixed with K63-linked tetra-ubiquitin and the cleavage efficiency after 1 and 4 hours was compared with a buffer control. GST-OTU11 had a higher cleavage efficiency in the samples with liposomes than in the buffer controls (Figure 44 C). The activity of the 6A1 variant was also slightly enhanced in the liposome-containing sample. However, the enhancement was higher for the wild-type version. The DUB assay with liposomes shows that liposome binding enhanced the catalytic activity of OTU11 against K63-linked tetra-ubiquitin and that PBM1 is important for this mechanism.
2.2.11. OTU11 and OTU12 are required for primary root growth in Arabidopsis seedlings

To analyze the influence of OTU11 and OTU11 on the growth and development of Arabidopsis seedlings, *otu11otu12* knockout mutants as well as OTU11 and OTU12 overexpressor (o/e) lines were generated. To generate *otu11otu12* mutants, TDNA-insertion mutants *otu11* (SALK_05296) and *otu12* (SALK_132515) were crossed (Figure 45 A) and homozygous *otu11otu12* lines were isolated with genotyping (Franziska Anzenberger, Technical University of Munich) (Figure 45 B). Tobias Bläské showed with an RT-PCR that OTU11 and OTU12 are not expressed in *otu11otu12* (Figure 45 C). The expression of GFP-OTU11 and GFP-OTU12 in the o/e lines was verified with an anti-GFP-blot (Figure 45 D).

**Figure 45: The TDNA insertion mutants *otu11* and *otu12* are knockout mutants.**

A) Scheme showing the intron and exon structure of OTU11 (At3g22260) and OTU12 (At3g02070) and the position of the T-DNA insertions. In the *otu11* line (SALK_05296), the insertion is positioned in the fifth intron shortly after the fourth exon, in the *otu12* line (SALK_132515) the insertion is positioned in the fourth exon.

B) Genotyping PCR of Col 0 and *otu11 otu12* mutant seedlings, showing that the mutant lines are homozygous for both alleles.

C) RT-PCR of Col 0 and *otu11 otu12* mutant seedlings, showing the knockout of OTU11 and OTU12 in the mutant lines.

D) Anti-GFP-blot of the total plant extract of 7-day old Col 0, 35S: GFP-OTU11.2-1 (KV30-1), 35S: GFP-OTU11.2-2 (KV30-2) and 35S: GFP-OTU12 (KV31) seedlings. Actin was used as a loading control. Black arrowhead: GFP-OTU11, white arrowhead: GFP-OTU12.
The length of the primary root was measured on ½ MS to analyze if OTU11 or OTU12 influence the growth or development of Arabidopsis seedlings. *otu11otu12* seedlings had significantly longer primary roots than wild-type seedlings (Figure 46 A+B, C+D). The difference is very small. It was, however, consistently observed in many experiments. The root length phenotype could be rescued by the OTU11 and OTU12 own promotor constructs. (Figure 46 A+B, C+D). The OTU11p:GFP-OTU11/otu11 otu12 seedlings had the same root length as Col-0 seedlings. Interestingly, OTU12p:GFP-OTU12/otu11 otu12 had significantly shorter roots than the wild-type seedlings.

**Figure 46:** *otu11otu12* seedlings have significantly longer primary roots than Col-0 seedlings.
A) Representative pictures of Col-0, *otu11 otu12* and OTU12:gOTU12 / *otu11otu12* seedlings.
B) Boxplot of the root length measurement of 7-day-old Col-0, *otu11otu12* and OTU12:gOTU12 / *otu11otu12*. *otu11otu12* had significantly longer primary roots than Col-0 seedlings, OTU12:gOTU12 / *otu11otu12* seedlings had significantly shorter primary roots than Col-0 seedlings. This results indicates, that OTU12:gOTU12 complements the root length phenotype of the *otu11 otu12* seedlings.
C) Representative pictures of Col-0, *otu11otu12* and OTU11:gOTU11 / *otu11 otu12* seedlings.
D) Boxplot of the root length measurement of 7-day-old Col-0, *otu11otu12* and OTU11:gOTU11 / *otu11 otu12* seedlings. *otu11otu12* had significantly longer primary roots than Col-0 seedlings, OTU11:gOTU11 / *otu11otu12* seedlings had the same root length as Col-0 seedlings. This results indicates, that OTU11:gOTU11 complements the root length phenotype of the *otu11 otu12* seedlings.
To analyze the individual influence of OTU11 and OTU12 on the root growth, the assay was repeated with the single mutants *otu11* and *otu12*. OTU12 seems to have a stronger influence on the root growth, as only *otu12* had significantly longer primary roots compared with the wild-type seedlings (Figure 47 A+B). In contrast, two *OTU11* o/e lines and one *OTU12* o/e line had significantly shorter primary roots than Col-0 (Figure 47 C+D). The overexpressor phenotype of OTU11 correlated with the expression level (compare Figure 45 D). Altogether, the root length assays show that both OTU11 and OTU12 have an influence on primary root growth of Arabidopsis seedlings.

**Figure 47:** Both OTU11 and OTU12 have an influence on the root length.

E) Representative pictures of Col-0, *otu11*, *otu12* and *otu11 otu12* seedlings.
F) Boxplot of the root length measurement of 7-day old Col-0, *otu11*, *otu12*, and *otu11 otu12* seedlings. *otu12* and *otu11 otu12* seedlings had significantly longer roots than Col-0 seedlings. *otu11* did not have significantly longer primary roots than Col-0 (A, p<0.05), *otu12* did not have significantly longer roots *otu11* (C, p<0.05) and *otu12* seedlings did not have significantly longer primary roots than *otu11 otu12* seedlings (B, p<0.05).
G) Representative pictures of Col-0, 35S:GFP-OTU11.2-1/2 and 35S:GFP-OTU12 seedlings.
H) Boxplot of the root length measurement of 7-day-old Col-0, 35S:GFP-OTU11.2-1, 35S:GFP-OTU11.2-2, and 35S:GFP-OTU12 seedlings. Both the OTU11 o/e and the OTU12 o/e lines had significantly shorter primary roots than Col-0 seedlings. 35S:GFP-OTU11.2-1 seedlings had significantly shorter roots than 35S:GFP-OTU11.2-2 and 35S:GFP-OTU12 seedlings (groups A, B, C, p<0.001).
There was no visible difference between 4-week-old wild-type and otu11otu12 plants grown on soil in continuous light, long-day or short-day conditions (Figure 48 A). As OTU11 and OTU12 are deubiquitylating enzymes, their influence on the abundance of ubiquitylated proteins was analyzed.

Figure 48: *otu11otu12* mutants do not show germination or growth defects or accumulated ubiquitylated proteins.
A) Representative pictures of 4-week old Col-0 and *otu11otu12* plants grown in continuous light, LD and SD conditions on soil in plant growth chambers (Sanyo chambers). There was no visible difference between wild-type and mutant plants.
B) Anti-ubiquitin blot of the total plant extract of 10-day old Col-0, *otu11otu12* and 35S:GFP-OTU11.2 seedlings. There was no visible difference in the ubiquitin content. An Anti-Actin antibody was used as a loading control, the presence of overexpressed OTU11 was confirmed with an anti-OTU11 antibody.
C) Representative pictures of a germination assay with Col-0 and *otu11otu12* on GM with and wild type and mutant.
D) Representative confocal images of propidium iodide stained root tips of Col-0 and *otu11otu12* seedlings.
E) Quantification of the meristem length and root width in the propidium iodide Col-0 and *otu11otu12* root tips. There was no significant difference between wild type and mutant.
In addition, there was no visible difference in the total ubiquitin-conjugates between the otu11otu12, 35S:GFP-OTU11-1, and wild-type seedlings (Figure 48 B). The knockout of OTU11 and OTU12 did not influence the germination of seedlings on GM regardless of the presence of the phytohormone abscisic acid (ABA), which inhibits germination (Figure 48 C). Either differences in the generation of new cells or the elongation of the cells could be the reason for the enhanced root growth. To analyze this, the meristem length was measured in propidium-iodide stained wild-type and otu11otu12 seedling roots (Figure 48 D). As there was no difference between Col-0 and otu11otu12 roots in the meristem length, it is improbable that the root length difference is caused by defects in the generation of new cells (Figure 48 E).

To test the possibility that OTU11 and OTU12 are important for the elongation of the cells, the root growth on media containing the synthetic auxin Indolacetic acid (IAA) and the auxin transport inhibitor Naphthylphthalamic acid (NPA) was analyzed. Treatment with IAA, a synthetic auxin that needs a carrier to be transported in the cell, leads to less primary root growth and an induction of lateral root growth. The auxin transport inhibitor NPA also leads to less primary root growth and defects in gravitropism (Casimiro et al., 2001; Rahman et al., 2007). To examine the sensitivity of otu11otu12 and Col-0 seedlings to IAA and NPA, they were grown on ½ MS for 7 days and then transferred to plates with different concentration of NAP and IAA (Figure 49 A). After 10, 12 and 14 days the primary root length was measured and divided by the value directly after the transfer. otu11otu12 and Col-0 seedlings that were transferred from normal growth conditions (Figure 49 B) to plates with different concentrations of IAA, did not show any differences in the sensitivity (Figure 49 C). In contrast to this, for otu11otu12 and Col-0 seedlings that were transferred from normal growth conditions (Figure 49 D) to plates with different concentrations of NPA there were subtle differences in the sensitivity (Figure 49 C). At the highest tested NPA concentration, 1.5 µM NPA, otu11otu12 seedlings were slightly more sensitive to the NPA treatment after 12 and 14 days (Figure 49 C). These results could indicate that OTU11 and OTU12 influence the primary root length via auxin signaling.
Figure 49: *otu11otu12* seedlings are slightly more sensitive to NPA than Col-0 seedlings.

A) Representative pictures of Col-0 and *otu11 otu12* seedlings grown in LD on ½ MS medium, 1 µM NPA and 0.2 µM IAA.

B) Boxplot of the root length measurement of 7-day old Col-0, *otu11otu12* seedlings before the IAA treatment. *otu11 otu12* had significantly longer primary roots than Col-0 seedlings.

C) Analysis of the IAA treatment effects on the root length of Col-0 and *otu11otu12* seedlings. Seedlings were grown on 0.025, 0.05 and 0.2 µM IAA. The treatment was started after 7 days. The length of at least 40 roots was measured after 7, 10, 12 and 14 days and normed on the 7-day root length at the start of the treatment. Col-0 and *otu11otu12* roots did not react differently on the IAA treatment.

D) Boxplot of the root length measurement of 7-day old Col-0, *otu11 otu12* seedlings before the NPA treatment. *otu11 otu12* had significantly longer primary roots than Col-0 seedlings.

E) Analysis of the NPA treatment effects on the root length of Col-0 and *otu11otu12* seedlings. Seedlings were grown on 0.5, 1 and 1.5µM NPA. Col-0 and *otu11otu12* roots did not react differently on the treatment with 0.5 and 1 µM NPA. However, the *otu11otu12* seedlings were significantly more sensitive to the treatment after 5 days (12 days in total) and 7 days (14 days in total) on 1.5 µM NPA.
2.2.12. Potential OTU11 and OTU12 interactors from a yeast two-hybrid screen are not localized at the PM

To find interacting proteins that might function together with OTU11 and OTU12, potential interactors were identified in a yeast two-hybrid screen (Braun Lab, Helmholtz Zentrum, Munich). One of the potential interactors, the Really Interesting New Gene (RING)-type-domain-containing protein (At1g17970) could be part of E3-Ligase complexes and therefore functionally connected with OTU11 and OTU12 (Lechner et al., 2006; Hua and Vierstra, 2011) (Figure 50 A). The RING-domain-containing protein interacted with GST-OTU11 and GST-OTU12 in vitro (Figure 50 B), and localized to the nucleus in Arabidopsis protoplasts (Figure 50 C). It is possible, that OTU11 and OTU12 function together with the RING-domain-containing in the nucleus.

A second interactor, Forked-like 5 / Pleckstrin-homomology domain containing protein 15 (FL5/PH15, At4g17350) contains a membrane-binding pleckstrin homology (PH) domain which could contribute to the binding of OTU11 or OTU12 to the PM (Figure 51 A). Full length MBP-FL5/PH15 interacted with GST-OTU11 and GST-OTU12 in vitro (Figure 51 B). As the N-terminus of GST-OTU11 interacted with the N-terminus of MB-FL5/PH15, the interaction site seems to be in the N-terminus of both proteins (Figure 51 C). In protoplasts FL5/PH15 localized to filaments, maybe the cytoskeleton (Figure 51 D).

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**Figure 50:** RING-domain-containing protein interacts with GST-OTU11 and GST-OTU12 in vitro and localizes to the nucleus.

A) Scheme of RING (Really Interesting New Gene (RING)-type domain-containing protein (At1g17970)).


C) Anti-MBP and anti-GST western blot of an in vitro binding assay with GST-OTU11, GST-OTU12 and an MBP-tagged version of the RING-domain-containing-protein.
Figure 51: The N-terminus of GST-OTU11 interacts with the N-terminus of MBP-FL5/PH15.
A) Scheme of the PH15/FL5 (At4g17350) full length and PH15/FL5 (N).
B) Selected confocal pictures of protoplasts expressing 35S:RFP-PH15/FL5. RFP-FL5/PH15 localized to filaments.
C) Anti-MBP and anti-GST western blot of an in vitro binding assay with MBP-FL5/PH15, GST-OTU11 and GST-OTU12. In the in vitro binding assay, 100 pmol of GST-tagged proteins was mixed with 500 pmol of MBP-PH15. Both GST-OTU11 and OTU12 bound to MBP-FL5/PH15. D) Anti-MBP and anti-GST western blot of an in vitro binding assay with MBP-FL5/PH15 (N) and GST-OTU11(N). In the in vitro binding assay 100 pmol of GST-tagged proteins were mixed with 500 pmol MBP-PH15 N-terminus. The N-terminus of OTU11 was able to bind to the N-terminus of PH15 in vitro.

It was also reported to localize to the Golgi in tobacco cells (Prabhakaran Mariyamma et al., 2018). In addition to this, there was no obvious difference between wild type and otu11otu12 in the vein pattern, a phenotype previously observed for FL proteins (Prabhakaran Mariyamma et al., 2018), Figure 52 A).

Figure 52: otu11otu12 has no forked-like or seed coat mucilage phenotype.
A) Representative microscope pictures of the vein pattern in Col-0 and otu11otu12 cotyledons. There was no difference in the vein pattern.
B) Microscope pictures of the seed coat mucilage of Col-0 and otu11otu12 untreated seeds and seeds treated with 50 mM EDTA or CaCl2 stained with ruthenium red. There was no difference between Col-0 and otu11otu12 seed coats.
C) Quantification of the seed coat mucilage area in comparison to the seed area in the untreated sample. There was no significant difference between Col-0 and otu11otu12.
This further indicates that OTU11 and OTU12 have no functional connection with FL5/PH15 in the plant cell. As FL5/PH15 was reported to localize to the Golgi a Golgi-related phenotype, the seed coat extrusion under different conditions, was analyzed. Differences in the seed coat formation can be caused by defects in Golgi-related trafficking processes (Voiniciuc et al., 2013; Saez-Aguayo et al., 2017). Seed coat extrusion is enhanced by the removal of Ca\(^{2+}\) from the media and reduced by CaCl\(_2\) treatment. As there was no difference in the seed coat mucilage area of between wild-type and otu1otu12 seeds in treated and untreated seeds (Figure 52 B +C), it is improbable that OTU11 and OTU12 function together with FL5/PH15 and influence Golgi-mediated secretion processes in Arabidopsis seeds.

2.2.13. Aquaporins are potential OTU11 targets

To identify potential OTU11 interactors, a GFP-IP with subsequent MS analysis was conducted (Julia Mergner, Küster Lab, TUM). In the GFP-OTU11 sample, 378 proteins were significantly enriched (Figure 53 A), 165 were enriched more than 8x. Among them were several proteins associated with the transport of membrane proteins. Examples are the COATOMER (COPI) subunits α, β, γ and ε, CLATHRIN-ASSOCIATED ADAPTOR PROTEIN COMPLEX 2 (AP-2) μ and DYNAMIN LIKE 1 (DL1).

![Figure 53: A part of the proteins that are significantly enriched in the GFP-OTU11 sample might be ubiquitylated.](image)

A) Cut-out of the volcano blot of the proteins that were significantly enriched in the GFP-IP with GFP-OTU11 (Julia Mergner, Küster Lab, TUM). The PM proteins H\(^+\)-ATPase 1 and 2 and the PLASMA MEMBRANE INTRINSIC PROTEINS (PIPs) 1A, 1B, 1C, 2A and 3 are marked in red. B) Venn diagram of significantly enriched proteins in the GFP-OTU11 sample and the K63- and TUBE-ubiquitylome. 36.4% of the proteins that are significantly enriched in the GFP-OTU11 samples were found in the ubiquitylomes.
Figure 54: Most of the proteins in the overlap between the GFP-IP with OTU11 and the two ubiquitylomes are PM proteins.

A) Subcellular analysis with the internet tool SUBA4 of the more than 8x enriched proteins in the GFP-OTU11 sample. Most proteins were found in the cytosol.

B) Subcellular analysis with the internet tool SUBA4 of the proteins in the overlap between the GFP-OTU11 IP dataset, the TUBE and the K63-ubiquitylome. Most proteins were PM-related proteins.

