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# Broad Utility of an Affinity-enrichment Strategy for Unanchored Polyubiquitin Chains

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## Abstract

Protein ubiquitination is a common post-translational modification where selected targets are covalently modified by the ubiquitin protein, often in the form of isopeptide-linked polyubiquitin chains. More recently, unanchored (i.e. non-substrate-linked) polyubiquitin chains have also been described and implicated in a range of biological processes. The development of Tandem-repeated Ubiquitin-Binding Entities (TUBEs), engineered repeats of ubiquitin-binding domains that interact non-covalently with polyubiquitin, has allowed strategies for the affinity-enrichment of ubiquitin-modified proteins to be established, in some cases with linkage specificity. Here, we demonstrate the utility of a Free Ubiquitin-Binding Entity (FUBE), based on an ubiquitin-binding domain with high specificity for the free C-terminus of ubiquitin (the Znf-UBP domain of human USP5). In contrast to TUBEs which do not distinguish conjugated or free polyubiquitin, the FUBE exclusively recognises ubiquitin in its unconjugated form, including endogenous unanchored polyubiquitin chains. Affinity-enrichments using the FUBE demonstrate that unanchored polyubiquitin chains are present in different mammalian cell lines and accumulate when the 26S proteasome is pharmacologically inhibited, being retained on the proteasome. The high conservation of the ubiquitin sequence permits the FUBE to also be applied to the purification of endogenous unanchored polyubiquitin chains from species as diverse as *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. The development and refinement of an affinity-enrichment strategy for unanchored polyubiquitin chains opens the way for more complete investigations into their biological significance.

**Keywords:** Ubiquitin; Unanchored polyubiquitin; Affinity-enrichment; Ubiquitin-binding domain; TUBE

**Abbreviations:** TUBE: Tandem-repeated Ubiquitin-Binding Entity; FUBE: Free Ubiquitin-Binding Entity; UBD: Ubiquitin-Binding Domain; DUB: Deubiquitinating Enzyme; WB: Wash Buffer; HB: Homogenising Buffer; NEM: N-Ethylmaleimide; DB: DUB Buffer; WT: Wild-Type

## Introduction

Ubiquitin typically exerts its effects through the post-translational covalent modification of specific target proteins [1]. Often isopeptide-linked polyubiquitin chains are appended to substrate proteins, linked *via* any one of the seven lysine (K) residues within the ubiquitin primary sequence to the C-terminal G76 residue of an adjacent ubiquitin (although linear peptide-linked chains involving M1-G76 bonds also possible). More recently, however, roles for unanchored polyubiquitin chains have also been proposed. In this context, polymers of ubiquitin that are free of substrates have been suggested to act within different biological systems, such as the regulation of 26S proteasome activity [2-4], as second messengers in NF-κB signalling pathways [5], regulators of innate immune signalling [6,7], regulators of aggresome biogenesis [8], and in various stress responses [9]. In each case, non-covalent recognition of the unanchored polyubiquitin chain by specific ubiquitin-binding domains (UBDs) found within ubiquitin receptor proteins is a critical event in potentiation of the ubiquitin signal.

The precise sources of unanchored polyubiquitin chains remain to be clarified, although in mammalian systems the E3 ubiquitin ligases TRAF6 [5], TRIM25 [6] and TRIM5 [7] contribute to their signal-induced assembly, and the deubiquitinating enzyme (DUB) ataxin-3 can generate unanchored chains *via* de-conjugation of substrates [8]. Some proteasome-associated DUBs possess polyubiquitin chain amputating activities, indicating the proteasome itself could potentially act as a source of unanchored polyubiquitin upon substrate degradation [10,11]. Polyubiquitin immunoreactivity is associated with purified proteasome fractions [11,12] and free tetra-ubiquitin, generated from chain amputation of the artificial proteasome substrate $Ub_4$-(K48)-UbcH10, can be retained on the proteasome *in vitro* [11]. Recent studies in yeast identified the HECT domain E3s UFD4 and HUL5 as enzymes capable of generating unanchored polyubiquitin chains [9]. In some instances, the isopeptide linkages that connect the ubiquitin moieties and define unanchored polyubiquitin chains with particular functions have been determined. For example, unanchored K63-linked polyubiquitin chains are responsible for activating TAK1 kinase [5] and RIG-I [6], whereas chains with linkages alternate to K63 (and K48) appear to activate the IKK complex [5]. In contrast, unanchored K48-linked polyubiquitin chains are presumed to make a contribution to the

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regulation of the 26S proteasome [2,4]; polyubiquitin chains are potent competitive inhibitors of the 26S proteasome *in vitro* [2], although this latter proposal has not been definitively proven in cells. Observations that accumulation of unanchored polyubiquitin in response to suppression of USP5 in cells is associated with inhibition of proteasome activity [3,4], supports a model in which unanchored polyubiquitin, produced upon substrate degradation by the 26S proteasome could subsequently exert auto-inhibitory effects. Thus, in the same way that conjugated polyubiquitin chains can be considered to represent an 'ubiquitin code', distinct sub-populations of unanchored polyubiquitin chains are likely to exert different biological activities.

