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Ubiquitin and its binding domains

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1. ABSTRACT

Post-translational modification by ubiquitin (ubiquitination, ubiquitylation, ubiquitinylation) is used as a robust signaling mechanism in a variety of processes that are essential for cell homeostasis. Its signaling specificity is conferred by the inherent dynamics of ubiquitin, the multivalency of ubiquitin chains, and its subcellular context, often defined by ubiquitin receptors and the substrate. Greater than 150 ubiquitin receptors have been found and their ubiquitin-binding domains (UBDs) are structurally diverse and include alpha-helical motifs, zinc fingers (ZnF), pleckstrin-homology (PH) domains, ubiquitin conjugating (Ubc)-related structures and src homology 3 (SH3) domains. New UBD structural motifs continue to be identified expanding the ubiquitin-signaling map to proteins and structural families not previously associated with ubiquitin trafficking. In this manuscript, we highlight several ubiquitin receptors from the multiple UBD folds with a focus on the structural characteristics of their interaction with ubiquitin.

2. INTRODUCTION: UBIQUITIN AND PROTEIN UBIQUITINATION

The 76-amino acid protein ubiquitin is attached covalently to other proteins to in turn, modify or expand their cellular activities. The most common sites of ubiquitin attachment are Lys epsilon-amino groups and the substrate's amino terminus, as reviewed in (1). In rare cases, ubiquitin can also be attached to Ser hydroxyl and Cys thiol groups (2, 3). Ubiquitination is tightly regulated and generally requires a 3-step catalytic cycle that includes E1 activating, E2 conjugating, and E3 ligating enzymes (Figure 1A). The human genome has been demonstrated to encode two E1 (Uba1 and Uba6), ~40 E2, and greater than 600 E3 enzymes, as reviewed in (4). E1 enzymes activate ubiquitin with a two-step ATP-dependent reaction to form an E1-thiolester~ubiquitin intermediate (5), from which ubiquitin is transferred to an E2 catalytic Cys by a thioester transfer reaction, as reviewed in (6, 7). The transfer of activated ubiquitin to a substrate generally requires an E3 ligase. Depending on the E2-E3 pair, ubiquitin is either

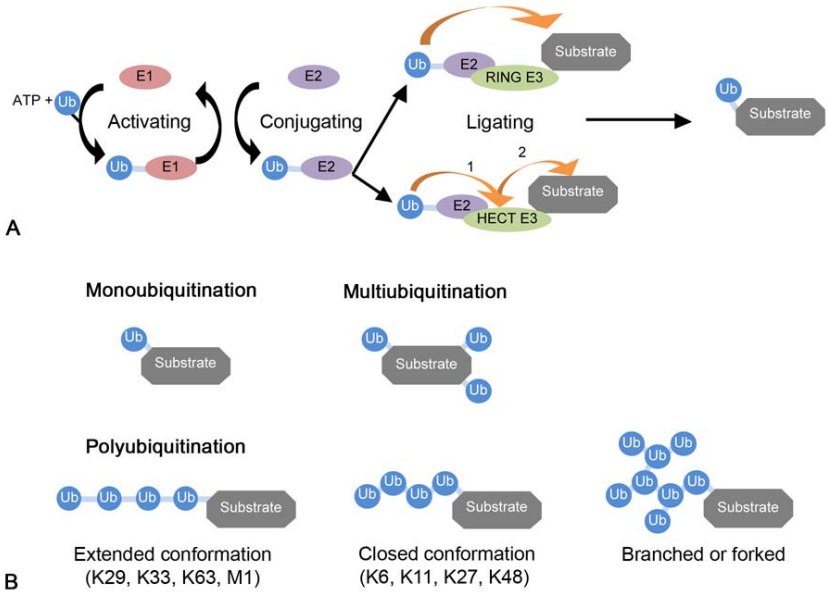

Figure 1. Ubiquitination: (A) E1-E2-E3 enzymatic cascade leading to substrate ubiquitination, (B) Diversity of ubiquitin modification.

transferred to the target substrate directly from the E2 or after passage to an E3 catalytic Cys. The former mechanism is performed by RING (Really Interesting New Gene) domain E3s, which are the most populated class of E3. These act as scaffolds by orienting E2s for ubiquitin transfer to a substrate, as reviewed in (4). HECT (Homologous to E6AP C-Terminus) domain E3s, by contrast, form a thioester bond with ubiquitin en route to its substrate transfer, as reviewed in (8). Recently, the mechanistic distinction between RING and HECT E3s has become blurred as two E3s that belong to the RING-in-between-RING (RBR) family were proposed to act as RING/HECT hybrids by forming a thiolester~ubiquitin intermediate via a conserved RING domain cysteine prior to ubiquitin transfer to substrate (9).

A single passage through an E1-E2-E3 enzymatic cascade results in a monoubiquitinated substrate. Additional passages yield multiubiquitinated substrates, with ubiquitin moieties at multiple sites, and/or polyubiquitinated substrates, with additional ubiquitins ligated to previously attached ones (Figure 1B). Ubiquitin moieties are linked by an isopeptide bond between a Gly76 carboxyl group and either a Lys sidechain primary amino group (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, or Lys63) or the ubiquitin amino terminus (Met1) (10-12). Quantitative mass spectrometry (MS) experiments in Saccharomyces cerevisiae (13) and mammalian cells (HEK293) (14) revealed differences in polyubiquitin linkage abundance. Although Lys48 and Lys63 linked chains are abundant in both cell types, their population is greater in HEK293 cells, as S. cerevisiae contained more Lys6 and Lys11 linkages (Table 1). It is possible that these differences are of physiological origin, but they may also be due to the use of different experimental procedures. The S. cerevisiae study captured His-tagged ubiquitin conjugates for MS analyses, whereas isotope enriched media was used to grow the HEK293 cells with total cell lysate used for subsequent MS experiments (13, 14).

