PURIFICATION AND CHARACTERISATION OF ENDOGENOUS UNANCHORED POLYUBIQUITIN

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Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

February 2017

ABSTRACT

The 76 amino-acid protein ubiquitin is peculiar in its ability to covalently modify substrate proteins. The complexity and diversity in structure and function, of this posttranslational modification is increasingly evident and has been extensively scrutinized. However, the existing notion for ubiquitin to be functionally relevant when covalently linked to substrates was recently found to be dispensable. Pioneering work by Chen's group has shown that unanchored (non-substrate bound) polyubiquitin chains are just as effective in regulating various cellular processes and presented compelling evidence about the influence of unanchored polyubiquitin chains in regulating cell signalling [1]. That said, not enough emphasis has been placed in developing tools for the direct isolation and characterisation of endogenous unanchored polyubiquitin chains.

Our group had previously developed a platform, which employs the Znf-UBP (BUZ) ubiquitin-binding domain of human Isopeptidase T enzyme, to (for the first time) directly purify cellular unanchored polyubiquitin chains of up to 15 ubiquitin moieties long from rat skeletal muscle tissue, also confirming the presence of K48 isopeptide linkages in such chains. Following on, the work described here details further refinement and optimisation of the purification protocol specifically from eukaryotic cell line (HEK293T). We could confirm by western blotting, the presence of K48 and K63 linked endogenous unanchored polyubiquitin chains, some 10 or more ubiquitin moieties long.

Our next step was to optimise existing standard protein processing protocols specifically for the study of cellular unanchored ubiquitome. Considering ubiquitin being resistant to trypsin digestion, we found a general lack of consensus in protocols best suited for processing and analysis of the cellular ubiquitome. Extensive optimisations of various protein digestion conditions were performed to arrive on the protocol best suited for unanchored polyubiquitin. The protocol was designed to address the specific problem of maximising (efficient) digestion of ubiquitin, minimising losses in the process to produce samples for quantitative analysis. The purchase of a QqQ mass

spectrometer by the group enabled quantitative estimation of polyubiquitin linkages by using AQUA standards although this required significant optimisations as well. Subsequently, we could successfully detect and estimate the abundance of isopeptide linkages purified from HEK293T cell line to be K63 (83%)>>K11 (9%)>K48, K27 (3.7-4.0%) at basal conditions. Moreover, during the course of trying to attain self-sufficiency in this area of work, our knowledge about the pitfalls and caveats in the area also improved.

Following the development of a robust in-house platform for the isolation and characterisation of unanchored polyubiquitin chains, we were eager to apply the protocol to study the endogenous unanchored polyubiquitin makeup of cells at basal conditions, and take a step further to determine the change in their levels upon activation of signalling, manipulation of possible regulators and in response to stress. While our attempt at absolute quantification of linkage abundance was not entirely successful, we present data which confirmed increase in K48 and K11 (but not K63) linked unanchored polyubiquitin chains upon proteasomal inhibition and also a strong indication of accumulation of all three linkage types upon suppression of IsoT. Finally, the study has improved our understanding of the regulation of the cellular unanchored ubiquitome.

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr Robert Layfield for the opportunity to work in his lab, and more so for all the support and advice he has offered over the years. I will remain forever grateful.

Dr David Tooth, thank you for your time, and all the help with experiments and otherwise, one day I will pay for my own or perhaps even your drink. I am grateful to Dr Joanna Strachan for all the help with techniques and advice she has offered, you are the perfect post-doc. Dr Alice Goode and Dr Daniel Scott, lab was always fun when you two were around. Matthew Carlile, I will always remember our debates about Kevin Pietersen.

I have had the pleasure of the company of a few wonderful people through the course of the PhD who at different points have made the experience a little less stressful. Dr Mar Marzo who remains my favourite Spaniard and my old friends Sid and Neha, who made Nottingham a little less alien. Also, my friends at GML especially Diane, Christine, Sally, Bob and Ray who put up with me when I was bitter, angry, stressed and sad (pretty much every day).

I cannot forget to mention the only constant for all these years – my parents who always have and continue to support me in any and every way possible. Thank you.

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ABBREVIATIONS

Amp	Ampicillin
AP-1	Activating Protein-1
APC/C	Anaphase Promoting Complex
APF-1	ATP Dependent Proteolysis Factor-1
AQUA	Absolute Quantification
ATP	Adenosine triphosphate
AUC	Area under curve
BARD1	BRCA1-Associated RING Domain Protein 1
BCA	Bicinchoninic acid
BRCA1	Breast Cancer Type 1 Susceptibility
BSA	Bovine Serum Albumin
CARD	Caspase Activation And Recruitment Domains
CID	Collision Induced Dissociation
CnBr-Sepharose	Cyanogen Bromide Activated Sepharose-4B
CYLD	Cylindromatosis
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
Doa4	Deubiquitinating Enzyme 4 / Ubiquitin Carboxyl-Terminal Hydrolase 4
DTT	Dithiothreitol
DUB	Deubiquitinating Enzyme
EDTA	Ethylenediaminetetraacetic Acid
ERAD	Endoplasmic Reticulum Associated-Degradation

FBS	Foetal Bovine Serum
GAB	Gel Application Buffer
GST	Glutathione S-Transferase
H2A	Histone 2A
HDAC6	Histone Deacetylase 6
HECT	Homologous To The E6-AP Carboxyl Terminus
HEK293	Human Embryonic Kidney 293 Cells
HEK293T	Human Embryonic Kidney 293 Cells Containing SV40 Large T Antigen
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
Hsp	Heat Shock Proteins
Hul5	HECT Ubiquitin Ligase 5
IFN	Interferon
IKK	Inhibitor of κB Kinase
IL-1R	Interleukin-1 Receptor
IL-1β	Interleukin-1β
IPTG	Isopropyl-1-Thio-β-D-Galactopyranoside
IRAK1	Interleukin-1 Receptor-Associated Kinase 1
IsoT	Isopeptidase T
IUIM	Inverted Ubiquitin Interaction Motif
I-κB	Inhibitor of κB
JAMM	JAB1/MPN/MOV34 Family
LB	Luria-Bertini Broth
LC	Liquid Chromatography

LC-MRM-MS/MS	Liquid Chromatography-Multiple Reaction Monitoring-Tandem Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
Lo	Load
LUBAC	Linear Ubiquitin Chain Assembly Complex
MAVS	Mitochondrial Antiviral Signalling
MDA5	Melanoma Differentiation-Associated Protein 5
MeCN	Acetonitrile
MEF	Mouse Embryo Fibroblasts
MMS	Methyl Methanesulfonate
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
NEM	N-Ethylmaleimide
NEMO	NF-κB Essential Modifier
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NZF	Nuclear Protein Localisation 4 Zinc Finger
oP	1,10-o-Phenanthroline
OTU	Ovarian Tumor Proteases
P/D	Pulldown
PAGE	Polyacrylamide Gel Electrophoresis
PAZ	Polyubiquitin-Associated Zinc Binding
PBS	Phosphate Buffered Saline
POH-1	Pad One Homologue-1

QqQ	Triple Quadrupole
Rfu1	Regulator Of Free Ubiquitin Chains 1
RIG-1	Retinoid Inducible Gene-1
RING	Really Interesting New Gene
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SiRNA	Small Interfering Ribonucleic Acid
SRM	Selected Reaction Monitoring
TAK1	Transforming Growth Factor B Activated Kinase-1
TBS	Tris-Buffered Saline
TEMED	Tetramethylethylenediamine
TLR	Toll Like Receptors
TNFα	Tumor Necrosis Factors A
TRAF6	Tumor Necrosis Factor Receptor-Associated Factor 6
TRIM25	Tripartite Motif E3 Ligase 25
TUBE	Tandem-Repeated Ubiquitin-Binding Entitie
UBA	Ubiquitin Associated Domain
UBAN	Ubiquitin Binding in ABIN and NEMO
UBD	Ubiquitin-Binding Domain
UBE3C.	Ubiquitin Protein Ligase E3C
Ubp14	Ubiquitin-Specific Protease
UIM	Ubiquitin Interaction Motif
USP2	Ubiquitin Carboxyl-Terminal Hydrolase 2
ZNF4	Zinc Finger 4
Znf-UBP	Zinc-Finger Ubiquitin Binding Domain

CHAPTER 1

INTRODUCTION

1.1. UBIQUITIN

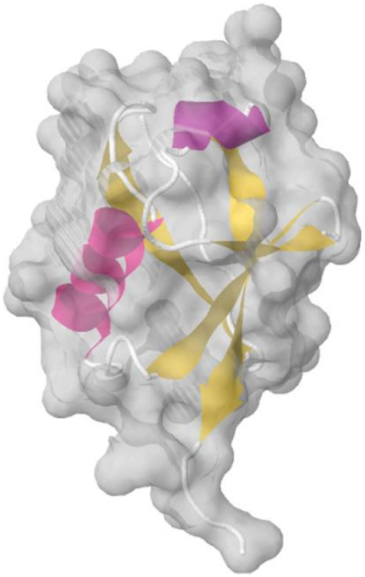
1.1.1. INTRODUCTION

Pioneering work carried out by through late 1970-80s by Avram Hershko's group on cellular pathways governing protein turnover led to the purification of a small (estimated at 9kDa) polypeptide remarkably stable to heat and proteolytic enzymes, then referred to as ATP-dependent proteolysis factor-1 or APF-1 [2]. The protein was later identified as ubiquitin, a highly conserved protein previously isolated from bovine thymus extracts [3, 4]. The molecule consisting of 76 amino-acid residues with a molecular weight of 8.565 kDa was first sequenced by Schlesinger and colleagues (bovine ubiquitin) [5]. Further, the essential requirement of ubiquitin conjugation (to other proteins) was implicated in ATP-dependent protein breakdown in reticulocytes [6, 7]. This was found to arise by the covalent attachment of ubiquitin C-terminal glycine (76^{th} residue) with lysine residues of target proteins, by means of an isopeptide linkage. The ATP-dependent protein breakdown was governed by a large multi-subunit protease complex, later purified and termed the proteasome [8-10].

The highly conserved nature of the protein was evident when the primary structures of ubiquitin was found to be conserved when extracted from range of animals [4], but differed in only three residues in that isolated from oat (*Avena sativa*) and baker's yeast (*Saccharomyces cerevisiae*) [11, 12]. Structurally, the molecule was found to be compact with 87% of the it involved in hydrogen bonding derived secondary structure [13]. The compact

globular structure (Figure 1.1) offers ubiquitin remarkable resistance to heat, changes in pH and treatment with trypsin or chaotropic agents [5, 14]. It is important to note that the primary structure of ubiquitin contains seven lysine residues, the significance of which is described later in the chapter.

A.



B.

```
        1                   20
Human - MQIFVKTLTG KTITLEVEPS
Yeast - MQIFVKTLTG KTITLEVESS
Oat   - MQIFVKTLTG KTITLEVESS
       21                   40
Human - DTIENVKAKI QDKEGIPPDQ
Yeast - DTIDNVKSKI QDKEGIPPDQ
Oat   - DTIDNVKAKI QDKEGIPPDQ
       41                   60
Human - QRLIFAGKQL EDGRTLSDYN
Yeast - QRLIFAGKQL EDGRTLSDYN
Oat   - QRLIFAGKQL EDGRTLADYN
       61                76
Human - IQKESTLHLV LRLRGG
Yeast - IQKESTLHLV LRLRGG
Oat   - IQKESTLHLV LRLRGG
```

Figure 1.1.
A. Depiction of ribbon and surface structure of human ubiquitin encompassing secondary structures displayed (Protein Data Bank Code - 1UBQ).

B. Comparison of primary structure of ubiquitin from three sources – human, yeast and oat. The 7 lysine residues have been highlighted (in bold and underlined) along with the 3 variations seen in primary structures of yeast and oat when compared to human (marked in red).

1.1.2. UBIQUITIN AS A POST-TRANSLATIONAL MODIFICATION

Ubiquitin is an abundant cellular protein accounting for 0.1%-5% ($^{w}/_{w}$) of total cellular proteomes [15-17]. Ubiquitin is synthesised (in yeast and higher eukaryotes) *de novo* by four genes [18]. Three of these in yeast and two in mammals produce a fusion product of ubiquitin with ribosomal polypeptide at the C-terminal, while the rest produce transcripts that code multiple copies of the molecule in a linear head to tail configuration through peptide bonds [18-20]. While ubiquitin is an abundant protein in cells, it was discovered to covalently modify substrate proteins by the formation of an isopeptide bond between the ε-amino group of lysine residues of substrates and C-terminal glycine of ubiquitin [6]. The first evidence of such modification reported was that of histone H_2A [21, 22]. Furthermore chains containing multiple (poly) ubiquitin moieties covalently linked to substrates were found to be necessary for proteasomal degradation [23, 24]. The ubiquitin moieties in such chains were linked *via* isopeptide bonds formed between C-terminal glycine of one ubiquitin and lysine residues at the 48^{th} position (K48) of the adjacent ubiquitin molecule.

Avram Hershko's group while noting the requirement of such a modification in protein degradation also discovered the ability of ubiquitin chain formation and postulated the mechanism for regulation of the process [24, 25]. The (reversible) attachment of ubiquitin to substrate proteins is governed by consecutive action of three sets of enzymes – ubiquitin activating enzymes E1, ubiquitin conjugating enzymes E2 and ubiquitin ligases or E3 (reviewed in [26]).

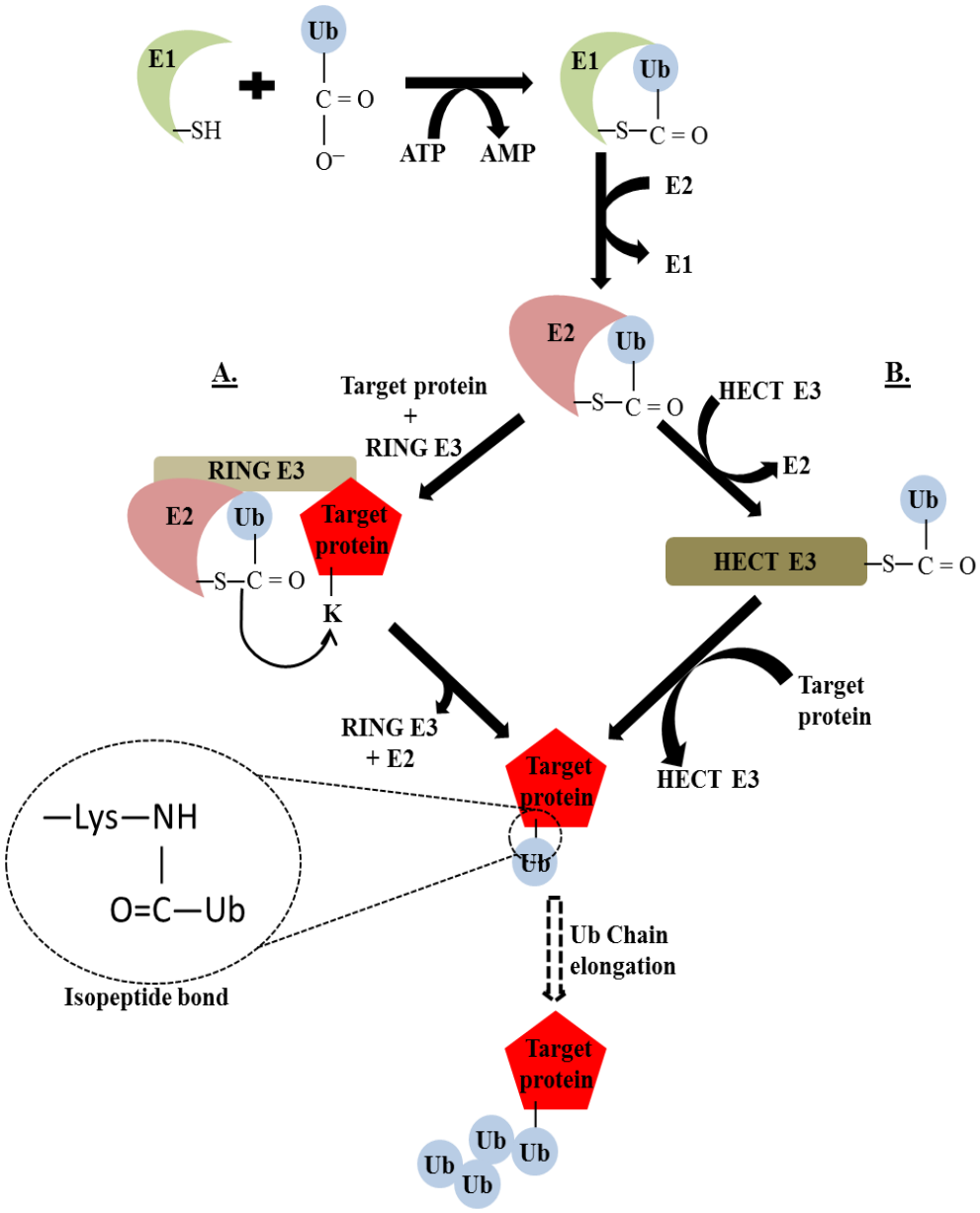


Figure 1.2. The ubiquitination machinery involves covalent attachment of the C-terminus of ubiquitin to lysine residues on protein substrates through the sequential action of ubiquitin activating enzymes (E1), conjugating enzymes (E2) and ligases (E3). E1 forms a thiolester bond between their active site cysteine and ubiquitin C-terminal at the expense of ATP, eventually transferring the ubiquitin to E2 enzymes (also linked through thiolester linkages). Based on the family of E3 involved, E2 can either directly transfer the ubiquitin on to lysine residues of target substrates as with the case of RING domain (and U-box) E3s (**A.**), or as seen in the case of HECT E3 may involve an intermediate step where the ubiquitin is covalently linked to the E3 before being transferred on to the substrates. The cycle can be repeated to produce polyubiquitinated substrates.

Briefly, ubiquitin in the presence of E1 and ATP is first adenylated and subsequently attached to the active cysteine residue of E1 enzyme by a process called thioesterification. The E1 now transfers the attached ubiquitin to an E2 enzyme through a tran-thioesterification reaction. E2 now interacts with a specific E3 enzyme which covalently modifies substrate proteins in a process that varies depending on the family of E3 enzyme involved. RING (really interesting new gene) domain containing E3 enzymes and the related U-box E3s interact with ubiquitin conjugated E2s, and facilitate the direct transfer of the ubiquitin molecule onto the ε-amino group of lysine residues of protein substrates. However, HECT (homologous to E6-AP C-terminus) E3 requires an additional intermediate step wherein ubiquitin, prior to being attached to substrate proteins, is first transferred onto the E3 itself by a trans-thioesterification reaction (Figure 1.2). The human proteome is considered to contain 2 E1s, over 40 E2s and over 600 E3s (reviewed in [27]).

It is important to note that with respect to polyubiquitin chain conjugation (with substrates) and elongation, there is a lack of absolute consensus on whether these chains are constructed *de novo* or whether pre-formed chains can be attached directly to protein substrates. The enzyme E2-25K is known for its ability to synthesize free (not-substrate linked) polyubiquitin chains *in vitro*, which in theory can be transferred *en bloc* to substrates by E3 enzymes [28]. It is thus easy to see how the process can ensure rapid ubiquitination if necessary by attachment of pre-formed ubiquitin chains [29]. However, it has been argued that due to abundant mono-ubiquitin in cells, construction of ubiquitin chains *de novo* may be the preferred method of chain elongation [30].

The second aspect of the ubiquitination pathway ensures that the process is reversible. A family of proteases termed deubiquitinating enzymes or DUBs regulate ubiquitin pathway by cleaving the isopeptide (or peptide) linkages between substrate proteins and ubiquitin, or that between two ubiquitin molecules. About 100 DUBs are known to exist in humans which have been divided into five families namely - ubiquitin C-terminal hydrolases or UCH, ubiquitin specific proteases USP/UBP, ovarian tumor proteases OTUs, Josephin domain, and the JAB1/MPN/MOV34 family JAMM domain metalloprotease [31, 32]. While the first four families are cysteine proteases displaying an active site cysteine residue, the JAMM domain DUBs are peculiar for the requirement of coordinated zinc ions (by histidine and aspartate residues) to catalyse breakdown of isopeptide linkages [33].

As expected, the activity of a DUB requires stringent controls in the form of regulators. The selectivity of the DUB for a particular substrate is governed by avidity of multiple substrate interacting domains, which upon binding cause conformational changes in the DUB thereby regulating activity and specificity of the enzymes [32, 34]. Functionally, DUBs act on ubiquitin precursors in the form of linear multi-ubiquitin or ubiquitin conjugated to ribosomal polypeptides, thus releasing free monoubiquitin. DUBs also act on ubiquitinated proteins, in the process regulating signals mediated by ubiquitination and also replenishing the cellular reservoir of ubiquitin [31, 35].

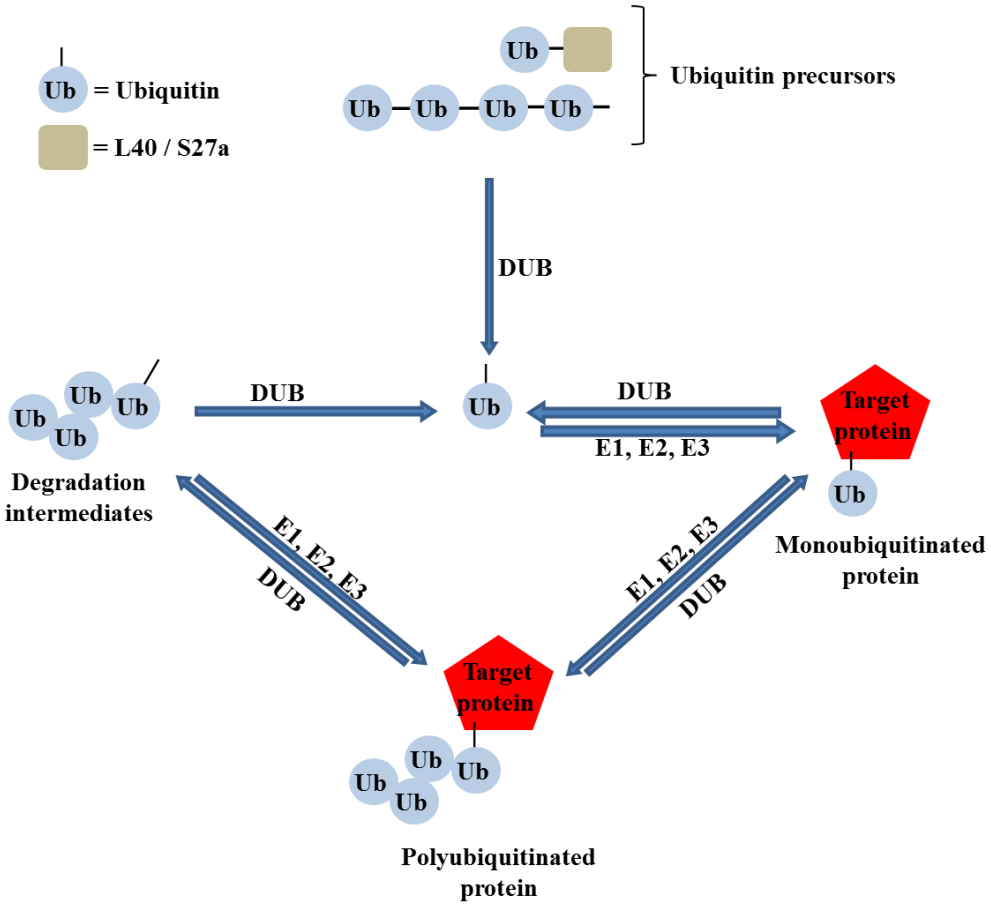


Figure 1.3. Diverse roles of DUBs in the ubiquitin system. Ubiquitin in mammals is coded by four genes, which produce either a fusion of multiple ubiquitin moieties in a linear fashion or monoubiquitin linked to ribosomal proteins L40 or S27a. An essential function of DUBs is the release of monoubiquitin from these precursors into the cellular ubiquitin pool. Ubiquitin is then utilised in (mono/poly) ubiquitination of substrates by the actions of E1, E2 and E3. These ubiquitination signals are either non-degradative in which case the substrates are deubiquitinated while the substrates are released. However, if the signal is degradative, the substrates are targeted to proteasomal degradation and the ubiquitin molecules restored to the pool. The release of ubiquitin back into the cellular pool is governed by the reservoirs of DUBs.

1.1.3. COMPLEXITY OF UBIQUITIN MODIFICATION – POLYUBIQUITIN CHAIN TOPOLOGIES

While covalent attachment of one or multiple molecules of ubiquitin to protein substrates was widely documented [6], the first instance of polyubiquitinated proteins (and certainly the best documented) involved the linkage of ubiquitin molecules via their lysine residues at the 48th position, commonly referred to as K48 linkage [23]. As mentioned previously, ubiquitin contains seven lysine residues with a possibility of any of these being involved in such isopeptide linkages. Preliminary experiments which sought to determine what lysine residues of ubiquitin were involved in isopeptide linkages leading to polyubiquitin chains, made use of ubiquitin constructs wherein lysine residues were systematically replaced by arginine, thereby blocking the site from being an acceptor of ubiquitin. The strategy in *Saccharomyces cerevisiae* revealed occurrence of polyubiquitin chain formation *via* K29, K63 and K48 (termed K29, K63 and K48 isopeptide linkages respectively) [36]. The essential role of K48 and K63 linkages became obvious when substitution of host ubiquitin with UbK48R and UbK63R mutants impacted cell cycle progression and DNA repair mechanism respectively [37, 38]. Existence of K6 and K11 linked polyubiquitin chains, synthesised by ubiquitin conjugating enzymes were also observed by the same strategy along with the affinity of proteasomal receptor (subunit 5 of the regulatory complex) for K48 and K11 linkages [39]. The implication of such studies was the existence of polyubiquitin chains of various linkage types synthesised by their corresponding ubiquitin conjugating enzymes, with each linkage type signalling specific cellular functions.

The recent advances in techniques and sensitivity of mass spectrometers enabled the confirmation of presence of all 7 ubiquitin isopeptide linkages in *Saccharomyces cerevisiae* [40, 41]. Similar techniques also confirmed the presence of all 7 ubiquitin isopeptide linkages in mammalian cells [42]. In addition to the 7 isopeptide linkages described, ubiquitin ligase complex LUBAC (linear ubiquitin chain assembly complex) displays the ability to synthesize linear ubiquitin chains (or M1 linked). Such linear chains lack isopeptide linkages but instead contain normal peptide linkages between C-terminal glycine and N-terminal methionine (Met1 linked) of adjacent ubiquitin residues formed post-translationally [43, 44].

Thus far, polyubiquitin chains described have been assumed to be homogenous regarding the linkage type (i.e. lysine used in chain formation), more specifically containing only one linkage type and hence termed homotypic chains. However to add to the diversity and complexity of the polyubiquitin signal, such chains can also be heterogeneous or heterotypic, wherein a single polyubiquitin chain could contain more than one linkage type (Figure 1.4). This could involve the presence of a branch point wherein a single ubiquitin molecule is bound to two or more ubiquitin moieties (*via* multiple lysine residues) thus termed branched or forked ubiquitin chains [40, 45]. The other form of heterotypic polyubiquitin chains would involve multiple types of ubiquitin linkages in a single linear unbranched chain [45]. The presence of linear (unbranched) mixed heterotypic chains would be harder to detect requiring middle-down or top-down mass spectrometry, thereby preserving ubiquitin linkage integrity [46].

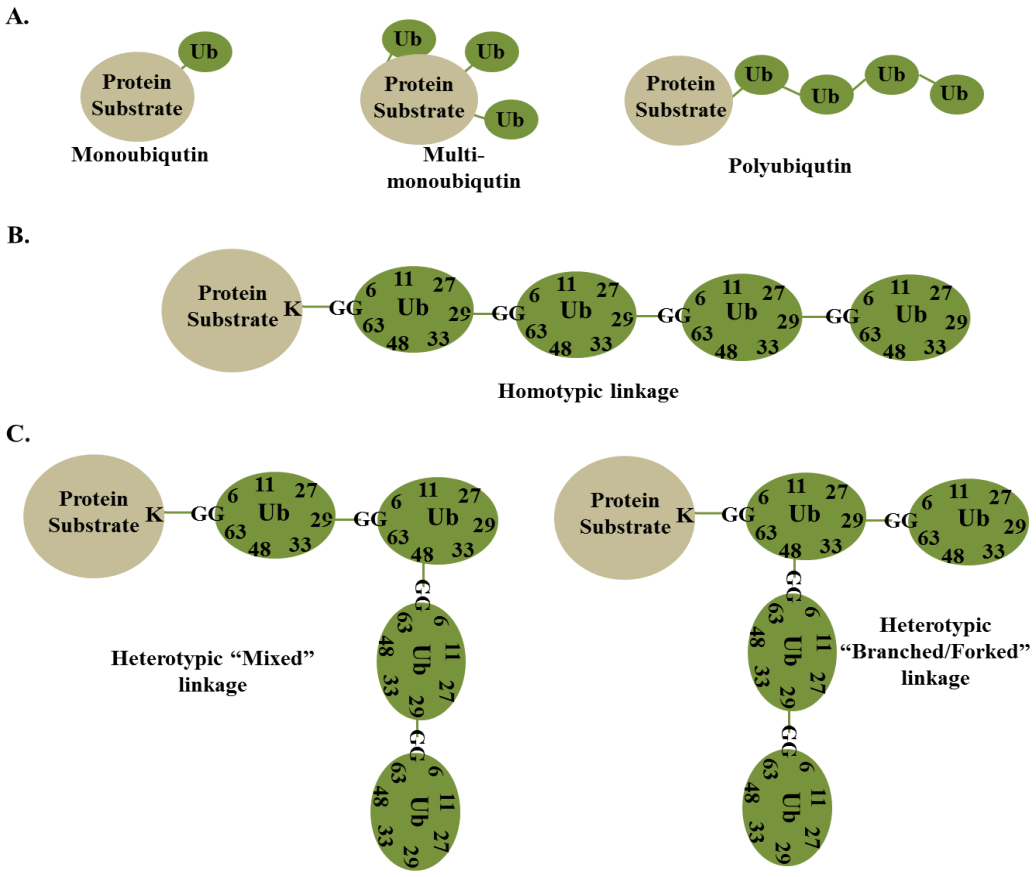


Figure 1.4. Diverse forms of substrate ubiquitination. Substrates can be monoubiquitinated, multi-monoubiquitinated or polyubiquitinated, based on the number of ubiquitin moieties covalently linked to substrate lysines (**A**). Polyubiquitin linkages can be classified as homotypic when all isopeptide linkages involve the same (ubiquitin) lysine residues (**B**). Heterotypic polyubiquitin linkages contain isopeptide linkages involving multiple ubiquitin lysine residues (**C**). These could either be present as an extended mixed chain or in some cases a branched or forked chain wherein one ubiquitin moiety is linked to ubiquitin moieties through multiple lysine residues

It is worth noting that the complexity of the ubiquitin system reviewed so far has overlooked the existence of unanchored (or non-substrate linked) polyubiquitin chains, which would be detailed in Section 1.2.

1.1.4. UBIQUITIN-BINDING DOMAINS IN UBIQUITIN RECEPTORS INTERPRET STRUCTURAL TOPOLOGIES INTO FUNCTION

The complexity of ubiquitin modifications demands a multitude of proteins that recognize and interact with topologies presented by such modifications, interpret signals and modulate downstream processes. Such proteins are termed ubiquitin receptors and the domains encompassed within, which aid in such interactions, are called ubiquitin-binding domains or UBDs [47, 48]. These UBDs are commonly between 20-150 amino-acid residues in size and are capable of (weak) non-covalent interactions with ubiquitin with K_D values ranging from 10-500 μM [49, 50]. Three distinct patches on the surface of ubiquitin molecule are seen to be the primary surfaces for such interactions with UBDs as illustrated in Figure 1.5. A hydrophobic patch centred around Ile44 is the surface that the majority of UBDs interact with [48]. All α-helical UBDs, which form the biggest subset of UBDs and includes the ubiquitin associated domain (UBA) which was the first UBD to be discovered [51], are seen to interact with the Ile44 patch that includes Leu8, Ile44, His68 and Val70 [52-54]. The second is an acidic (polar) patch around the Asp58 residue (encompassing residues Arg54, Thr55, Ser57, Asp58, Tyr59, and Asn60), a site for interactions with the inverted ubiquitin interaction motif (IUIM) and A20 like zinc finger (A20 Znf) domain [55, 56]. The third such interacting surface is the C-terminal tail (consisting of residues Leu71, Arg72, Leu73, Arg74, Gly75, Gly76), a known interacting surface for the polyubiquitin-associated zinc binding (Znf-UBP or PAZ) domain, as noted in case of unanchored polyubiquitin chains (discussed in detail later) [57].

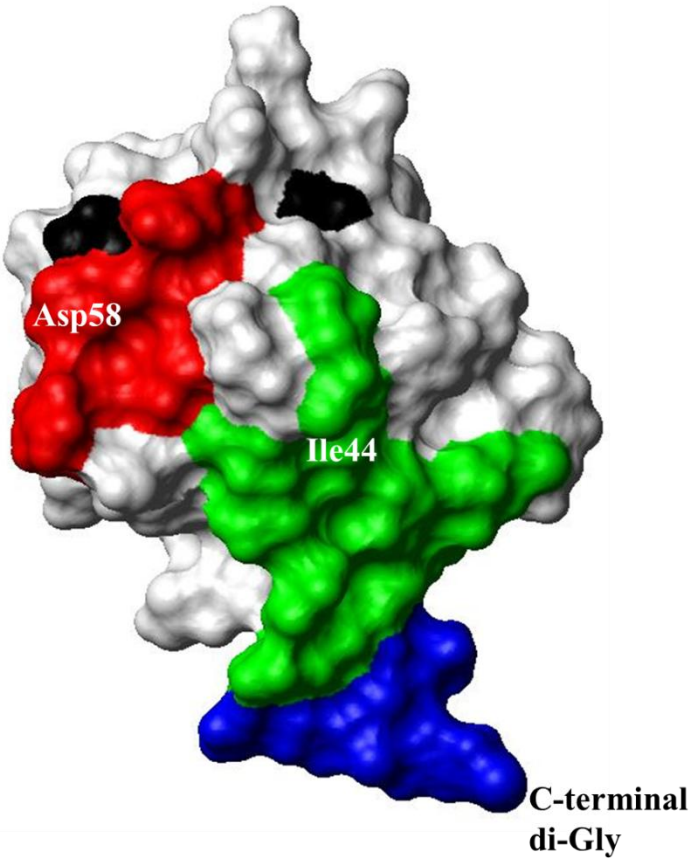


Figure 1.5. The three major interaction surfaces of ubiquitin have been depicted. The hydrophobic Ile44 surface (green), acidic Asp58 (red) and C-terminal diglycine motif (blue) have been indicated. (The patches highlighted in black are serines which could potentially be modified by phosphorylation).

While the major interaction surfaces on ubiquitin have been detailed herein, it is however worth noting that additional interaction patches have also been detected in the ubiquitin code. This may involve an UBD interacting with ubiquitin at a motif in the vicinity of, or surrounding the three patches described. A patch centred on Phe4 (composed of residues Gln2, Phe4 and Thr14) was first studied for being essential for cell survival in *Saccharomyces cerevisiae* [58], and was later shown to play a key role in the interaction with Met1 linked polyubiquitin chains produced by LUBAC [59, 60]. Another such hydrophobic patch is presented by Ile36 (along with Leu8, Leu71 and Leu73),

and due to its proximity to the C-terminal tail, the Znf-UBP domain of the DUB Isopeptidase T (IsoT) is an interacting partner [57, 61]. The domain has also been shown to participate in associating with HECT E3 enzymes [62]. It is worth noting that the Leu8 residue which is part of the flexible β1-β2 loop, could contribute to interactions pertaining both the Ile44 and the Ile36 patches based on the conformation of the loop [61]. The last of these known interaction patches termed the TEK box (consisting of residues Lys6, Lys11, Thr12, Thr14 and Glu34) plays a vital role in K11 linked ubiquitin chain elongation and mitotic progression [63, 64].

While the ubiquitin binding patches described thus far could explain interaction of proteins with single ubiquitin molecules, interactions with polyubiquitin chains are far more complex. Firstly, polyubiquitin chains of different isopeptide linkages assume corresponding unique conformations, depending on the possibility of surface interactions (involving the hydrophobic Ile36 and Ile44 patches [65]) between adjacent ubiquitin moieties in the polyubiquitin chain [66]. As a result, such chains can assume "compact" or "closed" conformations as in the case of K6, K11 and K48 linkages [67-70]. However, Met1 or K29, K33 and K63 linked polyubiquitin chains adopt a more "relaxed" or "open" conformation with the only contact between adjacent ubiquitin moieties being the isopeptide (or normal peptide linkage in case of Met1 linked chains) linkage [67, 71, 72]. It is also interesting to note that discrepancies can be observed when observing the conformations of polyubiquitin chains in crystal or in solution, indicating the possibility of intrinsic flexibility in conformations adopted [68, 73, 74]. The presence of

such dynamic conformations affects the relative positioning or orientation of interacting surfaces of ubiquitin moieties on polyubiquitin chains. UBDs of ubiquitin receptors can thus discriminate between polyubiquitin chains of different linkages (linkage selectivity) based on their conformations, while such interactions (between ubiquitin receptor and polyubiquitin chains) themselves can further remodel or stabilise polyubiquitin chain conformations as needed [66, 75]. This process ensures specificity of interactions resulting in the complex ubiquitin signal governing vast array of cellular processes.

Since the binding affinities of UBDs with monoubiquitin are relatively weak, it is not uncommon for the existence of multiple binding sites, thereby leading to higher binding affinities through avidity [52]. The protein A20 contains seven zinc finger domains, out of which (ZNF4) has been seen to bind specifically to K63 linked polyubiquitin chains [55]. This affinity for K63 linkage is due to the interaction of ZNF4 with the Ile44 patch, the TEK-box, and residues surrounding Asp58 of ubiquitin moieties in a tri-ubiquitin chain [55]. Similarly, ubiquitin receptors may also contain multiple ubiquitin binding domains, thereby strengthening the interactions with ubiquitinated substrates. The DUB IsoT contains a Znf-UBP and two UBA domains and together they bestow specificity of the enzyme towards unanchored polyubiquitin chains [57, 76]. Similarly, the 2 UIM domains of RAP80 protein are spaced so as to bind specifically to the Ile44 patches of ubiquitin moieties linked by K63 linkage in polyubiquitin chain, thereby ensuring linkage specificity [77]. It was interesting to note that the same study also proved that a shorter linker between

the two UIMs of Ataxin-3 provided the protein specificity for K48 linked polyubiquitin chains [77].

1.1.5. CHARACTERISATION OF CELLULAR UBIQUITOME (TOTAL CELLULAR UBIQUITIN) – COMPOSITION AND FUNCTION

Ubiquitin is an abundant cellular protein essential for regulating numerous physiological processes. Based on the accumulating evidence regarding the ubiquitin pathway, it is possible to estimate the amount and distribution of ubiquitin in cells. A large variation was obtained when attempts were made to quantify cellular ubiquitin in various mammalian cell lines. Human embryonic kidney or HEK293 cells, known for their abundant protein levels were seen to contain 500 pmol/mg of ubiquitin in cells [78]. The same study revealed ubiquitin content of mouse embryo fibroblasts (MEF cell line) to be almost half of that in HEK293 cells [78]. Further studies on various human cell lines revealed the total cellular ubiquitin content to be ~100 pmol/mg [79]. Furthermore, the distribution of ubiquitin in cells has been studied by quantifying distinct pools that exist in cells – free ubiquitin (including thiolester linked activated ubiquitin) and conjugated ubiquitin (monoubiquitinated and polyubiquitinated). In this regard, it was revealed that the majority (over 60%) of cellular ubiquitin (in HEK293 and MEF cell lines) exists in the form of conjugated monoubiquitin [78]. The same study estimated the amount of free ubiquitin to ~23%, while polyubiquitinated chains made up the least abundant fraction making up ~11% of cellular ubiquitin.

While conjugated polyubiquitin comprises the least abundant fraction of the cellular ubiquitome, available data on this pool certainly sheds some light on

the complexity. This is evident firstly by the identification of increasing number of substrate ubiquitination sites, but also in the makeup of polyubiquitin isopeptide linkages present. The identification of ubiquitination sites involves a novel technique to enrich peptides with modified lysines in the typsinolysed ubiquitome using an antibody specific for the C-terminal diglycine remnant which is indicative of ubiquitination [78], followed by quantitative MS [80, 81]. Regarding the linkages, as previously mentioned (Section 1.1.3) it is well established that all eight types (seven isopeptide and Met1 linked chains) of polyubiquitin chain linkages exist in cells. Initial reports based on relative abundance suggested the distribution of ubiquitin linkages (in yeast) to be K48 > K63 and K11 >> K33, K27 and K6 [40]. The distribution in wild type *S.cerevisiae* was later revised and quantified to be 29.1 ± 1.9% (K48), 28.0 ± 1.4% (K11), 16.3 ± 0.2% (K63), 10.9 ± 1.9% (K6), 9.0 ± 0.1% (K27), 3.5 ± 0.1% (K33) and 3.2 ± 0.1% (K29) [82]. This was later contradicted by a study which found K48, K63 and K29 linkages to be the most abundant in yeast with very low levels of K11 [83]. In mammalian cells (HEK293), these were estimated at 52% (K48) and 38% (K63), 8% (K29), 2% (K11) and 0.5% for (K6) [42]. The contribution of K27, K33 and Met1 linkages were each found to be less than 0.5%. A study soon after concluded that K48, K63 and K11 to be the most abundant linkages in HEK293 cells, with negligible levels of K6, K27, K29 and K33 [78]. The discrepancies in the analysis of polyubiquitin chain composition could arise due to factors including growth stage of cells, method of sample preparation including lysis, purification and enrichment (tags, antibodies or UBDs) and equipment used

and its sensitivity. The data so far also highlights the lack of consensus on methodologies used to quantify polyubiquitin linkages.

To date K48 linked polyubiquitin chains have been the best characterised, in terms of its structure, interactions and function. As previously mentioned, K48 linked chains are one of the most abundant among cellular polyubiquitin chains, and its role in targeting substrates to proteasomal degradation was the first to be established [23]. K48 linked tetraubiquitin chains have been shown to be necessary and sufficient to signal proteasomal degradation of the conjugated substrate [84]. Experiments involving proteasomal inhibition suggested that all but the K63 (and Met1) linked chains were found to accumulate in *S.cerevisiae* and mammalian neuronal cells, thereby indicating their possible role in mediating proteasomal degradation [82, 85]. K63 linked polyubiquitin chains however have been long known for their role in an array of non-degradative processes including DNA repair, kinase activation, protein trafficking, cell signalling and cellular immune response pathways [38, 86-88]. The mediation of cytokine signalling and NF-κB activation by K63 linked polyubiquitination has been described in Section 1.2.2. Considering there being very little literature about physiological roles of K6, K27, K29 and K33 linkages, a brief summary of known cellular functions of such "non-canonical" or "atypical" ubiquitin linkages is also included below.

- Met1 linked – The interaction of Met1 linked polyubiquitin chains with the NF-κB essential modifier (NEMO) protein via the latter's ubiquitin binding in ABIN and NEMO (UBAN) domain are essential for inhibitor of κB kinase (IKK) activation and thus cell signalling [59].
- K6 – The tumour suppressor breast cancer type 1 susceptibility (BRCA1) in association with BRCA1-associated RING domain protein 1 (BARD1) forms an E3 complex known to assemble K6 linked polyubiquitin chains [89, 90]. The BRCA1-BARD1 complex ubiquitinates itself and its substrates with K6 polyubiquitin chains which are then recognized by ubiquitin receptors such as RAP80 in response to DNA damage [91]. It can thus be presumed that K6 linked polyubiquitin chains may have a role mediating DNA repair.
- K11 – Homotypic K11 chains are assembled by anaphase promoting complex (APC/C) in higher eukaryotes, and are found to target substrates for proteasomal degradation thereby regulating mitotic progression [92, 93]. The linkage is also associated with the endoplasmic reticulum associated-degradation (ERAD) pathway [82] and cytokine signalling [94]. K11 (and K63) linked heterotypic chains are necessary for internalisation of cell surface immunoreceptors [95, 96].
- K27 – Mitochondrial damage prompts the E3 parkin to conjugate substrates with K27 linked polyubiquitin chains thus mediating their clearance by autophagy (mitophagy) [97, 98].

- K29/K33 – While the DUB TRABID shows selectivity to K29 and K33 linked polyubiquitin chains [99], very little is known about the involvement of these chains in cellular processes.

In addition to polyubiquitin chains, monoubiquitin also has well defined physiological roles. Monoubiquitin has previously been considered to be sufficient to signal proteasomal targeting of smaller proteins [100, 101]. However, monoubiquitination is widely acknowledged to mediate endocytosis or cell surface receptors [102, 103], transcriptional activation governed by the ubiquitination of histones [104] and DNA damage response [105]. Furthermore, multi-monoubiquitin is regarded as a signal for receptor internalisation and lysosomal degradation [106].

1.2. NON SUBSTRATE LINKED "UNANCHORED" (POLY)UBIQUITIN CHAINS

1.2.1. INTRODUCTION

While thus far, discussions about the cellular ubiquitome was in reference to ubiquitin modified substrates, unanchored polyubiquitin as the name suggests refers to a subset of the ubiquitome which is not covalently attached to substrates, and as a result exhibits a free C-terminal diglycine (-$G^{75}G^{76}$) motif. It is important to note the distinction that although free monoubiquitin exhibits a diglycine motif as well, in this study unanchored ubiquitin would solely refer to chains containing two or more ubiquitin moieties which are not conjugated to protein substrates (Figure 1.6).

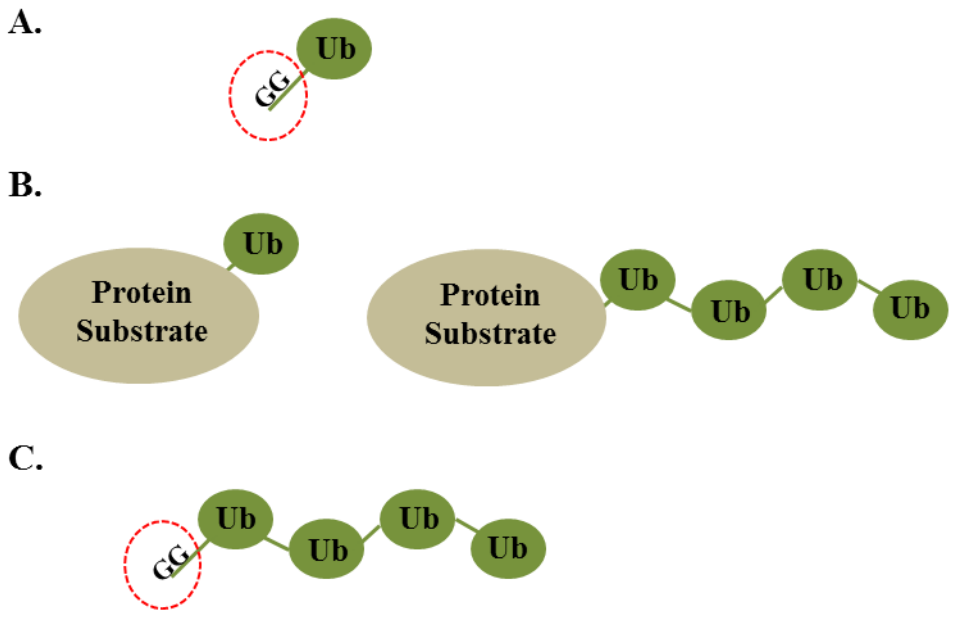


Figure 1.6. Unanchored polyubiquitin chains (**C**) being depicted as characteristically distinct species in cellular ubiquitome, when compared to free monoubiquitin (**A**) or substrate linked (mono or poly) ubiquitin (**B**). The free C-terminal diglycine motifs ($G^{75}G^{76}$) displayed by unanchored polyubiquitin (and monoubiquitin) but not by conjugated (to protein substrate) ubiquitin have been highlighted in red.

Though the presence of this cellular unanchored polyubiquitin species has been acknowledged for some time [107], until recently very little effort has been devoted towards their study. Some of the earlier accounts include the ability of short unanchored polyubiquitin chains to competitively inhibit proteasome function [107]. The study discovered an increase in unanchored polyubiquitin chains in *S. cerevisiae* upon deletion of DUB Ubp14 (ubiquitin-specific protease, homolog of human IsoT), leading to defects in proteasomal processing. Not only did the study discover a role of cellular unanchored polyubiquitin chains, it also highlighted the presence of a modulating enzyme whose function was to specifically regulate unanchored polyubiquitin chains [107]. The finding was supported by the observation of exogenously synthesised polyubiquitin chains inhibiting processing of ubiquitinated substrates by purified mammalian 26S proteasomes [108]. Discovery of cellular machinery which specifically regulates level of endogenous unanchored polyubiquitin chains was yet another indication of the significance of the latter. Firstly, the mammalian DUB IsoT was discovered to specifically target and disassemble unanchored polyubiquitin chains [57, 76]. Significance of the enzyme was once again highlighted when knockdown of IsoT levels in cultured mammalian cells resulted in the stabilisation of ubiquitinated p53 by competitive inhibition of the proteasome [109]. Also significant was the discovery of Rfu1 (regulator of free ubiquitin chains 1), an inhibitor of yeast DUB Doa4 (ubiquitin carboxyl-terminal hydrolase 4) involved in regulation of homeostasis between levels of cellular ubiquitin and unanchored polyubiquitin chains [110].

While there was enough evidence to suggest a vital role of unanchored polyubiquitin chains in cellular processes, the most definitive evidence of this was provided by a study conducted by Xia *et al* [1]. The findings of this study have been discussed in detail in the next section.

1.2.2. UNANCHORED POLYUBIQUITIN IN CELLULAR PROCESSES - THE STORY SO FAR

While the effect of unanchored polyubiquitin chains on proteasomes has been known for some time, a host of recent studies have linked these chains to directly modulating cellular processes thereby challenging the existing dogma of the ubiquitin system. TRAF6 (tumor necrosis factor receptor-associated factor 6) is a ubiquitin ligase with an essential role in propagating IL-1R (Interleukin-1 receptor) and TLR (Toll-like receptors) and mediated activation of NF-κB and MAPK [111-114]. In the context of IL-1β mediated activation of IL-1R, TRAF6 in association with ubiquitin conjugating enzymes UBC13/UEV1A was thought to dimerise and undergo autoubiquitination *via* K63 linkage [115, 116]. It is this K63-linked polyubiquitin signal which acts as a scaffold, and leads to recruitment and activation of downstream kinases including TAK1 or Transforming growth factor β activated kinase-1 (as depicted in Figure 1.7) [116]. The K63-linked polyubiquitin chains associate with the TAB2 and TAB3 subunits of the complex (*via* their ubiquitin-binding nuclear protein localisation 4 zinc finger or NZF domain) thereby leading to the activation of TAK1 [117]. Activated TAK1 further phosphorylates and activates IKK, thus leading to phosphorylation (and eventual degradation) of the inhibitors of κB (IκB) and nuclear translocation of NF-κB [86, 118].

However, a study by Zhijian Chen's group proved that autoubiquitination of TRAF6 was dispensable for the propagate of such signalling [1], with a similar observation reported previously [119]. This was found to be true when the pathway was reconstituted *in-vitro*, as well as experiments performed using HEK293T cells stably expressing IL-1R which were stimulated with IL-1β. The same was found to be true with the activation of IKK, where unanchored polyubiquitin chains synthesised by TRAF6 in association with E2 Ubc5 activated IKK [1]. This was the first study to provide definitive proof of the significance of unanchored polyubiquitin chains in cell signalling (aspects of the experiment were replicated and dealt with in further detail in Chapter 6). It is interesting to note that the unanchored nature of polyubiquitin chains in the study was inferred indirectly by making use of IsoT, a DUB known to selectively disassemble unanchored polyubiquitin chains [57].