Despite these observations, knowledge of the range of fundamental processes that may be regulated by unanchored polyubiquitin chains, as well as the precise molecular composition of these ubiquitin polymers (i.e. different isopeptide linkages present), is lacking. Insights in to the (poly)ubiquitin-modified proteome and by extension the biological significance of different ubiquitin modifications, have been provided from affinity-enrichment strategies that make use of Tandem-repeated Ubiquitin-Binding Entities (TUBEs), engineered repeats of UBDs that interact non-covalently with polyubiquitin chains [13,14], in some cases with linkage selectivity. We recently reported an affinity-enrichment strategy using a variant of TUBEs, a so-called Free Ubiquitin-Binding Entity (FUBE), which in contrast to TUBEs which do not distinguish conjugated or free polyubiquitin recognises ubiquitin exclusively in its unconjugated form, including endogenous unanchored polyubiquitin chains [15]. Our FUBE affinity-enrichment strategy is based on the use of a high-affinity ubiquitin linkage-independent UBD [16], the Znf-UBP domain from the USP5 enzyme, the principal DUB that disassembles unanchored polyubiquitin chains. The Znf-UBP domain is highly selective for unanchored over conjugated polyubiquitin [17], allowing polyubiquitin chains to be captured in a substrate-free form [15]. Here, we demonstrate the broad applicability of this FUBE affinity-enrichment strategy to different biological samples, and present further insights in to the molecular composition of endogenous unanchored polyubiquitin chains.

## Materials and Methods

### Proteasome inhibition

To pharmacologically inhibit proteasome activity, mammalian cells (U20S or HeLa; 80% confluency) cultured in 10 cm dishes were treated with 1 μM MG-132 or vehicle only (DMSO) for 16 hours. Affinity-enrichments using the Znf-UBP domain (our FUBE) were performed as described below.

### Proteasome purification

For proteasome purifications, a HeLa cell line was generated that stably expresses S13/Rpn11-HTBH; S13/Rpn11 is a proteasome subunit and the HTBH tag includes a $His_6$ tag, tobacco etch virus protease site, *in vivo* biotinylation sequence and a further $His_6$ tag [18]. The original plasmid was a gift from Dr. Lan Huang at UC Irvine [19]. A 'mild' buffer was utilised to affinity purify the 26 S proteasome and preserve associating (including ubiquitinated) proteins [18].

### Znf-UBP domain affinity-enrichment (mammalian cells)

Recombinant Znf-UBP (also known as BUZ) domain (residues 163-291) of human USP5 represents our archetypal FUBE and was produced and purified, as previously described [15]. Thrombin-cleaved Znf-UBP protein was covalently immobilized using cyanogen bromide-activated Sepharose 4B (CNBr Seph 4B) (GE Healthcare; protein on beads at 10 mg/mL). Unanchored ubiquitin/polyubiquitin from a range of biological samples was captured on Znf-UBP Sepharose beads (pre-washed in wash buffer (WB); 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% (v/v) NP-40, 1 mM DTT). An overview of the FUBE affinity-enrichment strategy is shown in Figure 1.

U20S and HeLa cells (untreated and proteasome-inhibited) were scraped into homogenising buffer (HB: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% (v/v) NP-40, 5 mM N-ethylmaleimide (NEM), 20 μM MG-132, 0.1% (v/v) mammalian protease inhibitor cocktail (Sigma, Dorset, UK)), typically 4×10 cm dishes in 6 mL of HB, and sonicated. The protein concentration of the supernatant (determined using a Thermo Scientific Pierce BCA Protein Assay Kit, according to the manufacturer's protocol; Thermo Fisher Scientific, Hemel Hempstead, UK) was typically 1-3 mg/mL after centrifugation (35,000 g, 30 min, 4°C). To remove excess NEM, DTT was incubated with clarified supernatant at a final concentration of 10 mM for 15 min at 4°C. The NEM-quenched supernatant was heated to 75°C for 20 minutes and centrifuged (as above), which preserves unanchored polyubiquitin, whilst reducing levels of conjugated polyubiquitin [15]. The heated supernatant was incubated with Znf-UBP-Sepharose (resulting protein derived from ~15 mg unheated supernatant captured on 100 μL beads; amounts previously optimised to ensure FUBE is not limiting), and rotated overnight at 4°C. Beads were washed thoroughly in WB, prior to washing in DUB buffer (DB: 50 mM Tris-HCl, pH 7.5, 1 mM DTT) to remove detergent that may impede downstream analyses. The remaining Znf-UBP-Sepharose-bound proteins were eluted with gel loading buffer and separated by SDS-PAGE for western blot using 5-20% gradient gels.

