How will I recognize you? Insights into endocytic cargo recognition in plants.

Andreas De Meyer[1,2], Peter Grones[1,2], Daniel Van Damme[1,2,*].

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium

[2]Center for Plant Systems Biology, VIB, 9052 Ghent, Belgium

* Corresponding author: daniel.vandamme@psb.vib-ugent.be

Abstract

The plasma membrane (PM) houses a wide variety of proteins, facilitating interactions between the cell and its surroundings. Perception of external stimuli leads to selective internalization of membrane proteins via endocytosis. A multitude of endocytic signals affect protein internalization, however their coordination and the exact mechanism of their recognition still remains elusive. In this review, we summarized the up-to-date knowledge of different internalization signals in PM cargo proteins and their involvement during protein trafficking.

Highlights

- Multiple cargo recognition signals function in parallel for most cargo proteins

- Ubiquitination emerges as the most prominent cargo internalization signal in plant CME

- Ubiquitination is independently recognized by several endocytic players

- Cargo ubiquitination is controlled by addition, chain length and competition

Abbreviations

ANTH: AP180 N-terminal homology domain; **AGFG1:** ARF-GAP DOMAIN AND FG REPEAT-CONTAINING PROTEIN 1; **AMSH3:** ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM 3; **AP-2**: ADAPTOR PROTEIN 2 complex; **BIK1:** BOTRYTIS-INDUCED KINASE 1; **BRI1**: BRASSINOSTEROID INSENSITIVE 1; **CME**: clathrin-mediated endocytosis; **CIE:** clathrin-independent endocytosis; **DUB**: Deubiquitinating enzyme; **EH**: EPS15 homology domain; **FLS2:** Flagellin-sensitive 2; **IRT1**: IRON-REGULATED TRANSPORTER 1;

PICALM: PHOSPHATIDYLINOSITOL BINDING CLATHRIN ASSEMBLY PROTEIN **PIN2:** PIN-FORMED 2; **PM**: plasma membrane; **OTU:** OVARIAN TUMOR PROTEASE; **PTM**: post-translational modification; **PUB:** Plant U-box; **SCAMP5**: Secretory Carrier Membrane Protein 5; **RGF1:** ROOT MERISTEM GROWTH FACTOR; **RGLG:** RING domain Ligase; **REN4:** ROP1 ENHANCER4; **SH3P2:** SH3 DOMAIN-CONTAINING PROTEIN 2; **SLiM**: short linear motif; **TGN/EE**: Trans-Golgi network/early endosome; **TASH3:** TPLATE-ASSOCIATED SH3 DOMAIN CONTAINING PROTEIN; **TOL:** TOM1-like; **TPC**: TPLATE complex; **UBP**: Ubiquitin-specific protease; **VAMP:** VESICLE-ASSOCIATED MEMBRANE PROTEIN; **WAV3:** Wavy growth 3

Main review text

1. Introduction

The plasma membrane (PM) consists of lipids and proteins. This cellular border carries out a wide variety of functions, ranging from hosting receptors of various signaling pathways [1–5] and channels to allow nutrient uptake [6–10], to machinery to perform cell wall formation [11,12]. Consequently, the PM content needs to be tightly regulated to ensure that plants can adapt to changing environments. The predominant way for cells to achieve PM homeostasis is by balancing secretion and endocytosis, wherein lipids and proteins are respectively deposited and internalized from the PM via vesicular transport [13]. Removal of material by endocytosis is essential to regulate signaling pathways [1–3,5], to maintain polarity [6,10], to recycle secretory machinery [14], or to remove surplus lipids during cell plate formation [15,16].