Ubiquitin's seven lysines can be used to synthesize homotypic chains of one linkage type or heterotypic chains with multiple linkage types, as reviewed in (15). Certain E2-E3 enzyme pairs have been shown *in vitro* to synthesize exclusively homotypic chains, as in the case of UbcH5-Nedd4, or heterotypic chains, as in the case of UbcH5-Mdm2 (16). UbcH5-Mdm2 has an added capacity of forming branched heterotypic chains (Figure 1B) *in vitro*. The physiological role and abundance of branched heterotypic chains is speculative, but they have been proposed to act as dominant negative inhibitors of the proteasome (16). Distinct functional roles have been associated with certain ubiquitin chain linkage types. Lys48-linked chains target their substrates for degradation by the 26S proteasome, as reviewed in (17), or lysosome, as reviewed in (18). Lys63 linked chains function in NF-kappaB signaling, DNA repair, and receptor endocytosis, as reviewed in (19, 20). Linear ubiquitin chains have also been implicated in the regulation of NF-kappaB activity, as reviewed in (21). Function specific to the other linkages has not yet been assigned, although some associations exist. Lys29-linked chains can target proteins for degradation by lysosome and are synthesized *in vitro* by the E3's AIP4 (Atrophin-1-Interacting Protein 4) and UBE3C/KIAA10, although both of these enzymes also synthesize Lys48-linked chains (22, 23). Heterotypic Lys29/Lys33-linked chains are found on ARK5/NUAK1 (AMPK Related Kinase 5) and MARK4 (Microtubule-Affinity-Regulating Kinase 4). These two substrates belong to the AMPK-related kinase family, which is involved in cell polarity and proliferation and blocking kinase activation by interfering with phosphorylation sites (24). Homotypic Lys11-linked

Table 1. Ubiquitin linkage abundance determined by quantitative mass spectrometry

HEK293 Mammalian Cells (14)	
Lys6	≤ 0.5%
Lys11	2%
Lys27	≤ 0.5%
Lys29	8%
Lys33	≤ 0.5%
Lys48	52%
Lys63	38%
***Saccharomyces cerevisiae* (13)**	
Lys6	10.9% ± 1.9%
Lys11	28.0% ± 1.4%
Lys27	9.0% ± 0.1%
Lys29	3.2% ± 0.1%
Lys33	3.5% ± 0.1%
Lys48	29.1% ± 1.9%
Lys63	16.3% ± 0.2%

chains are associated with many cellular processes, including ERAD (Endoplasmic Reticulum Associated Degradation), endocytosis, TNF (Tumor Necrosis Factor) signaling, WNT signaling, and cell cycle control, as reviewed in (25). Lys6- and Lys27-linkages are the least well characterized; however, Lys6 may regulate DNA repair (26) and Lys27 may function in stress response pathways (27).

3. UBIQUITIN: A PROTEIN MODIFIER

3.1. Monoubiquitin: placidity for multifaceted recognition

Ubiquitin adopts a compact globular fold that is formed by a 5-stranded beta-sheet, a short 3_{10} helix, and a 3.5-turn alpha-helix (Figure 2A). This fold is also the foundation of a larger family of ubiquitin-like (UBL) domains, which will not be discussed in this manuscript, but are reviewed in (28, 29). The majority of UBDs interact with a hydrophobic patch formed on the surface of ubiquitin's beta-sheet by Leu8, Ile44 and Val70 (Figure 2A). The amino acids surrounding this hydrophobic triad are disparate in terms of charge and size enabling diverse UBD binding modes to this common surface (Figure 2B) (30). For example, TSG101's UEV (Ubiquitin-conjugating enzyme E2 Variant) domain directly contacts ubiquitin's Gln62 (31), whereas this amino acid is not contacted by the Hrs DUIM (Double-sided Ubiquitin-Interacting Motif) (32). Comparison of multiple UBD:ubiquitin complexed structures demonstrates differences in ubiquitin with its various binding partners. This variability is not generated by UBD binding, but rather present in free ubiquitin due to its intrinsic motions, according to NMR experiments (33). UBDs thus appear to select for their optimal conformer from ubiquitin molecules that have variable amino acid sidechain exposure for the canonical UBD recognition surface, including Leu8 and the beta1-beta2 loop (Figure 2A) (33). Despite its conformational placidity, ubiquitin is a very stable protein that retains its fold at pH 4 up to ~75°C (34); for comparison, ubiquitin-like protein SUMO (Small Ubiquitin-like MOdifier) of similar fold and size denatures at ~52°C (pH 5.6) (35). Ubiquitin's placidity, coupled with its multifaceted recognition surface, enables a broad spectrum of structural folds to be used as UBDs, 24 of which are listed in Table 2.

3.2. Polyubiquitin: diversity in chains

Ubiquitin chains contribute an additional layer of diversity to the ubiquitin modifier, as these vary in both length and linkage type, as discussed in Section 2. They also expand the placidity present in monoubiquitin, as the linker region connecting ubiquitin moieties is flexible and allows diverse configurations for UBD-bound complexes (Figure 3). For example, Lys63-linked diubiquitin adopts a more compact conformation when bound to a Lys63-linkage specific antibody (36) than when bound to AMSH (Associated Molecule with the Src Homology 3 domain of signal-transducing adapter molecule), an endosome-associated ubiquitin isopeptidase (37). When not bound to a UBD, Lys48-linked tetraubiquitin exchanges (38) between a compact configuration with its ubiquitin moieties packed tightly against each other (39) and a more extended one (40) . The canonical UBD-binding surface is not accessible in the compact form of Lys48-linked diubiquitin (41) (Figure 3B) or tetraubiquitin (39) and UBDs are therefore expected to bind moieties in the extended form. For example, hHR23a's C-terminal UBA (UBiquitin-Associated) domain interacts with the hydrophobic patches of both ubiquitin moieties from Lys48-linked diubiquitin (Figure 4C), which are inaccessible in its compact form (42).

Steric clashes prevent the ubiquitin moieties of Lys63-linked and linear polyubiquitin from packing against each other (43) (Figure 3C and D), whereas this configuration is possible for Lys11-linked chains as demonstrated by X-ray crystallography (44, 45) (Figure 3A) and predicted by molecular modeling (46). Lys6 and Lys27 linkages are predicted to enable a compact conformation, whereas Lys29 and Lys33 linkages are not (46). It is possible that the isolation of ubiquitin hydrophobic patches that occurs in compactly configured ubiquitin chains prevents them from engaging in non-specific interactions.

4. UBIQUITIN-BINDING DOMAINS (UBDs)

4.1. Structural diversity of UBDs

No general ubiquitin binding consensus element has been identified, requiring all UBDs to be discovered experimentally. As a group, UBDs use all structural elements to bind ubiquitin, and new UBDs continue to be identified. UBDs can be alpha-helical, ZnF, PH, Ubc-related, SH3, or WD40 beta-propellers. The following sections describe binding modes and functions of various UBD structural folds (summarized in Table 2).