FUBE affinity-enrichment of unanchored polyubiquitin associated with purified 26S proteasomes followed the same protocol, with some

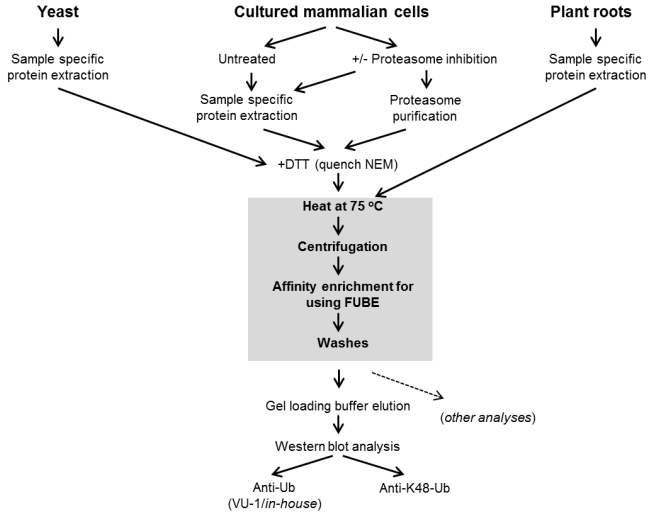


**Figure 1:** Overview of FUBE affinity-enrichment strategy applied to a range of biological samples.
Proteins were extracted from yeast, cultured mammalian cells, or plant roots, as detailed in the Materials and Methods section. The shaded box highlights the common stages of the purification, which included a heating step and removal of precipitated proteins by centrifugation. The composition of the purified unanchored polyubiquitin was analysed by western blot analyses following elution with gel loading buffer. Ubiquitin and K48-polyubiquitin linkage specific antibodies were used for western blot analysis.

modifications. Proteasome fractions (having been extracted in a 'mild' buffer [18]) were diluted 100-fold in HB to a final volume of 10 mL (~ 3 µg/mL), and were further purified using 100 µL of Znf-UBP-Sepharose. Purified proteins eluted from the equivalent of 25 µL of Sepharose with gel loading buffer were loaded per lane for SDS-PAGE and western blot analysis.

### Znf-UBP domain affinity-enrichment (plants)

To investigate the utility of the FUBE affinity-enrichment in the purification of unanchored polyubiquitin chains from different species, the method was applied to plant tissue. *Arabidopsis thaliana* roots (~25 g wet weight) were crushed in liquid nitrogen and re-suspended in 100 mL of EB1 buffer (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 1% (v/v) plant protease inhibitor cocktail (Sigma, Dorset, UK)). The homogenate was filtered through microcloth and centrifuged (2800 g, 20 min, 4°C). The supernatant (nuclei-depleted) was frozen at -20°C; prior to purification it was thawed and then heated at 75°C for 20 minutes and centrifuged again (35,000 g, 30 min, 4°C) to remove heat-precipitated proteins. Different volumes of extracted plant proteins were incubated with 50 µL of Znf-UBP-Sepharose overnight at 4°C, and washed (as described above), prior to elution of bound proteins in gel loading buffer. Proteins eluted from the full 50 µL of Znf-UBP-Sepharose were loaded per lane for SDS-PAGE and western blot analysis.

### Znf-UBP domain affinity-enrichment (yeast)

For the capture of unanchored polyubiquitin from yeast, *Saccharomyces cerevisiae* strains that express wild-type (WT) UBP14 (yeast equivalent of human USP5) or lack the UBP14 gene were compared. Isogenic WT UBP14 and ΔUBP14 yeast strains [3] were grown at 30°C in 2 L of YPD medium, starting from OD 0.05 and harvested in the logarithmic phase of growth. Yeast extracts were prepared by grinding cells blast-frozen in a 1:0.7 ratio of cells to buffer U (50 mM Tris-HCl, pH 7.2; 50 mM KCl; 10 µM NEM, 10 µM MG-132), followed by thawing in an ice-cold water bath and centrifugation (15,000 g, 30 min, 4°C). Samples were frozen at -80°C, until required. Prior to purification, they were thawed quickly and a further centrifugation step was performed (15,000 g, 20 min, 4°C). Yeast lysate (3 mL, ~ 40 mg total protein) was diluted 8-fold in modified HB (mHB: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% (v/v) NP-40, 5 mM NEM), 20 µM MG-132, 0.1% (v/v) yeast protease inhibitor cocktail (Sigma, Dorset, UK), 0.1% (v/v) phosphatase inhibitors (Sigma, Dorset, UK)). The FUBE affinity-enrichment procedure and washes were performed as above (24 mL diluted lysate to 350 µL Znf-UBP-Sepharose; equivalent of 50 µL of beads eluted with gel loading buffer was analysed per lane on SDS-PAGE and western blot). Samples of Znf-UBP-Sepharose with captured proteins were also incubated overnight at 37°C, with the catalytic core of the USP2 DUB (ENZO Life Sciences, Exeter, UK) or full-length human USP5 (ENZO Life Sciences, Exeter, UK), diluted to 25 ng/µL or 10 ng/µL, respectively, in DB (equal volume solution to beads), to confirm the presence of unanchored polyubiquitin chains. In this case, each lane for western blot analysis again represents proteins derived from 50 µL of Znf-UBP-Sepharose beads.