They could be either targets of OTU11 or involved in the transport of OTU11 to and from the membrane. Initially, the GFP-IP was conducted to find potential interactors of OTU11. However, among the interactors could be also ubiquitylated OTU11 targets. Either OTU11 interacted with the ubiquitin chain attached to the target protein or with its target protein directly. To test if potential OTU11 targets were isolated in the GFP-IP, the dataset was compared with publishes ubiquitylomes, K63-Sensor ubiquitylome and the TUBE ubiquitylome (Kim et al., 2013; Johnson and Vert, 2016). 36.4 % of the proteins enriched by GFP-OTU11 were found in the ubiquitylomes (Figure 53 B). 15 proteins were found in both ubiquitylomes. The observation that the proteins were found in both ubiquitylomes is a hint that they are ubiquitylated. They could therefore be target proteins of OTU11. The potential OTU11 targets had a different distribution of subcellular localization than proteins in the complete dataset (Figure 54 C+D). The amount of cytosolic proteins decreased from one third to one sixth, whereas the amount of PM proteins increased from one eighth to one third. Among them were the PLASMA MEMBRANE INTRINSIC PROTEINS (PIP) 1C, 1B, and 2A which are also called aquaporins and the H+-ATPase isoforms 1 and 2.

In further experiments, the binding of GFP-OTU11 to PIP1 was verified with a GFP-IP followed by a immunoblot (Figure 55 A). To test, if there is a functional connection between GFP-OTU11 and PIP1 and H+-ATPase isoforms a P100/S100 fractionation of Col-0 and otu11otu12 extract was performed. There was about twice more PIP1 and about four times more H+-ATPase in the membrane-containing P100 fraction of the mutant, than in the wild type (Figure 55 B). In contrast to this, there was nearly no difference in the abundance of the cytosolic UGPase. The result indicates that the PIPs do not accumulate at PM, as they are accumulating in the endosomes.
Figure 55: Aquaporins are potential OTU11 targets.

A) Anti-PIP1, anti-GFP-blot and whole protein staining of a GFP-IP with GFP-OTU11. GFP was used as a negative control. The PIP1 monomer (31 kDa) was bound to the GFP-OTU11 beads.

B) Anti-PIP1, anti-UGPase and H+-ATPase-blot of a P100/S100 fractionation of Col0 and otu11otu12 seedling extract. In the P100 fraction of otu11otu12 there was slightly more PIP1 (monomer: 31kDa, dimer: 62 kDa) and H+-ATPase than in the P100 fraction of Col-0.

C) Representative microscope pictures of the lateral root emergence stages II to >VIII observed in Col-0 and otu11otu12 roots after a gravistimulus.

D) Quantification of the lateral root stage distribution in Col-0 and otu11otu12 roots 48 h after a gravistimulus. The experiment was repeated three times with different number off roots (n=40, n=64/62, n=100). There was no significant difference between Col-0 and otu11otu12 for each individual root stage.

The functional connection between PIP and OTU11 was also analyzed with a phenotypical experiment. pip2;1 (pip2A) mutants and PIP2 overexpressor lines are reported to have a delayed lateral root emergence (Péret et al., 2012). The lateral root development of Col-0...
and *otu11otu12* seedlings was examined, and the lateral root growth stages were identified 48 h after a gravistimulus in three independent experiments (Figure 55 C+D). There was no significant difference in the distribution of the lateral root growth stages.

*PIP* overexpressor seedlings have, similar to the *otu11otu12* mutants, longer primary roots than wild-type seedlings (Wang et al., 2019; Li et al., 2021). *H*-ATPase overexpressor lines do not have longer primary roots under normal growth conditions. Both the *PIP* and the *H*-ATPase overexpressor lines, however, have an enhanced tolerance to salt or osmotic stress (Wang et al., 2013a; Wang et al., 2019; Li et al., 2021). To analyze if OTU11 and OTU12 are also involved in salt or osmotic stress responses the root growth on different concentrations of NaCl, Mannitol and the PIP inhibitor AgNO₃ (Niemietz and Tyerman, 2002) was measured. Both the NaCl and Mannitol-treatment were stress conditions that led to reduced primary root growth (Figure 56 A+B, D+E). To examine the sensitivity towards the treatments the root length measured on the treatment plates was divided by the root length under standard conditions. *otu11otu12* seedlings were more sensitive to NaCl–induced salt stress and less sensitive to the PIP inhibitor AgNO₃ (Figure 56 C). However, there was no sensitivity difference between *otu11otu12* and Col-0 seedlings in the Mannitol treatment (Figure 56 F). The sensitivity to NaCl-treatment implies that OTU11 and OTU12 have biological functions which becomes important under stress conditions. If there is a functional connection between the OTU11 and OTU12 and the aquaporins or the H*-ATPases must be analyzed in further experiments.
Figure 56: *otu11* *otu12* seedlings are more sensitive to salt stress than Col-0 seedlings.
A) Representative pictures of Col-0 and *otu11* *otu12* seedlings grown in LD conditions on ½ MS medium with 50 µM AgNO₃ and 100 and 150 mM NaCl.
B) Boxplot of the root length measurement of 7-day old Col-0, *otu11* *otu12* seedlings grown on ½ MS medium with AgNO₃ and NaCl. *otu11* *otu12* had significantly longer primary roots than Col-0 seedlings on ½ MS and 1 µM AgNO₃. In the other conditions there was no significant difference between wild type and mutant.
C) Analysis of the effect of NaCl and AgNO₃ treatment on the root length of Col-0 and *otu11* *otu12* seedlings. The root length measured in (B) was normed on the root length under control conditions. *otu11* *otu12* seedlings reacted more sensitive to 100 and 150 mM NaCl and less sensitive to 50 µM AgNO₃.
D) Representative pictures of Col-0 and *otu11* *otu12* seedlings grown in LD conditions on ½ MS medium with 100, 200 and 300 mM Mannitol.
E) Boxplot of the root length measurement of Col-0, *otu11* *otu12* seedlings grown on ½ MS medium with 100, 200 and 300 mM Mannitol. *otu11* *otu12* had significantly longer primary roots than Col-0 seedlings in all conditions.
F) Analysis of the effect of Mannitol treatment on the root length of Col-0 and *otu11* *otu12* seedlings. The root length measured in (B) was normed on the root length under control conditions. There was no sensitivity difference between Col-0 and *otu11* *otu12* seedlings.
2.2.14. OTU11 and OTU12 influence the endocytic degradation of PM proteins

To test if OTU11 and OTU12 are able to influence the degradation of PM proteins, the artificial endocytosis substrate 2x25S:GFP-Plasma membrane ATPase (PMA)-Ub (Herberth et al., 2012) was co-expressed in protoplasts with 35S:RFP-OTU11, 35S:RFP-OTU12 and the catalytic-site mutant of 35S:RFP-OTU12 C90R. PMA-GFP and the 35S:RFP-SYP121 were used as controls. Both GFP-PMA and PMA-UB localized to the vacuole, endosomes and the PM (Figure 57 A). As expected, GFP-PMA was localized at PM in nearly all protoplasts in the control, in the RFP-OTU12, and RFP-OTU12 C90R sample (Figure 57 B). In the RFP-SYP121 and the RFP-OTU11 sample, the PM localization was significantly reduced. This observation is difficult to explain. It could be an artefact caused by the competition for membrane binding sites.

In the PMA-Ub experiment, both the buffer and the SYP121 control had approximately the same localization patterns with approximately 50% of the protoplasts showing PM localization of GFP-PMA-UB. The number of protoplasts with PM localization was significantly increased by the co-expression of 35S:RFP-OTU12, 35S:RFP-OTU12 C90R and 35S:RFP-OTU11 (Figure 57 C). This shows that both RFP-OTU11 and RFP-OTU12 stabilized PMA-GFP-Ub at the PM. Surprisingly, the catalytic site mutant of OTU12, OTU12 C90R, also had a stabilizing effect. A possible explanation is that the catalytic activity of OTU12 is not essential for its stabilizing effect. Altogether, the results show that OTU11 and OTU12 are able to influence the degradation of PM proteins in vivo.

To test if OTU11 and OTU12 also influence the degradation of PM proteins in planta, the Brefeldin A (BFA)-dependent endocytosis of PIN2-GFP was analyzed. PIN2-GFP-expressing wild-type and otu11otu12 seedlings were treated with 50 µM of the endocytosis inhibitor BFA. BFA inhibits ER-Golgi trafficking and leads to the aggregation of endosomes and TGN to BFA bodies (Chardin and McCormick, 1999). The PM-localized auxin transporter PIN2-GFP is internalized in the BFA bodies (Leitner et al., 2012). The effect of BFA on the internalization of PIN2-GFP in otu11otu12 and Col-0 seedlings was analyzed at different time points. In the otu11otu12 cells, BFA bodies with PIN2-GFP were observed at earlier time points than in the Col-0 cells (Figure 58 A). After 30 to 40 min BFA bodies were formed in otu11otu12 root cells. In Col-0, the first BFA bodies were observed after 60 min. In contrast to the experiment with PIN2-GFP there were no visible differences between Col0-0 and otu11otu12 cells in the BFA treatment with another endocytic cargo, the styryl dye FM4-64 (Figure 58 B).
Figure 57: RFP-OTU11 and RFP-OTU12 stabilized GFP-PMA-Ub at the PM of protoplasts.

A) Representative confocal pictures of protoplasts transformed with the artificial endocytosis substrate GFP-PMA-Ub and the GFP-PMA control.

B) Distribution of localization patterns of GFP-PMA in protoplasts that were co-transformed with GFP-PMA, and 35S:RFP-OTU12, 35S:RFP-OTU11, and 35S:RFP-OTU12 C90R. 35S:RFP-SYP121 was used as a negative control. The RFP-OTU12 did not influence the localization of PMA-GFP. In the RFP-SYP121 and the RFP-OTU11 sample, the amount of protoplasts with PM localization was significantly reduced.

C) Distribution of localization patterns of GFP-PMA-Ub in protoplasts that were co-transformed with GFP-PMA, and 35S:RFP-OTU12, 35S:RFP-OTU11, and 35S:RFP-OTU12 C90R. 35S:RFP-SYP121 was used as a negative control. The overexpression of RFP-OTU12, RFP-OTU12 C90R and RFP-OTU11 enhanced the number of protoplasts with PM localization significantly.

The significance was tested with a Fisher Test in R (*p>0.05, **p>0.01, ***p>0.001). Vac=Vacuole, End=Endosomes. The with PM category includes protoplasts with the following localization patterns: PM + vacuole, PM, endosomes + vacuole, PM + endosomes and PM.
Figure 58: OTU11 and OTU12 influence the endocytosis of the PM protein PIN2-GFP in Arabidopsis seedlings.

A) Selected confocal pictures of epidermis cells of PIN2:PIN2-GFP / Col-0 and PIN:PIN2-GFP / otu11otu12 seedlings treated with the endocytosis inhibitor Brefeldin A (BFA). BFA bodies were formed faster in the otu11otu12 mutant, after 15 min there were already visible aggregates in the mutant. In Col-0 first BFA bodies were visible after 60 min. After 90 min, the BFA bodies had the same size and abundance in both wild-type and mutant samples.

B) Selected confocal pictures of epidermis cells of Col-0 and otu11otu12 seedlings, that were pre-stained with FM4-64 and treated with BFA. Both in Col-0 and the mutant, BFA bodies were formed after 15 min.

Altogether, the cell biological experiments show that OTU11 and OTU12 are able to stabilize PM proteins in vivo and might have a stabilizing effect on some endocytic cargo proteins. OTU11 and OTU12 are probably involved in fine-tuning processes that regulate the abundance of PM proteins.
3. Discussion

3.1. AMSH1 targets various proteins

The aim of my study was to elucidate the function of deubiquitylating enzymes involved in the degradation of membrane proteins in *A. thaliana*. The first attempt to obtain an ubiquitylome with the affinity purification of ubiquitylated protein with the ubiquitin binding GST-2xGAT constructs and subsequent N-NTA-purification did not lead to identification potential AMSH1 targets as there was too much non-specific binding. In the second affinity purification experiment, it was not possible to assess the influence of *AMSH1*. The third affinity purification experiment with GST-2xGAT was conducted with three replicates for each genotype. In this experiment, the abundance of 40 proteins was significantly influenced by the knockdown of *AMSH1*. In both affinity purification experiments without a N-NTA step, there was an overlap with the Arabidopsis K63-ubiquitylome (Johnson and Vert, 2016) and the TUBE ubiquitylome (Kim et al., 2013). As both experiments are based on an affinity purification method, also non-ubiquitylated proteins that are not covalently to the ubiquitylated proteins were co-purified. In the TUBE experiment, however, the amount of non-ubiquitylated proteins was reduced by the denaturing Ni-NTA step. So the occurrence in the overlapping region is a strong indication of ubiquitylation.

PLEIOTROPIC DRUG RESISTANCE PROTEIN 6 (PDR6) was found both in GST-TUBE and GST-2xGAT samples in the second proteomics dataset and the K63-ubiquitylome and is a potential AMSH1 target. PDR6 is important for the transport of a secondary metabolite involved in pathogen defense (Khare et al., 2017). Furthermore, PDR6 belongs to a family of multi-pass ATP-binding cassette transporters with members such as PDR8 that are known to localize to the PM in Arabidopsis cells and are involved in pathogen defense (Stein et al., 2006). Two other PM-proteins that are involved in plant immunity, the BR-SGNALLING KINASE 1 (BSK1) (Yan et al., 2018) and the MAC/PERSFORIN-DOMAIN CONTAINING PTOTEIN (CAD1) (Morita-Yamamuro et al., 2005) were enriched both in the GST-2xGAT and the GST-TUBE samples and found in the TUBE ubiquitylome (Kim et al., 2013). Considering that *amsh1* mutants were more susceptible to infections with fungal pathogens (Katsiarimpa et al., 2013), it is plausible that potential AMSH1 targets are involved in plant immunity. Along this line, it is of high interest that the AMSH1 homologues AMSH3 and the *L. japonicas* AMSH1 are involved in plant immunity and infection processes (Malolepszy et al., 2015; Schultz-Larsen et al., 2018). Further studies have to be conducted to elucidate the potential connection between AMSH1 and PM-proteins involved in plant immunity.
Three Proteins that might be associated with membrane protein trafficking processes were also found in the overlap between the proteins enriched in the \textit{amsh1} mutant by GST-2xGAT, GST-TUBE sample and the TUBE ubiquitylome (Kim et al., 2013). One of the proteins, the WD-40 REPEAT FAMILY PROTEIN was found to be ubiquitylated at lysine 535 in the TUBE ubiquitylome (Kim et al., 2013). The WD-40 REPEAT FAMILY PROTEIN is a BEIGE AND CHEDIAK-HIGASHI (BEACH)-DOMAIN PROTEIN that contributes to the transport of proteins to the protein storage vacuole in seeds (Teh et al., 2015). Another protein, the TGN-localized protein ADAPTOR PROTEIN-1 (AP-1) is involved in clathrin-mediated endocytosis and post-Golgi trafficking (Yan et al., 2021). The third enriched protein was the RAS-RELATED PROTEIN GDPASE HOMOLOG B1C (RAB-BC1). The homologues RAB-A2 and RABA-A3 of RAB-B1C are localized at the TGN and contribute to the formation of the cell plate (Chow et al., 2008). Another member of this family, the RAB-D2b, was significantly enriched in the wild-type sample of the third affinity purification experiment with only GST-2xGAT. In the third experiment, further proteins associated with membrane-trafficking processes were found to be influenced by the amount of AMSH1. One protein enriched in the wild-type sample was the protein ALA-INTRACTING SUBUNIT 3 that interacts with a flippase, an enzyme responsible for the flipping of phospholipids across cellular membranes (López-Marqués et al., 2010). Additionally, in the wild-type sample, three PM-localized proteins that belong to the same family and are known to be involved in membrane-trafficking processes were significantly enriched. One of these proteins was found in the K63-ubiquitylome. As the proteins were significantly enriched in the wild-type sample, AMSH1 could have a stabilizing effect on some proteins associated with membrane-trafficking processes.

In the third affinity purification experiment with only GST-2XGAT, 30% of the proteins enriched in the \textit{amsh1} sample were ribosomal proteins. Ribosomes are degraded by proteasome-mediated degradation and selective autophagy in mammals (reviewed in (An and Harper, 2020)). As DUBs are important at multiple steps of selective autophagy (Tian et al., 2021), they could also be involved in ribophagy as shown for the \textit{S. cerevisiae} DUB Ubp3 (Kraft et al., 2008). In Arabidopsis, it has been shown that rRNA is degraded by autophagy (Floyd et al., 2015; Marshall and Vierstra, 2018), though the molecular details are yet unclear. Altogether, the MS results imply that AMSH1 is versatile DUB that has a stabilizing or de-stabilizing effect on various kinds of target proteins.
3.2. OTU11 and OTU12 could have a function at the PM

In Arabidopsis, the AMSH proteins and UBP12/13 are known to influence the degradation of PM-proteins (Isono et al., 2010a; Katsiarimpa et al., 2013; Luo et al., 2022). In this study, two additional proteins, OTU11 and OTU12, were identified that could have an influence on the degradation of PM-proteins. As OTU11 and OTU12 are localized at the PM in Arabidopsis protoplasts and cells and are able to bind directly to PIPs \textit{in vitro}, they could target proteins at the PM.