4.1.1. Alpha-helical motifs

Ubiquitin binding surfaces are most commonly formed by alpha-helices (Table 2). A single alpha helix can define a UBD, as in the case of the UIM (Ubiquitin Interacting Motif), IUIM/MIU (Inverted UIM/Motif Interacting with Ubiquitin), UMI (UIM- and MIU-related) and DUIM (Double-sided UIM) domains. Ubiquitin binding is localized to a single surface of UIMs, MIUs, and the UMI. Experimentally derived structures of UIMs and an MIU have been solved complexed with ubiquitin; however, no experimental structure is available for the

Table 2. UBD structural folds

Structural Fold	Subclass	Example Proteins	Ubiquitin Signaling Role	References
Alpha-helical	UIM	Rap80	DNA repair	(57, 58)
		Vps27	Endocytosis	(55)
		S5a/Rpn10	Proteasomal degradation	(53, 54, 130)
		STAM	Endocytosis	(82)
		Epsins	Endocytosis	(131)
		Ataxin 3	Deubiquitination	(56)
	IUIM/MIU	Rabex-5	Endocytosis	(90, 91, 132)
		RNF168	DNA repair	(50)
	DUIM	Hrs	Endocytosis	(32)
	UBM	Polymerase iota	DNA repair	(133, 134)
		Reversionless 1	DNA repair	(133)
	UBAN	NEMO	NF-kappaB signalling	(63, 65)
		ABIN1/2/3	NF-kappaB signalling	(135)
	UBA	hPLIC/Dsk2	Proteasomal degradation	(136, 137)
		hHR23/Rad23	Proteasomal degradation	(75, 137, 138)
		NBR1	Autophagy	(139)
		p62	Autophagy	(140)
	CUE	Vps9	Endocytosis	(70)
	GAT	TOM1	Endocytosis	(79, 141)
		GGA3	Endocytosis	(80, 142)
	VHS	STAM	Endocytosis	(81, 83)
		GGA3	Endocytosis	(143)
	UMI	RNF168	DNA repair	(47)
Zinc-finger (ZnF)	UBZ	Polymerase eta	DNA repair	(144)
		Polymerase iota	DNA repair	(145)
		TAX1BP1	NF-kappaB signaling	(146)
	NZF	TAB2/3	NF-kappaB signaling	(85, 86, 88)
		Vps36	Endocytosis	(87)
		Npl4	ERAD	(89)
	ZnF A20	Rabex-5	Endocytosis	(90, 91, 132)
		A20	NF-kappaB signalling	(147)
	ZnF UBP	IsoT/USP5	Deubiquitination	(92)
		HDAC6	Autophagy	(148, 149)
Pleckstrin-homology (PH)	PRU	Rpn13	Proteasomal degradation	(94, 95)
	GLUE	Eap45	Endocytosis	(93, 96, 97)
Ubiquitin conjugating (UBC)-related	UEV	TSG101	Viral budding and VPS	(31, 150)
		Mms2	DNA repair	(151)
	UBC	UbcH5c	Ubiquitin conjugation/chain assembly	(103)
Src homology (SH)	SH3	Sla1	Endocytosis	(108, 110)
		CIN85	Endocytosis	(109)
WD40	WD40 beta-propeller	PLAA/Doa1	ERAD	(112)
		Cdc4	Ubiquitin chain assembly	(112)
Additional UBDs	PFU	PLAA/Doa1	ERAD	(115, 152)
	JAB1/MPN	Prp8	Pre-mRNA processing	(153)
	DC-UbP_N	DC-UbP	Ubiquitinated substrate delivery	(111)
	MDA-9/ syntenin UBD	MDA-9/syntenin	Endocytosis	(113)

Abbreviations: UIM (Ubiquitin-Interacting Motif), IUIM/MIU (Inverted UIM/Motif Interacting with Ubiquitin), DUIM (Double-sided UIM), UBM (Ubiquitin Binding Motif), UBAN (Ubiquitin Binding in ABIN and NEMO), UBA (UBiquitin Associated), GAT (GGA And TOM), CUE (Coupling of Ubiquitin conjugation to Endoplasmic reticulum degradation), VHS (Vps27/Hrs/STAM), UMI (UIM- and MIU-related), UBZ (Ubiquitin-Binding ZnF), NZF (Npl4 (Nuclear protein localization 4) Zinc Finger), ZnF A20 (Zinc Finger A20), ZnF UBP/PAZ (Zinc Finger UBiquitin-specific Protease/Polyubiquitin Associated Zinc finger), PRU (Pleckstrin-like Receptor for Ubiquitin), GLUE (Gram-Like Ubiquitin-binding in Eap45), UEV (Ubiquitin-conjugating enzyme E2 Variant), UBC (UBiquitin-Conjugating), SH3 (Src Homology 3), PFU (PLAA Family Ubiquitin binding), Jab1/MPN (Jun Activation-domain Binding protein 1/Mpr-Pad1-N-terminal), DC-UbP_N (Dendritic Cell-derived UBiquitin-like Protein N-terminal domain), WD40 beta-propellers (WD40 beta-Prps), MDA-9/syntenin (Melanoma Differentiation Associated gene 9), ERAD (Endoplasmic Reticulum Associated Degradation), VPS (Vacuolar Protein Sorting)

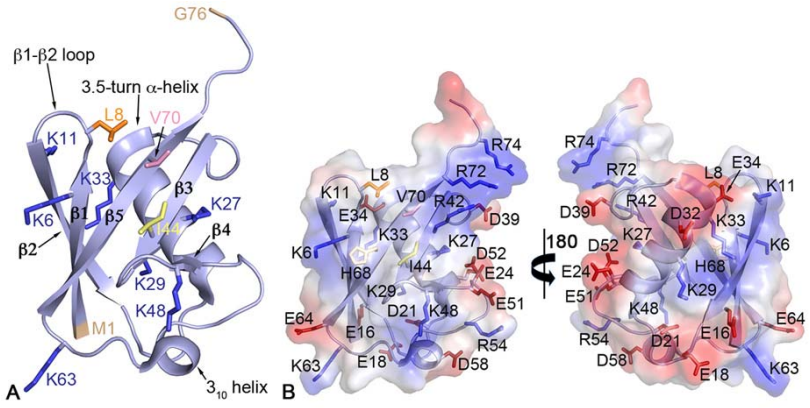

Figure 2. Features of monoubiquitin: (A) Ribbon diagram of monoubiquitin (PDB 1D3Z) highlighting its seven lysines (blue), hydrophobic triad (Leu8, orange; Ile44, yellow; Val70, pink), and termini (Met1 and Gly76, beige). Secondary structural elements are labeled. (B) Electrostatic surface diagram of monoubiquitin rendered over its ribbon diagram with positive and negative charges displayed on the surface in blue and red, respectively, and hydrophobic regions in white. Heavy atoms of several charged amino acids, as well as those mentioned in the text are rendered as sticks and labeled.

UMI, which is expected to be alpha-helical based on its sequence composition and so-named because of its sequence similarity to UIMs and MIUs (47). UIMs use a conserved LeuXXAlaLeu motif to bind the hydrophobic patch of ubiquitin, reviewed in (48, 49), whereas MIUs bind in an opposite orientation (LeuAlaXXLeu). The RING-finger ubiquitin ligase RNF168 contains two MIUs and a UMI (47, 50), which uses a dileucine motif and not the conserved Ala residue of UIMs and MIUs to interact with ubiquitin (47). DUIMs harbor two ubiquitin-binding surfaces at opposite sides of a helix, as displayed in the X-ray crystallographic structure of Hrs (Hepatocyte growth factor-Regulated tyrosine kinase Substrate) complexed with two monoubiquitin molecules (32); Hrs functions in protein sorting during endocytosis, as reviewed in (51, 52).

