Targeting plant UBX proteins: AI-enhanced lessons from distant cousins

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Across all eukaryotic kingdoms, ubiquitin regulatory X (UBX) domain-containing adaptor proteins control the segregase cell division control protein 48 (CDC48), and thereby also control cellular proteostasis and adaptation. The structures and biological roles of UBX proteins in animals and fungi have garnered considerable attention. However, their counterparts in plants remain markedly understudied. Since 2021, the artificial intelligence (AI)-based algorithm AlphaFold has provided predictions of protein structural features that can be highly accurate. Predictions of the proteomes of all major model organisms are now freely accessible to the entire research community through user-friendly web interfaces. We propose that the combination of cross-kingdom comparison with AF analysis produces a wealth of testable hypotheses to inspire and guide experimental research on plant UBX domain-containing (PUX) proteins.

CDC48 segregase and UBX adaptors

By extracting and unfolding proteins from various locations in the cell, CDC48 assures a balanced proteome (see Glossary) and serves as a fast-response mechanism to changing conditions [1]. CDC48 is essential for all eukaryotes, and CDC48A is the major isoform in Arabidopsis thaliana (Arabidopsis). CDC48 is also called p97 or valosin-containing protein (VCP) in mammals; we use CDC48/p97 to refer to all orthologs. CDC48/p97 is a hexameric AAA-ATPase. Its sequence and function as a segregase and unfoldase are highly conserved across the plant, fungal, and animal kingdoms. The combination of CDC48/p97 with specific adaptor proteins allows it to participate in diverse biological functions, such as promoting cancer cell growth in humans or reshaping lipid droplets in tobacco pollen tubes [2,3]. In addition, post-translational modifications can also contribute to the regulation of CDC48/p97 [4].

The UBX domain-containing proteins constitute the largest and most heterogeneous group of adaptor proteins for CDC48/p97 [5]. UBX proteins have a modular architecture where the combination of building blocks (i.e., domains and motifs) can produce various functions. Remarkably, despite almost a billion years of distinct evolution, UBX proteins across all eukaryotic kingdoms still use the same building blocks and combinations thereof (Box 1 and Figure 1). These building blocks are connected through extended and poorly conserved regions that have a high probability of being intrinsically disordered. Unfortunately, the resulting dynamic ‘beads-on-a-string’ architecture makes it challenging to experimentally determine how the 3D structural features of full-length UBX proteins synergize to control the catalytic activity and ligand engagement of CDC49/p97. However, the AI-driven structure prediction algorithm AlphaFold may now help to close this knowledge gap.

AI-assisted grouping

For most protein sequences, AF can predict the structure of folded domains and the presence of disordered regions with high accuracy. Moreover, the AlphaFold’s-predicted aligned error

Highlights

Ubiquitin regulatory X (UBX) domain-containing adaptor proteins guide and control the AAA-ATPase cell division control protein 48 (CDC48) to mediate an astonishing variety of fast-response mechanisms.

Recent structural and functional studies have clarified the biological roles of many UBX proteins from animals and fungi, but most plant UBX domain-containing (PUX) proteins remain poorly understood.

Despite their evolutionary distance, many domains and their combinations are conserved across eukaryotic UBX proteins, suggesting that they form functionally coherent ‘work packages’.

The artificial intelligence (AI)-based program AlphaFold is now able to accurately predict protein 3D structures and connections between structural units.

The combination of a cross-kingdom comparison and AlphaFold analysis reveals conserved motifs and higher-order features for PUX proteins that collectively provide testable hypotheses to efficiently guide future experimental research.

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(PAE) plots can reveal contacts between residues distant in the primary sequence [6] (Box 2). Therefore, AlphaFold can be used to identify connections between protein building blocks and reveal secondary structure elements located in disordered protein regions. If these connections and structural elements are conserved across orthologs, they may indicate functionally coherent modules and novel interaction motifs, respectively. The AlphaFold team already made predictions for the proteomes of most model organisms, including Arabidopsis, rice, yeast, and human, and ultimately aims to cover all sequences in the UniProt database [7]. These predictions are freely available at uniprot.org and alphafold.ebi.ac.uk where interactive web interfaces allow their analysis without requiring in-depth knowledge of structural biology.