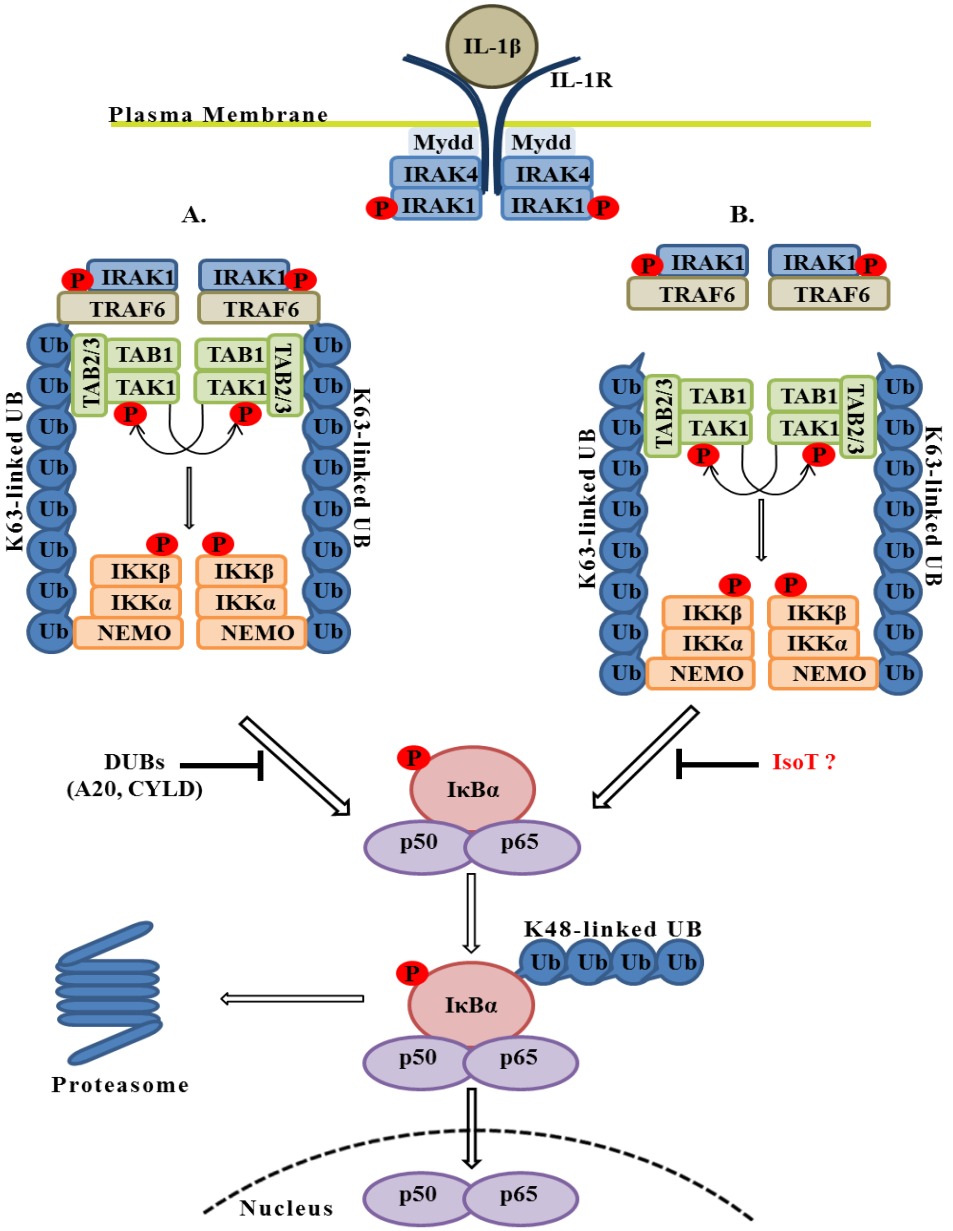


Figure 1.7. Conjugation of polyubiquitin chains to substrates was not a requisite to IL-1β mediated NF-κB activation. Stimulation of IL-1R by IL-1β led to recruitment of MyD88-IRAK4-IRAK1 complex, and subsequent phosphorylation of IRAK1. The latter associates with E3 TRAF6 which (in association with Ubc13 and Uev1A) catalyses the synthesis of K63 linked polyubiquitin chains bound to itself (A) or which are unanchored (B). The consequence of both events is the binding to TAB2 (of TAK1 complex) and autophosphorylation (and activation) of TAK1. Activated TAK1 phosphorylates IKKβ of IKK complex, which is thus activated to phosphorylate IκBα and thus signal its proteasomal degradation (post K48 polyubiquitin binding). The NF-κB complex (p50/p65) is now free to translocate into the nucleus and control expression of downstream genes. It is also important to mention the inhibitory effects of DUBs such as A20 and CYLD which may prevent IKK activation. The specificity of IsoT for unanchored polyubiquitin chains may also suggest its possible role in NF-κB regulation. (Adapted from [120]).

A subsequent study by Chen group also indicated the role of unanchored polyubiquitin chains in the initiation of interferon mediated immune response. Briefly, activated retinoid inducible gene-1 or RIG-1 (in the presence of viral RNA) leads to the interaction between its own caspase activation and recruitment domains (CARD) and that of mitochondrial antiviral signalling (MAVS) on the outer mitochondrial membrane [121, 122]. The interaction signals a downstream cascade which results in the activation of transcription factors IRF3 and eventually the expression of type 1 interferons (IFN) [123]. Ubiquitination is well documented as an essential requirement at several steps of multiple immune response and signalling cascades [124]. In this case, activation of RIG-1 was previously thought to arise on account of its ubiquitination (of K172 residue) with K63-linked chains synthesised by a member of the tripartite motif family of E3 ligases - TRIM25 [125]. However, the covalent attachment of polyubiquitin chain to RIG-1 was not a prerequisite to its activation when the pathway was reconstituted *in vitro* [126]. Experiments conducted by the group noted that the interaction of unanchored K63-linked polyubiquitin chains with CARD domains of RIG-1 (in the presence of viral RNA) was sufficient to signal its activation [126]. Furthermore, co-purification of what was shown to be unanchored polyubiquitin chains from HEK293T cells by the group further substantiated the argument of their existence in cells and possible involvement in the regulation of cellular processes. A follow-up study by the group further highlighted the mechanism in IFN expression, involving the oligomerization and subsequent activation of RIG-1 and (also) MDA5 (melanoma

differentiation-associated protein 5), the latter mediated by binding of unanchored polyubiquitin chains [127].

Unanchored polyubiquitin was also recorded to modulate immune response culminating in the activation of AP-1 (activating protein-1) and NF-κB mediated signalling, upon virus invasion [128]. Another member of the tripartite motif family – TRIM5 (much like TRAF6), was also seen to interact with the viral capsid and synthesise unanchored K63-linked polyubiquitin chains in the presence of E2 enzymes UBC13/UEV1A. These unanchored polyubiquitin chains were found to be necessary and sufficient for the activation of the TAK1 complex (through *in-vitro* reconstitution experiments) and initiation of downstream signalling cascade [128]. Furthermore, unanchored polyubiquitin chains were also found to play an essential role in aggresome formation. Aggresomes are inclusion bodies composed of detergent insoluble (misfolded) ubiquitinated protein aggregates, formed as a result of proteasomal processivity being challenged [129]. The formation of aggresomes is governed by histone deacetylase 6 or HDAC6 [130], an enzyme comprising of a Znf-UBP domain [131] which selectively binds to the ubiquitin C-terminal diglycine motif [132, 133]. Action of DUB ataxin-3 on ubiquitinated proteins in aggregates leads to free C-terminals of (now unanchored) polyubiquitin chains being exposed [133]. HDAC6 binds to these free C-terminals of unanchored polyubiquitin chains and facilitates the transport of such aggregates to the aggresomes using cellular microtubule network. Unanchored (K63-linked) polyubiquitin chains produced by proteasome associated DUB

POH-1 (pad one homologue) were also found necessary for HDAC6 mediated clearance of aggresomes [134].

1.2.3. ISOPEPTIDASE T AND UBIQUITIN PROTEIN LIGASE E3C - REGULATORS OF ENDOGENOUS UNANCHORED POLYUBIQUITIN

In the context of our study of potential regulators of cellular unanchored polyubiquitin chains, we have considered two candidates. One of the first proteins to be linked to regulation of the cellular unanchored polyubiquitin and perhaps the best characterised was IsoT [135]. IsoT belongs to the ubiquitin specific processing proteases (UBP/USP) class of DUBs, known for its ability to disassemble unanchored polyubiquitin chains of K48, K63, K6/29, K11 and linear linkages [69, 72, 135, 136]. Alternate splicing results in production of two functional isoforms, a short containing 835 amino-acids (93.3 kDa) and a long isoform containing 858 amino-acids (95.7 kDa) [137]. Both isoforms of IsoT contain four UBDs; a Znf-UBP, two UBA and a catalytic UBP domain [57]. While there are differences in expression of the isoforms in tissues [137, 138], it is not entirely clear if they vary in function. Although, considering that the site of insertion of the 23 amino-acid sequence in the long isoform lies between the catalytic UBP and the first UBA domain, it is argued this may affect the preference of polyubiquitin recognition off the two isoforms [76].

As noted previously, the significance of the enzyme was discovered when accumulation of unanchored polyubiquitin and furthermore, aberrant proteasomal processing (resulting from competitive binding of unanchored polyubiquitin chains [108]) were observed upon deletion of the *UBP14* gene in *S.cerevisiae* [107]. UBP14 was also established as a homologue of mammalian

IsoT. Developmental defects resulting from IsoT (homologue) mutants were observed in *Dictyostelium.discoideum* [139] and *Arabidopsis.thaliana* [140], with accumulation of unanchored polyubiquitin chains being a common feature. Furthermore, higher eukaryotes too display proteasomal processing abnormalities resulting from aberrant IsoT expression. Suppression of IsoT resulted in an increase of unanchored polyubiquitin chains in mammalian cell lines, leading to increase in stability of p53 (due to inhibition of proteasomal degradation) [109]. An increase in ubiquitinated proteins, specifically p53 was also reported upon the inhibition of IsoT by a small molecule DUB inhibitor WP1130 [141]. *Drosophila* IsoT homologue Leon, was found to be necessary for development and survival, with abnormal ubiquitin profiles specifically increase in unanchored polyubiquitin (and also substrate bound polyubiquitin) chains reported [142].

IsoT is particularly useful to our study as its structural features enabled (for the first time) the capture of cellular unanchored polyubiquitin chains. It is the (ubiquitin C-terminal binding) Znf-UBP domain of IsoT which provides the selectivity for unanchored polyubiquitin and acts as the activation domain of the enzyme [57]. This selectivity of Znf-UBP for unanchored polyubiquitin is a consequence of the penetration of the ubiquitin C-terminal ‘tail’ residues L71 through G76 (LRLRGG) into the hydrophobic pocket of the Znf-UBP domain of IsoT as shown in Figure 1.8 [57]. It is this key domain which we have utilised in our *in-house* setup to characterise the cellular unanchored ubiquitome. The structure of the enzyme and its application has been described in detail in Chapter 3.

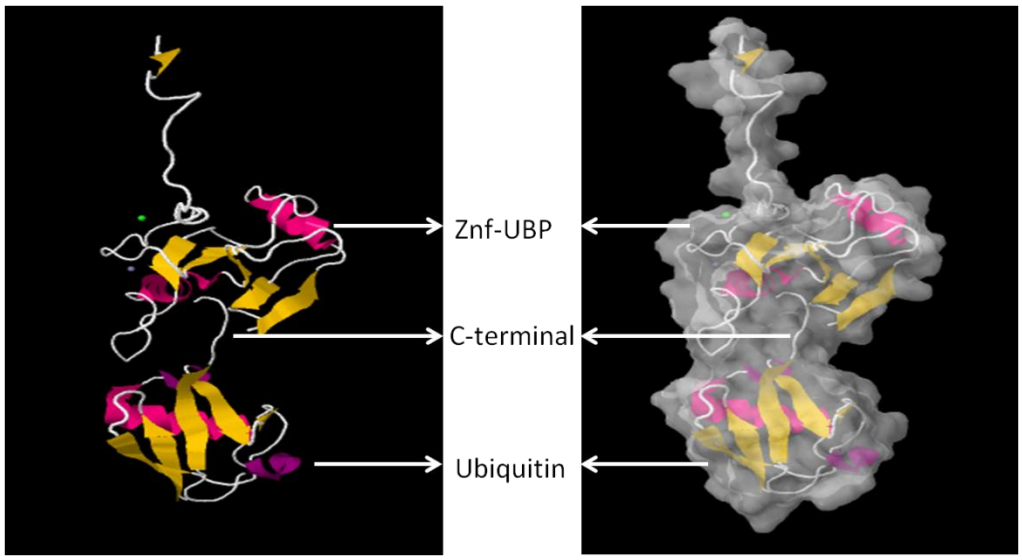


Figure 1.8. Ribbon (left) and surface (right) representation of the co-crystal structure of Znf-UBP domain of human IsoT in complex with ubiquitin (Protein Data Bank Code – 2G45). The interaction of ubiquitin C-terminal motif with Znf-UBP domain is essential for substrate specificity (unanchored polyubiquitin) and deubiquitinating activity of IsoT.

The second regulator of cellular polyubiquitin chains utilised in our study is the proteasome associated ubiquitin protein ligase E3C or UBE3C (also referred to as KIAA10 or RAUL) [143, 144]. The 125 kDa UBE3C is seen to associate with the 19S regulatory subunit of proteasome by means of its N-terminal domain both *in vivo* and *in vitro*, while its HECT domain is seen to synthesise K48 and K29 linked polyubiquitin chains [145]. UBE3C and its yeast homologue HECT ubiquitin ligase 5 (Hul5) have a vital role in proteasomal degradation, wherein deletion of either enzymes resulted in the accumulation of proteins which were only partially degraded by the proteasome [146, 147]. Studies also suggest Hul5 might primarily be involved in the elongation of chains, thereby stabilising ubiquitinated proteins at the proteasome against the opposing effects of proteasomal DUB Ubp6 [148]. An important role of Hul5 was also seen in the heat shock response resulting in the (increased) ubiquitination of aberrant and misfolded proteins [149, 150]. Hul5 is thus seen to contribute towards proteasomal functioning and in the regulation of misfolded cytosolic proteins, with specific roles in DNA damage and heat shock response [149-151].

IsoT being one of the most well characterised DUBs, a wealth of information was readily available to us. However in comparison, very little work has been done to establish a direct relation of the E3 with unanchored polyubiquitin chains. The first indication of a possible role of UBE3C in the regulation of the unanchored ubiquitome was revealed when the enzyme from purified rabbit reticulocyte extract was seen to assemble homogenous unanchored polyubiquitin chains containing K29 or K48 linkage [152]. The significance of

UBE3C was evident when deletion of yeast *Hul5* resulted in significantly reduced proteasomal processing of short-lived proteins [148]. More importantly, the study also reported the extension of ubiquitin chains catalysed by Hul5 *in vitro*, in the presence of proteasome (holoenzyme), E1, E2, ubiquitin and a primer (tetraubiquitin) [148]. Finally, the most comprehensive study thus far in the direct purification of unanchored polyubiquitin and its regulation by Hul5 was carried out by Aaron Ciechanover's group [151]. The study employed the expression and purification of tagged version of ubiquitin (Ub^{VV}) in *S.cerevisiae*. As part of the experiment, a basal level of such purified unanchored polyubiquitin was compared with that upon deletion of *Hul5*. While the study reported no significant change in ubiquitin levels upon over-expression or deletion of Hul5, DNA damage induced my methyl methanesulfonate (MMS) lead to increase in levels of yeast Hul5 and more importantly an increase in level of unanchored polyubiquitin [151]. However, besides the use of tagged versions of ubiquitin, it must be noted that the study employed western blotting (not quantitative MS) as means of confirming results [151].

Considering the evidence thus far suggesting a plausible role of UBE3C in the regulation of endogenous unanchored polyubiquitin chains, we decided to explore such a possibility in mammalian systems.

1.2.4. CONDITIONS AFFECTING CELLULAR UBIQUITIN PROFILES – HEAT SHOCK AND PROTEASOMAL INHIBITION

Changes to total ubiquitin content and linkage profiles of conjugated ubiquitin (to proteins) in response to various forms of proteolytic stress have previously been documented. Two conditions were selected to investigate their effect on endogenous unanchored polyubiquitin chains; heat shock response (increase of temperature for a short period) and proteasomal inhibition by treatment with carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, commonly referred to as MG132.

The heat shock response, first observed in *Drosophila* salivary glands [153], refers to a generic mechanism that allows cells to cope with increase in temperature, hypoxia, nutrient deprivation and other forms of stress (environmental and chemical) [154]. The immediate effect of heat shock was the denaturation of labile and short lived proteins which are rapidly degraded as a result [155]. However, upon prolonged stress these proteins can accumulate and lead to activation of a highly conserved family of heat shock proteins (Hsp), which aid in recovery of cells by stabilising cellular proteins [156, 157]. It was noted that being subjected to increase in temperature beyond the permissive causes a change (irreversible depending on the magnitude and duration of the increase) in the cellular ubiquitin profiles. Some of the earliest of such studies (using HeLa cells and chicken embryo fibroblasts) showed an increase in conjugated ubiquitin, and rates of protein turnover while causing a concomitant decrease in free (mono) ubiquitin levels [158-161]. A detailed study of the effects of heat shock using MS analysis allowed for the accurate determination of change in ubiquitin linkages in polyubiquitinated conjugates from HEK293 cell extract [42]. The study confirmed increases in K11, K48

and K63 linkages post heat shock. Since the study involved direct analysis of ubiquitin linkages in cell extracts, no distinction could be made between unanchored or substrate bound (conjugated) chains. Also, the study was limited to characterisation of ubiquitin conjugates of molecular weights over ~80 kDa, leading to a loss of information from smaller ubiquitinated substrates or unanchored chains [42]. Furthermore, a targeted study observed an increase in endogenous unanchored polyubiquitin in yeast upon incubation at 40^{o}C for 1 hour [151]. However, this was concluded based on affinity capture of haemagglutinin tagged ubiquitin ($Ub^{G75,\ 76V}$-HA) in transformed yeast, with no information on the composition of such chains with respect to linkage type. A recent study detailed the accumulation of Hsp90 at aggresomes upon MG132 treatment of human lung carcinoma cells (A549) [162]. The study further established the role of Hsp90 in aggresome clearance and more importantly, unanchored polyubiquitin chain formation [162].

The second form of stress induced was proteasomal inhibition by MG132. The proteasome complex is part of the cellular machinery primarily involved in the recognition (regulatory subunit), unfolding and degradation (catalytic subunit) of ubiquitinated proteins. As a result, the proteasome plays an obvious role in regulating protein turnover, and as a consequence several biological processes including transcription, signalling, and immune response while replenishing the cellular amino-acid pool [163]. An estimated 20% of total proteasomes in cells are actively involved in substrate processing at basal conditions (absence of external stress) [164]. MG132 acts as a proteasomal inhibitor affecting the catalytic activity of the proteasome [165]. One of the earlier accounts about the

effect of MG132 was about the inhibition of the NF-κB response, by hindering the processing of the p50 subunit precursor (p105) and also by stabilising ubiquitinated IκB [166]. Proteasomal inhibitors (including MG132) have been observed to elicit many aspects of the heat shock response [167], including the accumulation of ubiquitinated proteins [168]. More detailed approaches revealed that such proteasomal inhibition caused an increase in all but K63 isopeptide linked polyubiquitin chains [42, 82, 169]. In other words, proteasomal targeting could primarily, but not entirely be attributed to K48 linked ubiquitin conjugated substrates.

One must note that in such studies involving the pharmacological inhibition of proteasome, only changes to conjugated and "free" monoubiquitin (but not unanchored polyubiquitin chains) were documented [78]. In addition to the proteolytic activity, the proteasome complex also contains chain amputating proteasomal DUBs (Rpn11/POH-1) which could possibly contribute to the cellular unanchored polyubiquitin pool [170]. MG132 treatment was capable of inhibiting the catalytic activity but had no effect on such DUB activity [171]. As anticipated, regarding the specific effects of MG132 treatment on endogenous unanchored polyubiquitin chains, our group was the first to document an increase in total and K48 linked unanchored polyubiquitin chains [172].

1.3. PURIFICATION AND ANALYSIS OF UNANCHORED POLYUBIQUITIN CHAINS – A SUMMARY OF THE WORK SO FAR

Although the existence and functional significance of endogenous unanchored polyubiquitin chains has received much attention in recent years such that they are now accepted to be physiologically relevant, their selective purification from cells which is requisite for detailed analysis of molecular composition has been challenging. The precise nature of such chains, including confirmation of isopeptide linkage types present, remains to be fully understood. In the course of their study thus far, experiments employed the use of (1D and 2D) gel electrophoresis followed by western blotting for confirmation of the nature of such ubiquitin species [107, 109]. The landmark publication by Chen's group was the first to predict a direct role of unanchored polyubiquitin chains in cell signalling [1]. However, the study utilised the susceptibility of affinity purified (unanchored) polyubiquitin species to the DUB IsoT in order to confirm these were not substrate bound. Alternatively, the ability of polyubiquitin chains to form thiolester intermediates upon incubation with E1 in vitro, a reaction requiring free C-terminal G76 residue of ubiquitin (as occurs in the proximal ubiquitin of an unanchored chain), has been taken as indirect evidence that endogenous polyubiquitin chains can exist in unconjugated forms [126]. Chen's group also developed methods to better purify such chains exploiting the stability of polyubiquitin chains to heat and modification of cysteines (by NEM, as ubiquitin lacks cysteines) [126]. The only group thus far which directly purified unanchored polyubiquitin chains from cells was Aaron

Ciechanover's. The group expressed tagged ubiquitin in yeast where glycine residues at the 75th and 76th positions were replaced by valine, hence enabling the tagged mutant ubiquitin to form unanchored chains only by accepting ubiquitin [151]. The same setup was used to monitor the presence of unanchored polyubiquitin chains in HEK293 cells, with their confirmation being made through western blotting [151]. Although the development of tandem-repeated ubiquitin-binding entities (TUBEs) making use of synthetic arrays of ubiquitin-binding domains to promote artificial avidity effects has revolutionised the affinity capture of (poly)ubiquitin-conjugated substrates [173], these reagents are incapable of distinguishing conjugated and unconjugated ubiquitomes [172]. The attempts described thus far show a lack of consensus in the purification of cellular unanchored polyubiquitin and quantitative analysis in the makeup with respect to constituent isopeptide linkages. Also, there is a general lack of knowledge of the possible functions of unanchored polyubiquitin or cellular regulators that govern their homeostasis.

Consequently, prior to the start of this project an approach was established that presented for the first time the opportunity to efficiently, selectively and rapidly capture endogenous unanchored polyubiquitin chains using a single-step affinity purification (discussed in Chapter 3) [174]. Previously this approach was applied to purification of endogenous unanchored polyubiquitin chains from rat skeletal muscle tissue, revealing the presence of K48 and K11-linked ubiquitin dimers as well as longer chains containing as many as 15 ubiquitin moieties.

1.4. MASS SPECTROMETRY (MS)

1.4.1. INTRODUCTION AND APPLICATION IN PROTEOMICS

While western blotting remains a robust and extensively used technique, proteomic analysis and inference using western blots would depend largely on the availability, reliability and sensitivity of antibodies. The advent of sensitive mass spectrometers has been a boon as it offers the ability to carry out accurate, efficient and high-throughput analysis of cellular proteome. This has especially been true in the detailed characterisation of cellular ubiquitome [40, 42, 82, 174, 175]. However, approaches designed to quantify isopeptide linkage profile of cellular polyubiquitin vary in the MS strategy, sample preparation and in instruments used for detection. It must be noted that the strategy discussed here is restricted to bottom-up proteomics, which involves MS analysis of peptides derived from proteins after being subjected to proteolysis by trypsin, chymotrypsin or similar enzymes [176, 177]. An alternative to this is top-down proteomics strategy which involves the analysis of intact proteins [176].

Briefly, MS can be described as an analytical technique wherein molecules (analytes) are ionised, separated and differentiated based on their mass-to-charge ratios (*m/z*) in the gas phase. With specific reference to protein chemistry, mass spectrometry involves the detection of positive ions derived from precursor molecules [178]. The basic principle of MS has been known for over a century and the sensitivity and reliability of some of the present instruments has been extensively used for accurate qualitative and quantitative

proteomics. A mass spectrometer consists of an ionisation source, a mass analyser which measures (and separates based on) the *m/z* of analyte ions and a detector which detects the number of ions at each *m/z* value (Figure 1.9).

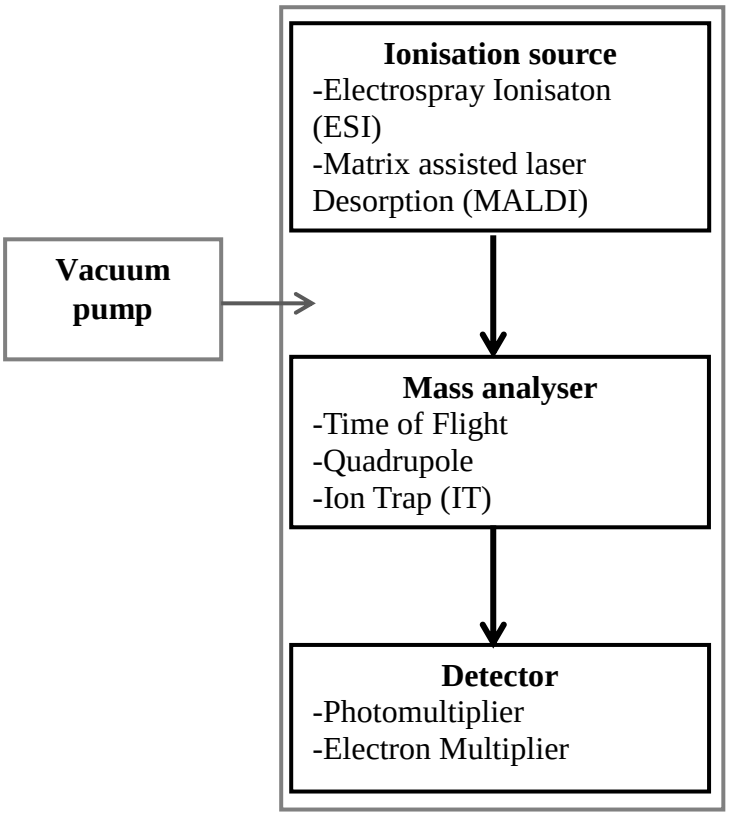


Figure 1.9. Schematic representation of the generic components of a mass spectrometer. A typical mass spectrometer includes an ionisation source, a mass analyser and a detector. While the ionisation source ensures that (part of) sample molecules are ionised to gas-phase, mass analysers measure such ions using electromagnetic fields with the presence of ions recorded by the detector.

The two classes of ionisation source employed are - matrix assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI). MALDI involves the ionisation of analyte crystals embedded in a matrix (of organic acid) by a high energy laser beam, with the ionised analytes directed to the

mass analyser in high vacuum [179, 180]. ESI on the other hand involves the ionisation of analyte molecules diluted in solution (such as MeCN gradient in water, with 0.1% formic acid) while continuously being sprayed into the mass spectrometer by a needle at high potential [180]. Instruments employing ESI are often used in combination with a HPLC system, which offers an added degree of separation of peptides in mixture. While MALDI is seen to produce singly charged ions, ESI results in analytes (some with multiple basic residues) competing for available protons, thus producing multiply charged ions [180].

The two mass analysers used by the mass spectrometers relevant to this study were time-of-flight (ToF) and quadrupole. Briefly, ToF analysers detect the time taken by accelerated ions (in vacuum) to travel from ion source to detector through a flight tube. Quadrupoles consists of four parallel rods, which act as a filter wherein an oscillating electric field permits only ions of specified *m/z* values to reach detector [180, 181]. While MALDI based instruments are best coupled to a ToF analyser, ESI based instruments are combined to quadrupoles, the latter also allows for obtaining product ion spectra (MS/MS) using collision-induced dissociation (CID) [179, 180]. The mass spectrometer used for an experiment would depend on the objective of the study and strategy employed. An invaluable tool in targeted quantitative proteomics is the triple quadrupole mass (QqQ) spectrometer. The instrument consists of three quadrupole mass analysers, with the first being an ion selector for precursor (intact peptide) ions, the second a collision cell and the third a mass analyser for fragment (product) ions [182]. The combination of *m/z* of precursor ion and of their corresponding fragment ion is called a transition, and

forms a unique identifier for the peptide of interest [181, 182]. The detection of one or many peptides using corresponding transitions is referred to as selected reaction monitoring (SRM) and is dealt in detail in Chapter 4.

1.4.2. QUANTITATIVE ANALYSIS OF ENDOGENOUS POLYUBIQUITIN LINKAGES - ABSOLUTE QUANTIFICATION (AQUA) STANDARDS

Confirmation of the presence of all seven ubiquitin isopeptide linkages in (*S.cerevisiae*) cells was first made by J Peng and colleagues in 2003 [40]. The method employed was only semi-quantitative (spectral counting) which allowed the estimation of relative abundance of each linkage type. The need for a more comprehensive quantitative analysis of endogenous ubiquitin linkage composition led to the generation and application (by S Gygi's group) of stable isotope labelled standards for this purpose, termed Absolute quantification or AQUA standards [175, 183]. AQUA standards are commercially available synthetic peptides which resemble native peptides in primary structure, but are labelled with heavy isotopes of carbon and nitrogen (^{13}C and ^{15}N), hence have a higher mass. As a result, they behave identically to their corresponding native peptides while handling - including in HPLC column and during ionisation, but would be separated from native peptides during MS due to their higher *m/z* values [183, 184]. Such peptide standards are introduced or 'spiked' into sample to be analysed by MS. With respect to quantitation of ubiquitin isopeptide linkages, AQUA standards are generated for "signatures" of each isopeptide linkage, such signatures being the product of trypsinolysis which is representative and exclusive to a particular linkage type [184]. SRM analysis using AQUA standards have been used extensively in the quantification of ubiquitin linkages in *S.cerevisiae* [82, 83], and in

human cell lines [42]. Figure 1.10 depicts the generation of a signature peptide from unanchored (K63 linked) polyubiquitin chains by trypsinolysis.

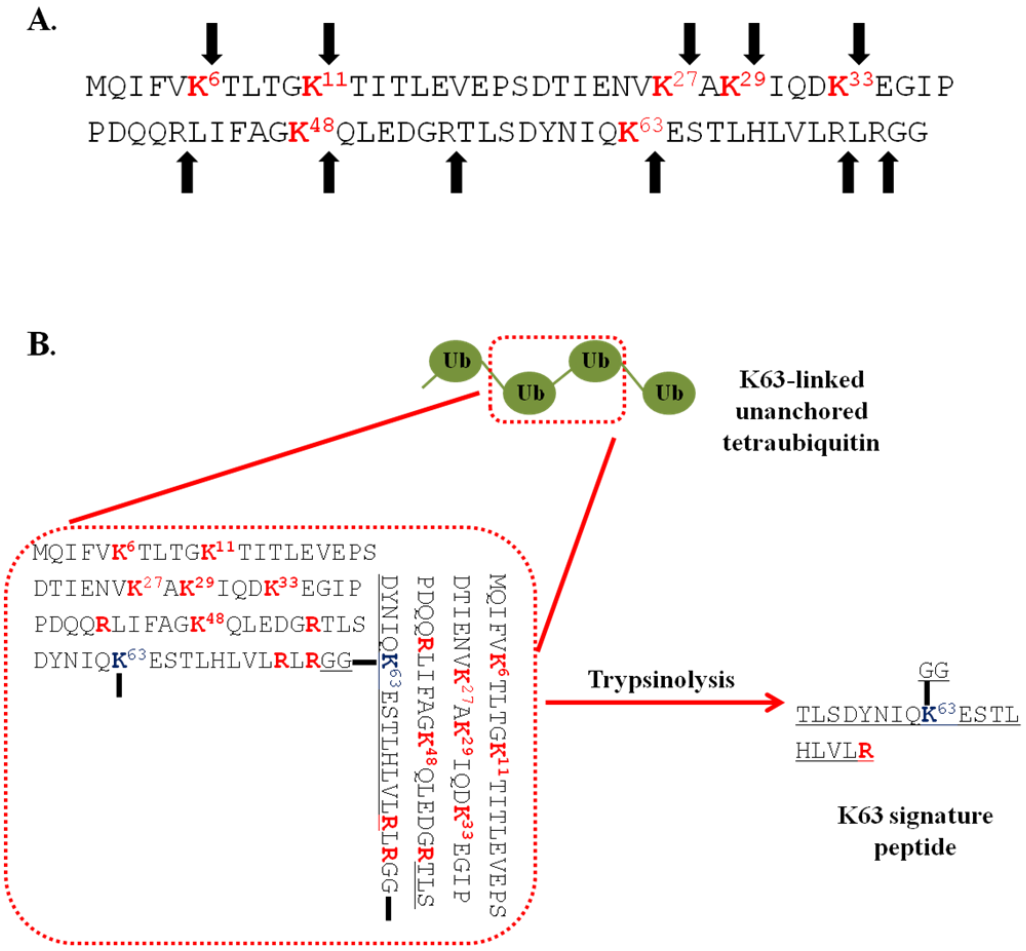


Figure 1.10. Linkage specific signature peptides are generated by the trypsinolysis of polyubiquitin chains.

A. Human ubiquitin sequence, with all lysine residues labelled in red. All the trypsin cleavage sites in ubiquitin have also been indicated by arrows.

B. Depiction of the generation of K63 signature peptide from unanchored K63 tetraubiquitin chain. The diglycine remnant attached to the acceptor ubiquitin at K63 residue is the characteristic feature of the K63 signature peptide. All the sites sensitive to trypsin cleavage are marked in red with the exception of the K63 residues (marked in blue) which are protected by the isopeptide linkage at the site.

However, an essential prerequisite to accurately determine polyubiquitin linkage information by bottom-up mass spectrometry, is efficient (complete) trypsinolysis to release (total) signature peptides present. The need for complete trypsinolysis and challenges encountered have been reviewed in [185]. Ubiquitin, by virtue of its small size and compact structure due to extensive hydrogen bonding [13], is highly resistant to proteolysis. Previously, groups which have attempted to characterise cellular polyubiquitin linkages in total ubiquitome [42, 78] or conjugated to specific proteins [175] using bottom-up mass spectrometry, have had to develop protocols specifically for the purpose. Methods previously used include digesting ubiquitin conjugated proteins in gel slices post separation by SDS PAGE, or digesting samples in-solution often accompanied by chaotropic agents or detergents [186]. In short there is no consensus protocol or guidelines and most studies in fact fail to acknowledge the problems of incomplete digestion.

1.5. OBJECTIVES

Considering the rather limited focus on purification and analysis of unanchored polyubiquitin till date (detailed in 1.3), the objectives of this study has been outlined below.

- To refine methods for purification of (total) endogenous unanchored polyubiquitin chains from mammalian cells.

- Establish in-house protocols for qualitative (isopeptide linkages present) and quantitative characterisation of purified unanchored polyubiquitin chains. This would first require development/refinement of methods to maximise trypsin digestion of polyubiquitin so as to maximise recovery of linkage specific signature peptides. During the course of the study, the group purchased a Triple Quadrupole mass spectrometer which was intended to utilise Absolute Quantification or AQUA ubiquitin standards to quantify polyubiquitin linkages. The setup had to be optimised so as to develop a robust platform for characterisation of unanchored polyubiquitin chains henceforth.

- Apply refined protocols to compare the changes in cellular pools of unanchored polyubiquitin between basal conditions or in response to modulation of signalling pathways and changes in levels of enzymatic regulators of ubiquitin system in order to gain an increased understanding of the possible role of such chains in various biological processes**.**

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. BACTERIAL STOCKS AND RECOMBINANT PROTEIN EXPRESSION

2.1.1.1. GENERATION OF BACTERIAL STOCKS – HEAT SHOCK TRANSFORMATION

- XL10-Gold ultracompetent cells (Stratagene, Cat No. 200314)
- Luria-Bertani Broth (LB) – 2.5% ($^{w}/_{v}$) LB in H_2O was autoclaved
- LB Agar (ampicillin) plates – 3.5% ($^{w}/_{v}$) LB Agar, 0.25% ($^{w}/_{v}$) Agar select in H_2O was autoclaved and 100 µg/ml ampicillin was added once cooled.
- 30% ($^{v}/_{v}$) Glycerol in H_2O was filter sterilised
- Plasmids with selectable markers. The regions coding for the 129 amino-acid Znf-UBP domain (residues 163 – 291 of human IsoT) was previously cloned in to the pGEX4T-1 vector allowing expression in E. coli with an N-terminal glutathione S-transferase (GST) fusion. The construct included a site for cleavage with thrombin protease between the fusion partners, and conferred resistance to ampicillin. The plasmid was designed and donated by Dr Joanna Strachan [174].

2.1.1.2. PURIFICATION OF PLASMIDS FROM BACTERIAL STOCKS

- QIAprep Spin Miniprep kit (QIAGEN, Cat No. 27104)
- NanoDrop 2000 spectrophotometer (Thermo-Scientific)

2.1.1.3. OVER-EXPRESSION AND PURIFICATION OF GST-TAGGED RECOMBINANT PROTEIN (ZNF-UBP) FROM BACTERIAL STOCKS

- LB-Amp - 2.5% ($^{w}/_{v}$) LB in H_2O was autoclaved, and 100 µg/ml ampicillin was added
- 200 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG)
- Tris-buffered saline – Triton X (TBS-Tx) – 10 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 0.1% ($^{v}/_{v}$) Triton X-100

2.1.1.4. AFFINITY PURIFICATION OF GST-TAGGED RECOMBINANT PROTEIN

- TBS-Triton (TBS-Tx) – 10 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 0.1% ($^{v}/_{v}$) Triton X-100.
- Glutathione sepharose 4B (GE Healthcare Life Sciences)

2.1.1.5. THROMBIN CLEAVAGE TO RELEASE BAIT PROTEIN

- Thrombin protease
- Thrombin cleavage buffer – 20 mM Tris-HCl pH 8.4, 150 mM sodium chloride, 2.5 mM calcium chloride dihydrate

2.1.1.6. ESTIMATION OF PROTEIN CONCENTRATION – BICINCHONINIC ACID (BCA) ASSAY

- Pierce™ BCA protein assay reagents (Thermo Fisher Scientific)
- 2 mg/ml bovine serum albumin (BSA) standards in thrombin cleavage buffer

2.1.2. MAINTENANCE AND MANIPULATION OF CELL LINES

2.1.2.1. CELL CULTURE

- Human embryonic kidney cells containing the SV40 Large T-antigen (HEK293T cells)
- Cell culture media – Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich D6429) supplemented with 10% ($^{v}/_{v}$) foetal bovine serum (FBS; Sigma-Aldrich F9665), and 1% ($^{v}/_{v}$) Penicillin-Streptomycin (Pen-Strep; Sigma-Aldrich P0781 containing 10,000 units/ml penicillin and 10 mg/ml streptomycin) - stored at 4^{o}C, brought to 37^{o}C
- 1X sterile-filtered Trypsin / EDTA (Sigma-Aldrich T3924, 0.5 g porcine trypsin and 0.2 g EDTA, 4Na per litre of Hanks' Balanced Salt Solution with phenol red) - stored at 4^{o}C and brought to 37^{o}C before use
- Dulbecco's phosphate buffered saline or PBS (Sigma-Aldrich D8537, sterile-filtered, contains no calcium or magnesium chloride) - stored at 4^{o}C, brought to 37^{o}C before use
- Freezing media – filter-sterile 10% ($^{v}/_{v}$) DMSO in FBS

2.1.2.2. TRANSFECTION OF PLASMIDS

- HEK293T cells (and cell culture media and reagents)
- Plasmid (in filter-sterilised H_2O, concentration known)
- Opti-MEM® (Thermo Fisher Scientific, 11058) - stored at 4^{o}C, brought to 37^{o}C before use

- TransIT®-LT1 transfection reagent (Mirus Bio LLC) - stored at 4^{o}C, and brought to 37^{o}C before use
- Improved Neubauer Haemocytometer

2.1.2.3. TRANSFECTION OF siRNA

- HEK293T cells and cell culture media and reagents
- DharmaFECT™ Duo Transfection Reagent (Thermo Scientific Dharmacon ®), stored at 4^{o}C, brought to 37^{o}C before use
- 5 nmol siGENOME SMARTpool siRNA (Thermo Scientific) specific for Human IsoT (8078), Human UBE3C (9690) and non-targeting control #2. 20 µM stock made by adding 250 µl of 1X siRNA dilution buffer. Stored at -80^{o}C until use.
- Opti-MEM® (Thermo Fisher Scientific, 11058) - stored at 4^{o}C, brought to 37^{o}C before use
- Improved Neubauer Haemocytometer

2.1.2.4. DUAL LUCIFERACE NF-κB REPORTER ASSAY TO CONFIRM ACTIVATION OF IL-1β MEDIATED SIGNALLING

- HEK293T cells, cell culture media and reagents
- Dual-Luciferase® Reporter Assay system (Promega, E1910)
- Luciferase plasmids - IL-8 promoter firefly luciferase plasmid, previously used by the group and detailed in [187]. Ubiquitin *Renilla* luciferase plasmid kindly donated by Prof Peter Shaw (The University of Nottingham).

- Opti-MEM® (Thermo Fisher Scientific, 11058) - stored at 4^{o}C, brought to 37^{o}C before use
- TransIT®-LT1 transfection reagent (Mirus Bio LLC) - stored at 4^{o}C, brought to 37^{o}C before use.
- HumanKine™ Interleukin-1β, human recombinant (Sigma-Aldrich, H6291). Reconstituted in sterile 0.1% ($^{w}/_{v}$) human serum albumin in PBS, stored at -20^{o}C
- 96 well white flat-bottomed microplate (Grenier Bio-One Ltd, 655075)
- Improved Neubauer Haemocytometer
- Graphpad Prism software

2.1.2.5. IL-1β TREATMENT OF CELLS FOR AFFINITY PURIFICATION OF ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS

- HEK293T cells, cell culture media and reagents
- HumanKine™ Interleukin-1β, human recombinant (Sigma-Aldrich, H6291). Reconstituted in sterile 0.1% ($^{w}/_{v}$) human serum albumin in PBS, stored at -20^{o}C. For use in activation of signalling, IL-1β (or vehicle) was added to DMEM culture media containing 1% Pen-Strep but lacking FBS.

2.1.2.6. PROTEASOMAL INHIBITION

- HEK293T cells, cell culture media and reagents
- Z-Leu-Leu-Leu-CHO or MG132 (Enzo® Life Sciences, PI-102) – 10 mM in DMSO (Sigma-Aldrich, D2650), kept at -80^{o}C.

2.1.2.7. HEAT TREATMENT

- HEK293T cells, cell culture media and reagents
- Cell culture incubators set at 37°C and 42°C (both 5% CO_2)

2.1.3. AFFINITY PURIFICATION OF UNANCHORED POLYUBIQUITIN

2.1.3.1. CELL LYSIS AND SAMPLE PREPARATION

- HEK293T cells, cell culture media and reagents
- Homogenising buffer - 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% ($^{v}/_{v}$) NP-40 (IGEPAL® CA630, Sigma-Aldrich®), 5 mM *N*-ethylmaleimide (NEM), 20 μM MG132, 0.1% ($^{v}/_{v}$) mammalian protease inhibitor cocktail were added. Homogenising buffer was left on ice.
- Glass wool
- Dithiothreitol (DTT)

2.1.3.2. COUPLING OF BAIT PROTEIN TO CNBR SEPHAROSE BEADS

- Cyanogen bromide activated sepharose 4B (CnBr Sepharose beads, GE Healthcare)
- 1 mM HCl
- Coupling buffer – 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3
- 1 M ethanolamine, pH 8.0
- Tris buffer - 0.1 M Tris-HCl pH 8.0, 0.5 M NaCl
- Acetate buffer - 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0
- Storage buffer - 25 mM Tris-HCl pH 7.4, 1 mM sodium azide

2.1.3.3. AFFINITY PURIFICATION

- Wash buffer – 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% ($^{v}/_{v}$) NP-40 and 1 mM DTT

2.1.3.4. DEUBIQUITINATION ASSAY OF PURIFIED UNANCHORED POLYUBIQUITIN CHAINS ON BEADS

- Affinity purified unanchored polyubiquitin chains bound to CnBr sepharose 4B beads
- DUBs – IsoT (long form) and Usp2 catalytic domain (both Enzo® Life Sciences)
- DUB buffer - 50 mM Tris-HCl pH 7.5, 1 mM DTT

2.1.4. PROTEIN VISUALISATION

2.1.4.1. SDS PAGE

- 30% ($^{w}/_{v}$) Acrylamide/Bis-acrylamide
- Buffer A – 1.1 M Tris-HCl pH 8.8, 0.1% ($^{w}/_{v}$) SDS, 30% ($^{v}/_{v}$) glycerol
- Buffer B – 1.1 M Tris-HCl pH 8.8, 0.1% ($^{w}/_{v}$) SDS
- Stacking gel buffer – 0.14 M Tris-HCl pH 6.8, 0.1% ($^{w}/_{v}$) SDS
- 0.1% ($^{w}/_{v}$) SDS
- 10% ($^{w}/_{v}$) ammonium persulfate (APS)
- Tetramethylethylenediamine (TEMED)
- Gel application buffer (GAB) – 0.15 M Tris-HCl pH 6.8, 8 M Urea, 2.5% ($^{w}/_{v}$) SDS, 20% ($^{v}/_{v}$) glycerol, 10% (w/$_{v}$) 2-mercaptoethanol, 3% ($^{w}/_{v}$) Dithiothreitol (DTT), 0.1% ($^{w}/_{v}$) bromophenol blue

- Electrode buffer – 0.025 M Tris, 0.192 M glycine, 0.1% ($^{w}/_{v}$) SDS – made up in deionised H_2O
- Coomassie blue stain – 50% ($^{v}/_{v}$) methanol, 20% ($^{v}/_{v}$) glacial acetic acid, 0.12% ($^{w}/_{v}$) Coomassie Brilliant Blue R-250
- Coomassie blue destain – 10% ($^{v}/_{v}$) methanol, 10% ($^{v}/_{v}$) glacial acetic acid
- Controls - Commercial polyubiquitin ladders (2-7 ubiquitin moieties) of K48 or K63 linkages (Boston Biochem), bovine monoubiquitin (Sigma-Aldrich)

2.1.4.2. WESTERN BLOTTING

- Hybond-C Nitrocellulose membrane (Amersham)
- Western transfer buffer
- Ponceau-S solution– 0.1% ($^{w}/_{v}$) Ponceau S, 10% ($^{v}/_{v}$) glacial acetic acid
- ECL hyperfilm (Amersham)
- Renaissance ECL solutions (Amersham)
- Developing solution (Calumet Photographic LTD 757314)
- Fixer solution (Calumet Photographic LTD 2000RT)
- Blocking reagent - 5% ($^{w}/_{v}$) Marvel or 1% ($^{w}/_{v}$) BSA in buffer (as required per antibody)
- Primary Antibodies – tabulated in Table 2.1
- Secondary antibodies – Peroxide-conjugated polyclonal anti-mouse immunoglobulin or Peroxide-conjugated polyclonal anti-rabbit immunoglobulin (both purchased from DAKO)

Antibody	Wash Buffer	Blocking conditions	1° Antibody conditions	2° Antibody conditions
Ubiquitin (In-house)	TBS	Autoclave membrane, block in 5% Marvel at RT for 1 hr	1/1000 at RT for 1 hr	Anti-rabbit, 1/2000 at RT for 1 hr
Ubiquitin, VU-1 clone (Lifesensors Inc)	TBS-Tween (0.1%)	5% Marvel at RT for 1 hr	1/1000 at 4°C overnight	Anti-mouse, 1/2000 at RT for 1 hr
K48, Apu2 clone (Millipore)	TBS-Tween (0.5%)	5% Marvel at RT for 1 hr	1/1000 at 4°C overnight	Anti-rabbit, 1/2000 at RT for 1 hr
K63, HWA4C4 clone (Enzo Biomol)	TBS-Tween (0.1%)	1% BSA at RT for 1 hr	HRP-conjugated, 1/2500 at 4°C overnight	n/a
K63, HWA4C4 clone (Enzo Biomol)	TBS-Tween (0.2%)	5% Marvel at RT for 1 hr	1/2500 at 4°C overnight	Anti-mouse, 1/2000 at RT for 1 hr
IsoT (Proteintech)	TBS-Tween (0.1%)	5% Marvel at RT for 1 hr	1/1000 at RT for 1 hr	Anti-rabbit, 1/2000 at RT for 1 hr
UBE3C (Sigma-Aldrich)	TBS-Tween (0.1%)	5% Marvel at RT for 1 hr	1/1000 at 4°C overnight	Anti-mouse, 1/2000 at RT for 1 hr
Actin (Sigma-Aldrich)	TBS	5% Marvel at RT for 1 hr	1/25000 at RT for 1 hr	Anti-mouse, 1/2000 at RT for 1 hr

Table 2.1. Antibodies and their conditions used for Western blotting

2.1.5. SAMPLE PREPARATION FOR MASS SPECTROMETRY

2.1.5.1. FORMIC ACID ELUTION OF AFFINITY PURIFIED UNANCHORED POLYUBIQUITIN CHAINS

- Affinity purified sample on bat tagged CnBr sepharose beads
- 0.1% ($^{v}/_{v}$) formic acid in deionised H_2O
- Micro-centrifugal filters

2.1.5.2. TRYPSINOLYSIS OF PROTEINS IN POLYACRYLAMIDE GEL (PAG) PIECES

- Digestion buffer – 0.2 M ammonium bicarbonate buffer (AmBic), pH 8.0
- Wash buffer – Equal volume of 0.2 M AmBic buffer pH 8.0 and acetonitrile or MeCN (HPLC gradient grade, Fisher-Scientific)
- 10 mM DTT in wash buffer
- 20 mM chloroacetamide or CAA in wash buffer
- 0.1 mg/mL sequencing grade Trypsin (Promega) stock was prepared in resuspension buffer (provided with Trypsin). Stock was diluted in digestion buffer prior to use such that an estimated enzyme:substrate ratio was a minimum of 1:10
- Positive and Negative controls - Regions of polyacrylamide gel either containing a standard (such as bovine serum albumin) or lacking any protein were excised and used as controls
- Extraction Buffer - 0.1% formic acid in 70% MeCN, HPLC gradient grade H_2O (Fisher-Scientific)

2.1.5.2. *IN SOLUTION* DIGESTION

- Digestion buffer – 1 M urea in a solution of 5% MeCN (HPLC gradient grade, Fisher-Scientific) in 0.1 M AmBic buffer, pH 8.0
- 50 mM DTT in digestion buffer
- 100 mM chloroacetamide or CAA in wash buffer
- 0.2 mg/mL sequencing grade Trypsin (Promega) in digestion buffer
- Positive control – 0.1% ($^{w}/_{v}$) BSA in digestion buffer
- Centrifugal ultrafiltration (UF) devices – 0.5 ml, 3-5 kDa MWCO
- Extraction Buffer - 0.1% formic acid (Fisher-Scientific) in 70% MeCN, HPLC gradient grade H_2O (Fisher-Scientific)

2.1.5.3. ABSOLUTE QUANTIFICATION (AQUA) STANDARDS

- 500 ng each of 10 heavy isotope labelled ubiquitin AQUA commercial standards (Enzo Life Sciences Inc), Table 2.2.
- Resuspension solution – 5% (v/v) MeCN, 0.1% (v/v) formic acid in H2O, all HPLC gradient grade (Fisher-Scientific), final concentration of commercial AQUA standards was 10 ng/µl

Signature Peptide	Sequence	AQUA standard mass (Da)	Native peptide mass (Da)
K6	MQIFVK^{GG}TLTGK	1385.80	1378.76
K11	TLTGK^{GG}TITLEVEPSDTIENVK	2408.40	2401.26
K27	TITLEVEPSDTIENVK^{GG}AK	2106.12	2100.10
K29	AK^{GG}IQDK	821.48	815.45
K33	IQDK^{GG}EGIPPDQQR	1642.83	1636.82
K48	LIFAGK^{GG}QLEDGR	1466.82	1459.78
K63	TLSDYNIQK^{GG}ESTLHLVLR	2250.21	2243.19
TLS	TLSDYNIQK	1087.56	1080.55
EST	ESTLHLVLR	1073.64	1066.61
TITLE	TITLEVEPSDTIENVK	1792.96	1786.92

Table 2.2. Commercial ubiquitin derived AQUA signature peptides have the same primary amino-acid structure but show an increase in molecular mass. Seven AQUA standards correspond to linkage specific signature peptide, while three correspond to internal ubiquitin (non-linkage) peptides.

2.1.6. MASS SPECTROMETERS AND SAMPLE PREPARATION

2.1.6.1. MALDI-TOF

- LaserToF TT (SAI Ltd)
- C-18 solid-phase tips (Supelco Analytical)
- 5% ($^{v}/_{v}$) MeCN, 0.1% ($^{v}/_{v}$) formic acid in HPLC gradient grade H2O (Fisher-Scientific)
- 70% ($^{v}/_{v}$) MeCN, 0.1% ($^{v}/_{v}$) formic acid in HPLC gradient grade H_2O
- α-cyano-4-hydroxy-cinnamic acid (α-CHC, Sigma)

2.1.6.2. Q-TOF

- Waters QToF2 with nanoelectrospray ion-source
- Phenomenex Jupiter® 4µm Proteo (0.075X150 mm) HPLC column, 5% and 90% MeCN in H_2O (both containing 0.1% formic acid)
- Waters MassLynx™ 4.0 software
- Mascot database (www.matrixscience.com)

2.1.6.3. TRIPLE QUADRUPOLE OR QQQ

- Waters Quattro Ultima
- Phenomenex Luna® 3 µm (2.1X40 mm) C18 column
- Waters MassLynx™ 4.0 software and Skyline v3.6 software (https://skyline.ms/project/home/software/Skyline/begin.view)

2.2. METHODS

All reusable glassware were subject to autoclaving at 121^{o}C for 20 minutes prior to use. Disposable materials were purchased in sterile condition and retained in their packaging until use. All work-spaces were disinfected periodically with Trigene so as to minimise contamination. Unless stated otherwise, all buffers and solutions were prepared using deionised H_2O and all reagents were purchased from Sigma-Aldrich®. All bacterial work was carried in the vicinity of a Bunsen burner, while cell cultures were handled in a Walker Class II Microbiology Safety cabinet.