### Antibodies and polyubiquitin standards

Ubiquitin was detected on western blots using *in house* pan anti-ubiquitin, commercial pan anti-ubiquitin (VU-1; LifeSensors, PA, USA), or anti-K48-polyubiquitin-specific (Apu2 clone; Millipore, Watford, UK) antibodies. All SDS-PAGE directed for western blot analysis were transferred to nitrocellulose (for 16 hours at 40 mA). For membranes probed with the *in house* ubiquitin antibody, membranes were autoclaved, blocked in 4% (w/v) milk powder in TBS for 1 hour at room temperature, followed by incubation with primary antibody (1:1000 dilution) for 1 hour at room temperature. Blots were washed extensively in TBS, detected with horseradish peroxidase-conjugated swine-anti rabbit antibody and developed with enhanced chemiluminescence (GE Healthcare, Amerham, Bucks, UK). Membranes probed with VU-1 and K48-polyubiquitin antibodies were blocked in 5% milk in TBS-0.1 % (v/v) Tween or TBS-0.05% (v/v) Tween (respectively) for 1 hour at room temperature, followed by incubation with primary antibodies (both 1:1000) overnight at 4°C. Primary antibodies were detected and developed as described above (for VU-1 using rabbit-anti mouse), but washed in TBS with 0.1% (v/v) or 0.05% (v/v) Tween (for VU-1/K48 antibodies, respectively). Commercial K48- and K63-linked polyubiquitin standards were from R&D systems (Abingdon, UK). When used as standards for *in house* ubiquitin and K48-polyubiquitin antibodies 0.25 µg commercial K48/K63-linked polyubiquitin per lane was used; for VU-1 blots, K48 and K63 lanes represent 0.05 µg of commercial polyubiquitin chains. Rpt5/S6a blotting (from here on referred to as Rpt5) of purified 26S proteasomes was performed, as described previously [18].

## Results

### Endogenous unanchored polyubiquitin chains can be purified from different mammalian cell lines and accumulate upon proteasome inhibition

We previously reported that endogenous unanchored polyubiquitin chains containing as many as 15 ubiquitin moieties can be affinity-enriched from skeletal muscle using our FUBE, the polyubiquitin linkage-independent Znf-UBP domain of human USP5 [15,16]. Consistent with the widespread distribution of unanchored polyubiquitin, we found that chains with similar ubiquitin-immunoreactive profiles (in particular, detected with anti-ubiquitin and anti-K48-polyubiquitin antibodies) to those in muscle can be readily captured from commonly used mammalian cell lines, for example U20S and HeLa (Figure 2A). Specifically, in addition to ubiquitin monomers, dimers and longer polyubiquitin chains were recovered from cell extracts using our FUBE affinity-enrichment strategy. We routinely observed that U20S yielded a lower recovery of chains relative to HeLa cells, as highlighted by lower detection with K48-polyubiquitin-specific antibodies.

To understand further the role of the 26S proteasome in the generation of unanchored polyubiquitin, we applied our FUBE affinity-enrichment strategy to cells, in which the 26S proteasome had been pharmacologically inhibited with MG-132. Treatment of cells with MG-132 led to an accumulation of unanchored polyubiquitin chains, as evidenced by affinity-enrichment using the FUBE (Figure 2B). MG-132 blocks proteolytic without affecting DUB activities of the 26S proteasome; we predict that proteolytic inhibition of the proteasome forces irregular deubiquitination of accumulating proteasomal substrates, including chain amputation by proteasome-associated DUBs (e.g. POH1) [11].

To investigate whether as is the case for polyubiquitin derived from the artificial proteasome substrate $Ub_4$-(K48)-UbcH10 [11], endogenous unanchored polyubiquitin chains reside and are retained on the 26S proteasome, we took advantage of a HeLa cell line, which stably expresses HTBH-tagged Rpn11 (a proteasome subunit),

permitting affinity purification of 26S proteasome fractions, whilst preserving associating proteins [18]. Affinity-purified proteasome fractions from these cells (purified on streptavidin resin followed by TEV-based elution) exhibited discrete characteristic banding patterns on SDS-PAGE, and by western blotting showed immunoreactivity for the proteasome subunit Rpt5 (Figure 3A). Consistent with previous reports [11,12], anti-ubiquitin western blotting of purified proteasome fractions demonstrated the co-purification of high molecular weight ubiquitin immunoreactive bands (Figure 3B, lane 5), with anti-K48-polyubiquitin antibodies also revealing discrete bands of lower molecular weight (Figure 3C, lane 5). These proteasome fractions were subjected to a second round of affinity-enrichment using the FUBE giving recovery of discrete unanchored polyubiquitin chains, as short as dimers (Figure 3B, lane 3), with chains containing 4-10 ubiquitins, particularly prominent when detected with anti-K48-polyubiquitin antibodies (Figure 3C, lane 3). Notably, we did not detect bands co-migrating with ubiquitin trimers. Ubiquitin immunoreactivity (both anti-ubiquitin and anti-K48-polyubiquitin) markedly increased in the proteasome fractions following MG-132 treatment (Figure 3B lane 6; 3C lane 6), consistent with previous observations [18], and likewise, in fractions representing unanchored polyubiquitin chains following further enrichment using the FUBE (Figure 3B, lane 4; 3C, lane 4). Together these data confirm that like conjugated polyubiquitin, endogenous unanchored polyubiquitin chains are associated with the 26S proteasome and their levels increase in this fraction when proteasome catalytic activity is inhibited with MG-132.