Endocytosis in plants occurs via two pathways. Depending on whether or not it relies on the scaffolding molecule clathrin, it is termed clathrin-mediated endocytosis (CME) or clathrin-independent endocytosis (CIE). Plant CIE is mechanistically not well understood and our knowledge mainly derives from observations of cargo internalization under conditions where CME is presumed to be inactive [5,17–20]. The plasma membrane localized FLOTTILIN1 likely functions in CIE as it localizes to ~100nm vesicles occurring in the vicinity of the PM that are devoid of clathrin. The internalization of FLOTILLIN1 requires sterols [21] and the dynamic behavior of FLOTILLIN1, visualized via variable-angle total internal reflection fluorescence microscopy, differs from that of clathrin [22]. The exact mechanism of how CIE operates remains however unclear. CME on the other hand is much better characterized in terms of its machinery as well as its mode of action and this review will focus exclusively on CME. In plant CME, the ADAPTOR PROTEIN 2 COMPLEX (AP-2) [11,23–25] and the TPLATE adaptor COMPLEX (TPC) [26,27], together with monomeric adaptors such as AP180 N-terminal homology (ANTH) domain containing proteins [14,28–30], recognize cargo [14,28,29,31–35] (i.e. membrane proteins and their associated ligands) and pack it into vesicles with the help of the scaffolding protein clathrin. These clathrin-coated vesicles are pinched off from the membrane and internalized [36,37]. As there seems to be a plethora of internalization signals that function in parallel and whose relative importance varies between cargoes, our view on cargo recognition in plants is becoming increasingly complex. In this review, we aim to provide insight into the different ways of cargo recognition in plants and we focus on the important role of ubiquitin in endocytosis.

2. Short linear motifs contribute to cargo internalization

Short linear motifs (SLiM), present in the cytoplasmic parts of the cargoes, are well-established for cargo recognition in mammalian model systems. The best known SLiMs are tyrosine (YxxΦ; Y=tyrosine, Φ=bulky hydrophobic residue) [38–40] and dileucine (D/ExxxLL; D=aspartic acid, E=glutamic acid, L=leucine) motifs [41]. They are recognized by the AP2M and AP2S subunits of the AP-2 complex respectively [38–41]. In plant cells, there is clear evidence for the role of tyrosine and dileucine motif-based endosomal trafficking for AP-1, AP-3 or AP-4 adaptor complexes [42]. The connection between these motifs and AP-2 is however less obvious (reviewed in [42]). The cytosolic kinase domain of the BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor contains several canonical tyrosine motifs, of which Y898 was shown to interact directly with AP2M. However, mutating this single motif only reduced, but did not abolish, BRI1 endocytosis [32]. Whether the Y961 motif, which is also not required for BRI1 phosphorylation [32] acts redundantly with Y898 remains to be determined. In contrast, the tyrosine motifs of the boron exporter BOR1 are not recognized by AP2M and this AP-2 subunit preferentially binds to the cytoplasmic C-terminal tail of BOR1, which is devoid of canonical tyrosine motifs [31].

In addition to SLiM recognition by AP-2, TPC can bind cargo directly through its AtEH1/Pan1 subunit [33]. AtEH1/Pan1 contains two EPS15 homology (EH) domains, the first of which was shown to facilitate binding to the double NPF (N=asparagine, P=proline, F=phenylalanine) motifs present in the Secretory Carrier Membrane Protein 5 (SCAMP5), thereby controlling its internalization [33]. The direct interaction between the receptor protein kinase CLAVATA1 and the N-terminal half of AtEH1/Pan1 presents another example of the cargo recognition capacity of AtEH1/Pan1. CLAVATA1, as opposed to SCAMP5, does however not contain NPF motifs in its cytoplasmic tail. The exact mechanism of interaction therefore remains unknown [35].

In conclusion, the role of SLiM adaptor-mediated trafficking downstream of the Trans Golgi Network/ Early Endosome (TGN/EE) in plants is rather well established [42], but its requirement during cargo internalization from the PM remains elusive. Based on our current knowledge, SLiM recognition by AP-2 seems to be cargo specific and not essential for internalization. Alternative signals therefore likely facilitate endocytic trafficking in parallel. In addition, there might also be other, so far uncharacterized SLiMs, facilitating cargo recognition. Moreover, besides AP-2 and TPC, other endocytic players could also be involved in SLiM recognition. For example, the mode of action and contribution of the monomeric adaptors to cargo internalization in plants leaves a lot of room for further exploration.

3. Post-translational modifications

a. Ubiquitin and phosphorylation as an internalization signal

Next to SLiMs, reversible post-translational modifications (PTM) represent an additional layer of regulation of PM cargo internalization. Phosphorylation and subsequent ubiquitination of cytoplasmic lysine residues of cargoes, are common endocytic internalization signals [43]. In mammalian cells, the most common ubiquitin forms serving as an internalization signal are multi-mono- and K63-linked poly-ubiquitin [43,44]. Analogously to mammals, the most common PTMs in plant that are linked to cargo internalization are also ubiquitin and phosphorylation [9,45–47].