We propose that grouping UBX proteins across kingdoms according to their domain composition and AlphaFold features will facilitate information transfer between orthologs, and can yield novel testable hypotheses for the functions of less well-studied UBX proteins. We illustrate this approach for the PUX proteins which are poorly understood compared with their animal counterparts. For each group, we briefly state what is known for individual members, implying that this knowledge may be transposed to other members. We also explain AlphaFold features that are conserved in each group, and propose functional hypotheses resulting from our approach. Owing to the modularity of UBX domains, grouping according to features different from those we use is possible and might provide additional insights.

**Group I: UBX-only ubiquitin-independent CDC48/p97 tethering factors**

We assigned proteins that only contain a UBX domain to group I (Figure 1). HsUBXN10 from this group localizes p97 to the cilia by binding to the intraflagellar transport B complex, which controls the anterograde traffic into cilia [8]. The HsUBXN10 UBX domain binds to p97, suggesting that the cilia-targeting motifs are located in the flexible N-terminal part of HsUBXN10. Although the AtPUX12 N-terminal has no sequence similarity to HsUBXN10, AtPUX12 may functionally copy HsUBXN10 in using this region to tether CDC48 to specific protein complexes in a ubiquitin-independent manner.

The AlphaFold 3D structural models and PAE plots suggest that a poly-proline type II (PPII) helix is located immediately upstream of the UBX domains of HsUBXN10 and AtPUX12. This helix binds back to the UBX domain through a proline-rich hxFPxLxLEEP motif (where h is a hydrophobic residue and x is any residue), which we named the PPII-EP flap (Box 3). Structurally similar PPII-EP flaps with an Lx[1,2]EP core motif are predicted to cover the UBX domains of several other plant and animal UBX proteins (Box 3 and later), suggesting functional relevance. The PPII-EP flap is away from the UBX region that binds to CDC48/p97-N and may provide structural support or regulate additional binding events.

**Group II: inhibition of CDC48/p97 through hexamer dissociation**

The hallmark of group II proteins is a long N-terminal UBX domain extension that binds back to this domain to form a helical lasso or lariat (abbreviated HL in Figure 1). When the UBX domain of HsASPL and AtPUX1 binds to CDC48/p97-N, the lariat latches around the CDC48/p97 monomer, thereby breaking up the catalytically required hexamer [9–11]. AlphaFold also predicts a lariat for ScUbx4, suggesting that all group II members inactivate CDC48/p97 through forced monomerization.

**Group III: PUB-based associations with CDC48/p97**

According to AlphaFold, several PUB domain-containing UBX proteins also harbor a UBX lariat. We assigned these proteins to group III. Their UBX lariat is very similar in length and amino acid composition to group II proteins, suggesting a CDC48/p97-disrupting function. However, HsUBXN6 from group III binds to p97 hexamers without dissociating them [12–14]. The inability
to disrupt p97 hexamers may be because the UBX domain of HsUBXN6 lacks a proline-based signature motif in its S3–S4 loop, which precludes it from binding to p97-N [15]. Instead, HsUBXN6 uses its PUB domain to recognize the C-terminal PUB-interacting motif (PIM) of p97, and it is the HsUBXN6 VCP/p97-interacting motif (VIM) that binds to p97-N (Box 1). AtPUX2 also has an atypical S3–S4 loop in its UBX domain, suggesting that it cannot bind to AtCDC48A-N [16], and AtCDC48A contains a PIM as a potential interaction partner for AtPUX2 PUB [16]. However, instead of a VIM, AtPUX2 contains a putative CDC48A-N-binding SHP box in its N-terminal. Therefore, we hypothesize that all PUB domain-containing UBX proteins from group III form an alternative bidentate association with CDC48/p97, thereby freeing the modified UBX domain for other targets for which their UBX lariat may be required.

