The Molecular Toolbox for Linkage Type-Specific Analysis of Ubiquitin Signaling

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Modification of proteins and other biomolecules with ubiquitin regulates virtually all aspects of eukaryotic cell biology. Ubiquitin can be attached to substrates as a monomer or as an array of polyubiquitin chains with defined linkages between the ubiquitin moieties. Each ubiquitin linkage type adopts a distinct structure, enabling the individual linkage types to mediate specific functions or outcomes in the cell. The dynamics, heterogeneity, and in some cases low abundance, make analysis of linkage type-specific ubiquitin signaling a challenging and complex task. Herein, the strategies and molecular tools available for enrichment, detection, and characterization of linkage type-specific ubiquitin signaling, are reviewed. The molecular "toolbox" consists of a range of molecularly different affinity reagents, including antibodies and antibody-like molecules, affimers, engineered ubiquitin-binding domains, catalytically inactive deubiquitinases, and macrocyclic peptides, each with their unique characteristics and binding modes. The molecular engineering of these ubiquitin-binding molecules makes them useful tools and reagents that can be coupled to a range of analytical methods, such as immunoblotting, fluorescence microscopy, mass spectrometry-based proteomics, or enzymatic analyses to aid in deciphering the ever-expanding complexity of ubiquitin modifications.

1. Introduction

Ubiquitin (Ub) is a highly conserved eukaryotic protein that is conjugated to other intracellular substrate proteins as a post-translational modification (PTM).[1] More than 110,000 ubiquitination sites in over 12,000 proteins have been reported in human cells.[2–6] With a median modification half-life of only ≈12 min,[7] ubiquitination is both one of the most pervasive and dynamic PTMs. But the key defining trait of the Ub system is the complexity of Ub modifications that can be formed on substrates. Ub can be attached to the substrates' amino acid side chains, most commonly lysine (K), as a single molecule. This is termed monoubiquitination. But Ub itself contains seven lysines as well as the N-terminal methionine (M1) that can serve as sites or linkage points for further ubiquitination. Ubiquitination of Ub itself leads to the formation of homotypic, mixed, or branched polyUb chains in which the linkage between the Ub moieties determines their discrete architectures and topologies (**Figure 1**). This vast array of modifications is referred to as the "Ub Code".[8] Remarkably, the different polyUb chain types are not only structurally distinct but they also have different functions in cells.[8,9] Ubiquitination was originally thought to exclusively be earmarking proteins for proteasomal degradation. It was work by Daniel Finley's laboratory[10] that led to the realization that the different polyUb chain types might represent functionally distinct signals in cells. Finley's team discovered that yeast cells expressing a Ub mutant, K63R, which rendered them incapable of forming K63-linked Ub chains, were fully proficient in proteolytic turnover but had a defective DNA repair response, indicating that K63-linked Ub chains had a signaling role during DNA repair that was independent of proteasomal degradation. The understanding of linkage-specific functions has advanced rapidly ever since. It is now clear that the individual Ub linkages can function as discrete signals in cells and that they each control important—but different—cellular mechanisms and functions. However, many open questions remain about the roles and functions of the different Ub chain types. This review focuses on the concepts and tools used to crack the code of chain type-specific Ub signaling.

1.1. The Ubiquitin System

The covalent attachment of Ub to a substrate is called ubiquitination. It requires a sophisticated, ATP-dependent, three-step cascade[11] using Ub-activating (E1) enzymes,[12] Ub-conjugating (E2) enzymes,[13] and Ub (E3) ligases[14–16] (Figure 1). Ubiquitin modifications on substrates are recognized by Ub-binding domains (UBDs),[17] which facilitate Ub-dependent signaling and the cellular outcomes of the modifications, and specialized proteases known as deubiquitinases (DUBs) cleave and remove the Ub modifications to terminate or modulate signaling.[18,19] Ub is most frequently conjugated to the ε-amino group of substrate lysines via its C-terminal glycine (Gly76).[1] But non-canonical protein ubiquitination via serine, threonine, or cysteine residues, as well as the α-amino group on the N-terminus in substrate proteins, also occur.[20,21] Intriguingly, increasing evidence shows that Ub is also conjugated to non-proteinaceous biomolecules,

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including bacterial lipopolysaccharide,[22] lipids,[23] sugars,[24] as well as ADP-ribose and nucleic acids,[25–27] vastly expanding the repertoire of potential ubiquitination substrates.

A hallmark of the Ub system is that Ub itself can be ubiquitinated, giving rise to structurally and functionally distinct types of polyUb chains, which should be considered distinct PTMs (Figure 1). Canonically, the first Ub added to a substrate (the proximal Ub) can be ubiquitinated on one of its seven lysines—K6, K11, K27, K29, K33, K48, or K63—or at the N-terminal M1, leading to the formation of amide bonds—isopeptide bonds for lysines and peptide bonds for methionine—between the Ub molecules. Recently, formation of ester-linked polyUb via serine and threonine residues in Ub was also identified.[28–30] Ester linkages through Thr12, Thr14, Ser20, and Thr22 have been identified in cell lysates,[30] while Thr55 linkages have been observed in vitro.[28,29] This brings the total number of Ub linkages identified in cells up to 12 (eight amide linkages and four ester linkages). These Ub linkages can form homotypic polyUb chains, that is, chains consisting of a single linkage type, or heterotypic chains. Heterotypic polyUb chains contain multiple linkage types and can be either "mixed" chains, in which one linkage type is extended with a different one, or they can be "branched", in which one or more Ub moieties are ubiquitinated on multiple side chains (Figure 1).[31]

Common to all Ub linkages is that the linkage residue (and hence type) determines the orientation of the Ub moieties in the chain relative to each other. The position of the linkage point on the proximal Ub determines the relative orientation of the Ub being conjugated (the distal Ub) to the proximal Ub and thus the architecture of the Ub chains (Figure 1) as well as the structures and dynamic conformations these distinct signals can adopt.[32,33] This is the very foundation of linkage type-specific functions of Ub chains. All Ub chains adopt distinct structures and have unique distributions of hydrophobic interaction patches, for example, the Ile44 patch, consisting of Leu8, Ile44, and Val70. The distinct structures allow the different polyUb chains to "encode" specific cellular outcomes. By being bound by specific or selective UBDs and Ub receptor proteins that can facilitate their individual outcomes, the different Ub modifications (Figure 1) function as individual, distinct PTMs.

1.2. Complexity and Function of Ub Modifications

The fate of a ubiquitinated protein is determined by the combined actions of Ub ligases (i.e., the polyUb linkage type(s) conjugated to the protein), DUBs that cleave or trim the Ub chains, and UBD-containing proteins that sense and facilitate the cellular outcomes. The structural and combinatorial diversity of the Ub modifications attached to substrates is immense. Substrates can be modified with one or several Ubs or with one or several of the 12 polyUb chain types in either homotypic, mixed, and/or branched chains of varying length and shape. The idea of a "Ub Code"[8] where these different Ub modifications and linkage types have distinct functions emerged from the different fates of substrate proteins ubiquitinated with proteasome-targeting

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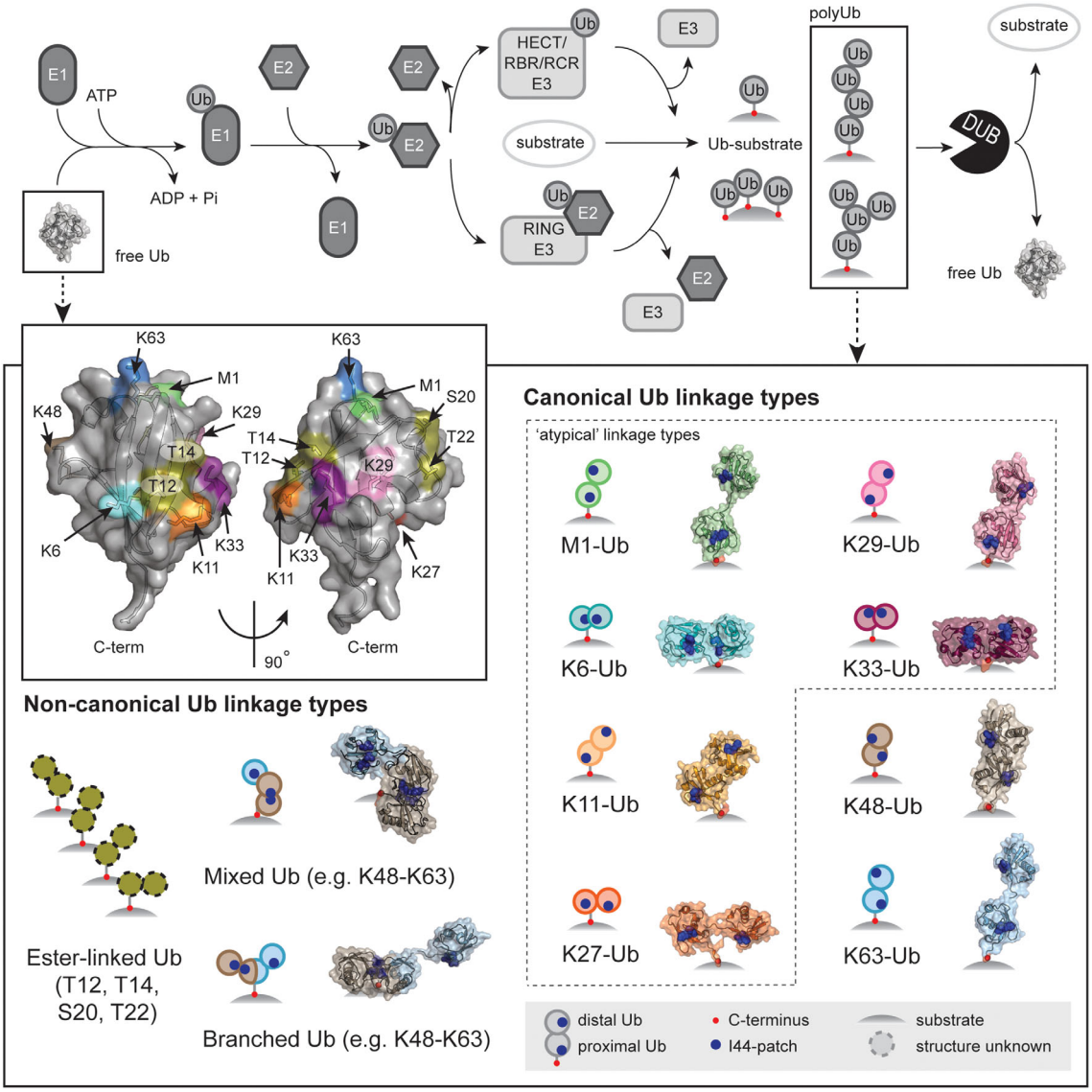