2.2.1. BACTERIAL STOCKS AND RECOMBINANT PROTEIN EXPRESSION

2.2.1.1. GENERATION OF BACTERIAL STOCKS – HEAT SHOCK TRANSFORMATION

100 µl competent cells were incubated with 1-10 µl plasmid (or control containing no plasmid) on ice for 30 minutes. Immediately after, this was transferred onto water-bath at 42^{o}C for 30 seconds. The cultures were then transferred onto ice for 5 minutes. The volume was made up to 1 ml with Luria Broth and incubated at 37^{o}C for 1 hour while shaking. The cultures were then plated on LB Agar plates (containing ampicillin), and incubated overnight at 37^{o}C. Cells from colonies grown on these plates were picked and transferred to 10 ml of LB broth and grown overnight at 37^{o}C. These cultures could then be used to purify plasmids or produce a stock for future use. 500 µl of culture was mixed with 500 µl of 30% glycerol to produce a bacterial stock for the plasmid of interest. These stocks were stored at -80^{o}C.

2.2.1.2. PURIFICATION OF PLASMIDS FROM BACTERIAL STOCKS

Plasmids were purified from bacterial cultures using the protocol provided with the QIAprep spin Miniprep kit. In short, cells were first pelleted from (10 ml) overnight cultures (in LB), resuspended in 250 µl buffer P1 and transferred to a microcentrifuge tube. 250 µl of buffer P2 was then added to the mixture to lyse cells, shaken and incubated for no longer than 2 minutes. 350 µl of buffer N3 was then added to neutralize the mixture and tubes inverted 4-6 times. The microcentrifuge tube was then centrifuged at 13,000 rpm on a table top centrifuge. The supernatant was then transferred to a spin column supplied with the kit. The column was now centrifuged for 60 seconds and flow-through discarded, followed by a wash with 750 ml buffer PE which was later discarded by centrifugation. The column was centrifuged for 1 minute to rid of remnants of buffer PE. Now the column was placed in a clean microcentrifuge tube with the addition of 30 µl of filter-sterilised deionised H_2O. Centrifugation of the tube containing the spin column resulted in the accumulation of plasmid (suspended in H_2O) in the microcentrifuge tube. The last step was repeated to obtain a total of 60 µl plasmid DNA. The concentration was estimated using a NanoDrop spectrophotometer.

2.2.1.3. OVER-EXPRESSION AND PURIFICATION OF GST-TAGGED RECOMBINANT PROTEIN (ZNF-UBP) FROM BACTERIAL STOCKS

10 ml LB-Amp was inoculated with bacterial stock culture and incubated overnight whilst shaking at 37^{o}C. The following day, these were transferred to conical flasks each containing 1 litre of LB-Amp and grown for 3 hours (from previous optimisation) at 37^{o}C while shaking. IPTG at final concentration of

200 mM was added to cultures to induce expression of recombinant protein. The cultures were left to incubate overnight at a temperature of 20^{o}C. The following day, these cultures were centrifuged at 4000 rpm for 15 minutes at 4^{o}C using a Cellsep centrifuge. While the supernatant was discarded, the pellet was resuspended in 10 ml TBS-Tx and stored at -80^{o}C.

2.2.1.4. AFFINITY PURIFICATION OF GST-TAGGED RECOMBINANT PROTEIN

Bacterial cell extracts stored in TBS-Tx were first sonicated for 15 seconds followed by incubation in ice for 30 seconds. This was repeated 7 times. This was then subjected to centrifugation at 19,000 rpm at 4^{o}C for 30 minutes using an Avanti J-25 centrifuge. The supernatant was collected for purification of protein. Meanwhile, 1 ml of Glutathione sepharose 4B beads was first equilibrated with TBS-Tx in a purification column. This was then incubated for 1 hour at 4^{o}C (while rotating) with the supernatant containing GST-tagged recombinant protein. The beads were then given 3X10 ml washes with TBS-Tx after which they were ready for cleavage with thrombin protease and recovery of recombinant protein.

2.2.1.5. THROMBIN CLEAVAGE TO RELEASE BAIT PROTEIN

Affinity purified protein tagged to glutathione sepharose beads (in column) were washed 3X10 ml with thrombin cleavage buffer. These were then incubated overnight while rotating at 4^{o}C, with 10 units of thrombin protease in 1 ml thrombin cleavage buffer. The purified protein was collected the following day and subjected to SDS-PAGE (2.2.4) to confirm cleavage, and its concentration was estimated using BCA assay.

2.2.1.6. ESTIMATION OF PROTEIN CONCENTRATION – BICINCHONINIC ACID (BCA) ASSAY

25 µl BSA standards ranging from 0-2 mg/ml were pipetted into wells of a 96 well glass plate as triplicates. The protein sample (with unknown concentration) was also pipetted in replicates with an additional sample of diluted material used if required. The Pierce BCA assay reagents A and B were mixed in a ratio of 50:1, with 200 µl of this solution was added into each well. The plate was then incubated at 37^{o}C whilst shaking for 30 minutes. A spectrophotometer set to measure absorbance at 562 nm was used to read the plate. A standard curve was plotted using readings from standards and the protein concentration of the sample was thus estimated.

2.2.2 MAINTENANCE AND MANIPULATION OF CELL LINES

2.2.2.1. CELL CULTURE

HEK293T is an adherent cell line grown and passaged in 75 cm^2 cell culture flasks in a humidified cell culture incubator set at 37^{o}C with 5% CO_2. Cells in flask were passaged every 2 days (80-90% confluent using light microscope), and supplemented with fresh media. Firstly, media in flask was discarded and the cells were gently washed with 10 ml sterile PBS which was subsequently discarded. Cells were then treated with 3 ml Trypsin-EDTA solution and incubated at 37^{o}C until they detach (~ 5 minutes). The Trypsin-EDTA was then diluted using DMEM media bringing the final volume to 10 ml. The cells were then usually split 1:4 and supplemented with 10 ml fresh media.

Cell lines (usually at low passage stage) were often frozen for future use. Cells in 75 cm^2 culture flasks were first subjected to a PBS wash followed by Trypsin-EDTA treatment and resuspension in 10 ml DMEM, as described before. The cells were gently centrifuged at 1200 rpm and pelleted. The pellets were washed with PBS and resuspended in 1 ml of freezing media. Cells were then transferred to cryovials and frozen at -80^oC overnight followed by transferring them into liquid nitrogen for storage. To thaw frozen cells, cryovials from storage in liquid nitrogen were thawed quickly and immediately transferred to a 75 cm^2 cell culture flask containing DMEM supplemented with 20% ($^v/_v$) FBS, 1% ($^v/_v$) Pen-Strep and incubated overnight. The cells were monitored the following day and were passaged upon reaching 80-90% confluency.

2.2.2.2. TRANSFECTION OF PLASMIDS

HEK293T cells which were 80-90% confluent were selected to seed for transfection. Optimisations of transfection were carried out in 24-well cell culture plates. Confluent cells were treated with Trypsin-EDTA and diluted with DMEM media as mentioned prior. Cell concentration (number per ml) was then estimated using a Neubauer haemocytometer. Cells were seeded on to the wells of a 24-well plate at a density of 1 X 10^5 cells per well in 500 µl media and left overnight at 37^oC. 24 hours after seeding cells, transfection mixture was prepared by first adding transfection reagent TransIT®-LT1 to 100 µl Opti-MEM® which was left to incubate for 5 minutes. DNA was then added to the mixture such that the DNA:reagent ratio was 0.2 µg:0.6 µl (optimisations were carried out with a range of concentrations). The mixture

was thoroughly mixed using a pipette and left to incubate for 15 minutes at room temperature. The transfection mixture was now gently added drop wise to each well and cells were left to grow for 48 hours at 37^{o}C. Following 48 hour incubation, cells were lysed (lysis buffer depended on the experiment).

2.2.2.3. TRANSFECTION OF siRNA

Optimisations of transfection were initially performed using 24-well plates with a range of siRNA concentration varying from 10 nM – 60 nM (0.25 µl – 1.5 µl siRNA stock) per well. 1 X 10^5 cells were seeded per well of the plate. Transfection mixture (for a single well) was prepared in two separate RNase free microcentrifuge tubes. Tube 1 contained 49 µl Opti-MEM® mixed with desired volume of siRNA. Tube 2 contained 49 µl Opti-MEM® with 1 µl DharmaFECT™ Duo transfection reagent. The two tubes were first incubated at room temperature for 5 minutes, following which the contents of both tubes were mixed together gently and incubated at room temperature for 20 minutes. Post-incubation, 400 µl of DMEM media was added to the mixture. Prior to addition of transfection mixture, existing media in wells was drained and cells were gently washed with sterile PBS. This was immediately followed by the gentle addition of transfection mixture into each well and the cell culture plate was left to incubate at 37^{o}C (5% CO_2 concentration) for 48 hours (72 if required) after which they were lysed and transfection and suppression of targeted protein was confirmed.

For affinity purification of endogenous unanchored polyubiquitin, HEK293T cells were seeded on 10 cm cell culture plates. The cell seeding density was 3

X 10^6 per plate, with the use of 300 µl of Opti-MEM® and 30 µl of DharmaFECT™ Duo transfection reagent per cell culture plate. The concentration of siRNA depended on results obtained from optimisations. 48 hours post transfection; cells were lysed in homogenising buffer as mentioned in 2.2.3.1.

2.2.2.4. DUAL LUCIFERACE NF-κB REPORTER ASSAY TO CONFIRM ACTIVATION OF IL-1β MEDIATED SIGNALLING

1 X 10^5 HEK293T cells were seeded per well of a 24-well plate. 24 hours later, cells were co-transfected with 100 ng of IL-8 promoter firefly luciferase and 0.4 ng ubiquitin *Renilla* luciferase plasmids per well (according to the protocol mentioned in 2.2.2.2). 42 hours after transfection, existing media was drained and fresh DMEM lacking FBS (cells were starved of cytokines) was added to cells and left to incubate for a period of 6 hours. Cells were then treated with a range of IL-1β concentrations (range 0 – 25 ng/ml) in DMEM lacking FBS, with one well being treated with vehicle alone (2.5 µl sterile PBS containing 0.1% (w/v) human albumin). Cells were incubated for a period of 4 hours after which they were lysed in 200 µl of 1X passive lysis buffer.

20 µl of each extract was transferred to wells of a 96 well white flat-bottomed microplate. Firefly and *Renilla* luciferase activity (NF-κB activity readout) was determined using the Dual-Luciferase Reporter assay according to the manufacturer's instructions on a GloMax-96 Microplate Luminometer (Promega). Luciferase activity was displayed as a ratio of firefly and *Renilla* luciferase and fold increase was measured relative to control. Experiments was carried out in triplicates and data was displayed as mean of averages ± SD.

Statistical analysis was carried out using the Graphpad Prism 6 software, using a one-way ANOVA and a Dunnett's test was to calculate level of significance between control and IL-1 β stimulated values [188]. The significance was set at $p<0.05$.

2.2.2.5. IL-1β TREATMENT OF CELLS FOR AFFINITY PURIFICATION OF ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS

HEK293T cells were seeded on 10 cm cell culture plates and incubated in media until they were ~90% confluent. Cells were serum starved for a period of 6 hours, by replacing media with DMEM lacking FBS. Following serum starvation, media from each plate was first discarded and replaced with 10 ml DMEM (lacking FBS) containing 20 ng/ml IL-1β or control (vehicle alone). Cells were left to incubate at room temperature for 90 seconds, following which media was removed and cells were gently washed in sterile PBS. IL-1β stimulated cells were now lysed in homogenising buffer (2.2.3.1) exactly 2 minutes from the time of IL-1β addition and extract was processed and endogenous unanchored polyubiquitin chains were affinity purified as detailed in Section 2.2.3.

2.2.2.6. PROTEASOMAL INHIBITION

HEK293T cells were grown on 10 cm cell culture plates and grown in DMEM media until they reached a confluency of ~80%. Following incubation, the media in each plate was replaced with 10 ml DMEM supplemented with either MG132 (1 µl from stock of 10 mM such that final concentration was 1 µM), DMSO (1 µl) or just media. Cells were incubated for 16 hours after which they

were lysed (in homogenising buffer, 2.2.3.1), processed and subject to affinity purification of unanchored polyubiquitin chains (as detailed in 2.2.3).

2.2.2.7. HEAT TREATMENT

HEK293T cells were grown on 10 cm cell culture plates and grown in DMEM media until they reached a confluency of ~90%. Following incubation, culture plates were either transferred to cell culture incubators set to 42^{o}C (5% CO_2) or retained in (control) incubators set to 37^{o}C (5% CO_2) for a total of 60 minutes. Following the 60 minute incubation at 42^{o}C (or 37^{o}C), cells were immediately lysed in homogenising buffer (2.2.3.1), processed and subject to affinity purification of unanchored polyubiquitin chains.

2.2.3. AFFINITY PURIFICATION OF UNANCHORED POLYUBIQUITIN

2.2.3.1. CELL LYSIS AND SAMPLE PREPARATION

Cells grown for affinity purification of unanchored polyubiquitin chains were first observed under light microscope to ensure lack of contamination and confluency. Once media was drained from culture plates, cells were then gently washed with 5 ml of sterile PBS which was discarded along with remnants of media. Culture plates were now kept on ice and were then lysed with 2 ml of homogenising buffer (per 10 cm culture plate). Homogenised cell extract thus obtained was subjected to sonication for 3 X 30 seconds with intermittent 30 second cooling in ice. Extracts were then centrifuged at 13,000 rpm at 4^{o}C for 10 minutes using a bench-top centrifuge and supernatant was passed through glass wool. The concentration of extracts was estimated using BCA protein assay. The extracts were now treated with DTT to bring the final

concentration to 10 mM followed by incubation at 4^{o}C whilst shaking for 10 minutes. The extracts were now transferred to a water bath at 75^{o}C for 15 minutes with intermittent shaking followed by centrifugation on a table top centrifuge at 13,000 rpm at 4^{o}C for 10 minutes. Supernatants were retained for affinity purification, with about 200 µl being retained for immunoblotting (load/input).

2.2.3.2. COUPLING OF BAIT PROTEIN TO CNBR SEPHAROSE BEADS

The bait protein (Znf-UBP domain) was immobilised to CnBr sepharose beads at a concentration of 10.7 mg/ml beads. CnBr sepharose beads were first weighed and hydrated with excess 1 mM HCl for 30 minutes in polypropylene columns. The HCl solution was drained and 10 ml of coupling buffer was added to the column along with the desired amount of bait protein followed by incubation at 4^{o}C for 3 hours whist shaking. Post coupling, the buffer was drained and the immobilised bait was first washed and left to incubate overnight in excess 1M ethanolamine. The following day, once ethanolamine was drained, CnBr sepharose tagged bait was subject to 4X alternate washes with excess Tris and acetate buffers. Beads were washed in and stored in storage buffer, for use within 2 days.

2.2.3.3. AFFINITY PURIFICATION

Affinity purification could be carried out once bait protein was tagged to CnBr sepharose (2.2.3.2) and cell extracts were subject to treatment described in 2.2.2. Immobilised bait was first equilibrated with wash buffer in a polypropylene column. The beads were then incubated overnight at 4^{o}C with

processed cell extract whilst rotating. The following day, extract was allowed to flow through the column while beads were retained and washed 3X10 ml with wash buffer. The sample was then transferred to a microcentrifuge tube and centrifuged at 13,000 rpm at 4^{o}C on a bench-top centrifuge for 5 minutes in order to discard buffer present. The beads were stored at -20^{o}C.

2.2.3.4. DEUBIQUITINATION (DUB) ASSAY OF PURIFIED UNANCHORED POLYUBIQUITIN CHAINS ON BEADS

Affinity purified material on beads which was first washed 3X with DUB buffer, was incubated with an equal volume of DUB buffer. This was either incubated overnight with IsoT, Usp2 catalytic core or control at 37^{o}C whilst shaking. The following day, samples were centrifuged at 13,000 rpm for 1 minute on a bench-top centrifuge and solutions were separated while the beads were analysed to assess deubiquitination through immunoblots.

2.2.4. PROTEIN VISUALISATION

2.2.4.1. SDS PAGE

Samples were visualised initially using polyacrylamide gradient gels, composed of 5-20% gradient resolving gel and 5% stacking gel. 10 ml each of 5% and 20% acrylamide solutions were prepared separately and poured using a gradient mixer to form the resolving gel. 5% gel was composed of 1.67 ml of 30% acrylamide, 3.33 ml buffer B, 100 µl of 10% SDS and 5 ml deionised H_2O while 20% gel was composed of 6.67 ml of 30% acrylamide, 3.33 ml buffer A and 100 µl of 10% SDS. 100 µl of 10% APS and 10 µl of TEMED were added to both solutions, to catalyse polymerisation, immediately before pouring. The gradient gel was poured between two gel plates; the surface of

the gel was covered by (water) saturated butanol. Meanwhile, stacking gel was prepared by mixing 1 ml of 30% acrylamide and 5 ml stacking gel buffer, with the addition of 100 µl of 10% APS and 10 µl of TEMED just before pouring. Once polymerisation of resolving gel was completed, butanol was washed away and stacking gel was poured on top of the resolving gel. A gel comb was used to form wells for samples.

Samples (including affinity purified polyubiquitin chains on beads) were prepared by adding GAB (usually 20 µl GAB to 50 µl of sample) with total amount of protein per well kept under 5 µg. The sample was then heated at 90^{o}C for 5 minutes before loading into wells. 2 µl of molecular weight marker (BLUeye prestained protein ladder, Geneflow Limited) was also loaded to estimate protein size. Gels were run using an electrophoresis unit filled with electrode buffer at 40 mA for ~2 hours using a power pack. Once proteins were resolved in gel, these could either be subjected to staining with Coomassie Blue or transferred on to a nitrocellulose membrane for immunoblotting (2.2.4.2). For staining, gels were first washed with deionised H_2O and incubated with Coomassie Blue for 1 hour while shaking after which they were left to destain overnight.

2.2.4.2. WESTERN BLOTTING

Gels containing resolved protein and the nitrocellulose membrane were first soaked in transfer buffer. These were then sandwiched together between 2 sheets of Whatman 3MM filter paper. The proteins on gel were transferred on to nitrocellulose membrane in a transfer tank filled with transfer buffer, at 40

mA overnight. The following day, the nitrocellulose membrane was retained and soaked in Ponceau-S solution to confirm transfer of proteins. Blots were washed with 10 mM TBS until the membrane was completely destained (3X5 minute washes). The membrane could now be subject to immunoblotting.

Immunoblotting was carried out by first incubating at room temperature for an hour in blocking buffer (depends on antibody used, Table 2.1), followed by incubation in primary antibody (suspended in blocking buffer). The membrane was then washed (3X5 minute washes with buffer), and incubated with secondary antibody in blocking solution for an hour at room temperature. The membrane was again washed (3X5 minute washes with buffer) and incubated in ECL mixture for 1 minute. The ECL solutions were drained and an ECL hyperfilm was exposed to the blot in a dark room for durations varying from 1 minute to up to 20 minutes (if required). The ECL hyperfilm was then immersed in developing solution, washed in water and immersed in fixer solution. The film was finally washed and dried.

2.2.5. SAMPLE PREPARATION FOR MASS SPECTROMETRY

2.2.5.1. FORMIC ACID ELUTION OF AFFINITY PURIFIED UNANCHORED POLYUBIQUITIN CHAINS

Affinity purified endogenous unanchored polyubiquitin chains bound to immobilised bait on CnBr sepharose beads had to be eluted prior to MS analysis. Affinity purified sample tagged to beads were washed 3X10 minutes (whilst rotating) with DUB buffer, followed by centrifugation at 13,000 rpm on a bench-top centrifuge to discard buffer. This was to minimise the amount of surfactants present in sample. The volume of DUB buffer used was 10X the

volume of beads. The sample (on beads) was incubated for 30 minutes at room temperature in 0.1% formic acid in deionised H_2O (10X volume of beads), with gentle intermittent agitation. The eluted material along with remnant CnBr sepharose beads was passed through a micro-centrifugal filter and the eluate was collected upon centrifugation on a bench-top centrifuge at 13,000 rpm for 1 minute. The eluate was left to completely dry in a vacuum centrifuge. The dried sample could then be resuspended in desired buffer for MS based analysis.

2.2.5.2. TRYPSINOLYSIS OF PROTEINS IN POLYACRYLAMIDE GEL (PAG) PIECES

Areas of (Coomassie stained) gel containing protein of interest were excised and their volumes were estimated. These were carefully transferred to microcentrifuge tubes pre-rinsed with wash buffer. Gel pieces were agitated in excess wash buffer, until Coomassie stain was completely cleared. Gel pieces were then agitated with 10X volume of 10 mM DTT in the dark, for a period of 30 minutes at room temperature, following which the solution was discarded. This was done to break disulphide bonds between cysteine residues in proteins (reduction). Next the gel pieces were agitated with 10X volume of 20 mM CAA in the dark, for 30 minutes at room temperature, following which the solution was discarded. Gel pieces were agitated for 20 minutes, first with 10X volume of wash buffer, followed by a wash in 10X volume of 100% MeCN.

Remnants of MeCN were allowed to air dry. Appropriate amount of trypsin (in digestion buffer) was added such that the volume was the same as that of the

gel piece (1X). An additional volume (1X) of digestion buffer was added to the gel piece to prevent drying. The gel pieces were incubated at 37^{o}C for up to 16 hours. Following incubation, the supernatant was collected in pre-rinsed tubes. The gel pieces were then agitated with 3X volume of extraction buffer for 10 minutes and collected. The process was repeated and all the recovered factions from a gel piece were pooled. The extraction buffer could then be evaporated and the peptides thus obtained could be subject to MS analysis.

2.2.5.2. *IN SOLUTION* DIGESTION

The section details existing generic optimised trypsinolysis protocols, which were used thus far to prepare samples for MS analysis. The protocols were further developed to suit the specific needs and demands of this project.

Samples suspended in respective buffer were transferred to UF devices. The buffer was drained and discarded by centrifugation at 13,000 rpm for 15 minutes on a bench-top centrifuge. The sample was washed (pulse vortex) with 400 µl digestion buffer and centrifuged to discard the filtrate. 100 µl of 50 mM DTT was added and vortexed, followed by incubation for 30 minutes in the dark at room temperature. The UF device was again centrifuged at 13,000 rpm for 15 minutes using a bench-top centrifuge. Samples were alkylated using 100 µl of 100 mM CAA and incubated for 30 minutes in the dark followed by a round of centrifugation. The samples were washed with 400 µl digestion buffer, vortexed and centrifuged at 13,000 rpm until the liquid phase had ultra-filtered. The buffer was discarded. 100 µl of 0.02 mg/ml sequencing grade Trypsin was introduced to the UF device, followed by a vortex mix and

incubated overnight at 37^{o}C for 12-24 hours. Post-incubation, the UF devices were subject to centrifugation at 13,000 rpm for 15 minutes, however the filtrate was retained as it contained trypsinolysed peptide fragments. An addition of 100 µl of digestion buffer was made followed by pulse vortex mix and an incubation of 30 minutes at room temperature. A final round of centrifugation at 13,000 rpm for 15 minutes was performed and the ultra-filtrates were pooled and allowed to dry using a vacuum centrifuge before subjecting to MS analysis.

2.2.5.3. ABSOLUTE QUANTIFICATION (AQUA) STANDARDS

Synthetic peptides corresponding to trypsin scissile products of various diglycine linked ubiquitin sequence with incorporated heavy isotopes of carbon (^{13}C) and nitrogen (^{15}N) were commercially produced for the purpose of quantification of ubiquitin linkages [175]. Table 2.2 details the primary structure of these peptides while comparing the molecular masses of AQUA and native signature peptides. While AQUA standards were generated for all seven isopeptide linkage specific signatures, three additional peptides were also generated which were ubiquitin specific but were not representative of isopeptide linkages. These three peptides were generated from trypsinolysis of monoubiquitin and polyubiquitin chains and lacked the diglycine motif.

2.2.6. MASS SPECTROMETERS AND SAMPLE PREPARATION

All MS experiments were carried out with the help of Dr David Tooth (The University of Nottingham). The section is a compilation of the instruments

available for MS analysis, the application of which has been previously detailed [189].

2.2.6.1. MALDI-ToF

The instrument was set to operate in positive ionisation setting in Reflectron mode and had a mass/charge (*m/z*) detection range of of 1000-20,000 Da. Samples to be analysed were first desalted (purified) by first adsorbing on to using C-18 solid-phase tips. These were washed in and were washed in 5% ($^{v}/_{v}$) MeCN, 0.1% ($^{v}/_{v}$) formic acid in HPLC gradient grade H_2O. The adsorbed peptides were then eluted in 70% ($^{v}/_{v}$) MeCN, 0.1% ($^{v}/_{v}$) formic acid in HPLC gradient grade H_2O. These were then directly spotted on to a matrix of α-cyano-4-hydroxy-cinnamic acid. The instrument was first calibrated using standards and the samples were ionised by gradually increasing the laser energy while focusing on a spot on the matrix for an average of 5-10 seconds. The signal obtained over a period of several minutes was automatically averaged and displayed as a spectrum.

2.2.6.2. Q-ToF

Protein mixtures were either first separated using SDS PAGE or directly digested with trypsin *in solution*. Samples were then suspended in 0.1% formic acid in H_2O for analysis using LC-MS/MS. The samples from HPLC column were eluted with a gradient of 5-90% MeCN in H_2O (0.1% formic acid). The instrument was used in data-dependant-acquisition mode where precursor ion scans were followed by product ion analysis so yielding sequence information of peptides in sample. Data analysis was performed using MassLynx 4.0

software and protein identifications from data derived from LC-MS/MS experiments were analysed using the Mascot database. Standard curves for 2+ and 3+ charged states were generated by analysing LC-MS data, measuring peak area of extracted ion chromatogram per peptide (using MassLynx 4.0) and plotting areas (abundance) against amount (ng) loaded.

2.2.6.3. QqQ

This instrument was coupled to an HPLC for fractionation of peptide mixtures prior to analysis. SRM transitions for AQUA standards have previously been published but could be calculated if required [175] and the local system acquisition parameters were optimised. Protein mixtures were first trypsinolysed and submitted for LC-MRM-MS/MS analysis using pre-designated (based on precursor and product ions) SRMs. Extracted ion chromatograms were generated from total ion chromatograms (TIC) after which integrated SRM transition peak areas were calculated. This was either plotted against amount in sample to generate standard curves or was used to quantify the amount of unknown peptide in sample. Data analysis was performed using Skyline software, except in the generation of Figures 5.4, 5.5, 5.7 and 5.8 which were analysed using MassLynx 4.0.

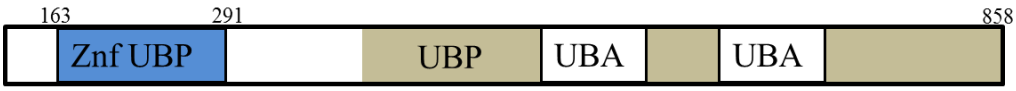
CHAPTER 3

REFINEMENT OF METHODS FOR THE PURIFICATION OF ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS

3.1. ISOLATED ZNF-UBP DOMAIN OF ISOPEPTIDASE T CAN BE USED AS "BAIT" TO AFFINITY PURIFY ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS

The DUB IsoT contains four distinct domains which mediate its ability to recognise and disassemble unanchored polyubiquitin chains: a catalytic UBP domain, two UBA domains and a Znf-UBP domain (Figure 3.1). Whereas the UBA domains recognise the hydrophobic I44-centred surface of ubiquitin, the Znf-UBP selectively binds to the diglycine motif exposed only by the proximal ubiquitin within an unanchored polyubiquitin chain, hence providing the selectivity for recognition of substrate-free over conjugated chains [57]. The affinity purification method previously established utilises this selectivity and high affinity binding (low micromolar K_d) of the Znf-UBP domain to capture unanchored polyubiquitin chains and free monoubiquitin [174]. As discussed in Section 1.3, this approach was previously used to capture endogenous unanchored polyubiquitin from rat skeletal muscle tissue and confirm the presence of K48 and K11 linkages.

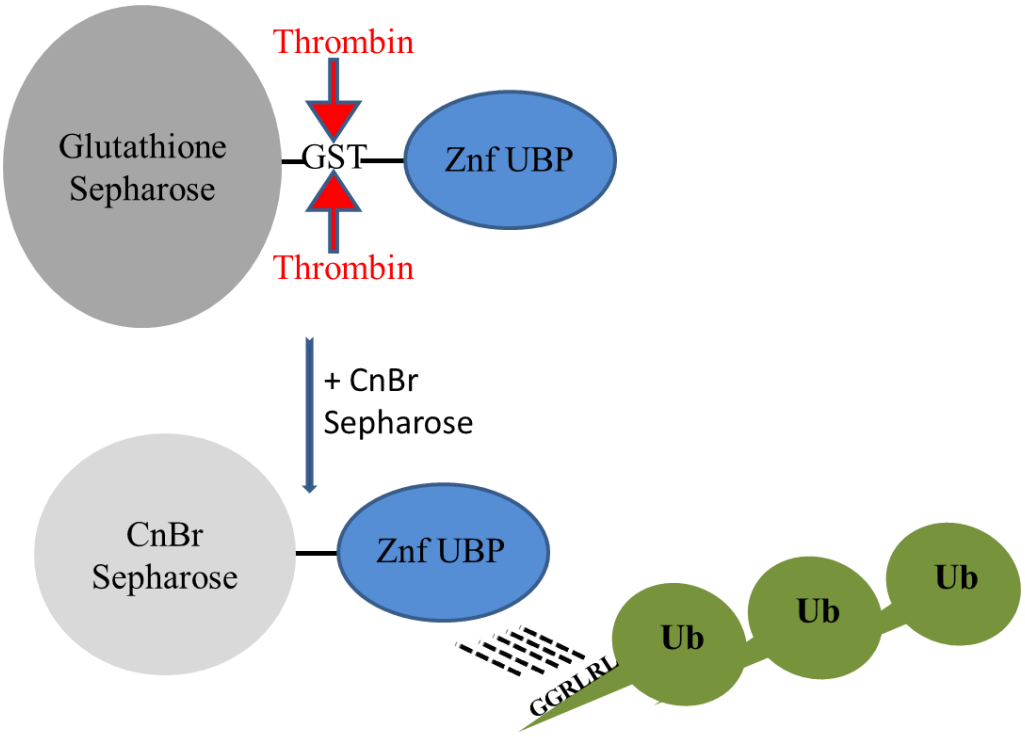
A.



B.

```
160      KQEVQAWDGEVRQVSKHAF    181
182   NLKQLDNPARIPPCGWKCSKCD    203
204   MRENLWLNLTDGSILCGRRYFD    225
226   GSGGNNHAVEHYRETGYPLAVK    247
248   LGTITPDGADVYSYDEDDMVLD    269
270   PSLAEHLSHFGIDMLKMQKTDK    291
```

C.



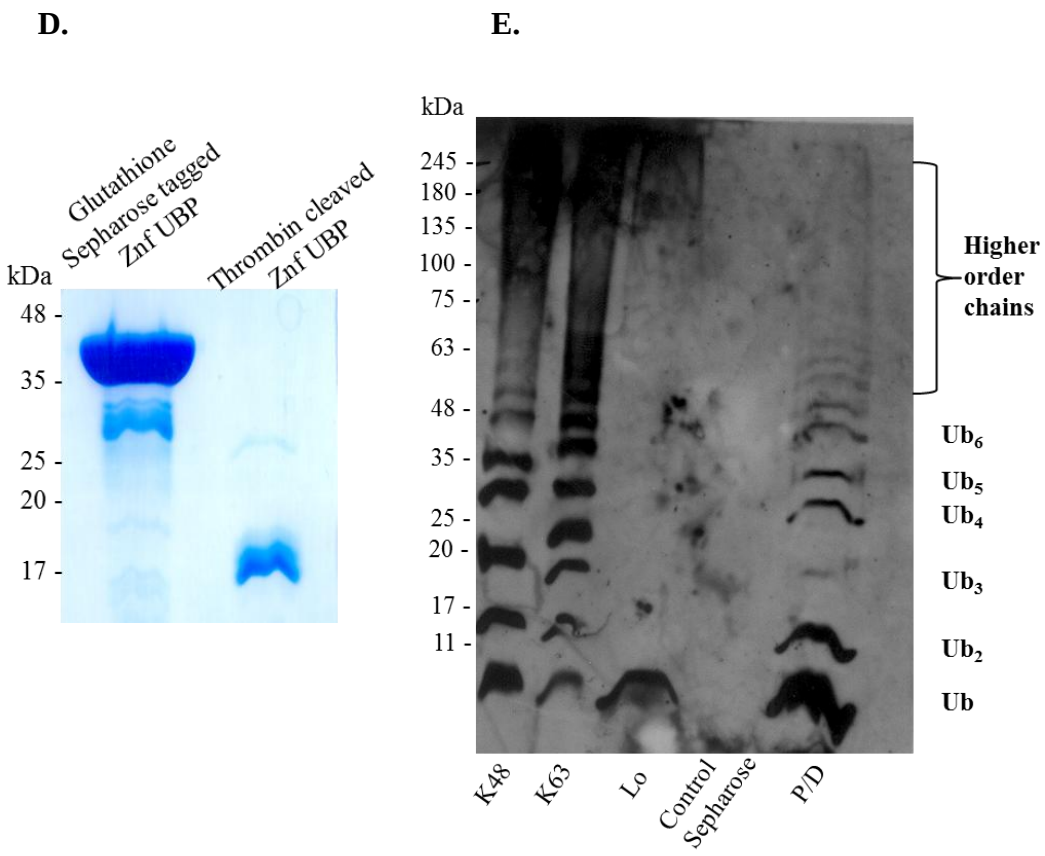


Figure 3.1. Summary of the previously established protocol for the purification of endogenous unanchored polyubiquitin chains.
A. Representation of the four domains in Isopeptidase T; Znf-UBP domain, two UBA domains and a catalytic UBP domain.
B. The amino acid residues (163 – 291, human sequence) constituting the Znf-UBP domain which were used as "bait" for purification of unanchored polyubiquitin chains. Amino acid residues associated with zinc-binding are highlighted in red.
C. GST tagged Znf-UBP was overexpressed in *E. coli* and purified using glutathione-Sepharose, and thrombin cleaved to remove the GST tag, then covalently linked to CNBr-activated Sepharose. The intrinsic affinity for the free C-terminus of the proximal ubiquitin in unanchored polyubiquitin chains (shown in green) was exploited for their affinity capture.
D. Coomassie stained gel loaded with [lane 1] 10 µl of glutathione-Sepharose purified GST-Znf-UBP (5 L of bacterial culture purified on 1 ml of beads) and [lane 2] 5 µg (confirmed by BCA assay) of thrombin cleaved purified Znf-UBP domain.
E. Affinity purified sample (labelled P/D) of unanchored polyubiquitin chains from 8 mg of total protein from HEK293T cell extract, were separated using SDS PAGE and subjected to western blotting. Also run in separate lanes were 250 ng commercial K48- and K63-linked polyubiquitin ladders, 50 µl load (labelled Lo, refers to input for purification) and proteins eluted from control Sepharose (lacking bait). The blot was probed with an *in house* ubiquitin antibody and confirmed the presence of ubiquitin chains in sample. The chain length was estimated after comparison with commercial ladders (displayed in figure). Control Sepharose sample appeared to lack purified (poly) ubiquitin.

The 129 amino-acid Znf-UBP domain (amino acid residues 163 – 291 of human IsoT) was previously cloned in to the pGEX4T-1 vector allowing expression in *E. coli* with an N-terminal GST fusion, with a thrombin protease site between the fusion partners. In order to confirm the utility of the Znf-UBP domain in the purification of unanchored polyubiquitin chains, the plasmid was used to over-express the GST-Znf-UBP protein which was then purified using glutathione-Sepharose affinity chromatography. Following thrombin cleavage, the released Znf-UBP domain was collected and covalently linked to CNBr-activated Sepharose-4B beads (CNBr-Seph). Successful isolation of the Znf-UBP domain was evident from SDS PAGE analysis (Figure 3.1.D).

The immobilised Znf-UBP domain was initially used to capture unanchored polyubiquitin chains from HEK293T cell extracts cultured under standard conditions (Figure 3.1.E) using the previously reported purification protocol [174]. HEK293T cells (~90% confluent) in 75 cm^2 cell culture flasks were lysed in homogenising buffer (2.1.3). The protein content was estimated (using a BCA assay) to be 2 mg/ml in a total final volume of 4.1 ml. The protein extract was then processed for purification by first incubating with DTT (final concentration of 10 mM) and subsequent heat treatment at 75°C for 20 minutes and affinity purified with 50 µl of immobilised Znf-UBP domain (10 mg/ml on beads). Half of this affinity-purified sample was subject to separation by SDS PAGE followed by anti-ubiquitin western blotting. Consistent with previous preliminary findings, this revealed distinct ubiquitin-positive bands, some corresponding to chains containing over 10 ubiquitin moieties (Figure 3.1.E) that co-migrated with commercial K48 or K63 polyubiquitin ladders run in

adjacent lanes of the blot. Given the known selectivity of the Znf-UBP domain, these were presumed to represent endogenous unanchored polyubiquitin chains. To rule out non-specific interactions, an equal amount of cell extract-derived proteins was incubated with 50 µl of CNBr-Sepharose beads lacking 'bait' protein. Any proteins on beads were eluted in gel-application buffer (GAB) and analysed in the same blot (labelled control Sepharose). Absence of ubiquitin immunoreactivity suggested lack of non-specific binding to CNBr-Sepharose.

3.2. REFINEMENT OF THE PURIFICATION STRATEGY FOR THE CAPTURE OF ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS FROM HEK293T CELLS

3.2.1. OPTIMISING AMOUNT OF ZNF-UBP BAIT REQUIRED FOR QUANTITATIVE CAPTURE OF ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS

Since the Znf-UBP domain affinity purification strategy had thus far been used to extensively characterise unanchored polyubiquitin chains from rat skeletal muscle, care was taken to optimise the approach to ensure quantitative capture of unanchored chains from HEK293T cells. Specifically it was important to ensure complete and quantitative capture of unanchored polyubiquitin chains i.e. that the Znf-UBP bait was not limiting. A caveat to the purification approach is the existence of high levels of free (unconjugated) monoubiquitin in cells [16, 78], which competes with unanchored polyubiquitin for binding to the Znf-UBP bait. Since the purification of monoubiquitin was unavoidable,

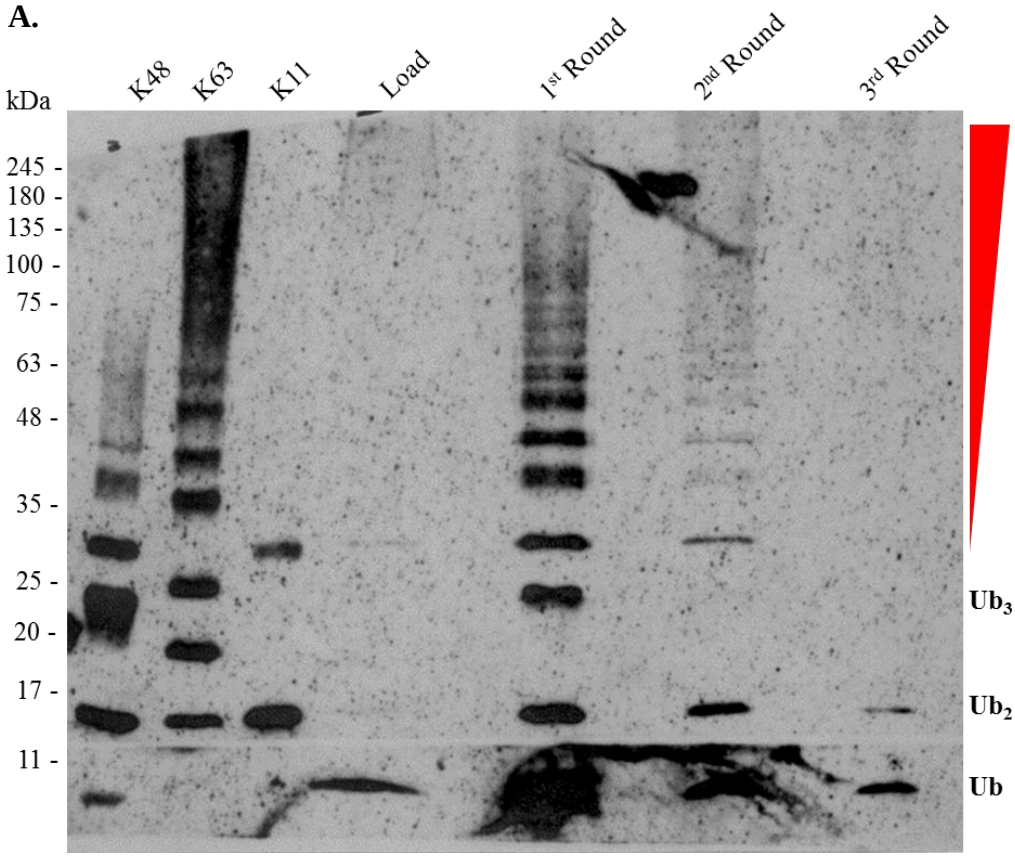
excess bait was required for the purification of the total unanchored polyubiquitin component from cells.

The experiment performed involved using of a fixed amount of soluble protein extract from HEK293T cells which was subject to sequential rounds of affinity purification on a series of Znf-UBP columns, until the captured material showed no evidence of further enrichment of unanchored polyubiquitin chains. HEK293T cells were homogenised as described in section 2.2.3. Post-centrifugation at 13,000 rpm at 4°C for 20 minutes, 40 mg of protein extract (as determined by BCA assay) in a total volume of 13 ml was processed for purification. The sample was first incubated overnight (16 hours) with 100 µl of immobilised Znf-UBP domain (concentration of 10 mg/ml on beads) in a polypropylene column. The flow-through was collected and further incubated overnight with a fresh 100 µl column of immobilised Znf-UBP. Finally, the flow-through was subjected to a third round of purification.

The affinity-purified samples (on beads) from all three rounds of affinity capture were eluted in GAB. Eluates (25% by volume) from all three rounds of purification were separated using SDS PAGE, alongside commercial K48 and K63 linked polyubiquitin ladders and also K11 dimers (as standards). Western blotting was performed using four ubiquitin antibodies (Figure 3.2); an *in house* ubiquitin polyclonal antibody and commercial antibodies specific for K48, K63 and K11 polyubiquitin linkages. The anti-ubiquitin and anti-K48 polyubiquitin antibodies clearly visualised unanchored polyubiquitin chains containing more than 12 ubiquitin moieties, with the blots also demonstrating

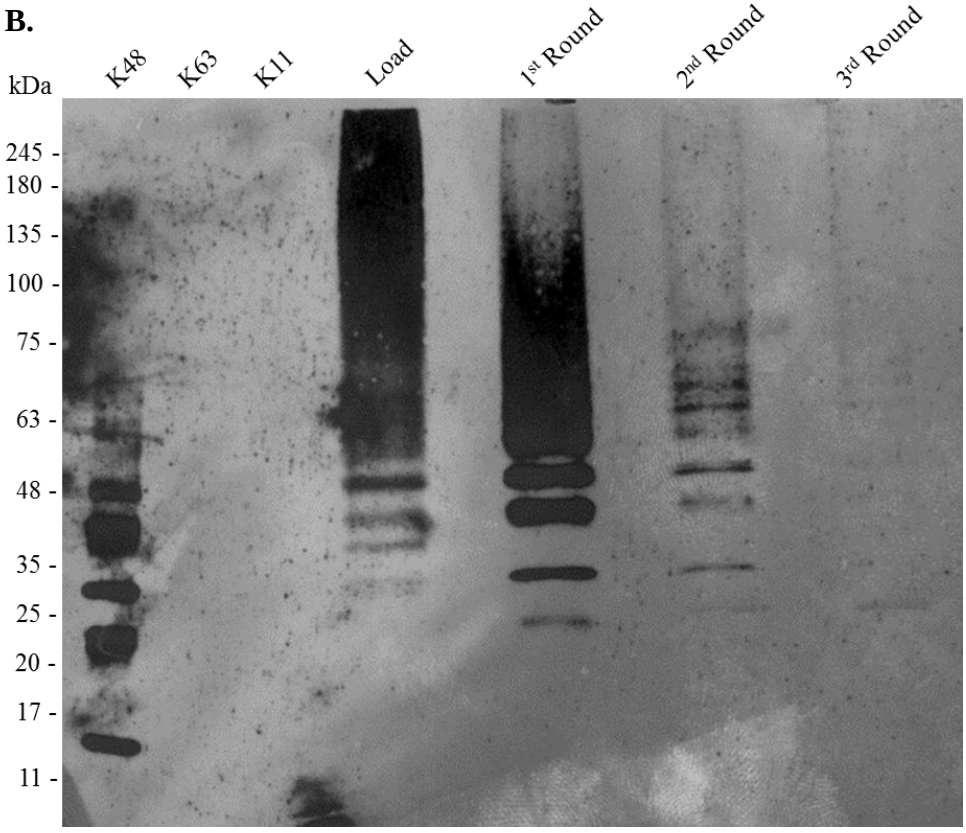
the capture of the majority of the chains during the first round of purification (Figure 3.2). While some polyubiquitin chains could be detected after the second round of purification, the samples from the third columns were effectively devoid of unanchored polyubiquitin. Thus, a volume of 250 µl immobilised Znf-UBP (at 10 mg/ml on beads) was proposed as being necessary to completely deplete unanchored polyubiquitin chains from the 40 mg of HEK293T protein extract, equivalent to 6.25 µl of bait required per mg of protein extract.

A.



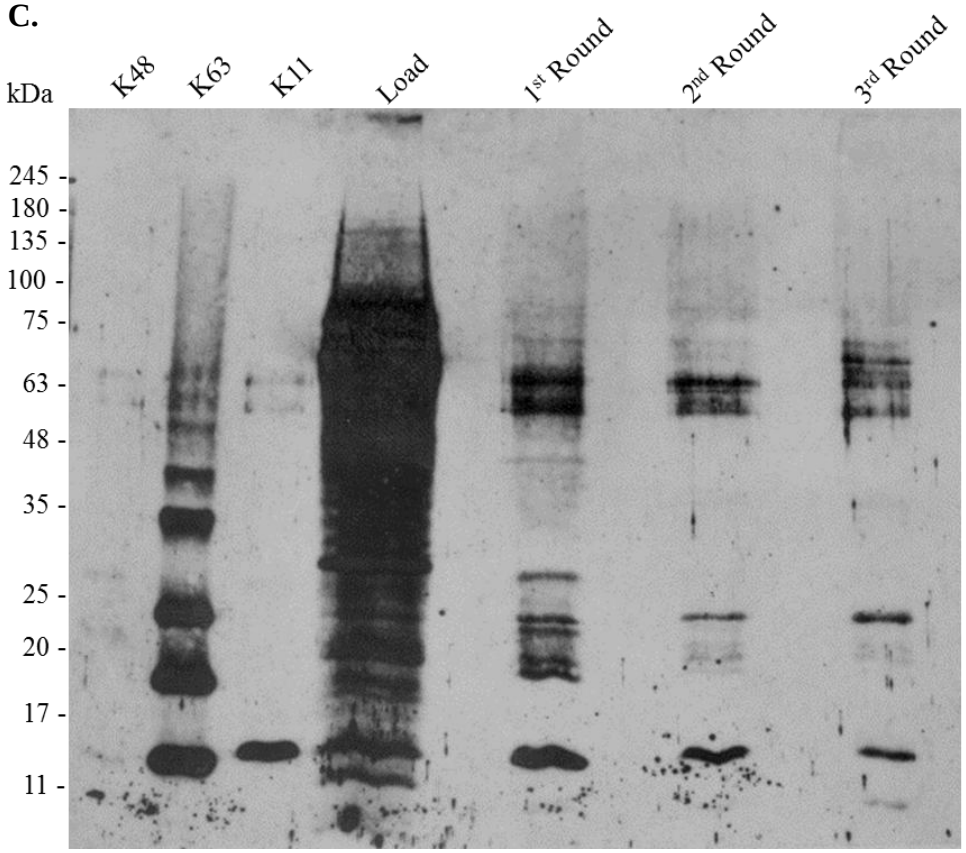
IB – Ubiquitin

B.



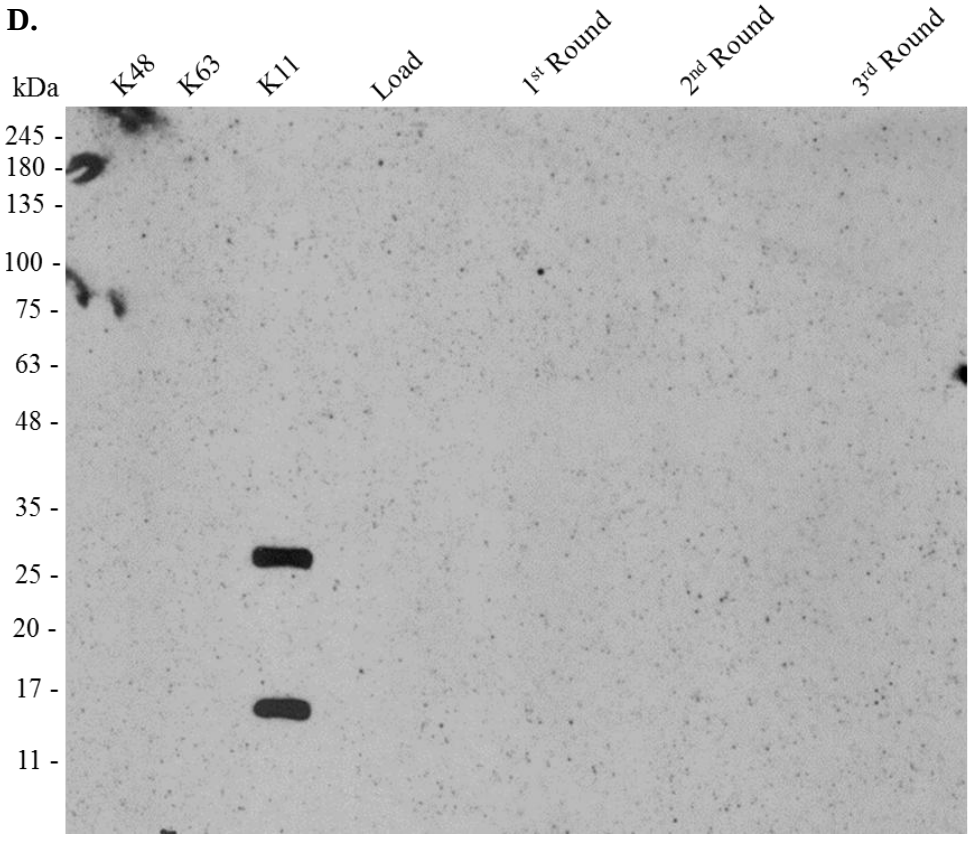
IB – K48

C.



IB – K63

D.



IB – K11

Figure 3.2. Determination of the amount of immobilised Znf-UBP bait required for complete capture of unanchored polyubiquitin chains from HEK293T cells. Cells were homogenised in 50 mM Tris-HCl 150 mM NaCl, 0.5% (v/v) IGEPAL (NP-40), 5 mM NEM, 20 µM MG132 and 0.1% (v/v) mammalian protease inhibitor cocktail. 40 mg of protein (estimated by BCA assay) was pre-treated and subject to three successive rounds of incubation with 100 µl Sepharose-immobilised Znf-UBP domain, leading to the gradual depletion in unanchored chains. 25% of sample from each round was eluted in GAB and used to obtain four western blots which were probed with four ubiquitin antibodies for **A.** ubiquitin, **B.** lysine 48 (K48), **C.** lysine 63 (K63) and **D.** lysine 11 (K11) polyubiquitin linkages.

In addition to the samples, 250 ng each of K48 and K63 commercial polyubiquitin ladders and K11 dimer (also observed to contain tetramer) were run in adjacent lanes and used as positive control and as an indicator of ubiquitin chain length in sample derived chains (higher order chains in ubiquitin immunoblots depicted with red symbol. 50 µl of load (input) mixed with 20 µl of GAB was also run in an adjacent lane (labelled load).

Subjective visual assessment of immunoblots suggested depletion of most unanchored polyubiquitin after two rounds of purification. However, while the K63 antibody was seen to lack selectivity, no reactivity (except against its control antigen) was seen with the K11 antibody. A volume of 250 µl Znf-UBP tagged CNBr-Seph was considered sufficient for complete capture of unanchored polyubiquitin chains from the 40 mg of extract proteins, hence a bead: protein ratio of 6.25:1 was used for future experiments.