Mono- or K63-linked poly-ubiquitination contribute to the internalization and enhanced vacuolar degradation of a plethora of cargoes such as the boron exporter BOR1 [46], the IRON-REGULATED TRANSPORTER 1 (IRT1) [7,9,48], the auxin efflux carrier PIN-FORMED 2 (PIN2) [49] and the brassinosteroid

receptor BRI1 [47,50]. While interference with ubiquitination leads to a total apparent arrest of internalization of IRT1 [7,9,48], this is not the case for other cargoes. For BOR1, rapid internalization upon high boron is abolished by interfering with ubiquitination. However, boron transport-defective variants of BOR1 with severely reduced ubiquitination and ubiquitin-dependent internalization still maintain their AP-2-dependent polar localization, indicating functional endocytosis [46]. Similarly, a brassinosteroid-binding-deficient version of BRI1, $BRI1^{Q}$, which is largely devoid of phosphorylation and ubiquitination maintains a similar endocytic rate compared to its wild type counterpart [51]. In contrast, a double mutant in two E2-ubiquitin ligases (UBC35 and UBC36) shows drastically reduced K63-linked ubiquitination of various PM proteins and this correlates with reduced BRI1 internalization [52]. The differential observations between BRI^{Q} and BRI1 in the ubc35/ubc36 double mutant appear to be contradictory. One possible explanation might be that BRI^{Q} still undergoes some BR-independent ubiquitination and that this is sufficient for internalization.

It is evident for BOR1 and BRI1 that both SLiM and PTM internalization signals function in their trafficking. This brings us to an important question why some cargoes require a multitude of different endocytic internalization signals, while others, like IRT1, seem to have only one predominant signal [7,9,48]. A potential reason is that this could reflect the downstream route and fate of the cargo, for example to discriminate between recycling and polarity establishment versus degradation. Nevertheless, IRT1 has both degradation and recycling modulated by ubiquitin moieties [7,9]. Why do other cargo proteins then also require alternative and parallel signals for their internalization? In addition, there are other layers of complexity that involve PTMs. BRI1 is SUMOylated, which negatively affects its internalization [53]. As ubiquitin and SUMO are both modifications on lysine residues, the above observation hints to a spatial competition between different PTMs. If this is a general mechanism, it would indicate a far greater complexity of the system and at the same time offer a lot of room to alter cargo recognition and internalization.

b. Balancing cargo ubiquitination: Ubiquitin ligases and deubiquitinating enzymes

Ubiquitination of cargo proteins (mono- or poly-ubiquitination) is performed through the joined action of E2- and E3-Ubiquitin ligases. As there are only about 37 predicted E2 ligases, but more than 1500 predicted E3-ligases, it is evident that specificity is mainly determined by the latter [54]. The broad range-action of the E2 ligases is evident from the observations made using the aforementioned *ubc35/ubc36* double mutant, where K63-linked ubiquitination of multiple plasma membrane proteins was strongly reduced [52].

Various E3-Ubiquitin ligases mark cargoes to affect their trafficking and/or degradation. For example, the receptor kinase ERECTA is ubiquitinated by Plant U-box (PUB) 30/31 [55], BOTRYTIS-INDUCED KINASE 1 (BIK1) by RING-H2 FINGER A3A [56], IRT1 by IRT1 DEGRADATION FACTOR 1 (IDF1) [9,57], the auxin exporter PIN2 by either Wavy growth 3 (WAV3) [58] or RING domain Ligase 1/2 (RGLG1/2) [49,59] and the receptor kinases BRI1 and FLAGELLIN-SENSITIVE 2 (FLS2) by PUB12/13 [50,60]. As some cargoes are associated with multiple E3-ubiquitin ligases, it is likely that plants use different E3-ubiquitin ligases to control both anterograde and retrograde trafficking decisions. Plant pathogens have also evolved to use E3-ubiquitin ligases to evade plant immunity. An example of this is the bacterial effector AvrPtoB from *Pseudomonas syringae*, which acts as an E3-ubiquitin ligase to degrade FLS2, thereby attenuating flg22-mediated immune signaling [61]. How it is decided which E3-ubiquitin ligase is recruited towards cargo under which conditions is not clear and requires further investigation. In the case of IRT1, BRI1, BIK1 and

FLS2 it seems that phosphorylation of the cargo [9,56] or the E3-ubiquitin ligase [50,60] is a prerequisite to ubiquitination. With respect to ubiquitin-dependent degradation of transmembrane receptors, the relative importance of endocytic versus proteasomal degradation pathways, how the proteasome can control degradation via endocytosis or whether MG132 treatment affects membrane trafficking remains currently unclear.