The PM-binding mechanism of OTU11 and OTU12 seems to include at least two different sequence motifs, PBM1 and PBM2. For PM-localized D6PK-related kinases polybasic motif was enough to mediate PM-association in protoplasts (Barbosa et al., 2016). In contrast to this, PBM1 was not essential for PM localization in protoplasts, it only reduced the PM localization. This is in accordance with the observation that most PM proteins do not only bind to the PM via a polybasic motif and require additional anchor or adaptor proteins (Simon et al., 2014). Although PBM2 led to the loss of PM localization \textit{in vivo}, it did not influence the binding to phospholipids \textit{in vitro}. Thus, PBM2 could be important for the trafficking to the PM or for binding to interacting proteins, in addition to the lipid-binding PBM1.

Both OTU11 and OTU12 had only a weak catalytic activity \textit{in vitro} and were able to cleave K63-linked ubiquitin only when added in excess, which is in accordance with previously published results (Radjacommare et al., 2014). The catalytic activity of OTU11 was enhanced by the binding to liposomes \textit{in vitro}. This observation suggests that the DUB activity of OTU11 is closely connected to its membrane localization. So far, similar mechanisms have not been shown for other DUBs. The PM-localized human DUB JosD1 was active \textit{in vitro} without lipid binding when it was activated by ubiquitylation (Seki et al., 2013). The ER-localized human USP35 without TM-domain and the USP domain of the TGN-localized human USP32 were also active \textit{in vitro} without lipid binding (Leznicki et al., 2018; Sapmaz et al., 2019). The only DUB-like enzyme that is known to directly bind to PIPs as OTU11 and OTU12 is RavZ, a protein of the intracellular human pathogen \textit{L. pneumophila}. RavZ imitates the function of the Ub-like-deconjugating enzyme Atg4 of its human host and blocks autophagy by irreversibly cleaving Atg8 from pre-autophagosomal structures. However, although RavZ binds to PI(3)P-lipids \textit{in vitro}, it does not require liposome binding for its \textit{in vitro} delipidation activity (Horenkamp et al., 2015). Thus, the activation by lipid binding seems to be a characteristic feature of OTU11 and maybe also of OTU12 which might restrict the DUB activity to the PM and maybe also other intracellular membranes.
A close connection of the catalytic activity of OTU11 with its PM-localization would be advantageous if OTU11 targets PM-proteins. As OTU11 and OTU12 cleave free tetra-ubiquitin, it is unlikely that their activity is dependent on the interaction with a specific target protein. It is, however, possible that OTU11 and OTU12 have a general fine-tuning effect on the abundance PM proteins. The experiments with the artificial endocytosis substrate GFP-PMA-Ub indicate that OTU11 and OTU12 could modulate the abundance of PM proteins. One potential target could be PIN2. The auxin transporter PIN2 is known to be K63-poly-ubiquitylated and the ubiquitylation influences the stability of PIN2-GFP at the PM. Ubiquitylation deficient PIN2-GFP is stabilized at the PM, but it is still internalized in BFA bodies upon BFA treatment (Leitner et al., 2012). OTU11 and OTU12 could influence the ubiquitylation status of PIN2-GFP at the PM and stabilize PIN2-GFP at the PM.

Other targets of OTU11 and OTU12 could be the PM-proteins which were significantly enriched in the GFP-IP with GFP-OTU11. The enriched H+-ATPase and aquaporin isoforms were partly also identified in the K63- and the TUBE ubiquitylome (Kim et al., 2013; Johnson and Vert, 2016). Furthermore, one of the identified H+-ATPase isoforms (AHA2) was reported to be ubiquitylated at Lysine 330 (Kim et al., 2013) and one of the identified aquaporins (PIP2A) was reported to be ubiquitylated at Lysine 276 (Chen et al., 2021), although in my experiments it could not be clarified if H+-ATPases or aquaporins are indeed targets of OTU11 or OTU12.

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*otu11otu12* seedlings had longer primary roots than wild-type seedlings. The root length difference could be caused by changes in the abundance H+-ATPases, aquaporins, or auxin transport protein PIN2 at the PM as the stabilization of H+-ATPases, aquaporins or PIN2 could enhance the primary root length of Arabidopsis seedlings (Rahman et al., 2007; Wang et al., 2013a; Wang et al., 2019). In further experiments, *otu11otu12* seedlings were more sensitive to the auxin transport inhibitor NPA and salt stress. The response to NPA could be influenced by changes in the abundances of PIN proteins, the salt stress response by changes in the abundance of H+-ATPases or aquaporins. The exact connection between the cell biological and physiological function of OTU11 and OTU12, however, remains to be elucidated.

### 3.3. OTU11 and OTU12 could have a function in the nucleus

As OTU11 and OTU12 are localized in the nucleus in Arabidopsis protoplasts and plant cells, they could also have a function in the nucleus in addition to their function at the PM. The finding that OTU11 is activated by membrane lipids *in vitro* indicates that OTU11 and
OTU12 require binding to membrane lipids for their activation. The cytosolic and nuclear fractions of OTU11 and OTU12 are probably not active. As the catalytic inactive version of OTU12 was able to stabilize PMA-GFP-UB at the PM, OTU11 and OTU12 could have a non-catalytic activity in the cytosol or the nucleus as described for human OTUB1 (Nakada et al., 2010) or human OTUD4 (Zhao et al., 2015).

However, it is also possible that OTU11 and OTU12 bind to nuclear membranes and thus are activated in the nucleus. OTU11 and OTU12 could interact with phospholipids in the nuclear envelope (Peeters et al., 2022). Currently it is not known if DUBs localize to the nuclear envelope. Human FAM105A/OTULINL, a catalytically inactive OTU protein, is the only reported example of a nuclear envelope-localized pseudo-DUB (Ceccarelli et al., 2019). PI(4,5)P₂ has been found in the nuclei of Arabidopsis seedlings and tobacco cells. Upon heat stress, a PI(4,5)P₂-binding protein accumulated at the nuclear envelope of tobacco BY-2 cells (Mishkind et al., 2009). Furthermore, the PI(4,5)P₂-binding domain 1xTUBBY-C (P15R/Y) localized both to the PM and the nucleus in Arabidopsis seedlings (Simon et al., 2014). The PI(4,5)P₂-binding OTU11 and OTU12 could have a function both at the PM and in the nucleus. A DUB that is bound to the nuclear envelope could have an impact on the abundance of proteins that influence gene expression or chromatin remodeling via the interaction with components of the nuclear lamina (Blank, 2020; Evans et al., 2020).

An explanation for the YFP-OTU11 and YFP-OTU12 signal in the interior of the nucleus could also be a localization to membrane-containing structures within the nucleus. In mammalian cells, the nucleus contains nuclear lipid islets, membrane-covered lipid structures which are associated with the RNA and the transcription machinery. PI(4,5)P₂ is found in the surface of the nuclear lipid islets and the amount of PI(4,5)P₂ in the surface of the lipid islets is important for the transcription activity of polymerase II (Sobol et al., 2018; Hoboth et al., 2021). As OTU11 and OTU12 bind to PI(4,5)P₂ they could interact with nuclear lipid islets in the nucleus if the potential plant nuclear lipid islets had the same composition as the mammalian structures. Whether OTU11 and OTU12 have a catalytic or non-catalytic function in the nucleus has to be analyzed in further experiments.

3.4. AMSH1 as well as OTU11 and OTU12 influence selective protein degradation processes

Both AMSH1 and OTU11/12 seem to influence the selective degradation of PM proteins (Figure 59). AMSH1 and AMSH3 influence the stability of PM proteins by their interaction
with ESCRT-III. AMSH1 probably influences the abundance of PM proteins by removing their K63-linked ubiquitin chain prior to the internalization in the endosomes. Additionally, AMSH1 might target components of the membrane-protein trafficking machinery and AMSH1 might have a role in the selective autophagy of cytosolic proteins.

OTU11 and OTU12 bind to the PM via a PIP-binding polybasic motif PBM1. The PM localization of OTU11 and OTU12 is also influenced by PBM2. PBM2 could be a binding site for an adaptor protein that stabilizes OTU11 and OTU12 at the PM. Additionally, OTU11 and OTU12 might be stabilized at the PM by the interaction with the K63-linked ubiquitin chain of potential PM-localized target proteins. OTU11 and OTU12 seem fine-tune the abundance of PM proteins modified with a K63-linked ubiquitin chain. Probably, OTU1 and OTU12 rescue PM-proteins from endocytic degradation by removing or shortening K63-linked ubiquitin chains attached to the PM-proteins. Furthermore, OTU11 and OTU12 might have a function in the nucleus. In the nucleus, OTU11 and OTU12 could interact with PIPs in the nuclear envelope or intra-nuclear structures and influence the abundance of nuclear proteins. However, if OTU11 and OTU12 have a function in the nucleus has to be analyzed in further experiments.

Figure 59: Both AMSH1 and OTU11/12 influence the selective degradation of proteins. Scheme showing the potential functions of AMSH1 and OTU11 and OTU12 in the plant cell. Grey: target proteins, blue: ubiquitin.
4. Material and Methods

4.1. Cloning procedures

4.1.1. RNA extraction and cDNA synthesis

Total RNA was extracted from Arabidopsis seedlings with the Nucleospin RNA-Kit for plant and fungi (MN) according to manufactures’ protocols. The RNA concentration was measured with the Nanodrop One photometer (Thermo-Fisher Scientific). cDNA was transcribed from RNA with M-MuLV Reverse Transcriptase (NEB) or SuperScript Transcriptase II (Thermo-Fisher Scientific). For the transcription with the MuLV-RT up to 1 µg RNA, 2 µL 50 µM Oligo-dT-Primer, 2 µL 10x buffer, and 4 µL 2.5 mM dNTP Mix were mixed with 1 µL M-MuLV-RT, filled up to 20 µL with water and incubated for 42°C at 1 hour. After the transcription, the Transcriptase was inactivated for 20 minutes at 65°C.

For the transcription with the SuperScript Transcriptase II, 20 µL RNA, 0.5 µL Random Primer and 2 µL 50 µM Oligo-dT-Primer were mixed and incubated for 5 min at 70°C. After cooling, 26.5 µL dH₂O, 20 µL 5x First-Strand Buffer, 10 µL 10 mM dNTP Mix (Roth), 10 µL 100 mM dithioerythritol (DTT, Roth), and 5 µL SuperScript II RT were added to the Primer-RNA-Mix. The Mixture was incubated for 2 hours at 42°C. After this step, additional 5 µL RT was added to the reaction and the mixture was incubated for 30 minutes at 55°C. The reaction was stopped by inactivating the reverse transcriptase for 15 minutes at 70°C.

4.1.2. PCR for Cloning

Primers for the cloning of Arabidopsis genes were designed with coding sequences (CDS) from TAIR (https://www.arabidopsis.org/) to have a melting temperature of 58°C and ordered from Merck. The coding sequence (CDS) was amplified with the proof-reading Phusion polymerase (NEB) from Arabidopsis cDNA or existing constructs. For a 50 µL PCR reaction 10 µL 10xBuffer, 1 µL 2.5 mM dNTPs, 1 µL 50 µM forward, 1 µL 50 µM reverse primer, and 3 µL template (undiluted cDNA, or an 1:10 to 1:100 diluted Miniprep) were mixed with 0.5 µL Phusion polymerase. The fragments were amplified with the following program in an Eppendorf thermocycler: 95°C, 30 s, 40x (95°C, 20s; 58°C, 30s; 72°C, 45s); 72°C, 3 min. The amplification was optimized by variation of the annealing temperature (56-62°C), template concentration, MgCl₂-concentration and cycle number. To analyze the amplification reaction a 5 µL sample of each PCR reaction was analyzed on an Agarose gel.
4.1.3. Agarose gel electrophoresis

DNA fragments were analyzed on a 0.8% or 1 % (w/v) Agarose gel. The Agarose was dissolved 0.5 L 1xTAE Buffer (40 mM Tris-base (Roth), 1 mM ethylene-diamine-tetra-acetic acid (EDTA, Roth), pH 8.0, 0.11% Acetic acid (Roth)) by heating. The gels were run at 135 V for 15 or 20 min in a Mupid electrophoresis chamber (Nippon Genetics) with 1xTAE as a running buffer. Before running them on a gel, DNA samples were mixed with 6x Loading dye (10 mM Tris-HCl, pH 7.5, 0.08 % (w/v) Orange G (Merck), 60 % (v/v) glycerol (Roth), 60 mM EDTA, pH 8.0). DNA Gels were stained with Midori-Green (Nippon Genetics), GelRed (Biotium) or SYBR Safe (Thermo-Fisher Scientific) DNA stains either by adding them directly to gel before pouring, or with a staining bath according to the instructions of the manufacturer.

4.1.4. Bacteria transformation and growth conditions

Chemically competent E. coli strain DH5alpha were transformed with 1 µL of Mini-prep or a complete cloning reaction with a heat shock. For this, a 100 µL Aliquot of bacteria was thawed on ice for 10 min, mixed with the DNA by tapping, incubated with the DNA on ice for 30 min, and shocked for 45s at 42°C. After the heat shock, 1 ml LB medium (10 g/L NaCl (Roth), 5 g/L yeast extract (VWR), 10 g/L peptone/tryptone (VWR)) was added to the tube and the tube was incubated with shaking at 37°C for 1 hour. The incubation with LB medium was not performed for Ampicillin resistant vectors. After the incubation step the cells were pelleted at 6000xg for 1min. About 800 µL supernatant was removed, the cells were re-suspended in the residual LB, and plated on appropriate resistance plates.

Plates were prepared by pouring 25 mL solid LB medium (10 g/L NaCl, 5 g/L yeast extract, 10 g/L peptone, 15 g/L agar-agar (Roth)) with antibiotics (50 µg/ml ampicillin (Roth), 20 µg/ml gentamicin (Duchefa), 50 µg/ml spectinomycin (Duchefa), 50 µg/ml kanamycin (Roth)) in round petri dishes (Ø 9,4 cm). Ampicillin, spectinomycin and kanamycin stocks were prepared in dH2O and stored at 4°C. To grow the bacteria, the plates were incubated overnight at 37°C or for two days at 30°C.

4.1.5. Mini - Prep

For Mini-Prep cultures, 3 mL of LB media with the appropriate antibiotics was filled in a sterile glass tube, inoculated with a single colony, and incubated 8 hours to overnight at
37°C, or for two days at 30°C. The cells were pelleted by centrifuging the culture twice in the same 1.5 mL tube at 6000x g for 1 min. The plasmids were either isolated with the Nucleospin Plasmid Easy Kit (MN) or hand-prep. For the hand-prep the cell pellets were re-suspended in 200 µL solution 1 (50 mM Tris-HCl, pH 7.5, 1 mM ethylene-di-amine-tetra-acetic acid (EDTA, pH 8.0). To destroy the cells 200 µL solution II (200 mM NaOH (Roth), 1% sodium dodecyl sulfate (SDS, Roth)) was added to the cell suspension, mixed by inversion, and the mixture was incubated at room temperature (RT) for 3 min or until the mixture was clear. Proteins were precipitated by the addition of 200 µL solution III (3 M potassium acetate (pH 5.5, Merck) and removed by centrifuging the mixture at 13,000x g for 5 min. The supernatant was transferred to a new 1.5 mL tube. To precipitate the DNA, 600 µL isopropanol (VWR) was added to the lysate. The lysate was mixed with isopropanol by inverting it several times. The precipitated DNA was pelleted by centrifugation at 13,000xg for 20 min. The supernatant was removed completely, and the DNA pellet was washed with 500 µL 70% ethanol (VWR) by centrifugation for 3 min at 13,000x g. After the washing, 70% Ethanol was removed completely, and the pellet was dried for at least 15 min in the 37°C incubator. The dried DNA pellet was dissolved in 50 µL 0.1 mg/mL RNase A and incubated for 15 min at 37°C to remove the RNA. The DNA concentration was measured with a Nanodrop photometer (Thermo-Fisher Scientific). If necessary, the hand-prepped plasmids were purified with a PCR purification Kit (MN).

4.1.6. Midi - Prep

For Midi-Prep, 50 to 100 mL of LB media with the appropriate antibiotics were inoculated with single cultures or a streak from the plate and incubated overnight at 37°C. The cells were pelleted for 10 min at 6000x g and the plasmids were isolated with the PureLink HiPure Plasmid Midiprep Kit (Thermo-Fisher Scientific) according to the instructions of the manufacturer. The Plasmid DNA was dissolved in 100 µL water, and the DNA concentration was measured with a Nanodrop photometer (Thermo-Fisher Scientific).

4.1.7. Restriction cloning

For restriction cloning, two different restriction sites that were suitable for the vector were inserted in the forward and reverse primers. PCR products and hand-prepped vectors were purified with the PCR purification Kit (MN) and digested with restriction enzymes (NEB) in a 50 µL reaction mixture (10 µL 5x CutSmart buffer, 2.5 µL enzyme 1, 2.5 µL
enzyme 2 and 5 µL vector or 20 µL PCR product) for 2 to 4 hours at 37°C. The digested DNA was purified with ethanol precipitation. For this, 125 µL ethanol and 5 µL 3 M sodium-acetate (Roth), pH 5.2 was added to the reaction. The reaction tube was incubated for 5 min at RT and the DNA was pelleted by centrifugation at maximum speed for 20 min at 4°C. The DNA pellet was washed with 200 µL 70 % ethanol (5 min, 13,000x g, 4°C), dried at 37°C for 30 min and dissolved in 10 µL dH₂O. If the purity was not sufficient, the PCR-Products and vectors were purified from an agarose gel with the PCR purification Kit (MN).