The blots also confirmed the presence of K48-linked unanchored polyubiquitin chains in the purified samples but failed to detect K11 linkages even though reactivity was seen against commercial K11-linked dimers (Figure 3.2). Although the reactivity of the K63-linked polyubiquitin antibody appeared to suggest abundance of this linkage type in the purified unanchored polyubiquitin chains, the cross-reactivity with K48 and K11-linked polyubiquitin ladders and also with Znf-UBP bait (data not shown) suggested a lack of selectivity (Figure 3.2). Hence the use of the anti-K63 (and anti-K11) antibody was generally discontinued in future experiments (subsequent to section 3.4). These experiments highlight the limitations of using linkage-selective antibodies to define polyubiquitin linkage types, and emphasise the need for the development of other analytical methodologies e.g. MS-based methods (see later).

Having established the optimum amount of bait required for purification of endogenous unanchored polyubiquitin chains from HEK293T cells, the incubation time for efficient affinity capture was also briefly investigated. Although such experiments have previously been performed for 16 hours at 4°C (as in Figure 3.2), a shorter incubation time of 1 hour was also tested. 8 mg of protein derived from HEK293T cells was split equally and incubated with 25 µl of immobilised Znf-UBP bait (10 mg/ml) each either for 1 hour or 16 hours. Purified samples were eluted in GAB and separated by SDS PAGE, then visualised by anti-ubiquitin western blotting (Figure 3.3). The experiment suggested more efficient purification of unanchored polyubiquitin chains at the 16 hour time point, and hence overnight incubation was continued hereafter.

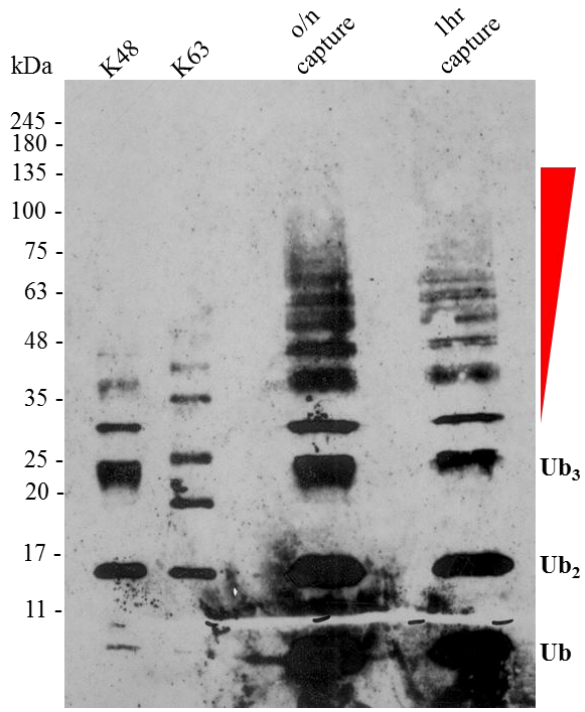


Figure 3.3. Determination of optimal incubation time for affinity purification. 25 µl of Znf-UBP tagged beads was incubated with 4 mg of protein derived from HEK293T cells at 4°C, for the duration of either 1 hour or 16 hours (overnight). The sample thus obtained was eluted in GAB and used for western blotting with ubiquitin specific antibody. 250 ng each of commercial K48 and K63 polyubiquitin ladders were used as positive controls. The numbers of ubiquitin moieties in chains obtained from samples have been labelled. Overnight capture gave a greater enrichment of unanchored polyubiquitin chains than one hour capture.

3.2.2. PROFILES OF ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS FROM HEK293T CELLS AS DETERMINED USING DIFFERENT UBIQUITIN ANTIBODIES

Polyubiquitin chain topologies vary depending on the linkages involved, which in turn determines recognition by ubiquitin-binding domains and proteins. Thus it is possible that different pan-anti-ubiquitin antibodies could show a bias towards chains of a specific chain linkage. The endogenous unanchored polyubiquitin profiles of chains purified from HEK293T cells were further probed using four different pan-ubiquitin antibodies in parallel; *in house* polyclonal, VU1 (LifeSensors, Inc.), FK2 (VIVA Bioscience Ltd) and P4D1 (Santa Cruz Biotechnology). 25 µl of affinity purified sample derived from HEK293T cells (prepared under optimised conditions, see 3.2.1.) were analysed by western blotting with the different antibodies (Figure 3.4).

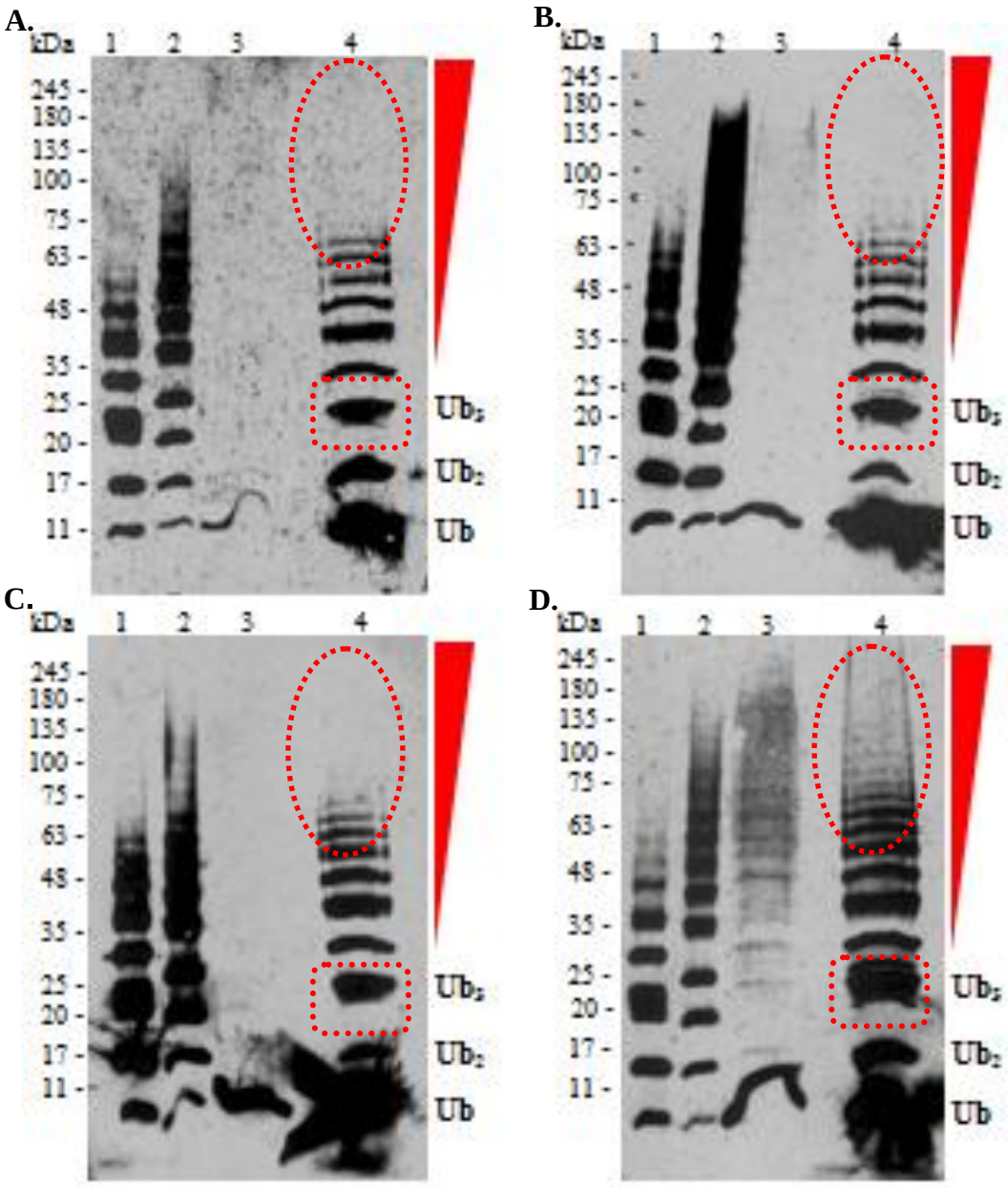


Figure 3.4. Comparison of efficiency of four ubiquitin-specific antibodies. 25% each of 100 µl affinity purified unanchored polyubiquitin chains from HEK293T cells on Znf-UBP tagged beads were eluted in GAB and subject to western blotting (lane 4) to produce four replicate blots. Each of these was probed with a separate ubiquitin antibody – **A.** the *in house* antibody, **B.** FK2, **C.** VU-1 and **D.** P4D1 clones. 50 µl of load (input for each immunoblot) in GAB was run in an adjacent lane (lane 3). Commercial K48 and K63 polyubiquitin ladders were used as positive control (lanes 1 and 2 respectively), however while 250 ng of each were used in blots probed with the *in house* antibody and the FK2 clone, only 100 ng was used in blots probed with VU-1 and P4D1 clones. The length of ubiquitin chains in sample, in terms of the number of moieties, has been indicated. Subtle differences in reactivity of antibodies have been highlighted in red.

As evident from the western blots, three of the antibodies, namely the *in house* antibody and the FK2 and VU-1 clones, showed very similar reactivity against the purified sample. Another feature to note was the difference in sensitivities; significantly less commercial polyubiquitin ladders were required for VU-1 and P4D1 antibodies to give comparable signals (250 ng each used for FK2 and *in house* antibodies whereas 100ng was used for VU-1 and P4D1). The P4D1 antibody was the most versatile of the four as apparent from its ability to recognise longer chains and also to reveal subtle differences in the (presumed) ubiquitin trimer within the purified sample. However, the P4D1 clone did not offer a significant advantage over using the other antibodies. Hence, the *in house* anti-ubiquitin and VU-1 clones (both readily available in the lab) were utilised for the majority of the subsequent western blotting assays.

3.3. DEUBIQUITINATING ENZYMES (DUBS) CONFIRM THE PURIFIED SAMPLES DERIVED FROM HEK293T CELLS CONTAIN UNANCHORED AND NOT SUBSTRATE-LINKED POLYUBIQUITIN CHAINS

Although the Znf-UBP domain from different proteins (including IsoT and HDAC6) has been previously reported to selectively bind unanchored polyubiquitin chains as opposed to conjugated polyubiquitin [57], DUBs were employed to confirm that the purified samples from HEK293T cells were indeed not covalently attached to protein substrates. Commercially available recombinant IsoT, an enzyme previously described for its selective disassembly of unanchored polyubiquitin chains [57] and the catalytic core of

USP2, a broad spectrum DUB seen to disassemble all polyubiquitin linkages [16, 190], were used to confirm integrity of the purified samples.

50 µl aliquots of affinity-purified endogenous unanchored polyubiquitin chains from HEK293T cells immobilised on beads, were incubated overnight (37^{o}C) with either of the two enzymes (2.2.3.4) or left to incubate in deubiquitination assay buffer alone. The three samples were divided equally and visualised with anti-ubiquitin or K48-linked polyubiquitin specific antibodies by western blotting (Figure 3.5). Disappearance of polyubiquitin reactivity in the sample upon incubation with IsoT confirmed the unanchored nature of the purified polyubiquitin chains. Similarly, USP2 efficiently disassembled the polyubiquitin chains (Figure 3.5). It was however unclear as to why both the enzymes spared the diubiquitin species (and tri-ubiquitin in case of USP2).

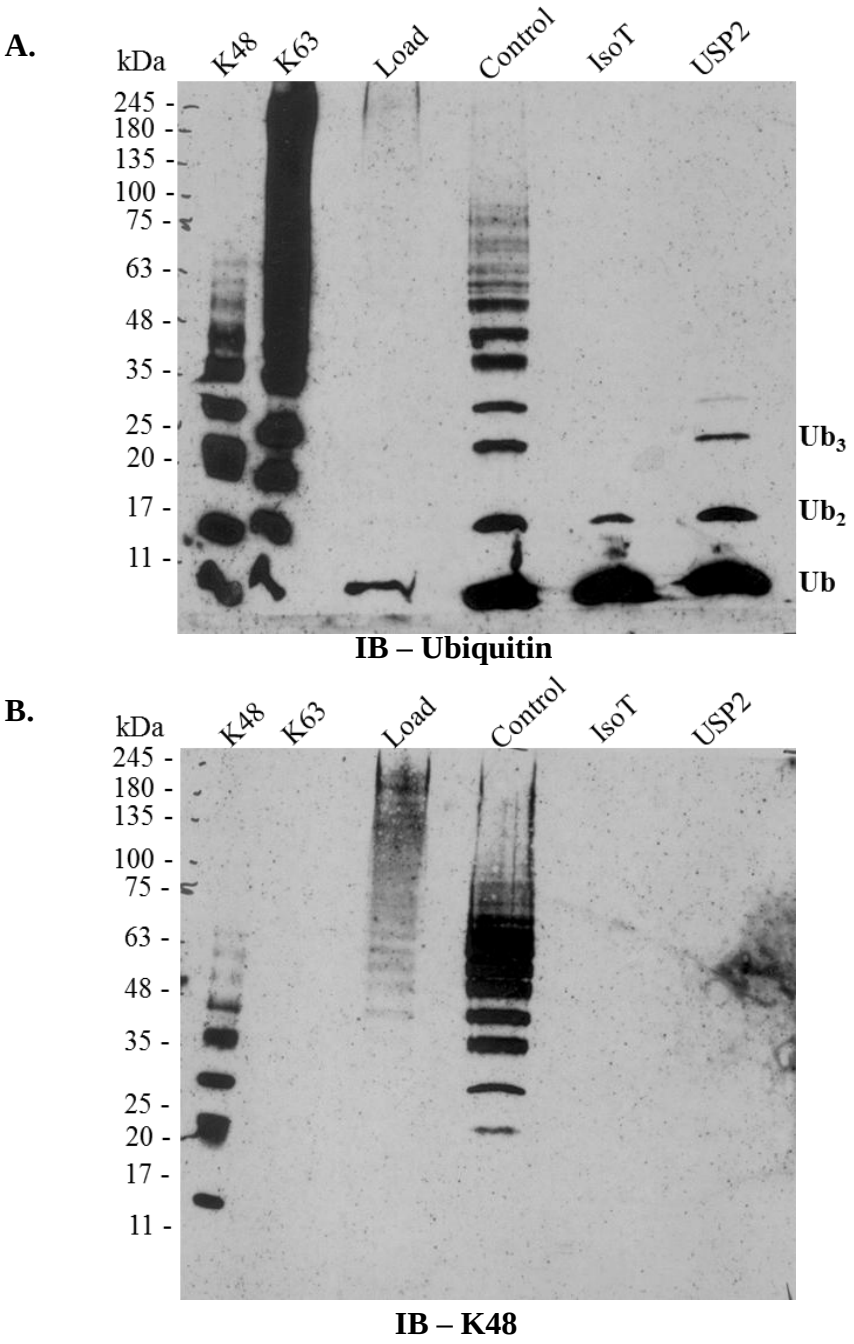


Figure 3.5. Sensitivity to DUBs was used to test if purified polyubiquitin chains from HEK293T cells were indeed unanchored. 50 µl samples on Znf-UBP tagged beads were incubated overnight at 37°C with either commercial long isoform of IsoT, catalytic core of USP2 (positive control) or in deubiquitination assay buffer alone (negative control). The supernatant buffer was discarded while the purified samples on beads were eluted in GAB, split equally and were subject to immunoblotting with **A.** ubiquitin and **B.** K48 polyubiquitin-specific antibodies. 50 µl of input mixed with 20 µl of GAB was run in adjacent lane (Load). Commercial K48 and K63 polyubiquitin ladders were used as positive controls for antibodies (100 ng for ubiquitin immunoblot and 250ng in case of K48 immunoblot). Disappearance of ubiquitin-reactive bands upon treatment with IsoT and USP2 confirmed that the purified chains were indeed unanchored polyubiquitin.

3.4. FURTHER ATTEMPTS TO DETECT ENDOGENOUS UNANCHORED K63-LINKED POLYUBIQUITIN CHAINS FROM HEK293T CELLS

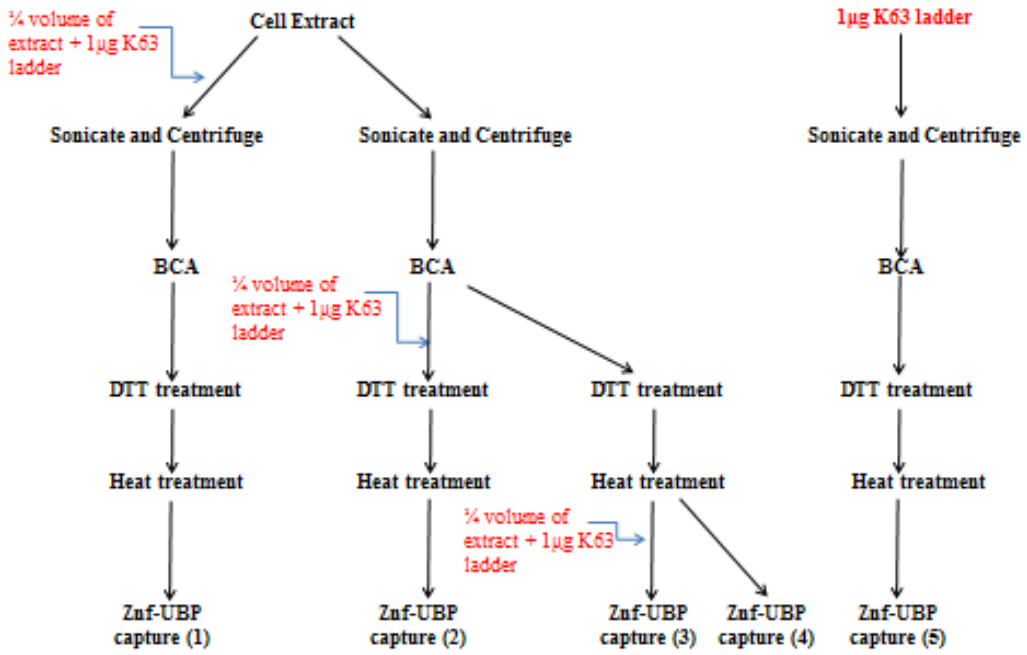
Unanchored K63-linked polyubiquitin chains have previously been shown to play a central role in the activation of ubiquitin-dependent kinases relevant to NF-κB signalling pathways [1]. However, in section 3.2 their presence in affinity-purified samples derived from HEK293T cells could not be confirmed by western blotting because of the unreliability of the anti-K63-linked polyubiquitin antibody (Figure 3.2). Recent evidence suggested the notion of K63-linked polyubiquitin being "shielded" by non-covalent interactions with certain cellular ubiquitin-binding proteins which could compete with their interactions with the Znf-UBP domain during affinity purification [126, 191]. Although the heat treatment step included in our purification method should be effective to deplete such ubiquitin-binding proteins (the unanchored polyubiquitin chains being heat resistant), heat-stable ubiquitin-binding proteins may exist which still compete for binding to the unanchored K63-linked chains. Therefore a study was designed in order to test the recovery of K63-linked polyubiquitin chains, both to estimate losses during various stages of our purification protocol but also to determine if their binding to the Znf-UBP domain might be blocked by associated cellular proteins.

The experiment involved the addition of 1 µg of commercial (exogenous) K63-linked polyubiquitin chains to HEK293T-derived cell extract at three separate stages [Samples (1), (2) and (3) in Figure 3.6.A] of the purification process,

prior to affinity capture with equal volumes (40 µl) of immobilised Znf-UBP domain. The recovery of K63-linked polyubiquitin chains was then assessed by western blotting (Figure 3.6.B). Two sets of controls were used in the setup; firstly a sample was prepared without the addition of commercial K63-linked chains (Sample 4). Similarly, a sample was prepared wherein only 1 µg of K63-linked chains (in homogenising buffer) was used as starting material, without any HEK293T cell-derived proteins (sample 5). The ratio of Znf-UBP bait to protein derived from HEK293T cell extract used in affinity purification was deliberately kept higher than previously found to be optimal to ensure excess bait available to capture the additional exogenous K63-linked unanchored polyubiquitin chains.

40 µl of immobilised Znf-UBP (10 mg/ml on beads) was used for the capture of extracts from each of the five samples (where exogenous K63-linked polyubiquitin chains were introduced at different points) from a starting material of 4mg of HEK293T protein extract. 50% of the purified material was eluted into GAB and analysed by western blotting with anti-ubiquitin antibodies (Figure 3.6.B). 100 ng of commercial K63-linked polyubiquitin ladder was used as a standard to compare relative recoveries.

A.


¼ volume of extract + 1µg K63 ladder
Cell Extract
1µg K63 ladder
Sonicate and Centrifuge
Sonicate and Centrifuge
Sonicate and Centrifuge
BCA
BCA
BCA
¼ volume of extract + 1µg K63 ladder
DTT treatment
DTT treatment
DTT treatment
DTT treatment
Heat treatment
Heat treatment
Heat treatment
Heat treatment
¼ volume of extract + 1µg K63 ladder
Znf-UBP capture (1)
Znf-UBP capture (2)
Znf-UBP capture (3)
Znf-UBP capture (4)
Znf-UBP capture (5)

B.

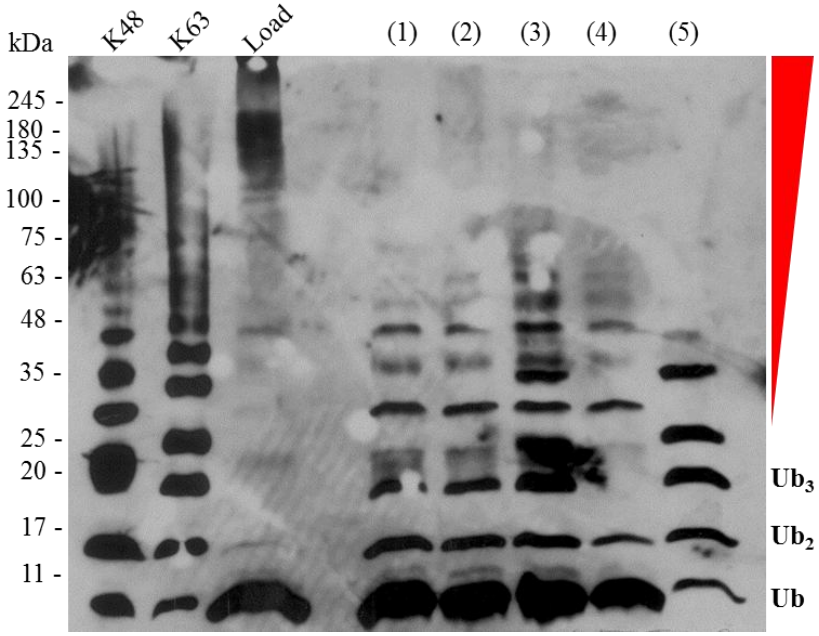

kDa
K48
K63
Load
(1)
(2)
(3)
(4)
(5)
245 -
180 -
135 -
100 -
75 -
63 -
48 -
35 -
25 -
20 -
17 -
11 -
Ub3
Ub2
Ub

Figure 3.6. Protocol employed to address the failure to recover / detect K63 linkages in samples of endogenous unanchored polyubiquitin chains purified from HEK293T cells.

A. HEK293T cells were lysed in homogenising buffer and 1 µg of commercial K63-linked chains were added to equal amounts of sample at three different stages of the purification strategy (indicated in red) to obtain samples (1), (2) and (3). A batch was purified without the addition of commercial polyubiquitin chains to obtain sample (4). The amount of cell-derived proteins was kept constant in all samples purified. In addition, 1 µg of K63-linked chains was also processed in parallel with the cell-derived samples, (5), to confirm losses of these chains during pre-treatment steps.

B. 50% of total purified material on beads (total of 40 µl), derived from the five conditions, was eluted into GAB and subject to western blotting. The blot was then probed with anti-ubiquitin antibody. In addition to the samples, lanes containing 100 ng of commercial K48 and K63 ladders (positive controls) and 50 µl load (input) were also included on the blot.
Subjective visual assessment of the blot and comparison with the positive control suggests loss of commercial K63-linked chains from sample (5) during the purification process. While samples (4), lacking commercial K63 chains was the least enriched, sample (3) appeared to be the most enriched upon comparison with samples (1) and (2), suggesting significant losses of K63-linked chains upon the heat treatment step.

Based on comparison of relative abundance of K63-linked polyubiquitin-positive bands in sample lanes on the western blot, the following observations were made:

- Lane 4 represents the control lacking "exogenous" K63-linked chains, whilst lane 5 represents capture of K63-linked chains alone (without HEK293T-derived proteins). When compared with the lane containing 100 ng commercial K63 standard (i.e. input), lane 5 (50% of Znf-UBP capture of processed 1 μg K63-linked chains) does reveal loss of polyubiquitin chains through the purification process.
- The blot reveals a marginal increase in the captured material in lanes 1 and 2 when compared to lane 4. These lanes contained samples wherein commercial K63-linked chains were added prior to heat treatment.
- Lane 3, containing sample wherein commercial K63 chains were added after heat treatment, appears to yield the most K63-linked polyubiquitin abundant sample. This implies that the hypothesis of K63-linked polyubiquitin chains being "shielded" from interacting with bait protein was not supported.
- Comparison of lane 3 with lanes 1 and 2 suggests that the higher order K63-linked polyubiquitin chains were more susceptible to losses during heat treatment than shorter chains.

To summarise the observations, unanchored K63-linked polyubiquitin chains may be susceptible to losses during the heat treatment step of the purification protocol. Similar observations were made in a recent study wherein polyubiquitin chains were seen to be increasingly heat labile with increase in

chain length, with K63 linked polyubiquitin chains being more sensitive to heat treatment than for instance K48 linked or linear chains [192]. However there is little evidence to suggest the possible "shielding" of K63-linked chains from Znf-UBP bait by other cell-derived proteins.

3.5. EFFECT OF HEAT TREATMENT ON RECOVERY OF ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS FROM HEK293T CELLS

An integral part of the purification strategy previously established for endogenous unanchored polyubiquitin chains was the heating of homogenised protein extract (prior to affinity purification) at 75°C for 20 minutes, which leads to denaturation of the majority of the proteins in cell extract including ubiquitin-binding proteins and ubiquitin conjugated substrates, whilst sparing unanchored polyubiquitin chains. However, it was unclear (and indeed not established) if this also leads to losses in unanchored polyubiquitin chains, either generally or of specific linkages (an observation described in the previous section). Hence, an alternate strategy involved the omission of the heating step and at the same time including the use of 1 mM 1,10-o-phenanthroline (oP) as an additional inhibitor of DUBs (specifically, metalloprotease class of DUBs) was investigated [191]. Note that the original purification strategy does include an inhibitor of cysteine-proteases (2.1.3), NEM, and this was also included alongside the oP treatment.

3.5.1. AMOUNT OF ZNF-UBP DOMAIN REQUIRED FOR PURIFICATION OF ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS IN THE ABSENCE OF SAMPLE HEATING

As seen previously when applying the purification approach to HEK293T cells, it was first necessary to determine the amount of bait (immobilised Znf-UBP domain) that was required to completely capture available unanchored polyubiquitin chains in the absence of sample heating, since the heating step was anticipated to selectively reduce total protein concentration. In an experiment similar to that described in section 3.2, 40 mg of proteins from HEK293T cell extract (prepared by omitting the sample heating step) were purified using in this case four successive Znf-UBP affinity columns (100 μl of beads with Znf-UBP at 10 mg/ml on beads). Purified samples were analysed by western blotting with anti-ubiquitin and K48-linked polyubiquitin antibodies (Figure 3.7). It was evident from the blots of the affinity-purified samples that in this case, even on the fourth round of purification there was capture of what was presumably endogenous unanchored polyubiquitin chains. It was concluded that there was a requirement for an increased amount of bait (500 μl of Znf-UBP beads was trialled) to that needed when the heat step was included, in order to more completely capture endogenous unanchored polyubiquitin chains from the 40 mg of cellular proteins used.

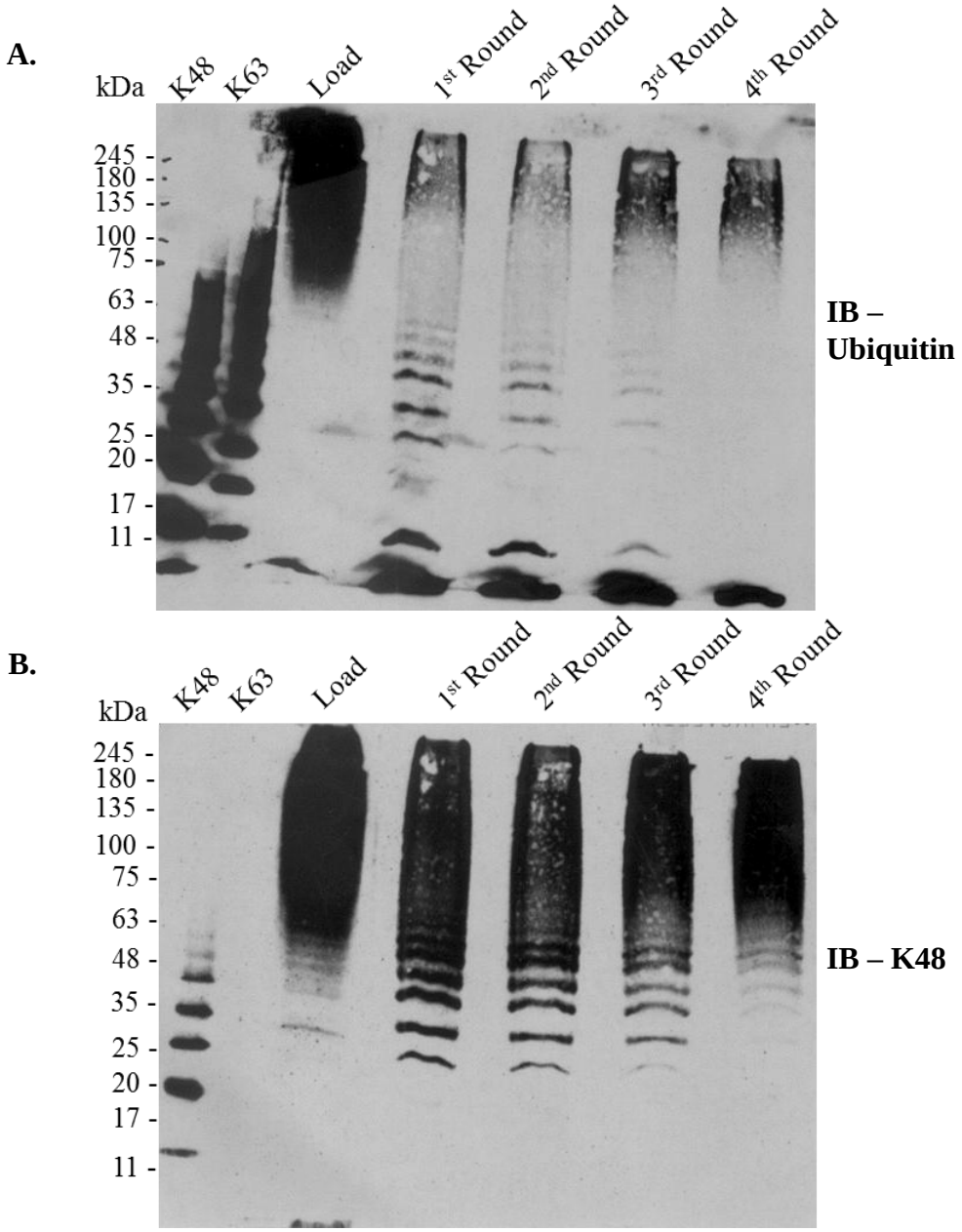
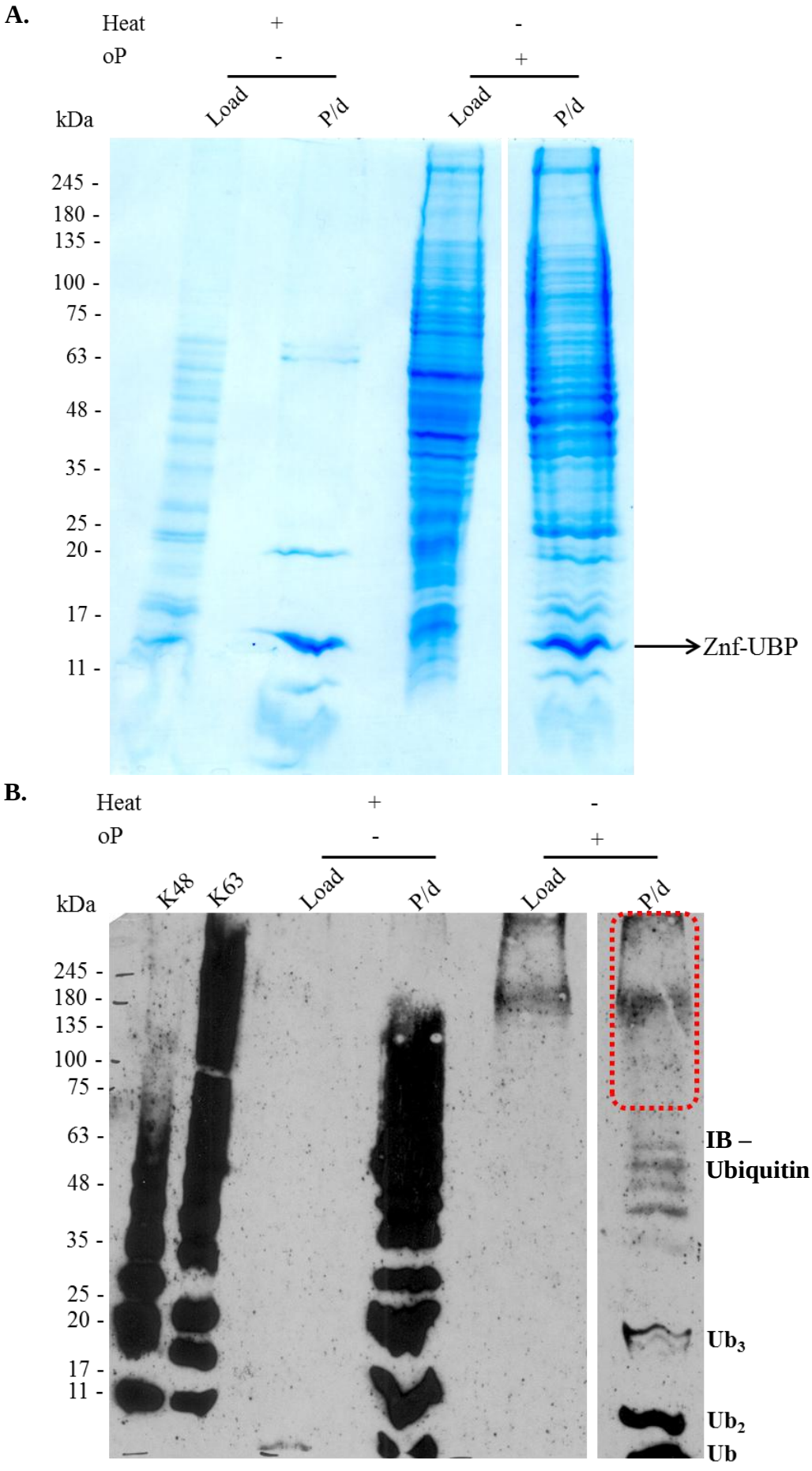


Figure 3.7. Determination of the amount of bait required for capture of unanchored polyubiquitin chains from HEK293T cells without sample heating. Cells were lysed in homogenising buffer supplemented with 1 mM oP. 40 mg of protein (estimated by BCA assay) was subject to four successive rounds of incubation with 100 µl Sepharose tagged Znf-UBP, leading to the gradual reduction in recovery of unanchored chains. 25% of sample from each round was eluted in GAB and used to produce four western blots which were probed with antibodies specific for **A.** ubiquitin, **B.** K48-linked polyubiquitin. In addition, K48 and K63-linked commercial polyubiquitin ladders (100 ng each in **A** and 250 ng in **B**) were run in adjacent lanes and used as positive control. 25 µl of load (input) mixed with 20 µl of GAB was also run. As evident, since the purified sample from the fourth round still contained unanchored polyubiquitin chains, a volume of 500 µl Znf-UBP tagged CNBr Seph was used for subsequent purification from 40 mg of starting material, representing a bead: protein ratio of 12.5:1.

A direct comparison of affinity-purified samples obtained from both methods (without and with sample heating) was then made and is presented in Figure 3.8. Samples purified using the two conditions (+heat/–oP and – heat/+oP) and eluted from 50 µl and 16.5 µl of beads (from a total volume of 100 µl sample on beads) were separated using SDS PAGE and visualised by Coomassie staining or by anti-ubiquitin western blotting, respectively. As apparent from the Coomassie-stained gel, there was a more complex banding pattern in the sample purified -heat/+oP. Specifically, the anti-ubiquitin antibody failed to consistently detect discrete ubiquitin-reactive bands in the –heat/+oP sample and on several occasions resulted in a smear of high molecular weight immunoreactivity (also evident with anti K48-polyubiquitin antibodies). Given their high molecular weight, we presumed these ubiquitin-reactive species may in fact represent conjugated polyubiquitin that was for some reason being captured from HEK293T cells by the Znf-UBP bait in the absence of sample heating.


A.
Heat
+
-
oP
-
+
Load
P/d
Load
P/d
kDa
245 -
180 -
135 -
100 -
75 -
63 -
48 -
35 -
25 -
20 -
17 -
11 -
Znf-UBP
B.
Heat
+
-
oP
-
+
K48
K63
Load
P/d
Load
P/d
kDa
245 -
180 -
135 -
100 -
75 -
63 -
48 -
35 -
25 -
20 -
17 -
11 -
IB – Ubiquitin
Ub3
Ub2
Ub

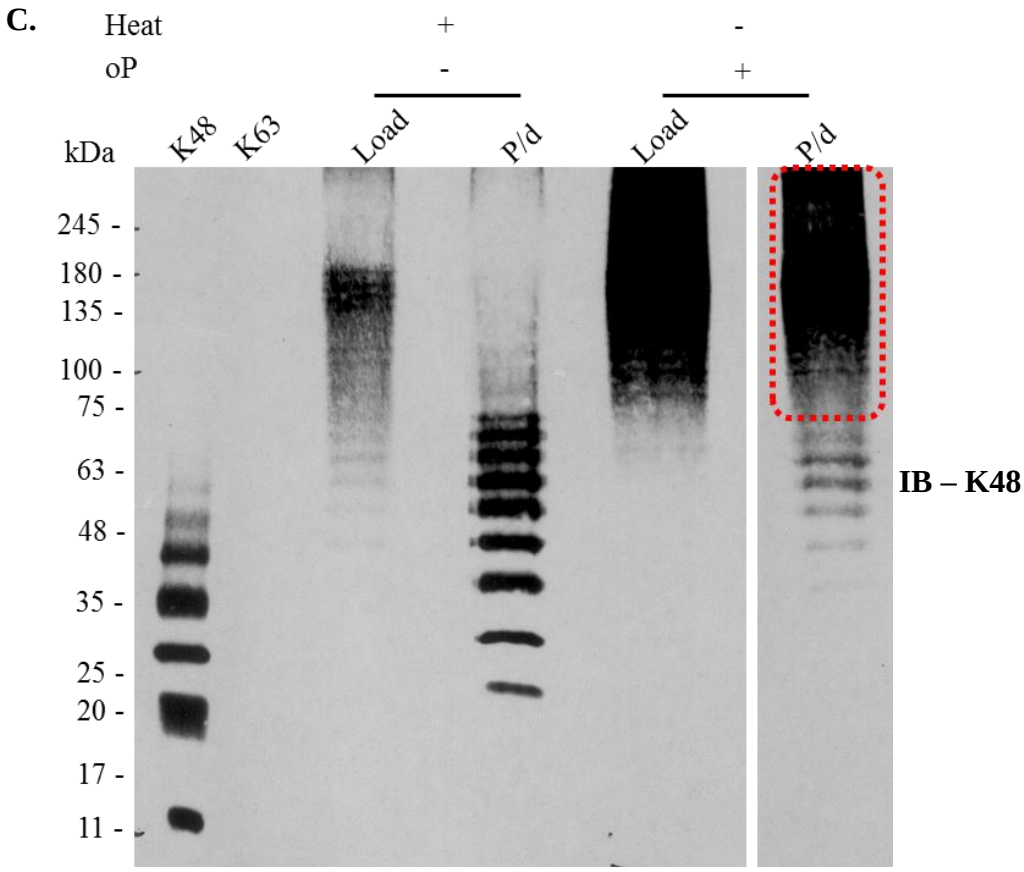


Figure 3.8. Direct comparison between Znf-UBP captures using two different sample preparation condition, the inclusion or omission of heat treatment (+Ht or -Ht) and use of 1,10-o-phenanthroline (+oP or -oP). HEK293T cells were lysed in homogenising buffer and subjected to heat treatment (+Ht/-oP) or were lysed in buffer supplemented with 1 mM oP and were processed without heat treatment (-Ht/+oP). 100 µl of affinity-purified sample was derived from 16 mg and 8 mg of HEK293T protein processed under +Ht/-oP and –Ht/+oP conditions, respectively. 50% of the material was eluted in GAB and separated using SDS PAGE and visualised using Coomassie blue (**A**). 16.5% each of sample was also subject to western blotting and probed with antibodies specific to ubiquitin (**B**) and K48-linked polyubiquitin (**C**). Lanes containing affinity purified samples were labelled P/d (for Pull-down). The amount of captured unanchored polyubiquitin was greater in the +heat/-oP sample; the unheated sample also showed evidence of a high molecular weight immunoreactive smear (indicated in red) presumed to represent conjugated polyubiquitin. Also evident was the marked increase of immunoreactive species in the load of sample not subjected to heat treatment compared to one that was heated.

3.5.2. NATURE OF THE UBIQUITIN-REACTIVE SPECIES IN THE ZNF-UBP-PURIFIED FRACTION GENERATED IN THE ABSENCE OF HEK293T SAMPLE HEATING

A deubiquitination assay (as described in Section 3.3) was performed on fractions purified from HEK293T cells in the absence of sample heating (+oP) using IsoT and the catalytic core of USP2. As apparent from anti-ubiquitin and anti-K48-polyubiquitin western blots (Figure 3.9), the inability of IsoT treatment (but not USP2) to reduce the high molecular weight immunoreactivity in the Znf-UBP-purified samples implied they most likely included substrate linked polyubiquitin chains. Thus, all future purifications reverted to the inclusion of the original sample heating step.

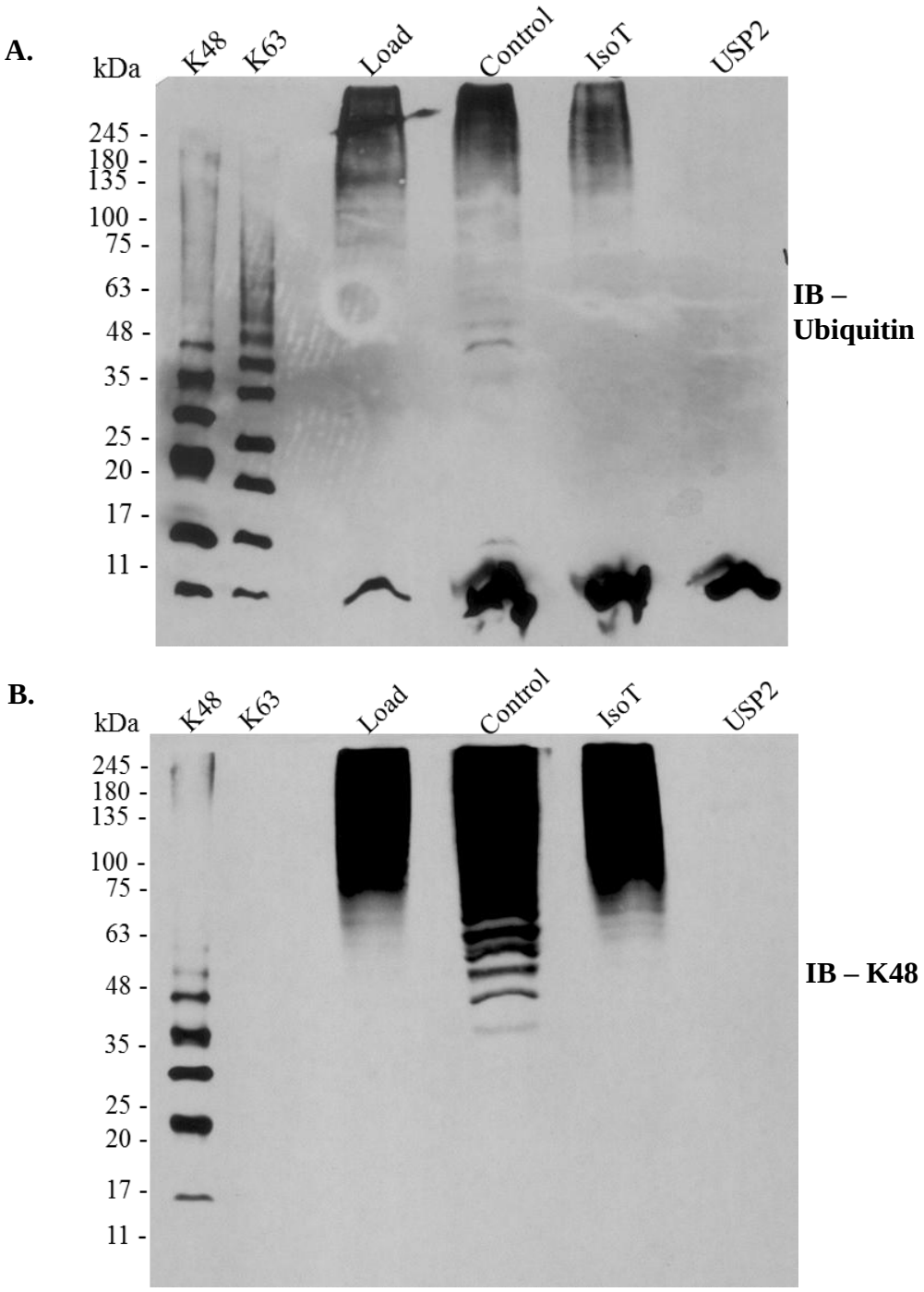


Figure 3.9. Deubiquitination assay of Znf-UBP affinity-purified samples from HEK293T cells obtained under condition of -heat/+oP. 50 µl of affinity-purified sample was either incubated with commercial long isoform of IsoT, catalytic core of USP2, or in deubiquitination assay buffer alone (control). The supernatants were discarded while the beads were eluted in GAB, split equally and were subject to immunoblotting with ubiquitin (**A**) and K48-linked polyubiquitin (**B**) antibodies. 25 µl of input with 20 µl of GAB was run in adjacent lane (load). Commercial K48 and K63-linked polyubiquitin ladders were used as positive control (100 ng for ubiquitin immunoblot and 250ng in case of K48-linked polyubiquitin immunoblot). Disappearance of ubiquitin reactive bands upon treatment with USP2 but not with IsoT confirmed the presence of substrate linked polyubiquitin chains in the purified sample.

CHAPTER 4

AVAILABLE MASS SPECTROMETERS WERE USED FOR COMPLEMENTARY ANALYSIS OF UBIQUITIN AQUA "SIGNATURE" STANDARDS

4.1. MALDI-ToF WAS LIMITED IN ITS APPLICATION TO DETECT AND MEASURE AQUA STANDARDS

The LaserToF TT instrument employs MALDI based ionisation of proteins or peptides coupled to a ToF analyser. The instrument was found to be useful in accurate mass determination of large peptides (or proteins) and peptide mass fingerprinting of either pure protein samples or a simple mix of proteins (post-trypsinolysis). However, the occurrence of ion suppression by competitive ionisation is a major drawback when attempting to use the instrument for analysis of complex mixture of proteins in sample [193]. The shortcoming of MALDI LaserToF became apparent when a mixture of AQUA standard peptides subjected to analysis did not yield reliable data. That said, the minimal requirement of sample and relative ease of sample preparation and analysis meant that the instrument could be used to confirm efficient trypsinolysis (of ubiquitin or polyubiquitin chains) by checking for the presence of (undigested) ubiquitin and polyubiquitin.

4.2. MULTIPLE CHARGED STATES OF AQUA STANDARDS WERE OBSERVED USING ESI QToF

The QToF2 equipped with a nanospray ion source was a very sensitive instrument for accurate qualitative analysis of peptides in complex mixtures. Until the purchase of QqQ instrument, the Waters QToF2 offered a platform for initial experiments to check for ionisation of AQUA signature standards. In addition to noting the ionisation of standards, we also tested the possibility of

carrying out quantitative (or semi-quantitative) proteomics using the instrument. Mixtures containing equal amounts of all seven ubiquitin (isopeptide) linkage specific AQUA standards, ranging from 0.3, 0.625, 1.25, 2.5, 5 and 10 ng (in 20 µl of 0.1% formic acid in HPLC gradient grade H_2O) were subjected to LC-MS to measure precursor ions. Data obtained confirmed the presence of multiple charged (M^{2+} and M^{3+}) states of all AQUA standards except that corresponding to K29 linkage. The lack of detection of the K29 AQUA standard was due to its inability to adsorb to the HPLC column as a result of its small size and hydrophilicity, an observation previously encountered [193]. Raw data was analysed and a standard curve was generated by plotting total ion current or TIC (value proportional to abundance) of each charged state of the standards (as seen in extracted ion chromatogram) against amount present in sample (Figure 4.1). The data obtained was then fitted to a linear equation (y=slope*x).

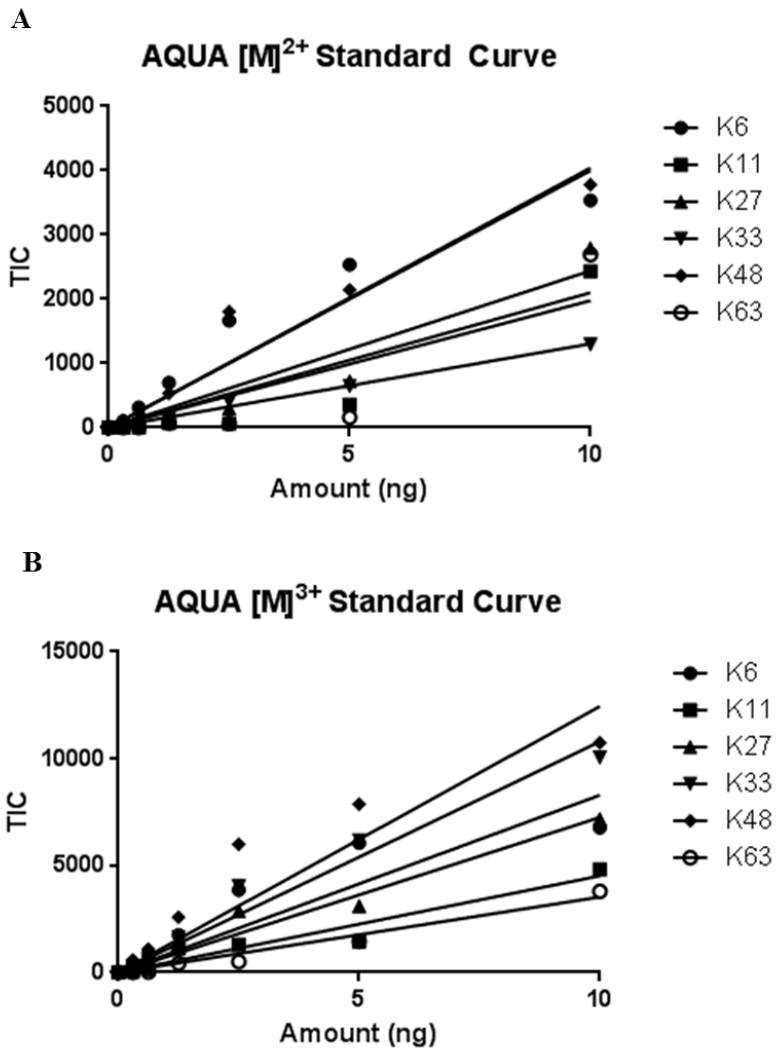


Figure 4.1. Data demonstrating the assessment of QToF platform for precursor ion Total Ion Count (TIC) of synthetic AQUA standards. Samples containing mixtures of 7 AQUA ubiquitin linkage-specific standards were analysed by LC-MS using the Waters QToF2. Both the lower limit of detection and data linearity were noted to be inferior to that necessary for quantitative analysis.

The standard curves plotted in Figure 4.1 does confirm the inability of the QToF for quantitation of ubiquitin isopeptide linkage signatures due to lower linear dynamic range offered by LC-MS analysis, a result of detector saturation [194]. Also, LC-MS analysis lacks structural (sequence) information, hence selectivity.