Intriguingly, deubiquitinating enzymes (DUBs) can counteract ubiquitination of cargoes at different positions of the endosomal pathway. ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM 3 (AMSH3) is a well-described plant DUB, which works at endosomal compartments [62,63]. At the TGN/EE, AMSH3 might function in deubiquitinating cargoes, possibly leading to cargo recycling back to the PM [62,63]. Similarly, UBIQUITIN-SPECIFIC PROTEASE (UBP) 12 and UBP13 stabilize BRI1 at the PM by removing K63-linked ubiquitin chains [64] and stabilize the Root Meristem Growth Factor receptor (RGF1) to maintain root cell sensitivity to its ligand [65]. However, whether these UBP proteins work at the PM and/or at endosomal compartments is not clear as GFP-fusions localize these UBPs in the cytoplasm and the nucleus [66]. Recently, DUB activity has been reported to take place at the PM. OVARIAN TUMOR PROTEASE (OTU) 11 and OTU12 have been shown to tune down the internalization of the auxin efflux carrier PIN2. Their DUB activity is stimulated by the presence of anionic lipids, which likely causes a conformational change to increase the accessibility of ubiquitin to the catalytic site [67]. The above result indicates that ubiquitination is counteracted at the PM. The co-occurrence of both ubiquitin ligases and DUBs points to a very complex regulation of the ubiquitination code of cargo proteins to finetune cargo internalization depending on the input of various signaling pathways. DUBs could remove ubiquitination signals from previous endosomal trafficking decisions such as secretion [58] or recycling [7,9]. Alternatively, the PM located DUBs could serve to correct promiscuous ubiquitination performed by the ubiquitin ligases or to limit chain length. For example, the BOTRYTIS-INDUCED KINASE 1 (BIK1), strictly undergoes mono-ubiquitination performed by the E3 ubiquitin ligase RING-H2 FINGER A3A [56]. Longer chains might therefore potentially be restricted by DUB activity.

c. Endocytic machinery that recognizes ubiquitin

Ubiquitin is an important internalization signal in plants, which is evident by the growing number of endocytic machinery that is discovered to recognize this modification. TOM1-LIKE (TOL) proteins and SH3 domain-containing proteins bind ubiquitin [34,68–70]. Both TOL2 and TOL6 bind mono-ubiquitin as well as K48- and K63-linked poly-ubiquitin with a preference for K63-linked poly-ubiquitin [68,70]. While the SH3 DOMAIN-CONTAINING PROTEIN 2 (SH3P2) has not been tested for mono-ubiquitin binding, it has a clear preference for K63-linked ubiquitin over K48-linked ubiquitin [69]. Lastly, the SH3 domain of the TPC subunit TPLATE-ASSOCIATED SH3 DOMAIN CONTAINING PROTEIN (TASH3) has a clear preference for poly- over mono-ubiquitin, but does not seem to differentiate between the different types of linkages [34].

While it is clear that ubiquitin-binding proteins can have preferences for ubiquitin configurations, there seems to be an apparent redundancy in ubiquitin detection, which raises the question of how exactly the specificity of the ubiquitin-interacting proteins during ubiquitinated cargo recognition is established? The first, most obvious potential reason is that ubiquitin recognition by endocytic machinery is an essential process that benefits from redundancy. In addition, there may also be temporal necessities for ubiquitin recognition during CME. This is evident in the case of TASH3 and SH3P2, where TASH3 is present from the start until the end of clathrin-coated pit formation [34], whereas SH3P2 apparently only arrives at the very end of the process [71]. The relative recruitment dynamics of TOLs versus the other endocytic players

have not yet been observed to our knowledge, thus it would be interesting to situate the temporal position of TOL, TASH3 and SH3P2 in ubiquitinated cargo recognition during CME.

How do the different ubiquitin binding proteins work together during cargo recognition? How do they differentiate between the different ubiquitin configurations? How exactly do they regulate the different ubiquitin-mediated endocytic routes? All these questions will require further research. It is also likely that there are still many unidentified ubiquitin-binding proteins. Among the possible candidates are the TWD40-1 and TWD40-2, subunits of TPC, as both have WD40 β-propeller domains [26,27], which can bind ubiquitin in yeast [72].