Group IV: SEP-containing substrate-degrading adaptors
We assigned the SEP domain-containing AtPUX3, 4, 5, and 6 to group IV with three different subgroups. The human orthologs in group IVa (Hsp37) and group IVb (Hsp47) bind to CDC48/
Figure 1. Domain architecture and proposed classification of ubiquitin regulatory X (UBX) domain-containing proteins. Folded protein domains are shown boxed with domain names. The first two letters of the protein name indicate the species: At, Arabidopsis thaliana; Ce, Caenorhabditis elegans; Hs, Homo sapiens, Sc, Saccharomyces cerevisiae. Alternative protein names are as follows: UBXN10 (UBXD3), ASPL (UBXN9/UBXD9), Ubx4 (Cui1p), UBXN1 (UBX2), p37 (UBXN2B), p47 (UBXN2C), Ubx1 (Shp1), UBXN7 (UBXD7), Ubx8 (Cui2p), Erasin (UBXN4/UBXD2), Ubx7 (Cui3p), and FAF2 (UBXN3B/UBXD8); aa, amino acids. The scale bar corresponds to the length of 50 residues. The full names and functions of domains and motifs are given in Boxes 1 and 3. Putative domains or motifs previously predicted but not confirmed experimentally are indicated by broken lines. The new elements resulting from this study are hashed in the figure and highlighted in the second inlay (see Box 3 for full description). The UBX domain of ScUbx2 has a 64-residue loop insertion.
Box 2. Guide to using predicted aligned error (PAE) plots

Even for scientists without in-depth knowledge of structural biology, the PAE plots produced by AF can suggest novel sequence motifs and reveal structural (and hence functional) connections between protein building blocks. In *Figure I*, we explain the use of PAE plots based on two examples, namely, PUX4 from group IV (left panels) and PUX10 from group VII (right panels).

**Studied domains/motifs:**
- A: SEP domain
- B: UBX domain
- C: Helix
- D: UBA domain
- E: UAS domain

**Novel-predicted motifs/module:**
- F: GG-SG hairpin
- G: SHP box-containing hairpin
- H: Helix-SHP-SEP module
- I: CC region
- J: CC-UBX module
- K: N-located helix of UAS
- L: Helix-UAS module

*Figure I.* Predicted aligned error (PAE) plots (top panels) and their relation to the 3D structure of the protein (bottom panel). For each position (x, y), the PAE plots estimate the expected position error at residue x when the predicted and true structures are aligned on residue y. Due to this alignment, the PAE plots are not perfectly symmetric about the main diagonal. The degree of confidence is expressed in shades of green, where dark green means that AlphaFold is certain about the relative position of two residues (i.e., the positional error is small) and light green means that AF cannot reliably estimate the distance between two residues. When AF predicts the 3D structure of a domain with high confidence (as expressed by a per-residue confidence score, predicted local distance difference test (pLDDT), of >70), then AF is also certain about the relative position of the amino acids that form this domain. Consequently, well-structured domains correspond to dark green squares situated on the diagonal of the PAE plot. The position and extent of these squares correspond to the amino acids that form the domain. In the PAE plots, we have highlighted the SEP, ubiquitin regulatory X (UBX), UBA, and UAS domains (labeled A, B, D, and E, respectively) present in AfPUX4 (left) or AfPUX10 (right). Secondary structural elements that are located outside of domains can also be identified, and may indicate protein motifs with a specific function, such as ligand binding. Isolated helices have a narrow dark green signature shape on the diagonal of the PAE plot (elements C, I, and K), and hairpins (F and G) produce a cross shape on the diagonal. If dark green off-diagonal patches link structural elements that are distant in sequence, then these elements are predicted to contact each other, and possibly synergize to form a structural unit with a joint biological function (H, J, and L). PAE segments of interest can easily be mapped onto the protein sequence and protein structure using the display options provided by alphafold.ebi.ac.uk. This website also offers a PAE tutorial. (Bottom panel) AF-predicted 3D protein structures, colored according to the elements annotated in the PAE plot. Note that 3D structures, pLDDT, and PAE are predictions and should only be taken as testable hypotheses, not as ground truth.

p97 via their C-terminal SHP box and UBX domain [17], suggesting canonical CDC48/p97 associations also for the other members of these subgroups. The biological functions of SEP domains remain incompletely understood [16].
Box 3. Novel motifs and modules predicted by AF

Applying comparative AF analysis to PUX proteins suggests conserved sequence motifs of currently unknown functions as shown in Table I. Our AF analysis also suggests structural units formed by several joint building blocks and/or motifs, as shown in Table II. Figure I shows the structure and sequence of these motifs and the predicted conformation of the joint hairpin–helix–SHP–SEP module.