4.3. LC-MS/MS ANALYSIS OF *IN GEL* DIGESTS OF AFFINITY PURIFIED ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS FROM HEK293T CELLS FAILED TO DETECT LINKAGE-SPECIFIC PEPTIDES

The QToF2 platform was routinely used by our group to carry out tandem MS/MS, followed by submitting data generated for analysis on Mascot database (Matrix Science). However, we were not certain if the process could be used to detect isopeptide linkages in trypsinolysed affinity purified samples of unanchored polyubiquitin chains. An experiment was thus conducted wherein 75 µl of unanchored polyubiquitin chains (tagged to Znf-UBP beads) were purified from HEK293T cell extracts obtained using two purification protocols that differed in the use of heat treatment (at 75^{o}C for 15 minutes) during sample preparation. As previously detailed in Section 3.5, endogenous unanchored polyubiquitin chains were purified from either 6 mg of HEK293T extract which was not subjected to heating (-heat/+oP) or 12 mg of extract post heat treatment (+heat/–oP). Purified material (75 µl on Znf-UBP beads) from both conditions was eluted into GAB, separated using SDS PAGE and visualised with Coomassie stain (Figure 4.2).

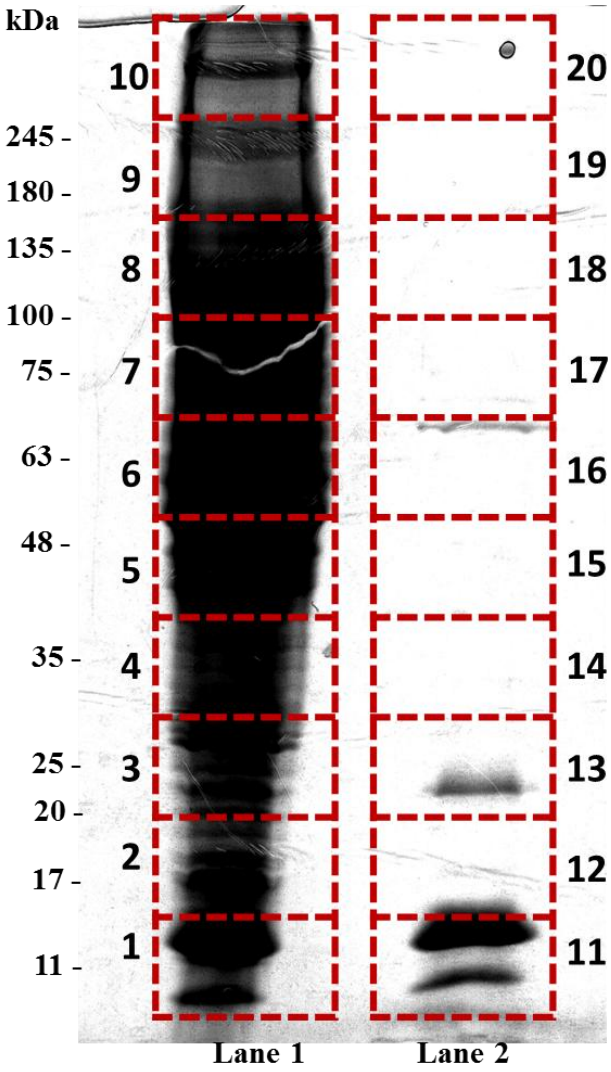
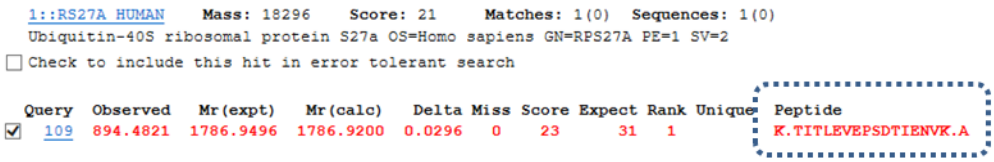


Figure 4.2. Affinity purified unanchored polyubiquitin from HEK293T cells obtained from two conditions was visualised by Coomassie staining. 75 µl of affinity purified unanchored polyubiquitin tagged to beads obtained from HEK293T cells processed under two conditions – without heating (Lane 1) and with heat treatment (Lane 2). As noted in Chapter 3, sample purified from extracts which were not heat treated contained far more protein than samples purified from extracts subjected to heat treatment. Both lanes in gel were cut into 10 pieces (per lane) and subject to *in gel* trypsinolysis for analysis by LC-MS/MS.

The two lanes of the gel were cut into 10 pieces as indicated in Figure 4.2. The gel pieces were separately trypsinolysed (as described in 2.2.5.2). 6.25% of digests derived from each band from both lanes, with the exception of bands 1 and 6 from Lane 1 and bands 11 and 16 from Lane 2, were pooled to provide

two samples. Both samples were submitted to LC-MS/MS analysis, and data was analysed on Mascot database. Unfortunately, not a single ubiquitin derived peptide was detected in heat treated sample (in Lane 2). The only ubiquitin derived peptide detected from the experiment was the TITLE peptide in sample from Lane 1 (Figure 4.3).


1::RS27A_HUMAN Mass: 18296 Score: 21 Matches: 1(0) Sequences: 1(0)
Ubiquitin-40S ribosomal protein S27a OS=Homo sapiens GN=RPS27A PE=1 SV=2
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
109	894.4821	1786.9496	1786.9200	0.0296	0	23	31	1		K.TITLEVEPSDTIENVK.A

Figure 4.3. Result query from a Mascot (Matrix Science) search confirming identity of a trypsinolytic peptide of ubiquitin. Mascot analysis of affinity purified unanchored polyubiquitin chains purified without heat treatment subjected to LC-MS/MS, confirmed the presence of TITLE fragment of ubiquitin (sequence highlighted). No other ubiquitin derived peptides were observed in the analysis.

Bands 1, 6, 11 and 16 contained abundant proteins, and were excluded from being pooled but were individually subjected to LC-MS/MS analysis, with ubiquitin related peptides not being detected in either. The experiment proved that analysis by LC-MS/MS on QToF followed by database analysis, was not an effective method for qualitative analysis of ubiquitin (isopeptide) linkages in affinity purified endogenous unanchored polyubiquitin chains. The data obtained could however be subjected to sequence analysis manually, which would be carried out in the near future.

4.4. LINEAR STANDARD CURVES WERE OBTAINED FOR AQUA STANDARDS USING TRIPLE QUADRUPOLE MASS SPECTROMETER

The triple quadrupole (QqQ) was the instrument most suited for quantitative MS. As noted earlier (1.4.1), the first and the third quadrupoles of the instrument act as mass filters for selecting the precursor ion and generating product ion spectras respectively, while the second contains the collision cell which produces product ions [181]. The SRM transitions thus generated for ubiquitin AQUA standards have previously been published [175] but could also be calculated and verified if required. The SRM transitions used for ubiquitin linkage quantitations have been tabulated in Table 4.1. SRM transition corresponding to K29 signature standard has not been tabulated due to the inability to detect the peptide. Also included in Table 4.1 are three ubiquitin derived (non-linkage) peptides EST, TLS and TITLE, whose sequences have been previously tabulated in Table 2.2.

	SRMs	
Peptide	**AQUA (Heavy)**	**Native (Light)**
[EST]$^{2+}$	537.8>644.4	534.3>637.4
[TLS]$^{2+}$	544.8>867.4	541.3>867.4
[K6]$^{2+}$	693.9>1014.6	690.4>1007.6
[OxMet-K6]$^{2+}$	701.9>1014.6	698.4>1007.6
[K11]$^{3+}$	803.8>1002.5	801.4>1002.5
[K27]$^{3+}$	703.0>947.5	701>944.0
[K33]$^{2+}$	822.4>746.4	819.4>740.4
[K48]$^{2+}$	734.3>621.3	730.9>617.8
[K63]$^{3+}$	751.7>1019.0	748.7>1015.7
[TITLE]$^{2+}$	897.5>1008.5	894.5>1002.5

Table 4.1. Selected reaction monitoring (SRM) transitions specific for ubiquitin AQUA (heavy) peptide standards and native (light) peptides. A similar study which used AQUA analysis to estimate relative abundance of cellular ubiquitin isopeptide linkages was consulted for selection of SRM transitions [175].

The instrument allows for multiple SRMs to be analysed in a single experiment, termed multiple reaction monitoring or MRM (both abbreviations being used interchangeably in literature). Using the SRMs tabulated (in Table 4.1), we subjected a range of samples containing serial dilution of AQUA standards (ranging from 0.8 ng to 10 ng per sample) to LC-MRM-MS/MS. Raw data obtained from one such experiment has been displayed in Figure 4.4.

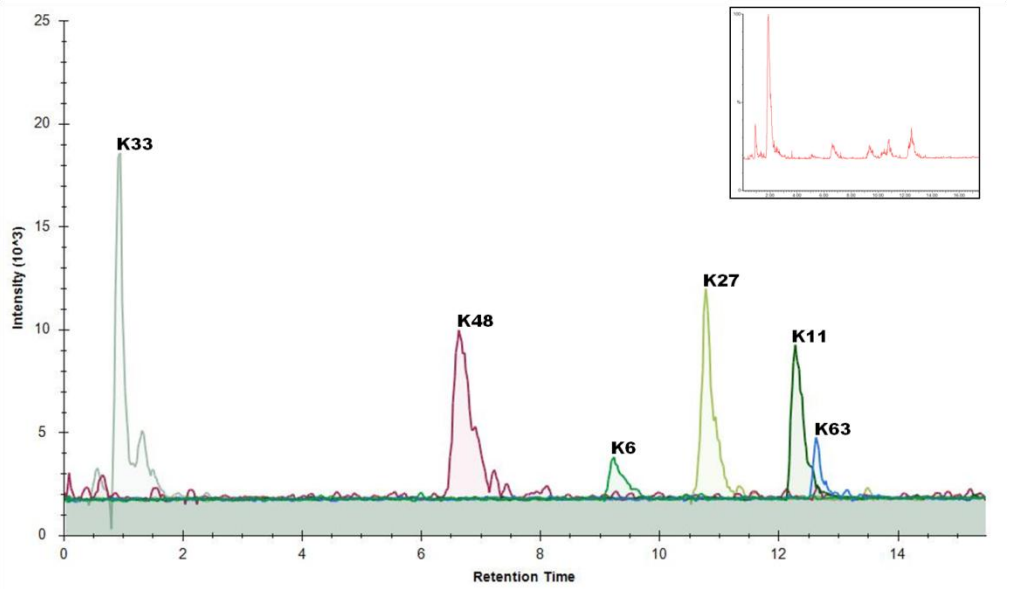


Figure 4.4. Extracted ion chromatogram obtained from processing sample containing 1 ng each of seven AQUA signature standards. Raw data obtained as Total ion chromatogram or TIC (inset) was processed to generate extracted ion chromatogram with peaks representing individual SRM transitions. The six peaks visible correspond to SRM transitions of six signature peptides with their abundance represented as intensity at respective retention times. Integrated peak areas were calculated per transition (signature peptide) and standard curve was generated. Note the absence of peak corresponding to K29 signature peptide due to its inability to adsorb to the HPLC column, a result of its small size and hydrophilicity.

Raw data from each LC-MRM-MS/MS experiment was obtained in the form of total ion chromatogram (TIC, described in further detail in Figure 5.12). As seen in Figure 4.4, extracted ion chromatogram of SRM transitions corresponding to AQUA signature standards were generated from TIC. This was done for every sample containing dilutions of the AQUA signature standard. An overlay of SRM transition peaks from extracted ion chromatograms generated for dilutions of K11 AQUA signature standard has been displayed in Figure 4.5.

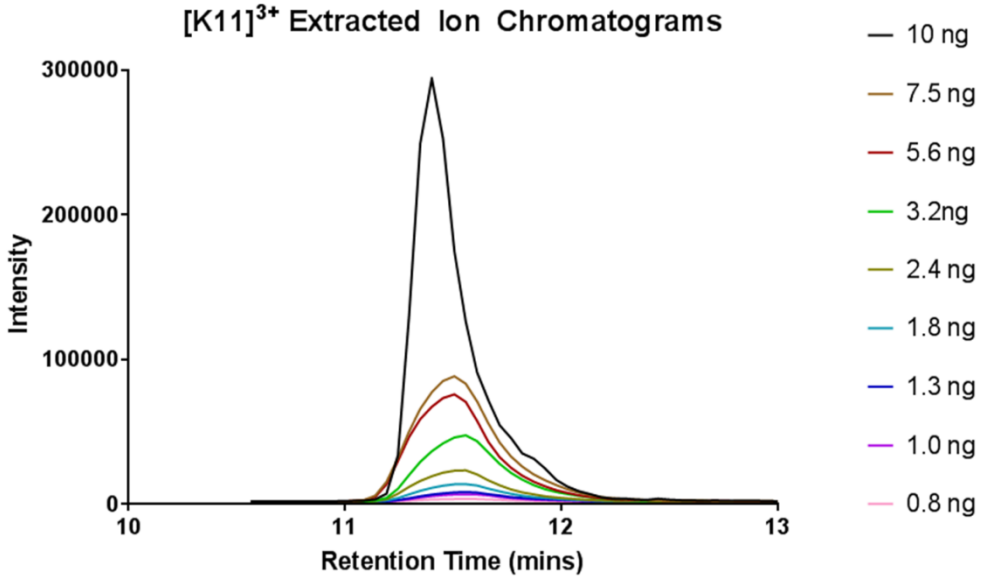


Figure 4.5. Compilation of SRM transition peaks generated by the overlay of extracted ion chromatograms, obtained by processing data from (multiple) samples containing serial dilutions of K11 AQUA standard.

LC-MRM-MS/MS data from each sample containing dilutions of AQUA standard peptides were processed to generate extracted ion chromatograms. Integrated peak area of SRM transitions for each AQUA standard were calculated (using Skyline software), and plotted against amount (ng) per sample to generate a standard curve in order to confirm linear dynamic range (Figure 4.6). In addition to standard curves corresponding to signature peptides of six isopeptide linkages (A), similar curves were generated for ubiquitin derived (non-linkage) peptides TLS and TITLE.

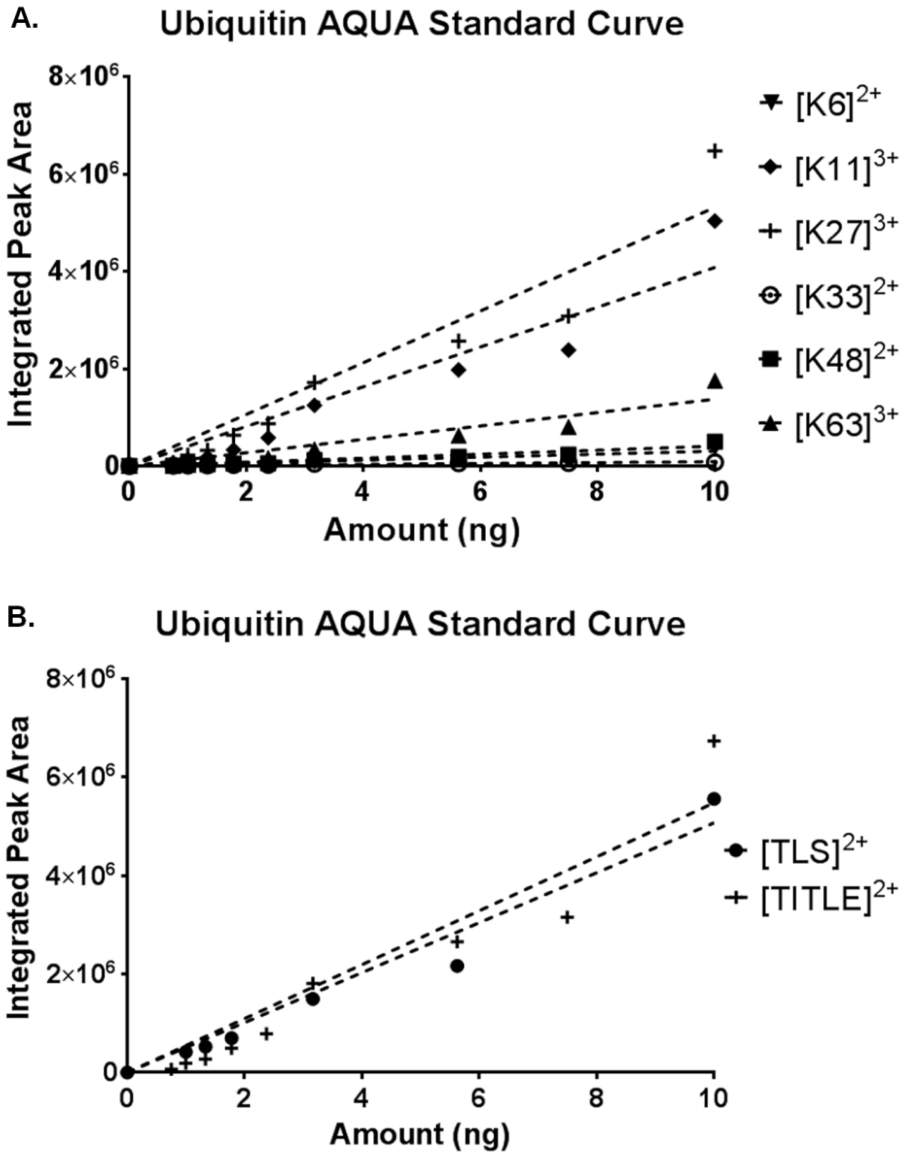


Figure 4.6. Linear dynamic range of QqQ instrument was confirmed at higher mass range (up to 10 ng) of AQUA signature standards. Standard curves were generated by plotting integrated peak areas per SRM transition against amount (in ng) per sample.

The confirmation of linear dynamic range at amounts up to 10 ng was an important prerequisite for quantitative analysis. However, we were also keen in testing the sensitivity of the instrument with respect to its lower limits of detection. Standard curves were also generated for serial dilutions of AQUA

signature standards with amount per sample ranging from 0.16 ng to 1.5 ng. As evident from Figure 4.7, AQUA signature standard curves confirmed detection and linear dynamic range at low mass range (<1 ng).

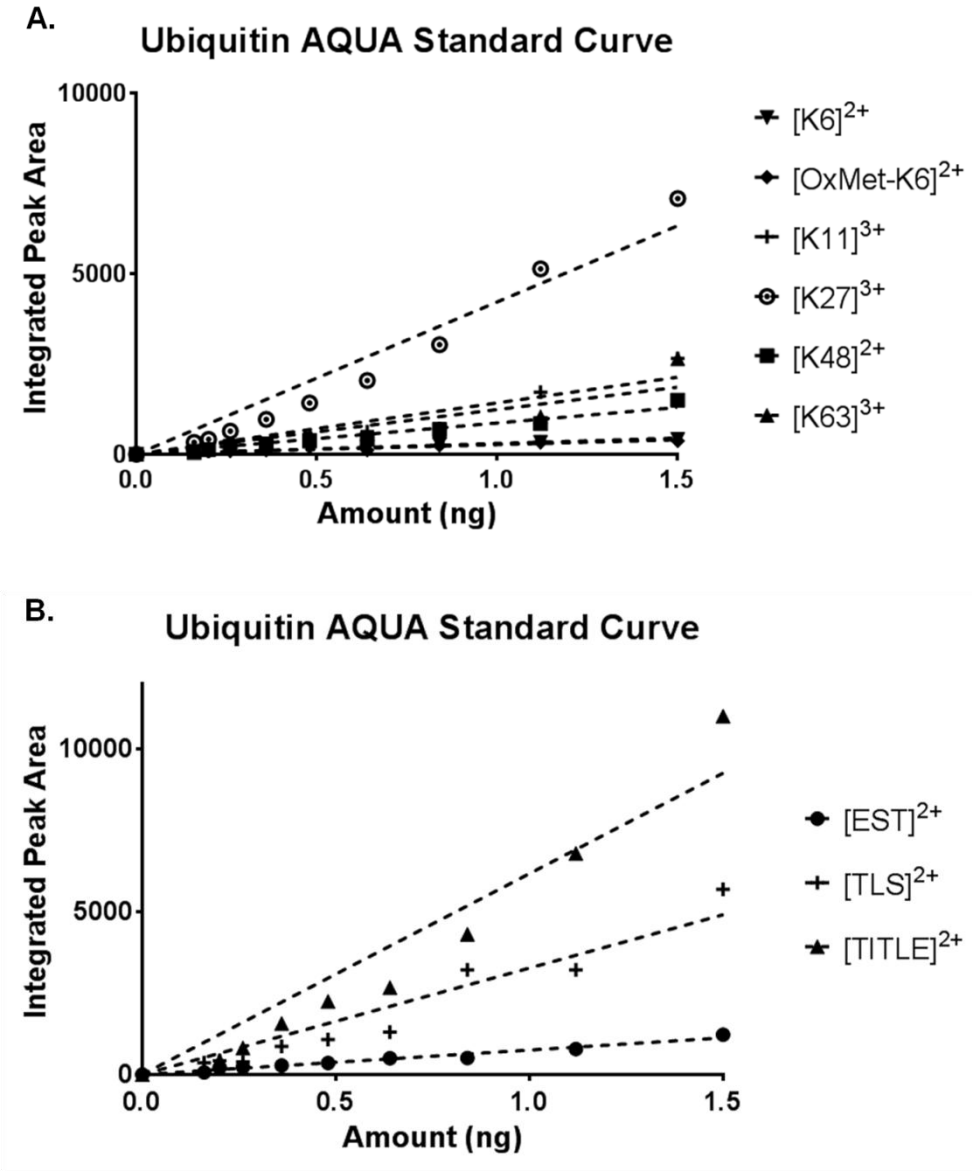


Figure 4.7. Linear dynamic range of QqQ instrument was confirmed at low mass range (<1 ng) of AQUA signature standards. Standard curves were generated by plotting integrated peak areas per SRM transition against amount (in ng) per sample. AQUA standards plotted display linear dynamic ranges and typically sub 0.2ng limits of detection.

CHAPTER 5

OPTIMISATION OF TRYPSINOLYSIS PROTOCOL TO ENSURE COMPLETE DIGESTION OF UNANCHORED POLYUBIQUITIN CHAINS AND RECOVERY OF SIGNATURE PEPTIDES FOR LC-MRM-MS/MS ANALYSIS

5.1. ASSESSMENT OF PREVIOUSLY ESTABLISHED PROTOCOL FOR IN-SOLUTION TRYPSINOLYSIS OF UBIQUITIN

Since our objective was to characterise the nature and relative amounts of isopeptide linkages present in affinity purified samples of endogenous unanchored polyubiquitin chains, we had to first ensure complete trypsinolysis of our samples. Hence, there was a need to verify if the protocol being used by our group could be directly applied to digest the samples of unanchored polyubiquitin chains generated. Optimisations were carried out using bovine (mono) ubiquitin in place of bovine serum albumin, (the latter) which was generally used as a substrate for standardising trypsinolysis protocols. The compact structure of ubiquitin by virtue of its relatively small size and extensive hydrogen bonding [13] was expected to offer some amount of resistance to (trypsin) enzymatic cleavage. Hence, the use of monoubiquitin as a substrate should closely match ubiquitin chains in terms of conditions which would be most effective for cleavage. Also, this was far more economical than using commercial polyubiquitin chains to test trypsinolysis efficiency initially.

Our first set of experiments in this regard was to test the already optimised (generic) protocol for *in solution* trypsinolysis of protein mixtures. As described in the Chapter 2, 1 M urea and 5% ($^{v}/_{v}$) acetonitrile (MeCN) in 100 mM ammonium bicarbonate buffer (pH 8.0) has routinely been used as digestion buffer. Since we were initially unaware of the efficiency (or thereby lack of) of the established protocol, trypsinolysis were carried out using

Promega sequencing grade trypsin. Also, reduction (with DTT) and alkylation (with CAA) steps were included in the protocol (described in 2.2.5) to break disulphide bridges and lock reduced cysteines in protein samples. However, since ubiquitin lacks cysteine residues, these steps were not required. Briefly, 4 µg of bovine ubiquitin was incubated with 4 µg or more of trypsin in the digestion buffer (Table 5.1). Since the digestion product was to be analysed exclusively by SDS PAGE, the reaction was carried out in microcentrifuge tubes (and not ultrafiltration devices, as mentioned in the protocol in the Methods), which were left to incubate at 37^{o}C overnight (16 hours).

Sample	Volume of digestion mixture	Amount of Trypsin	Amount of ubiquitin	Trypsin – Substrate ratio
1	100 µl	4 µg	4 µg	1:1
2	100 µl	5 µg	4 µg	1.25:1
3	100 µl	6 µg	4 µg	1.5:1
4	100 µl	7 µg	4 µg	1.75:1
5	100 µl	8 µg	4 µg	2:1
Control	100 µl	-	4 µg	-

Table 5.1. Details of setup used to test effect of increasing trypsin amounts on trypsinolysis efficiency of monoubiquitin. 4 µg of bovine monoubiquitin was incubated in 100 µl of digestion buffer containing 1 M urea and 5% ($^{v}/_{v}$) acetonitrile (MeCN) in 100 mM ammonium bicarbonate (pH 8.0). Varying amounts (4, 5, 6, 7 or 8 µg in samples 1, 2, 3, 4 or 5 respectively) of Promega sequencing grade trypsin were added to the digestion mixture which was incubated overnight at 37^{o}C. Control contained 4 µg monoubiquitin in buffer but lacked trypsin.

Post the incubation time, the reaction mixture was allowed to dry in a vacuum centrifuge, mixed with GAB and 25% from each sample (digestion product from 1µg ubiquitin) was separated using SDS PAGE. Samples on gel was then

visualised by Coomassie staining (Figure 5.1). Surprisingly, even the highest concentration of trypsin (2:1 ratio) failed to completely (or even substantially) digest monoubiquitin. This was surprising as the protocol had been routinely used to degrade protein mixtures.

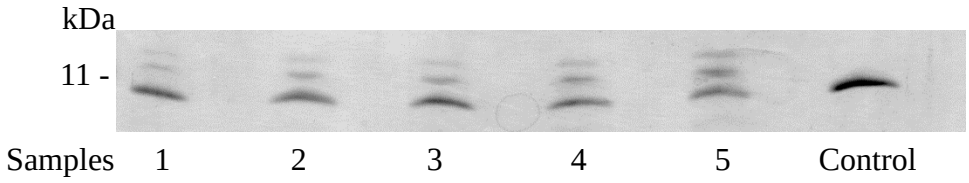


Figure 5.1. Efficiency of trypsinolysis was verified by visualising Coomassie stained gels containing trypsin digested monoubiquitin. Five samples of 4 µg of bovine monoubiquitin were incubated with between 4 – 8 µg Promega sequencing grade trypsin (as detailed in Table 5.1) in 100 µl digestion buffer. The digestion products were dried, treated with 50 µl GAB, subjected to SDS PAGE and visualised using Coomassie staining. As evident, the ubiquitin bands failed to disappear even after treatment with 8 µg trypsin (when compared to control sample). Samples displayed bands in addition to monoubiquitin, which presumably were the digestion products of small amounts of diubiquitin present in starting material.

One of the possible reasons for low trypsinolysis was thought to be the gradual loss of activity of trypsin and subsequent autocatalysis after a short period of incubation. To test this, the experiment was repeated however, this time making two separate additions of trypsin to our reaction mixture followed by 8 hour incubation times each (Table 5.2).

Sample	Volume of digestion mixture	Amount of Trypsin		Amount of ubiquitin	Trypsin – Substrate ratio
		First addition	Second addition		
1	100 µl	2 µg	2 µg	4 µg	1:1
2	100 µl	2 µg	3 µg	4 µg	1.25:1
3	100 µl	3 µg	3 µg	4 µg	1.5:1
Control	100 µl	-	-	4 µg	-

Table 5.2. Details of setup used to test effect of two separate additions of trypsin on trypsinolysis efficiency of monoubiquitin. 4 µg of bovine monoubiquitin was incubated in 100 µl of digestion buffer containing 1 M urea and 5% ($^{v}/_{v}$) acetonitrile (MeCN) in 100 mM ammonium bicarbonate (pH 8.0). two separate additions of Promega sequencing grade trypsin were made (as tabulated) after incubation time of 8 hours and the digestion mixture was incubated at 37^{o}C. Control used contained 4 µg monoubiquitin in buffer but lacked trypsin.

As with the previous experiment, samples in microcentrifuge tubes were dried and mixed with GAB. 25% of recovered reaction mixture was then subjected to SDS PAGE and visualised after Coomassie staining. Although the gel was damaged during the staining process, it was evident that ubiquitin in sample was not completely digested post two separate additions (Figure 5.2). This implied that increasing trypsin-substrate ratio and multiple additions of trypsin were not the sole requisites for complete trypsinolysis of ubiquitin. Hence, before we could proceed with the characterisation of purified endogenous unanchored polyubiquitin chains, there was a requirement to develop a protocol that would ensure complete trypsinolysis of the sample and efficient recovery of total signature peptides for analysis using MS.

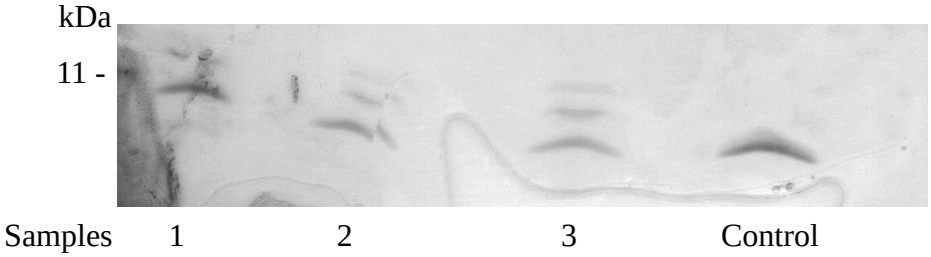


Figure 5.2. Efficiency of trypsinolysis was verified by visualising Coomassie stained gels containing trypsin digested monoubiquitin. Three samples of 4 µg of bovine monoubiquitin were incubated after two additions of Promega sequencing grade trypsin (as detailed in Table 5.2) in 100 µl digestion buffer. The digestion products were dried, treated with 50 µl GAB, subjected to SDS PAGE and visualised using Coomassie staining. The ubiquitin bands failed to disappear even after 2 rounds of trypsin addition (when compared to control sample). Results indicated that trypsin activity did not account for incomplete digestion. It notable that samples displayed bands in addition to monoubiquitin, which presumably were the digestion products of small amounts of diubiquitin present in starting material.

5.2. OPTIMISATION OF PROTOCOL FOR COMPLETE TRYPSINOLYSIS OF UNANCHORED POLYUBIQUITIN CHAINS

Since the use of trypsin (and no other) protease was essential for work, we set out to find the optimal conditions – buffer composition, salt (calcium chloride to prevent autolysis of trypsin [195]), presence of chaotropes (urea and Guan-HCl), acid-labile surfactant (RapiGest™) and organic solvents (MeCN) in combination with increasing trypsin concentration, some of which were previously detailed [196-198]. Initial experiments provided some information on the conditions which could be best for the purpose. A summary of the digestion conditions used is as follows.

- All digestions were carried out in microcentrifuge tubes containing 5 µg of bovine ubiquitin in 50 µl buffer used (0.1 µg/µl).
- Two buffers were made use of for the experiment – 50 mM Tris-HCl pH 7.6 or 200 mM AmBiC, pH 8.0 which were both supplemented with 10 mM $CaCl_2$.
- Since, the optimisation experiments would require the use of large amounts of trypsin, lower grade TPCK treated trypsin was preferred to the sequencing grade trypsin used in the previous section. Trypsin was suspended in the buffer used to produce three stocks of 1 µg/µl, 0.5 µg/µl and 0.1 µg/µl. Trypsin-substrate ratios of 1:1, 1:2 and 1:10 were used, and the use of 3 stocks ensured that the volume of the digestion mixture remained constant. Incubations time, when not mentioned was

16 hours (overnight). Also, the digestion mixtures were incubated at 37^{o}C.

- Guan-HCl was used by Hervey *et al* [196] at a concentration of 6 M (buffer used was 50 mM Tris-HCl pH 7.6) before being diluted to 1 M prior to trypsin addition. Briefly, 60 µg bovine ubiquitin was suspended in 100 µl of 6 M Guan-HCl in buffer. The sample was heated at 60^{o}C for 1 hour. Once brought back to room temperature, the sample was diluted 6-fold, thereby reducing the concentration of Guan-HCl to 1 M and that of ubiquitin to 0.1 µg/µl.
- Acid labile surfactant RapiGest™ was also used at a concentrations recommended by the manufacturer – 0.1% or 0.2% ($^{w}/_{v}$). Initial experiments showed that the surfactant worked best in the presence of Guan-HCl. 50 µl of 0.1 µg/µl bovine ubiquitin suspended in 1 M Guan-HCl, 50 mM Tris-HCl pH 7.6 was supplemented with either of the two concentrations of RapiGest™ and heated to 100^{o}C for 5 minutes. This was brought back to room temperature prior to addition of trypsin. It was important to note that the RapiGest™ was first resuspended in 50 mM Tris-HCl pH 7.6 to produce a stock of 20 µg/µl. Addition of RapiGest™ to the digestion mixture increased the volume to 60 µl (0.1% $^{w}/_{v}$) or 70 µl (0.2% $^{w}/_{v}$). This was taken into consideration while analysing extent of digestion.
- We also made use of organic solvent MeCN at concentrations of 40% and 80% (in Tris-HCl pH 7.6), the latter being their most efficient condition for the digestions carried out by Hervey *et al* [196]. The study also detected complete digestion in as little as 1 hour, which

prompted us to include a condition wherein trypsin digestion was carried out for 2 hours (instead of 1 hour).

- 1 M urea has previously been used for in-solution trypsinolysis, but a concentration of 4 M urea showed promise when dissolved in 200 mM AmBic pH 8.0. Also, 3 additions of trypsin (1:10 trypsin-substrate ratio) were made, followed by 4 hours of incubation, except the last addition which was left to incubate for 16 hours.

The conditions used for the digestions have been summarised in Table 5.3.

Sample	**Condition**	**Trypsin-substrate**	**Incubation**
1	Guan-HCl	01:10	16 hours
2	Guan-HCl	01:02	16 hours
3	Guan-HCl	01:01	16 hours
4	Guan-HCl + 0.1% RapiGest™	01:10	16 hours
5	Guan-HCl + 0.1% RapiGest™	01:02	16 hours
6	Guan-HCl + 0.1% RapiGest™	01:01	16 hours
7	Guan-HCl + 0.2% RapiGest™	01:10	16 hours
8	Guan-HCl + 0.2% RapiGest™	01:02	16 hours
9	Guan-HCl + 0.2% RapiGest™	01:01	16 hours
10	40% MeCN	01:10	16 hours
10A	40% MeCN	01:10	2 hours
11	40% MeCN	01:02	16 hours
11A	40% MeCN	01:02	2 hours
12	40% MeCN	01:01	16 hours
12A	40% MeCN	01:01	2 hours
13	80%MeCN	01:10	16 hours
13A	80%MeCN	01:10	2 hours
14	80%MeCN	01:02	16 hours
14A	80%MeCN	01:02	2 hours
15	80%MeCN	01:01	16 hours
15A	80%MeCN	01:01	2 hours
16	4M Urea in 200 mM AmBic	3 additions of 1:10	4 hours + 4 hours + 16 hours

Table 5.3. Details of setup used to test effect of different digestion conditions on trypsinolysis efficiency of monoubiquitin. 5 µg of bovine monoubiquitin was incubated in 50 µl of one of 16 digestion buffers. 0.5, 2.5 or 5 µg TPCK treated trypsin was added to mixture thus giving trypsin-substrate rations of 1:10, 1:2 and 1:1 respectively. Digestion mixtures were incubated at 37^{o}C for times indicated in table.

Post-incubation, each digestion was stopped by adding formic acid (1% $^{v}/_{v}$) except in the case of samples containing RapiGest™. The latter was treated with 0.5 µl TFA, incubated for 1 hour at 37^{o}C and centrifuged at 13,000 rpm for 10 minutes. The surfactant was broken down as a result and the supernatant was carefully isolated and used to check digestion efficiency. Efficiency of digestion was estimated by visualisation of disappearance of ubiquitin in Coomassie stained gels containing samples and by also subjecting samples to analysis by LC-MRM-MS/MS. 20% of each digest was set aside to be used for SDS PAGE analysis, while the rest was dried using a vacuum centrifuge and resuspended in 0.1% formic acid (in deionised H_2O) for analysis by MS.

20% of all samples (trypsinolysis product derived from 1 µg ubiquitin) were mixed with 25 µl GAB and separated using SDS PAGE and Coomassie stained (Figure 5.3). A control lane containing 1 µg bovine ubiquitin was also used for comparison. Complete digestion of ubiquitin (interpreted by disappearance of ubiquitin band) was observed in lanes containing samples 2 and 3 (both in Guan-HCl), 13, 13A, 14, 14A, 15, 15A (all containing 80% MeCN). In addition, substantial reduction in ubiquitin levels were observed with all samples except 16 (4M urea in AmBic), 10 and 10A (both in 40% MeCN).

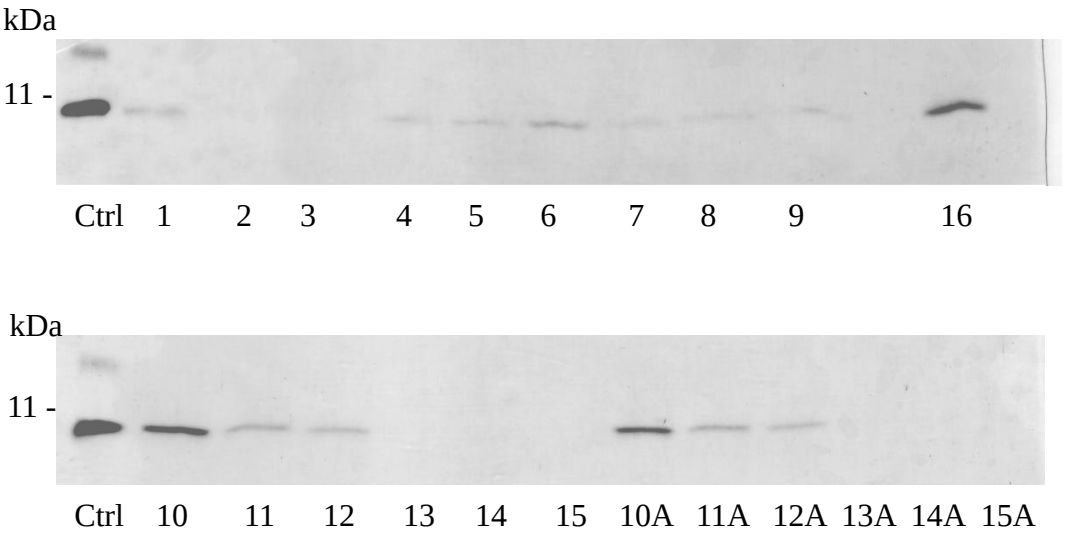


Figure 5.3. Efficiency of trypsinolysis was verified by visualising Coomassie stained gels containing trypsin digested monoubiquitin. 22 samples of 5 µg of bovine monoubiquitin were incubated in 50 µl of 22 trypsinolysis conditions with TPCK treated trypsin, as summarised in Table 5.3. Post digestion, 20% of digested products from all samples were mixed with 25 µl GAB and subjected to SDS PAGE. The gels were Coomassie stained to detect the ubiquitin band and compare digestion efficiency against control (containing 1 µg bovine monoubiquitin). Lanes containing samples 2, 3, 13, 13A, 14, 14A, 15, 15A appeared to show lack of ubiquitin thereby implying complete trypsinolysis.

Confirmation of digestion efficiency was also made using the LC-MRM-MS/MS to track the formation of ubiquitin digestion product. Three transitions of the most abundant charged states of ubiquitin derived peptides were selected. The advantage of using these specific transitions was that they correspond to a peptide released from ubiquitin N-terminal (TITLE), C-terminal (EST) and one which was internal (EGI). Hence, these transitions allowed maximum coverage for tracking effective digestion and release of peptides throughout the ubiquitin molecule (Table 5.4). All three transitions were monitored for each of the samples using LC-MRM-MS/MS.

	SRM transition	Sequence	Abbreviation
1	894.5 > 1002.4	TITLEVEPSDTIENVK	**TITLE**
2	534.3 > 636.4	ESTLHLVLR	**EST**
3	520.3>740.4	EGIPPDQQR	**EGI**

Table 5.4. SRM transitions used to detect 3 ubiquitin derived trypsinolysis products to test trypsinolysis efficiency using monoubiquitin. The three products were chosen as they were derived from the N-terminal (TITLE), C-terminal (EST) and internal peptide (EGI), thereby allowing for complete coverage of digestion of substrate. The abundance of three peptides per digestion mixture were detected using LC-MRM-MS/MS.

20% of digested material derived from each sample was subjected to MRM analysis for the three peptides mentioned. It is important to note that though Tris-HCl buffer is not recommended for use with MS, the reversed-phase chromatography desalts the sample introduced and elutes into a compatible mobile phase. Area of each of the three peaks (corresponding to the three SRM transitions) in the chromatogram (sample chromatogram displayed later in the chapter in Figure 5.12) of samples were measured, and levels for each transition was normalised with the sample producing the most abundant area for the corresponding transition (set to 100). Thus, the maximum score for a sample was set to 300, provided all three digestion products recovered were the most abundant. The abundance of digestion products thus calculated was plotted and displayed in Figure 5.4.

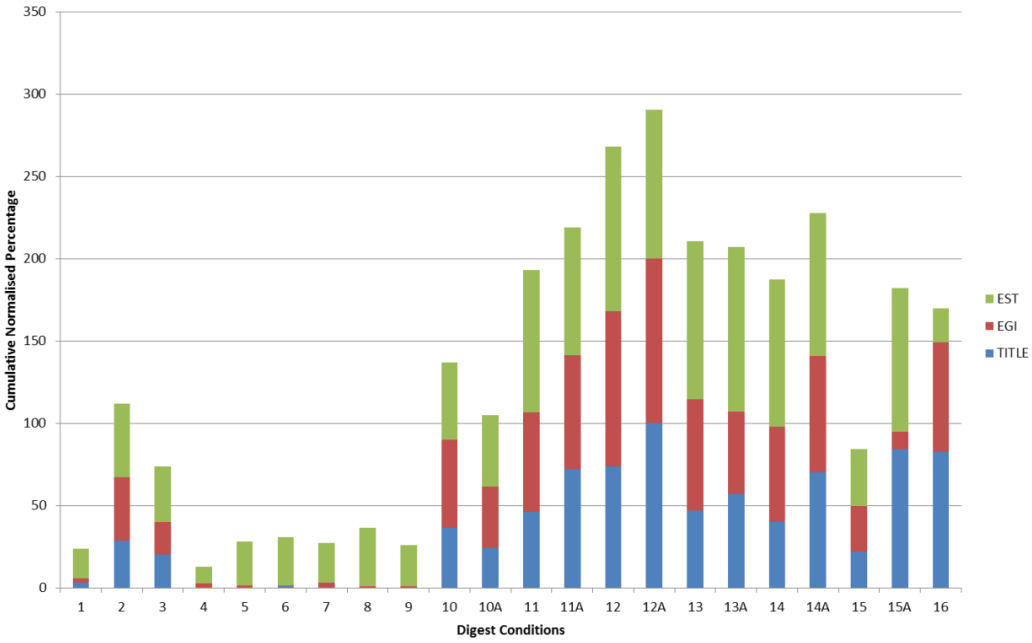


Figure 5.4. Trypsinolysis efficiency of monoubiquitin using 16 digest conditions displayed as cumulative normalised percentages of three ubiquitin derived peptides EST, EGI and TITLE. 5 µg bovine monoubiquitin were incubated with either 0.5, 2.5 or 5 µg TPCK treated trypsin overnight at 37^{o}C. 20% each of the digestion products were submitted to analysis using LC-MRM-MS/MS to quantify 3 ubiquitin derived peptides. Abundance of each of the three peptides were calculated by measuring signal derived from using corresponding SRMs. Signal from each peptide was normalised to sample showing highest signal for the peptide, which was set to 100. The values thus obtained for all three peptides per sample (or condition) were added and displayed as a cumulative signal with 300 being the highest possible signal per sample. As evident, condition 12A displayed maximum cumulative signal, thereby implying that it was the most efficient digest condition.

The results obtained from MRM analysis were surprising as this did not correlate entirely with what was observed upon visualising samples in Coomassie stained gels. This however highlighted the advantage of analysing data using two separate methods. For example, the 'disappearance' of ubiquitin in digest conditions containing 80% MeCN, as evident from Figure 5.3 could result partly from the precipitation of the protein.

Also notable was the low abundance of ubiquitin peptides in samples trypsinolysed in the presence of Guan-HCl, especially when supplemented with RapiGest™. The probable reasons for this have been discussed later (see Discussion), but low yield of peptides upon use of RapiGest™ although surprising, was not entirely disappointing. Use of the detergent would add to the number of steps involved in the trypsinolysis process, increase losses incurred and would not have been very economical considering the number of samples to be generated for analysis. Clearly the best performing condition for trypsinolysis was found to be 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$. What was surprising was the trend of maximum recovery of peptides upon shorter incubation time of 2 hours when compared to 16 hours.

Another important observation was the formation of visible precipitate (possibly calcium bicarbonate) upon addition of 10mM $CaCl_2$ into 200 mM AmBic. Since the use of 4 M urea previously had shown promise, and the fact that it was seen to digest ubiquitin to a substantial degree, we decided to test the condition against 40%MeCN, this time as a time-course experiment.

5.3. ACETONITRILE WAS NECESSARY FOR EFFICIENT AND COMPLETE TRYPSINOLYSIS OF BOVINE MONOUBIQUITIN

As seen in the previous section, use of 40% MeCN (in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$) appeared to be the best condition ensuring maximum recovery of monoubiquitin derived peptides following trypsinolysis. However, this was not obvious upon visual inspection of Coomassie stained gels containing the digested material. Hence, we wanted to confirm total digestion of ubiquitin and also the time it would take to do so. Also as previously stated, the presence of $CaCl_2$ in 200 mM AmBic pH 8.0, may explain the lower peptide yield seen while using the condition. The experiment was designed to ensure these questions were addressed. Briefly, 20 µg bovine ubiquitin was digested in 150 µl of either 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$ or 4M urea in 200 mM AmBic pH 8.0 (the latter lacking $CaCl_2$). 3 aliquots of 1:10 sequencing grade Promega trypsin (2 µg trypsin) were added followed by a 4 hour incubation period at 37^{o}C, except the last addition which was followed by a 16 hour (overnight) incubation – this was mainly done to provide a convenient time to stop trypsinolysis. Since the trypsin used was modified by reductive methylation, the treatment prevented autolysis and thereby retaining its activity over longer incubation times. 10% or 15 µl (corresponding to 2 µg ubiquitin derived product) was extracted post every incubation period (treated with 1% TFA to stop digestion) and used for MRM analysis. Thus we were left with a total of 6 samples to analyse, 3 each from

both conditions tested. Data was analysed as described in the previous section and has been displayed in Figure 5.5.

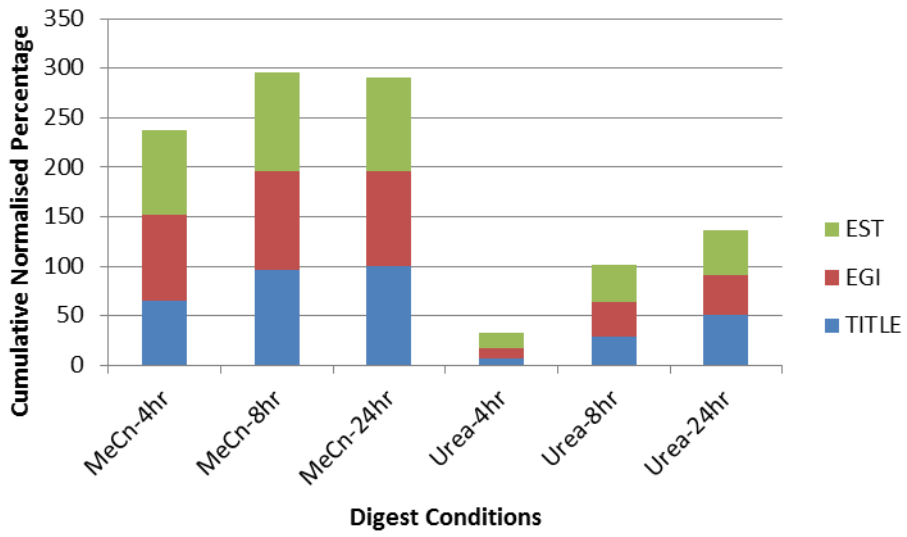


Figure 5.5. Direct comparison of trypsinolysis efficiency between time-courses of two digest conditions after multiple additions of trypsin. Digestion efficiency of monoubiquitin was tested using either 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$ (labelled MeCN) or 4M urea in 200 mM AmBic pH 8.0 (labelled Urea). 3 separate aliquots of trypsin were added and incubated for 4 hours (after first and second addition) and 16 hours (final incubation), and 10% (15 µl) of digested products were extracted after each incubation to track progress of trypsinolysis, by quantifying three ubiquitin derived peptides. Abundance of each of the three peptides were calculated by measuring signal derived from using corresponding SRMs. Signal from each peptide (EST, EGI and TITLE) was normalised to sample showing highest signal for the peptide, which was set to 100. The values thus obtained for all three peptides per sample (or condition) were added and displayed as a cumulative signal with 300 being the highest possible signal per sample. It was evident that digest condition containing 40% MeCN promoted efficient and possibly complete trypsinolysis when compared to that containing 4M urea. Since there was no increase in measured values of peptides after the third addition (when compared to the second), it could also be assumed that trypsinolysis was complete when incubated in 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$.

As evident from the results of the analysis, trypsinolysis of bovine monoubiquitin was much greater in the presence of organic solvent 40%MeCN than 4M urea. Also, trypsinolysis of ubiquitin appeared to near completion after only 8 hours of incubation in 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$ while in comparison trypsinolysis was not complete even after incubating for 24 hours in 4M urea in 200 mM AmBic, pH 8.0.

5.4. FURTHER INSIGHT INTO THE BUFFER THAT OFFERS GREATER EFFICIENCY OF DIGESTION

The results described in the previous sections clearly favoured the use of 40% MeCN in 50 mM Tris-HCl pH 7.6 with 10 mM $CaCl_2$. This was a surprising find also because it outperformed what was a routinely used condition to carry out trypsinolysis (1 M urea in 200 mM AmBic, pH 8.0). While it may not have been premature to assume that we had arrived on the most appropriate trypsinolysis condition, we were keen to investigate further the specific component responsible for such efficiency. The experiment described in this section was specifically designed to isolate the chief contributor towards complete trypsinolysis.

A total of 14 conditions were tested, which included the two buffers mentioned above, and 12 other conditions which were a derivative of the buffers. Table 5.5 summarises the conditions used along with information about the effect of particular components that directly impact trypsinolysis efficiency. Briefly, 5 µg of bovine ubiquitin was subject to trypsinolysis by making 3 additions of

0.5 µg each of Promega sequencing grade trypsin in a reaction volume of 100 µl. Each addition was followed by an incubation time of 4 hours, except the final addition which was followed by 16 hour incubation. The digestion products were subject to visual assessment using Coomassie stained gels and later subjected to LC-MRM-MS/MS analysis.

Sample	40% MeCN	4M Urea	Buffer	pH	10 mM $CaCl_2$	Changes
T1	Yes	No	50 mM Tris-HCl	7.6	Yes	Test Condition 1
C1	No	No	50 mM Tris-HCl	7.6	Yes	Aqueous only
C2	Yes	No	50 mM Tris-HCl	8.0	Yes	pH
C3	Yes	No	50 mM AmBic	7.6	Yes	Effect of Buffer
C4	Yes	No	200 mM Tris-HCl	7.6	Yes	High Salt
C5	Yes	No	50 mM Tris-HCl	7.6	No	No CaCl2
C6	No	No	200 mM AmBic	8.0	Yes	Aqueous only
T2	No	Yes	200 mM AmBic	8.0	Yes	Test Condition 2
C7	No	Yes	200 mM AmBic	7.6	Yes	pH
C8	No	Yes	200 mM Tris-HCl	8.0	Yes	Buffer
C9	No	Yes	50 mM AmBic	8.0	Yes	Low Salt
C10	No	Yes	200 mM AmBic	8.0	No	No CaCl2
C11	Yes	No	200 mM AmBic	8.0	Yes	Buffer from Test 2
C12	No	Yes	50 mM Tris-HCl	7.6	Yes	Buffer from Test 1

Table 5.5. Details of setup used to determine effect of each component of digest conditions on trypsinolysis efficiency of monoubiquitin. 5 µg of bovine monoubiquitin was incubated in 100 µl of one of 14 digest conditions with 0.5 µg sequencing grade Promega trypsin. Digestion mixtures were incubated overnight (16 hours) at 37^{o}C.

An equivalent of 1.5 µg of digestion product (30%) was run on SDS PAGE and Coomassie stained (Figure 5.6). As evident, along with T1 (which was expected), the conditions which appeared to lead to complete disappearance of ubiquitin were C8, C9 and C12, while samples C2, C4, C6 and C10 too seemed to degrade ubiquitin efficiently but were unable to drive complete trypsinolysis.