Alpha-helical ubiquitin-binding elements are invariably just one functional module within a multi-domain ubiquitin receptor, which can have multiple UBDs that are used together in novel ways to increase affinity for ubiquitin chains and in some cases, to define ubiquitin chain linkage specificity. Proteasome component S5a (53, 54), endosomal sorting protein Vps27 (55), deubiquitinating enzyme Ataxin-3 (56), and DNA repair protein Rap80 (57, 58) are examples of ubiquitin receptors with multiple UIMs. In all cases, the UIMs are separated by flexible linker regions that are exploited to fit the signaling needs of the ubiquitin receptor. S5a's two UIMs are poorly defined relative to each other when unbound or complexed with monoubiquitin (53). In binding to Lys48-linked diubiquitin, however, S5a's flexible linker region adapts to enable each UIM to contact a ubiquitin moiety simultaneously and thereby confer a significant increase in affinity compared to that for monoubiquitin (8.9 microM versus 350 microM to UIM1 and 73 microM to UIM2) (54) (Figure 4A). The pliability of the region linking S5a's UIMs likely contributes to its ability to also bind Lys63-linked chains with similar binding affinity (53, 59) and Lys29-, Lys6-, and Lys11-linked chains, albeit with lower affinity (59-61). The adaptability of S5a's UIM region may contribute to the proteasome's capacity to degrade substrates of all ubiquitin chain linkages, an ability indicated by mass spectrometry-based experiments in S. cerevisiae (13). By contrast, the region linking Rap80's UIMs undergoes a conformational switch to generate a continuous helix that spans its two UIMs and orients them optimally to bind Lys63-linked chains (57, 58). The resulting specificity for Lys63-linked chains is consistent with Rap80's role in DNA damage response (DDR) pathways, as reviewed in (62).

UBDs can also gain ubiquitin linkage specificity by oligomerization. The UBAN (Ubiquitin Binding in ABIN and NEMO) domain of NEMO (NF-kappaB Essential MOdifier) (63-65) forms a coiled-coil homodimer that binds preferentially to Lys63-linked (64-67) and linear ubiquitin chains (43, 63, 64). Its affinity for linear ubiquitin chains however is 100-fold higher than Lys63-linked chains (64), as the UBAN monomers bind in a bipartite manner to neighboring ubiquitin moieties of linear chains. Hydrophobic amino acids of one monomer interact with the Leu8-Ile44-Val70 triad of the distal ubiquitin while polar amino acids of the other monomer interact with a surface adjacent to the proximal ubiquitin's hydrophobic patch (63) (Figure 4B). When binding to Lys63-linked chains, NEMO recapitulates the interactions of the hydrophobic residues only, resulting in a single site-binding mode (65). The bipartite binding mode of UBAN to linear ubiquitin chains appears to be important for NEMO's function as an NF-kappaB activator, as mutation of either ubiquitin-binding site leads to the loss of IkappaBalpha degradation following TNF-alpha induction (63).

Multiple alpha-helices can also define a UBD, as in the case of the UBM (Ubiquitin Binding Motif), CUE (Coupling of Ubiquitin conjugation to Endoplasmic reticulum), GAT (GGA and TOM), UBA (UBiquitin Associated), and VHS (Vps27/Hrs/STAM) domains. The UBM domains of the Upsilon DNA polymerase iota (Pol iota) and Reversionless 1 (Rev1) are the smallest multiple