To estimate the amount of PCR product and vector, 1 µL sample (Ethanol Prep), or 5 µL sample (from the gel) was analyzed on an Agarose gel. For the ligation reaction, PCR product and vector were mixed in an approximate molar ratio of 10:1, with 0.5 µL T4 Ligase (NEB), 1 µL 10x T4 Ligase buffer, and dH₂O in a total volume of 10 µL. The reaction was incubated for 4 hours at 21°C or overnight at 16°C. The complete reaction volume was transformed to chemically competent DH5 alpha and the transformed colonies were selected on the appropriate resistance plates (pGex-6P-1: Amp).

4.1.8. Gateway cloning

For Gateway-Cloning the attB sites were included in the primers. PCR products and hand-prepped entry or destination vectors were purified with the PCR purification Kit (MN). For the BP-reaction, 0.5 µL pDonr207 (150 ng/µL) was mixed with up to 75 ng PCR-Product, and the reaction was filled up with dH₂O to 2 µL. To start the reaction, 0.5 µL BP Clonase II (Thermo-Fisher Scientific) was added and the reaction was incubated for 4 to 6 hrs or overnight at RT. The complete reaction volume was transformed to chemically competent DH5 alpha and the transformed colonies were selected on Gentamycin plates.

For the LR-reaction 0.5 µL destination vector (150 ng/µl), 0.5 µL entry clone (150 ng/µL), 2 µL dH₂O, and 0.5 µL LR-Clonase II were mixed and incubated at RT for at least 2 hrs. The complete reaction volume was transformed to chemically competent DH5 alpha and the transformed colonies were selected on appropriate resistance plates (pFastR06: Spec, pExTag-YFP/RFP: Amp).

4.1.9. Verification of cloning products

To analyze the efficiency of the restriction cloning, 4 to 12 Mini-prep cultures were inoculated with single colonies from the selection plates. The cloning products were
isolated by hand prep. The success of the cloning was analyzed by restriction digest and sequencing. First, the cloning products were digested with a combination of restriction enzymes, which cuts the plasmids into easily distinguishable fragments. The test digest was performed by mixing 0.5 µL enzyme 1, 0.5 µL enzyme 2, 1 µL 10x Cutsmart-buffer, and 5 µL water with 3 µL plasmid, and incubating the mixture for 1 hours at 37°C. The fragment size was analyzed on a 0.8% Agarose gel.

To verify the sequence of the insert, a construct with the expected fragment size was purified with a PCR purification Kit (MN) and sent to sequencing (Eurofins). For sequencing, approximately 500 ng of plasmid was mixed with 1 µL 50 µM sequencing primer and the mixture was filled up with dH₂O to 10 µL.

4.1.10. Expression constructs for Arabidopsis

The coding sequences of UBP7 (At3g21280), UBP17 (At5g65450), UBP22 (At5g10790), UBP24 (At4g30890), UBP23 (At5g57990), UBP25 (At3g14400), OTU9 (At5g04250), OTU10 (At5g03330), OTU12 (At3g02070) and OTU11 (At3g22260) were amplified from cDNA with primers containing attB-sites [KV1/2 (UBP7), KV13/14 (UBP22), KV15/16 (UBP23), KV17/18 (UBP25)] and cloned in the Gateway entry vector pDonr207 (Invitrogen) [entry clones: pKV16 (UBP7), pKV18 (UBP25), pKV21 (UBP23), pKV23 (UBP22), pKV64 (OTU9), pKV65 (OTU10)].

Constructs for OTU11.2 and OTU12 were created by Chris Gude with primers CG17/18 (OTU11) and CG19/20 (OTU12) [OTU11.2 (entry clone: pCG2, expression clone: pCG21), OTU12 (entry clone: pCG3, expression clone: pCG22)]. To generate constructs for both isoforms of OTU11, OTU11.1 and OTU11.2 the CDS was amplified with the same primer pair (CG17/18) and transferred to pDonr207. After the cloning, single colonies were picked and sequenced to identify the second isoform [entry clones: OTU11.1 (pKV130), OTU11.2 (pKV131)].

Gateway entry clones based on pENTR223.1 with the coding sequence of UBP6 (At1g51710, pKV3), UBP10 (At4g10590, pKV4), UBP12 (At5g06600, pKV5), UBP18 (At4g31670, pKV6), UBP20 (At4g17890, pKV7), UBP27 (At4g39370, pKV8) were obtained from ABRC and given to us from the Braun Lab.

To generate 35S CamV-promotor driven N-terminal YFP-tagged fusion constructs for protoplasts the coding sequences were transferred to the Arabidopsis destination vector pExTag-YFP (MPI Köln) for protoplast by Chris Gude and me [OTU11.2 (pCG21),
OTU11.1 (pKV255), OTU12 (pCG22), UBP6 (pKV10), UBP10 (pKV11), UBP12 (pKV12), UBP18 (pKV13), UBP20 (pKV14), UBP27 (pKV15), UBP7 (pKV19), UBP25 (pKV20), UBP23 (pKV22), UBP22 (pKV24). To generate 35S CamV-promotor driven N-terminal GFP-tagged fusion constructs for plant transformation the coding sequences were transferred to the Arabidopsis destination vector pFastR06 (Shimada et al., 2010) [OTU11.2 (pKV30), OTU12 (pKV31), OTU11.1 (pKV151)]. To generate N-Terminal RFP-tagged versions of OTU11.1, OTU11.2, and OTU12 the coding sequences were transferred to pExTag-RFP (derived from pExTag-YFP, generated by Franziska Anzenberger) [OTU11.1 (pKV173), OTU11.2 (pKV174), OTU12 (pKV214)]. As a control for the PMA-GFP-UB and PMA-GFP experiment the PM protein SYP121 was amplified with primers KV274/275 and transferred with Gateway cloning to pExTag-RFP (entry clone: pKV129, expression clone: pKV137).

To analyze the location of the truncated versions or the membrane mutants of OTU11 and OTU12 the sequences were amplified with Primers containing attB-sites (CG17/KV102 for OTU11 N-Terminus (N, aa 1-100), CG19/KV103 for OTU12 – N-terminus (N, aa 1-78), KV231/KV232 for the OTU11 domain (OTU, aa 93-225), KV233/KV234 for the OTU12 domain (OTU, aa 79-193) and KV233/CG20 for OTU12 C-terminus (CT, aa 79-219)). The fragments were cloned in pDonr207 [entry clones: OTU11 N (pKV101), OTU11 OTU (pKV102), OTU12 N (pKV103), OTU12 OTU (pKV229), OTU12 C (pKV235)] and transferred to pFastR06 [OTU11 N (pKV113), OTU11 OTU (pKV114)] or pExTag-YFP [OTU12 N (pKV231), OTU12 OTU (pKV232), OTU12 C (pKV240)].

4.1.11. In vitro expression constructs of OTU11 and OTU12

To express N-terminal GST-tagged recombinant proteins the ORFs of OTU11 and OTU12 were amplified with primers containing BamHI/EcoRI (OTU11, CG46/47) and BamHI/Xhol (OTU12, CG48/CG49) recognition sites. N-terminal fragments of OTU11 (aa 1-100) and OTU12 (aa 1-78) were amplified from full length constructs with primers containing EcoRI/Sall (OTU11, CG46, KV104) and BamHI/Sall (OTU12, CG48/KV105) restriction sites, the OTU domain of OTU11 (aa 93-225) and OTU12 (aa 79-193) were amplified with primers containing EcoRI/Sall (OTU11, KV47/KV363) and EcoRI/NotI recognitions sites (OTU12, KV48/46), the C-Terminal part of OTU12 with primers containing EcoRI/Xhol (KV48/CG49) recognition sites. All PCR products were digested and ligated into the pGex-6P-1 (Cytiva) E. coli expression vector [OTU12 (pKV26), OTU11.1 (pKV118), OTU11.2 (pKV119), OTU11 OTU (pKV186), OTU11 N (pKV67), OTU12 OTU (pKV28)].
4.1.12. Membrane-binding mutant constructs of OTU11 and OTU12

Potential membrane binding sites were identified with the BH-search (https://hpcwebapps.cit.nih.gov/bhsearch/, (Brzeska et al., 2010)) internet tool (window=10). Mutations in the potential membrane binding sites were introduced with site directed mutagenesis and Overlap-PCR. For 6A1 OTU11, lysines and arginines in the longest potential membrane binding region (PBM1, aa 153 to 164) were exchanged to alanines with primers LH9/10 in the OTU domain and the full length version of OTU11. The product was amplified with primers CG46/47 (OTU11 FL) or KV47/KV363 (OTU11 OTU) and ligated it into pGex-6P-1 [OTU11 6A1 (pKV191), OTU11 (OTU 6A1) (pKV185)]. The corresponding region in OTU12 (PBM1, aa 131 to 141) was mutated with primers KV421/422. The mutated versions of OTU11 and OTU12 were transferred with Gateway cloning to the pExTag-YFP vector [OTU11.1 6A1 (entry clone: pKV164, expression clone: pKV190), OTU12 6A1 (entry clone: pKV171, expression clone: pKV216)].

To further analyze the membrane binding mechanism, mutations in the second potential polybasic motif at the C-Terminus (PBM2, OTU11: aa 236 to 242, OTU12: aa 210 to 216) of the proteins were introduced with a one-step PCR. For GST-OTU11 6A2, the mutation was introduced with Primers containing BamHI/EcoRI sites [OTU11 6A2, (CG46/KV417)] and the mutated gene was transferred to pGex-6P1 [OTU11 6A2 (pKV243)]. For YFP-fusion constructs, the mutations were introduced with primers CG17/KV456 (OTU11) and CG19/KV455 (OTU12) the genes were transferred to pExTag-YFP/RFP [OTU11.2 6A2 (entry clone: pKV256, expression clone: pKV258, OTU12 6A2 (entry clone: pKV244, expression clone: pMU2)].

Double mutants with 6A1 and 6A2 mutations in OTU11 (M3) were created with the respective primers and transferred to pGex-6P-1 [OTU11.2 M3 (pKV246)] or pExtag-YFP/RFP [OTU11.2 M3 (entry clone: pKV257, expression clone: pKV259). For OTU12 M3 the 6A1 and 6A2 mutation were introduced with respective primers. An additional potential membrane binding site was mutated with primers KV423/424 (aa 189 to 192). The mutated version of OTU12 was transferred to pExTag-YFP (entry clone: pKV252, expression clone: pKV254).
4.1.13. Active site mutants

The active site cysteine in full length OTU11 was exchanged with arginine (C112R) with primers KV355/356, the active site cysteine in full length OTU12 was exchanged with arginine (C90R) with primers KV359/KV360 with an Overlap-PCR. The mutated versions were amplified with CG46/CG47 (OTU11), ligated into pGex-6P-1 [OTU11.1 C112R (pKV187)], or amplified with attB-site containing primers CG19/20 (OTU12), and transferred with Gateway-cloning in to pExTag-RFP [OTU12 C90R (entry clone: pKV210, expression clone: pKV215)].

4.1.14. Phosphorylation mutants

By searching the database PhosPhAt 4.0 (https://phosphat.uni-hohenheim.de/) we found that OTU1 is phosphorylated at two positions in the peptide containing the potential phosphorylation sites S64, S64 and T72 (Wang et al., 2013b; Lin et al., 2015; Roitinger et al., 2015). In the Arabidopsis proteome from Mergner et al. (2020b) additional phosphorylation sites T14, S52, and S93 in the sequence of OTU11 were found. In the same experiment OTU12 was found to be phosphorylated at S31 which aligns with S52 in the OTU11 sequence. As OTU11 S64 is conserved in OTU12 at position S42, we speculated that it might also be phosphorylated.

In first mutant constructs the potential phosphorylation sites S68, S64 and T72 of OTU11 were changed with primers KV159/KV160 to alanine, to create a phospho-dead mutant, or with primers KV191/192 to glutamine, to create a phospho-mimic mutant. The phosphorylation sites T14, S52, and S93 were changed with primers KV392/393 (T14), KV237/KV238 (S52), and KV243/KV244 (S93) to alanine, and primers KV395/396 (T14), KV239/KV240 (S52), and KV245/KV246 (S93) to glutamine. The phosphorylation sites S31 and S42 of OTU12 were changed with primers KV388/KV389 to alanine and KV386/387 to glutamine. The mutant sequences of OTU11.2 and OTU12 were cloned with restriction cloning in pGex-6P-1 and with Gateway cloning in pExTag-YFP [OTU11.2 phospho-dead (entry clone: pKV219, expression clone: pKV221, phospho-mimic (entry clone pKV220, expression clone: pKV222)] or pExTagRFP [[OTU12 phospho-dead (entry clone: pKV200, expression clone: pKV202), phospho-mimic (entry clone: pKV199, expression clone: pKV203)].
4.1.15. Interactor constructs

For the yeast two-hybrid screen full length OTU11 and OTU12 was transferred with Gateway cloning to the yeast expression vector pDest-pPC97 [OTU11 (KV51), OTU12 (KV52)]. To generate MBP-tagged fusion proteins for in vitro binding assays the CDS of potential interactors from the yeast two-hybrid screen was amplified with primers containing BamHl/EcoRl sites KV114/KV115 (At1g17970, RING-domain containing protein) and BamHl/Sall sites KV208/209 (At4g17350, FL5/PH15). The PCR products were digested and ligated into the pMal-2p V087 E. coli expression vector [RING-domain containing protein (pKV66), FL5/PH15 (pKV90)]. The N-Terminal part of FL5/PH15 was amplified with primers containing BamHl/Sall sites KV254/KV255 and cloned in V087 (pKV188).

For the expression in protoplasts the CDS of potential interactors from the yeast two-hybrid screen was amplified with primers containing attB-sites [RING-domain containing protein (KV139/KV140), FL5/PH15 (KV147/KV148)] and cloned with Gateway cloning in pExTag-YFP/RFP [RING-domain containing protein (entry clone: pKV69, expression clone: pKV70 (YFP)), FL5/PH15 (entry clone: pKV86, expression clone: pKV93 (RFP))].

4.2. Plant material and growth conditions

4.2.1. Plant lines

To analyze the influence of OTU11 and OTU12 on plant growth, Arabidopsis T-DNA mutant lines otu11 (SALK05296) and otu12 (SALK13251) in the Columbia-0 (Col-0) background, were identified with the T-DNA Express Arabidopsis Gene Mapping Tool (SIGnAL) and obtained from NASC. The lines were genotyped and crossed to obtain homozygous otu11 otu12 double mutants. The knockout was verified with RT-PCR. The purification of ubiquitylated proteins from plant extract was performed with the T-DNA mutant line amsh1-1 (CSHL_ET8678) in the Landsberg erecta (Ler) background, that was already established in the Lab (Katsiarimpa et al., 2013). Pre-experiments were performed with a Dexamethasone (Dex)-inducible AMSH3-AXA overexpressor line (Isono et al., 2010b). For the tandem affinity purification procedure a 6xHis-Ubiquitin (6His-UBQ)—expressing Col-0 plant line (Saracco et al., 2009) was crossed with amsh1 and AMSH3 AXA plants by Marie-Kristin Nagel.

35S:GFP-OTU11.1 (pKV151), 35S: GFP-OTU11.2 (pKV30), 35S: GFP-OTU12 (pKV31), 35S: GFP-OTU11 NT (aa 1-100, pKV113) and 35S: GFP-OTU11 OTU (aa 93-225,
Arabidopsis lines were created by transforming the constructs with the floral dip method in Col-0 plants. To analyze the membrane localization, KV30 and KV31 plant lines were crossed with the PI4P sensor line P5R (UBQ10prom::2xCherry-1xPH(FAPP1))(Simon et al., 2014). For complementation assays, own promotor driven OTU11 (OTU11p: gGFP-OTU11, pTB39) and OTU12 (OTU12p: GFP-gOTU12, pTB114) were transformed to Col-0 and otu11 otu12 plants by Tobias Bläske. To analyze the influence of OTU11 and OTU12 on the endocytic degradation of membrane proteins, the PIN2:PIN2-GFP (Xu and Scheres, 2005) plant line given to us from the Friml Lab was crossed with the otu11otu12 double mutant line. Homozygous PIN2:PIN2-GFP/otu11otu12 mutant lines were identified by genotyping and fluorescence microscopy (Epifluorescence microscope, Nikon) in the F2 generation.

4.2.2. Seed sterilization

Arabidopsis seeds were surface sterilized with 1 % NaOCl and 0.1 % Silwett (BASF) or 1 % NaOCl for phenotypical experiments. The seeds were sterilized by incubating them for 10 min with 1 mL NaOCl-solution (Merck) in a 1.5 mL tube or 10 mL NaOCl-solution in 15 mL tube (Sarstedt) for 10 min on a rotating wheel (Labinco). After the sterilization step, NaOCl was removed by washing the seeds 5 time with 0.05 % Silwett in sterile water. If not plated directly, sterilized seeds were kept in water in the fridge for up to 3 days and stratified in this way. Alternatively, the seeds were stratified on the plates for 1 to 3 days at 4°C in the cold room. Seeds for physiological experiments were always stratified for 3 days on plate.

4.2.3. Growth conditions

Arabidopsis seedlings were grown on ½ MS (2.15 g/L Musharige&Skoog (MS) medium with vitamins (Duchefa), 250 mg/L MES (Roth), pH 5.8) or Growth medium (GM, 4.3 g/L MS, 250 mg/L MES, 1 % Sucrose (Roth), adjust pH to 5.8 with KOH) medium under long day (LD) or continuous light conditions at 21°C for 5 to 14 days in plant growth chambers (Sanyo). Agar plates for Arabidopsis seedlings were prepared in 12x12 cm square petri dishes (Greiner) and round Ø 9,4 mm petri dishes under the sterile hood.