Figure 1. The ubiquitin system. An ATP-dependent, three-enzyme cascade of Ub-activating (E1) enzymes, Ub-conjugating (E2), and Ub (E3) ligases controls modification of biomolecular substrates, mainly proteins, but also sugars, fatty acids, and nucleotides, with Ub. Ub modifications are removed by deubiquitinases (DUBs). The complexity of the Ub system arises from the many possible linkage points that exist in Ub for further ubiquitination and generation of polyUb chains. Ub itself can be ubiquitinated by conjugation of the C-terminal Gly on one Ub molecule to an α- or ε-amino group-containing residue on another Ub, giving rise to the canonical amide (isopeptide or peptide) linkages through M1 (also known as linear chains), K6, K11, K27, K29, K33, K48, and K63. The resulting polyUb chain can be homotypic (i.e., consisting of a single linkage type) or heterotypic (i.e., consisting of multiple linkage types). Heterotypic chains can be further divided into "mixed" or "branched" polyUb chains. In mixed chains, one linkage type is extended with a different one, but no Ub moieties are ubiquitinated on more than one residue. In branched chains, one or more Ub moieties are ubiquitinated on multiple side chains. Apart from mixed and branched chains, ester linkages through T12, T14, S20, or T22 have recently been discovered as an additional, non-canonical type of Ub chain. The linkage type (or point) between two Ub moieties determines their relative spacing and orientation and thereby the structure, dynamics, and distribution of interaction patches, for example, the Ile (I) 44 patch (blue; from hereon indicated by I44 in structures), of the polyUb chain type. This allows the different polyUb linkages to function as individual PTMs and to bind specific UBDs that facilitate their individual functions. The depicted Ub chains are illustrated as diUb (the minimal functional unit of a polyUb chain); however, in cells, these chains are typically longer. Crystal structures from PDB are of Ub PDB: 1UBQ,[177] M1-linked diUb PDB: 2W9N,[97] K6-linked diUb PDB: 2XK5,[178] K11-linked diUb PDB: 2XEW,[179] K27-linked diUb PDB: 6QML,[180] K29-linked diUb PDB: 4S22,[70] K48-linked diUb PDB: 5GOI,[181] K63-linked diUb PDB: 2JF5,[97] K48-K63 mixed triUb PDB: 5O44,[182] and K48-K63 branched triUb PDB: 7NPO.[152] Abbreviations: DUB, deubiquitinase; E1, Ub-activating enzyme; E2, Ub-conjugating enzyme; E3, Ub-ligase; HECT, Homologous to the E6-AP Carboxyl Terminus; RBR, RING between RING; RCR, RING-Cys-Relay; RING, Really Interesting New Gene.

K48-linked Ub chains and K63-linked Ub chains, which mainly facilitate non-proteolytic signaling functions. We now appreciate that multiple Ub chain types can lead to degradation, even K63-linked chains via the lysosome.[34,35] But a large array of different, non-proteolytic functions, including protein recruitment, kinase activation, and substrate localization,[8,9] has been established as cellular outcomes for the different Ub modifications.

Among the canonical Ub chains (Figure 1), K48-linked and K63-linked chains are by quite a margin the best characterized polyUb modifications. They are also the most abundant chain

types with K48-linked chains constituting on average ≈40% of cellular Ub linkages and K63-linked chains constituting on average ≈30%.[36–39] Proteins decorated with K48-linked chains are targeted for proteasomal degradation,[34] whereas K63-linked chains are mainly involved in non-degradative signaling in the DNA damage response, immune signaling, and protein trafficking.[40] The functions of the remaining six so-called "atypical" linkage types (M1 (also known as linear chains), K6, K11, K27, K29, and K33) are less well understood both functionally and mechanistically, but they play important roles in processes such as cell cycle regulation, proteotoxic stress, and immune signaling.[9,41–43] Recently, the Ub Code has expanded significantly with the addition non-canonical, oxyester-linked serine- and threonine-linked Ub chains (Figure 1).[28–30] Although there are indications of a role in regulation of immune signaling,[44,45] the roles and functional importance of the oxyester-linked Ub chains remain largely unknown.

Given that the different Ub chain types play important roles in proteostasis and cell signaling and can give rise to distinct cellular outcomes, characterizing the substrates, dynamics of formation and removal, localization, and architecture of the individual Ub linkage types is vital for understanding their individual cellular function. Yet, we are still far from understanding the functions of individual Ub chains, particularly atypical and non-canonical chains, in cellular signaling.

Herein, we review the reagents, tools, and experimental approaches used to decipher the complexity of the Ub Code and the signaling roles of the individual Ub chains in linkage type-specific manner. We summarize the state-of-the-art and recent advances in affinity-based reagents for linkage-specific polyUb enrichment, their applications for methods such as immunoblotting, microscopic analysis, and proteomics analysis, and discuss the challenges and opportunities for further reagent development to uncover the functions and cellular importance of the individual Ub linkages.

2. Linkage Type-Specific Analysis of the Ub Code

2.1. Enrichment and Detection of Ub Chains

Without linkage type-specific tools and reagents able to enrich or detect specific Ub chains, especially low-abundant chains such as most atypical chain types, it is virtually impossible to analyze these modifications and their substrates beyond an *in vitro* setting. To understand the cellular function of the different Ub chains, we are often interested in knowing the substrates of the modification to understand the pathways it regulates. An unambiguous way to investigate the attachment of Ub chains to a substrate protein is to enrich the ubiquitinated form of the substrate of interest from cell lysates using an affinity reagent specific for a defined Ub chain type (**Table 1**) followed by immunoblotting with antibodies specific for the substrate protein of interest.[46] Such enrichments may also be coupled to mass spectrometry-based proteomics workflows instead of immunoblotting,[47,48] or one may be interested in enriching chains to probe their architecture on a substrate or use fluorescence microscopy for instance to investigate the localization of a specific Ub chain type to an organelle or cellular structure. The ability to specifically enrich or detect the individual Ub linkage types is critical for all these analyses. Many efforts and resources have therefore been invested in developing reagents and methods that allow this. Over the past two decades, a range of tools for specific detection and enrichment of individual Ub modifications has been developed, including different types of affinity reagents and molecular biology approaches relying on Ub variants engineered with tags or mutations, enabling the spatiotemporal analysis of the Ub system.

2.2. Linkage Type-Specific Affinity Reagents

Affinity reagents are molecules or molecular tools that are used to specifically recognize and bind a target molecule, particularly proteins or peptides. Affinity reagents are often antibodies. That is true for affinity reagents used to bind Ub chains as well. But other types of molecules are also used for recognizing, binding, or enriching specific Ub linkages. As of today, more than 30 linkage-specific Ub affinity reagents have been described in the literature, including antibodies, antibody fragments, single-domain antibodies (sdAbs), engineered UBDs, engineered and catalytically inactive DUBs, affimers, and macrocyclic peptides (Table 1). A selection of different tools and reagents exist for each of the canonical and abundant K48 and K63 linkages. The "atypical" linkages (M1/linear, K6, K11, K27, K29, and K33) generally have a much smaller range of available reagents, with M1-linked Ub being the exception. For mixed, branched, and ester-linked chains, few or no specific affinity reagents have been described.

2.3. Achieving Specificity

The key challenge in developing affinity reagents toward Ub is not to make them Ub-specific. It is to make them Ub linkage type-specific. Conceptually and molecularly, it is a difficult task: generating or developing a binder that can not only recognize Ub specifically among the ≈15,000 cellular proteins but specifically recognize one of 12 very similar but distinct polymers of Ub, all composed of identical monomers.

The affinity reagents achieve specificity by recognizing unique features of the various Ub chain types (**Figure 2**), including the distance between Ub molecules and binding patches (primarily the Ile44 patch, Figure 1), Ub chain architecture and relative orientation of the Ub moieties in a chain, or the actual linkage and the molecular environment around it[8] (Figure 2A). Different reagents exploit different features, and even reagents within the same class bind through distinct mechanisms, such as the UBDs where multiple specific binding modes are exploited[17] (Figure 2). One key feature on Ub that many reagents bind the is the Ile44 patch. This surface-exposed, hydrophobic patch, consisting of Leu8, Ile44, and Val70, is located in Ub's β-sheet (Figure 2A). It is the main interaction site for most naturally occurring UBDs, as well as the proteasome, and it is essential

Table 1. Ub linkage-specific affinity reagents. List of linkage type-specific affinity reagents sorted by the linkage they bind. The list only includes reagents validated in peer-reviewed publications.

Linkage	Reagent	Type	References
M1/linear	NEMO UBAN	UBD (helical)	[56,72]
	OPTN and ABIN1 UBANs	UBD (helical)	[72]
	HOIL1L-NZF and SHARPIN-NZF	UBD (ZnF)	[73]
	OTULIN (aa 80-348, C129A)	inactive DUB	[57,105]
	1E3	Fab/IgG	[55]
	LUB9	IgG	Validated in [183]
K6	K6-Affimer	Affimer	[119]
	LotA	inactive DUB	[111]
K11	2A3/2E6	Fab/IgG	[136]
	K11-Affimer (cross-reactive for K33)	Affimer	[119]
K27	EPR17034	IgG	Validated in [162]
K29	TRABID NZF (also selective for K33)	UBD (ZnF)	[70,74]
	sAB-K29	Fab	[137]
K33	TRABID NZF (also selective for K29)	UBD (ZnF)	[70,74]
	K33-Affimer (cross-reactive for K11)	Affimer	[119]
K48	RAD23B	UBD (helical)	[70,88]
	MINDY-1 MIU	UBD (helical)	[75]
	Met4 UIMLx2	UBD (helical)	[77]
	Apu2	Fab/IgG	[54]
	Ub4a and related peptides	Cyclic peptide	[128,129,131]
K63	Rap80 tUIM	UBD (helical)	[61,71,184]
	EPSIN1 tUIM	UBD (helical)	[71]
	TAB2 ZNF	UBD (ZnF)	[93,94]
	TAB3 ZNF	UBD (ZnF)	[93]
	Apu3	Fab/IgG	[54]
	Cyclic peptide 2	Cyclic peptide	[127]
Branched	NbSL3.3Q (K48-K63-Ub3)	sdAb	[152]
Bispecific	K11/K48 bispecific Ab	IgG	[139]
	K63-Lin bispecific Ab	IgG	[140]
	RIP1-K63 bispecific Ab	IgG	[140]
	RIP1-Lin bispecific Ab	IgG	[140]
	RIP2-K63 bispecific Ab	IgG	[140]
	RIP2-Lin bispecific Ab	IgG	[140]

Abbreviations: ABIN1, A20-binding inhibitor of NF-κB activation 1; Fab, fragment antigen binding; HOIL-1, Heme-oxidized IRP2 ubiquitin ligase 1; IgG, Immunoglobulin G; LotA, *Legionella* ovarian tumor-like protein a; lin, linear; MINDY-1, MIU-containing novel DUB family 1; NEMO, NF-κB essential modifier; NZF, Npl4 zinc finger; OPTN, Optineurin; OTULIN, OTU DUB with linear linkage specificity; RAD23B, UV excision repair protein RAD23 homolog B; RIP1, Receptor-interacting protein kinase 1; RIP2, Receptor-interacting protein kinase 2; Rap80, Receptor-associated protein 80; SHARPIN, shank-associated RH domain-interacting protein; TAB2, TGF-β-activated kinase 1-binding protein 2; TAB3, TGF-β-activated kinase 1-binding protein 2; tUIM, tandem UIM; TRABID, TRAF-binding domain-containing protein; UBAN, Ub binding in ABIN and NEMO; UIM, Ub-interacting motif; ZnF, zinc finger.

for growth in yeast.[49–51] Other hydrophobic interaction patches exist, including the Ile36 patch (Ile36, Leu71, and Leu73), which can mediate interactions between Ub moieties in chains and is bound by HECT E3s, DUBs, and some UBDs, and the Phe4 patch (Gln2, Phe4, and Thr12) that is bound by USP DUBs and some UBDs,[8] for example, the Ub-binding in NEMO and ABIN (UBAN) domain.[52] Many linkage-specific affinity reagents, both the ones based on natural domains as well as synthetic binders selected from libraries, rely on binding these surfaces on Ub, particularly the Ile44 patch (Figure 2).