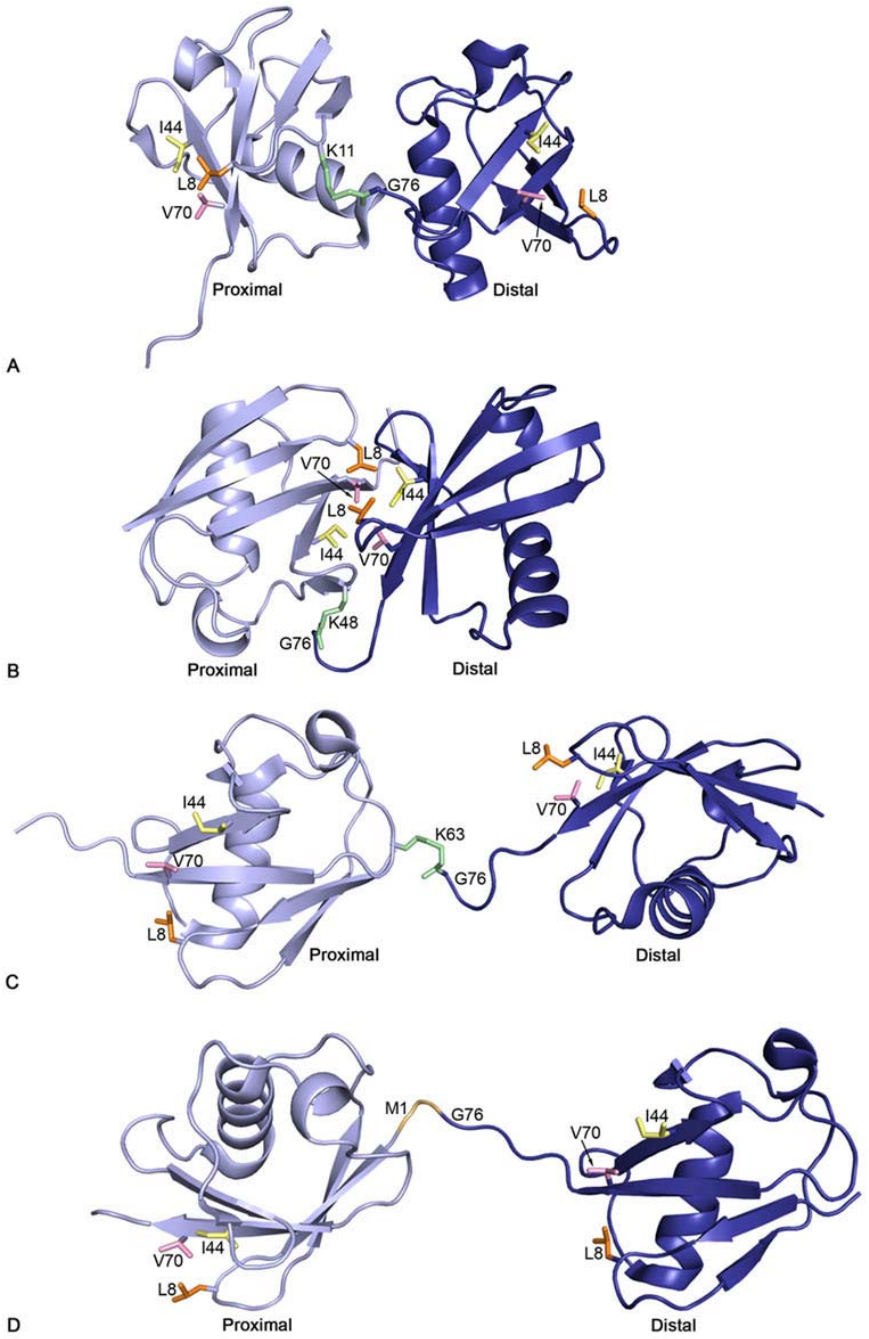

Figure 3. Diversity of ubiquitin polymers: Ribbon representation of diubiquitin with the ubiquitin moieties linked via Gly76 and (A) Lys11 (PDB 2XEW), (B) Lys48 (PDB 1AAR), (C) Lys63 (PDB 2JF5), and (D) Met1, so-called linear (PDB 2W9N). In all cases, the ubiquitin moiety with its Gly76 available for conjugation to a substrate is displayed in light blue whereas the more distal moiety is dark blue. The hydrophobic triad is colored as in Figure 2A and the lysine ligated to Gly76 is displayed in green for Lys11, Lys48, and Lys63 linkages or beige for Met1.

helical UBDs with two amphipathic helices that bind a ubiquitin surface centered on Leu8 (68, 69). CUE, GAT, and UBA domains make up the bulk of the multiple alpha-helical domains and are comprised of a three-helix bundle. CUE and UBA domains are structurally similar, despite low sequence identity (17% between Vps9p CUE and Rad23 internal UBA (UBA1)) (70). Both use their alpha1 and alpha3 helices to bind ubiquitin's hydrophobic patch, but UBA domains use a conserved Met/Leu-Gly-Phe/Tyr motif (71), whereas CUE domains use an Met-Phe-Pro motif (70). UBA domains are diverse in their preferences for ubiquitin chains of certain length and linkage type (59).

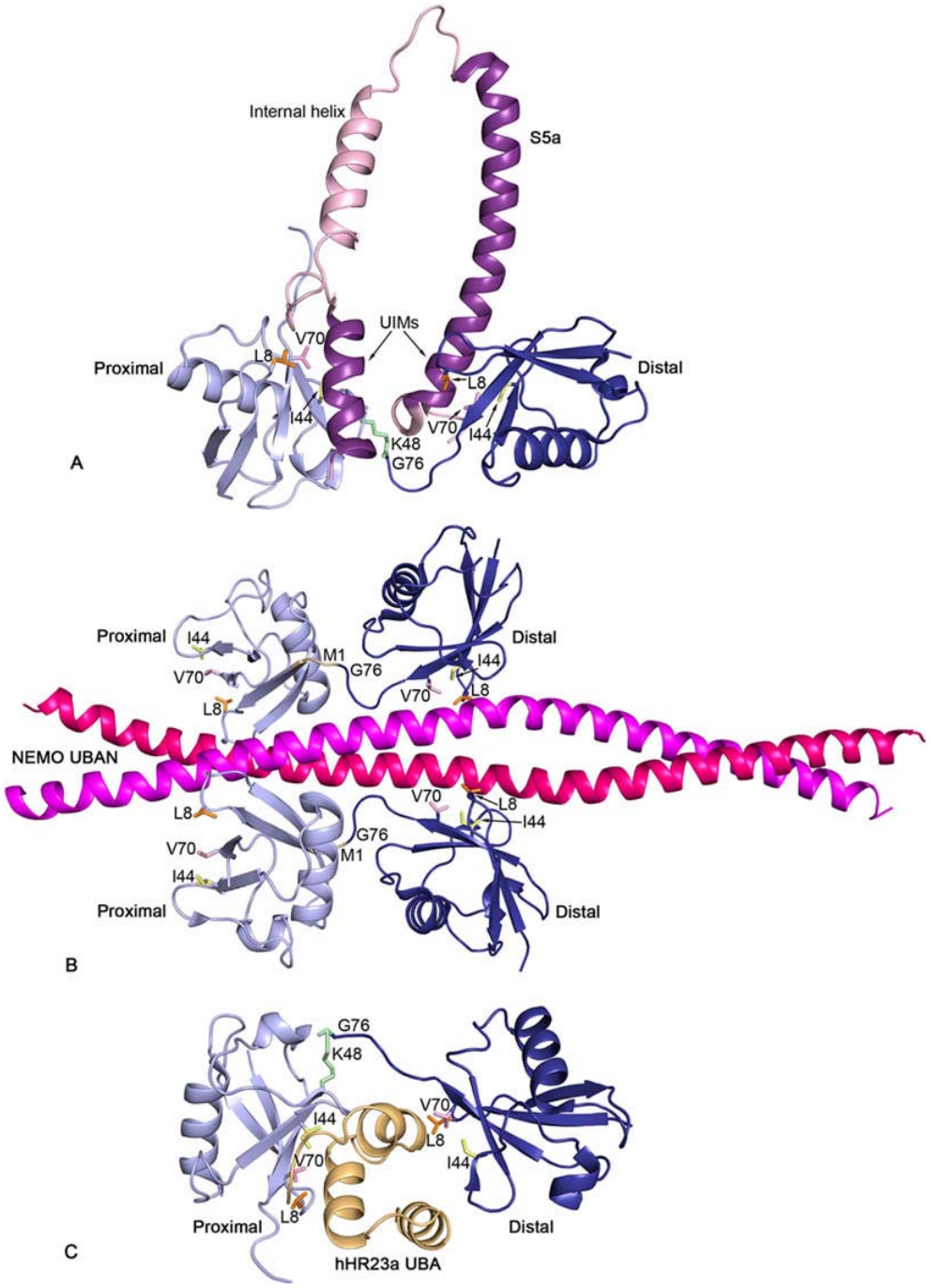

Figure 4. Alpha-helical ubiquitin-binding motifs: Ribbon representation of (A) S5a's UIM region (purple and pink) bound to Lys48-linked diubiquitin (PDB 2KDE), (B) NEMO's UBAN domains (pink and magenta) bound to two linear diubiquitins (PDB 2ZVO), and (C) hHR23a's UBA domain (gold) sandwiched between the two ubiquitin moieties of Lys48-linked diubiquitin (PDB 1ZO6). The coloring scheme for diubiquitin follows that of Figure 3.