For microscopy, single seeds were pipetted in one row on Ø 9,4 cm plates in upper third of the plate or two rows with about 5 cm space on the 12x12 cm square plates to allow a vertical growth of the seedlings. For protein extraction, droplets of approximately 100
seeds were pipetted in one or two rows on the plates. The seedlings were grown vertically to facilitate the removal of the seedlings. For selection, seeds were streaked out with a pipet tip, distributed equally, and grown horizontally.

After 10 days, Arabidopsis seedlings were transferred to wet soil. Alternatively, seeds were sown directly on soil, and the pots with the seeds were stratified 1 to 3 days at 4°C. Plants for seed propagation or phenotypical analysis were grown as single plants in 4x4 cm pots. For plant transformation, about 20 seeds were sown in Ø 15 cm pots. Arabidopsis plants were grown in long day or continuous light at 21°C in plant chambers (Sanyo, BOGA) or in a long day greenhouse of the botanical garden of the University of Konstanz. If the plants were grown in the botanical garden, they were cultivated by Gerd Rönnebeck and the other members of the botanical garden team. Plants were watered twice a week. After 6 to 8 weeks, the plants were bound together to reduce the loss of seeds. If the plants had red rosette leafs, they were fertilized. Nematodes and predatory mites were used for pest control. When the plants were completely dry, approximately 3 months after sowing, seeds were collected from the plants and separated from dry plant material with a sieve. Dry seeds were kept in paper bags at RT.

4.2.4. Plant transformation and selection

Arabidopsis lines expressing GFP-OTU11 and GFP-OTU12 were created with the Agrobacterium tumefaciens (GV3101) mediated floral dip method. All constructs used were based on pFastR06 or pBB10 and transformed with the A. tumefaciens strain GV3101.

For Agrobacteria transformation, 5 µL of a Mini-prep were added to a 200 µL aliquot of electro competent Agrobacteria. The mixture was filled in to an electro cuvette (d=2 mm) and treated with an electric pulse (2000 V) in the Eporator (Eppendorf). After the shock, the cells were cooled on ice and incubated with 1 mL LB for 1 hours at 30°C with shaking. After the incubation, the bacteria were pelleted for 1 min, 6000xg. About 800 µL supernatant was removed, the cells were re-suspended in the residual LB and plated on rifampicin (Duchefa, 25 µg/mL) / gentamicin (20 µg/µL) / spectinomycin (100 µg/µL) containing LB-Agar-plates. Gentamicin stocks were prepared in water and stored at 4°C, rifampicin stocks were prepared in DMSO and stored at -20°C. To grow the bacteria, the plates were incubated for 3 days at 30°C.

To prepare pre-cultures for plant transformation, several colonies were picked and transferred to 20 mL LB medium with Rif/Gent/Spec in a sterile 50 mL glass flask. The pre-
cultures were incubated overnight with shaking at 30°C. To prepare main cultures, the pre-cultures were added to 200 mL medium with Rif/Gent/Spec in a 500 mL flask. The main cultures were grown for two days with shaking at 30°C. For plant transformation, the Agrobacteria were pelleted for 20 min at 6000x g at RT. The pellets were re-suspended in 200 to 500 ml transformation solution (2,2 g/L MS+Vitamins, 500 mg/L MES, 50g/L glucose (Roth), 10 µL/L 6-BA (Merck), 200 µL/L Silwett and filled in beakers or glass jars. About 6 week old Arabidopsis plants with many buds were turned upside-down and dipped for about 30s in the bacteria suspension. The transformed plants were put tray covered with a plastic lid, which was removed the next day.

T0 Seeds transformed with pFastR06 based constructs (KV30, KV31, KV151, KV113, KV114) and the golden gate construct TB114 (OTU12p: GFP-gOTU12), were selected with an RFP-filter equipped fluorescent binocular (Olympus). T0 Seeds transformed with the golden gate construct TB39 (OTU11p: GFP-gOTU11) were selected by spraying seedlings on soil with 1000x diluted Basta solution (200 g/L glufosinate-ammonium / phosphinotricin, Bayer). The selected seeds were propagated on soil. The heterozygous T1 seeds were analyzed with the confocal microscope (LSM700) and propagated, if there was a visible fluorescent signal. In the T2 generation, homozygous plant lines were selected with the binocular (pFastR06, TB114) or on phosphinotricin (Duchefa) plates (15 µg/µL, TB39).

4.2.5. Crossing

To combine to alleles, two Arabidopsis lines with different alleles were crossed with each other. The crossing worked best with flowers from the main inflorescence. Side inflorescences and siliques were removed from the main inflorescence of the first plant with a scissors. After that step, sepals, petals, and stamen were removed from three flowers of this plant with a fine forceps. Stamen were removed from a flower of the second plant, and the pollen from the stamen was transferred to the pistil of the first plant with the help of a binocular (VWR). Pollinated pistils were sprayed with water and marked. After ripening, the crossed siliques were harvested to obtain the F1 seeds. The heterozygous F1 seeds were propagated and the success of the crossing was analyzed with genotyping or fluorescence microscopy. In the F2 generation, homozygous plants were selected by genotyping and fluorescence microscopy.
4.3. DNA extraction

For the extraction of DNA from Arabidopsis seedlings, stems, and leafs, the tissue was either mashed with a mortar in Eppendorf tubes or disrupted with glass beads (0.1 or 2.7 mm. Roth) by a bead mill (Tissue Lyzer, Qiagen) after freezing in liquid nitrogen. For the extraction of DNA from Arabidopsis seeds 20 to 100 seeds were filled in an Eppendorf tube and mashed with a pestle. After the disruption step 300 µL DNA extraction buffer (200 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, pH 8.0, 0.5% SDS) was added to the tissue, and the tubes were incubated for 20 min at 65°C to inactivate DNAse. To remove cell debris, the samples were centrifuged at 13.000x g for 5 min. For the extraction of DNA from seedlings or leafs, 200 µL supernatant was removed from the reaction tube and mixed with 200 µl isopropanol to precipitate the DNA. The DNA was pelleted at 13.000x g for 20 min and the supernatant was completely removed. To remove residual salts, the DNA pellet was washed with 500 µL 70% ethanol and centrifuged at 13.000xg for 3 min. The supernatant was removed, the pellet was dried at 37°C for one hour or RT overnight and the dried DNA pellet was dissolved in 200 µL dH2O.

To extract DNA from seeds it was necessary to clarify the extract with Phenol/Chloroform before the DNA precipitation to remove storage proteins and fat. For this step 200 µL Phenol/Chloroform (Roth) was added to the extract. The phases were mixed by vortexing and separated by centrifugation (5 min, 13.000x g). The upper phase (200 µL) was transferred to a new Eppendorf tube and mixed with 200 µL isopropanol. If necessary, residual phenol was removed by washing with 100% chloroform before the addition of isopropanol.

4.4. Genotyping PCR

4.4.1. General protocol of the genotyping PCR

The insertion of TDNA in the Arabidopsis genome was confirmed with genotyping PCR with Primer combinations for the wild-type and the mutant line. Primers for Genotyping were designed with the gDNA from TAIR to have a melting temperature of 58°C. To verify the existence of the wild-type gene the forward and reverse primer pair were designed to amplify a 500 to 1500 bp fragment from the gene. The TDNA insertion was verified with the TDNA LB primer (SALK-Lines: LBB3.1) and the gene specific forward primer, if the TDNA insertion was reversed in comparison to the gene, or the gene specific reverse
primer, if the insertion was in the same direction as the gene. The gene fragments were amplified with home-made Taq-Polymerase. For a 30 µL reaction, 3 µL 10x PCR Buffer (200 mM Tris-HCl, pH8.2, 500 mM KCl, 25 mM MgCl₂), 3 µL 2.5 mM dNTPs, 0.4 µL Taq, 0.4 µL forward, and reverse primers were mixed with 5 µL gDNA. The fragments were amplified with the NL58 program in an Eppendorf thermocycler: 94°C, 4 min; 40 x (94°C, 45 s, 58°C, 30 s, 72°C, 45 s); 72°C, 4 min. The PCR products were analyzed on a 0.8% agarose gel or with capillary gel electrophoresis (QIAxcel Advanced, Qiagen).

### 4.4.2. Genotyping primers for T-DNA lines

In the *otu11* (SALK05296) line, the wild-type gene was amplified with primers FA134/135, designed by Franziska Anzenberger, and the insertion was confirmed with left border LBb1.3 and the gene specific forward primer (FA134). In the *otu12* (SALK13251) line, the wild-type gene was amplified with primers FA130/131, designed by Franziska Anzenberger, and the insertion was confirmed with left border LBb1.3 and the gene specific reverse primer (FA131). In the *amsh1* line, the wild-type gene was amplified with AMSH1 fw2 and AMSH1 rv2 primer (Katsiarimpa et al., 2013) and the insertion was confirmed with the T-DNA specific primer Ds3.1 and the gene specific reverse primer (AMSH1 rv2). In the *amsh3* line, the wild-type gene was amplified with EI40/41 (Isono et al., 2010b) and the insertion was confirmed with the T-DNA specific primer p745 and the gene specific reverse primer (EI41). 6His-UBQ was detected with primers KV20/KV21, the Dex-inducible AMSH3-AXA construct EI19 with primers RBCS/PTA7002.

### 4.5. RT-PCR

The transcript level of *OTU11* and *OTU12* in *otu11otu12* mutants was analyzed with RT-PCR. For this, RNA was extracted from mutant and wild-type seedlings and transcribed to cDNA as described for the cloning PCR. Full length OTU11 and OTU12 amplified with primers CG46/47 (*OTU11*) and CG48/CG49 (*OTU12*) from the cDNA by Tobias Bläske. SYP121 primers KV288/289 were used as a control. To analyze the different splicing forms of OTU11, a OTU11 fragment was amplified with primers KV352/KV245 at 62°C from cDNA and pKV118 (*OTU11.1*) and pKV119 (*OTU11.2*).
4.6. Plant cell culture and protoplast transformation

4.6.1. Plant cell culture

Arabidopsis suspension culture was kept with constant shaking at 21°C in Arabidopsis cell culture medium (0.87 g/200 mL Murashige and Skoog (MS) medium including Gamborg B5 vitamins (Merck), 6 g/200 mL sucrose (Merck), 200 μL of 1 g/L 2,4-dichlorophenoxyacetic acid stock solution (2,4 D, Duchefa), 666 μL/200 μL KP-buffer, adjust to pH 5.8 with KOH). The KP-buffer with a pH of 5.8 is prepared by mixing 850 μL 1M K₂HPO₄ (Roth) with 9.15 mL 1M KH₂PO₄ (Roth) and 3.92 mL dH₂O. For the cultivation, 50 mL of the medium was sterile filtered with a 0.2 µm filter (VWR), filled in a 250 mL glass flask which was closed with membrane containing lid to allow aeration, and autoclaved. The culture was inoculated with 10 mL cell slurry and maintained by transferring 10 mL cell slurry to a fresh flask with medium every week.

4.6.2. Protoplast transformation

The protoplasts were transformed with pExTag-YFP or –RFP based constructs to analyze the localization of fusion proteins in Arabidopsis cells. Constructs for protoplast transformation were prepped with a Midi-prep Kit (Thermo-Fisher Scientific).

Protoplastation and protoplast transformation was performed as described in (Nagel et al., 2019). About 10 mL of the root cell culture was transferred to a 15 mL tube. the medium was removed by centrifugation for 1 min at 300x g at RT. The remaining cell pellet with a volume of about 2 mL was washed once by centrifugation at 300x g for 1 min with 10 mL Solution I (400 mM Mannitol (Merck), 5 mM EGTA (Merck), pH 8.2). To prepare the enzyme solution, 125 mg Cellulase (Serva) and 25 mg Macerozyme (Serva) were dissolved in 10 mL solution I and pre-treated at 50°C for 10 min. The cell wall was removed by incubating root cells the enzyme solution in a 15 mL tube for 2 hours with shaking at room temperature. The efficiency of the digest was checked with a microscope (Nikon Eclipse Ni) and the incubation time was prolonged if necessary. After the digest, the protoplasts were pelleted for 1 min at 100x g and washed twice with Solution A (400 mM Mannitol, 70 mM CaCl₂ (Merck), 5 mM MES, pH 5.7). To prepare the protoplasts for DNA uptake, they were incubated in 1 mL MMM solution (400 mM Mannitol, 15 mM MgCl₂ (Merck), 5 mM MES, pH 5.7) for 30 min on ice in a 2 mL tube. During the incubation time, the protoplasts were counted with a Thoma cell chamber (Brand) and the volume was adjusted. The aim was a cell concentration of about 1x10⁷ cells/mL.
Afterwards, 100 µL of protoplasts were transformed with 20 - 40 µg of plasmid by mixing the protoplasts and the DNA carefully, drop by drop with constant mixing, with 400 µL DNA uptake solution (400 mM Mannitol, 100 mM Ca(NO₃)₂ (Merck), 4 g PEG 6000 (Merck), and incubating them for 30 min on ice in a 2 mL tube. The DNA uptake solution was removed by washing the protoplasts drop by drop with constant mixing with 1 mL Dilution Solution (400 mM Mannitol, 125 mM CaCl₂, 5 mM KCl (Merck), 5 mM glucose, 1.5 mM MES, pH 5.7). The supernatant was removed completely and the protoplasts were washed once more with 1 mL Dilution solution. The supernatant was removed completely and 0.5 to 1 mL MS+Mannitol solution (400 mM Mannitol, 4.6 mg/mL MS) were transferred to the 2 mL tube. The fusion proteins were expressed overnight at 21°C in the dark.

The localization of the fusion proteins was analyzed with an epifluorescence microscope (Olympus) or confocal laser microscopes (Olympus (TUM), Zeiss LSM700 (Bioimaging center Konstanz)) with RFP- and YFP-filters. The expression of the fusion proteins was verified with a western blot. For the western blot analysis, the protoplasts were pelleted for 1 min at 3000 x g, washed once with 1x Phosphate Buffered Saline (PBS, 139 mM NaCl, 2.7 mM KCl, 1.8 mM KH₂PO₄, 12.5 mM Na₂HPO₄), suspended in 50 µL 1x Laemmli-buffer, and boiled at 98°C for 5 min. The samples were stored at -20°C if they were not directly analyzed on the gel.

4.7. Expression of recombinant proteins in *E. coli*

Proteins used for *in vitro* assays were expressed in *E. coli* Rosetta DE3, Rosetta Gami 2, or Rosetta Gami B strains (Invitrogen) that are optimized for the expression of eukaryotic proteins. The proteins were expressed with expression constructs (pGex-6P-1 for GST-tagged protein and pMal-p2p (NEB) for MBP-tagged proteins), in which the expression of the proteins is driven by an IPTG (isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible lac-Operon.

For the protein expression, a 100 µL aliquot of chemically competent E. coli cells was transformed with 1 µL plasmid with a heat shock, plated on appropriate antibiotic plates, and incubated overnight (DE3, Gami 2) or for two days (Gami B) at 37°C. Rosetta DE3 cells were grown on Cm plates (chloramphenicol (Roth), 25 µg/mL), Rosetta Gami 2 cells on Cm (25 µg/mL) / Tet (tetracycline (Roth), 2.5 µg/mL) plates and Rosetta Gami B on Cm (100 µg/mL) / Tet (2.5 µg/mL) / Kan (15 µg/mL). In addition to the antibiotics needed for the strains, 50 µg/µL ampicillin was added for the expression plasmid. Chloramphenicol stocks (25 mg/mL) were prepared in 100 % Ethanol and stored at 4 °C or for long term
storage at -20 °C. Tetracycline stocks (5mg/mL) were prepared in 90 % Ethanol, protected from light with aluminum foil, and stored at -20 °C.

To prepare the pre-cultures, 25 mL to 100 mL (10 % of the final volume) of LB medium with the appropriate antibiotics was filled in glass flasks with the double nominal volume, and several colonies were picked from the plate and transferred to the medium. The pre-cultures were incubated overnight at 37°C with shaking.

For the main culture, 250 mL to 1 L of LB medium with the appropriate antibiotics was filled in flasks with at least the double nominal volume. The main culture was inoculated with the pre-culture and incubated for 4 to 6 hours at 37°C (OD600 ≥ 1). Protein expression was induced with 0.5 mM IPTG. The proteins were expressed at 18°C overnight (approximately 18 hours) or at 37°C for 3 hours with shaking. Prior to the induction at 18 °C, the cultures were pre-cooled in an ice bath or cold room. To harvest the cells, the cultures were centrifuged for 10 min at 6000 x g at 4 °C. The supernatant was removed, the bacteria pellet was re-suspended in 1x PBS, transferred to 50 mL tubes, and centrifuged again for 10 min at 6000 x g at 4°C. The supernatant was removed and the pellets were stored at -80°C.

4.8. Purification of recombinant proteins from E.coli

To purify recombinant proteins from E. coli pellets, the cells were lysed with B-PER complete reagent (Thermo-Fisher Scientific), sonification or lysozyme treatment. To facilitate the break-up of the cells, the pellets were frozen at -80°C for at least 1h.