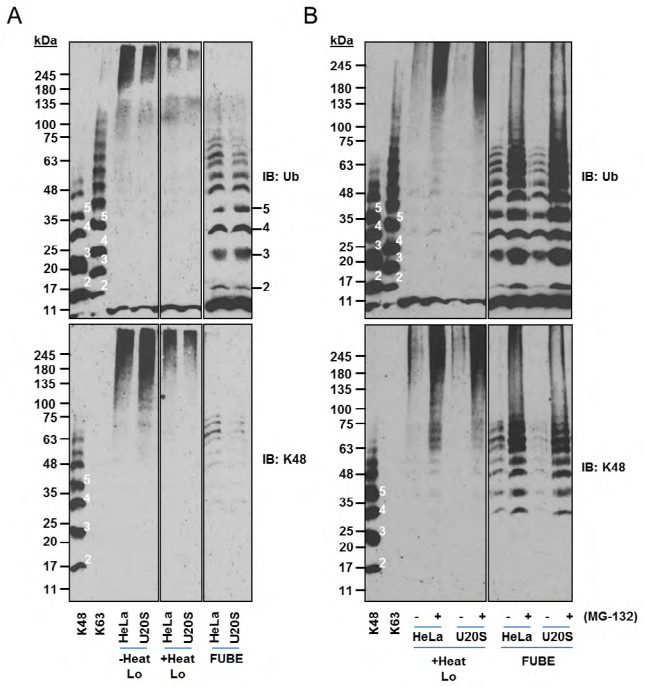


**Figure 2:** Endogenous unanchored polyubiquitin chains can be purified from different human cell lines and increase upon proteasomal inhibition.
(A) Unanchored polyubiquitin chains captured from U20S or HeLa cells using the FUBE enrichment and detected by western blotting (anti-ubiquitin (VU-1) and anti-K48 polyubiquitin specific antibodies). FUBE lanes represent purified proteins from 30% (30 μL) of beads, captured from lysates heated to 75°C (Materials and Methods). "-Heat" load (Lo) represents 1% (150 μg) of the lysate, subsequently heated before ubiquitin capture. "+Heat" Lo represents all of the protein recovered from that 150 μg after heating, and is therefore, equivalent to 1% of the heated cell lysate loaded onto 100 μL beads. Note that only heated lysate was applied to FUBEs. Although U20S cells yield a lower recovery of unanchored polyubiquitin chains than HeLa cells, the predominant bands in both co-migrate with commercial unanchored polyubiquitin chain standards (K48 and K63 lanes, 0.05 or 0.25 μg per lane for VU-1 or K48 antibodies, respectively). (B) FUBE affinity-enrichment was applied to MG-132-treated cells to investigate the changes in unanchored polyubiquitin upon proteasomal inhibition. Cells were treated with 1 μM MG-132 or vehicle only (DMSO) for 16 hours, prior to lysis and purification. An increase in total polyubiquitinated proteins is apparent in the load upon MG-132 treatment; increased levels of unanchored polyubiquitin chains that are not substrate-bound and purified by the FUBE were evident upon proteasomal inhibition. Numbers indicate ubiquitin moieties in the chains.

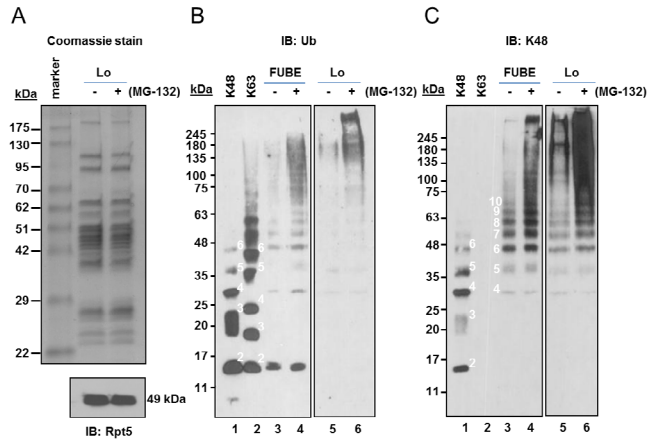


**Figure 3:** Unanchored polyubiquitin chains are associated with the 26 S proteasome and this fraction increases after proteasomal inhibition.
26 S proteasomes (and their associated proteins) were purified from HeLa cells stably expressing Rpn11-HTBH. Prior to extraction, cells were treated with proteasome inhibitor MG-132 (+) or vehicle only (-). (A) 5 μg of purified proteasomes were visualised by SDS-PAGE and 1 μg immunoblotted for the proteasomal subunit Rpt5 (confirming successful enrichment of proteasomes). Western blot analysis of proteasomes from MG-132-treated HeLa cells (load (Lo); ~ 1 μg per lane) with (B) anti-ubiquitin (*in house* antibody), and (C) anti-K48 linkage antibodies reveals an increase in ubiquitin/K48-linked polyubiquitin immunoreactivity (lanes 6), relative to untreated proteasomes (lanes 5). Unanchored K48-linked polyubiquitin is captured from purified proteasome fractions using the FUBE, detected by both (B) ubiquitin (lane 3) and (C) K48-specific antibodies (lane 3). Figure 2 illustrated that proteasomal inhibition by MG-132 augments the levels of FUBE-captured unanchored polyubiquitin chains, and these chains are, at least in part, associated with the proteasome (Figure 3B and 3C, lanes 4). K48 and K63 (lanes 1 and 2 in (B) and (C)) represent commercial K48-and K63-linked polyubiquitin chains. Lo (lanes 5 and 6 in (B) and (C)) represents ~ 15% of proteasomes applied to the FUBE. Numbers (on blots) indicate ubiquitin moieties in the chains.