Table I. Novel AF-predicted motifs

| Name         | Motif   | Features                                      | Occurrences |
|--------------|---------|-----------------------------------------------|-------------|
| PPII-EP flap | Lx(1,2)EP | PPII helix covering the UBX N terminus       | AtPUX7–9, 12–16; HsUBXN10; ScUbx5 |
| GG-SG hairpin| GGx(2,3)SG | Upstream of the helix–SHP–SEP module         | AtPUX3–5; Hsp47; ScUbx1 |
| FGG helix    | FGG     | Acidic amphipathic helix                      | AtPUX8, 9, 13 |

Table II. AF-derived functional modules

| Composition            | Possible functions                  | Occurrences |
|------------------------|------------------------------------|-------------|
| GG-SG hairpin–helix–SHP–SEP | Ubiquitin-independent binding        | AtPUX3–5; Hsp47 |
| UAS–CC–UBX              | Self-association                    | AtPUX10; Hs/CeErasin; HsFAF2; ScUbx2, 7 |

Figure I. 3D structures and sequence alignments of novel motifs and of a joint structural module found in plant ubiquitin regulatory X (UBX) domain-containing (PUX) proteins. (Left) The AlphaFold-predicted 3D structures were downloaded from alphafold.ebi.ac.uk and visualized using PyMOL (pymol.org). The side chains of key residues are shown and labeled with their one-letter code. (A) The UBX domain of PUX12. Green, UBX domain; red, PPII-EP flap region. (B) Superimposition of the GG-SG hairpin–helix–SHP–SEP modules from AtPUX3, 4, and 5. Cyan, GG-SG hairpin; red, helix; orange, SHP box; blue, SEP domain. The structure to the right focuses on the GG-SG hairpin, and superimposes this element from AtPUX3 (dark blue), AtPUX4 (cyan), and AtPUX5 (light blue). (C) Superimposition of the FGG helix from AtPUX8 (red), AtPUX9 (green), and AtPUX13 (blue). (Right panels) Sequence alignments corresponding to the novel motifs. The alignments were produced by T-Coffee [36], and displayed by ESPript 3.0 [37].
Groups IVa and IVb contain both a short helix and a predicted second SHP motif upstream of their SEP domain [16,18]. For both subgroups, AlphaFold suggests that this helix folds back onto the SEP domain and that the helix–SHP–SEP fragment in these orthologs is, in fact, a single structural unit (Boxes 2 and 3). However, functional differences may exist between members of groups IVa and IVb because only the latter harbor a UBA domain. In Hsp47, the UBA domain binds to ubiquitinated substrates in a ‘degradation’ manner [19], and the Hsp47 SEP–UBX module dimerizes in the absence of CDC48/p97 [20], suggestive of autoregulatory functions. Conversely, group IVa lacks a ubiquitin-binding domain. Instead, the helix–SHP–SEP fragment of Hsp37 serves as a ligand-binding module that allows the ubiquitin-independent degradation of substrates by p97 [17,18].

All group IVa and IVb members, except for Hsp37, also have a predicted β-hairpin element immediately upstream of the helix–SHP–SEP unit. This β-hairpin has conserved features, namely, a GGx(2,3)SG motif, a charged tip, and hydrophobic top and bottom surfaces, suggesting that it may have a conserved function, presumably ligand binding. We refer to this element as the GG-SG hairpin (Box 3). The roles of the AtPUX6 tandem SEP domains, which lack upstream elements, remain to be determined, prompting us to assign it to group IVc. AlphaFold suggests that the tandem SEP domains form a joint structural unit. However, this AlphaFold prediction is of low confidence [predicted local distance difference test (pLDDT) <70].