Whereas the UBA from hPLIC1 (ubiquilin1) shows little preference between Lys29-, Lys48-, and Lys63-linked polyubiquitin (59, 72), hHR23a's C-terminal UBA domain (UBA2) prefers Lys48 linkages over Lys63 linkages, as it sandwiches between neighboring ubiquitin moieties of Lys48-linked chains (Figure 4C) (42, 59, 73). Full length hHR23a has two UBA domains that can bind concomitantly to ubiquitin (74), and its UBA1 also exhibits stronger affinity for Lys48-linked chains (75). Whereas dimeric UBA domains become monomeric to bind ubiquitin (76), CUE domains can bind to monoubiquitin as homodimers, as in the case of the Vps21p GTPase exchange factor Vps9p (70). CUE domains are published to prefer monoubiquitin, based on S. cerevisiae two-hybrid

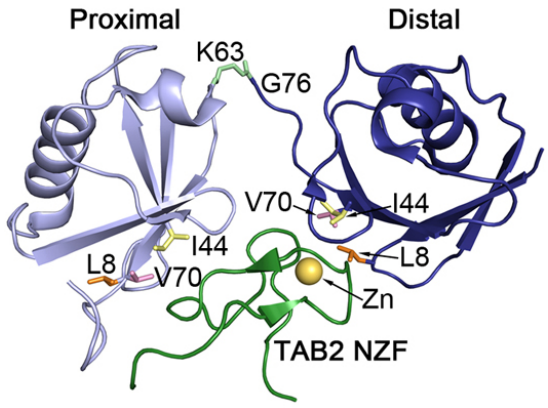

Figure 5. Zinc-finger domain of TAB2: Ribbon diagram of TAB2's NZF domain (green) complexed with K63-linked diubiquitin (PDB 3A9J). TAB2's single zinc ion is rendered as a gold sphere and the coloring for diubiquitin follows that of Figure 3.

screens with ubiquitin mutants that lack primary sites for ubiquitin chain formation (77). They have however been found to bind to Lys48-linked chains *in vitro* (78). In contrast to CUE and UBA domains, GAT domains have been reported to bind two ubiquitin molecules through two distinct binding sites, or one ubiquitin molecule and an additional binding partner, as in the case of ESCRT (Endosomal Sorting Complex Required for Transport) pathway proteins Tom1 (Target of Myb1) and GGA3 (Golgi-localized, Gamma-ear-containing, ARF-binding protein 3) (79, 80).

VHS domains contain the most alpha helices of a UBD known to date with eight alpha-helices, and this UBD has recently been found to have Lys63-linkage specificity (81, 82). A surface that is primarily composed of amino acids from alpha2 and alpha4 is used by the VHS domains of ESCRT proteins Stam1 (83, 84) and Stam2 (81) to bind ubiquitin.

4.1.2. Zinc finger (ZnF)

The second most abundant UBD structural fold is the ZnF domain, which is relatively small and stabilized by its coordination of one or more Zn ions. These have been shown to bind to three different regions of ubiquitin. Some bind to the Leu8-Ile44-Val70 hydrophobic patch, such as UBZ (Ubiquitin-Binding ZnF) and NZF (Npl4 (Nuclear protein localization 4) Zinc Finger) domains. NF-kappa B activator proteins TAB2 (TAK1-Binding protein 2) and TAB3 (TAK1-Binding protein 3) contain NZF domains that contact the Ile44-centered surface of neighboring ubiquitin moieties of Lys63-linked chains simultaneously (85, 86) (Figure 5) to preferentially bind this chain type (21, 85-88). The distal ubiquitin moiety binds a Thr-Phe dipeptide motif that is highly conserved among NZF domains and also used by Npl4's NZF domain to bind monoubiquitin (85-87, 89). Direct contact to the Lys63 isopeptide linkage is not made by these NZF domains; however, the placement of the ubiquitin's other lysines and Met1 demonstrates that concurrent contact to the two distinct binding surfaces is likely to be prohibited for chains formed by linkages other than Lys63 (Figure 5).

The ZnF UBD of the E3 ligase Rabex-5 (Rab5 guanine exchange factor) binds to a ubiquitin surface centered on Asp58 (Figure 2B) to which its Ser36 forms two hydrogen bonds (90, 91). Key contacts are also formed by two aromatic amino acids (Tyr25 and Tyr26) of Rabex-5 to ubiquitin's Arg54, Thr55, Ser57, Asp58, Tyr59 and Asn60 (90, 91). This dityrosine motif plays an integral role in Rabex-5's ability to bind to a ubiquitin-loaded E2 (UbcH5c), as binding is lost by its mutation to Ala (91).

Deubiquitinating enzymes disassemble ubiquitin chains by catalyzing the hydrolysis of the isopeptide bonds that link the individual moieties together. These linkage sites use ubiquitin's C-terminal Gly76, which was demonstrated to be required for interaction between ubiquitin and the ZnF UBP/PAZ of deubiquitinating enzyme isopeptidase T (IsoT/USP5) (92). This UBD has a deep pocket into which Gly75 and Gly76 insert and IsoT amino acids that interact with these glycines are required for its catalytic activation (92). A newly deposited crystal structure of HDAC6's UBP/PAZ domain bound to ubiquitin (PDB 3PHD) demonstrates ubiquitin's C-terminal amino acids to be similarly sequestered into a deep pocket. IsoT/USP5 and HDAC6 use analogous contacts to ubiquitin's C-terminal tail, as all but two of the seven interacting amino acids are conserved or functionally equivalent (92).

4.1.3. Pleckstrin-homology (PH)

Pleckstrin-homology (PH) domains are comprised of a 7-stranded beta-sheet with a C-terminal alpha-helix and many bind phosphoinositides (PIs) within membranes to function in intracellular signaling. Two PH domains have been reported to bind to ubiquitin, including that within ESCRT-II Eap45, termed GLUE (Gram-Like Ubiquitin-binding in Eap45) (93), and the proteasome component Rpn13, termed PRU (Pleckstrin-like Receptor for Ubiquitin) (94, 95). A region of Eap45's GLUE domain that contains the C-terminal end of its alpha-helix, beta5, beta6, and the beta6-beta7 loop binds to the Ile44 centered surface of ubiquitin, as demonstrated by X-ray crystallography (96, 97) (Figure 6A). This ubiquitin-binding surface is at an opposite location relative to the

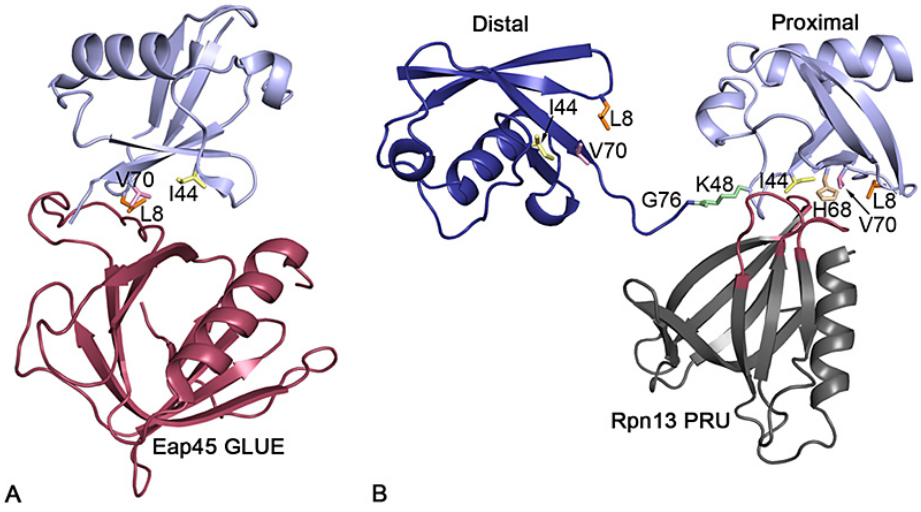

Figure 6. Pleckstrin-homology domains: Ribbon representation of (A) Eap45's GLUE domain (red) bound to monoubiquitin (PDB 2DX5) and (B) Rpn13's PRU domain (grey with ubiquitin-binding loops in red) bound to Lys48-linked diubiquitin (generated from PDB 2Z59 and 1D3Z). The ubiquitin coloring is as described in Figure 3, but with His68 included in beige.

proposed site that binds PIs, thereby suggesting that these two surfaces are used together to bring ubiquitinated cargo to the endosomal membrane (96-99).