## Endogenous unanchored polyubiquitin chains can be purified from a range of biological samples

The Znf-UBP domain, which is the basis of our FUBE affinity-enrichment strategy, exerts specificity for unanchored (poly)ubiquitin, as it recognises residues within the unmodified free C-terminus of ubiquitin and inserts this in to a deep binding pocket [17]. We noted that ubiquitin contact residues in the structure of the USP5 Znf-UBP domain and mammalian ubiquitin [17] are completely conserved in ubiquitin sequences, as diverse as from yeast and plants (Figure 4A), indicating that it should be possible to directly purify unanchored polyubiquitin chains from non-mammalian sources. To test this notion, we first attempted purification of unanchored polyubiquitin chains from *Arabidopsis thaliana*. Unanchored polyubiquitin chains are known to be abundant in plants [20], although their functional significance is unclear. A single previous report highlighted the purification of an ubiquitin dimer from wheat germ by column

chromatography, which was shown (by Edman degradation) to contain the K48 linkage [20]. Using our FUBE affinity-enrichment, we were able to purify unanchored polyubiquitin chains from *Arabidopsis thaliana* root extracts (Figure 4B), with western blot profiles indicating that the complexity of the unanchored polyubiquitin pool in plants is likely to rival that of mammalian systems. Note that enrichments from increasing volumes of root extract were associated with corresponding increases in ubiquitin immunoreactivity on western blot (Figure 4B), indicating the capture method is sensitive to changes in abundance of unanchored polyubiquitin chains.

We further demonstrated the utility of the FUBE affinity-enrichment with the purification of unanchored polyubiquitin chains from yeast (*Saccharomyces cerevisiae*). As well as the capture of substantive levels of monoubiquitin, we observed low levels of unanchored polyubiquitin chains (principally dimer, detected with anti-ubiquitin) in purified fractions from wild-type yeast in the Log phase of growth (Figure 4C). Consistent with the central role for yeast Ubp14 (USP5 equivalent) in the disassembly of unanchored polyubiquitin [3], purifications from yeast cells, in which the UBP14 gene was deleted, gave rise to significantly increased levels of unanchored polyubiquitin chains (Figure 4C), reflecting the striking accumulation of free polyubiquitin chains previously reported. From cells in the stationary phase, we recovered little or no unanchored polyubiquitin (not shown). Anti-ubiquitin western blots readily detected $Ub_2$-$Ub_4$ chains in the samples purified from Ubp14 deleted cells in Log phase (Figure 4C), which were confirmed as being unanchored due to their disassembly upon incubation with the broad specificity DUB USP2 (catalytic core) and purified human USP5 (Figure 4D), the latter which has previously been shown to be capable of functionally replacing Ubp14 [3]. Notably, anti-K48-polyubiquitin antibodies also detected chains as long as $Ub_6$ in purified fractions, as well as a distinct band (asterisk) below the position of the presumed ubiquitin tetramer, which could be disassembled with USP2, but curiously was not amenable to processing by purified USP5 (Figure 4D). We also found that this species bound avidly to the A20 Znf domain (of ZNF216, data not shown), a UBD we have utilised previously to capture polyubiquitinated proteins [15]. However, the precise identity of this ubiquitin-reactive species remains to be determined, as MS/MS approaches only recovered ubiquitin sequence.

## Discussion

In this study, through the use of a FUBE-based affinity-enrichment strategy, we have extended previous observations related to muscle tissue [15], to demonstrate that unanchored polyubiquitin chains exhibit a widespread distribution in mammalian cell lines; specifically, chains containing 10 or more ubiquitin moieties and as short as dimers were recovered from HeLa and U20S cells. Further, we show that endogenous unanchored polyubiquitin chains are like conjugated polyubiquitin [11,12], associated with the 26 S proteasome. This unanchored polyubiquitin fraction increases when the 26 S proteasome is inhibited by MG-132, we presume as a result of impaired proteasome catalytic activity abrogating substrate degradation and promoting chain amputation by proteasome-associated DUBs (e.g. POH1) [11]. Overall, these studies support the notion that proteasome-associated DUBs may contribute to the generation of the cellular pool and proteasome-associated fraction of unanchored polyubiquitin chains, and are consistent with a model in which substrate degradation by the 26S proteasome could, in some circumstances, generate unanchored polyubiquitin chains with auto-inhibitory effects. Notably, in fractions of unanchored polyubiquitin generated from purified