The lysis with B-PER complete was performed according to the instructions of the manufacturer. For the sonification method the cells were res-suspended in 5 mL/g pellet Buffer A (50 mM Tris, 100 mM NaCl, 10 % glycerol, adjust pH to 7.5 with HCl) with 0.2 % Triton-X-100 (Merck). Additionally, 1 mM EDTA was added to the lysis buffer to de-stabilize the outer membrane of the bacteria. The cell suspension was sonified for 15 min at a low intensity (5-15 %, 15s on, 30s off, Sonopuls (Bandelin)) on ice. After the sonification, cell debris was removed by centrifugation (10,000 x g, 10 min, 4 °C), and the supernatant was transferred to a new 50 mL tube. For the lysosome treatment the cells were re-suspended and incubated for 30 min at 4 °C in 5 mL/g pellet lysozyme solution (1 mg/ml lysozyme (Roth), 0.2% Triton-X-100 and 1 mM EDTA in Buffer A). The DNA was destroyed with 3 min of sonification (10-15 %, 15 seconds on, 30 seconds off, Sonopuls (Bandelin)) on ice and the lysate was cleared by centrifugation at 16.000x g for 10 min at 4°C. The supernatant was transferred a new 50 mL tube.
To isolate the tagged proteins, the lysate was incubated with 100 µL/2.5 g pellet Protino Glutathion-S-Transferase (GST)-Agarose 4B (MN), 200 µL/2.5 g pellet magnetic GST-beads (Thermo-Fisher Scientific) or 100 µL/2.5 g pellet Maltose-Agarose (NEB) for 1 to 2 hours at 4°C in 50 mL tube with shaking. Before use, the affinity beads were washed three times with 1 mL Buffer A by centrifugation for 15-30 s at 1000 x g. After the incubation step, the beads were collected at the bottom of the 50 mL tube by centrifugation (5 min, 3000 x g, 4 °C) with a swing out rotor (Eppendorf centrifuge 5702/R, Rotor: A-4-38). The supernatant was removed carefully with a 25 mL pipette, and the beads were transferred to a 1.5 mL tube or mini-spin column. The beads were washed three times with 1 mL Buffer A (1000 x g, 30 s) to remove non-specifically bound proteins.

After washing, the proteins were kept on beads or they were eluted in 100-200 µL/100 µL beads of 40 mM Glutathione, pH 7.2 (GST-tag) or 100-200 µL/100 µL beads of 10 mM Maltose (Roth, Maltose binding protein (MBP)-tag) by rotating the on a spinning wheel for 15 min at RT. Alternatively, the GST- or MBP-tag was cleaved off by incubating the beads with 100-100 µL/100 µL with 1U/100µl PreScission protease (GE Healthcare) in PreScission buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.0) on a spinning wheel overnight. Beads were stored in Buffer A in the fridge. The eluted proteins were either stored in the fridge or at -80°C.

OTU11 without GST-tag (28 kDa, pI 8.97) was polished with cation exchange chromatography columns (SP HP, 1 mL, GE Healthcare) on an Äkta™ purifier system (Cytiva). The protein was bound to the column in 50 mM Tris, pH 7.5 (ΔpH > 1). In this buffer system in OTU11 is positively charged and should therefore bind to the cation exchange column. The bound protein was eluted with a continuous NaCl gradient (0 to 1 M NaCl in 20 min), monitored with a 280 nm fluorescent detector, and collected in 1 mL fractions which were analyzed on a protein gel.

4.9. SDS-PAGE

Recombinant proteins and proteins from plant extract were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected with Western Blot or protein staining. In SDS-PAGE gels denatured and equally loaded proteins are separated by their molecular weight. Most proteins (25 to 100 kDa) were analyzed on 10% or 12.5% gels. For the 16 kDa protein di-ubiquitin 15% gels were used, for proteins ≥100 kDa 7.5% gels.
Polyacrylamide gels for the SDS-PAGE were prepared with the SureCast-System (Thermo-Fisher Scientific) for 1.5 mm gels. The glass plates for the back and the front were assembled with a spacer in the casting stand.

The separating gel was prepared by mixing 2 mL separating gel buffer (1.5 M TRIS, 0.4 % SDS, pH 8.8 with 37 % HCl) with different amounts of acrylamide: bisacrylamide (37.5:1, Rotiphorese Gel 30 (Roth), 2.6 mL for a 10 % gel, 3.2 mL for a 12.5 % gel, 4 mL for a 15 % gel, and 2 mL for a 7.5 % gel), water (3.4 mL for a 10% gel, 2.8 mL for a 12 % gel, 2 mL for a 15 % gel, and 4 mL for a 7.5 % gel) and 50 µL of 10 % ammonium persulfate (APS, Roth). Directly after the addition of 8 µL tetra-methyl-ethylene-diamine (TEMED) (Roth) which started the polymerization, the mixture was shortly mixed and poured between the glass plates. The separating gel was overlaid with 0.6 mL isopropanol and incubated at RT until it was solid, approximately 15 min. The stacking gel was prepared by mixing 1 mL stacking gel buffer (0.5 M TRIS, 0.4 % SDS, pH 6.8 with 37 % HCl) with 0.6 mL Rotiphorese Gel, 2.4 mL water, 50 µL of 10 % APS, and 8 µL TEMED. After the removal of the isopropanol, the stacking gel was poured on top of the separating gel and a 15-, 12-, or 10-well comb was put on top of the stacking gel. The gel was again incubated for approximately 15 min at RT, until the stacking gel was solid. The comb was removed, the wells were washed carefully with water, and the gel was put into a Mini Gel Tank (Thermo-Fisher Scientific).

Protein samples for SDS-PAGE were mixed with ¼ of the sample volume of 5x Laemmli-buffer (310 mM Tris-HCl pH 6.8, 50 % Glycerol, 10 % SDS, 0.5 % bromophenolblue, 3.5 % β-mercaptoethanol) and boiled for 3 min at 98°C. Samples with membrane proteins like H+-ATPase were incubated for 10 min at 70°C. After boiling, the samples were centrifuged for 30 s at maximum speed and loaded on a polyacrylamide gel. To determine the size of the proteins, 1 to 5 µL of a pre-stained protein marker PageRuler™ Plus (Thermo-Fisher Scientific) were loaded in parallel to the samples. Protein quantification was performed by loading 5 µL of the unstained BenchMark™ protein ladder (Thermo-Fisher Scientific). In 5 µL of Benchmark, there were 1 µg of protein in the 50 kDa and the 20 kDa band and 0.5 µg of protein in the other bands. After addition of the sample, the gels were run at 40 mA and 300 V for 50 min in 1x SDS Running buffer (25 mM TRIS, 192 mM glycine (Roth), 0.04% SDS). After the run, the gel was removed carefully from the glass plates and either stained with total protein stain, phosphostain or used for western blot. Nupage gradient gels (4-12%, Invitrogen) were run in 1x MES Running buffer (0.975 g/L MES, 0.06 g/L Tris Base, 1 g/L SDS, 0.3 g/L EDTA) at 500 mA and 200 V for 35 min.
4.10. Protein staining

Total proteins were stained with an 2,2,2-Trichloroethanol (TCE) dependent UV staining (Ladner-Keay et al., 2018) or Coomassie staining for ≥0.5 µg proteins. Smaller amounts of proteins were visualized with a silver staining kit (SilverQuest, Invitrogen) and commercially available fluorescence staining Kits (Krypton, SYPRO-RUBY, Thermo-Fisher Scientific).

For UV staining of total proteins, 40 µL of TCE was added to the separating gel prior to the pouring step. After the run, the gels were activated for 3 min at a 302 nm UV table (Benchtop 2 UV Illuminator) and the proteins were detected with an UV detector (Amersham 600 Imager, GE Healthcare). Since TCE binds to the amino acid tryptophan, Ubiquitin and other proteins which do not contain tryptophan in their sequence could not be stained with this method.

For Coomassie-Staining, the gels were transferred to a plastic box with Coomassie-Staining solution (2.5 g/250 mL Coomassie brilliant blue R250 or G250 in 7 % acetic acid, 40 % ethanol, 53 % H₂O). The Gels were heated for 20 s at 600 W in a microwave and incubated with shaking for at least 20 min at RT. After the staining step, the staining solution was removed. Residual staining solution was washed away with water and the gel was de-stained with shaking for 1h at RT in de-staining solution (7 % acetic acid, 40 % ethanol, 53 % H₂O). The gels were imaged with a trans-illuminator camera (Amersham 600 Imager, GE Healthcare). The silver staining was performed as described by the manufacturer, and the gels were imaged with a trans-illuminator camera. The commercial fluorescence staining kits (Krypton, SYPRO Ruby) were used according to the protocol of the manufacturer with adaptions. The staining was performed with only 15 mL staining solution instead of 60 mL as recommended by the manufacturer. To avoid shearing of the gel which could happen with small amounts of staining solution the gels were incubated for 10 min in water before staining. Ubiquitin was stained with the SYPRO Ruby Kit as the Krypton Kit did not work well for ubiquitin. The Krypton-stained gels were imaged with the 520 nm epifluorescence camera of the Amersham 600 Imager. The SYPRO Ruby stained gels were imaged with the UV camera or the 480 or 520 epifluorescence camera of the Amersham 600 Imager.

Phosphorylated proteins were stained with the ProQ™ Diamond Phosphoprotein Gel Stain (Thermo-Fisher Scientific) according to the instructions of the manufacturer. The gels were imaged with Typhoon FLA9200 equipped with an excitation wave length of 532 nm and an LPG filter (≥ 575 nm).
4.11. Western Blot

Specific proteins in the plant extract and in vitro experiments were detected with a western blot. For each western blot, one PVDF membrane slide (0.5 cm longer and broader than the gel (0.2 µm, Immobilon-PSQ®, Merck-Millipore) and four slides of Whatman® paper (1 cm longer and broader than the gel, thickness 1.5 mm, Roth) were prepared. The paper slides were soaked in SD buffer (25 mM Tris-Base, 192 mM glycine, 20% methanol (VWR), 1.3 mM SDS, adjust pH to 8.3 with NaOH) and the membrane was activated for 30 s in methanol. The western blot sandwich was assembled by putting two Whatman® papers on the base of the blotting chamber (VWR), then the membrane, the gel, and the two other Whatman® papers. Bubbles were removed by rolling a tube over the sandwich. The blotting chamber was closed, and the proteins were blotted on the membrane for 1.5 hours at 1 to 2 mA/cm² and 300 V (Power supply EV2310, Consort). Nupage gradient gels (4-12%, Invitrogen) were blotted for 25 to 40 min at 300 mA and 15 V with 1x Nupage Transfer Buffer (8.16 g/L Bicine, 10.48 g/L Bis-Tris, 0.6 g/L EDTA, 10% Methanol). Mono-ubiquitin blots were boiled for 5 min in cooking water on a heating plate (Bomann) to facilitate binding of the ubiquitin antibody to mono-ubiquitin. After the blotting, the membrane was transferred to approximately 1 % milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl₂ x 6 H₂O, pH 7.8 with 0.5 % Tween (VWR) and incubated at RT for 15 min. After blocking, the blot was incubated with the primary antibody diluted in milk in TBS-T for 1 to 2 hours at RT or overnight at 4 °C. After the incubation with the primary antibody, the blot was washed three times for 10 to 30 min in TBS-T. Then the blot was incubated with the secondary antibody diluted in TBS-T for 1 hour at room temperature or overnight at 4°C. The blot was washed again 3x for 10 min in TBS-T. All incubation steps were performed with constant shaking.

Binding of HRP-coupled secondary antibodies was detected with ECL or SuperSignal (Thermo-Fisher Scientific) substrate solutions and imaged with a chemiluminescence camera at the Amersham 600 Imager. Binding of Dylight 650 nm anti-rabbit and the Dylight 480 nm anti-mouse fluorescence secondary antibodies was imaged with the respective epifluorescence option (480 nm or 650 nm) of the Amersham 600 imager.

The following primary antibodies were used in the indicated dilutions: anti-GFP (rat/rabbit, 1:1000, Chromotek), anti-tagRFP (Sigma, 1:1000), anti-GST (rabbit, made by Tobias Bläske, Eurogentec, 1:1000), anti-OTU11 (made by Tobias Bläske, Eurogentec, 1:1000), anti-Ubiquitin (P4D1 SCBT, 1:500 – 1:2000), anti-MBP (NEB, 1:1000), anti-UGPase (Agrisera, 1:3000), anti-H⁺-ATPase (Agrisera, 1:5000), anti-PIP1 (Agrisera, 1:1000), anti-
Sec21 (Agrisera, 1:1000), anti-PDR8 (Agrisera, 1:1000) and anti-Actin (from mouse hybridoma cells, 1:50).

The following secondary antibodies were used in the indicated dilutions: mouse-Dylight 488 (Invitrogen, 1:5000), rabbit-Dylight 650 (Invitrogen, 1:5 000), rabbit-Horse-Radish-Peroxidase (HRP), Merck/Sigma, 1:100.000), rat-HRP (Al1:10.000), mouse-HRP (Sigma, 1:80.000).

Total proteins on the membranes were stained for approximately 10 min with 0.1 % PonceauS in 5 % acetic acid, washed in water for 5 min, dried, and imaged with the epi-illuminator camera of the Amersham 600 imager.


The total protein concentration of plant extract was measured with a Bradford assay (ROTI® Quant, Roth). The standard for the Bradford assay was a Bovine serum albumin (BSA) dilution series (1, 0.8, 0.6, 0.4, 0.2, 0.1, 0 mg/mL BSA in water). For the assay, 200 µL of 1:5 diluted Roti-Quant solution was mixed with 4 µL of 1:5 to 1:10 diluted sample in a flat-bottom transparent 96-well plate (Sarstedt), and the absorption was measured at 595 nm with the Infinite F50 plate reader (Tecan). If it was necessary to have an equal protein concentration in all samples, e. g. for the Mass Spec experiment, the volumes were adjusted with Buffer A with Triton-X-100.

4.13. Affinity purification of ubiquitylated proteins from amsh1 and Ler seedlings

Ubiquitylated proteins were isolated from wild-type (Ler) und amsh1 seedlings with a tandem ubiquitin binding domain constructs (pSK16: GST-2xGAT, TUBE (Kim et al., 2013)) and analyzed by Mass Spectrometry by Julia Mergner of the Küster Lab (TUM) and Eva Höllmüller from the Stengel Lab (University of Konstanz). The GST-2xGAT construct were designed and cloned by Marie-Kristin Nagel and Steffi Klein with the ubiquitin binding GAT domain of TOL6 (At2g38410) (Moulinier-Anzola et al., 2020).
4.13.1. Preparation of protein beads

The GST-proteins used for the purification of ubiquitylated proteins from proteins were either kept on GST-beads or covalently bound to Affigel 15 beads (Biorad). The primary amines of the proteins will form a stable amino bond with the active groups of the Affigel beads. To couple the proteins on Affigel beads, the Tris-buffer was exchanged with dialysis (Snake Skin dialysis tubing, 10 kDa Cut-off) overnight or a buffer exchange column (PD SpinTrap, G-25, GE Healthcare) to 50 mM MOPS-KOH (Roth), pH 7.5 as Tris contains primary amine groups that bind to the Affigel beads. After the buffer exchange, the proteins were quantified with SDS-PAGE and incubated with 1 mL Affigel beads/5 mg proteins overnight at 4°C with shaking. Before the binding, the Affigel-beads were washed 3x with water. The binding reaction was stopped and remaining active groups on the beads were blocked by incubating them with 1/10 of the reaction volume of 1 M Tris-HCl, pH 7.5 for 1 hours at 4 °C. The supernatant was removed by centrifugation and analyzed by SDS-PAGE or Bradford assay to monitor the efficiency of the binding reaction. The protein decorated beads were washed 3x with EB (50 mM Tris-HCl, pH 7.2, 200 mM NaCl) and stored in EB at 4 °C until use.

4.13.2. Affinity purification of ubiquitylated proteins from Arabidopsis seedlings

For the large scale affinity purification, 40 g of 10-day old seedlings were grinded in a mortar with liquid nitrogen until the plant material was a homogenous powder. The powder was filled in a 50 mL tube on ice and dissolved in 20 mL EB with Triton-X-100 and protease inhibitors (50 mM Tris-HCl, pH 7.2, 200 mM NaCl, 0.25% Triton-X-100, 2 mM PMSF (Roth), 1 mM NEM (Merck), 5 mM EDTA, 1x Protease Inhibitor cocktail (Roche)). The mixture was further homogenized with an electric homogenizer (IKA T-25 Ultra-Turrax) for 1 min at 1000 rpm in the cold room and centrifuged for 20 min at 16.000x g at 4°C. To remove any remaining solid particles, the supernatant was filtered with a PVDF, 0.45 µm syringe filter or a 0.2 µm nylon mesh in a new 50 mL tube, and mixed with 5 mg GST, GST-2xGAT, or GST-TUBE on Glutathione Agarose beads or Affi-Gel beads. The supernatant was incubated for 6 hours at 4 °C on a shaker or rotating wheel with the protein beads. After the incubation, the tubes were centrifuged for 5 min at 4°C at 3000x g in a swing out rotor and the supernatant was discarded. The beads were transferred to a minispin column, washed 3x with 1 mL EB with Triton-X-100, 2x, with 2 M NaCl in EB, and 3x with 1 mL EB without Triton-X-100. After washing, the proteins were eluted 1x SDS
in EB by boiling them a 98 °C or 5 min. The efficiency of the purification was analyzed with an ubiquitin western blot and total protein staining.

4.13.3. Tandem affinity purification

The first large scale experiment was performed with 6His-Ub / amsh1and 6His-Ub / Col-0 with a previously described tandem affinity purification (Kim et al., 2013). The method combines the affinity purification with a Ni-NTA-Purification under denaturing conditions. To compare both methods, the experiment was performed with in-solution and in-gel digest. Col-0 without 6His-Ub was used as a negative control.