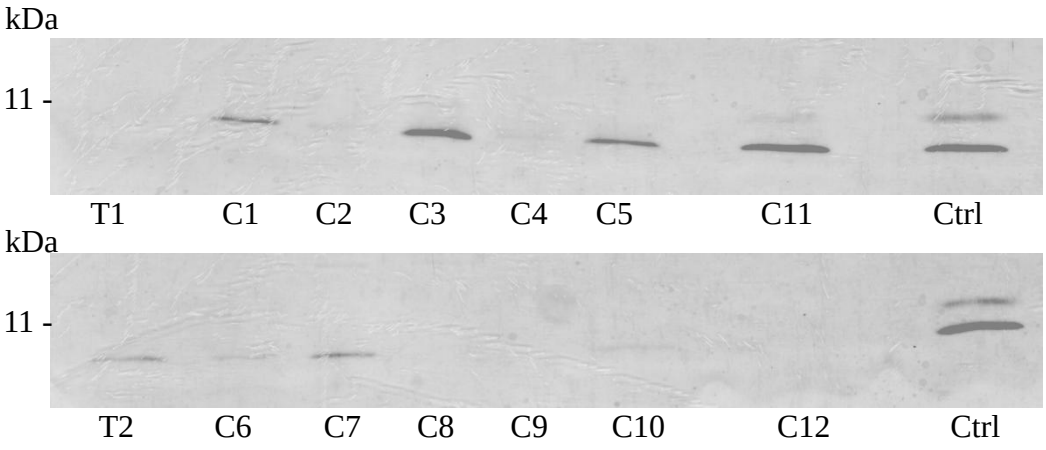


Figure 5.6. Coomassie stained gels were used to verify trypsinolysis efficiency by detecting disappearance of ubiquitin band. Post digestion, 30% of digested products from 14 conditions were mixed with 25 µl GAB and subjected to SDS PAGE. The gels were Coomassie stained to detect the ubiquitin band and compare digestion efficiency against control (containing 1.5 µg bovine monoubiquitin). Lanes containing samples T1, C8, C9 and C12 appeared to show lack of ubiquitin thereby implying complete trypsinolysis.

A further 30% of the digests were subjected to MRM analysis to measure ubiquitin derived peptides. Surprisingly, the most efficient condition thus detected was that containing 4M urea in 50 mM AmBic pH 8.0, 10 mM $CaCl_2$. As mentioned previously, addition of $CaCl_2$ was seen to lead to visible precipitate when used with 200 mM AmBic, but the reduction in salt levels appeared to have minimised this effect. While previously established test condition T1 did fare well, and appeared to produce the maximum yield of TITLE peptide, maximum yields of EST and EGI were produced upon digestion using C9.

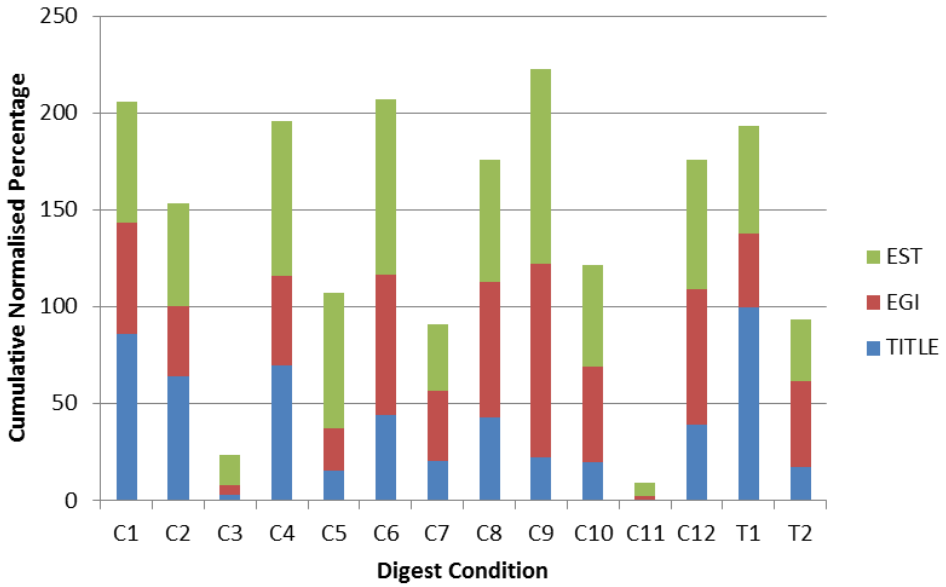


Figure 5.7. Trypsinolysis efficiency of monoubiquitin in 14 digest conditions, displayed as cumulative normalised percentages of three ubiquitin derived peptides EST, EGI and TITLE. 5 µg bovine monoubiquitin were incubated with 0.5 µg sequencing grade Promega trypsin overnight at 37°C. 30% each of the digestion products were submitted to analysis using LC-MRM-MS/MS to quantify 3 ubiquitin derived peptides. Abundance of each of the three peptides were calculated by measuring signal derived from using corresponding SRMs. Signal from each peptide was normalised to sample showing highest signal for the peptide, which was set to 100. The values thus obtained for all three peptides per sample (or condition) were added and displayed as a cumulative signal with 300 being the highest possible signal per sample. Overall, ubiquitin appeared to efficiently trypsinolysed in condition C9. Also, while digest condition C9 promoted maximum recovery of peptides EST and EGI, condition T1 displayed maximum recovery of peptide TITLE.

While our initial assumption to proceed with trypsinolysis in 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$ may have been justified, the experiment described in this section proved that there may be a possibility of a more refined condition for the purpose. We discovered (possibly) improved trypsinolysis efficiency with a few conditions and hence decided to perform a final round of optimisations while comparing the conditions which worked best thus far. This was also recommended appropriate considering all optimisation experiments described thus far were carried out only once and the final round of optimisation would more robustly establish our findings. The experiment was identical to that described earlier in this section and involved the addition of 3 aliquots of 0.5 µg Promega sequencing grade trypsin to 5 µg bovine monoubiquitin while incubating in 100 µl of one of 7 buffers (described in Table 5.6) at 37^{o}C. The addition of trypsin was followed by incubation times of 4 hours except the last addition which was followed by 16 hour incubation.

Sample	40% MeCN	4M Urea	Buffer	pH	CaCl2	Changes
T1	Yes	No	50 mM Tris-HCl	7.6	Yes	Test 1
C1	No	No	50 mM Tris-HCl	7.6	Yes	Aqueous only
C6	No	No	200 mM AmBic	8	Yes	Aqueous only
C9	No	Yes	50 mM AmBic	8	Yes	Low Salt
C12	No	Yes	50 mM Tris-HCl	7.6	Yes	Buffer from Test 1
T3	80% MeCN	No	50 mM Tris-HCl	7.6	Yes	Increased MeCN
T4	No	No	50 mM AmBic	8	Yes	No urea, Low salt

Table 5.6. Details of setup used for final round of optimisation of trypsinolysis efficiency of monoubiquitin. 5 µg of bovine monoubiquitin was incubated in 100 µl of one of 14 digest conditions with 0.5 µg sequencing grade Promega trypsin. Digestion mixtures were incubated overnight (16 hours) at 37^{o}C.

30% of the digested product was subjected to MRM analysis, and the results generated have been described in Figure 5.8. T1 once again showed very high yields of the three ubiquitin derived peptides, in this case it proved to be the best digest condition. Aside from providing the highest cumulative abundance of all three peptides, it also produced the highest amount of TITLE and EGI peptides among the conditions tested. However, condition C9 though promising in earlier analysis, fared poorly. Even more surprising was the efficiency of trypsinolysis in C1, containing 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$. The condition was responsible in providing the highest yield of peptide EST. The trypsinolysis efficiency achieved by the use of C1 suggested that the use of Tris-HCl based buffer might be the way forward. The optimised

trypsinolysis condition to use for all future experiments was confirmed to be 40% MeCN in 50 mM Tris-HCl pH 7.6 with 10 mM $CaCl_2$. The condition consistently provided efficient trypsinolysis through the optimisations and did not involve multiple manipulations thereby minimising losses during trypsinolysis.

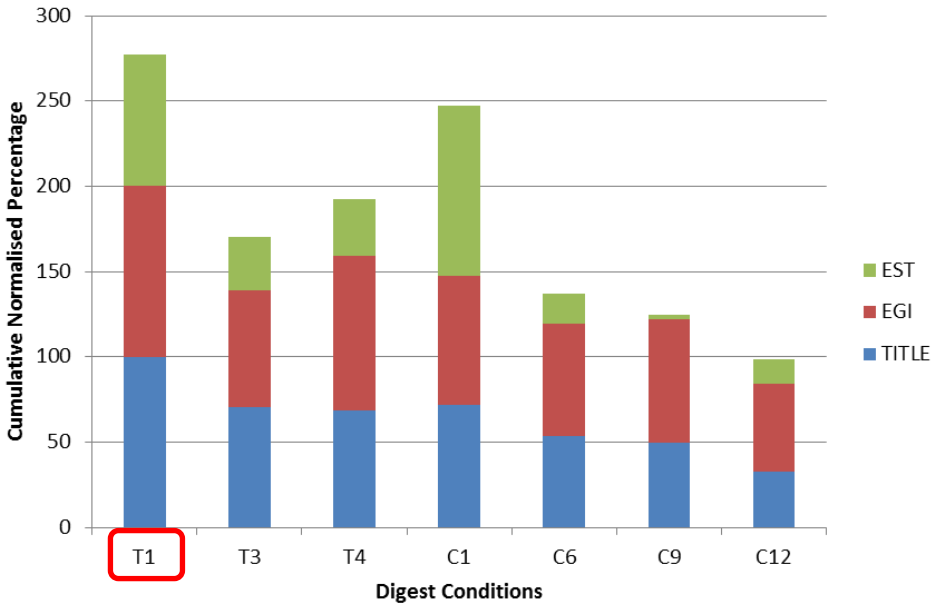


Figure 5.8. Trypsinolysis efficiency of monoubiquitin using 7 digest conditions, displayed as cumulative normalised percentages of three ubiquitin derived peptides EST, EGI and TITLE. 5 µg bovine monoubiquitin was incubated with 0.5 µg sequencing grade Promega trypsin overnight at 37°C. 30% each of the digestion products were submitted to analysis using LC-MRM-MS/MS to quantify 3 ubiquitin derived peptides. Abundance of each of the three peptides were calculated by measuring signal derived from using corresponding SRMs. Signal from each peptide was normalised to sample showing highest signal for the peptide, which was set to 100. The values thus obtained for all three peptides per sample (or condition) were added and displayed as a cumulative signal with 300 being the highest possible signal per sample. The experiment confirmed that T1 (highlighted) was the most efficient condition for complete trypsinolysis of monoubiquitin.

Although each of the experiments (with multiple trypsinolysis protocols) may lack replicates, it is however important to note that the developmental nature of the work meant that the best conditions were replicated as an extension of

experiments. Hence, the efficiency (of a protocol) was only confirmed after multiple digestions were carried out using the protocol.

5.5. VALIDATION OF OPTIMISED TRYPSINOLYSIS CONDITION BY USE IN DIGESTING COMMERCIAL POLYUBIQUITIN CHAINS

Extensive optimisations revealed 40% MeCN in 50 mM Tris-HCl pH 7.6 with 10 mM $CaCl_2$ to be the condition best suited for trypsinolysis of monoubiquitin. However, our characterisation of cellular unanchored polyubiquitin profiles would rely on the efficient trypsinolysis of purified polyubiquitin chains and the release of linkage specific signature peptides. Hence, we had to ascertain if our optimised condition was suited for the purpose. This was carried out by testing trypsinolysis efficiency of commercial polyubiquitin chains that were available. Commercial K48 and K63 polyubiquitin ladders (previously used as positive controls in immunoblots) along with K33 and K11 dimers were the substrates used for this experiment. 5 µg of each of the commercial ubiquitin substrates were incubated (at 37^{o}C) in 100 µl of trypsinolysis buffer containing 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$. As with previous optimisation experiments, 3 X 1 µl aliquots of 0.5 µg Promega sequencing grade trypsin were added and followed by 4, 8 and 16 hour incubations. An additional aliquot of trypsin was added to ensure that trypsinolysis was completed. After each round of incubation, 20% of the digestion mixture (equivalent to trypsinolysis product from 1 µg) was extracted and subjected to LC-MRM-MS/MS analysis of isopeptide linkage

specific signature peptides. The transitions which were selected for the purpose have been tabulated in Table 5.7.

	Linkage	SRM transition	Signature Peptide Sequence
1	**K11(Ub_2)**	801.4>1002.5	TLTG**K**^{GG}TITLEVEPSDTIENVK
2	**K33(Ub_2)**	819.4>740.4	IQD**K**^{GG}EGIPPDQQR
3	**K48 (Ub_n)**	730.9>617.8	LIFAG**K**^{GG}QLEDGR
4	**K63 (Ub_n)**	748.7>1015.7	TLSDYNIQ**K**^{GG}ESTLHLVLR

Table 5.7. SRM transitions used to detect signature peptides corresponding to 4 polyubiquitin linkages (K11, K33, K48 and K63), released upon trypsinolysis. The abundance of these signature peptides in digestion mixtures were detected using LC-MRM-MS/MS.

The levels of signature peptides collected at the end of each incubation were measured, normalised and plotted (Figure 5.9). It seemed evident that trypsinolysis was completed even prior to the final addition of trypsin. This was true with K11, K33 and K48 chains. On first inspection, K63 chains appeared to require 4 additions of trypsin for completion of trypsinolysis. However, considering the levels of signature peptide recovered after the second addition was nearly 100 (90%), it was likely that complete trypsinolysis was probably achieved after the third addition. Reduction in signature peptide recovery after final addition could be a consequence of depletion of their reservoir in the digestion mixture over the course of the experiment.

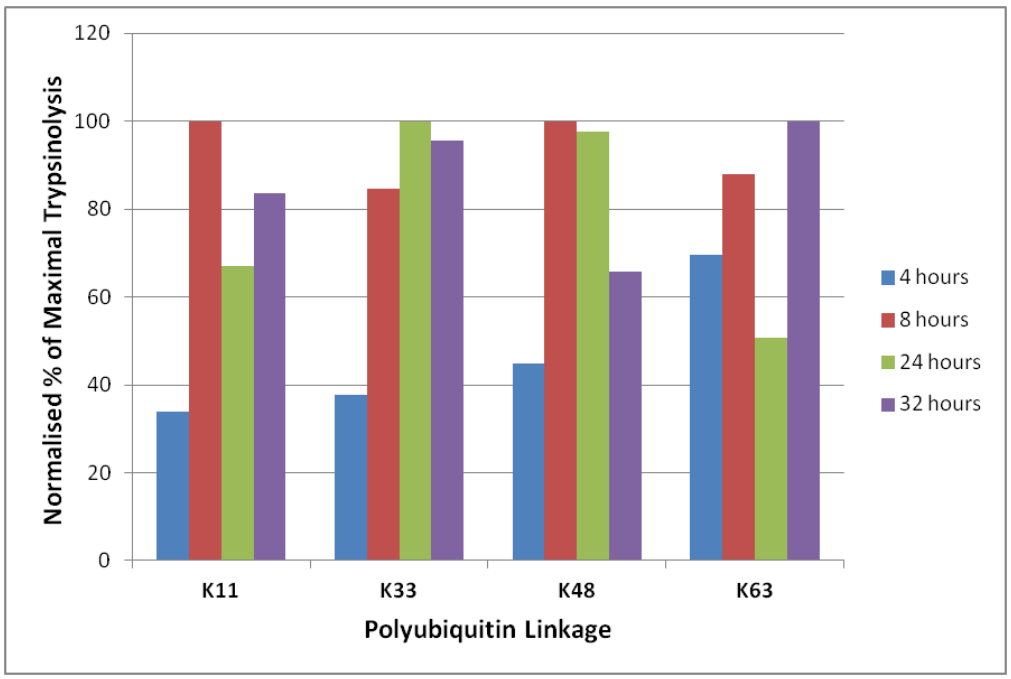


Figure 5.9. Optimised digest condition containing 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$, was shown to efficiently promote complete trypsinolysis and release of linkage specific signature peptides from 4 commercial polyubiquitin chains. 5 µg of either K48 or K63 (polyubiquitin chains) or K11 or K33 linked diubiquitin were digested using 4 additions of trypsin followed by incubations at 37^{o}C. All but K63 linked polyubiquitin chains appeared to be completely digested by the third round of trypsinolysis, evident by no increase in measured signal derived from signature peptides extracted after the fourth incubation. In case of K63 linked chains, 90% signature peptides were released after 2 rounds of trypsinolysis, implying complete digestion by possibly after the third round.

In addition to the MRM analysis, 1% of each of the digested product (after 4 rounds of trypsinolysis) was also subjected to Western blotting with ubiquitin specific antibody to exclude the presence of undigested commercial chains. As positive controls, 0.05 µg of each of the commercial standards were used (equivalent of 5% of starting material). The immunoblot has been displayed in Figure 5.10, as evident there were no obvious remnants of commercial chains (or ubiquitin positive trypsinolysis intermediates) visible in the blot.

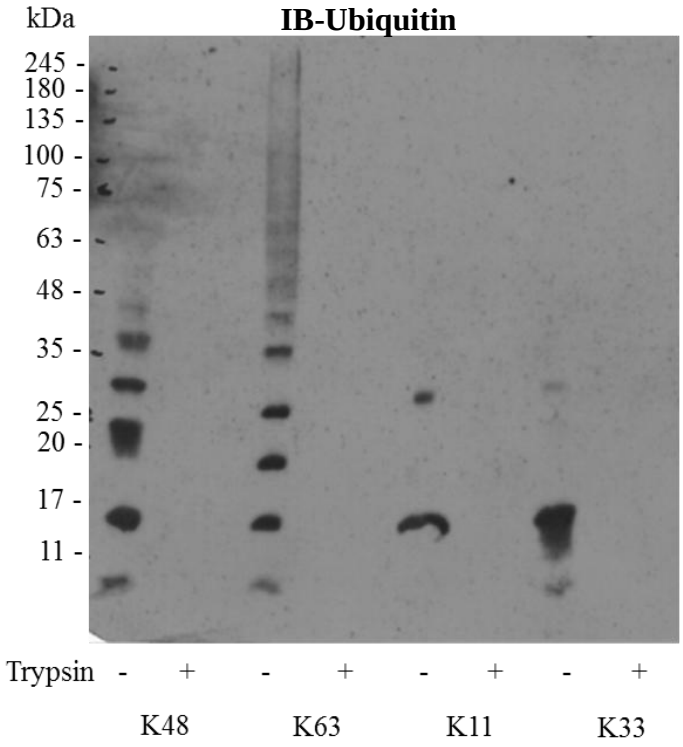


Figure 5.10. Western blotting confirmed complete digestion of commercial polyubiquitin (and diubiquitin) standards after 4 rounds of trypsinolysis. 5 µg of either K48 or K63 (polyubiquitin chains) or K11 or K33 linked diubiquitin were digested using 4 additions of trypsin followed by incubations at 37^{o}C. 1% of the digested material from each sample after 4 rounds of trypsinolysis were extracted, mixed with 25 µl GAB and subjected to western blotting with ubiquitin specific antibody. As positive controls, 50 ng (equivalent to 1% starting material) were run on adjacent lanes for comparison. Disappearance of ubiquitin positive bands post trypsinolysis suggested complete digestion.

The experiments described in the section thus far led us to believe that the trypsinolysis condition used ensured complete digestion of polyubiquitin chains. With respect to amount of trypsin and incubation times required, it was decided to make 3 additions of 1 µg Promega sequencing grade trypsin to our samples of purified endogenous unanchored polyubiquitin chains to ensure trypsin was not a limiting factor in digestion of affinity purified endogenous unanchored polyubiquitin. This was particularly useful as it was difficult to predict the amounts of unanchored polyubiquitin chains in each purified

sample. Also, an incubation time of 8 hours (at 37^{o}C) was used after each trypsin addition.

5.6. APPLICATION OF OPTIMISED TRYPSINOLYSIS PROTOCOL FOR COMPLETE DIGESTION OF PURIFIED ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS AND ANALYSIS USING MS

5.6.1. USE OF *IN HOUSE* PLATFORM ALLOWED FOR THE FIRST TIME ESTIMATION OF ABUNDANCE OF PURIFIED UNANCHORED POLYUBIQUITIN CHAINS FROM HEK293T CELLS

After extensive optimisations we had finally arrived upon a trypsinolysis protocol which was successfully applied to completely digest monoubiquitin and commercial ubiquitin ladders (K48 and K63 polyubiquitin chains and dimers of K11 and K33 linkages). The next step was to apply the protocol in our affinity purification pipeline and obtain an estimate of linkage composition in purified sample of endogenous unanchored polyubiquitin. 450 µl of affinity purified unanchored polyubiquitin chains (on Znf-UBP beads) were captured from 66 mg HEK293T cell extract protein. In order to eliminate remnants of detergent (NP-40) used during cell lysis, the affinity purified sample (on beads) was subject to washes with DUB buffer (Section 2.2.5). ~150 µl of sample on beads were set aside for further qualitative analysis in collaboration with the University of Leicester. Out of the remainder, ~50 µl was used to test the efficiency of (0.1%) formic-acid elution, and any losses incurred as a result. This was split into two halves and while 50% (25 µl sample on Znf-UBP beads) was eluted directly into GAB, 50% was first eluted into 250 µl of

0.1% formic acid (in deionised H_2O). As discussed in the Methods, centrifuge filters were used to separate the Znf-UBP tagged beads from the eluate. The latter was then dried using a vacuum centrifuge and then mixed with GAB. Both these fractions were separated using SDS PAGE and subject to western blotting using ubiquitin specific antibody. 100 µl each of commercial K48 and K63 linked chains were also run as positive control. Also, the separated beads (post elution) were mixed with GAB and analysed to test for remnant unanchored polyubiquitin chains. The blot thus obtained has been displayed in Figure 5.11. Remarkably, when compared with sample on beads, losses during formic acid elution, appeared minimal – mostly restricted to higher order chains. However, the beads showed little signs of ubiquitin positive bands, which could indicate nearly complete elution of material but loss of some amount of higher order unanchored polyubiquitin chains which may have adhered to the centrifuge tubes (surface adsorption).

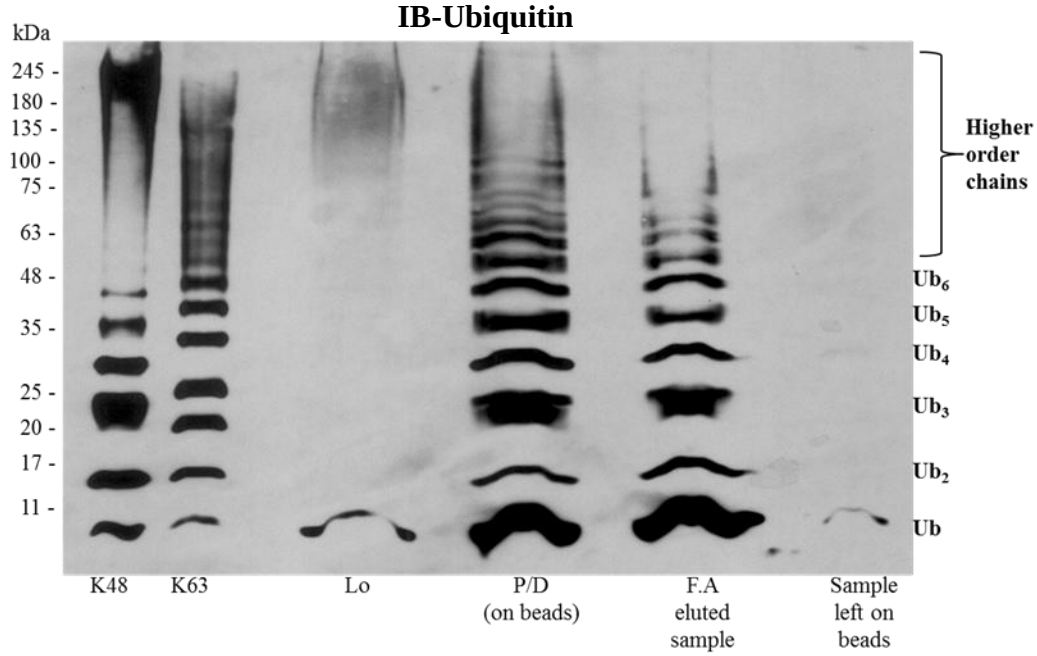


Figure 5.11. Western blotting confirmed efficient elution of purified endogenous unanchored polyubiquitin chains from Znf-UBP tagged sepharose, into 0.1% ($^{v}/_{v}$) formic acid (in H_2O). 50 µl purified cellular unanchored polyubiquitin sample was split into two, with half of it being eluted into GAB directly while the other half was first eluted into 250 µl 0.1% formic acid (in H_2O), dried and then mixed with GAB. Both samples were subjected to western blotting and labelled with ubiquitin specific antibody. 100 µg commercial K48 and K63 linked chains were used as positive control. Any remnants of purified unanchored polyubiquitin chains on immobilised beads were eluted into GAB and also used as sample for the western blot. Recovery of majority of purified unanchored polyubiquitin chains was confirmed being eluted into 0.1% formic acid, with minimal material left on beads after elution.

The remaining 250 µl of affinity purified sample was eluted in 2.5 ml of 0.1% formic acid (in deionised H_2O). This was dried (in vacuum centrifuge) and resuspended in 100 µl optimised trypsinolysis buffer (40% MeCN, 50mM Tris-HCl pH 7.6, 10 mM $CaCl_2$). The sample was subject to trypsin digestion by making 3 additions of 1 µg Promega sequencing grade trypsin, each addition followed by 8 hours incubation at 37^{o}C. An advantage of this technique is that post-elution, all additions to the sample are made while these remain in the same tubes (microcentrifuge tubes), thus minimising losses

occurring due to surface adsorption. The trypsin digested sample was first dried using a vacuum centrifuge and resuspended in 100 µl of 0.1% formic acid ($^{v}/_{v}$ in HPLC grade H_2O). However, to check reproducibility, two runs containing 25 % each of the digests were submitted for LC-MRM-MS/MS (2 repeats of the experiment). This was followed by a third run wherein the digests were diluted to 50%, to test if the estimate after analysis was also halved. Two AQUA standard dilutions containing a mixture of 0.5 ng or 2 ng each of all seven signature peptides and ubiquitin derived TLS and TITLE sequences were analysed to generate standard curves from which it was possible to estimate amounts of native signature peptides (corresponding to linkages) present in our digests. The SRM of each signature peptide used in the analysis has been tabulated in Table 4.1.

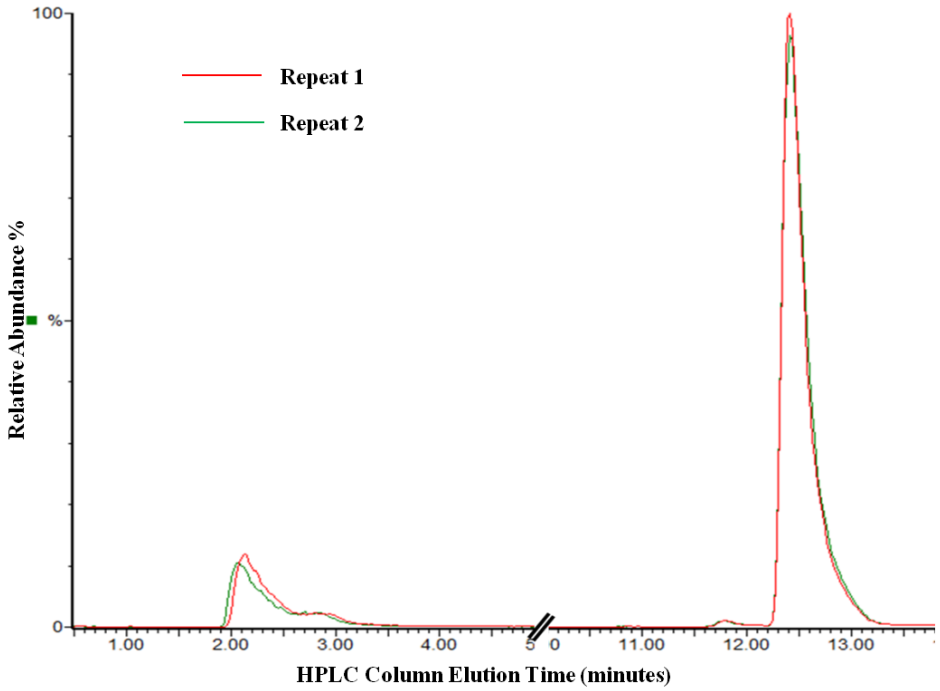


Figure 5.12. Reproducibility of LC-MRM-MS/MS experiments was initially confirmed by overlay of chromatograms produced by the submitting 2 repeats of the same sample of trypsinolysed endogenous unanchored polyubiquitin chains. The TIC indicates a combination of nine individual SRM transitions. Seven of these transitions correspond to seven isopeptide linkage derived signature peptides, with two SRM transitions corresponding to ubiquitin derived (non-linkage) TITLE (TITLEVEPSDTIENVK) and TLS (TLSDYNIQK) peptides.

The chromatograms obtained from the repeats have been displayed as an overlay in Figure 5.12. As evident, both chromatograms (obtained from repeats of the sample) appear to overlap completely. SRMs (for each signature peptide) obtained from both the chromatograms were calculated and concentration of each of the peptides were estimated by comparison with standard curves obtained for the experiment. These have been tabulated in Table 5.8.

	TLS (pmoles)	K11 (pmoles)	K27 (pmoles)	K48 (pmoles)	K63 (pmoles)	TITLE (pmoles)
25% Purified Sample Repeat 1	102.18	1.09	0.44	0.42	10.04	3408.14
25% Purified Sample Repeat 2	93.27	1.15	0.48	0.48	10.70	3499.94
12.5% Purified Sample	30.23	0.58	0.24	0.29	5.32	1795.50

Table 5.8. LC-MRM-MS/MS analysis of trypsinolysed endogenous unanchored polyubiquitin chains form HEK293T cells (at basal conditions) confirmed presence of K48, K63 and K11 linkages and allowed for the first time, quantification of the same. 250 µl affinity purified cellular polyubiquitin chains (from 40 mg HEK293T cell extract) were eluted and trypsinolysed and 25% of sample was subjected to LC-MRM-MS/MS using SRM transitions for 7 linkage derived signature peptides and 2 ubiquitin derived peptides. While we could not detect K6, K29 and K33 linkages, we could confirm the presence of K11, K27, K48 and K63 linkages. Surprisingly, K63 linkage contributed the majority isopeptide linkage in purified unanchored polyubiquitin fraction, with the proportions being K63>>K11>K48, K27. The reliability of such analysis was also corroborated; as amounts of peptides measured when 12.5% was submitted was nearly half of what was measured in 25% of the sample.

The experiments firstly proved the results obtained from the MRM analysis were indeed reproducible. Secondly, these experiments for the first time provided an estimate of the linkages that make up cellular unanchored polyubiquitome. To our surprise, the concentration of K48 (and K27) chains were the least abundant out of the linkages detected, while K63 chains were the most. This was even more surprising considering the challenges encountered in detecting K63 linked chains using western blotting. The relative abundance of isopeptide linkages present in cellular unanchored

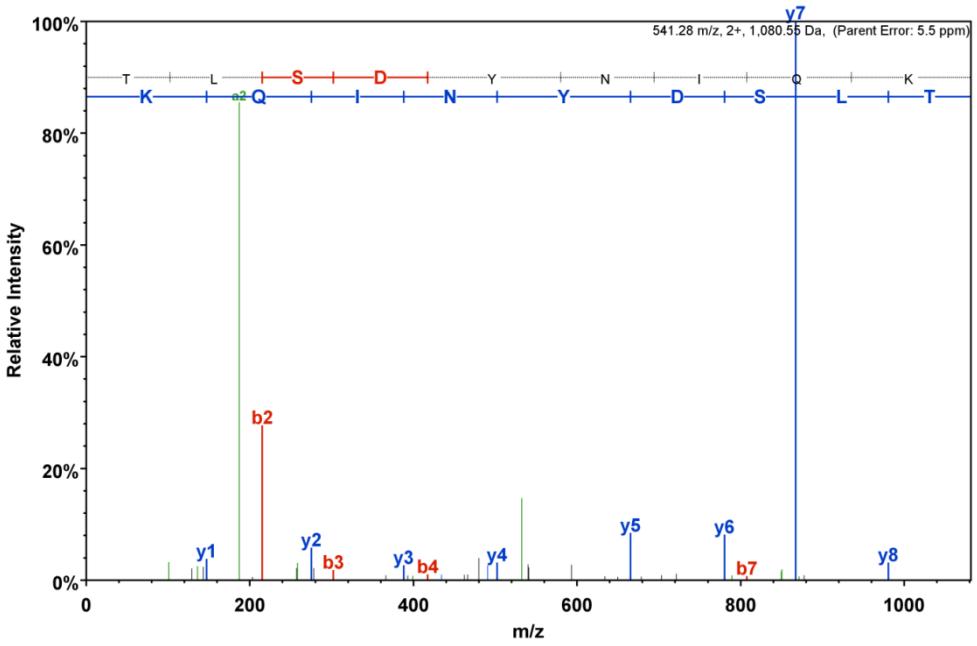
polyubiquitin chains were K63 (83%)>>K11 (9%)>K48, K27 (3.7% - 4.0%). Also, the samples which were diluted to 50% prior to submitting to analysis using the MS, were seen to contain the same linkages but at amounts which were about 50% of those detected from undiluted samples. The results of the experiment provided us with great confidence that our platform to affinity purify (summarised in Chapter 3) and now estimate endogenous unanchored polyubiquitin chains was optimised.

5.6.2. QUALITATIVE ANALYSIS USING LTQ-ORBITRAP CONFIRMED THE PRESENCE OF K48, K63 AND K11 LINKAGES WERE IN ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS PURIFIED FROM HEK293T CELLS

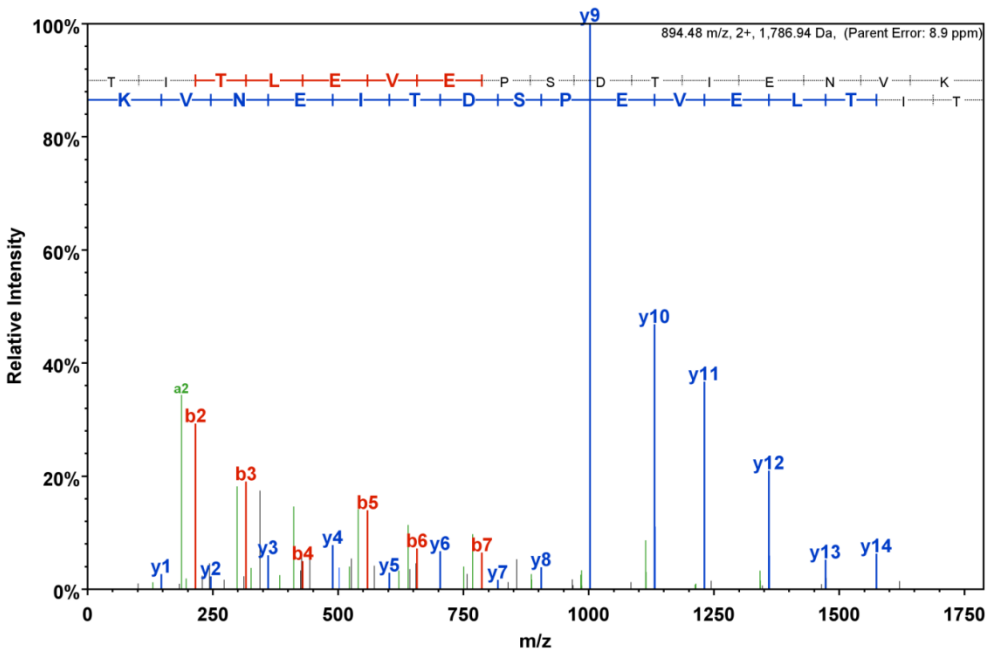
As mentioned in the previous section, 150 µl of endogenous unanchored polyubiquitin chains were trypsinolysed, dried to residue and set aside for qualitative analysis at the University of Leicester. LC-MS/MS was performed using RSLCnano HPLC system (Dionex) used LTQ-Orbitrap-Velos with ETD (Thermo Scientific), which offered a more sensitive platform to verify the presence of polyubiquitin isopeptide linkages. Using micro-capillary HPLC with pre-column trapping (Dionex, C18), a reversed phase column (PepMap C18) was eluted with a binary gradient of 5-90% acetonitrile, 0.1% ($^{v}/_{v}$) formic acid into a Nanoelectrospray ion-source using a PicoFrit^{TM} (New Objectives) column-emitter in positive ion mode [199]. Product ion spectra were generated automatically using data-dependant-acquisition (DDA), and data was processed using Scaffold (version 4.0.5, Proteome Software) which are shown in Figure 5.13. As expected, the analysis confirmed the presence of K48, K63 and K11 linkages (along with ubiquitin derived TITLE and TLS peptides) in

the sample of endogenous unanchored polyubiquitin chains purified from HEK293T cells (at basal growth condition).

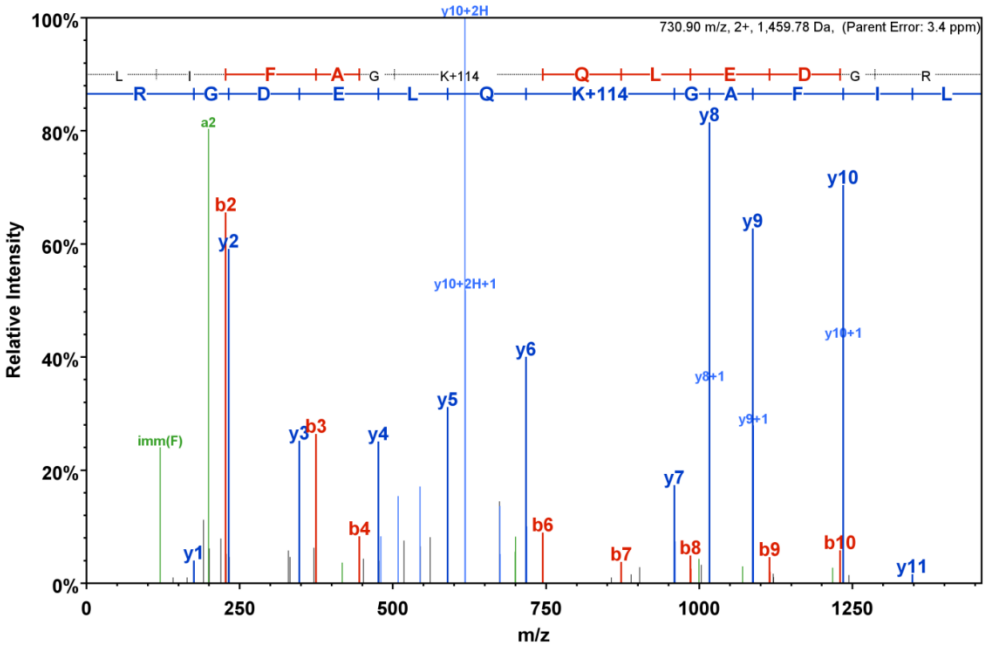
A



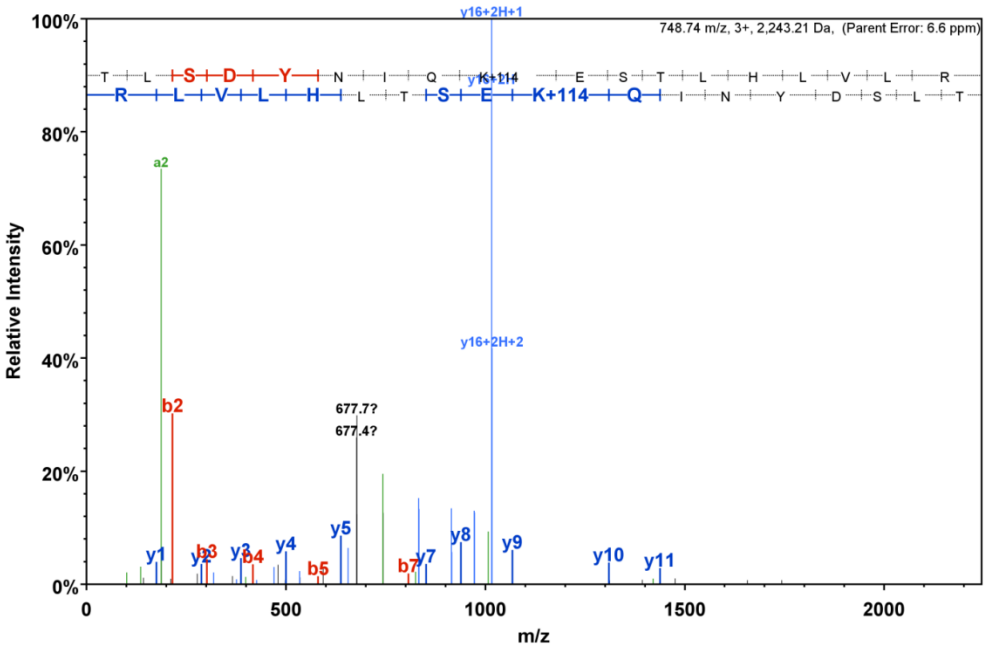
B



C


730.90 m/z, 2+, 1,459.78 Da, (Parent Error: 3.4 ppm)
L I F A G K+114 Q L E D G R
R G D E L Q K+114 G A F I L
Relative Intensity
m/z
y10+2H
y10+2H+1
a2
b2
y2
y8
y10
y9
y6
y5
y3
b3
y4
imm(F)
b4
y1
b6
y7
b7
b8
b9
b10
y11
y8+1
y9+1
y10+1

D


748.74 m/z, 3+, 2,243.21 Da, (Parent Error: 6.6 ppm)
T L S D Y N I Q K+114 E S T L H L V L R
R L V L H L T S E K+114 Q I N Y D S L T
Relative Intensity
m/z
y16+2H+1
y16+2H+2
a2
b2
677.7?
677.4?
y1
y2
b3
y3
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y8
y9
y10
y11

E

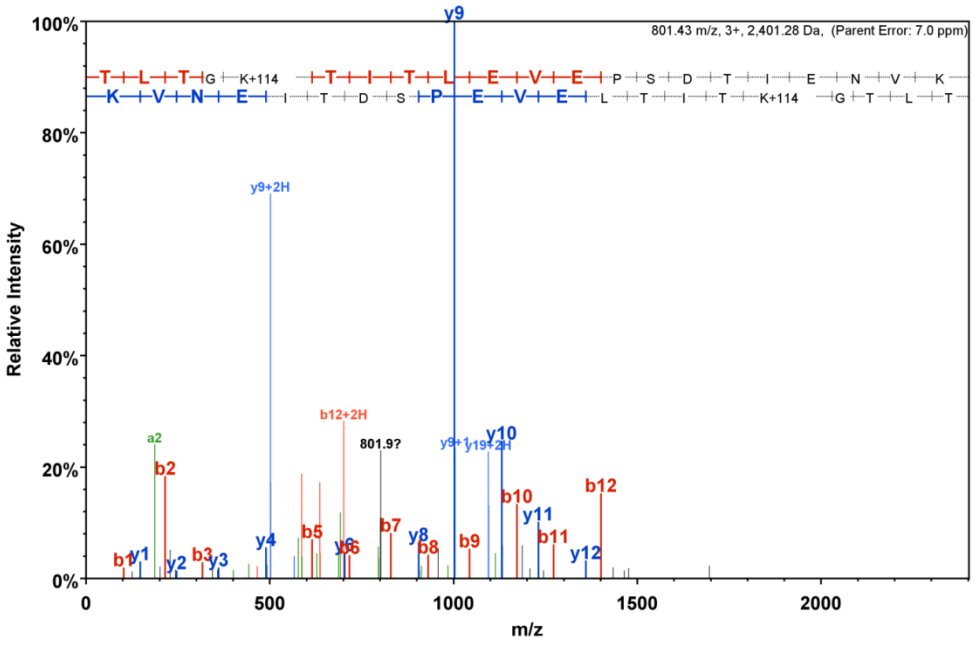


Figure 5.13. Spectra generated from qualitative analysis by LC-MS/MS using LTQ-Orbitrap and processed with Scaffold software confirmed presence of K48, K63 and K11 linkages in purified endogenous unanchored polyubiquitin chains from HEK293T cells. Trypsinolysed sample of purified unanchored polyubiquitin chains from HEK293T cells were seen to contain signature peptides corresponding to ubiquitin derived peptides TITLE (A) and TLS (B), in addition to linkage specific signature peptides K48 (C), K63 (D) and K11 (E). The diglycine residues (bound to lysine) in linkage specific signature peptides have been represented as K+114 indicating the molecular weight of the residues. All spectra were displayed as relative intensity of ions against corresponding mass/charge (*m/z*) values.

CHAPTER 6

VARIATION IN ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAIN PROFILES OF HEK293T CELLS IN RESPONSE TO ACTIVATION OF SIGNALLING, MODULATION OF REGULATORS AND STRESS

6.1. IL-1β MEDIATED ACTIVATION OF CELL SIGNALLING IN HEK293T CELLS

6.1.1. CONFIRMATION OF ACTIVATION OF SIGNALLING IN HEK293T CELLS UPON IL-1β TREATMENT

Since the Xia *et al.* study linking unanchored polyubiquitin chains to immune signalling was carried out in HEK293 cells stably expressing the IL-1R receptor, we first had to establish if our HEK293T cells could also respond to IL1β stimulation, and if so determine the cytokine level required to elicit a signalling response. An effective method to determine this was to carry out NF-κB reporter assays. Cells were transfected with firefly and *Renilla* luciferases as described in 2.2.2.4. Post-transfection and incubation, cells were lysed 4 hours post IL-1β treatment and analysed using a microplate luminometer. The method has previously been used in the lab and is described in [187, 200].

The NF-κB family of proteins act as transcription factors that regulate genes involved in the immune response [201, 202]. In unstimulated cells, the NF-κB dimer is maintained in an inactive state by being sequestered in the cytoplasm to the inhibitor of κB proteins (IκB) [203]. Stimulation by IL-1β (through IL-1R) leads to a rapid sequence of recruitment and activation of proteins eventually leading to the (K48 linked) ubiquitination and proteasomal degradation of IκB, thus enabling the nuclear translocation of the now unsequestered NF-κB and modulation of gene expression [203, 204]. One of the genes whose expression is governed by activated NF-κB is the chemokine

IL-8 [205, 206]. The expression of firefly luciferase, engineered in to a plasmid containing the NF-κB responsive promoter element, can be a determinant of the degree of NF-κB activation. The ubiquitin-*Renilla* luciferase kindly donated by Professor Peter Shaw (The University of Nottingham), was used as an internal control. Ubiquitin is found in abundance in cells and its levels and production of *de novo* ubiquitin are closely regulated in response to stress [18]. Hence, in theory the levels of *Renilla* luciferase would remain constant in response to IL-1β addition, which has been verified in preliminary experiments.

Luciferase activity was measured with the Dual-Luciferase assay system using a Glomax-96 Microplate Luminometer. The readout from the firefly luciferase activity was first normalised to that of the *Renilla* luciferase activity (for each sample), and the fold increase was calculated relative to control, which was set to 100. Results have been displayed in Figure 6.1. IL-1β concentrations ranging from 10 - 25 ng/ml (media) were used, along with a sample where cells were treated with the vehicle alone (2.5 µl sterile PBS containing 0.1% ($^{w}/_{v}$) human albumin). The experiment was carried out in triplicates and data was displayed as mean of averages ± SD. Statistical analysis was carried out using the Graphpad Prism 6 software as described in [188], using a one-way ANOVA and a Dunnett's test was to calculate level of significance between control and IL-1 β stimulated values. The significance was set at $p<0.05$.

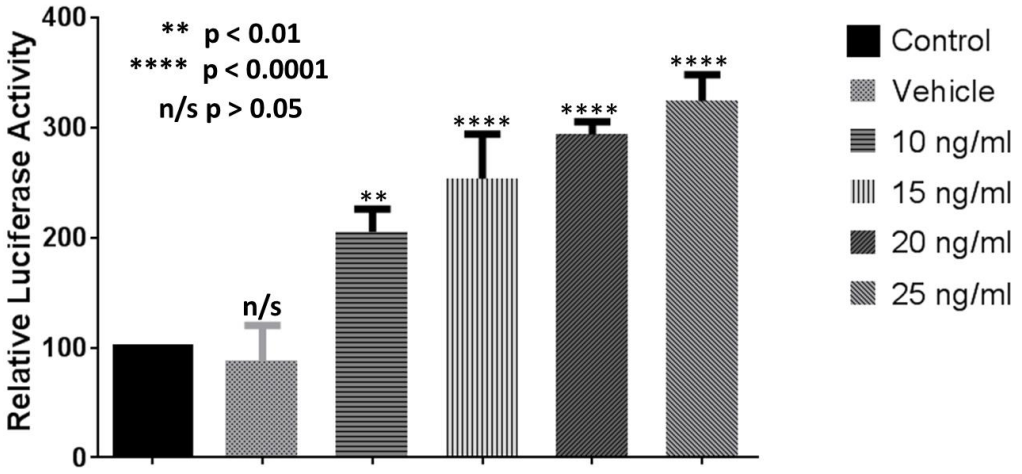


Figure 6.1. Luciferase reporter assay indicating NF-κB activity upon IL1β stimulation of HEK293T cells. Cells were co-transfected with 100 ng IL8 promoter-firefly luciferase plasmid and 0.4 ng ubiquitin promoter *Renilla* luciferase plasmid (amounts used to transfect 10^5 cells). 48 hours post transfection, cells were stimulated with increasing concentrations of IL1β (range 10 – 25 ng/ml, for a period of 4 hours) preceded by a 6 hour period of serum starvation (media was deprived of 10% FBS for the period). NFκB activity was measured and expressed in terms of the firefly luciferase activity, normalised to the *Renilla* luciferase activity. The experiment was repeated three times, the graph expresses the changes relative to control. As evident, the HEK293T cells respond to IL1β addition in a dose dependent manner. 20 ng/ml was considered sufficient to elicit a considerable response and was the concentration was used in future experiments. The data is presented as mean of average from three repeats of the experiment ± SD, with p values indicating the significance of difference when compared with control.

The reporter assay proved that the HEK293T cells were responsive to IL-1β treatment. While the IL-1β concentration of 10 ng/ml (of media) was used for stimulation by Xia *et al*. [1]; here, the concentration of 20 ng/ml IL-1β was seen to produce a significant (three-fold) increase in NF-κB activity and was used for all IL-1β stimulation experiments hereafter.

6.1.2. AFFINITY PURIFICATION OF ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS POST IL-1β TREATMENT OF HEK293T CELLS

HEK293T cells were grown in DMEM supplemented with 10% FBS and 1% Pen-Strep. Prior to IL-1β treatment, HEK293T cells which were ~90% confluent were first serum starved for a period of 6 hours, by replacing media with DMEM lacking FBS [200]. Longer duration of serum starvation was also tested but the cells were seen to detach from surface upon washing, thereby resulting in losses. Cells were then treated with 20 ng/ml IL-1β in DMEM (lacking FBS), were washed (after discarding media) in sterile PBS, and 2 minutes post treatment, lysed in homogenising buffer (2.2.3) which in addition to components previously mentioned also contained 0.2% ($^{v}/_{v}$) mammalian phosphatase inhibitor. As a control, cells were treated with equal volume of vehicle only [sterile PBS, 0.1% ($^{w}/_{v}$) human serum albumin] and were then washed in sterile PBS and homogenised. A BCA protein assay was then carried out after which 24 mg of proteins from each extract (IL-1β treated and control) was processed for affinity purification by incubating with 150 µl immobilised Znf-UBP domain (10 mg/ml bait per volume of beads) in polypropylene columns. A small volume (150 µl) of load was retained and used for western blotting.

Bead with captured polyubiquitin chains were then subjected to three washes with wash buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% ($^{v}/_{v}$) IGEPAL (NP-40)]. 50 µl samples on beads were retained for western blotting while the rest was used for MS analyses. Samples on beads for blotting were eluted into GAB and 33% each was used to generate western blots to be probed with anti-

ubiquitin and anti-K48 polyubiquitin antibodies. The blots generated also included commercial K48 and K63-linked polyubiquitin ladders and 50 µl loads from each sample.

The experiment was repeated three times and representative blots are shown in Figure 6.2. The blot probed with ubiquitin antibody did not show significant changes in purified IL-1β-treated samples when compared to control (even though the sample load suggested an increase in ubiquitin reactive species). However, visual inspection of the anti-K48 polyubiquitin blots did indicate an increase in endogenous polyubiquitin chains containing this linkage. This observation required further investigation using MS.