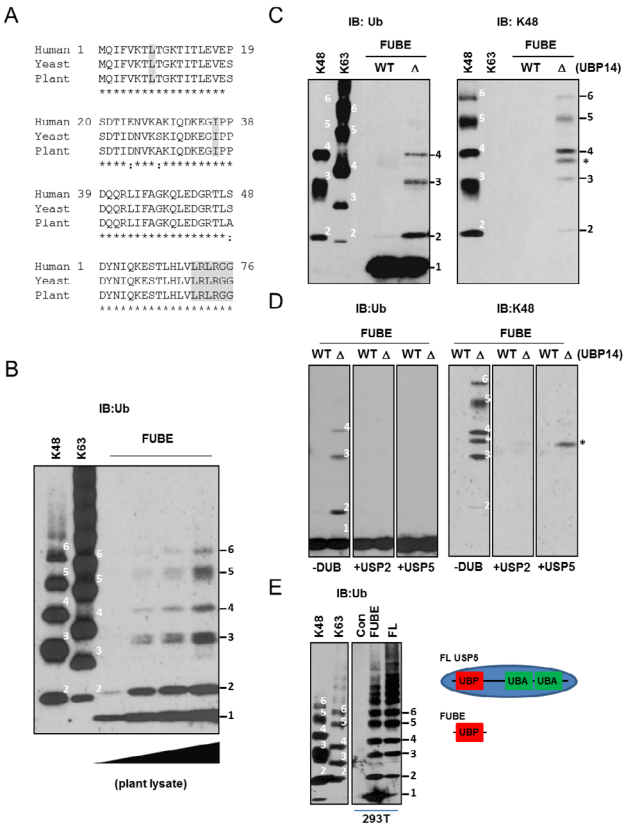


**Figure 4:** The high conservation of the ubiquitin sequence permits FUBE affinity-enrichment of unanchored polyubiquitin from diverse species.
(A) The ubiquitin sequence is highly conserved across Animalia, Fungi and Plantae kingdoms, demonstrated by the comparison of human (*Homo sapiens*), yeast (*Saccharomyces cerevisiae*) and plant (*Arabidopsis thaliana*) ubiquitin sequences (residues 1-76). Identical residues are denoted by asterisks (*), while highly similar amino acids are denoted by a colon (:). Note that only a single residue (P/S19) shows no similarity in amino acid properties between yeast and plants *vs.* human. The ubiquitin residues that contact the Znf-UBP domain of USP5 (our FUBE) are denoted by shading. Importantly, the Znf-UBP contact residues are 100% conserved across human, plants and yeast. (B) Unanchored polyubiquitin is captured from plant root tissue using the FUBE, revealing an increase of ubiquitin immunoreactivity (*in house* antibody), with increasing volumes of plant lysate and a fixed volume FUBE (0.5, 2.5, 5 and 10 mL of plant lysate). (C) The FUBE enriches more unanchored polyubiquitin chains from a ΔUBP14 (Δ) strain relative to an isogenic wild type UBP14 (WT) strain, as detected by anti-ubiquitin (VU-1) and anti-K48 polyubiquitin antibodies. Immunoblotting reveals unanchored K48-linked polyubiquitin species as long as $Ub_6$ from the ΔUBP14 strain. A K48-immunoreactive band that does not co-migrate with commercial unanchored K48-linked polyubiquitin chains was also purified on the FUBE (denoted by an asterisk; *) that migrates just below the commercial K48 ubiquitin tetramer. (D) FUBE-purified yeast unanchored polyubiquitin was characterised by treatment with either the broad specificity USP2 catalytic core (USP2), or full length USP5 (the human homolog of yeast Ubp14). Only the band denoted by the asterisk was resistant to USP5 treatment (but not USP2), whilst FUBE-purified polyubiquitin that co-migrates with commercial K48-linked standards was readily disassembled by both USP2 and USP5. (E) Unanchored polyubiquitin can be captured from an additional mammalian cell line, HEK293Ts (293T), using immobilised-FUBE (Znf-UBP domain of USP5). However, longer unanchored polyubiquitin (and less monoubiquitin) can be captured in pull-downs using a catalytically inactive mutant (C335A) of full-length USP5 (FL), which contains additional UBDs (at least two UBA domains). Control (Con) lane represents CNBr Seph 4B that has no protein immobilised. Purifications were performed essentially, as described for U20S and HeLa cells (Materials and Methods). In this case ~ 500 μg of HEK293T cell extract was captured on 30 μL each of Con-, FUBE- or FL-USP5-Sepharose and 100% of captured protein was loaded per lane. K48 and K63 lanes represent 0.25 μg of commercial standards and blot was detected using VU-1 anti-ubiquitin antibody. Numbers indicate ubiquitin moieties in the chains.

proteasome fractions (i.e. proteasome-associated free polyubiquitin), we detected chains as short as dimers. We speculate that these could be generated by chain trimming of ubiquitin-modified substrates engaged with degradation, which then dissociate from proteasome-associated ubiquitin receptors, and are 'amputated' by the proteasome-associated DUB Rpn11. Interestingly, despite both the high affinity of FUBEs for monoubiquitin and the co-purification of ubiquitin dimer, we observed a complete lack of monoubiquitin associated with proteasome fractions. This may indicate that proteasomal DUBs fail to cleave polyubiquitin into monomers, or simply (and more plausibly) that any monoubiquitin generated does not bind avidly enough to remain associated with the proteasome. The association of ubiquitin dimers with the proteasome is of particular interest, given the dogma that the minimum signal for efficient proteasomal targeting is tetraubiquitin, a chain length proposed to promote the required affinity for proteasome binding [21]. Notably, however, more recent studies have indicated that the ubiquitin proteasomal-targeting signal is adaptive, and in some instances, the proteasome can even degrade monoubiquitylated proteins [22]. Thus, free ubiquitin dimers may well have the ability to regulate proteasome activity *in vivo* (in parallel to the regulation of certain non-proteasomal DUBs [23]), although, at least *in vitro* K48-linked ubiquitin dimers have only minimal inhibitory effects on proteasome activity [2]. Additionally, we noted the absence of detectable ubiquitin trimer in proteasome-associated fractions, a species readily purified from whole cell lysates (Figure 2A). This may indicate an inability of the proteasome to bind to ubiquitin trimers, perhaps reflecting conformational differences between ubiquitin trimers and chains of alternative lengths.