Specific binding domains or affinity tools recognize polyUb through conformations, orientation, and interaction patch spacing that are unique to each linkage type (Figures 1 and 2).[17] Just as a key must align perfectly with the contours of a lock to turn, these binders engage at least two consecutive Ub moieties only when the topology, spacing, and orientation of the Ubs—particularly of the Ile44 patch—match the binder's interaction surfaces. Linkage type dictates the three-dimensional arrangement of Ub moieties (Figure 1). For example, K63-linked chains adopt an open conformation with exposed interaction surfaces, whereas

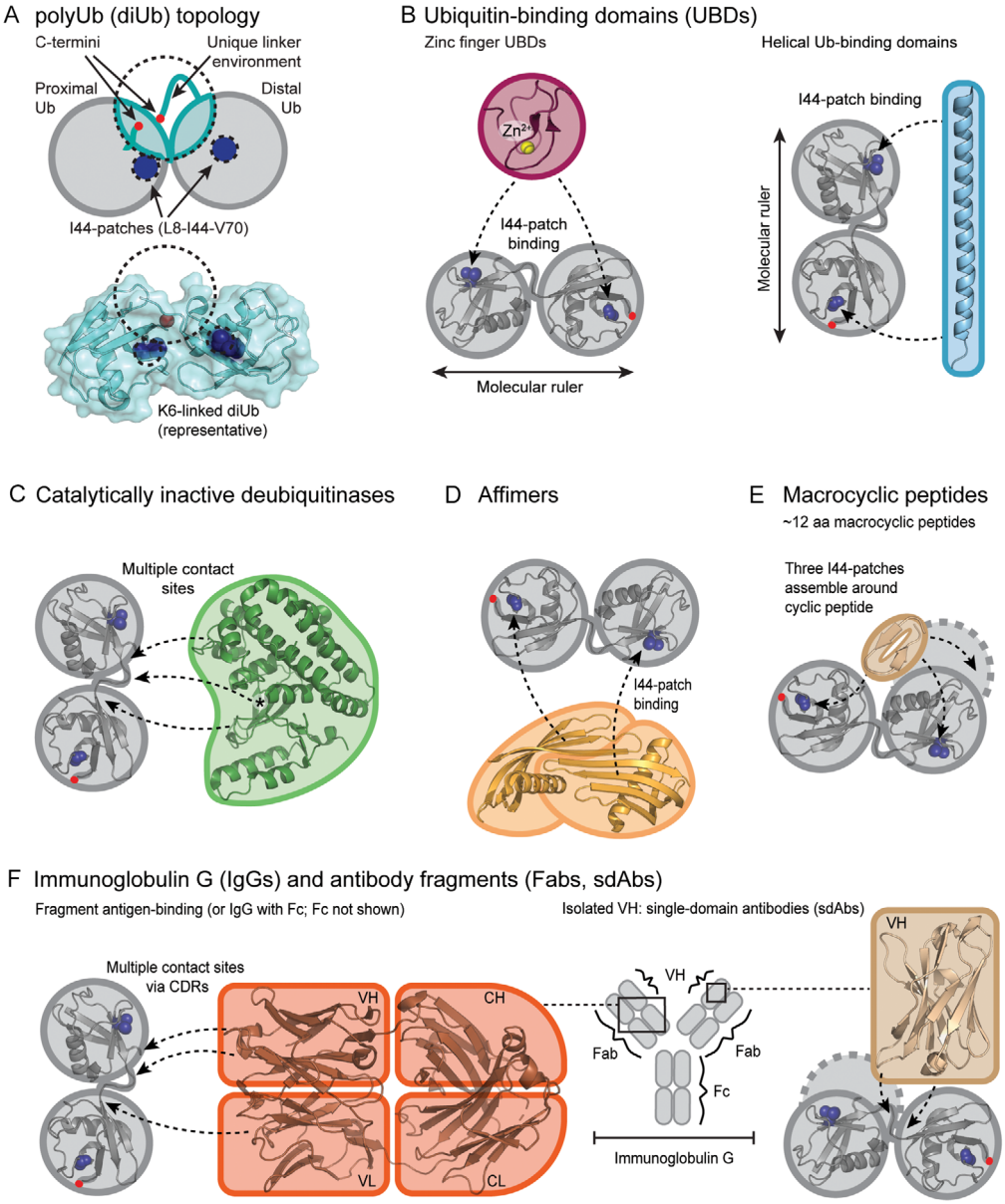

Figure 2. Classes of Ub linkage type-specific Ub affinity reagents. A) Each diUb (here: K6-diUb, 2XK5[178]) adopts distinct confirmations determined by the relative distance between the Ub moieties, the position of the linkage point, and the torsion angle of the linker, leading to a specific distribution of interaction patches, including the I44 patch (blue) for binding proteins to recognize. B) Examples of linage type-specific binding modes for two classes of UBDs, ZnFs and helical domains, which can both work as molecular rulers, mostly relying on spacing and orientation of the I44 patches for recognition. Structures shown are TRABID NZF (PDB: 5AF6[74]) and Rap80 UIM (PDB: 3A1Q[71]). C) DUBs, upon inactivation, can provide a very high level of specificity toward a defined linkage type. Their mode of binding is comprised of a large surface area centered around the linker, where they would cleave if left active, with multiple contacts made between DUB and Ub. Structure shown is catalytically inactive OTULIN-C129A (PDB: 3ZNZ[60]). D) Affimers are conceptually similar to antibody fragments, forming a surface area upon dimerization that binds to a unique configuration of the Ub linkage type. Structure shown is the K33-Affimer (PDB: 5OHV[119]). E) Macrocyclic peptides are small, versatile molecules that can be selected to bind specific Ub chains. Structure shown is the K48-specific Ub4a macrocyclic peptide (PDB: 8F1F[131]). F) Antibodies employ their hypervariable complementary-determining regions (CDRs) to bind the polyUb antigen. Smaller fragments of antibodies like Fabs, sdAbs, or nanobodies can be selected from libraries and have also been used as linkage-specific Ub affinity tools. Structures shown are Apu2.16 (PDB: 3DVN[54]) and NbSL3.3Q (PDB: 8A67[152]). The gray, representative diUb used as a schematic is PDB: 6N5M.[72] Abbreviations: CDR, complementary-determining region; CH and CL, constant heavy and light chains; Fab, fragment antigen-binding; Fc, crystallizable fragment; sdAb, single-domain antibody; VH and VL, variable heavy and light chains.

K48-linked chains fold compactly, shielding key residues. A binder, for example a UBD, achieves specificity only if its binding sites align precisely with the Ub moieties' topology, orientation, and spacing of hydrophobic patches. If the hydrophobic patches are oriented incorrectly or are spaced either too close or too far apart, the binder may engage only a single Ub, leading to weak or unstable interactions.[8,49] The development and engineering of some affinity reagents exploits this principle by fine-tuning the spatial organization of binding interfaces, effectively "reshaping the lock" to accommodate only one specific Ub linkage type. Ultimately, specificity depends on the precise interplay of distance, orientation, and surface compatibility, allowing only

the cognate Ub chain architecture (or the one intended by design or selection) to stably interact with a given binder.

The specific enrichment of a Ub linkage type is compromised if multiple different linkage types share the ability to adopt the conformation needed for binding to the affinity reagent.[53] This results in reagents that are not specific but can be "selective" or "non-selective", depending on the range of linkages that can be bound by the reagent. Enrichment with selective or cross-binding (i.e., binding to unintended linkage types) affinity reagents can be useful, but the resulting data must be interpreted with care if the intention is to draw conclusions about only one linkage type.

When considering the specificity (or selectivity) of a reagent, the dissociation constant (K_d) of the reagent to the linkage of interest compared with one or several other linkages is often used as a good measure: the affinity reagent should have a low K_d (high affinity) for the target linkage, and no or low affinity (high K_d) for other linkages. For example, specific reagents like the K48-specific Apu2 antibody and the K63-specific Apu3 antibody (Table 1) have very high affinities (single-digit nanomolar K_d) and no detectable binding to K63-linkages or K48-linkages, respectively.[54] Other aspects to review when considering the specificity of a reagent is how many linkage types it has been tested against for cross-binding. All eight amide linkages exist in cells,[36–38] but not all reagents have been tested for cross-binding toward all possible off-targets, partly because the complete panel of all eight in vitro assembled amide Ub chains was not available at the time of testing. Moreover, it is important to consider for which applications the specificity has been tested. Specificity might depend on application and experimental conditions. The M1-linkage-specific 1E3 antibody, for example, was M1-specific on a panel of immobilized chains for immunoblotting but also immunoprecipitated K63-linked chains, and to a lesser extent K48-linked chains, from solution unless the immunoprecipitation was done in high concentrations of urea.[55]

One caveat to consider, particularly regarding atypical linkages, is that the concentration of the atypical linkages in cells, perhaps with the exception of K11 linkages, is usually considerably lower than the most abundant linkages types, K48 and K63; sometimes one to two orders of magnitude lower for some linkages, such as M1, K27, K29, and K33.[36–39] As a result, affinity reagents specific for low-abundant chain types, for instance M1-linkages, but with slight cross-binding to high-abundant linkages like K48 and K63, struggle or are disproportionately disadvantaged in cells or lysates where the off-target linkages are present at much higher concentrations than the cognate target. For example, M1-linked chains constitute $<0.5\%$ of cellular polyUb[37–39] and are often enriched using either the UBAN UBD of NF-κB essential modifier (NEMO) or a catalytically inactive version of the M1-linkage-specific DUB OTULIN[56–58] (Table 1). Both reagents bind M1-linked Ub with ≈100-fold preference over K63-linked chains.[52,59,60] In cell lysates where K63-linkages may be 10–100 times more abundant than M1-linkages,[36–39] there is a risk that enriching M1-linkages can lead to co-enrichment of K63-linkages; not because the affinity reagents are intrinsically poor or unspecific, but because of the substantial excess of the off-target linkage compared with the cognate M1-linkage. It is therefore important to understand the strengths and limitations of the linkage-specific affinity reagents used in any given experiment, whether for enrichment or detection by immunoblot or microscopy, and to consider potential pitfalls, validation experiments, and controls when assessing the role and function of a single Ub linkage type.

2.4. Classes of Affinity Reagents Used for Ub Chains

The reported reagents (Table 1) can be categorized into UBD-derived reagents, DUB-derived reagents, affimers, macrocyclic peptides, as well as antibodies (IgG), antibody fragments and derivatives (e.g., fragment antigen-binding regions (Fabs) and single-domain antibodies (sdAbs)/nanobodies) (Figure 2). Generally, UBD/DUB-derived reagents are mostly used for enrichment (affinity precipitation), but some might in principle work for detection as well if engineered and tagged accordingly for fluorescence or chemiluminescent detection and if they work under the respective experimental conditions. Affimers, cyclic peptides, and sdAbs/nanobodies are very versatile and can potentially be used for multiple applications. Antibodies are preferred for detection but can also be used for enrichment.