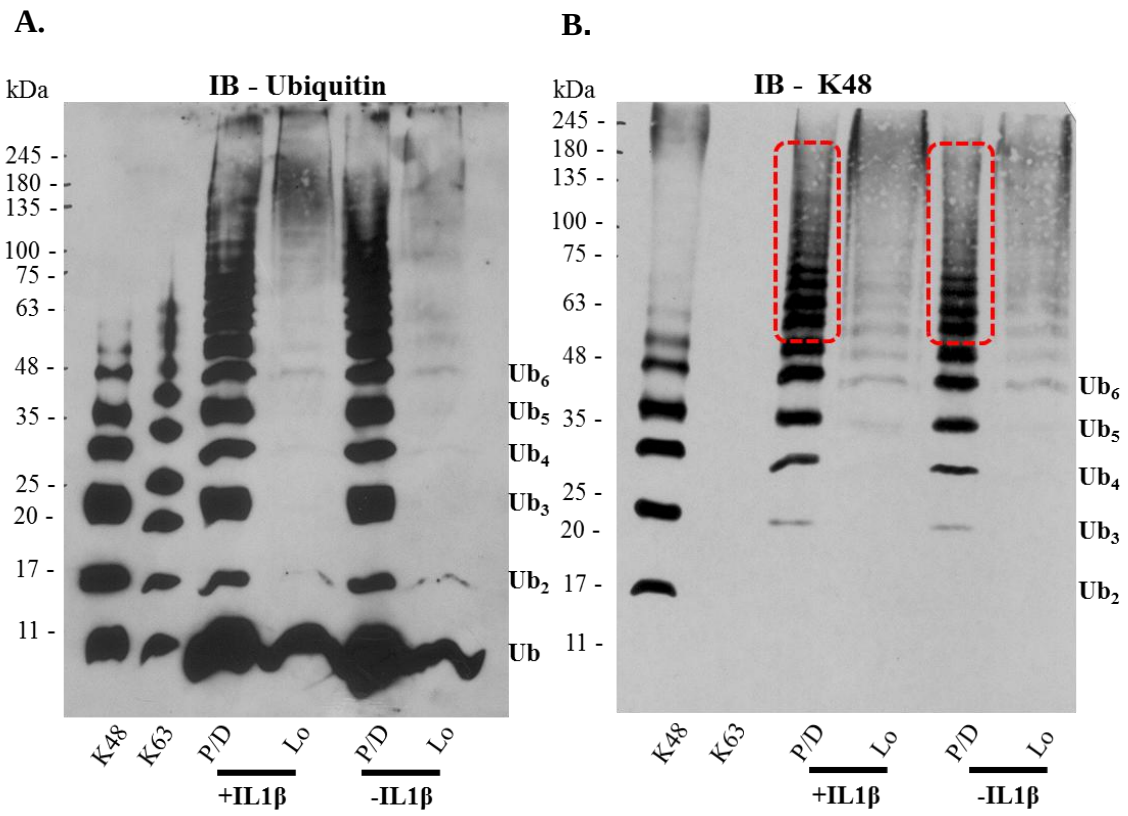


Figure 6.2. Immunoblotting of affinity captured endogenous unanchored polyubiquitin post IL-1β stimulation of HEK293T cells. Cells which were ~90% confluent were serum starved for a duration of 6 hours and then treated with IL-1β (20 ng/ml DMEM lacking FBS). Cells were homogenised 2 minutes post treatment in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% ($^{v}/_{v}$) IGEPAL (NP-40), 5 mM NEM, 20 µM MG132, 0.1% ($^{v}/_{v}$) mammalian protease inhibitor cocktail and 0.2% ($^{v}/_{v}$) phosphatase inhibitor. 24 mg proteins from cell extract was pre-treated (~ 2.5 mg/ml concentration of cell extract) and was incubated with 150 µl Znf-UBP tagged Sepharose (10 mg/ml bait per volume of beads) in a polypropylene column overnight. 40 µl samples on beads were eluted in GAB and 33% used for western blotting with **A.** ubiquitin and **B.** K48-linked polyubiquitin specific antibodies, representative blots are shown here (n=3). Lanes containing affinity purified samples in western blots were labelled P/D (pull-down). Also used in the blot were K48 and K63-linked commercial polyubiquitin ladders and 50 µl each of the sample loads (Lo). Subjective visual assessment of the blots appear to show an increase in longer unanchored polyubiquitin chains in the IL-1β treated sample (indicated in red), evident with the K48 antibody.

6.1.2.1. DETECTION OF K63-LINKED UNANCHORED POLYUBIQUITIN CHAINS AND POSSIBLE CHANGES IN PROFILES IN RESPONSE TO IL-1β

Detection of endogenous unanchored K63-linked polyubiquitin chains has been a challenge, as previously described in section 3.2. However, the availability at a later stage of the project of a non HRP-conjugated HWA4C4 clone of the K63-polyubiquitin specific antibody allowed us to attempt to detect the presence of K63 linkage in purified samples of unanchored polyubiquitin chains. However, the specificity of the antibody had to first be verified. The HRP conjugated antibody used in experiments thus far, was shown to cross-react to commercial K48 chains and also Znf-UBP bait, with the apparent reactivity to commercial K11-linked dimer being explained by the presence of K63 dimers in the sample. 250 ng of commercial K48 and K63-linked polyubiquitin ladders and 5 μg of (uncoupled) Znf-UBP domain were treated with GAB and separated using SDS PAGE. The western blot thus developed was probed with 'new' K63-linked polyubiquitin antibody (Figure 6.3.A.). The antibody showed reactivity to the K63-linked chains alone and non-specific interactions with the K48 ladder or bait were not detected.

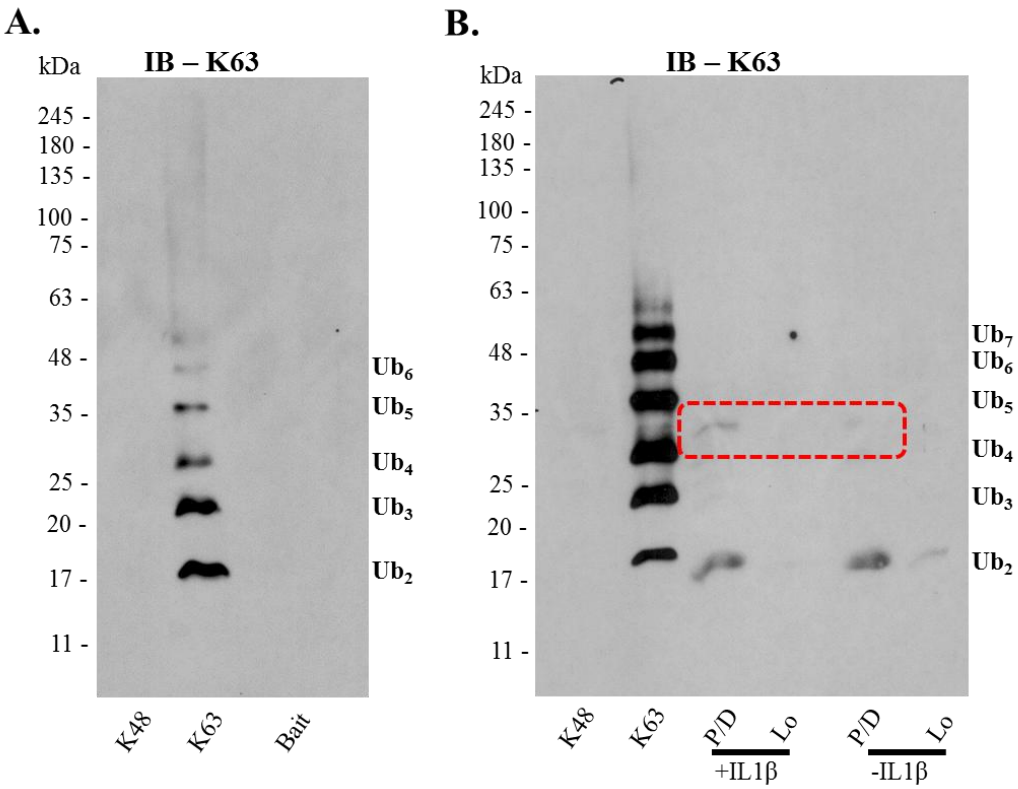


Figure 6.3. Detection of K63 linkage in purified unanchored polyubiquitin chains from HEK293T cells using the (HRP) untagged primary mouse HWA4C4 clone.
A. 250 ng of commercial K48 and K63-linked polyubiquitin ladders and 5 µg of Znf-UBP bait protein were separated using SDS PAGE and transferred on to a western blot. This was probed with the HWA4C4 clone, which showed no cross-reactivity with either K48-linked polyubiquitin or just the bait protein, in contrast to that observed with the previous antibody (Figure 3.2).
B. 33% of affinity-purified endogenous unanchored polyubiquitin sample that was eluted from 40 µl beads (as used to obtain Figure 6.2.) from HEK293T cells treated with (or without) 20 ng/ml IL1β was separated using SDS PAGE, transferred on to a blot and probed using the HWA4C4 antibody. Aside from the diubiquitin species, the level of K63-linked polyubiquitin in sample was possibly too low to be detected by the antibody. Although the level of the diubiquitin species does not appear to vary between control and IL1β treated sample, surprisingly the latter was also characterised by the appearance of a tetraubiquitin species (indicated in red). Lanes containing affinity purified samples in western blots were labelled P/D (pull-down). Also used in the blot were K48 and K63-linked commercial polyubiquitin ladders and 50 µl each of the sample loads (Lo).

Hence, the antibody was now used to probe western blots containing 33% of the eluted samples from IL-1β treated and control cells, along with respective

loads and K48 and K63-linked polyubiquitin ladders (Figure 6.3). Visual inspection of the blot only revealed detection of diubiquitin and possibly tetra-ubiquitin species precluding investigation of changes in longer chains. Interestingly, work by Z Chen and colleagues emphasised on the role on short unanchored polyubiquitin chains (up to 6 ubiquitin moieties in length) in activation of kinases [126]. A possible explanation could be that the levels of longer unanchored K63-linked polyubiquitin chains in cells may be too low for detection using western blotting (discussed in Chapter 3 and Chapter 7). However, it was interesting to note that the IL-1β treated cells consistently showed an increased level of the apparent tetra-ubiquitin band.

6.1.3. QUANTITATIVE ANALYSIS OF CHANGES IN HEK293T-DERIVED UNANCHORED POLYUBIQUITIN CHAINS IN RESPONSE TO IL-1β TREATMENT BY MASS SPECTROMETRY

Due to the limitations of validation of changes in unanchored polyubiquitin chain levels through western blotting, quantitative analyses using the triple quadrupole mass spectrometer were even more vital. Approximately 110 µl of affinity purified sample on beads from IL-1β treated cells and control (from three independent repeats) were first subjected to three washes in DUB buffer (50 mM Tris-HCl pH 7.5, 1mM DTT) to rid the sample of remnants of detergent (IGEPAL) used during homogenisation. The samples were then eluted in 0.1% ($^{v}/_{v}$) formic acid (in deionised H_2O) and beads separated using centrifugation columns with attached filters. Samples containing eluted polyubiquitin chains were completely dried down in a vacuum centrifuge. Samples were then resuspended in digestion buffer (40% MeCN in 50mM Tris-HCl pH 7.4, 10mM $CaCl_2$) and subjected to three rounds of trypsinolysis

using 1 μg sequencing grade trypsin for each round (all methods optimised and described in Chapter 5). The trypsinolysed samples were then dried, resuspended in 0.1% ($^{v}/_{v}$) formic acid, 50% of which were introduced into the mass spectrometer set to perform MRM.

An important aspect of the analyses involved deviating from the previously described strategy (Chapter 5) to present data obtained from mass spectrometry. The use of AQUA standards for quantitative analysis of ubiquitin linkages relies on the generation of standard curves for each AQUA signature standard. However, such standard curves could not be generated as the quality of MS data generated from AQUA standards was not acceptable to determine the abundance of signature peptides data. Therefore due to lack of confidence in some of the AQUA standards run during the experiment, an alternate strategy was used wherein the relative change in each linkage compared to control was determined. The AQUA standards did prove valuable in determining the retention times of each signature peptide – this was used to ensure that the correct corresponding light (i.e. sample-derived) signature peptide peak was selected. Once the area under the peak for each peptide was calculated, ideally the amount of each isopeptide linkage could be determined by comparing with the AQUA standard curves obtained. Due to the lack of reliability of AQUA standard curves, we decided to normalise the areas of polyubiquitin linkage signal peptides to linear internal ubiquitin peptides as standards namely the “TITLE” and “TLS” peptides i.e. normalise to total ubiquitin purified.

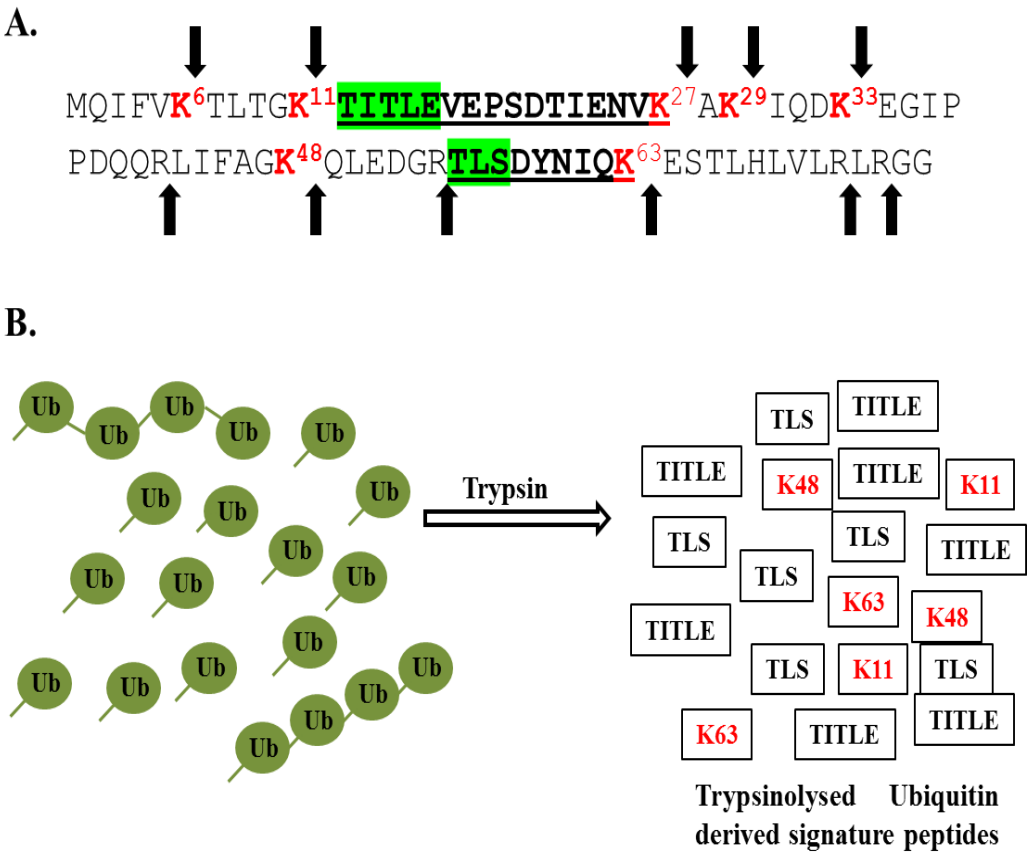

A.
MQIFVK6TLTGK11TITLEVEPSDTIENVK27AK29IQDK33EGIP
PDQQRLIFAGK48QLEDGRTLSDYNIQK63ESTLHLVLRLRGG
B.
Ub
Trypsin
TLS
TITLE
K48
K11
K63
Trypsinolysed Ubiquitin derived signature peptides
C.
K11 linked unanchored tetraubiquitin
MQIFVK6TLTGK11TITLEVEPS
DTIENVK27AK29IQDK33EGIP
PDQQRLIFAGK48QLEDGRTLS
DYNIQK63ESTLHLVLRLRGG
Trypsinolysis
GG
TLTGK11TITLEVEPS
DTIENVK27
K11 signature peptide

Figure 6.4. Trypsinolysis of monoubiquitin and polyubiquitin chains produce characteristic "signature" peptides.
A. Human ubiquitin sequence, with all lysine residues labelled in red. The location of "TITLE" and "TLS" peptides has been highlighted. All the trypsin cleavage sites in ubiquitin have also been indicated by arrows.

B. Depiction of relative abundance TLS and TITLE peptides (when compared to linkage derived signature peptides) being produced from cellular pools of unanchored polyubiquitin chains and monoubiquitin.

C. Depiction of the generation of K11 signature peptide from unanchored K11 tetraubiquitin chain. All the sites sensitive to trypsin cleavage are marked in red with the exception of the K11 residues (marked in blue) which are protected by the isopeptide linkage at the site.

As evident in Figure 6.4, the TITLE peptide is produced upon trypsinolysis only when the lysine residues at positions 11 and 27 of ubiquitin are not blocked or modified by (K11, K27) isopeptide linkages. Similarly, the TLS peptide is only released when lysine residue at position 63 is unmodified. Also worth noting is the fact that both these peptides are produced when mono-ubiquitin is subject to trypsinolysis. It is important to note that ubiquitin accounts for a large proportion of eukaryotic protein [20] most of this being mono-ubiquitin [16, 17], also evident from our immunoblots of affinity purified unanchored ubiquitin. Hence, complete trypsinolysis of purified samples yields abundant TITLE and TLS peptide signatures, especially when compared to (isopeptide) linkage-containing signature peptides. Hence, for the analysis strategy used, the generation of TITLE and TLS have been assumed to be largely independent of presence of K63 or K11/K27 linkages respectively.

Thus, the peak area in the chromatogram corresponding to a linkage signature peptide (measured by their corresponding transition) was first calculated, along

with that for TITLE and TLS for every given sample and control. The peak areas of each linkage relative to TITLE and TLS were then noted, and percentage change compared to control was calculated for each linkage. This has been used as a semi-quantitative measure in determining fold-changes of each linkage. As mentioned previously, since the presence of K48, K63 and K11 linkages have been validated; only changes to these linkages have been reported. Furthermore, these three linkages, along with the K29 linkage, have been found to be the most abundant in the HEK293 ubiquitome [42].

Based on the analysis displayed in Figure 6.5, it is evident that there appeared to be an increase in all three isopeptide linkages, K48, K63 and K11, relative to total ubiquitin in unanchored chains purified following IL-1β treatment. The increase in the K63 linkage may be predictable, with our data suggesting a possible 20%-30% increase due to activation of signalling. The K48 linkage showed a similar increase, which might account for the production of K48 and K63 mixed linkages discussed in Section 1.2.2. Another further reason for the increase could be release of K48-linked chains from proteins (I-κB) that are targeted for proteasomal processing upon activation of signalling. The increase in K11-linked endogenous unanchored chains is of interest as the possible role of this linkage in NF-κB mediated immune response has only recently been postulated [94].

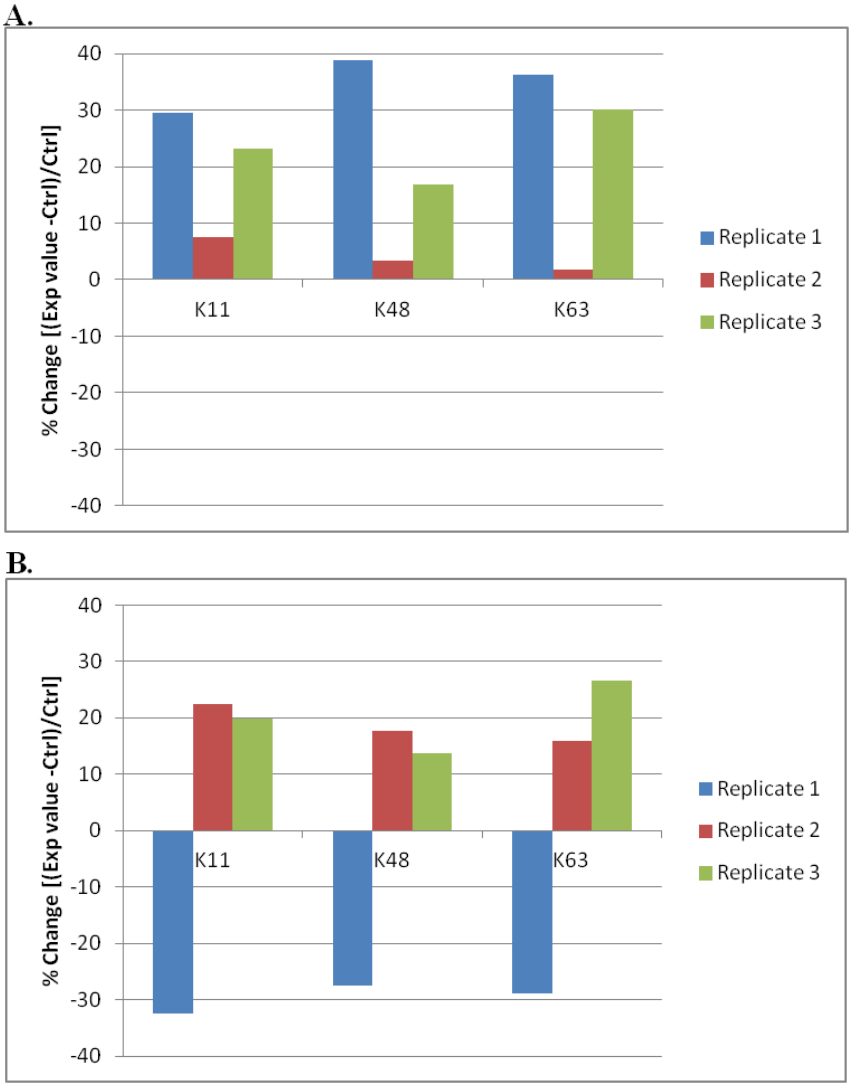


Figure 6.5. Analysis of data derived using SRM allows detection of relative changes in signature peptides corresponding to three different isopeptide linkages upon IL-1β stimulation in HEK293T cells. 100 µl affinity purified endogenous unanchored polyubiquitin chains on bait were first eluted in 0.1% formic acid. The eluate was dried, resuspended in trypsinolysis buffer and subjected to three rounds of trypsin digestion with 1 µg sequencing grade Promega trypsin. The digests were analysed using MRM mode on QqQ LC-MS/MS. AUC values for each signature peptides were measured from corresponding chromatograms. These were normalised with either values for ubiquitin derived "TITLE" (TITLEVEPSDTIENVK) or "TLS" (TLSDYNIQK) peptides for the same sample (as described in 6.1.3). The normalised values for each of the three linkage peptides were then displayed as percent change when compared to their corresponding controls. Relative changes in three linkages – K11, K48 and K63 - have been displayed for the three biological replicates of the experiment, which have either been normalised with **A.** TITLE or with **B.** TLS peptides. Mass spectrometry data obtained reveals a trend where all three linkages K11, K48 and K63, appear to show a marginal increase (compared to control) brought upon by IL-1β treatment.

6.2. KNOCKDOWN OF KNOWN AND NOVEL REGULATORS OF UNANCHORED POLYUBIQUITIN IN HEK293T CELLS

6.2.1. KNOCKDOWN OF ISOPEPTIDASE T

6.2.1.1. AFFINITY PURIFICATION AND VISUALISATION OF UNANCHORED POLYUBIQUITIN CHAINS USING WESTERN BLOTTING AFTER ISOT KNOCKDOWN IN HEK293T CELLS

In this study, we were keen on determining the immediate effects of the suppression of the enzyme on the unanchored polyubiquitin profiles and not its downstream effect on cellular processes. Since the initial attempts to silence or suppress the expression of the enzyme using single siRNAs did not sufficiently suppress IsoT, an siRNA pool (described in 2.1.2.3) was used for the purpose. Although the siRNA pool was confirmed to target both isoforms of IsoT, only the shorter isoform was expressed in HEK293T cells and was hence targeted, the long isoform was not detectable by western blotting.

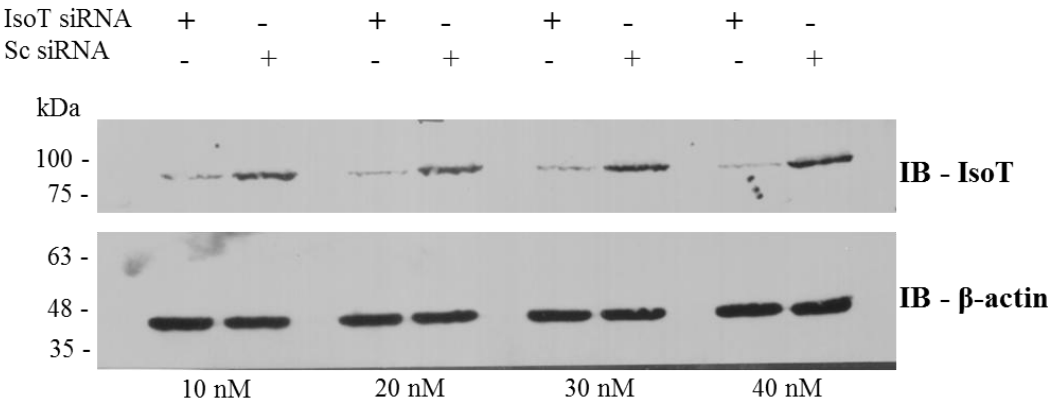


Figure 6.6. Optimisation of siRNA (pool) concentrations for effective suppression of IsoT levels in HEK293T cells. $1X10^5$ cells were seeded in each well of a 24-well plate. These were transfected with either IsoT or control (scrambled or Sc) siRNA pools at concentrations of 10, 20, 30 and 40 nM, 24 hours post seeding using DharmaFECT Duo Transfection reagent. 48 hours post transfection, cells were lysed directly in GAB and 25% volume of each cell extract was subject to western blotting, and probed with an IsoT specific antibody. An immunoblot using anti β-actin antibody was carried out as loading control. The western blot confirmed suppression of IsoT levels, and the siRNA concentration of 20 nM was found to be effective in significantly reducing IsoT levels

The knockdown itself was first optimised in 24 well plates, using a range of siRNA concentrations from 10 nM to 40 nM (Figure 6.6). A treatment of 20 nM siRNA for a period of 48 hours was deemed sufficient to substantially reduce the IsoT levels in HEK293T cells. The experiment was then scaled up, requiring the use of 10 cm cell culture plates (2.2.2.3). Cells were lysed in homogenising buffer, 48 hours post transfection. 50 µg cell extract was used for immunoblotting with IsoT antibody to confirm knockdown, with anti-β-actin being used as loading control (Figure 6.7). 20 mg of protein (verified by BCA assay) at a concentration of ~2.5 mg/ml, from both siRNA-treated cells and control were subjected to affinity purification using 125 µl each of

immobilised Znf-UBP domain (concentration of 10 mg/ml on beads) in polypropylene columns, overnight.

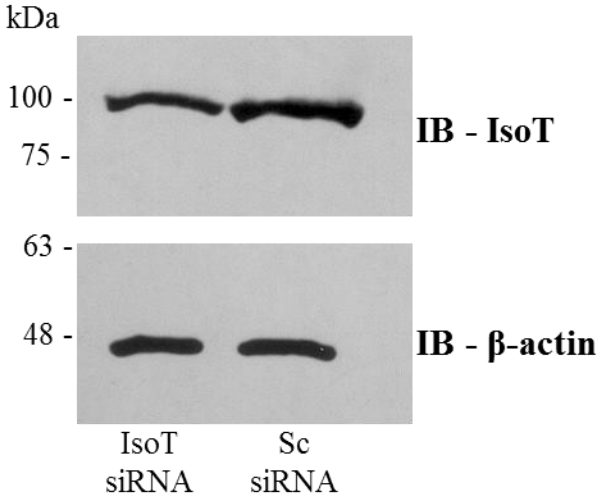


Figure 6.7. Confirmation of IsoT knockdown in HEK293T cells. HEK293T cells were treated with 20 nM IsoT siRNA or negative control scrambled (sc) siRNA and were lysed 48 hours post transfection. 50 µg of cell extract proteins from each sample were separated using SDS PAGE, transferred on to western blots and probed with antibodies specific for IsoT and β-actin (loading control). The western blot confirmed knockdown of IsoT. Efficiency of knockdown was estimated using densitometry analysis carried out by ImageJ 1.47, wherein the protein level was suppressed to ~55% that of control expression level. Three repeats of the experiment were carried out after which, samples were then subjected to affinity purification for unanchored polyubiquitin chains.

40 µl affinity purified sample on beads were retained for western blotting, while the rest was subjected to MS analysis. 33% of the 40 µl sample was eluted using GAB and separated using SDS PAGE, transferred to western blots and probed with ubiquitin and K48-linked polyubiquitin specific antibodies. The gels also contained K48 and K63-linked polyubiquitin ladders as positive

controls and 50 µl loads from each sample. The experiment was carried out three times and representative blots are shown in Figure 6.8.

The western blots suggest an increase in capture of endogenous unanchored polyubiquitin chain following IsoT knockdown, especially of longer chains, an observation requiring further validation using MS. Another point to note was that although the efficiency of knockdown was higher when carried out in 24 well plates, the decrease in expression levels achieved after scaling up the process was restricted to 45-50% (based on densitometry analysis using ImageJ software).

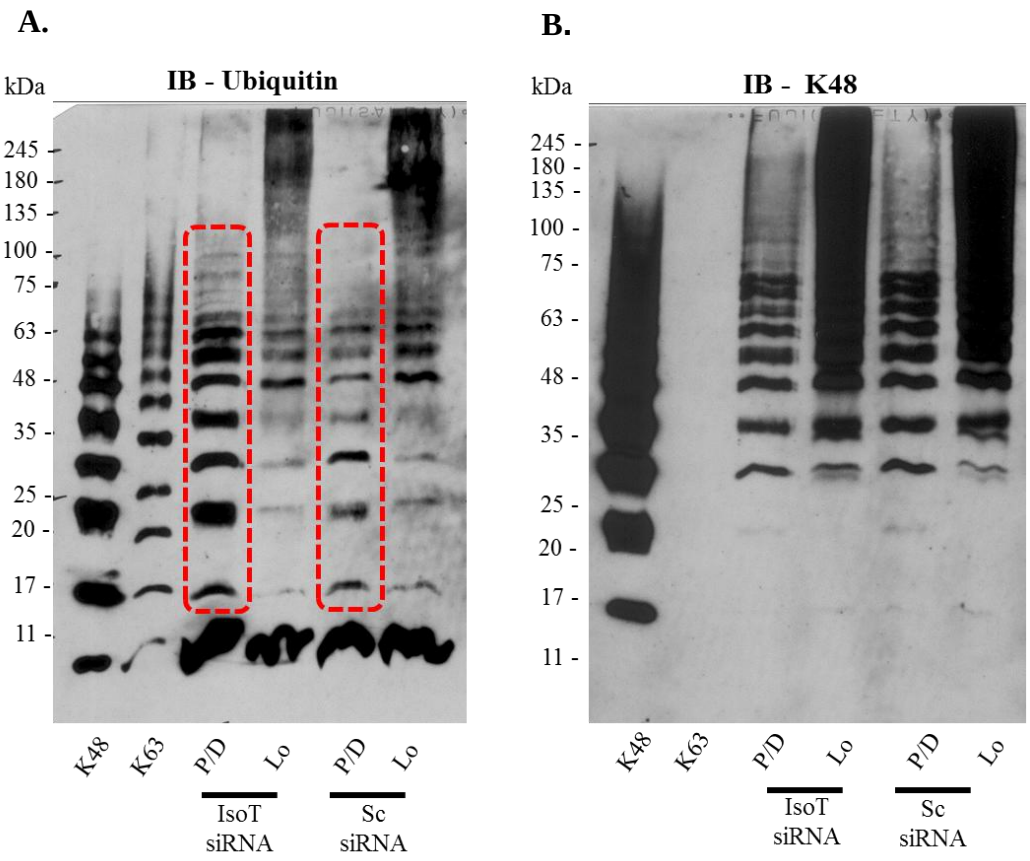


Figure 6.8. Immunoblotting of affinity captured endogenous unanchored polyubiquitin after IsoT knockdown in HEK293T cells. 3 X 10^6 HEK293T cells were plated on 10 cm culture plates and were transfected 24 hours later with 20 nM IsoT siRNA or negative control scrambled (sc) siRNA. Cells were lysed 48 hour post transfection in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% ($^{v}/_{v}$) IGEPAL (NP-40), 5 mM NEM, 20 µM MG132, 0.1% ($^{v}/_{v}$) mammalian protease inhibitor cocktail. Once knockdown of IsoT was verified, 20 mg proteins from cell extract was pre-treated (~ 2.5 mg/ml concentration of cell extract) and was incubated with 125 µl Znf-UBP domain tagged Sepharose (10 mg/ml bait per volume of beads) in a polypropylene column overnight. 40 µl sample on beads were eluted in GAB and 33% used for western blotting with **A.** ubiquitin and **B.** K48-polyubiquitin specific antibodies, representative blots are shown here (n=3). Also used in the blot were K48 and K63-linked commercial polyubiquitin ladders and 50 µl each of the sample loads (Lo). Subjective visual assessment of the blots appeared to show an increase in total endogenous unanchored polyubiquitin (6.8.A) when IsoT is suppressed (red box)

6.2.1.2. QUANTITATIVE ANALYSIS OF CHANGES IN HEK293T DERIVED UNANCHORED POLYUBIQUITIN CHAINS IN RESPONSE TO ISOT KNOCKDOWN

As previously described in section 6.1.3, the same technique was applied to analyse the relative changes in K48, K63 and K11 signature peptides upon IsoT knockdown. As seen in Figure 6.9, despite the large variability in the data obtained, it is possible that while K48 and K63 levels showed an increase as anticipated, a definite trend could not be confirmed with respect to levels of K11. While IsoT has previously been reported to disassemble unanchored K11 linked chains [69], results from a separate study looking at DUB activity suggested lower efficiency of degradation of K11 diubiquitin by recombinant IsoT [207]. Considering the variability in results from such *in vitro* experiments, the uncertainty of the data from our *in vivo* study may not be unexpected.

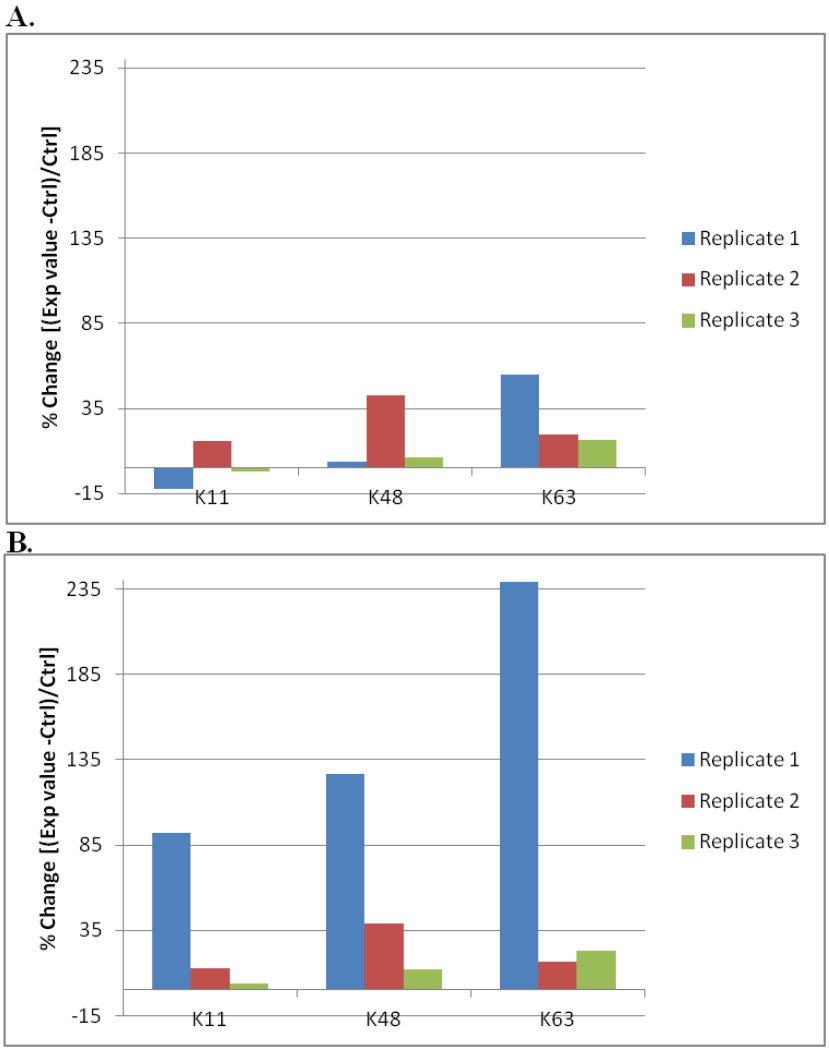


Figure 6.9. Analysis of data derived using SRM allows to detect relative changes in signature peptides corresponding to three selected ubiquitin isopeptide linkages post knockdown of IsoT in HEK293T cells. ~85 µl affinity purified endogenous unanchored polyubiquitin chains on bait were first eluted in 0.1% formic acid. The eluate was dried, resuspended in trypsinolysis buffer and subjected to three rounds of trypsin digestion with 1 µg sequencing grade Promega trypsin. The digests were analysed using MRM mode on QQQ LC-MS/MS. AUC values for each signature peptides were measured from corresponding chromatograms. These were normalised with either values for ubiquitin derived "TITLE" (TITLEVEPSDTIENVK) or "TLS" (TLSDYNIQK) peptides for the same sample as described in 6.2.4. The normalised values for each of the three linkage peptides were then displayed as percent change when compared to their corresponding controls. Relative changes in three linkages – K11, K48 and K63, have been displayed for the three replicates which have either been normalised with **A.** TITLE or with **B.** TLS peptides. Mass spectrometry data obtained consistently revealed accumulation of K48 and K63 linked chains upon IsoT knockdown. However with the case of K11, it was difficult to interpret the variation in levels.

6.2.2. KNOCKDOWN OF UBIQUITIN PROTEIN LIGASE E3C

6.2.2.1. AFFINITY PURIFICATION AND VISUALISATION OF UNANCHORED POLYUBIQUITIN CHAINS USING WESTERN BLOTTING AFTER UBE3C KNOCKDOWN IN HEK293T CELLS

Before we could attempt to knockdown the UBE3C levels, we were faced with the challenge of detection of the enzyme in HEK293T cells. UBE3C levels, while high in skeletal muscle, was found to be considerably low in most other tissue (including kidney) [145]. However, by making use of rat skeletal muscle tissue, and high amount of cell extract load (~60 μg), we were able to detect the enzyme (data not shown). The next step was to knockdown the levels of UBE3C, which was determined by using siRNA pool concentrations ranging from 10 μM – 60μM in 24 well plates. A concentration of 40 μM was shown to produce a significant knockdown in expression levels. This was scaled up to 10 cm cell culture plates as described in 2.2.2.3. Cells were lysed in homogenising buffer (2.2.3), 48 hours post transfection.

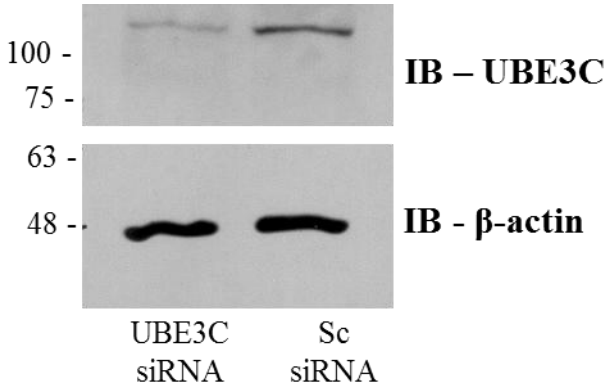


Figure 6.10. Confirmation of Ube3c knockdown in HEK293T cells. HEK293T cells were treated with 40 nM UBE3C siRNA or negative control scrambled (sc) siRNA and were lysed 48 hours post transfection. 50 µg of cell extract proteins from each sample were separated using SDS PAGE, transferred on to western blots and probed with antibodies specific for UBE3C and β-actin (loading control). The western blot confirmed knockdown of UBE3C. Efficiency of knockdown was estimated using densitometry analysis carried out by ImageJ 1.47, wherein the protein level was suppressed to ~30% that of control expression level. Three repeats of the experiment were carried out after which, samples were then subjected to affinity purification for unanchored polyubiquitin chains.

60 µg of proteins (based on BCA assay) in GAB were subjected to western blotting and probed with antibodies against UBE3C and actin (loading control). A representative immunoblot thus obtained is displayed in Figure 6.10. As evident, a significant reduction in levels of UBE3C was achieved. Densitometry analysis using ImageJ 1.47 determined the levels of UBE3C in knockdown sample as being typically ~30% that of control. 6 mg of proteins from HEK293T cell extract treated with and without siRNA was then subjected to pre-treatment and affinity purification with 100 µl immobilised Znf-UBP domain on Sepharose beads (10 mg/ml). The affinity-purified sample was then washed three times in wash buffer and 40 µl of sample on beads was retained for immunoblotting while 60 µl was separated for MS analysis. The

UBE3C knockdown could only be confirmed twice, because of shortage of siRNA during the third repeat.

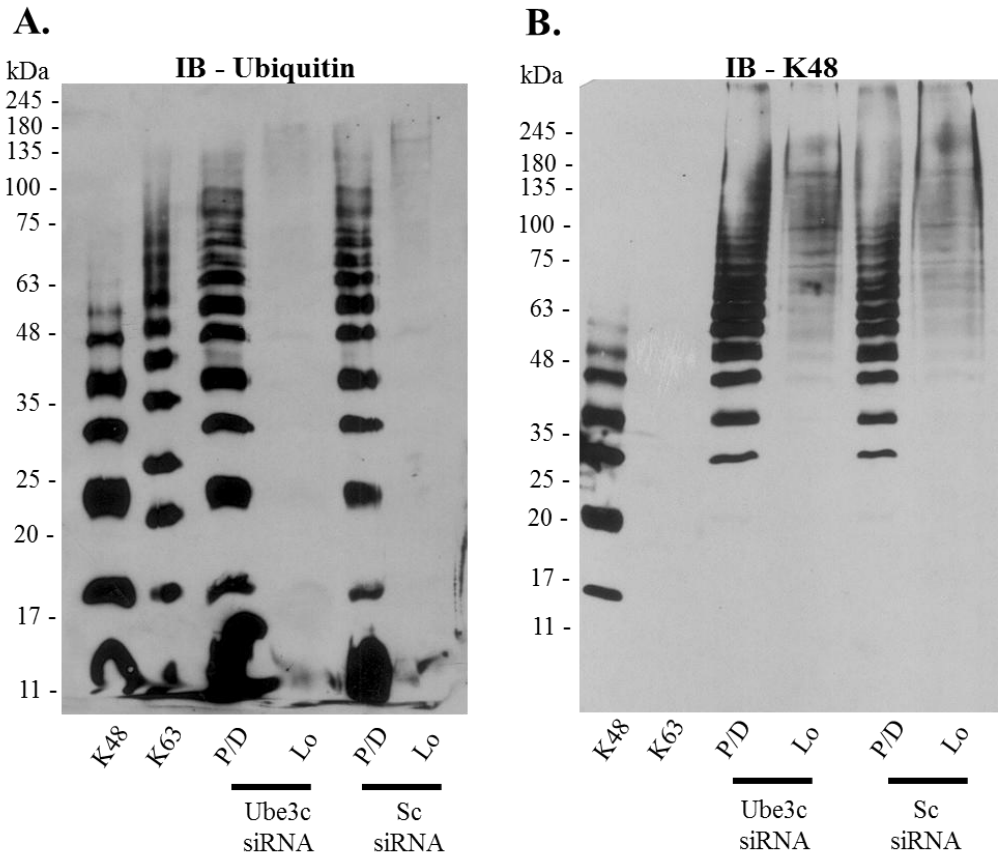


Figure 6.11. Immunoblotting of affinity captured endogenous unanchored polyubiquitin after Ube3c knockdown in HEK293T cells. 3 X 10^6 HEK293T cells were plated on 10 cm culture plates and were transfected with 40 nM Ube3c siRNA or negative control scrambled (sc) siRNA, 24 hours later. Cells were lysed 48 hour post transfection in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% ($^{v}/_{v}$) IGEPAL (NP-40), 5 mM NEM, 20 µM MG132, 0.1% ($^{v}/_{v}$) mammalian protease inhibitor cocktail. Once knockdown of Ube3c was confirmed, 16 mg cell extract was pre-treated (~ 2.5 mg/ml concentration of cell extract) and was incubated with 100 µl Znf-UBP domain tagged Sepharose (10 mg/ml bait per volume of beads) in a polypropylene column overnight. 40 µl sample on beads were eluted in GAB and 33% used for western blotting with **A.** ubiquitin and **B.** K48-linked polyubiquitin specific antibodies, representative blots are shown here (n=2). Lanes containing affinity purified samples in western blots were labelled P/D (pull-down). Also used in the blot were K48 and K63-linked commercial polyubiquitin ladders and 50 µl each of the sample loads (Lo). There were no obvious differences noted in the endogenous unanchored polyubiquitin profiles upon inspection of the blots.

The 40 µl purified sample on beads were eluted in GAB and 33% of each sample (knockdown and control) were subjected to western blotting along with K48 and K63 ladders and 50 µl each of corresponding loads. The results of probing these blots with ubiquitin and K48-linked polyubiquitin specific antibodies are displayed in Figure 6.11. Subjective visual assessment of the blots did not reveal significant differences in endogenous unanchored polyubiquitin profiles.

6.2.2.2. QUANTITATIVE ANALYSIS OF CHANGES IN HEK293T DERIVED UNANCHORED POLYUBIQUITIN CHAINS IN RESPONSE TO UBE3C KNOCKDOWN

Another setback was encountered while obtaining MS data from samples obtained from HEK293T cells subjected to UBE3C knockdown. Of the three attempts to knock down UBE3C, MS data from only one set could be used for analysis. While one repeat failed to sufficiently knockdown UBE3C (as mentioned previously), data from another could not be submitted to analysis due to failure of the LC system, confirmed by the abnormal chromatogram obtained for the corresponding sample. Hence the relative change of K48, K63 and K11 signature peptides from only one of the samples has been shown in Figure 6.12. UBE3C being a proteasome associated E3 ligase, the knockdown of the enzyme may in theory lead to a decrease in unanchored polyubiquitin chain, which was supported by Figure 6.12. The decrease in K48 and K11 linked chains was much greater than that seen for K63 chains.

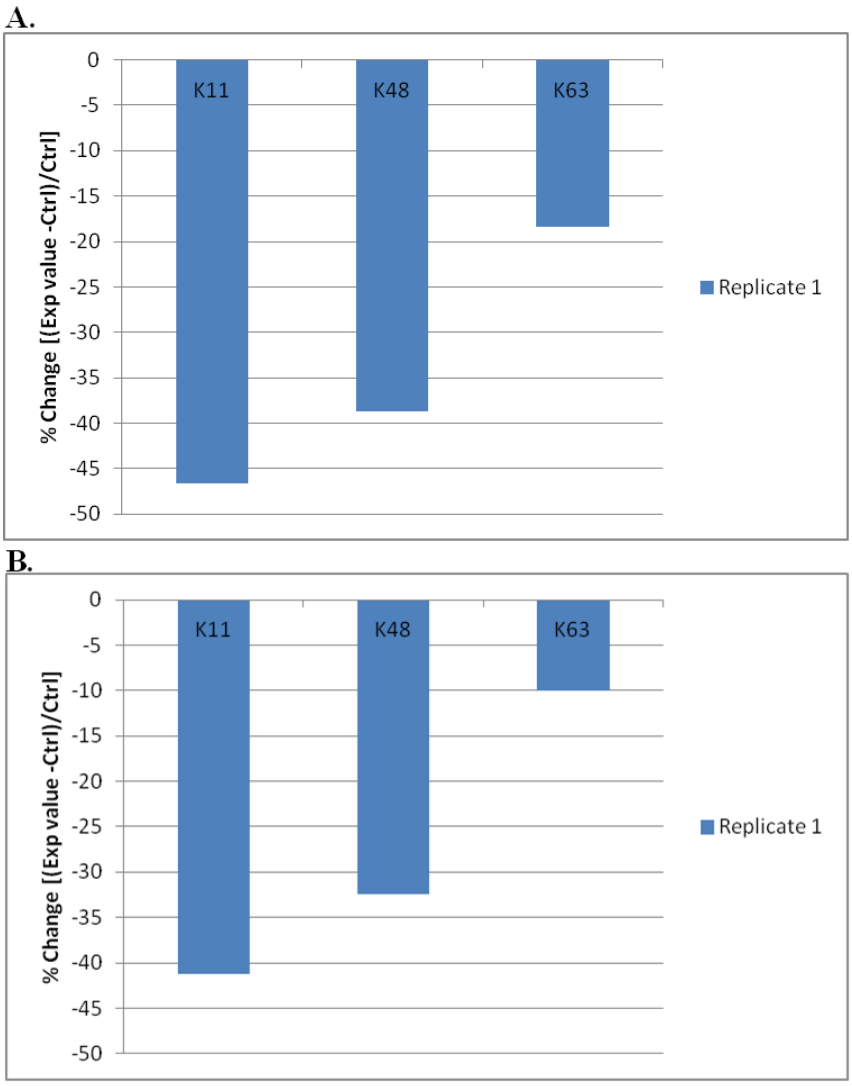


Figure 6.12. Analysis of data derived using SRM allows to detect relative changes in signature peptides corresponding to 3 linkages post knockdown of Ube3c in HEK293T cells. ~60 µl affinity purified endogenous unanchored polyubiquitin chains on bait were first eluted in 0.1% formic acid. The eluate was dried, resuspended in trypsinolysis buffer and subjected to three rounds of trypsin digestion with 1 µg sequencing grade Promega trypsin. The digests were analysed using MRM mode on QQQ LC-MS/MS. AUC values for each signature peptide were measured from corresponding chromatograms. These were normalised with either values for ubiquitin derived "TITLE" (TITLEVEPSDTIENVK) or "TLS" (TLSDYNIQK) peptides for the same sample (as described in 6.2.4). The normalised values for each of the three linkage peptides were then displayed as percent change when compared to their corresponding controls. Relative changes in three linkages – K11, K48 and K63, have been displayed which have either been normalised with **A.** TITLE or with **B.** TLS peptides. Data for only one of three replicated could be obtained for reasons explained in Section 6.2.2. Mass spectrometry data suggests reduction in levels of unanchored K48, K63 and K11 linked chains in response to reduction in UBE3C levels.

6.3. HEK293T CELLS SUBJECTED TO CONDITIONS OF THERMAL AND CHEMICAL STRESS

6.3.1. HEAT SHOCK TREATMENT OF HEK293T CELLS

6.3.1.1. AFFINITY PURIFICATION AND VISUALISATION OF UNANCHORED POLYUBIQUITIN CHAINS USING WESTERN BLOTTING POST INDUCTION OF HEAT SHOCK RESPONSE

HEK293T cells grown (in 10 cm cell culture plates) in DMEM supplemented with 10% FBS and 1% Pen-Strep at were allowed to attain ~90% confluency. These cells were grown in an incubator at 37^{o}C and 5% CO_2 concentration. An hour prior to lysis, culture plates were moved to an incubator with temperature set at 42^{o}C (and 5% CO_2). The specific temperature was selected as it matched the experimental condition of a similar study mapping effect of heat shock on ubiquitin linkages among protein conjugates [42]. An equal number of culture plates, corresponding to the control set, were retained at 37^{o}C. Once cells were exposed to elevated temperatures for 60 minutes, media from these plates was first discarded and cells were gently washed in sterile PBS. Cells were then immediately lysed in homogenising buffer and protein content for both heat treated sample and control were estimated using a BCA assay. A total of 24 mg of protein (2.6 mg/ml) were used for processing, subjected to heat treatment and affinity purification with 150µl immobilised Znf-UBP domain (at concentration of 10 mg/ml). Three repeats of the experiment were performed.