**Group V: linking to autophagy with UBA and UIM**

This group assembles proteins with one or two UIM sequences. For AtPUX7, 8, 9, 13, and ScUbx5, it was shown that the UIM sequences target these proteins to autophagosomes, where they mediate the clearance of nonfunctional CDC48A/Cdc48 [21]. The UBX domains of HsUBXN7 and AtPUX7 bind to CDC48/p97 [22,23], suggesting that members of this group have canonical CDC48/p97-N binding UBX domains. The mechanism through which specifically inactive CDC48/p97 is recognized remains elusive, but the presence of a UBA domain supports the involvement of ubiquitination.

AlphaFold suggests that group Va PUX proteins contain an additional acidic amphipathic helix that terminates in the sequence motif FGG (residues 353–370, 326–343, and 323–340 in PUX8, 9, and 13, respectively) (Box 3). This ‘FGG’ helix is situated between the most C-terminal UIM motif and the UBX domain. A BLAST search identifies the FGG helix in group Va orthologs from other plants, but not in other AtPUX paralogs, suggesting that it is specific for the function of group Va members. Conversely, group Vb proteins contain a UAS domain of unknown biological function.

AtPUX7, 8, 9, 13, and ScUbx5 also contain a predicted PPII-EP flap, which emerges from a long helix in AtPUX8 and 13. The sequences of these helices conform with a coiled-coil (CC) pattern, suggesting they mediate self-association. HsUBXN7 also contains a motif that is structurally reminiscent of the PPII-EP flap, but where the EP motif is replaced by a DV sequence (residues 407–408, corresponding to residues D38 and V39 in PDB id 1WJ4).

**Group VI: plant-specific UBA–UAS–UBX proteins with unknown function**

We grouped AtPUX14, 15, and 16 which contain UBA, UAS, and UBX domains in group VI. None of them has been functionally characterized, and we failed to identify orthologs with the same domain composition in fungi or animals. AlphaFold predicts that the UBA, UAS, and UBX domains are flexibly linked and that all UBX domains contain a PPII-EP flap. Based on these features, we speculate that PUX14, 15, and 16 are involved in plant-specific ubiquitin-independent protein degradation.
Group VII: UAS and membrane anchor to extract lipid-bound substrates

UBX proteins with a UAS domain and a membrane-anchoring hydrophobic patch (HP) form our group VII. Subgroups VIIa and VIIb contain an N-terminal UAS domain, and their HP is positioned in a long C-terminal extension. The HP localizes HsErasin (group VIIb) to the endoplasmic reticulum (ER), for its function in ER-associated degradation (ERAD) [24]. Group VIIc contains an additional N-terminal UBA domain. The HP of this group is situated between the UBA and UAS domains, and serves as an anchor for lipid droplets (case of AtPUX10 and HsFAF2) [28] and/or the ER/Golgi (HsFAF2, ScUbx2) [26].

The UBX domains of ScUbx6 (group VIIa), ScUbx7, and HsErasin (both group VIIb) bind to CDC48/p97-N [24,27]. Two group VII UAS domains have been investigated previously, but their function remains incompletely understood. The UAS domain of HsFAF2 (group VIIc) may sense fatty acids [28], whereas the HsErasin UAS domain is located within the amino acid region that binds to the UBA-containing ubiquitin–proteasome shuttle protein ubiquilin, inferring that the UAS domain helps HsErasin to recruit ubiquitinated substrates indirectly via the UBA domain of ubiquilin [29]. Hence, group VIIc proteins are expected to use their UBA domain for substrate recognition, whereas group VIIa/b proteins may use their UAS domain to recruit ubiquitinylated substrates indirectly.

AlphaFold predicts that group VIIa members feature a prominent helical extension of the C-terminal UAS helix, whereas group VIIb members contain a CC upstream of their UBX domain. These structural elements are expected to have functional repercussions and may, for example, explain certain differences in the way that ScUbx6 and ScUbx7 affect yeast sporulation [27]. For group VIIc, AlphaFold suggests that the UAS helical extension is fused with the CC, linking the UAS and UBX domains into one structural element (Box 2). According to AlphaFold, the second β-strand of the group VIIc UAS associates with an N-terminally located helix, possibly constituting an autoregulatory element (E, K, and L in Box 2).