Once a linkage type-specific binder has been identified, either from an endogenous gene, for example a UBD or a DUB, or a protein identified from a library, such as an affimer, they need to be "functionalized"; that is, turning them from a Ub-binding protein into a research tool. How is that done? The general strategy is to identify a linkage-specific Ub-binding of interest, obtain the DNA sequence, and then clone it into a suitable expression vector, for example, a plasmid for expression in *E. coli* for and for subsequent purification. Some engineering can be done at this stage, for example, optimizing the binding properties by mutation or making a tandem design where multiple copies of the same unit, for example a UBD, are inserted into the vector to be produced as one multidomain protein with linkers between the domains.[61] The cloning stage is also where epitope tags, for example, polyhistidine (His), FLAG, human influenza hemagglutinin (HA), HaloTag or Glutathione-S-transferase (GST) tags, or biotinylation sites are added for the purification after expression and for the actual functionalization, that is, the handle through which the reagent will be enriched or detected. Different affinity or epitope tags have certain strengths and weaknesses, and the choice might be influenced by user preference or specific application. Notably, the GST-tag has been shown to cause dimerization, possibly introducing an avidity effect, which in some cases might lead to changes in specificity or binding for some reagents.[46,61] Some reagents (e.g., OTULIN) need a specific inactivating mutation to work as a binder.[57,60] That must be mutated at this stage as well. Most reagents, except the antibody reagents, are suitable for standard recombinant expression in *E. coli* strains and subsequent protein purification, whereas others, like antibodies, require expression in mammalian cell systems.[46,62]

Tools for nonspecific Ub binding and enrichment are also available. Beyond non-selective anti-Ub antibodies, some of the most used reagents for non-selective Ub chain enrichment are tandem-repeated ubiquitin-binding entities (TUBEs)[63] based in the UBA domain of Ubiquillin,[63] MultiDsk (Dsk2 UBA

tandems),[64] or OtUBD,[65] which are all based on tandem constructs of non-selective UBDs.[48] These reagents bind common, broadly adoptable polyUb conformations or bind to each individual Ub moiety in a flexible manner, often relying on avidity.[49,53]

2.5. UBD-Based Reagents

UBDs are modular domains that bind non-covalently to Ub modifications, usually with relatively low affinity ($K_d \approx 20–500\ \mu M$).[17] The human genome encodes multiple UBDs with naturally occurring preference of specificity for certain Ub linkages (Figure 2B). Some of these have been exploited and engineered in several ways and are widely used as affinity reagents for Ub chain enrichment (affinity precipitation) and as labeled sensors in fluorescence microscopy.

UBDs can be categorized structurally into groups[49,66] defined as α-helical (e.g., Ub-interacting motif (UIM), VPS27, HRS, and STA domain (VHS)), Ub-associated domain (UBA), zinc fingers (e.g., Ub-binding ZnF domain (UBZ), Npl4 ZnF domain (NZF), Pleckstrin-homology (PH) domain), Ub-conjugating domain (UBC)-like, and several unique domain types, notably the UBAN domain.[17]

While most UBDs appear promiscuous,[67] binding to multiple linkage types, some UBDs are highly specific and bind almost exclusively to a defined linkage type.[17] Some of these domains have been engineered as affinity tools by cloning, expressing, and purifying them as isolated domains fused to epitope tags (e.g., FLAG-, GST-, or HaloTag) or biotinylated for detection and enrichment. UBDs can, when isolated and engineered as affinity reagents, suffer from low affinity. Endogenously, protein multi- or oligomerization in combination with single proteins containing multiple UBDs, the presence of multiple UBDs within a protein complex, and multiple Ub-binding surfaces in individual UBDs, form the basis for multivalent Ub-binding that can enhance avidity and improve binding to Ub.[68] To overcome low affinity of endogenous UBDs, fusing multiple identical domains in tandem can enhance the avidity and binding significantly over the isolated UBDs.[61–63,69] For this approach to be successful, however, some engineering of spacing and linker lengths between the individual domains may be required. This is illustrated, for example, by the Rx3(A7) Rap80 tUIM construct, in which linker engineering between the three Rap80 UIMs fused in tandem improves their relative orientation and spacing for binding to the target K63-linked chains, leading to enhanced affinity toward K63 chains and increased specificity over M1-linkanges.[61] As of today, only helical and zinc finger UBDs have been used as UBD-based linkage-specific affinity reagents (Table 1).

PolyUb- or linkage type-selective/specific UBDs often make contacts to one or more hydrophobic patches on at least two Ub moieties, mainly the Ile44 patch but also the Ile36 the Phe4 patches[8,17] (**Figure 3**A–C). Specificity in naturally occurring as well as engineered UBDs is mainly achieved through spacing and orientation of the interaction surfaces on the UBD with only specific Ub chain topologies and hydrophobic patch distribution being able to bind the UBD's interaction surfaces.[70,71] Some UBDs "selectively" bind to a small repertoire of linkage types that can adopt similar confirmations and hydrophobic patch distributions,[72–74] while others are non-selective.

The structurally simple α-helical UIMs of receptor-associated protein 80 (Rap80) (Figure 3A), vacuolar protein sorting-associated protein 27 (Vsp27), and EPS-15-interacting protein 1 (EPSIN1) have been exploited as linkage-specific affinity reagents, engineered in a tandem-UIM format (tUIM) fused to both with GST- or HaloTag, for K63-specific enrichment and localization studies by fluorescence microscopy.[57,61,71,75,76] The Rap80 Rx3(A7) tUIM construct[61] is the most widely used UIM-based reagent. Rap80 specifically recognizes K63-linked Ub chains through the UIMs each binding to an Ile44 patch of Ub (Figure 3A). UIM1 interacts with the proximal Ub Ile44 patch via a hydrophobic surface centered around Ala88, and UIM2 interacts with the distal Ub Ile44 via a similar surface centered around Ala113.[71] For the design of the Rx3(A7) tUIM construct, the linker length and linker composition between the three UIMs placed in tandem needed careful optimization for ideal spacing of the key Ala residues to improve the binding constants and K63/off-target binding ratios.[61] Following the same principle, engineering one of the two motif interacting with Ub (MIU) domains of MIU-containing novel DUB family 1 (MINDY-1) into the tandem-MIU2 domains yielded a potent K48-specific binder that prefers binding longer chains.[75] Another tandem-reagent is UIMLx2, engineered from the UIM-like (UIML) domain of the yeast transcription factor Met4 onto a SUMO/His/Avi-Biotin protein scaffold, enabling K48-selective enrichment.[77]

The UBAN domains of NEMO, A20-binding inhibitor of NF-kappa-B activation 1 (ABIN1), and Optineurin (OPTN) are structurally more complex, dimerizing α-helixes.[52,72] They have a strong preference for binding M1-linked polyUb (100-fold preference over K63 linkages)[52,59] via formation of a surface patch on a slightly intertwined, parallel coiled-coil dimer, called coil-zipper (CoZi)[52,78] (Figure 3B). Similar to the Rap80 UIMs, NEMO UBAN binds primarily to the Ile44 patch of the distal Ub of M1-linked diUb through hydrophobic interactions around Val293 and Ala296 but also makes contacts to the Ile36 patch.[52] In contrast, NEMO UBAN makes extensive polar contacts to the Phe4 patch on the proximal Ub via residues around Arg308 and Arg309 (Figure 3B). Unlike the Rap80 tUIMs, the NEMO UBAN has not undergone any engineering beyond fusion to a GST-tag for the use as an affinity reagent for enrichment of M1-linked chains.[56] Despite less optimization of the binding for this reagent termed the M1-linkage-specific Ub binder (M1-SUB), it has successfully been used to enrich and analyze M1-linked chains from cells, flies, and mouse tissues.[56,79,80]

While the extended helical Rap80 UIMs and NEMO UBAN domains recognize the distribution of hydrophobic patches in K63-linked and M1-linked open-confirmation Ub chains, respectively, much like molecular rulers (Figure 3A,B), other chain types adopt more compact structures (Figure 1) that other domains structures are better suited for recognizing. In contrast to the extended helical UIM, MIU, and UBAN domains, UBA domains consist of compact three-helix bundles. The UBA domains of UV excision repair protein RAD23 homolog A (RAD23A) and RAD23B (also known as hHR23A and hHR23B, respectively) are highly selective for K48 linkages.[70,81–83] The structure of