4. Recognition by monomeric adaptors

Besides adaptor complexes, there is also a variety of monomeric adaptors that can recognize a sub-selection of PM cargoes. In mammals, PHOSPHATIDYLINOSITOL BINDING CLATHRIN ASSEMBLY PROTEIN (PICALM) and ARF-GAP DOMAIN AND FG REPEAT-CONTAINING PROTEIN 1 (AGFG1) are necessary for SNARE recycling [73–75], while EPS15 and EPSIN recognize ubiquitinated cargo through their ubiquitin-interacting motifs [43,76]. Plants EPSINs appear not to possess these ubiquitin-interacting motifs and are therefore unlikely to bind ubiquitinated cargo directly [77]. In plants, only a few monomeric PM-localized adaptors are functionally characterized so far, all of which belonging to the PICALM family of ANTH-domain containing proteins. PICALM5a and PICALM5b serve as adaptors for ANXUR kinase tip-localization in pollen tube elongation [28]. Similarly, PICALM9b functions in CME of ROP1 ENHANCER 4 (REN4) in the pollen tube [29]. Lastly, PICALM1a and PICALM1b were shown to facilitate internalization of the R-SNARE VESICLE-ASSOCIATED MEMBRANE PROTEIN 721 (VAMP721) [14]. The interdependency of VAMP721 and its adaptor seems very strict as VAMP721 internalization is completely arrested in the *picalm1a/picalm1b*. But what happens to cargo whose internalization is inhibited? It appears unlikely that cargo can be deposited continuously at the PM, as this would lead to overcrowding. Therefore, there must be other mechanisms responsible for clearing the PM of unwanted proteins. It will be interesting to answer this question and to see further research into monomeric adaptors in plants, to identify which cargoes they internalize, how they recognize them and how they function in concert with the multimeric adaptor complexes.

5. Conclusion

For a long time, cargo recognition in plants was thought to be mainly facilitated by SLiMs recognized by AP-2, similar to the other mammalian model systems. However, cargo recognition in plants has proven to be more complex than initially thought. Reversible PTMs such as phosphorylation and ubiquitination have throughout time achieved a far more prominent role in cargo internalization. Ubiquitin provides various means of regulation as the action of ubiquitin ligases and DUBs at the PM likely result in a specific code of mono- and poly-ubiquitination prior to internalization and will contribute to a highly tunable and reversible process. Adding to this complexity, different endocytic proteins recognize ubiquitin. Although we lack evidence, these proteins will likely be able to differentiate between the type of ubiquitin linkage, which might control the internalization and maybe even the fate of the cargo proteins.

Overall, cargo internalization is orchestrated by a complex mixture of different signals leading to parallel endocytic recognition, offering flexibility and in many cases ensuring that the cargo can be internalized in response to different stimuli. In light of this, it would be interesting to see if BRI1 internalization can be effectively blocked by mutating the functional tyrosine motif in $BRI1^{Q}$. Next to specific recognition, the

multitude of signals could also simply be required to stabilize the initial contacts between certain cargoes and the internalization machinery. Future work will no doubt identify novel cargo internalization signals and their interplay as well as additional components of the endocytic machinery that orchestrate cargo internalization in plants.