Concluding remarks and future perspectives

Unraveling how UBX proteins control the CDC48/p97 segregase is important for understanding how plants, fungi, and animals rapidly adapt to changes and maintain a healthy proteome. We propose here that UBX proteins can be grouped based on their building block combinations and AlphaFold features, which jointly constitute conserved ‘work packages’. By providing a framework for transposing knowledge across eukaryotic lineages, such a classification may accelerate future experimental research on UBX proteins to explore domain function, autoregulation, or higher-order assemblies.

We applied this approach to investigate the PUX protein family, for which experimental information remains sparse. We identified several novel features (summarized in Box 3) whose putative biological roles warrant experimental investigations (see Outstanding questions). This approach also highlights areas where filling knowledge gaps would be particularly beneficial for enhancing our understanding of UBX proteins in general (e.g., what is the function of the UAS domain and of the group III UBX lariat?) or of PUX proteins in particular (e.g., what is the function of group VI members?) (see Outstanding questions).

Moreover, grouping UBX proteins across kingdoms reveals assembly rules that may reflect functionally coherent operations. For example, (i) UBX proteins can include either PUB, SEP, or UAS domains, but not a combination of these domains; (ii) the two linear interaction motifs UIM and SHP do not occur in the same protein and are either found alone or in combination with either the SEP (in the case of SHP) or the UBA (in the case of UIM) domain; and (iii) UBX proteins with an HP also contain a UAS domain.

Outstanding questions

The ultimate goal is to understand how the different features of PUX proteins synergistically control the biological functions of CDC48A, and hence contribute to plant adaptation and homeostasis. We propose that the following questions are the most important towards this goal:

What are the biological functions of the newly identified conserved structural features (PPI-EP flap, GG-SG β-hairpin, the FGG helix, and the putative CC regions)?

How do the newly identified multicomponent modules (β-hairpin–helix–SHP–SEP and UAS–CC–UBX) synergize to perform biological tasks?

What is the role of the UBX domain lariat from group III? Why is it present even though the UBX domains of this group neither bind to CDC48/p97 nor disrupt its hexamers?

What are the ligands and biological roles of the UAS domain? This domain is found in several PUX proteins and orthologs from other kingdoms, but its function remains unclear.

What is the biological role of group VI PUX proteins (i.e., AtPUX14, 15, or 16)? Despite their relatively simple UBA–UAS–UBX architecture, they do not have orthologs in other kingdoms, and their function in plants is unknown.

How are PUX proteins regulated, for example, through homo- or heteromeric interactions, post-translational modifications, or controlled expression and degradation?

Are PUX proteins drivers of LLPS? Indirect functional and structural evidence hints at the role of PUX proteins in biomolecular condensates. However, direct involvement in LLPS has not yet been demonstrated.
Experimentally clarifying the biological significance of features identified herein will help in exploring the mechanisms underlying the (auto)regulation of PUX proteins, including post-translational modifications and the formation of homo- or heteromultimers. AlphaFold predicts that PUX proteins comprise multiple interaction modules connected through long, flexible linkers; this is a typical characteristic of proteins involved in biomolecular condensates through **liquid–liquid phase separation (LLPS)**. PUX proteins have not yet been linked directly to LLPS; however, LLPS is involved in the autophagic clearance of nonfunctional CDC48 by nitrogen starvation in plants [30], and animal orthologs of the UBA domain can produce LLPS in p97-containing nuclear foci [31]. An enticing possibility is thus that PUX proteins promote the formation of biomolecular condensates. Beyond the applications discussed herein, AlphaFold may also help in designing stable protein fragments and provide structural templates for in silico ligand identification (however, current AlphaFold models do not contain ligands or cofactors). AlphaFold models and scores are, of course, only predictions and should be used to inspire experiments, not to replace them.

In conclusion, we propose that the combination of AlphaFold features and a cross-kingdom comparison provides a wealth of testable hypotheses to efficiently guide future experimental research. For PUX proteins, the information obtained can help to reveal the mechanisms that these proteins use to control CDC48A. The resulting enhanced understanding of the role of the UBX proteins in eukaryotic homeostasis and adaptation may have broader implications in various fields ranging from human health to food security.

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**Declaration of interests**

None are declared.

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