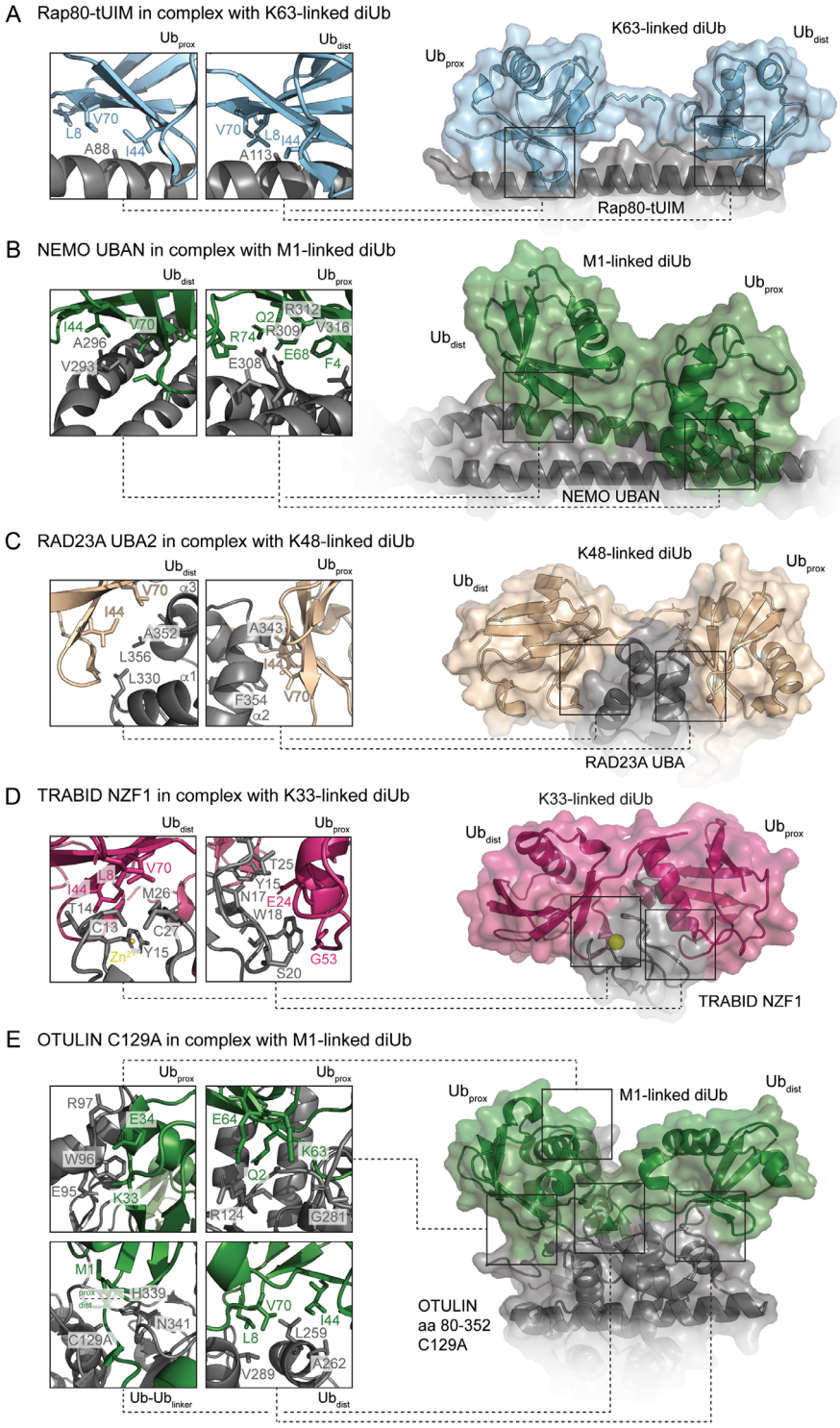

Figure 3. Binding modes of Ub linkage-specific Ubiquitin-binding domains and deubiquitinases that can be engineered into affinity reagents. A) Rap80-tUIM in complex with K63-linked diUb (PDB: 3A1Q[71]). The spacing between the UIMs and the Ub moieties works as a molecular ruler with the UIMs spaced exactly with the distance of the I44 patches in K63-linked Ub. B) NEMO UBAN in complex with M1-linked diUb (PDB: 2ZVO[52]). The extended helices of the NEMO UBAN domain work as a molecular ruler, similar to Rap80 UIM, but primarily contact the Ile44 patch of the distal Ub and Phe4 patch on the proximal Ub. C) RAD23A UBA2 in complex with K48-linked diUb PDB: 1ZO6[82]). The UBA2 domain binds the compact K48 diUb by slotting in between the two Ub moieties, making contacts to the Ile44 patch on each moiety. D) TRABID NZF1 domain in complex with K33-linked diUb (PDB: 5AF6[74]). NZF1 interacts with the Ile44 patch on the distal Ub and the α-helix on the proximal Ub. NZF1 recognizes K29-linked diUb by a similar mode of interaction, explaining how this domain binds both chain types.[70] E) Catalytically inactive OTULIN (aa 80-352, C129A) in complex with M1-linked diUb (PDB: 3ZNZ[60]). OTULIN makes extensive contacts with up to 50 residues binding to M1-linked diUb, mostly hydrogen bonds to either or both Ub moieties, thereby forming a large interaction surface around the linker region. Abbreviations: NEMO, NF-κB essential modifier; NZF, Npl4 zinc finger; OTULIN, OTU deubiquitinase with linear linkage specificity; RAD23A, UV excision repair protein RAD23 homolog A; Rap80-tUIM, Receptor-associated protein 80-tandem Ub-interacting motif; TRABID, TRAF-binding domain-containing protein; UBAN, Ub-binding domain in ABINs and NEMO.

RAD23A UBA2 in complex with K48-linked diUb shows that UBA2 recognizes the compact K48 chain by slotting in between the two Ub moieties (Figure 3C). The UBA2 makes contacts primarily to the Ile44 patch on each moiety. The UBA2-α2 helix binds the Ile44 patch on the proximal Ub via residues around Ala343, while the UBA2-α3 helix makes contacts to the Ile44 patch in the distal Ub through a patch around Ala352 (Figure 3C). In the widely accepted model of K48-linked Ub, the Ub moieties interact via their Ile44 patches[84–86] (Figure 1), which means that it requires dynamic opening of the compact K48-linked chain for the UBA2 domain to insert itself in between the two Ile44 patches.[82,87] Exploiting this mechanism and specificity, the UBA domains of RAD23B, which shares 78–95% sequence identity with the RAD23A domains, have been used to enrich for K48-linked chains, both as Halo- and GST-tagged versions.[70,88]

Lastly, zinc finger domains (ZnFs) adopt a compact fold coordinated around a zinc(II) ion, forming a versatile and highly abundant interaction domain.[89,90] ZnFs usually, but not exclusively, form a hydrophobic interface against the Ile44 patch (Figure 2B and 3D), often with a structure resembling the Npl4 ZnF (NZF).[91] As affinity reagents, the isolated, GST-tagged NZF-domains of Heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1) and Shank-associated RH domain-interacting protein (SHARPIN), both components of the linear Ub chain assembly complex (LUBAC),[43] were shown to preferentially bind M1-linked polyUb, with a cross-binding of SHARPIN's NZF toward K63-linked polyUb.[73,92] The NZF-domains of TGF-β-activated kinase 1-binding protein (TAB)2 and TAB3 bind selectively to K63-linked polyUb[93–95] and have been used as affinity reagents coupled to Strep-tag, GST and HaloTag.[73] Another intriguing reagent is the Halo- or GST-tagged TRAF-binding domain-containing protein (TRABID) NZF1, which binds K29- and K33-linked Ub chains.[70,74] TRABID has three NZF domains (NZF1-3). The NZF1-3 module binds K29/33-linked chains, but the specificity of this UBD module can be attributed entirely to NZF1. In complex with K33-linked diUb, the NZF1 interacts with the Ile44 patch on the distal Ub via hydrophobic interactions primarily with Thr14, Tyr15, and Met26 (Figure 3D).[74] Unlike other NZFs, TRABID NZF1 does not bind either the Ile44 or Phe4 patches on the proximal Ub. Instead, the NZF1 contacts residues in the proximal Ub α-helix, particularly Glu24, which is bound by Tyr15, Asn17, Trp18, and Thr25 in TRABID (Figure 3D).[74] This unusual binding to the proximal Ub explains how TRABID and the TAB2 and HOIL-1 NZFs achieve specificity. Each domain orients the proximal Ub differently. While TRABID NZF1 binds the proximal Ub α-helix,[74] TAB2 binds via the Ile44 patch,[93,94] and HOIL-1 uses a helical NZF extension to bind the Phe4 patch of the proximal Ub.[92] The structure of TRABID NZF1 in complex with K29-linked diUb reveals a mode of interaction similar to K33-linked Ub, explaining how this domain binds both chain types.[70] TRABID NZF1 can be used for pulling down K29/33-linked chains[70,74] or detecting them in signaling puncta in cells via fluorescence microscopy.[74] Overall, UBDs are the backbone of the current set of linkage-specific affinity reagents and with additional domains likely to be discovered, some may even be critical to investigate non-canonical Ub-chains,[20] for which there are no reported tools for enrichment.

2.6. Inactive DUBs

DUBs are specialized proteases that hydrolyze and cleave Ub moieties from each other and/or their target protein, thereby terminating or modulating the signal from the Ub modification.[96] There are seven DUB families: Ub-specific proteases (USPs), Ub C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Machado-Joseph deubiquitinases (MJDs), motif interacting with ubiquitin-containing novel DUB family (MINDYs), zinc finger-containing Ub peptidase 1 (ZUP1), and Josephins and JAB1/MPN/MOV34 DUBs (JAMMs; also known MPNs). USPs, UCHs, OTUs, MJDs, MINDYs, and ZUP1 are cysteine proteases, while the JAMMs are zinc-dependent metalloproteases.[19,97] While some, or likely most, DUBs are non-selective or cleavage a large subset of Ub linkages, some are highly specific for a certain linkage type.[98] DUBs specific or selective for virtually all canonical Ub linkages have been identified.[19] This has been exploited to enzymatically cleave and remove the targeted Ub linkage type(s) both in analytic assays (e.g., UbiCRest)[99] as well as in the enzymatic (dis)assembly of defined Ub-chains in vitro.[100]

Some DUBs rely on auxiliary UBDs to bind Ub, which usually leads to a preference for binding and cleaving longer chains,[101] for example, the MIU domains of MINDY-type DUBs set it up to trim down longer K48-chains,[102] or a preference for linkage-type by orienting the substrate linkage for hydrolysis by the catalytic domain, such as TRABID that requires its AnkUBD (ankyrin-repeat ubiquitin-binding domain) for preferentially cleaving K29- and K33-linked chains.[103] Many specific or selective DUBs, however, achieve their linkage preference intrinsically in their catalytic domain.[98]

This intrinsic ability of certain DUBs to distinguish between Ub chain linkages suggests that, if catalytically inactive DUBs retain their binding and specificity toward Ub chains, inactivating and engineering DUBs for the development of linkage type-specific reagents could hold great potential. The idea of turning inactivated DUBs into linkage-specific binders (essentially a UBD) was pioneered for the M1-specific DUB OTULIN.[60] It was observed both biophysically and in cells that mutating OTULIN's catalytic Cys129 residue to Ala (Cys129Ala) did not abrogate the binding of OTULIN to M1-linked chains, but rather caused M1-linkages to accumulate in cells, suggesting that the OTULIN-Cys129Ala mutation converted OTULIN from a DUB into a high-affinity ($K_d \approx 100$ nM) M1-specific UBD through its extensive interaction interface with M1-linked diUb.[56,60] OTULIN binds M1-linked diUb via extensive contacts between the OTU domain and both the distal and proximal Ub moieties (Figure 3E).[60] OTULIN binds both Ile36 and Ile44 patches of the distal Ub through hydrophobic interactions, particularly around Leu259 (S1 site), to the Ile44 patch.[60] A wide array of contacts that orients M1 toward the catalytic center is made between OTULIN and the proximal Ub. Particularly Trp96 (S1′ site) makes contacts to the α-helix of the proximal Ub and two loops create a binding pocked for K63, which is spatially close to M1, wedging it away from the catalytic center, likely contributing to OTULIN's specificity.[60] The importance of this part of the S1′ site is highlighted by a Gly281Arg mutation found in an OTULIN-related autoinflammatory syndrome (ORAS) patient, which lowers OTULIN's affinity—and thus activity—for M1-linked diUb by approximately two

orders of magnitude.[39] As OTULIN is a DUB, it of course also makes several contacts to the Ub–Ub linker between Met1 and Gly76 via the catalytic center consisting of Cys129, His339, and Asp341 (Figure 3E). These residues are critical for catalytic activity, but not for binding to M1-linked diUb.[60] But in the case of the catalytically inactive OTULIN-Cys129Ala mutant, the contact to the linker is important. The Cys129Ala mutation prevents catalysis and thereby release of hydrolyzed Ub, thus turning OTULIN into a high-affinity M1-binding UBD.[56,60] This principle was successfully exploited with the development of the M1-affinity purification (M1-AP) reagent (recombinant OTULIN aa 58–352, Cys129Ala, with a HaloTag), which was used to enrich M1-linked chains from cells.[57] A similar reagent, called the M1-Trap, which is also based on OTULIN-Cys129Ala, but which lacks the C-terminal ETSL-motif responsible for binding a range of PDZ domain-containing proteins,[104] can also be used for M1-purification from cells.[105]

In addition to OTULIN, Morrow and colleagues demonstrated that the principle is more broadly applicable. Catalytic inactivation by Cys-to-Ala mutations of OTU and USP DUBs can convert them from DUBs to UBDs (Figure 2C), and even in some cases increase their affinity for Ub.[106] However, the principle is not universally applicable. Locking a DUB in an inactivate state or conformation might keep it from binding Ub the same as if active, especially if the mode of specific binding depends on structural rearrangements during the catalytic cycle, as it is, for example, the case for the K11-specific DUB Cezanne.[107] Consequently, retention of binding is not guaranteed after the DUB is mutated for deactivation. This will have to be individually evaluated for each DUB.

Although OTULIN could appear to be a "lucky find" among the human linkage-specific DUBs, having been the only deactivated DUB used as a linkage-specific affinity reagent for years, recent findings could indicate possible avenues for extending the repertoire of these reagents. In recent years, many bacterially encoded DUBs have been identified. Despite not having a Ub system of their own, some pathogenic bacteria encode DUBs, or even E2 or E3 enzymes, which are secreted into host cells to counter or modulate the host's polyUb signals as part of the response to infection.[108] Intriguingly, some of these bacterial enzymes exhibit remarkable specificity toward individual Ub linkages,[109] which could make them useful molecular tools. One example is the *Legionella* ovarian tumor-like protein A (LotA) DUB from *Legionella pneumophila*,[110] the bacterium causing Legionnaire's disease. LotA's N-terminal OTU domain ($LotA_N$) has remarkable K6-specificity,[111,112] and an isolated and catalytically inactivate (Cys13Ala) $LotA_N$ domain is reportedly able to enrich K6-linked Ub chains from cells for further analysis.[111] As more bacterial DUBs are identified, the repertoire of potentially engineerable, linkage-specific DUBs that could be developed into affinity reagents or tools increases.