Rpn13's PRU domain by contrast does not appear to bind to PIs, but rather uses a surface opposite to its ubiquitin-binding one to dock into the proteasome (95) (Figure 6B). It uses three loops to contact ubiquitin (Figure 6B), including its beta4-beta5 loop, which contains two Asp's (Asp78 and Asp79) that form hydrogen bonds with ubiquitin's His68. Phe76 from this loop interacts with ubiquitin's Ile44, Gln49, and Val70 and its mutation to Arg abrogates Rpn13's ability to bind to ubiquitin (95). Rpn13's PRU has a high affinity for ubiquitin (300 nanoM for monoubiquitin, 90 nanoM for Lys48-linked diubiquitin) (94) compared to S5a (54), the proteasome's other ubiquitin receptor. This affinity however is reduced in the full length protein due to interactions between its PRU domain and its C-terminal Uch37-binding domain (100), as discussed further in Section 4.2. When binding to a Lys48-linked chain, Rpn13 prefers the proximal subunit (Figure 6B), most likely due to the loss of charge on its Lys48 (95). The use of loops to bind ubiquitin highlights the difficulty in predicting UBDs based on sequence information alone and the discovery that Rpn13 is a ubiquitin receptor in the proteasome (94) was 14 years after S5a was reported to be one (101).

4.1.4. Ubiquitin-conjugating (Ubc)-related

Ubc (UBiquitin-Conjugating) domains are found in E2 enzymes and are comprised of ~150 amino acids that fold into four standard helices and a 4-stranded antiparallel beta-sheet, as reviewed in (102). Generally, Ubc domains contain a conserved catalytic Cys that forms a thiolester bond with ubiquitin, as discussed in Section 2. All E2's interact with ubiquitin covalently, but noncovalent interaction surfaces have also been identified, as in UbcH5c (103). UbcH5c's non-covalent ubiquitin-binding surface is opposite to the E2 catalytic Cys (Figure 7A) and is essential for the processive nature of BRCA1 (BReast CAncer type 1)-mediated polyubiquitination, allowing multiple ubiquitination cycles to a bound substrate (103). UbcH5a and UbcH5b also use this non-covalent ubiquitin-binding surface (104), and UbcH5a has an additional ubiquitin-binding surface that is proposed to govern Lys11-linked chain formation, as a surface adjacent to its catalytic Cys interacts with a ubiquitin surface that flanks its Lys11 (105).

The UEV (Ubiquitin-conjugating enzyme E2 Variant) domain has an alpha/beta fold similar to Ubc domains, but has an additional N-terminal helix, an extended beta-hairpin linking beta1 and beta2 and lacks Ubc's two C-terminal helices (31). UEV domains are not E2's as they lack the catalytic Cys residue, but they can bind ubiquitin non-covalently, as in the case of TSG101, which functions in Human Immunodeficiency Virus-1 (HIV-1) budding and MVB (MultiVesicular Body) sorting (31). TSG101's UEV domain and UbcH5c bind ubiquitin differently (Figure 7), as the extended beta-hairpin is used by this UEV as well as the loop that follows beta4 (Figure 7B) (31, 103).

4.1.5. Src homology 3 (SH3)

Approximately 300 SH3 domains are encoded in the human genome (106). They are formed by ~60 amino acids, which assume a beta-barrel fold and tend to promote protein-protein interactions, most often by binding to proline-rich regions (PxxP) (107). SH3 domains of S. cerevisiae Sla1 (108), its mammalian orthologue CIN85 (109), and amphiphysin (110) are reported to bind to ubiquitin. Sla1's third SH3 (SH3-3) domain uses complementary hydrophobic residues from a shallow groove present in all SH3 domains to bind the hydrophobic patch of monoubiquitin, as revealed by solution NMR (108) (Figure 8). This shallow groove is typically used to

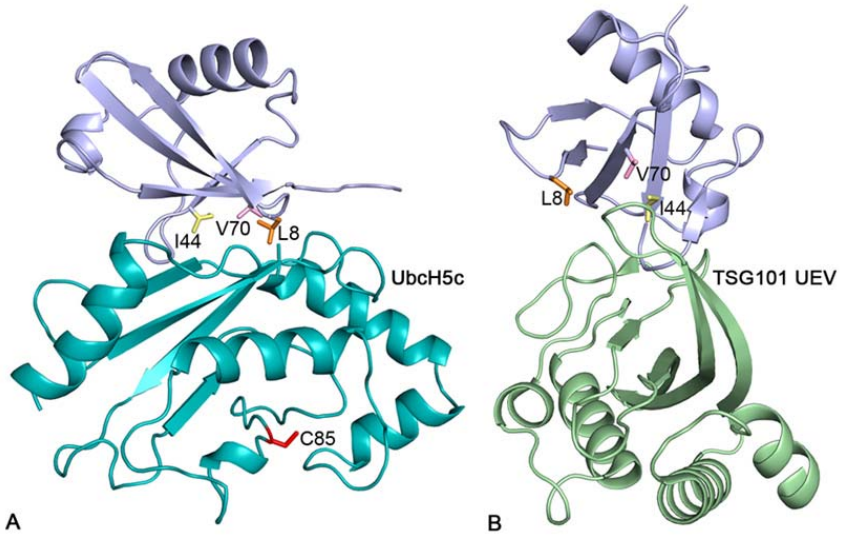

Figure 7. Ubc-like domains complexed with ubiquitin: Ribbon representation of (A) UbcH5c (cyan, PDB 2FUH) and (B) TSG101 UEV (green, PDB 1S1Q) bound to monoubiquitin colored as in Figure 3. UbcH5c's catalytic cysteine (Cys85) is displayed in red.

bind ProXXPro or ProXXProArg motifs (108-110), suggesting a competition between ubiquitin and ProXXPro/Arg binding partners. Like its yeast orthologue, CIN85 contains three SH3 domains, but in contrast to Sla1, all three bind ubiquitin (109, 110).