The affinity purification was performed as described in 4.13.2 with 25 g of seedlings. After the affinity purification, the proteins were eluted from the Affi-Gel beads by denaturing them with 10 mL Buffer G (7 M guanidine-HCl (Roth), 100 mM NaH2PO4 (Roth), 10 mM Tris, pH 7.4) for 30 min at RT on the spinning wheel (eluate 1). The eluate was mixed with 1 mL Ni-NTA-beads and incubated on the spinning wheel overnight at 4 °C. After the incubation, the beads were washed 1x in Buffer G with 0.2 % Triton-X-100, 1x in Urea buffer (8 M urea (Roth), 100 mM NaH2PO4, 10 mM Tris-HCl, pH 8.0) with 0.2 % Triton-X-100, 2x in Urea buffer with 20 mM imidazole and 3x in Urea buffer. The His-tagged proteins were eluted in 300 µL urea buffer with 400 mM imidazole (Merck, eluate 2).

The efficiency of the purification was analyzed with ubiquitin western blot and on silver staining gel. Before they were put on the gel, the eluate 1 samples were precipitated with Trichloroacetic acid (TCA), as Guanidine-HCl interferes with SDS-PAGE. For the TCA precipitation, 250 µL of the sample was mixed with 1 mL 100 % TCA incubated 2 hours on ice and centrifuged for 15 min at 16,000x g. The pellets were washed 2x with 100 % EtOH (cooled to -20°C) and re-suspended in 25 µL of 1xSDS in Urea buffer. The western blot samples were heated for 10 min at 70°C.

For the MS analysis, the eluate 2 samples were given to Julia Mergner of the Küster Lab, who performed the following steps. The eluates (285 µL) were lyophilized with a Speedbag, dissolved in 75 µL 50 mM Tris-HCl, pH 8.5, 1 mM CaCl2, treated with 10 mM DTT for 1 hours at RT, and alkylated with 55 mM Chloroacetamide (CAA) for 30 min at RT in the dark. Then, the samples were divided, 50 µL were run on a gel and 25 µL were used for in solution digest. For the in solution digest, the samples were diluted 1:16 with 50 mM Tris-HCl pH 8.5, 1 mM CaCl2, digested with Trypsin (1:50) for 2 hours at 37°C at 700 rpm, acidified with formic acid (FA) to pH 2-3, and desalted with Stage Tip desalting chromatography (5xC18; Buffer A: 0.1 % FA; Buffer B: 0.1 % FA, 50 % Acetonitrile (ACN)).
The samples were dissolved in 20 µL 0.1% FA, separated on a Dionex Ultimate 3000 LC (Buffer A: 0.1% FA, 5% DMSO in H2O, Buffer B: 0.1% FA, 5% DMSO in ACN, 1 hour gradient), and measured with the Q-Exactive HF mass spectrometer (method: Fullproteome_70min_4to32_100ms_top20). The samples for the in gel digest were lyophilized (Speedvac), dissolved in 40 µL 2xLDS (0.666 g/10 mL Tris-HCl, 0.682 g/10 mL Tris base, 0.8 g/10 mL LDS, 0.006 g/10 mL EDTA, 4 g/10 mL Glycerol, 0.75 mL/10 mL 1% Serva Blue G250, 0.25 mL/10 mL 1% Phenol Red) sample buffer (NuPage), incubated 45 °C, 20 min, sonicated 3 x 1 min, and 60 µL were loaded on a NuPage (4-12% Bis-Tris) gel. Each lane of the gel was cut in four pieces, avoiding the prominent bands at 50 and 35 kDa that were present in every sample, digested in the gel, and analyzed in the same way as the in solution samples.

4.13.4. Large scale experiment with GST, GST-TUBE and GST-2xGAT

In the second large scale experiment amsh1 and Ler was mixed the GST, GST-2xGAT and GST-TUBE to compare the purification efficiency of the different constructs. The affinity purification was performed as described in 4.13.2. A His-Tag purification was not performed. The experiment was performed with one replicate of each genotype and for each GST-protein. The experiment was performed with Affi-Gel beads and the proteins were eluted with 100 µL 1xSDS in EB.

The eluted samples were given to Julia Mergner of the Küster Lab, who performed the following steps. For the MS-Analysis 40 µL of the eluate were reduced by the addition of 10 mM DTT and incubation for 1 hour at 50°C, alkylated with 55 mM CAA for 30 min at RT in the dark, and separated on a Nupage gel (200 V, 5 min) gel. Each lane of the gel was cut in 6 equally sized pieces (>250, 100-250, 65-100, 45-65, 30-45, <30 kDa) which were digested with trypsin in-gel digestion. The digested samples were dissolved in 15 µl 0.1% FA, separated with Dionex Ultimate 3000 Liquid chromatography system (Buffer A: 0.1%FA, 5% DMSO in H2O, Buffer B 0.1%FA, 5% DMSO in ACN) with an 1 hour gradient, and analyzed with LUMOS2 (method: 60min_4to32_Top20_sensitive).

4.13.5. Large scale experiment with GST-2xGAT

For the third large scale experiment, 40 g of amsh1 and Ler seedlings were mixed with GST-2xGAT. The experiment was performed with three replicates of each genotype. Each
replicate was measured twice in the MS analysis. The affinity purification was performed as described in 4.13.2 without coupling to Affi-Gel beads. GST-2xGAT was kept on the GST-Agarose beads. After the affinity purification, the proteins were eluted in 150 µL 1xSDS in EB. For the MS-Analysis, 100 µL of the eluate were separated on a 12 % Gel in cooperation with Eva Höllmüller from the Stengel Lab at the University of Konstanz, who also did the following sample preparation steps and the MS analysis. The samples were cut from the gel, avoiding the prominent GST-2xGAT band and digested in the gel.

4.13.6. Data analysis

The initial data analysis of the MS data was performed by Julia Mergner and Eva Höllmüller with MaxQuant and Perseus. The Perseus software was used to insert annotations from TAIR, do statistical tests (t-tests), volcano blots, and clustering. Subcellular localization was analyzed with the SUBA4 internet tool (https://suba.live/). To compare different proteomic experiments, Venn diagrams were created with a free Venn diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) and Venn diagram plotter (http://omics.pnl.gov).

4.14. Homology models

Homology models were performed with Swissmodel (https://swissmodel.expasy.org), trRosetta (https://yanglab.nankai.edu.cn/trRosetta/) and visualized with Pymol (https://pymol.org/2/). Template structures were downloaded from PDB (https://www.rcsb.org/).

4.15. GFP-IP

GFP-tagged proteins were isolated from 10-day old Arabidopsis seedlings grown on GM or ½ MS under long day conditions. An appropriate amount of Arabidopsis seedlings that were expressing a GFP-fusion protein (e. g. 5 g of GFP-expressing seedlings and 10 g of GFP-OTU11 expressing seedlings for MS analysis) were grinded with, or without liquid nitrogen in the cold room with a mortar. After the first grinding step, 1 mL / g tissue Buffer A with Triton-X-100 (50 mM Tris, pH 7.5, 100 mM NaCl, 10 % glycerol, 0.2 % Triton-X-
(100) was added to the shredded tissue and the grinding was continued until the mixture looked homogenous. The extract was centrifuged at 13,000 g to remove cell debris.

At this point, the total protein concentration of the extract was measured with a Bradford and the volume was adjusted to achieve an equal protein concentration in all samples. The supernatant was mixed with an appropriate amount of GFP-magnetic agarose (Chromotek) (e.g. 10 µl beads for 5 g of GFP expressing seedlings and 20 µl for 10 g of GFP-OTU11 expressing seedlings) and incubated with the beads for 30 min at 4°C on a rotating wheel. After the binding step, the supernatant was removed, and the beads were washed three times with extraction buffer without Triton-X-100. For MS analysis, the bound proteins were eluted in 50 µl of 1xLaemmli in Buffer A. For phosphatase treatment or DUB Assays, the proteins were kept on beads.

4.16. P100/S100

Microsomal membranes were isolated with ultracentrifugation as described in (Tamura et al., 2005). In short, about 3 to 10 g of 10-day old seedlings were ground with or without liquid nitrogen in the cold room with mortar and pestle and dissolved in 1.5 to 5 mL P100/S100 buffer (100 mM HEPES-KOH (Roth), pH 7.5, 0.3 M sucrose, 5 mM EGTA, 5 mM MgCl₂ and protease inhibitor cocktail (Roche)). Cellular debris was removed by centrifuging the extract at 8000 g for 10 min at 4°C. After this step, the protein concentration was measured in the supernatant (S8) with a Bradford assay and the volume was adjusted to achieve an equal protein concentration in all samples. The S8 samples were filled in thick wall tubes (Beckmann) and centrifuged for 1h at 100,000xg (55 000 rpm) in the MLA-130 rotor (Beckmann) in an Optima Max Ultracentrifuge (Beckmann). After the centrifugation, the S100 supernatant that contained the soluble proteins was completely removed. A sample was kept for the western blot and rest of the supernatant was discarded. The pellet was washed twice with P100/S100 buffer to remove all soluble proteins. To dissolve the P100 pellet, it was incubated for 30 min at RT in 1xSDS in P100/S100 buffer without bromphenolblue. After the incubation, the P100 pellet was re-suspended with a syringe and the samples were stored at -20°C. For the western blot, bromphenolblue was added to the P100 samples and the S8, S100 and P100 samples were heated for 10 min at 70 °C. Since the volume was adjusted prior to the ultracentrifugation, the same volume of S8, S100 and P100 was loaded on the gel. The UGPase antibody was used as a marker for soluble proteins, the H⁺-ATPase or the PIP1 antibody as a marker for PM proteins.
4.17. Simplified PM enrichment with Brij-58

The simplified PM enrichment with Brij-58 was performed as described in (Collins et al., 2017) with small adaptations. About 3 to 6 g of seedlings were transferred to a pre-cooled mortar filled with 1.5 mL Buffer H (250 mM sucrose, 50 mM HEPES-KOH, pH 7.5, 5 % (v/v) glycerol, 50 mM sodium pyrophosphate (Merck) x 10 H2O, 10 mM EDTA, 0.5 % (w/v) PVP-10 (Merck), 3 mM DTT, 1x protease inhibitor cocktail). The seedlings were grinded without liquid nitrogen in the cold room for 3 min. The extract was transferred to a 2 mL Eppendorf tubes on ice and centrifuged for 10 min at 8000x g. A sample of the S8 supernatant was kept for the western blot and Bradford Assay. All samples were kept on ice during the enrichment procedure or directly mixed with 5x SDS and stored at -20°C. The rest of the S8 was transferred to thick wall tubes (Beckmann) and centrifuged for 30 min at 100.000xg (55 000 rpm) in the MLA-130 rotor (Beckmann) in the Optima Max Ultracentrifuge (Beckmann). The S100 supernatant was removed completely and a sample was kept for the western blot and the Bradford Assay. The pellet was washed twice with Buffer H, to remove all contaminants from the S100. After the washing the P100 pellet was re-suspended 100 - 200 µL buffer H and the protein concentration was measured in all fractions with the Bradford Assay. About half of the P100 fraction was kept for the western blot. The rest of the P100 fraction was mixed with 5x Brij-58 (0.01 g Brij-58 (Sigma) in 10 mL Buffer H) and Buffer H, so that the final mixture contained 2 µL 1x Brij-58 / 1 µg protein. The protein solution was incubated for 30 min on ice and centrifuged for 30 min at 100.000x g at 4°C. The supernatant was removed and discarded. The pellet was re-suspended in the same volume of 1 x Brij-58 that was used in the first incubation step, incubated for 30 min on ice, and centrifuged again for 30 min at 100.000x g at 4°C. The supernatant was removed completely, the pellet was re-suspended in 1 mL Buffer H, and centrifuged for 30 min at 100.000x g to remove the Brij-58. The supernatant was removed completely and the enriched plasma membrane (ePM) pellet was re-suspended in 50 to 100 µL Buffer H. The protein concentration was measured with the Bradford assay. The rest of the fraction was mixed with 5xSDS and stored at -20 °C until use. Before loading them on the gel, the western blot samples were heated for 10 min at 70 °C, and 20 to 40 µg of total proteins of each fraction was loaded on the gel. The UGPase antibody was used as a marker for soluble proteins, the H+-ATPase or the PIP1 antibody as a marker for plasma membrane proteins.
4.18. **In vitro binding assays**

To test if two proteins were able to bind to each other *in vitro*, an *in vitro* binding assay was performed. For the *in vitro* binding assays, 100 pmol of GST-OTU11 and GST-OTU12 kept on magnetic beads were mixed with 100 to 500 pmol of MBP-tagged interactor proteins in 400 µl MST buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 10 mM MgCl₂, 0.05% Tween). Bead volumes were equalized with GST magnetic beads blocked in milk. The reaction mixture was incubated for 4 hours at 4°C. After the incubation, the supernatant was removed, the beads were washed four times with MST buffer, and the bound proteins were eluted in 30 µl of 40 mM glutathione.

4.19. **Ubiquitin binding assay**

To test, if a protein was able to bind to free di- or tetra-ubiquitin, an ubiquitin binding assay was performed. For the ubiquitin binding assays, 100 pmol of recombinant GST-tagged protein from *E. coli* that was kept on magnetic GST-beads was mixed with 100 pmol of commercially available free di- or tetra-ubiquitin (R and D, UbiQ) in 400 µl MST buffer. The Bead volume for the assays with the recombinant proteins was equalized with GST magnetic beads blocked in milk. The reaction mixture was incubated for 4 hours at 4 °C. After the incubation, the supernatant was removed, the beads were washed 4x with 1 mL MST-buffer, and the bound proteins were eluted in 30 µL 40 mM glutathione, pH 7.2. GST was used as a negative control. For the binding assays with ubiquitylated proteins from the plant extract the procedure described section 4.13.2 was scaled down. For the binding assay with GST-2xGAT and *amsh1* seedlings, 400 µg of protein was mixed with the extract of 2 g of seedling material.

For Ubiquitin binding assays with active GFP-OTU11 isolated from plant extract, 100 pmol of non-hydrolysable K48-linked and K63-linked di-ubiquitin synthesized by the Marx Lab at the University of Konstanz was mixed with the protein decorated beads in 400 µL MST-Buffer. GFP was used as a negative control. The mixture was incubated for 2 hours at 4°C on a spinning wheel. After the incubation, the beads were washed 4x with MST-buffer and the bound proteins were eluted in 37.5 µL 1 x SDS in MST-buffer. Samples were analyzed on SDS-PAGE gels (15 % gels for di- or tetra-ubiquitin) with anti-GST, anti-GFP- and anti-ubiquitin western blots.
4.20. De-Ubiquitylation (DUB) assays

DUB assays with recombinant OTU11 and OTU12 were performed as described previously with modifications (Kalinowska et al., 2016). If not stated otherwise, 200 pmol GST-OTU11 or OTU12 was incubated with 250 ng of di-ubiquitin (16 pmol, UbiQ or Biotechne) or tetra-ubiquitin (7.81 pmol, Biotechne) 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 1 to 4 hours. For DUB assays with OTU11 isolated from plant extract by GFP-IP, the protein decorated beads, optionally treated by phosphatase, were divided equally and the supernatant was removed completely. The protein beads were incubated with 14 µL assay buffer and 250 ng di or tetra-ubiquitin for 1 to 3 hours at 21 °C or 30 °C in 15 µL DUB assay buffer. The reactions were stopped by adding 5 µL 5x Laemmlii-Buffer (310 mM Tris-HCl, pH 6.8, 50 % glycerol, 10 % SDS, 0.5 % bromphenolblue, 3.5 % ß-mercaptoethanol). For 0 hour samples, the 5xSDS buffer was added before the addition of the ubiquitin. The Samples were analyzed on Nupage Gradient gels (4-12%) or standard SDS-PAGE gels (GST: 10-12%, Ubiquitin: 15%) with western blot or total protein staining (SYPRO-Ruby or silver staining).

The DUB-Assay with TAMRA-K63-linked di-ubiquitin (Biotechne) was performed as described previously (Mevissen et al., 2016). For the assay, 7.5 µL of enzyme solution (1.06 pmol/µL, 8 pmol) was mixed with 7.5 µL of TAMRA-di-ubiquitin-solution (33.3 ng/µL, 250 ng, 16 pmol) in TAMRA-Buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 2 mM DTT, 0.1 mg/ml BSA) in a black, flat-bottom a 384-well plate (Corning). The increase of fluorescence was measured with an Infinite M Plex plate reader (Tecan) at room temperature (ex = 540 nm/em = 590 nm). The Assay was performed in quadruplicates.

4.21. Pip Strip assays

The binding of recombinant OTU11 to different kinds of phospholipids was analyzed with Pip Strip™ membranes (Thermo-Fisher Scientific) according to the instructions of the manufacturer. Pip Strip membranes were blocked for 6 hours with 3% fatty acid free BSA (Roth) at RT or overnight at 4°C and incubated with the 0.5 µg/µL (experiments with the truncated versions) or 1µg/µL (experiments with mutants) GST-tagged protein for 1 hour at room temperature or overnight at 4 °C. The binding of the GST-tagged proteins was analyzed with an anti-GST-blot. The washing steps after the primary antibody incubation step were performed with TBS-T without fatty acid free BSA.
4.22. Phosphorylation Assay

The phosphorylation status of GFP-tagged OTU11 isolated from the plant extract was performed as described previously (Vogel and Isono, 2020). In short, the protein decorated beads were treated with active and inactive lambda phosphatase (NEB) for 30 min at 30°C. The phosphorylation status of the protein was compared between both samples on a standard SDS-PAGE gel with phosphostain (ProQ®-Diamond) or on a MN²⁺ - Phos-tag™ gel (7.5 % Acrylamide, 20 mM Phos-Tag™).