2.7. Affimers

Apart from naturally occurring domains like UBDs and DUBs, synthetic proteins and scaffolds also make up a sizeable part of the available reagents (Table 1). Their main advantage is often that they can be selected or screened from large libraries in a systematic and high-throughput manner. One type of synthetic reagent is the affimer. Affimers are synthetic non-antibody proteins that mimic the binding and recognition characteristics of antibodies through highly variable loops (**Figures** 2D and **4**A). Affimers consist of a soluble, rigid β-sheet scaffold that holds multiple hypervariable loops in place for antigen binding.[113] Similar to antibodies and other synthetic binders, affimers can be selected through phage display.[113,114] The core molecular structure is derived from the cysteine protease inhibitor cystatin, and affimers serve in general as a good example of how to design screenable, highly variable binding sites onto a stable scaffold.[114,115] While affimers can be advantageous over other antibody and antibody-like proteins in some aspects, particularly around pH and temperature stability as well as protein production,[113,116] antibody fragments are very similar to affimers regarding size, affinity range, and functionalization.[117,118]

Two high-affinity (K_d in low nM to pM range) affimers developed against K6- and K33-linked Ub (Table 1) illustrate the power and usefulness of these reagents. Both affimers dimerize and bind their cognate Ub targets in conceptually similar ways by engaging the Ile44 patches of Ub.[119] As exemplified by the symmetric K6 affimer dimer, Leu74, Ile105, Met107, and Met110 in the variable loops of each monomer create a hydrophobic surface that interacts with the Ile44 patch of Ub (Figure 4A). As such, the two affimer molecules in the dimer each bind one Ub molecule, and each affimer, through their variable loops, thereby binds two Ub moieties of K6-linked diUb with a defined distance and relative orientation between them, leading to specific, high-affinity binding (Figure 4A).[119] The affimers can be used for detection (immunoblotting) and the K6 affimer has been employed for fluorescent confocal microscopy analysis and enrichment of K6-linked chains from cells.[119]

Interestingly, the K6 and K33 affimers dimerize differently, leading to distinct modes of diUb binding. The K6-specific affimer forms a symmetric dimer via β-strand swaps through its variable loops, similar to naturally occurring cystatin, whereas the K33-selective affimer, which binds K11 linkages with only slightly lower affinity than K33 linkages, dimerizes via formation of an intermolecular β-sheet.[119]The rather unique conformation and binding mode of the K33-selective affimer shows the flexibility and versatility of the affimer scaffold and indicates that affimers could be a source of binders for targets that might be structurally inaccessible for other types of affinity reagents. This underscores the importance of having access to a diverse set of scaffolds and reagent types.

2.8. Macrocyclic Peptides

A structurally and conceptually very different synthetic scaffold for the development of Ub- and linkage-specific affinity reagents is the macrocyclic peptide. Macrocyclic peptides are, as the name implies, circular peptides with potential use as small-molecule inhibitors as well as affinity reagents (Figure 2E).[120] They are singular, circularized, hypervariable peptides ranging in size

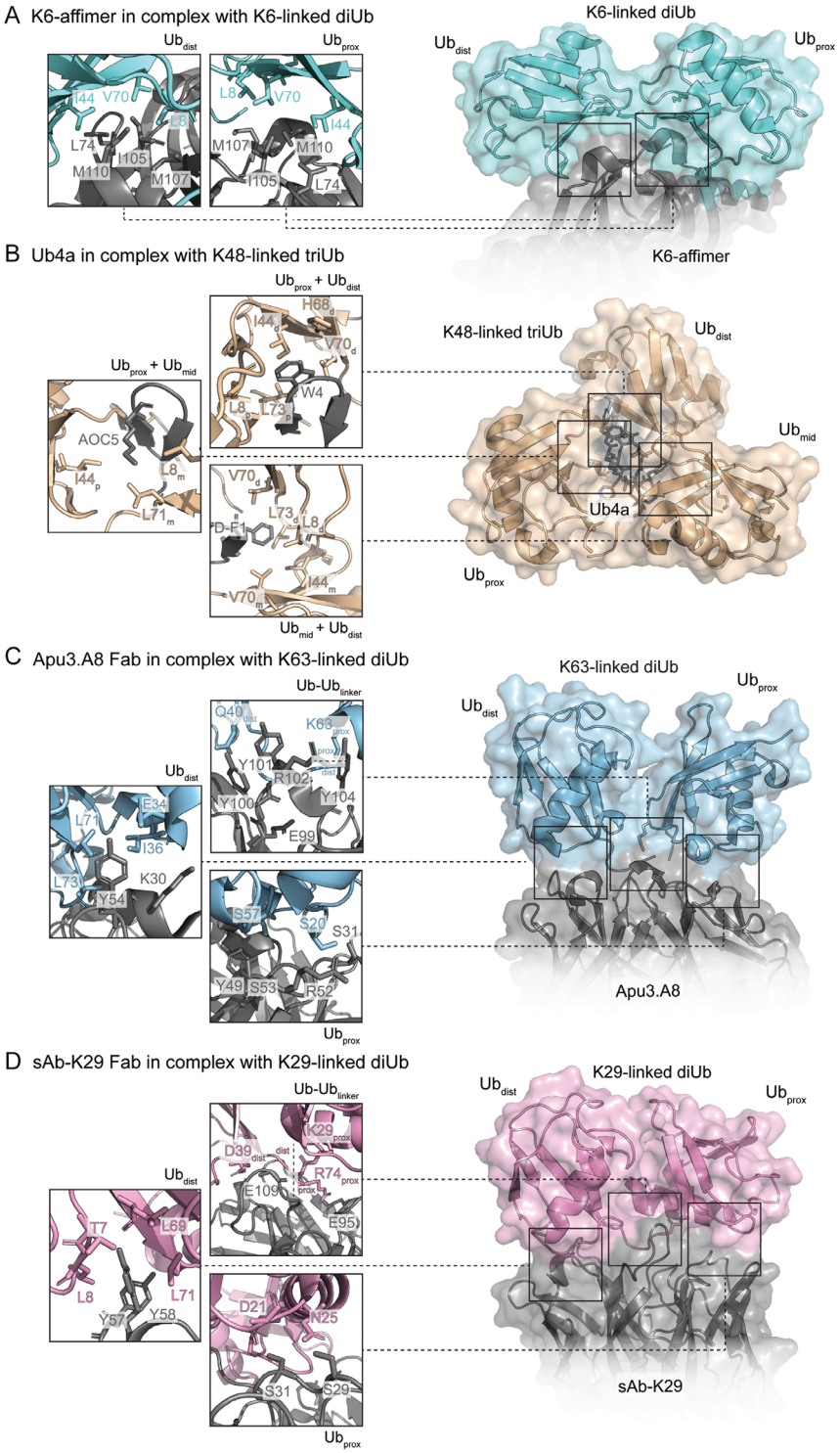

Figure 4. Binding modes of Ub linkage-specific affinity reagents derived from synthetic libraries. A) K6-Affimer in complex with K6-linked diUb (PDB: 5OHL[119]). In the symmetric K6 affimer dimer, each monomer binds the I44 patch of one Ub molecule through their variable loops, and as such the dimer works as a molecular ruler recognizing the specific distance and relative orientation between the I44 patches in K6-linkned Ub, leading to specific binding. B) Ub4a macrocyclic peptide in complex with K48-linked triUb (PDB: 8F1F[131]). The K48-specific cyclic peptide Ub4a gets fully embedded and surrounded by the K48-linked trimer, employing a mix of van der Waals and hydrogen bonds. The orientation of the I44 patches toward the peptide locks the K48-Ub3 in a unique confirmation, enabling the specific recognition. C) Apu3.A8 Fab in complex with K63-linked diUb (PDB: 3DVN[54]). Apu3 makes contacts to the Ile36 patch on the distal Ub, the α-helix on the proximal Ub, and to the context of the Ub-Ub linker, but not the isopeptide linkage itself. D) sAB-K29 Fab in complex with K29-linked diUb (PDB: 7KEO[137]). The interaction is dominated by hydrogen bonds conferred exclusively by the Fab's CDRs, which bind to both Ub moieties and the linker region. V_H CDR3 is the main contributor, looping into the linker region of the K29-diUb. Abbreviations: AOC, 2-aminooctanoic acid; CDR, complementary determining region; Fab, Fragment antigen-binding; V_H, variable heavy chain.

between peptides and proteins at 1–10 kDa.[121,122] They are locked covalently into a circular configuration, providing rigidity and structural constraints, which is important for specific binding.[123] In general, macrocyclic peptides offer certain advantages over screenable proteins, such as enhanced diversity due to the possibility of incorporating unnatural amino acids, increased library sizes used in mRNA display, and potentially increased therapeutic potential through membrane penetration.[124] In fact, macrocyclic peptides have seen heavy use as therapeutics already, often as inhibitors or blockers of epitopes or protein–protein interaction surfaces.[125,126]

In the context of Ub, several specific macrocyclic peptides have been selected against K63-linked chains[127] and K48-linked chains.[128–130] The length of the peptides range between 12 and 16 amino acid residues with high affinities ($K_d \approx 1$–40 nM). The K48-specific, 12 amino acid residue circular peptide Ub4a[130] has been crystallized with K48-linked triUb.[131] The structure reveals that Uba4 uses almost all its residues to bind the three Ub moieties in the K48-linked triUb, wrapping the K48-linked triUb chain tightly around the peptide in a compact ring[131] (Figure 4B). Uba4 exposes its hydrophobic elements, which engages the Ile44 patches on each of the three Ub moieties in the K48-linked chain. Due to the compact packing of the Ub chain around the peptide, multiple of Ub4a's residues are able to make contacts to two Ile44 patches: for example, the unnatural 2-aminooctanoic acid reside 5 (AOC5) makes contacts to Ile44 of the proximal Ub and Leu8 and Leu71 of the middle Ub, while D-Phe1 makes contacts to Ile44 and Val70 in the middle Ub and Leu8, Val70, and Leu73 in the distal Ub (Figure 4B).[131]

Specific macrocyclic peptides have been selected for both K48- and K63-linked Ub,[127,128] but structural information about the peptide-Ub binding mode is only available for the K48-binding Ub4a peptide.[131] It is therefore unclear what range of binding modes may exist for this class of reagents. K48-linked chains adopt compact folds with the moieties packing closely together,[132] whereas K63-linked chains adopt extended conformations, akin to beads on a string[97] (Figure 1). The fact that peptides specific for binding either have been identified suggests that different binding modes, which engages the chains in different conformations, exist.

The linkage-specific macrocyclic peptides have been used to block proteasomal degradation,[128,130,131] for imaging and localization studies in cells coupled to the fluorophores TAMRA[127] or fluorescein,[128] as well as biotinylated for enrichment and subsequent mass spectrometry analysis.[127] This range of applications highlights the versatility of macrocyclic peptides for the use as linkage-specific Ub binders, especially given the emerging tools allowing more straightforward in silico design of macrocyclic peptides.[133]

2.9. Immunoglobulin (IgG) and Fragment Antigen-Binding (Fab)

An obvious choice for the generation of specific affinity reagents, including linkage-specific Ub affinity reagents, is of course antibodies or antibody-based formats. And ≈40% of the linkage-specific reagents reported in the literature are antibodies or antibody-based molecules (Table 1). Many more linkage-specific antibodies are commercially available from a range of vendors, but as the testing and validation data for these are limited, they are not included in this review.