Our work also highlights the broad applicability of the FUBE affinity-enrichment strategy; unanchored polyubiquitin chains could be readily recovered from non-mammalian samples (yeast and plants), and indeed in a range of different sample-type specific extraction/binding buffers. This utility is reflective of the high conservation of the ubiquitin sequence (contact residues of ubiquitin with the Znf-UBP domain are completely conserved), and presumably also the relatively high ubiquitin-binding affinity of this particular UBD [17]. Indeed inspection of ubiquitin sequences from other species suggests that the FUBE should be useful for the purification of unanchored polyubiquitin from most eukaryotes.

The development and refinement of an enrichment strategy for unanchored polyubiquitin chains opens the way for more complete analyses into their biological significance, including, for example, a detailed consideration between polyubiquitin isopeptide linkage type(s) and biological activities. To date, K48 and K63-linked unanchored polyubiquitin chains have been confirmed to be physiologically relevant [5-7], although non-K63/K48-linked chains are also thought to exist [5], and we have provided evidence from mass spectrometry that the K11 linkage is utilised, at least in unanchored ubiquitin dimers [15]. A case in point is in *Arabidopsis* roots, where unanchored polyubiquitin chains are abundant ([20] and this work), but their composition and functions are completely uncharacterised. The phenotypes of the EMS-mutagenized *Arabidopsis* line, *per1*, which has a reduced abundance of the Ubp14 protein (USP5 equivalent), indicate unanchored polyubiquitin chains play a role in sustaining proper root responses to Pi deprivation [24]. In the future, the FUBE affinity-enrichment strategy should allow the physiological roles of unanchored polyubiquitin in plants to be defined.

Our observations from application of the FUBE affinity-enrichment to yeast are consistent with previous studies; deletion of the yeast UBP14 gene was associated with significantly increased levels of unanchored polyubiquitin chains [3], although we have been able to purify rather than simply observe these chains. Our purification using the Znf-UBP domain also, unexpectedly, revealed a ubiquitin-immunoreactive band migrating close to the position of the presumed ubiquitin tetramer, which could be disassembled with USP2 (suggesting it to be a genuine ubiquitin polymer), but resistant to processing by purified USP5 (suggesting it does not have a free proximal ubiquitin, which is inconsistent with the observed FUBE binding). The identity of this ubiquitin-reactive species is currently not known, although it could represent a ubiquitin-modified protein substrate that accumulates upon UBP14 suppression. We speculate this may be because the depletion of free monoubiquitin that occurs as unanchored polyubiquitin accumulates results in reduced activity of specific Znf-UBP domain-containing DUBs, which have been proposed to be activated by binding of ubiquitin monomers [25]. As an exemplar, the histone-specific human USP3 and USP16 DUBs are proposed to be regulated in this way [25], and yeast lacking both UBP8 (a Znf-UBP containing DUB, which is also histone specific) and UBP10 accumulate mono and diubiquitinated histones [26]. To determine whether this species represents a modified histone, we attempted identification *via* MS/MS, but only recovered ubiquitin sequences. Additionally, we used a candidate approach by probing with histone-specific antibodies, but failed to positively identify this species. Combined with the prior knowledge that the FUBE provides a high specificity for the purification of unanchored over conjugated polyubiquitin [15,16], the precise identity of the protein species remains unclear.

We envisage that in the future, FUBEs, such as the (USP5) Znf-UBP domain will provide additional insights into the physiological significance of unanchored polyubiquitin chains. One of the limitations of the current FUBE approach is the lack of selectivity for polyubiquitin chains over ubiquitin monomers, the latter of which is particularly abundant (e.g. evident prominent bands in blots shown in Figure 2 and 4), and compete for FUBE binding of polyubiquitin chains if the Znf-UBP domain is not in excess. We speculate that fusing an isopeptide linkage-selective UBD in tandem with the Znf-UBP domain should generate a reagent that not only favours unanchored polyubiquitin chains over ubiquitin monomers, but also favours chains of defined isopeptide linkage(s). As an exemplar, preliminary analyses using a catalytically inactive mutant of full-length USP5, which contains in addition to the Znf-UBP domain at least two other UBDs in tandem [3], can under certain conditions preferentially bind to longer unanchored polyubiquitin chains (Figure 4E). Such reagents could also be easily adapted for the generation of 'sensors' to selectively visualise unanchored polyubiquitin chains in living cells [27].

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