Declaration of interest: none

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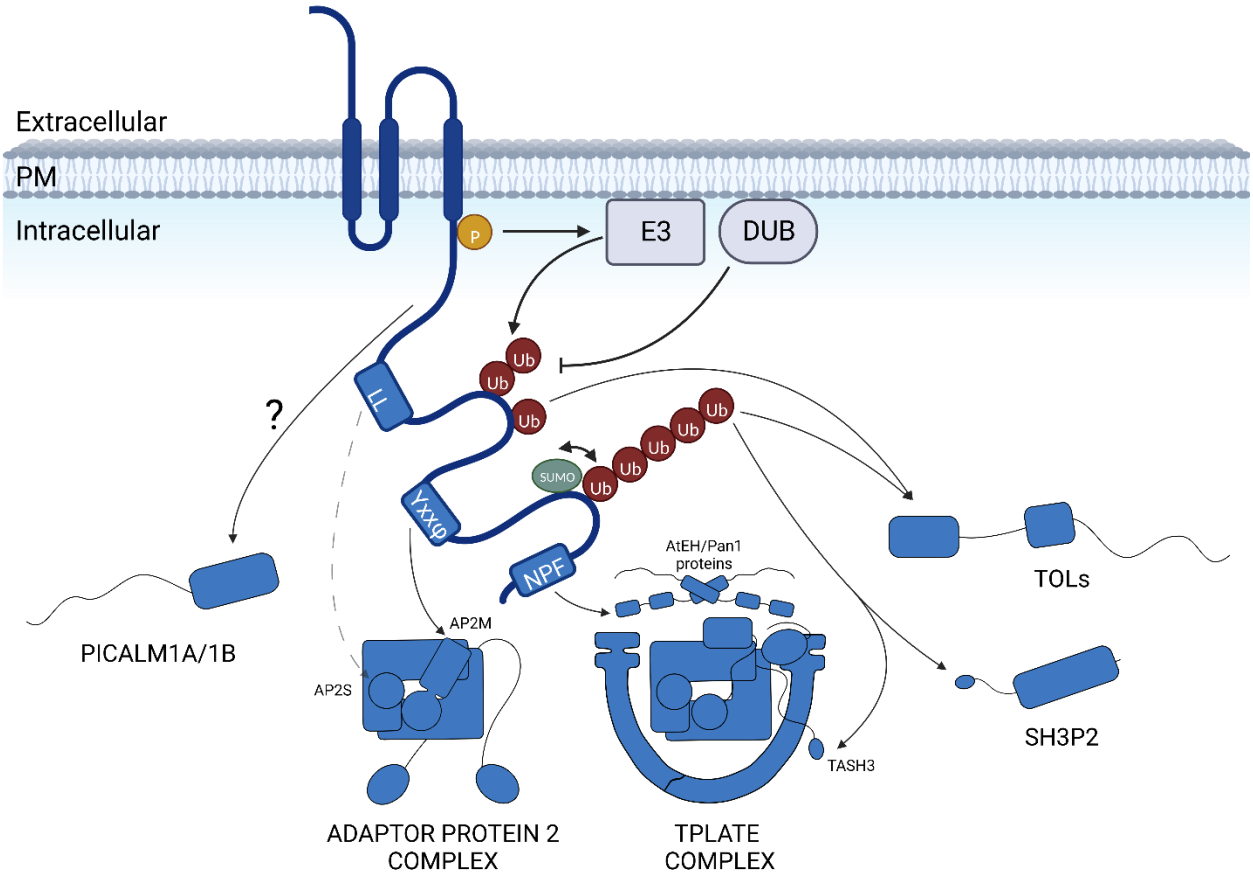

Figure1: Overview of the currently known endocytic internalization signals in plants. Short linear motifs in the cytoplasmic tails of cargo proteins present a first group of signals. Dileucine (LL) motifs are presumed to be recognized by the small subunit of the AP-2 complex (AP2S), tyrosine (YxxΦ) motifs are recognized by the medium subunit of Adaptor protein complex 2 (AP2M) and NPF motifs bind to the first EH domain of the AtEH1/Pan1 subunit of the TPLATE complex (TPC). Post-translational modifications such as phosphorylation and ubiquitination represent a second group of internalization signals. Whether or not phosphorylation can act as a signal on its own or whether it functions mainly as a prerequisite for ubiquitination is not clear. Endocytosis-linked ubiquitination ranges from mono- to various lengths of K63-linked ubiquitin. E3-Ubiquitin ligases (E3) and deubiquitinating enzymes (DUB) control the chain length. TOM1-LIKE (TOL) proteins recognize mono-ubiquitin and K63-linked poly-ubiquitin, whereas the SH3 domains of TPLATE-ASSOCIATED SH3 DOMAIN CONTAINING PROTEIN (TASH3) and SH3 domain-containing protein2 (SH3P2) are so far only connected to poly-ubiquitin. The PTM SUMOylation potentially negatively affects cargo recognition by competing with ubiquitination. Lastly, monomeric adaptors (e.g. PICALM) also recognize specific cargo through various yet unknown mechanisms. Arrows indicate connections. Question marks and the dashed arrow indicate currently limited knowledge. Structured domains of the proteins are schematically represented in blue shapes, whereas unstructured parts are indicated by a black line. The proteins are not drawn to scale. Image created with BioRender.com.