The main formats of antibodies used as affinity reagents are the full-length immunoglobulin G (IgG) and fragment antigen-binding (Fabs) (Figure 2F). IgGs are comprised of a crystallizable fragment (Fc) and two Fabs, which interact with the antigen via their hypervariable loops, called complementary-determining regions (CDRs), on the variable heavy (V_H) and light chains (V_L) (Figure 2F).[134,135]

The validated linkage-specific antibodies or Fabs target M1, K11, K27, K29, K48, and K63 linkages (Table 1). The antibodies are either generated via Fab-based phage display or, more rarely, animal immunization. Some of the specific binding modes have been elucidated through crystal structures. Generally, the antibodies or Fabs make multiple contacts to both the proximal and distal Ub, and in some cases to the actual linkage to obtain specificity, with the CDRs on V_H often making the majority of the contacts[54,55,136,137] (Figure 2F). For example, the V_H of the K63 linkage-specific Apu3 (Apu3.A8 clone) antibody makes extensive contacts to the Ile36 patch on the distal Ub and to the context of the Ub-Ub linker where Glu99, Tyr100, and Tyr101 binds the distal Ub moiety and Arg102 and Tyr104 contacts the proximal Ub moiety[54] (Figure 4C). V_L also binds Arg74 in the linker, which points away from the diUb interface, through Tyr91 and Ser96 and makes contacts to the Ser20- and Ser57-containing loops (Figure 4C). Similar to Apu3, the V_H of sAb-K29 makes considerable contacts to the distal moiety of K29-linked diUb, including via Tyr57 and Tyr58 binding to residues around both the Ile44 and Ile36 patches[137] (Figure 4D). The Ub-Ub linker and interface are contacted by both the V_H and V_L. V_H makes several contacts via multiple aromatic residues, including Trp111 that slots in between the K29-Gly76 isopeptide linkage and the V_L, and through Glu109 that forms a hydrogen bond with Asp39 on the distal Ub[137] (Figure 4D). The V_L binds Arg74, which, similar to Apu3, points away from the Ub-Ub linker and interface (Figure 4D). In addition, V_L contacts the 20's loop of the proximal Ub via multiple hydrogen bonds between Ser29 and Ser31 and surrounding residues (Figure 4D). Compared with free K29-linked diUb or K29-linked Ub bound to TRABID NZF,[70] sAb-K29 bends the K29 diUb into a compact conformation and rotates the proximal Ub to bind a unique confirmation.[137]

For antibodies selected as Fabs in phage display, the Fabs are grafted onto an IgG scaffold to produce a full, bivalent antibody,[54,55,136] but both the reported Fabs and full-length IgGs exhibit high specificities and high affinities (low, often single-digit, nM range) toward their cognate targets in a range of applications, including detection (immunoblot), immunoprecipitation, and fluorescence microscopy.[54,55,136,137] Generally, for these reagents, the strategy to achieve high specificity in the selection and development process of these antibodies has been to include off-target linkage types for negative or counter-selection early in the selection process. While working very specifically for immunoblotting on purified polyUb chains, some reagents are challenged when exposed to complex mixtures of Ub chains

or having to bind Ub in solution rather than Ub adsorbed to a membrane. For example, the specificity in immunoprecipitations for the M1-specific 1E3 clone, the K11-specific 2A3/2E6 clone, the Apu2 K48-specific clone, and the Apu3 K63-specific clone is enhanced considerably by performing the immunoprecipitation in the presence of 4–7 M urea.[54,55,136] This underscores the need for proper validation of linkage-specific antibodies and shows that the performance in immunoblotting cannot necessarily be extended to other applications. Rather, individual antibodies likely need optimization and specificity for each individual application.

Apart from just recognizing defined, homotypic linkages, bispecific antibodies have been engineered to recognize heterotypic chains and substrates modified with specific Ub linkages. A K11/K48-bispecific antibody was designed by using knobs-into-holes technology,[138] creating an antibody with one arm being the Apu2 K48-specific Fab and the other arm being the 2E3/2A6 K11-specific Fab.[139] This first bispecific anti-Ub antibody was used to elucidate the role of K11/K48 heterotypic chains in protein quality control.[139]

Recently, a new type of bispecific antibody was published, recognizing a specific Ub linkage type attached to a specific substrate. Goncharov and colleagues report the generation of bispecific antibodies for the two proteins Receptor-interacting protein kinase (RIP)1 and RIP2[140] (Table 1), which are heavily ubiquitinated with K63- and M1-linkages during innate immune signaling.[43,141,142] The RIP1-K63, RIP1-Lin, RIP2-K63, and RIP2-Lin antibodies were also generated by the knobs-into-holes approach and used for immunoblotting, immunoprecipitation, and fluorescence microscopy.[140] Antibodies or reagents like these may aid in unraveling linkage-specific Ub signaling in specific contexts in the same way histone- and modification-specific antibodies were important for elucidating the histone code and epigenetic mechanisms.[143]

When using IgGs as affinity reagents in the context of the Ub system, the limit is usually not availability of reagents, as it is the case with, for example, naturally occurring UBDs or DUBs, but rather the ability to find linkage-specific reagents. Many commercially available IgG antibodies marketed as Ub linkage-specific lack extensive validation and proper documentation for their specificity and sensitivity. Unspecific antibodies in general has become a point of focus and criticism from researchers in recent years.[144–147] Ub linkage type-specific antibodies, for the conceptual reasons explained earlier and by Emmerich and Cohen, require even more thorough validation, at least for applications beyond detection of isolated, recombinant Ub chains, than antibodies against targets that can be validated genetically.[46] Some commercially available linkage-specific antibodies were validated for their specificity retrospectively in publications and are cited as such in Table 1. But it is also well-known that different anti-Ub antibodies do not bind and detect all linkage types equally.[46] With often limited validation of commercially available linkage-specific antibodies, there is a risk of similar inconsistencies in their actual specificity versus their claimed specificity. Failure to achieve specificity can cause not only practical and experimental challenges but also problems with data interpretation.

2.10. Single-Domain Antibodies

Single-domain antibodies (sdAbs), also referred to as nanobodies, are small 12-15 kDa antibody fragments consisting of a single monomeric variable antibody domain containing three CDRs that have emerged as important therapeutics and affinity reagents over the last decades.[148] Heavy-chain antibodies were originally discovered to be naturally evolved in camelids (V_HH) and cartilaginous fish (V_{NAR} fragments) are termed nanobodies.[149,150] Since then, the field expanded into the use of heavy chains from human antibodies, sdAbs, which have been isolated and engineered as smaller alternatives to common antibody formats, like IgGs or Fabs, offering screening, enhanced design flexibility, cell penetration, and simpler production.[151] These features alone qualify sdAbs as a great format for linkage-specific Ub affinity reagents. Intriguingly, analyzing contacts between linkage-specific IgGs and Fabs and their cognate targets usually reveals that V_H makes substantially more contacts to the Ub molecule than the V_L. For example, for the sAB-K29 Fab and the M1-specifi 1E3 IgG, the V_H makes ≈80% and ≈74% of the contacts, respectively, to the diUb[55,137] (Figure 3E). This indicates that using V_H-based sdAbs could be a viable strategy for generating new linkage-specific affinity reagents. A recent study by Yogesh Kulathu's laboratory provides proof-of-concept for this notion as they succeeded in selecting and optimizing a specific, high-affinity ($K_d \approx 0.2$ nM) K48-K63 branched triUb binder through yeast surface display.[152] The high specificity and affinity of the NbSL3.3Q sdAb underscores the potential of this scaffold for the development of affinity reagents.

2.11. Ubiquitin Mutants and Ubiquitin Replacement Strategies

In the absence of high-affinity or high-specificity linkage-specific reagents suitable for a specific application, the use of mutated or tagged Ub has been a widely adopted method for studying ubiquitination. Ub can be fused to various affinity tags, including His-tag, AviTag for biotinylation, and epitope tags like HA, Strep, or FLAG, which are typically fused to the N-terminus of Ub. The tagged Ub can be ectopically expressed in cells or tissues where it becomes incorporated into Ub chains and conjugated to substrates. These ubiquitinated proteins can then be captured using affinity purification methods specific to the tag, enabling their enrichment and subsequent analysis by, for example, immunoblotting or mass spectrometry.[153–155] This approach is often used to study the involvement of specific linkage types in substrate ubiquitination and signaling by ectopically expressing Ub mutants with one or several lysine residues exchanged by arginine (R), referred to as either K-to-R mutants (e.g., Ub-K48R), in which K48 has been exchanged for arginine, or K-only mutants (e.g., K48only), in which all Lys residues but K48 has been exchanged for arginine. These mutants are used to assess the dependence on a specific lysine linkage for ubiquitination of a substrate, often by tag-based enrichment and immunoblot analysis, thereby inferring the linkage type conjugated to the substrate. For example, if expression of Ub-K48R results in reduced

ubiquitination of a substrate compared with wildtype Ub, this indicates that the substrate is modified with K48-linked chains. While this approach is valuable, ectopic- or overexpression of Ub and Ub mutants can result in excessive, non-physiological ubiquitination of cellular proteins, potentially altering cellular responses,[156] and K-to-R or K-only mutations may cause structural changes in Ub.[157] Discrepancies in linkage type-specific analyses exist between cells expressing Ub mutants and the biochemical evidence of the specificities of the E3 Ub ligases involved in the ubiquitination process. For example, using expression of tagged Ub mutants, the E3 ligases HECT, UBA, and WWE domain-containing protein 1 (HUWE1) and E3 ubiquitin-protein ligase NEDD4-like (NEDD4L) are reported to assemble K27- and K29-linkages, respectively, during immune signaling.[158,159] But enzymatically, HUWE1 assembles a mix of K6-, K11-, and K48-linkages[119] and NEDD4L specifically assembles K63 linkages.[160] Careful consideration must therefore be given to data interpretation and potential pitfalls when employing overexpression strategies.

To mitigate potential pitfalls and artacts associated with Ub overexpression, an alternative approach involves the stable exchange of endogenous Ub with an epitope-tagged version.[161] In this Ub replacement system, endogenous Ub is knocked down using shRNA, while an shRNA-resistant Ub variant, for example, a tagged Ub or Ub mutant, is expressed to a similar level as endogenous Ub in an inducible manner, thereby enabling the exchange of endogenous, wildtype Ub with for example an N-terminally tagged K-to-R mutant of choice.[161,162] This system can further be modified to include an internal His tag, inserted between Ser65 and Thr66 within the Ub sequence, positioning it between K63 and the C-terminus.[163] In addition to allowing for analysis of M1-linkages (by not blocking the N-terminus with a tag) and for nickel immobilized metal affinity chromatography (IMAC)-based, denaturing His-tag pulldown for enrichment, the internal tag also serves as a useful tool for mapping precise ubiquitination sites in substrates using mass spectrometry by enabling efficient enrichment of ubiquitinated peptides.[163]

2.12. Proteomics Workflows and "the Proteomics Problem"

Systematic, cell-wide identification of substrates modified with specific Ub chain types using mass spectrometry-based proteomics is an attractive approach toward understanding the signaling networks and cellular processes controlled by individual Ub chain types. However, there are multiple challenges to this approach, including that the ratio of modified to unmodified substrates (modification occupancy) is usually very low (below 1% for 99% of analyzed ubiquitination sites) and that the half-life of the modifications is very short (≈12 min).[7] But the past 10–15 years have seen substantial advances in the analysis of Ub modifications by mass spectrometry-based proteomics techniques to decipher cellular Ub signaling.[47,164] These developments have identified a plethora of ubiquitination events in virtually all cellular pathways. However, one major disadvantage of these mass spectrometry-based methods for understanding the roles and functions of the individual Ub chain types is that all current mass spectrometry-based workflows eliminate any information about which Ub chain type was attached to the substrate protein.