4.23. Phenotypical analysis

4.23.1. Root length assays

For standard root length assays, seedlings were grown for 5 to 10 days on ½ MS medium in LD conditions as described in section 4.2. Root length was measured by re-drawing the roots with the freehand tool in Fiji (https://imagej.net/software/fiji/). Averages and standard deviations were calculated with Excel, boxplots were drawn with R (https://www.r-project.org/). The statistical significance of the difference between two genotypes or treatments was analyzed with a T-Test in Excel (two-sided (“2”), two random samples with different variance (“3”)). For the plant hormone treatment, drugs (0.5, 1, 1.5 µM NPA; 0.025, 0.05, 0.2 µM NAA, 1 µM, 5 µM, 10 µM ABA (+1% Sucrose) were added in the indicated concentrations to the medium before pouring the plates.

4.23.2. Physiological experiments

Germination assays were performed with and without stratification on filter paper soaked with water under LD conditions or plates with 0.5 µM and 1 µM ABA (+Sucrose) under LD and SD conditions. The number of germinated seedlings was counted at different time points until all seeds germinated.

The meristem size and the number of cells in the meristem was analyzed with propidium iodide staining. For this, 7 to 10 day old seedlings were stained for 10 min at RT in 10 mg/mL propidium iodide (Merck) in liquid ½ MS, washed by dipping them in ½ MS without propidium iodide and imaged with the confocal microscope (LSM700, Zeiss).
The seed coat mucilage was analyzed with Ruthenium red staining. For this, seeds were pre-treated with 800 µL of water, 50 µM EDTA or 50 mM CaCl₂ for 2h at RT on the spinning wheel. After the incubation, the solution was removed and 800 µL of 0.1 % Ruthenium red was added. The seeds were incubated for 1 hour at RT on the spinning wheel, washed 3 x with water and analyzed with a microscope (Axiozoom, Zeiss).

The leaf vein pattern in cotyledons was analyzed with a microscope (Axiozoom, Zeiss) in cotyledons of seedlings grown on GM as described previously (Prabhakaran Mariyamma et al., 2018). Before microscopy, the leaves were removed from the seedlings and bleached with 1% NaOCl for 2 hours at RT. To analyze the lateral root emergence after gravistimulus, seedlings were grown on ½ MS in LD conditions and turned 90°C around after 3 days. Lateral root emergence was analyzed with a microscope (Nikon) after 18 hours and 42 hours or 48 hours. Seedlings were mounted in 1% NaOCl. Lateral root emergence stages were assigned as described previously (Péret et al., 2012).

4.23.3. Cell biological assays

To analyze, if the overexpression of OTU11 and OTU12 influences the endocytosis of plasma membrane proteins in the plant cell, 35S promotor driven N-Terminally RFP-tagged versions (in pExTag-RFP) of the proteins were co-expressed with the artificial endocytosis substrate GFP-PMA-Ub in Arabidopsis protoplasts (Herberth et al., 2012). The localization pattern (PM + endosomes + vacuole, vacuole, endosomes, vacuole + endosomes) of the PMA-GFP-UB was analyzed in protoplasts with and without 35S:RFP-OTU11 and 35S:RFP-OTU12. The distribution of localization patterns was compared with a Fisher-Test (R, https://www.r-project.org/). 35S:PMA-GFP and 35S:RFP-SYP121 were used as negative controls.

The influence of OTU11 and OTU12 on the endocytosis of PIN2-GFP was tested with BFA Brefeldin A (BFA) treatment. PIN2:PIN2-GFP / Col-0 and PIN2:PIN2-GFP / otu11otu12 seedlings were treated with 50 µM BFA (Bre50 mM stock in DMSO) in liquid ½ MS for up to 90 min. Mock treatments were performed with DMSO. The emergence of the BFA bodies was analyzed with a confocal microscope (LSM700).

Additionally, the influence of OTU11 and OTU12 on the endocytosis of the styryl dye FM4-64 (Invitrogen) was analyzed. Col-0, otu11otu12 and OTU12p:gOTU12 / otu11 otu12 seedlings were stained for 10 min with 2 µM FM4-64 in liquid ½ MS. The stained seedlings were transferred to 50 µM BFA in liquid ½ MS and incubated for up to 90 min. The emergence of the BFA bodies was analyzed with a confocal microscope (LSM700).
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Acknowledgement

During this PhD project a lot of people supported and helped me in many different ways. Without them it would have been not possible to carry out my experiments and write my thesis. Especially, I want to thank my supervisor Prof. Dr. Erika Isono for giving me the chance to perform my PhD project in her Lab. I want to thank for her constant support, patience, her many helpful explanations, scientific discussions and useful tips for presenting my data. No matter how small my issues were I could always discuss it with her and she would know a solution, even when I was very desperate. I am very thankful for her dedicated supervision. Many thanks also for the chance to participate in conferences and for proof-reading my thesis. Additionally, I want to thank Prof. Dr. Peter Kroth for being my co-supervisor in the GBS and for being the second referee of my thesis and Prof. Dr. David Schleheck for being the chair of my PhD committee.

Furthermore, I want to thank Dr. Marie-Kristin Nagel who always had an open ear for my problems and who helped me a lot with my experiments and all other aspects of the Lab life during the whole time of my PhD. Special thanks go to Tobias Bläske who did essential experiments for the OTU project and Franziska Anzenberger who established the mutan line. I also want to thank Dr. Kamila Kalinowska-Brandt for her help during the one and a half years of my PhD project in Freising. Many thanks also to all other past and present colleagues for their interest in my work und their scientific input. Especially, I want to thank Dr. Svetlana Boycheva-Woltering, Niccolò Mosesso, Marcel Bhattacharai, Niharika Savant-Lerner, Felix Groh, and Konstanze Meergans. I also want to thank Ms. Gimmi for her help with administrative issues. Furthermore, I want to thank all past and present colleagues from the Kroth Lab and the Schleheck Lab for the nice and collaborative atmosphere.

Aditionally, I want to thank Prof Dr Claus Schwechheimer for having me in his Lab for the first part of my PhD and I want thank all people from the Schwechheimer Lab for the nice atmosphere during this time. Many thanks go to Dr Julia Mergner from the Küster Lab and Dr. Eva Höllmüller from the Stengel Lab for the MS analysis and Dr. Melina Altmann from the Braun Lab for the Y2H-Screen. Many thanks also go to the gardeners of the botanical garden at the University of Konstanz, especially Gerd Rönnebeck, for growing the Arabidopsis plants for this project and the team of the Bioimaging Center of the University of Konstanz for their help in using the microscopes. Thanks also to the Vierstra Lab for giving us the TUBE construct, the Friml Lab for giving us the PIN-GFP-line and PD Dr. Dietmar Funck for giving us the GFP- line. Thanks also to the DFG (SFPB924, SFPB969), the GBS, and the TUM Graduate School for their support.
Particulary, I want to thank all students who contributed to this work as VTK or bachelor students, especially to Feng Liu, Sneha Prakash, Melina Dietzer, Mona Urban, Felix Eglindörfer, Özge Delikaffa, Michael Lüders, Laura Kayser, Aline Schöllkopf, and Colin Strasdat. I also want to thank Chris Gude who contributed to my reserach project as a master student and Dennis Dembinski who contributed to my PhD project as VTK and master student. Special thanks go to the one and only hero, Lea Held, for her contribution to this thesis as bachelor student, Hiwi and her huge moral support. Of course I also want to thank all other Hiwis for their help in the Lab. Special thanks go to Sina Bergemann for her help in the Lab and her great moral support thanks to her positive attitude.

Last but not least, I want to thank my parents for their constant moral support and believing in me. I also want to thank my grand-parents and all other family and friends who helped me to stay in a good mood during my PhD.

Thanks a lot to everyone, let`s stay healthy and remain peaceful!
### Appendix

#### Appendix table 1: Constructs used in this study

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<td>At3g02070</td>
<td>GST-OTU12 peak1 (aa 131 bis 141) in 6xA, peak3 (aa 189 bis 192) and peak2 (aa 236-242)</td>
<td>pGex-6P-1</td>
<td>May 2021</td>
</tr>
<tr>
<td>cCG2</td>
<td>At3g22260</td>
<td>OTU11 gateway entry</td>
<td>pDonr207</td>
<td>02.09.2015</td>
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<td>cCG3</td>
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<td>OTU12 gateway entry</td>
<td>pDonr207</td>
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<td>pExTag-YFP</td>
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<td>pExTag-YFP</td>
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<td>OTU11p: OTU11 genomic</td>
<td>BB10</td>
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</tr>
<tr>
<td>TB114</td>
<td>At3g02070</td>
<td>OTU12p: OTU12 genomic</td>
<td>BB10</td>
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<td>pSK16</td>
<td>At2g38410</td>
<td>GST-2xGAT</td>
<td>pGex-6P1</td>
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### Appendix table 3: Primers used in this study

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<td>KV1</td>
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<tr>
<td>KV2</td>
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<tr>
<td>KV13</td>
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<td>KV14</td>
<td>GGGGACCACCTTTGTACAGAGAGCCTGTtcaGCAATCACGCA AAG</td>
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<td>KV15</td>
<td>GGGGACAAGTTTTGTACAAAAAAGCAGGCTTgGAGGTTGCTAC GAG</td>
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<tr>
<td>KV16</td>
<td>GGGGACCACCTTTGTACAGAGAGCCTGTtcaTACCTGTAG GCTGTCT</td>
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<tr>
<td>KV17</td>
<td>GGGGACAAGTTTTGTACAAAAAAGCAGGCTTgGGATTAAACT GCAGAT</td>
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<td>Gene ID</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>KV18 UBP25</td>
<td>GW N rv</td>
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<tr>
<td>KV20 6xHis</td>
<td>fw</td>
</tr>
<tr>
<td>KV21 6xHis</td>
<td>rv</td>
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<tr>
<td>KV31 RBCS</td>
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<tr>
<td>KV32 PTA7002</td>
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<td>KV47 OTU11</td>
<td>fw</td>
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<tr>
<td>KV48 OTU12</td>
<td>fw</td>
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<tr>
<td>KV102 OTU11 N-term GW</td>
<td>rv</td>
</tr>
<tr>
<td>KV103 OTU12 N-term GW</td>
<td>rv</td>
</tr>
<tr>
<td>KV104 OTU11 N-term SalI</td>
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</tr>
<tr>
<td>KV105 OTU12 N-term SalI</td>
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<tr>
<td>KV114 At1g17970</td>
<td>BamHI</td>
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<tr>
<td>KV115 At1g17970</td>
<td>EcoRI</td>
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<tr>
<td>KV139 At1g17970</td>
<td>GW</td>
</tr>
<tr>
<td>KV140 At1g17970</td>
<td>GW</td>
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<td>KV148 Auxin can protein</td>
<td>GW</td>
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<td>KV233 OTU12 domain</td>
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<td>KV234 OTU12 domain</td>
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<td>KV237 OTU11</td>
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<td>S52E</td>
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<td>KV239 OTU11</td>
<td>S52A</td>
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<td>KV240 OTU11</td>
<td>S52A</td>
</tr>
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<td>KV243 OTU11</td>
<td>S93E</td>
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### Appendix Table 4: Primers from Other People Used in This Study

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<tr>
<th>Name</th>
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<tr>
<td>CG46 OTU11 pGEX fw BamHI</td>
<td>AAGGGGATCCATGGATGAAAAACCATAGGA</td>
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<tr>
<td>CG47 OTU11 pGEX rev EcoRI</td>
<td>AAGGGAAATCCATGAGAGCAATGCTGTTGCTTC</td>
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<tr>
<td>CG48 OTU12 pGEX fw BamHI</td>
<td>AAGGGGATCCATGGAGACTCTTGAGTTT</td>
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<td>CG49 OTU12 pGEX rev Xhol</td>
<td>AAGGCTCGGAGCTGAACACCAATGTTTTCTCT</td>
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<tr>
<td>CG13 OTU9 GW N fw</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAGGCTtgGGGTATGAGCCGTA</td>
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<td>CG14 OTU9 GW N rev</td>
<td>GGGGACCCTTTGTACAAGAGAGCTTGATGAAATAGAGAAC</td>
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<td>CG15 OTU10 GW N fw</td>
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<td>CG16 OTU10 GW N rev</td>
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<td>CG18 OTU11 GW N rev</td>
<td>GGGGACAATTTGTACAAAAAAGCAGGCTtgGTGACATGAGAGAC</td>
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<td>CG19 OTU12 GW N fw</td>
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<tr>
<td>CG20 OTU12 GW N rev</td>
<td>GGGGACAATTTGTACAAAAAAGCAGGCTtgGTGACATGAGAGAC</td>
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<tr>
<td>FA130 Salk_129340/_132515 fw</td>
<td>AGAGTGTCGTTCTATGTGATAAG</td>
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<tr>
<td>FA131 Salk_129340/_132515 rv</td>
<td>ATTCAAGAGATCTCTCCACTAAGG</td>
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<td>FA134 Salk_052963/_005987 fw</td>
<td>CTGATCGAGGCTGATCTCAG</td>
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<td>FA135 Salk_052963/_005987 rv</td>
<td>AGGAAGTGAGCTAAGCTCT</td>
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<td>GP20 LBb1.3 mod</td>
<td>ATTTGCGGATTTCCGGGATAGCAT</td>
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<td>LH9 FW 6xAla OTU11</td>
<td>TATGTTGCTAATGGCTACGTGAGGCCGGAAGTTGAGG</td>
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<td>LH10 RV 6xAla OTU11</td>
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### Appendix Table 5: Antibodies Used in This Study

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<th>Antibody</th>
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<th>Clone</th>
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<tbody>
<tr>
<td>anti-OTU11</td>
<td>rabbit</td>
<td>Eurogentec/Tobias Bläske</td>
<td>polyclonal</td>
<td>ZDE18063</td>
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<tr>
<td>anti-GST</td>
<td>goat</td>
<td>Sigma-Aldrich</td>
<td>polyclonal</td>
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<tr>
<td>anti-GST</td>
<td>rabbit</td>
<td>Eurogentec/Tobias Bläske</td>
<td>polyclonal</td>
<td>ZDE18062</td>
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<tr>
<td>anti-H+-ATPase</td>
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<td>Agrisera</td>
<td>polyclonal</td>
<td>AS07260</td>
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<tr>
<td>anti-UGPase</td>
<td>rabbit</td>
<td>Agrisera</td>
<td>polyclonal</td>
<td>AS05086</td>
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<td>anti-Sec21p</td>
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<td>Agrisera</td>
<td>polyclonal</td>
<td>AS08327</td>
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<td>anti-PDR8</td>
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<td>Agrisera</td>
<td>polyclonal</td>
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<tr>
<td>anti-GFP</td>
<td>rat</td>
<td>Chromotek</td>
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### Antigen-specific antibodies

<table>
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<td>anti-RFP</td>
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<tr>
<td>anti-UB</td>
<td>mouse</td>
<td>Santa Cruz</td>
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<tr>
<td>anti-Actin</td>
<td>mouse</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>anti-MBP</td>
<td>mouse</td>
<td>NEB</td>
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<tr>
<td>anti-FLAG</td>
<td>mouse</td>
<td>Sigma-Aldrich</td>
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<td>anti-rat-HRP</td>
<td>goat</td>
<td>Roche</td>
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<tr>
<td>anti-mouse-HRP</td>
<td>rabbit</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>anti-rabbit-HRP</td>
<td>goat</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>anti-goat-HRP</td>
<td>goat</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>anti-mouse IgG, Dylight 488</td>
<td>goat</td>
<td>Thermo Fisher Scientific</td>
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<tr>
<td>anti-rabbit IgG, Dylight 650</td>
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<td>Thermo Fisher Scientific</td>
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### Appendix table 6: Plant lines used in this study

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<td>otu11</td>
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</tr>
<tr>
<td>otu12</td>
<td>SALK13251</td>
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<td>SALK05296 / SALK13251</td>
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<td>35S:GFP-OTU11.2</td>
<td>KV30-1-2/KV30-2-6</td>
<td>pFastR06</td>
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<tr>
<td>35S:GFP-OTU12</td>
<td>KV31-2-2/3-4/4-10</td>
<td>pFastR06</td>
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<td>35S:GFP-OTU11.1</td>
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<td>pFastR06</td>
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<td>pFastR06</td>
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<td>35S:GFP-OTU11.2 (D)</td>
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<td>pFastR06</td>
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<td>OTU11p:GFP-gOTU11</td>
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<td>OTU12p:GFP-gOTU12</td>
<td>TB114-4-6</td>
<td>BB10</td>
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<tr>
<td>35S:GFP-OTU12 / UBQ 10prom:2xCherry-1x PH(FAPP1)</td>
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Publications


Talks / posters

- Poster: GBS poster session, 07/2019, Konstanz, Germany
- Poster: X-ZOMES, 02/2019, Akko, Israel
- Poster: SFB969 Symposium, 10/2018, Konstanz, Germany
- Poster: SFB924 Conference, 10/2017, Freising, Germany
- Poster: ISE-G annual meeting, 09/2017, Konstanz, Germany
- Talk: Deubiquitinases - from structure to physiology, 06/2017, Oxford, UK
- Talk: SFP924 Seminar, 04/2017, Regensburg, Germany
- Poster: ENPER, 09/2016, Bordeaux, France