4.1.6. Additional UBDs

Many other structural folds have evolved ubiquitin-binding surfaces. In the past year, novel UBD structural folds have been described in the N-terminal domain of DC-UbP (111), WD40 beta-propellers (112), and MDA-9/syntenin (113). DC-UbP's N-terminal ubiquitin-binding domain forms an alpha-alpha-alpha-beta-beta pattern with a short C-terminal alpha-helix (111). It binds to ubiquitin's hydrophobic patch and C-terminal residues (Leu71, Arg72, and Leu72) by using amino acids from the alpha1-alpha2 loop, alpha2, alpha3, and the beta1-beta2 loop (111). The C-terminal region of DC-UbP contains a UBL domain (114), thereby linking it to the UBL-UBA family of proteins, which include hHR23 and hPLIC, discussed in Section 4.1.1.

WD40 beta-propellers from functionally diverse proteins bind to ubiquitin's hydrophobic patch through a conserved surface made up of loops; these include components of SCF ubiquitin E3 ligases and AAA ATPase Cdc48/p97 adaptor Doa1/Ufd3 (112). Doa1/Ufd3 also contains a PFU domain (PLAA family Ub-binding domain), which binds to ubiquitin (115). It is not yet known whether Doa1's UBDs are used synergistically to confer specificity for ubiquitin chain linkage type, however it appears to bind preferentially to longer ubiquitin chains (116).

4.2. UBD regulation

There are many mechanisms to keep UBDs in check, which have perhaps arisen due to their widespread use and abundance. UBD regulation can occur through intramolecular activities involving the ubiquitin receptor or alternatively, by modification of the substrate. Intramolecular interactions between UBDs and other domains within a ubiquitin receptor or that involve a ubiquitin conjugated to the receptor itself can reduce a receptor's affinity for ubiquitinated substrates. Ubiquitin receptors hRpn13 and hHR23a, which function in proteasome-mediated protein degradation, have additional domains that interact with their UBDs. hRpn13's PRU binds its C-terminal Uch37-binding domain, which reduces the exposure of its ubiquitin binding surface and in turn, its affinity for ubiquitin (100). hRpn13 docking into the proteasome appears to abrogate this intramolecular interaction, thereby functionally coupling its activity as a ubiquitin receptor to its localization to proteasome (100). hHR23a's UBA and UBL domains similarly interact to reduce ubiquitin accessibility (117). Moreover, monoubiquitination of UBD-containing proteins causes the UBD to interact with its own conjugated ubiquitin rather than a substrate-attached one. This process, termed coupled monoubiquitination (118, 119), regulates Eps15 (UIM), Sts1 (UBA), Sts2 (UBA), Hrs (DUIM) and the MDA-9/syntenin UBD (118, 120, 121). Intermolecular interactions involving UBDs can also regulate ubiquitin receptor binding to ubiquitinated substrates, as in the case of p47, which is unable to bind monoubiquitin via its UBA domain unless it is complexed with p97 (122, 123). Post-translational modifications of substrates can also regulate UBD activity, including selective methylation of Lys residues, phosphorylation (as reviewed in (124)), and conjugation with ubiquitin-like proteins (such as SUMO, as reviewed in (29, 125)). Methylation of lysine side-chains prevents ubiquitination, causing loss of UBD interaction and prolonged protein half-life (126). A recent MS study done on the S. cerevisiae 26S proteasome suggests that 43% of Lys methylation sites in over 40 proteins are also sites for ubiquitination (127).

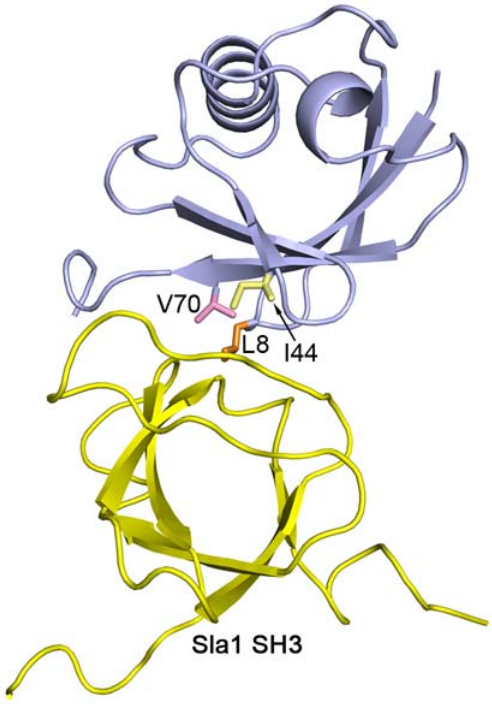

Figure 8. SH3 domain of Sla1: Ribbon representation of the SH3 domain of Sla1 (yellow) bound to monoubiquitin colored as in Figure 3 (PDB 2JT4).

5. PERSPECTIVE: SUMMARY AND FUTURE

Ubiquitin's signaling prowess was first discovered for its ability to target substrates for degradation by proteasome (128, 129), but has now expanded to encompass a myriad of processes including regulatory roles in DNA repair, transcription, pre-mRNA splicing, endocytosis and autophagy, as reviewed in (18). Its function as a signaling molecule in such a broad spectrum of activities is tightly coupled to its multilingualism for a plethora of diverse receptors, which is contributed by monoubiquitin's dynamic sampling of slightly different conformers, the diversity of the region connecting moieties within a ubiquitin chain, and the variations available in ubiquitin chain length and linkage. Structures of UBDs complexed with ubiquitin polymers have provided valuable insights into how ubiquitin signals for such a large repertoire of events with specificity. Some questions remain however. With the notable exception of ZnF domains, which can bind to three different ubiquitin surfaces, the vast majority of UBDs seek a common surface on ubiquitin, thus suggesting a packing problem as ubiquitin is shuttled from one receptor to another, or as multiple receptors are present and available for binding. It is tantalizing to speculate that these potential hurdles have been evolutionarily exploited, such as by preventing two receptors from distinct pathways from battling over where to shuttle their commonly bound substrate. Yet, the mechanisms that prevent such battles for substrates conjugated with long ubiquitin polymers, such as octaubiquitin, are difficult to fathom. New UBDs and mechanisms for their regulation continue to be discovered and it is interesting that so many structural domains can form surfaces that bind ubiquitin. Yet, it is not always clear how one domain type assumed ubiquitin-binding capacity for some of its members. Finally, it is often challenging to extrapolate the intricacies of a ubiquitin signaling cascade from the snapshots provided by UBD:ubiquitin complexes. It is hopeful that the future will offer atomic resolution images of ubiquitinated substrates complexed with their ubiquitin receptors as they are shuttled through a ubiquitin signaling pathway. Such information would provide a greater appreciation of how individual UBDs are integrated into the ubiquitin-signaling network.

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