Most conventional workflows for analyzing Ub modifications are based on bottom-up proteomics, where proteins are digested with proteases, typically trypsin, to generate smaller peptides for mass spectrometry analysis.[47,164] As the C-terminus of Ub ends in Arg-Gly-Gly, and trypsin cleaves after Arg, digestion of ubiquitinated proteins with trypsin generates peptides containing a Gly-Gly remnant on the side chain of the lysine residue modified by Ub. This Gly-Gly remnant (also referred to as a diGly remnant) is readily detectable by mass spectrometry.[165] Gly-Gly-specific antibodies can enrich Gly-Gly-modified peptides prior to mass spectrometry analyses. Combined with similar remnant-enrichment approaches, such as the UbiSite method,[4] this has led to the identification of over 100,000 ubiquitination sites in cellular proteins.[2,3,5,6] However, due to the trypsin digest, which cleaves off the polyUb modification on the substrates, Gly-Gly analyses cannot determine the type of Ub chain attached to the substrate; only that Ub was attached to it in some form. Identification of substrates in a linkage type-specific manner therefore relies on enrichment with linkage type-specific reagents prior to mass spectrometry analysis.[48,66] This enrichment is typically achieved using linkage type-specific antibodies, engineered UBDs, or other reagents as described earlier. The enriched material can then either be analyzed directly by bottom-up mass spectrometry, revealing proteins associated with the enriched Ub chain type (essentially an interactome analysis) or be further processed and analyzed for Gly-Gly remnants (although this is uncommon). Such analyses can provide insights into potential substrates modified with specific Ub linkages. While exceedingly useful, the mass spectrometry analyses alone still cannot confirm the presence of a specific chain type on a substrate. Validation of potential substrates identified after enrichment requires analyses by complementary methods coupled to the enrichment, such as immunoblotting or Ub chain restriction (UbiCRest) analysis.[99] Thus, identifying, analyzing, and understanding linkage-type-specific Ub signaling events is a complex and challenging task that requires an array of experimental approaches that depend on effective enrichment and specific detection of individual linkages.

In addition to analysis of ubiquitination sites, mass spectrometry-based methods can analyze the absolute levels of individual Ub linkages as well as probe chain architecture. Absolute quantification (AQUA) analysis of total Ub levels and linkage types can be achieved through spike-in of labeled Ub peptide standards into analyses of tryptic digests from most sample types and can be used in conjunction with antibodies or other reagents enriching ubiquitinated peptides to provide quantitative insights into the absolute concentration of different Ub linkages in the sample.[47,166,167] In addition, Ub-Clipping is a recently developed mass spectrometry-based method that allows probing chain architecture.[168] The method takes advantage of an engineered, viral protease, Lb^{pro*}, which cleaves all types of Ub linkages after Arg74, generating a truncated distal moiety spanning residues 1–74 and a Gly-Gly-modified proximal Ub 1–74.[168] This allows for analysis of the number of modification sites on each Gly-Gly-modified Ub, revealing the proportion of branches in polyUb chains.

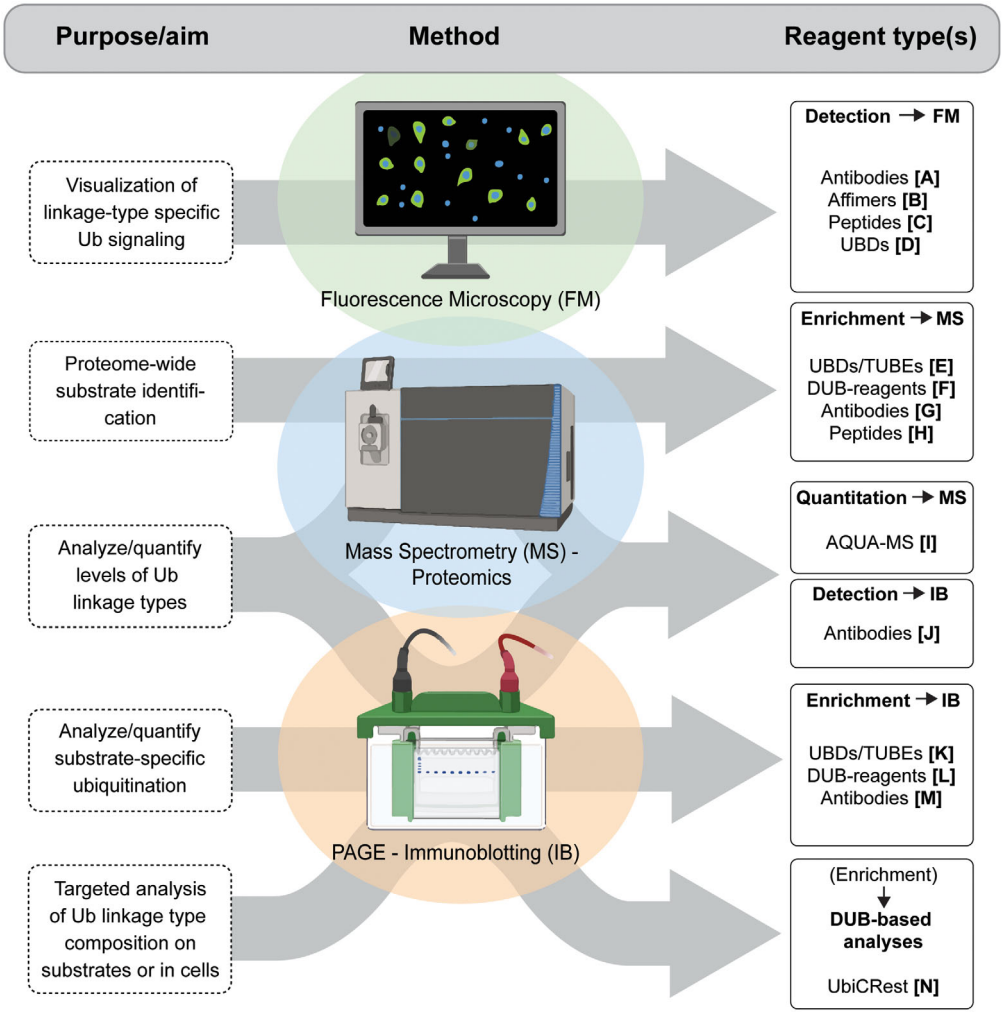

Figure 5. Ub analysis workflows. Overview of Ub analysis workflows associated with types of linkage-specific affinity reagents used for those applications. References belonging to reagents qualifying for each method (see also Table 1) **A:**[54,55,136,139,152] **B:**[119] **C:**[127,128] **D:**[57,61,71,75] **E:**[56,67,69,77] **F:**[57] **G:**[54,55,136,139] **H:**[127] **I:**[166] **J:**[54,55,136,139,162,183] **K:**[52,72] **L:**[57] **M:**[54,55,136,139,152] **N:**[99] Abbreviations: AQUA, absolute quantification, DUB, deubiquitinase; TUBE, tandem ubiquitin-binding entity; UBD, ubiquitin-binding domain; UbiCRest, ubiquitin chain restriction analysis.

A complementary method for further validating and investigating potentially linkage-specifically modified substrates, for example, identified by the mass spectrometry workflows described earlier, is UbiCRest analysis.[99] In UbiCRest, ubiquitinated substrates or polyUb chains undergo parallel treatment with a panel of linkage-specific DUBs followed by gel-based analysis, for example, immunoblotting or protein stains such as silver or Coomassie staining, to reveal which linkage type(s) are present on ubiquitinated substrates or to assess the architecture of heterotypic chains.[99,169] UbiCRest can, for example, be employed after substrate or Ub linkage type-specific enrichment to identify or validate the presence of a linkage type on the substrate(s).[56,58,170,171]

Overall, these methods provide great synergy with linkage-specific affinity reagents to probe linkage-specific modification of substrates and the linkage landscape in cells and tissues (**Figure 5**), thereby enabling a better understanding of linkage type-specific Ub signaling.

3. Summary and Outlook

Over the past couple of decades, a wide range of reagents, now covering all amide Ub linkages, have been generated (Table 1). These reagents, particularly the first generation of antibodies and the UBD-based reagents, have been key for developing the field. In recent years, an increasing number of new classes of reagents have been developed, taking advantage of the development in molecular biotechnology, including affimers, macrocyclic peptides, and sdAbs. While many reagents are available for some linkage types, like K48- and K63-linkages, few or only a single are available for others. This highlights the ongoing need to develop additional linkage type-specific reagents to expand the repertoire and possible applications by which the individual linkages can be investigated. We have also started to see reagents targeting more unconventional modifications emerge, including mixed and branched chains. The key to ensuring that new reagents keep moving the field

forward is validation. Careful validation of the specificity of new reagents and their applications, both *in vitro* and in cellular systems, if applicable, will make sure that new ground can be broken in the pursuit of understanding linkage-specific Ub signaling.

Even with new classes of reagents emerging, there are still modifications that remain "invisible". It is currently not possible to specifically detect or enrich for the recently discovered ester-linked Ub chains (Thr12, Thr14, Ser20, Thr22, and Thr55). Moreover, Ub modifications, aside from with Ub itself, is another set of modifications that are currently difficult to explore. Almost as an additional level of code on top of polyUb linkages, Ub can be phosphorylated (Thr7, Thr12, Thr14, Ser20, Thr22, Thr55, Ser57, Tyr59, Ser65, and Thr66), acetylated (K6, K11, K27, K33, K48, and K63), SUMOylated, and NEDDylated.[9,172] Yet, very few reagents exist that allows for mechanistic or functional investigation of the interplay of these modifications with the Ub linkages and how that affects signaling outcome. The field would benefit substantially from development of more affinity reagents allowing specific and effective enrichment and detection of the ester-linkages and Ub modifications to reveal their function. Similar to the amide linkages, we envision that multiple classes of reagents, maybe even some entirely new yet-to-be developed or discovered, may be necessary to generate reagents covering the entire range of low-abundant and highly dynamic (and in some cases chemically labile) Ub modifications and linkages.

Development of new, specific affinity reagents targeting these understudied types of Ub modifications may be challenging, even given the spectrum of scaffolds that exist. Given that it took almost nearly 14 years from the development of first linkage-specific antibodies until we had reagents covering all eight amide linkages gives an indication of how challenging the task may be. Recent advances in artificial intelligence (AI) and deep learning-assisted de novo protein design have revealed powerful workflows and technologies for designing high-affinity binding proteins.[173] De novo protein design can now generate specific, high-affinity binders to structured proteins and peptides.[174–176] Maybe AI/ML-assisted de novo protein design holds the potential to generate specific binders to Ub modifications and linkages from structural data? As an orthogonal or alternative approach to current workflows based on rational engineering of endogenous proteins or library-based selection campaigns, it is interesting to speculate that perhaps de novo protein design—or even AI/ML-based optimization or binders derived through other methods—could provide specific binders for very challenging targets or binders with bespoke characteristics, for example, targeting longer chains through more than two binding sites or binding specifically in certain conditions such as under denaturing conditions.

In summary, the last 15 years of research have provided an array of reagents that have paved the way for our current understanding of linkage-specific Ub signaling. But there is still a great and unmet need for development of Ub-binding affinity reagents for unraveling the Ub code.

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Conflict of Interest

The authors declare no conflict of interest.

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