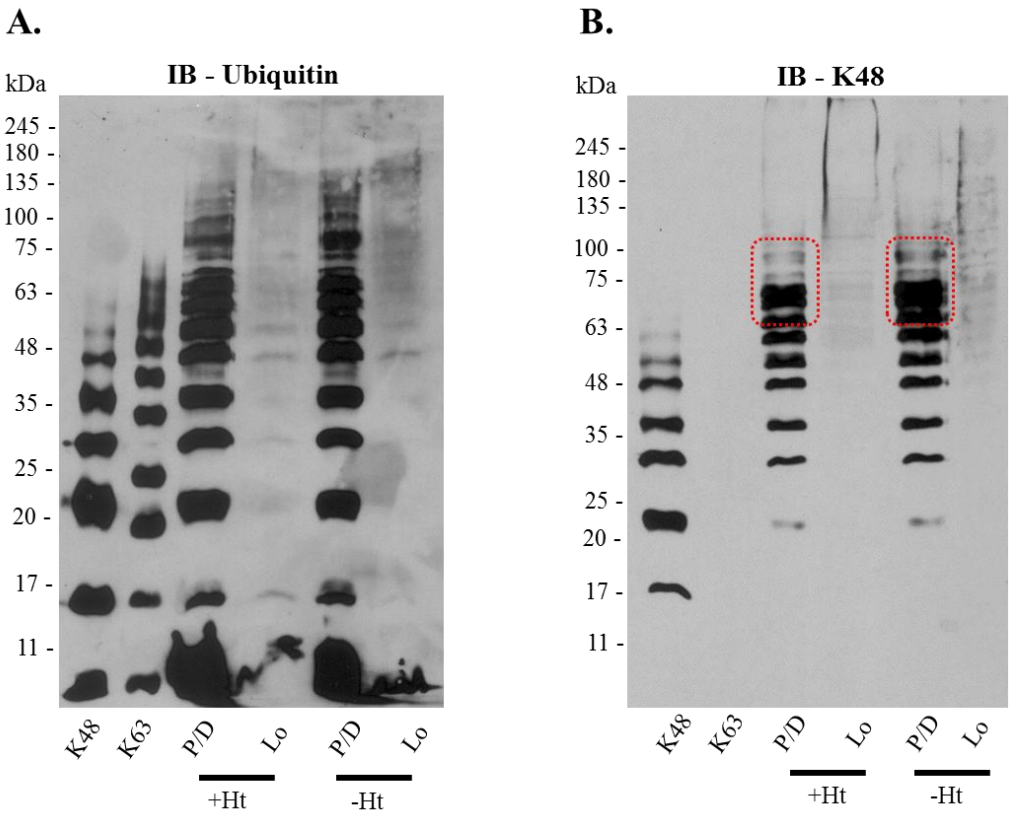


Figure 6.13. Immunoblotting of affinity purified endogenous unanchored polyubiquitin chains from HEK293T cells subjected to heat shock treatment (at 42^{o}C for 1 hour). Cells which were ~90% confluent were either moved to an incubator set at 42^{o}C or left at 37^{o}C an hour prior to lysis (CO_2 level of 5%). Cells were then homogenised in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% ($^{v}/_{v}$) IGEPAL (NP-40), 5 mM NEM, 20 µM MG132, 0.1% mammalian protease inhibitor cocktail. 24 mg proteins from cell extract was pre-treated (~ 2.5 mg/ml concentration of cell extract) and was incubated with 150 µl Znf-UBP tagged Sepharose (10 mg/ml bait per volume of beads) in a polypropylene column overnight. 50 µl sample on beads were eluted in GAB and 33% each used for western blotting with **A.** ubiquitin and **B.** K48 specific antibodies. Representative blots are displayed here (n=3). Lanes containing affinity purified samples in western blots were labelled P/D (pull-down). Also used in the blot were K48 and K63 commercial ladders and 50 µl each of the sample loads (Lo). Subjective visual assessment of the ubiquitin probed blot could not confirm change in unanchored polyubiquitin profiles. However, a consistent change was observed in K48 linked subset of these chains, wherein there was a reduction of K48 chains containing 10-14 ubiquitin moieties (~65 – 100kDa, marked in red).

While 50 µl purified sample on beads was eluted in GAB and subjected to western blotting, 100 µl was eluted in 0.1% formic acid and used for quantitative analysis using MS. Western blots generated using 33% each of eluted sample (and respective controls) in GAB were probed with ubiquitin and K48 polyubiquitin specific antibodies. The blots also included commercial K48 and K63 ladders as positive controls. Visual assessment of representative anti-ubiquitin immunoblot (out of three repeats, Figure 6.13.A.) was not conclusive in revealing consistent differences in cellular polyubiquitin levels. However, the western blot probed with K48 linkage specific antibody consistently displayed reduction in K48 linked endogenous unanchored polyubiquitin pools, specifically the bands between 65 – 100 kDa (highlighted in Figure 6.13.B). The bands in question would correspond to chains containing between 9-13 ubiquitin moieties.

6.3.1.2. QUANTITATIVE ANALYSIS OF CHANGES IN HEK293T DERIVED UNANCHORED POLYUBIQUITIN CHAINS IN RESPONSE TO HEAT SHOCK

Analysis of unanchored polyubiquitin chains purified from HEK293T cells subjected to heat shock (incubation at 42^{o}C for 1 hour) has been displayed in Figure 6.14. While the decrease in K11 and to a great extent in K63 chains was evident from MS analysis, K48 linkage surprisingly showed what could be interpreted as an increase. The analysed mass spectrometry data seemed to contradict the conclusion derived from visualising immunoblots.

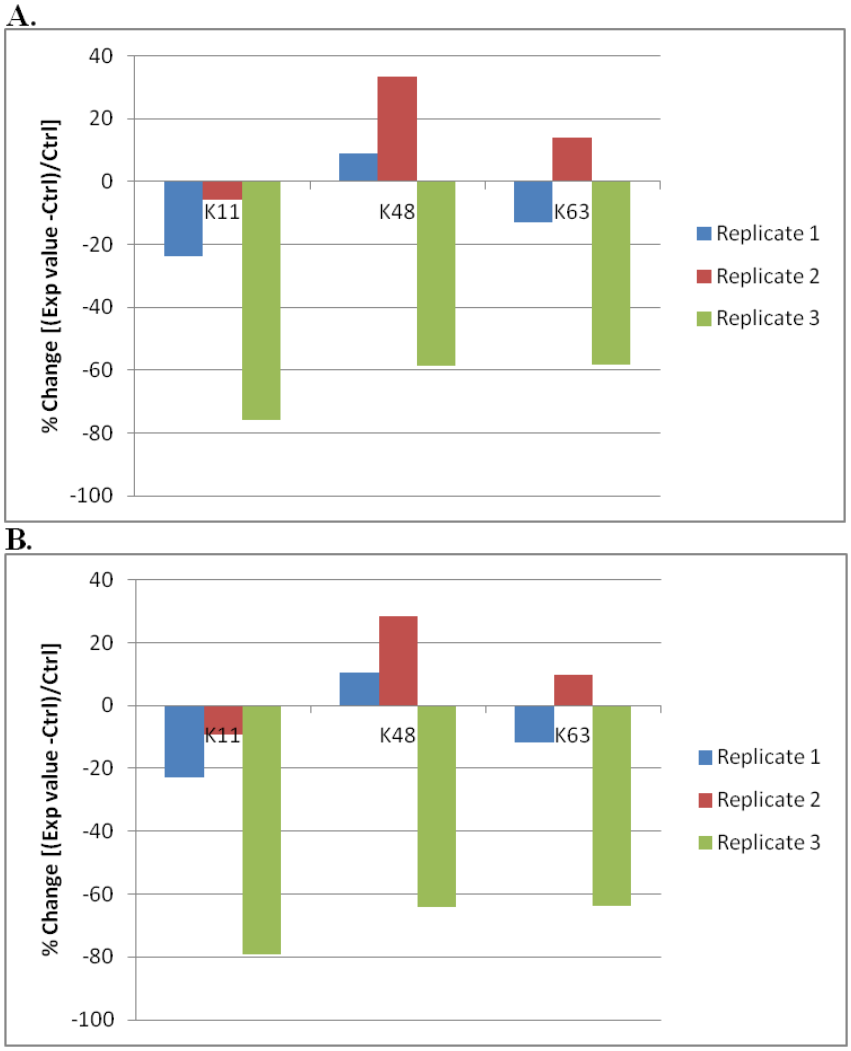


Figure 6.14. Analysis of data derived using SRM allows detection of relative changes in signature peptides corresponding to 3 linkages post heat shock treatment (42oC for 1 hour) of HEK293T cells. ~100 µl affinity purified endogenous unanchored polyubiquitin chains on bait were first eluted in 0.1% formic acid. The eluate was dried, resuspended in trypsinolysis buffer and subjected to three rounds of trypsin digestion with 1 µg sequencing grade Promega trypsin. The digests were analysed using MRM mode on QQQ LC-MS/MS. AUC values for each signature peptides were measured from corresponding chromatograms. These were normalised with either values for ubiquitin derived "TITLE" (TITLEVEPSDTIENVK) or "TLS" (TLSDYNIQK) peptides for the same sample (as described in 6.2.4). The normalised values for each of the three linkage peptides were then displayed as percent change when compared to their corresponding controls. Relative changes in three linkages – K11, K48 and K63, have been displayed which have either been normalised with **A.** TITLE or with **B.** TLS peptides. While levels of K11 linked chains consistently showed a reduction following heat shock treatment, the pattern of change in K48 and K63 linked endogenous unanchored chains were not as clear. The levels of K48 chains showed a possible increase while K63 linked unanchored polyubiquitin levels appeared to drop as a result of heat shock treatment.

6.3.2. TREATMENT OF HEK293T CELLS WITH PROTEASOMAL INHIBITOR MG132

6.3.2.1. AFFINITY PURIFICATION AND VISUALISATION OF UNANCHORED POLYUBIQUITIN CHAINS USING WESTERN BLOTTING POST MG132 TREATMENT

This experiment involved the treatment of cells with proteasomal inhibitor MG132. DMEM media from cell culture plates containing HEK293T cells at ~80% confluency was first discarded and replaced with 10 ml media containing either 1 µM MG132 (from a 10 mM stock in DMSO, 1 µl used), 1 µl DMSO (volume used during MG132 addition), or media alone. Cells were incubated in media containing MG132 (and controls) were incubated at 37^{o}C for a duration of 16 hours, as described in [172]. Post incubation, the media was discarded and cells were gently washed with sterile PBS. Cells were then lysed in homogenising buffer, and protein content estimated. BCA assays revealed the total protein content in MG132 treated cells were between 0.6 – 1.0 mg/ml lower than control, an indicator of the induction of cell death by apoptosis [208]. 16 mg proteins derived from each of the cell extracts of HEK293T cells were processed further and affinity purified using 100 µl immobilised Znf-UBP domain (at 10 mg/ml).

A volume of ~40 µl from total (100 µl purified sample on immobilised bait) affinity purified sample was eluted into GAB, and subject to immunoblotting with ubiquitin and K48 linkage specific antibodies, while the rest of the sample was devoted to MS analysis. The experiment was repeated three times and representative blots have been displayed in Figure 6.15. Surprisingly, although the loads derived from MG132 treated samples confirm accumulation of abundant ubiquitinated species (when compared to controls, indicated in red),

similar increase in unanchored polyubiquitin was not visible in the ubiquitin probed blot (Figure 6.15.A). This was contrary to what was observed previously upon MG132 treatment of U2OS and HeLa cells [172]. However, western blots probed with K48 specific antibody did confirm an increase in K48 linked unanchored polyubiquitin chains as a consequence of such proteasomal inhibition. A possible explanation about the discrepancy occurring with western blotting could be an error in normalising the starting material (cell extracts). As mentioned previously, MG132 treatment caused a decrease in protein content (presumably due to induction of apoptosis) compared to control.

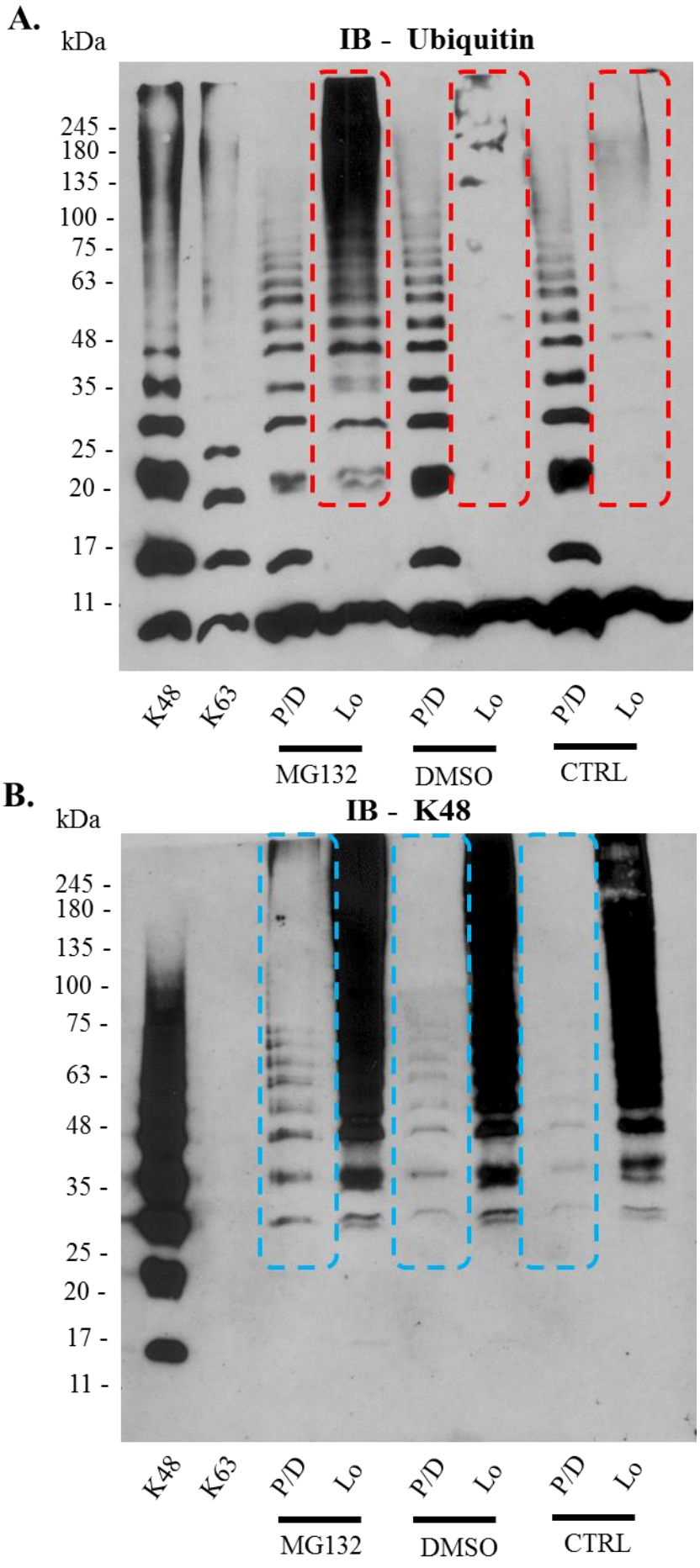

A.
kDa
IB - Ubiquitin
245 -
180 -
135 -
100 -
75 -
63 -
48 -
35 -
25 -
20 -
17 -
11 -
K48
K63
P/D
Lo
P/D
Lo
P/D
Lo
MG132
DMSO
CTRL
B.
kDa
IB - K48
245 -
180 -
135 -
100 -
75 -
63 -
48 -
35 -
25 -
20 -
17 -
11 -
K48
K63
P/D
Lo
P/D
Lo
P/D
Lo
MG132
DMSO
CTRL

Figure 6.15. Immunoblotting of affinity captured endogenous unanchored polyubiquitin from HEK293T cells, upon proteasomal inhibition. Cells which were 80-90% confluent were incubated in media containing with 1 μM MG132, vehicle (DMSO) or neither (negative control, media alone). Cells were homogenised 16 hours post treatment in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% ($^{v}/_{v}$) IGEPAL (NP-40), 5 mM NEM, 20 μM MG132, 0.1% mammalian protease inhibitor cocktail. 16 mg proteins from each cell extract was pre-treated (~ 2.0 mg/ml concentration of cell extract) and was incubated with 100 μl Znf-UBP tagged Sepharose (10 mg/ml bait per volume of beads) in a polypropylene column overnight. 40 μl sample on beads were eluted in GAB and 33% used western blotting with **A.** ubiquitin and **B.** K48 specific antibodies, representative blots are shown here (n=3). Lanes containing affinity purified samples in western blots were labelled P/D (pull-down). Also used in the blot were K48 and K63 commercial ladders and 50 μl each of the sample loads (Lo). Surprisingly, the ubiquitin immunoblot showed lower amount of total ubiquitin, even though the lanes containing load from MG132 treated samples showed considerably higher amounts of ubiquitin reactive species (indicated in red). However, the K48 immunoblot showed accumulation of endogenous unanchored polyubiquitin chains upon MG132 incubation (indicated in blue).

6.3.2.2. QUANTITATIVE ANALYSIS OF CHANGES IN HEK293T DERIVED UNANCHORED POLYUBIQUITIN CHAINS IN RESPONSE TO MG132 TREATMENT

Analysis of mass spectrometry derived data involved the comparison of signature peptide levels between MG132 treated samples and control (media alone). The results confirmed a marked increase (>90%) increase in K48 linkage (Figure 6.16). A significant increase of was visible with K11 linkages as well, but the change in K63 chains was not as clear with a possible increase of a much lower magnitude. The results obtained track what was seen with studies on conjugated ubiquitin which showed increase in K48 and K11 linkages but not K63[82]. MG132 is shown to act on the catalytic activity of the proteasome but not directly on the catalytic activity of proteasome associated DUBs, hence proteasomes could continue to contribute to the K48 (or even K11) linked endogenous unanchored polyubiquitin reservoir [171].

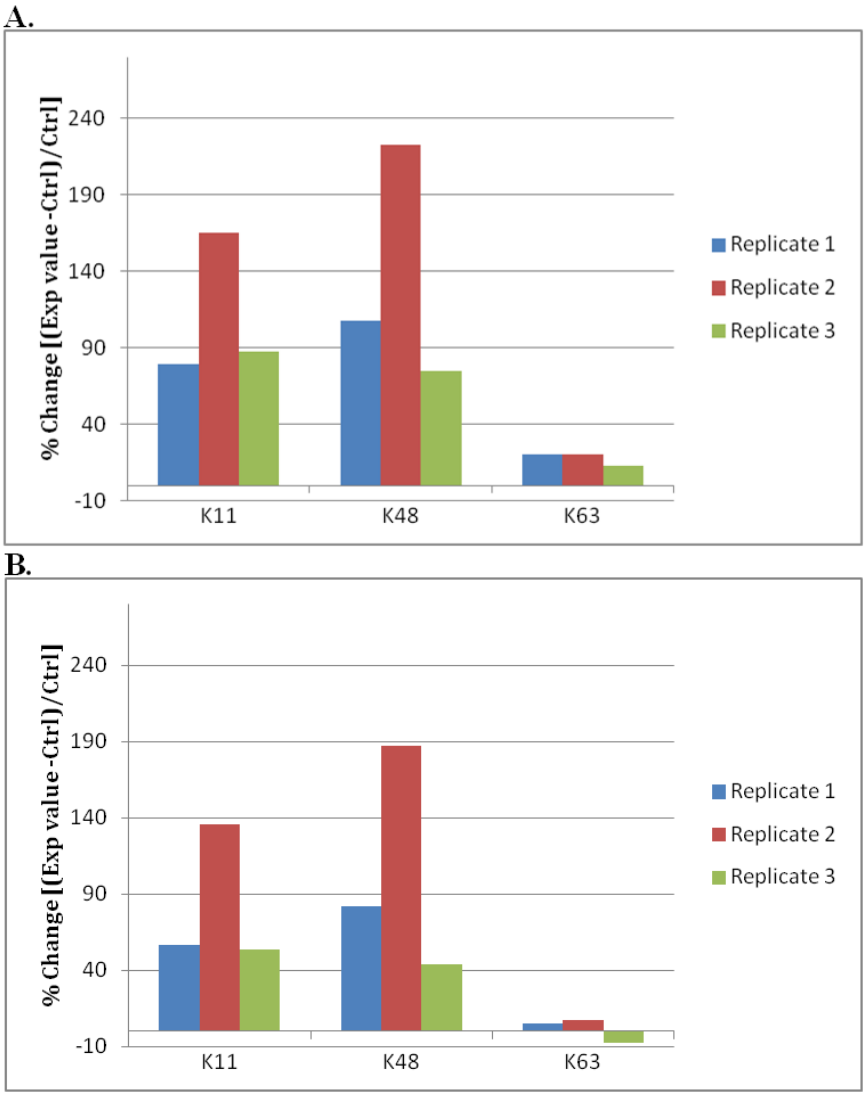


Figure 6.16. Analysis of data derived using SRM allows to detect relative changes in signature peptides corresponding to 3 linkages upon MG132 treatment of HEK293T cells. ~60 µl affinity purified endogenous unanchored polyubiquitin chains on bait were first eluted in 0.1% formic acid. The eluate was dried, resuspended in trypsinolysis buffer and subjected to three rounds of trypsin digestion with 1 µg sequencing grade Promega trypsin. The digests were analysed using MRM mode on QqQ LC-MS/MS. AUC values for each signature peptide were measured from corresponding chromatograms. These were normalised with either values for ubiquitin derived "TITLE" (TITLEVEPSDTIENVK) or "TLS" (TLSDYNIQK) peptides for the same sample (as described in 6.2.4). The normalised values for each of the three linkage peptides were then displayed as percent change when compared to their corresponding controls (media alone). Relative changes in three linkages – K11, K48 and K63, have been displayed for the three replicates which have either been normalised with **A.** TITLE or with **B.** TLS peptides. As anticipated, MG132 caused a marked elevation of K48 and K11 linked unanchored polyubiquitin. The effects on K63 linked chains were much more subtle and showed possibly a minor increase.

Since this setup varied from previous experiments discussed in this chapter for the use of two controls (DMSO and media alone), we had to confirm if DMSO itself contributed to changes observed upon MG132 treatment (since DMSO was used as the solvent). A comparison of levels of peptides (corresponding to each linkage) in DMSO treated cells confirmed that this was not the case and the observed changes were indeed a resultant of MG132 treatment.

CHAPTER 7

DISCUSSION

7.1. WESTERN BLOTTING CONFIRMED PURIFICATION OF TOTAL ENDOGENOUS UNANCHORED POLYUBIQUITIN FROM HEK293T CELLS

Chapter 3 summarises the methodology developed and refined for the capture of unanchored polyubiquitin chains from HEK293T cells, along with proof of their integrity (i.e. that they are non-substrate linked) and (using selective antibodies) confirms the presence of at least one isopeptide linkage (K48) in the purified samples. The chapter also presents limitations encountered during characterisation of unanchored polyubiquitin chains using western blotting with the current commercially-available antibodies.

7.1.1. COMPARISON WITH OTHER WORK UNDERTAKEN FOR THE STUDY OF UNANCHORED POLYUBIQUITIN

While the existence of unanchored polyubiquitin chains and their effects on proteasome function and ubiquitin-dependent kinase activity is well-established [107, 108], surprisingly there has been relatively little emphasis on their purification and detailed analysis of their molecular composition. Prior to our groups development of a strategy to purify unanchored polyubiquitin, confirmation of their nature was previously determined by simply subjecting cell extracts to 1D or 2D gel electrophoresis under denaturing conditions, immunoblotting with a ubiquitin-specific antibody and comparing spot mobility to a sample of *in vitro* synthesised polyubiquitin ladder [107, 109].

Indeed the significance and relevance of unanchored polyubiquitin chains in kinase activation was initially proposed in part based on indirect evidence based on their sensitivity to IsoT treatment [1]. The same group later

developed a strategy to release cellular unanchored polyubiquitin chains from proteins shielding them (such as RIG-1), by utilising the stability of ubiquitin to NEM (due to lack of cysteine residues) and heat treatment [126]. As a result majority of proteins (including DUBs) in the cell extract were denatured and precipitated.

Based on these previous studies, homogenising buffer used in our *in house* method was supplemented with NEM to effectively inhibit DUBs. The excess NEM is later quenched using DTT for reasons explained below. The other aspect of our protocol influenced by previous work was incubating cell extract at 75^{o}C for 20 minutes (i.e. heat treatment) followed by centrifugation, thus clearing the extract from heat labile proteins while having little detrimental effect on (heat stable) unanchored polyubiquitin chains. Significantly our purification protocol relies on the use of the Znf-UBP domain from the DUB IsoT, which has been recognised for its selectivity and high affinity binding to the diglycine motif at the 75th and 76th positions of ubiquitin. Since the motif is only presented by free monoubiquitin and unanchored polyubiquitin chains, affinity purification with this domain only captures these fractions of the cellular ubiquitome, while ubiquitinated proteins are either precipitated by heat treatment or unable to bind to the immobilised bait protein. Quenching of excess NEM by DTT treatment was essential to prevent cysteine residues of bait protein being modified by residual NEM. The validity of the technique was confirmed by western blots (Figure 3.1.E), which showed unanchored polyubiquitin chains can be purified from HEK293T cells. An indicator that the purified ubiquitin-modified material was indeed unanchored polyubiquitin

was the presence of discrete bands as opposed to smears equivalent to mixtures of ubiquitinated substrate proteins (incidentally, as seen when sample preparation does not include the heating step). The number of ubiquitin moieties in the purified polyubiquitin chains could also be deduced by comparison with migration in comparison with commercial polyubiquitin ladders (K48 and K63-linked) used in parallel.

7.1.2. EXPLORING THE HEK293T-DERIVED UNANCHORED POLYUBIQUITIN CHAIN PROFILE THROUGH WESTERN BLOTTING

The refinement of the purification strategy and successful purification of unanchored polyubiquitin chains from HEK293T cells has been detailed in sections 3.2 and 3.3. This was the first time the strategy was applied to capture of endogenous unanchored polyubiquitin chains from cultured cells. We also employed the use of deubiquitination assays to confirm the nature of purified material, and investigated if heat treatment was a contributing factor to loss of K63-linked polyubiquitin chains. The amount of bait protein required for complete capture of unanchored polyubiquitin chains was estimated (3.2.1.) to avoid this being a limiting factor for quantitative capture. Polyubiquitin chains containing in excess of 10 ubiquitin moieties in purified fractions (Figure 3.2) could be confirmed by western blotting, consistent with what was seen previously for the capture of unanchored polyubiquitin chains from mouse skeletal muscle tissue [174]. All western blots of Znf-UBP affinity purified samples were derived from HEK293T cells under 'basal' conditions i.e. prepared under standard growth conditions, implying the presence of a reservoir of such chains in mammalian cells. The source of these polyubiquitin chains is not entirely clear, with possibility of being assembled *de novo* by E3

ligases [1, 126], generated as a product or processing of ubiquitinated substrates (e.g. by proteasome-associated chain amputating DUBs) [170, 171] or through the novel mechanism of aggresome assembly by the action of DUB ataxin-3 and their clearance by proteasome associated POH-1 [133, 134]. A recent study also confirmed the role of POH-1 in producing unanchored polyubiquitin chains en masse (from ubiquitinated substrates at the proteasome) with preference for K63 linkage [162].

Western blots also confirmed presence of K48 the linkage in the unanchored polyubiquitin chains, which perhaps not surprising as several global ubiquitin isopeptide linkage analyses have described this linkage to be especially abundant *in vivo*. For example studies carried out in yeast suggest the K48 linkage to be the second most abundant [184], or even the most abundant [40, 82] among all polyubiquitin chain types. Similar results have been seen in HEK293 cells [42, 78]. However it is important to note that these estimates were derived by the use of mass spectrometry-based methods.

One point that was especially noteworthy was the length of the purified endogenous unanchored polyubiquitin chains, composed in excess of 10 ubiquitin moieties. This is of interest since the minimum requirement for proteasomal recognition of substrates for degradation is covalent attachment of a ubiquitin chain of only four moieties [84]. However, monoubiquitination of smaller peptides has also been shown to be sufficient to initiate proteasomal processing [209, 210]. Hence these unanchored polyubiquitin chains being

derived from substrate linked ubiquitin chains post proteasomal processing is debatable, as longer chains can be argued to be dispensable.

Probing for the presence of K63 and K11 polyubiquitin linkages in the purified sample was inconclusive. While the commercial K63-specific antibody seemed to lack selectivity, the K11 antibody failed to detect presence of any unanchored polyubiquitin chains containing the linkage (Figure 3.2.), even though chains of the topology have been observed in both yeast (relatively abundant) and in mammalian cells. The inability to confirm or detect the presence of such linkages further reaffirms the requirement of high sensitivity mass spectrometry-based approaches.

The validation of the *in house* method as being specific for unanchored polyubiquitin chains was made by performing DUB assays with IsoT and the catalytic core of the USP2 DUB. While the former selectively disassembles unanchored polyubiquitin chains [57], the latter lacks such selectivity and targets both unanchored and substrate bound polyubiquitin chains [16]. As seen in Figure 3.5., the purified chains from HEK293T cells were sensitive to both IsoT and USP2 treatment which confirmed that these were indeed unanchored polyubiquitin.

7.1.3. ATTEMPTS TO STABILISE AND MAXIMISE RECOVERY OF UNANCHORED POLYUBIQUITIN CHAINS FROM HEK293T CELLS

The apparent lack of K63 or K11-linked polyubiquitin chains in affinity-purified samples suggested a possibility of certain chain topologies being labile and perhaps susceptible to precipitation during heat treatment [192]. In

addition, recent work on the role of phosphorylation of ubiquitin moiety has yielded interesting results [211]. The difficulty in assembling polyubiquitin chains by the cellular machinery, especially with respect to K63 linked chains, and the decreased activity of DUBs on phosphorylated polyubiquitin chains may explain the relative lack of abundance of K63 chains [211].

Thus far, confirmation of presence of unanchored polyubiquitin chains (and isopeptide linkage analysis) was only carried out through western blotting analyses. Hence, the lack of the linkage types may have been a result of the protocol or sensitivity of detection. Section 3.4 details an attempt to verify the possibility of loss of "exogenous" unanchored polyubiquitin chains at various stages of the affinity purification strategy. As described, loss of exogenous K63-linked chains was seen during the course of the purification. However, heat treatment was considered to bring about the greatest losses of exogenous K63-linked unanchored polyubiquitin chains, which may have explained the lack of detection of endogenous K63-linked chains derived from HEK293T cell extract. A possible solution was to bypass the heat treatment step completely and the use of an additional DUB inhibitor during affinity purification. Initial attempts to capture unanchored polyubiquitin chains using the refined strategy appeared satisfactory with the requirement of double the amount of bait to completely deplete unanchored chains (as observed by western blotting). However direct comparisons between samples purified using both strategies suggested the possibility of capture of substrate linked polyubiquitin chains when the heating step was omitted, an observation that came about because of the appearance of high molecular smear in

immunoblots (as opposed to the expected individual bands). The fact that samples purified without heat treatment were not exclusively unanchored was confirmed from deubiquitination assays (Section 3.5.2). The complication of such non-specific interactions, resulting in enrichment of substrate linked polyubiquitin chains, prompted us to revert to retaining the heat treatment step in our affinity purification strategy. Further confirmation and characterisation of unanchored polyubiquitin chains using mass spectrometry approaches is discussed in Chapters 5 and 6.

7.2. MASS SPECTROMETERS ENABLED QUANTITATIVE ANALYSIS OF UBIQUITIN LINKAGES

Our group was fortunate to have access to three mass spectrometers. Since, the study focuses little on the general design and working principle of MS *per se*, but more on their application in our specific area of interest, the former has not been explored in detail. Chapter 4 instead focuses on the mass spectrometers available for use in our study, their possible applications and limitations in the context of qualitative and quantitative characterisation of endogenous unanchored polyubiquitin chains. The mass spectrometers available for our study were – MALDI-ToF and ESI based QTof and QqQ. In addition, the chapter includes a detailed description of the AQUA strategy which was used for estimating abundance of isopeptide linkages in purified unanchored polyubiquitin chains. Also, data generated from two mass spectrometers have been analysed and presented.

7.2.1. LASERTOF AND QTOF COMPLEMENT DATA GENERATED BY LC-MRM-MS/MS ANALYSIS USING QQQ

Each of the mass spectrometers used - LaserToF, QToF and QqQ were useful for different aspects of our study. The LaserToF was not be used for quantitations and had limitations when used for qualitative analysis of peptide mixtures due to ion suppression. However, analysis by the instrument required very little sample which once analysed, could again be reanalysed at a later date. As a result, samples could be subjected to analysis by LaserToF immediately post-trypsinolysis, to possibly check for the presence of signature peptides, missed cleavages or undigested ubiquitin.

Both the ESI based instruments namely the QToF and QqQ were extensively used for our study. The QToF offered a sensitive and accurate means to detect presence of ions (of analyte) by LC-MS, but lacked linear dynamic range for quantitative analysis. Tandem MS/MS experiments were previously carried out by our group, but the technique had limited applications with respect to detection of isopeptide linkage-specific signature peptides in a complex mixture such as affinity purified cellular unanchored polyubiquitin chains. The data however could be subjected to sequence analysis manually. The accurate mass determination of precursor and fragment ions (produced by CID) were however useful in determining SRMs for use with the triple quadrupole.

The triple quadrupole may lack the mass range of the LaserToF or the sensitivity and resolution of the QToF, but offers remarkable selectivity for parent and product ions using corresponding SRMs (section 4.4). This accompanied by the linear dynamic range makes it a valuable tool for

quantitative proteomics. The application of the QqQ for quantitation was evident from the AQUA standard curves generated which displayed linearity over a wide range of dilutions (amount per sample). These standard curves (obtained while analysing samples) could now be used for accurate estimation each signature peptide thus estimating each linkage type in samples of affinity purified endogenous unanchored polyubiquitin chains.

The caveats noted in LC-MRM-MS/MS analysis were the inability of the QqQ to detect K29 peptides and a proportion of K6 signature peptides being oxidised (at N-terminal methionine). While a separate SRM for oxidised K6 peptides was used to negate loss of signal due to oxidation, a possible fix to the problem would be to treat the samples with H_2O_2 to promote complete oxidation of K6 [185]. Detection of K29 though would require the use of TFA in place of FA [212]. Also, the occasional problems with separation by HPLC during LC-MRM-MS/MS analysis could result in lack of credible data.

7.3. EXTENSIVE OPTIMISATION OF TRYPSINOLYSIS ALLOWED FOR THE ESTIMATION OF ISOPEPTIDE LINKAGES IN PURIFIED CELLULAR UNANCHORED POLYUBIQUITIN CHAINS BY LC-MRM-MS/MS ANALYSIS

Chapter 5 is a compilation of challenges encountered and experiments undertaken to optimise one of the crucial steps in our *in house* platform for the purification and characterisation of endogenous unanchored polyubiquitin chains – efficient and complete digestion of unanchored polyubiquitin chains.

7.3.1. COMPLETE DIGESTION OF UBIQUITIN COULD NOT BE ACHIEVED BY OUR STANDARD *IN HOUSE* (*IN SOLUTION*) TRYPSINOLYSIS PROTOCOL

The relative inefficiency of established generic trypsinolysis protocols in use in our lab (and more widely) to completely (or close to completion) digest ubiquitin chains was surprising to begin with. We set out to drive complete trypsinolysis purely for MRM analysis; hence amount of trypsin would not affect analysis as any trypsin derived peptides would be ignored. Also, we had to minimise loss of purified sample and digested products thus formed.

Groups around the world which have previously worked on elucidating and quantifying cellular polyubiquitin linkages have employed both *in gel* trypsinolysis [42, 82, 175] and *in solution* [78] trypsinolysis protocols to suit their objectives. *In gel* digestion involves separation of samples by SDS PAGE prior to being treated with trypsin. While this offers an added dimension of separation, the method was avoided as it involved multiple manipulations, was prone to contamination while handling and resulted in losses [213, 214]. Hence, we set out on developing a protocol which would specifically enable complete digestion of polyubiquitin chains and release of signature peptides for quantification. The first step was to apply the generic *in house* protocol routinely being used for in-solution digests (Section 5.1). As evident, the increase in amount of or multiple additions of trypsin did not result in complete trypsinolysis of monoubiquitin. The contribution of buffers used or treatments like heating had to be looked into in order to achieve complete trypsinolysis.

7.3.2. EXTENSIVE TRYPSINOLYSIS OPTIMISATIONS CONDUCTED REVEALED 40 % MECN ($^{v}/_{v}$ IN 50 mM TRIS-HCl pH 7.6) CONSISTENTLY AND COMPLETELY DIGESTED UBIQUITIN AND POLYUBIQUITIN CHAINS.

We began our trypsinolysis optimisations by using bovine monoubiquitin as substrate for digestion using a total of 16 conditions (Section 5.2). These included chaotropic agents - urea and guanidine-HCl, organic solvent MeCN (at 40% and 80% $^{v}/_{v}$) and even the use of acid labile surfactant RapiGEST™. Another parameter included in these optimisations was the amount of trypsin which, with a trypsin-substrate ratio of 1:10, 1:2 and 1:1 being tested. The efficiency of digestion was measured first by visualising disappearance of substrate (ubiquitin) in Coomassie stained SDS PAGE gels, and by measuring release of ubiquitin derived peptides (TITLE, EST and EGI) upon trypsinolysis by LC-MRM-MS/MS. The latter required measurement of signal of each peptide, normalising to the maximum signal achieved for the peptide among all digest conditions tested and expressing digestion efficiency as a cumulative normalised percentage value. Thus the maximum score for a condition would be 300, in case it could release yield maximum amounts of all three peptides. Disappearance of ubiquitin in gels alone was not considered as a confirmation of complete trypsinolysis, as this could possibly be a resultant of loss of protein (possibly by heating, sample handling).

Based on visualising Coomassie stained gels (Figure 5.3) and outcome of MRM analysis (Figure 5.4), it was obvious that presence of organic solvent MeCN allowed for efficient digestion of ubiquitin. What was also noteworthy

was the yield which was higher at lower incubation times (2 hours). The experiment also suggested the use of higher trypsin concentrations increased trypsinolysis, suggesting use of trypsin in excess to avoid the possibility of it being a limiting factor. We were surprised at the limited digestion achieved by the use of RapiGEST™, however the number of steps involved would not have made the use of the surfactant very efficient. The same argument would hold true for the use of 6M Guan-HCl. Use of urea in digestions did look promising during initial experiments carried out by our group (and suggested in [185]), hence the extent of digestion achieved was surprising.

A follow-up experiment (Section 5.3) was carried out to verify if the use of 40% MeCN did indeed provide a significant advantage over the use of urea. This time, we also tracked the progress of digestion over time. As with the previous setup, it appeared that efficient (and possibly complete) trypsinolysis was achieved using 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$ as compared to 4 M urea in 200 mM AmBic, pH 8.0. As the value of recovered peptides (when digested in condition containing MeCN) did not increase after the third addition of trypsin, it could be argued that trypsinolysis was complete.

While it did initially appear as though we had arrived on the best condition for (trypsin) digesting and quantifying purified unanchored polyubiquitin chains, we decided to ascertain the specific component that contributes towards effective digestion. The two buffer conditions 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$ (referred to as T1) and 4 M urea in 200 mM AmBic, pH 8.0 (referred to as T2) along with 12 others which were derivatives of T1 and

T2 were tested for efficiency of trypsinolysis (Section 5.4). The study revealed some rather surprising results. C9 (4M urea in 50 mM AmBic pH 8.0, 10 mM $CaCl_2$) outperformed T1 by producing the maximum yield of 2 of the three ubiquitin derived peptides, even though T1 did result in high yield. Also surprising was the yield achieved upon trypsinolysis with the buffers (50 mM Tris-HCl pH 7.6 or 200 mM AmBic pH 8.0), without the addition of MeCN or urea. The experiment was repeated, this time narrowing the number of digest conditions to 5 of the best from the previous experiment – T1, C1, C6, C9 and C12). We also decided to test digestion in 50 mM AmBic pH 8.0 and also increasing the concentration of MeCN to 80% $^{v}/_{v}$, the latter did provide high yield of tryptic peptides when previously used in Section 5.2. The outcome of the experiment (Figure 5.8), prompted us to settle on T1 as our final optimised digest condition as it promoted complete, consistent and efficient trypsinolysis.

Through the course of the optimisations, the limitation of the mass spectrometers was also made obvious. We did encounter experiments where digest conditions did not yield expected results. The apparent inconsistency of the instrument could not be avoided and must be taken into account when discussing results obtained.

Before progressing to digesting purified unanchored polyubiquitin chains, we were keen on testing our optimised digestion condition to trypsinolyse commercial ubiquitin chains at our disposal (Section 5.5). These included polyubiquitin ladders of either K48 or K63 linkages or diubiquitin containing either K11 or K33 linkages (Table 5.7). Unlike the previous experiments, this

linkage specific signature peptides released on trypsinolysis were quantified over the course of four rounds of trypsinolysis. Trypsinolysis appeared to reach completion after the third round in case of K11, K33 and K48 linkages, while K63 appeared to require a fourth addition (Figure 5.9). However upon further scrutiny, it was noted that 90% digestion was achieved after the second round, thereby suggesting that digestion could well have been completed after the third round in case of K63 linked chains as well. Disappearance of ubiquitin positive bands upon western blotting of digested material also suggested complete trypsinolysis of samples (Figure 5.10).

7.3.3. *IN-HOUSE* PROTOCOLS ALLOWED FOR THE FIRST TIME, CONFIRMATION OF AND QUANTIFICATION OF CELLULAR UNANCHORED POLYUBIQUITIN CHAINS AND THE LINKAGES CONTAINED

Thus far we had successfully optimised the protocols leading up to LC-MRM-MS/MS, namely affinity purification process (Chapter 3) and complete trypsinolysis. What remained was to apply the setup on purified samples, thereby leading to qualitative and quantitative analysis of purified unanchored polyubiquitin chains from HEK293T cells (Section 5.6). For this purpose, we first purified 450 µl of unanchored polyubiquitin chains from HEK293T cells. Elution of purified chains (using 0.1% $^{v}/_{v}$ in H_2O) was first confirmed by western blotting (Figure 5.11). Though there were small losses particularly in the higher order chains, majority of the sample appeared to have been eluted, confirmed also by the immobilised bait lacking significant ubiquitin positive material post-elution.

250 µl of sample on immobilised bait was then eluted, dried and trypsinolysed in 40% MeCN in 50 mM Tris-HCl pH 7.6, 10 mM $CaCl_2$. The digests were then submitted to LC-MRM-MS/MS using SRMs corresponding to linkage specific signature peptides. The experiment was also used to test reproducibility of results obtained using the mass spectrometer and also the linearity which was important in estimating quantity of each signature peptide. An overlay of chromatograms of repeats obtained by submitted 25 % of digested material (twice) for MRM analysis suggested reproducibility (Figure 5.12). Reproducibility was also confirmed looking at the estimated amounts of each signature peptide in sample (Table 5.8). This was for the first time that we could confirm detection of K11, K27, K48 and K63 linkages in unanchored polyubiquitin fraction of HEK293T cells. K63 accounted for over 80% of linkages purified from HEK293T cells at basal growth conditions. This was surprising considering all studies measuring ubiquitin linkages in total ubiquitome estimate the relative proportion of K48 to be far greater than K63 in both *Saccharomyces cerevisiae* [40, 82] and cultured cell lines including HEK293 [42, 78]. The proportion of all detected linkages were K63 (83%), K11 (9%), K27 and K48 (3.7-4.0%). This was the only attempt at quantifying isopeptide linkages in purified cellular unanchored polyubiquitin chains, hence there was no published data for comparison. The data though does fit in well with the possible role (possibly requirement) of unanchored K63 polyubiquitin chains in cell signalling [1]. It was also important to note that K6, K29 and K33 linkages were not detected in our analysis. Also, it is worth noting that in a previous study, substrate linked K63 and K33 were not completely degraded when trypsinolysed in solution [41]. Another significant observation was that

although we could confirm the presence of four isopeptide linkages, the magnitude of ubiquitin derived peptide (TLS and TITLE) was much greater. This would indicate that when compared to unanchored polyubiquitin chains, cellular free monoubiquitin were the dominant ubiquitin species.

For further validation of our findings, a fraction of purified unanchored polyubiquitin chains from HEK293T cells was trypsinolysed and analysed by the University of Leicester on a collaborative basis using the LTQ-Orbitrap instrument, which offered greater sensitivity. Independent qualitative analysis by their group confirmed the presence of ubiquitin derived peptides (TLS and TITLE), along with presence of K48, K63 and K11 linkages (Figure 5.13). We now had indisputable evidence of the presence of unanchored polyubiquitin chains of at least three different linkage types in cultured HEK293T cells, and also that we could confirm the effectiveness of our *in-house* platform in their characterisation.

7.3.4. CAVEATS AND INTRODUCTION OF ERROR IN QUANTIFICATION OF ENDOGENOUS UNANCHORED POLYUBIQUITIN CHAINS.

While the successful application of in-house platform in purifying and characterising cellular unanchored polyubiquitin chains was indeed an exciting achievement, it was essential to acknowledge the possible caveats or inconsistency of results that may arise at various stages. These have been described in this section.

- To avoid possible fluctuations in detected unanchored polyubiquitin chain linkages, care was taken to lyse HEK293T cells at the same stage

(determined by cell confluency – usually kept at ~90%, usually achieved 48 hours after seeding).

- Affinity purification of unanchored polyubiquitin chains from cells required multiple manipulations to sample resulting in loss of sample. Multiple rounds of centrifugation and washing of sample on immobilised bait were the steps which were most likely to result in sample losses.
- Washing of affinity purified endogenous unanchored polyubiquitin chains (on beads) was critical for two reasons. Firstly, it eliminated (minimised) non-specific interactions. Secondly, remnants of detergent (NP-40) if present in affinity purified samples would adversely affect analysis by MS [215]. Hence, three rounds of washes was performed with wash buffer (as described Chapter 3) followed by 3 rounds of washes with deubiquitination assay buffer.
- Sample was always eluted in excess 0.1% ($^{v}/_{v}$) formic acid (10X the volume of sample on beads), to minimise sample losses. Also, the number of surfaces encountered by the polyubiquitin chains was kept to a minimal.
- Trypsin digestion of eluted sample of affinity purified unanchored polyubiquitin chains were carried out in the same microcentrifuge tube these samples were eluted into (in 0.1% formic acid). All manipulations leading to analysis of digested products on MS were carried out in the same tubes.

7.4. ELUCIDATION OF CHANGES IN UNANCHORED POLYUBIQUITIN POOLS HEK293T CELLS AT NON-BASAL CONDITIONS

The aim of the exercise was to develop *in house* capability for the complete purification of endogenous unanchored polyubiquitin chains from cells and eventually their qualitative and quantitative characterisation. While we have been successful in the purification of such chains from HEK293T cells (Chapter 3) and the successful identification and estimation of isopeptide linkages contained (Chapter 5). Chapter 6 was an attempt to measure changes occurring when cells were exposed to various stimuli – including activation of signalling, modulation of known and novel regulators of unanchored polyubiquitin chains and proteolytic stress. Briefly, unanchored polyubiquitin chains were purified from HEK293T cells in response to stimulation with IL-1β (activation of signalling), modulation of known and novel regulators of unanchored chains – IsoT and UBE3C and in response to stress including heat shock and proteasomal inhibitor MG132. It is important to note that the volume of immobilised Znf-UBP bait on beads used varied (as indicated) in some experiments, depending on amounts of starting materials (influenced by the availability of reagents). Three repeats of each condition were performed and affinity purified samples were divided to be analysed separately by western blotting (using antibodies specific for ubiquitin and K48 and in case of activation of signalling, K63 as well) and mass spectrometry (to monitor changes in levels of K48, K63 and K11). However, though there were some

promising observations, the attempt exposed inconsistencies and caveats in the platform developed.

The most important of these which affected data generated from all conditions was the inability to produce reliable mass spectrometry data. This was rather frustrating as considerable resources were directed towards optimisation of the process. What made this even more surprising was previous successful implementation of ubiquitin linkage specific AQUA standards for similar analysis in studies about complete ubiquitome [42, 78, 175]. A point to note was that the sensitivity and reliability of the instruments used for the studies mentioned was superior to what was available. While our data was generated using a QqQ LC-MS/MS, the studies mentioned were performed using mass spectrometers with an ion trap mass analyser. Also, the platform is highly sensitive to the presence of surfactant (derived polymers) and requires a large amount of starting material especially considering the relatively low abundance of some linkages. While the homogenising buffer used to lyse cells contained non-ionic detergent NP-40, all samples subjected to analysis using MS were thoroughly washed to completely deplete traces of it. Also, in many cases, scaling-up of the experiment would have required considerably greater amounts of resources.

Therefore due to lack of confidence in the AQUA standards run during the experiment, the MS analysis has been presented as a “semi-quantitative” comparison of different linkages with respective controls as opposed to absolute quantification of each linkage type.

Unfortunately, though concrete conclusions could not be drawn from these experiments attempted, the observations made in our experiments and the shortcomings have been discussed. It was also important to note that since there have not been many such previous studies on unanchored polyubiquitin for comparison; most of the data generated was novel. Also, we have retained enough trypsinolysed affinity purified chains from each condition to attempt a second mass spectrometry analysis in the future.

7.4.1. ACTIVATION OF SIGNALLING

Activation of IL-1β mediated signalling is regulated by a mechanism that involves post-translational modifications of several downstream proteins prior to nuclear translocation of transcription factor NF-κB including the rapid assembly of unanchored polyubiquitin chains (Section 1.2.2). Our attempt at elucidating the effect of activation of signalling on endogenous unanchored chains involved IL-1β mediated NF-κB activation. While evidence of the role unanchored polyubiquitin in cell signalling was first elucidated by a similar experiment using HEK293T cells stably (over) expressing IL-1R [1], we have attempted to replicate the conditions but by making use of normal HEK293T cells. An advantage of this approach was that we avoid changes which could possibly be an artefact of the overexpression as a result of such receptors being constitutively active. Activation of IL-1β mediated signalling in HEK293T cells was confirmed by measuring NF-κB activity, a strategy previously employed by our group [187, 188]. Titrations with IL-1β were carried out to measure response using a Dual-Luciferase Reporter assay thus providing a

direct indicator of NF-κB activity. A marked increase in NF-κB activity was observed upon treatment with a concentration of 20 ng/ml (of media) and this concentration was selected for treatment prior to purification of endogenous unanchored polyubiquitin chains (Figure 6.1).

For our attempts at capturing endogenous unanchored polyubiquitin chains from IL-1β treated cells, we had to consider the findings by Xia *et al* that such chains were assembled rapidly post activation, after which they immediately disappear [1]. The optimal time post IL-1β treatment to aid in maximum recovery of chains was observed to be 2 minutes. Hence, our strategy involved incubation of HEK293T cells in media substituted with IL-1β and immediately homogenising cells after 2 minutes. Also, to ensure that the affinity purified unanchored polyubiquitin chains were a recovered were a direct consequence of activation of signalling by IL-1β, cells were incubated in media lacking FBS thereby depleting cytokines contained. It was interesting to note that though the though changes in total unanchored ubiquitome were not obvious by Western blotting, however there was a consistent increase in the total ubiquitin positive species (post IL-1β treatment) evident by comparing the respective sample loads (Figure 6.2.A). Additionally we did notice an increase in K48 linked unanchored chains (Figure 6.2.B) upon stimulation. The latter could be a consequence of activation of NF-κB, which would involve proteasomal targeting of K48 conjugated IκB. The source of these K48 chains may possibly be a result of amputation of such chains by proteasomal DUBs (Section 7.1.2). Furthermore, the availability of a reliable antibody specific for K63 linkages allowed for the detection of unanchored K63 linked chains in our purified pool

(Figure 6.3). An interesting observation was the increase in K63 positive band that co-migrated close to tetra-ubiquitin (perhaps containing mixed linear or branched linkages). Absence of higher order chains was surprising as these species were described to be the activators of signalling while K63 chains of shorter length (Ub_3 and Ub_4) were deemed inhibitors [1].

Section 6.1.3 describes problems encountered when affinity purified unanchored polyubiquitin chains were subject to analysis by mass spectrometry. While the analysis of data generated did indicate an increase in K48, K63 and K11 linkages when compared to controls, it was difficult to quantify such an increase although the magnitudes of such response were not substantial, although may still be biologically relevant. While a case for increase in K63 and even K48 chains upon IL-1β stimulation can be made, increase in K11 chains were more surprising. However, K11 chains were previously described to participate in NF-κB activation upon stimulation by TNFα [94].

7.4.2. KNOCKDOWN OF REGULATORS OF ENDOGENOUS UNANCHORED POLYUBIQUITIN

7.4.2.1. KNOCKDOWN OF ISOT

The specificity of IsoT for unanchored polyubiquitin chains has been well established [57]. The significance of the enzyme in regulating cellular processes was observed when deletion of the gene or suppression of enzyme levels lead to an increase in unanchored polyubiquitin chains which competitively inhibited proteasomal processing [107, 109]. These findings indicated an essential role of IsoT in regulating the cellular unanchored

polyubiquitome. Hence, we were keen on further substantiating the characterising the effects of reduced levels of IsoT on endogenous unanchored polyubiquitin chains.

Optimisation of IsoT knockdown using siRNA pools were performed with a range of concentrations (Section 6.2.1.1), with significant reduction in levels seen upon use 20 nM of siRNA. This was subsequently scaled up to maximise protein recovery post transfections. Curiously, visual assessment revealed knockdown of IsoT levels to be more efficient when performed in 24 well cell culture plates than when we scaled up to 10 cm cell culture plates. Nevertheless, sufficient reduction (~45%) in their levels was observed prior to affinity purification of cell extracts. As expected, suppression of IsoT led to increased levels of total endogenous unanchored polyubiquitin chains (Figure 6.8). The trend was the same with respect to both short and higher order chains. Though the suppression of IsoT in the study by Dayal *et al* was much more prominent than what we could achieve, we could directly infer specific increase in unanchored polyubiquitin chains as a consequence of the knockdown [109]. Visual assessment of immunoblots could not confirm change in levels of K48 linked chains.

As mentioned previously, IsoT is known to disassemble polyubiquitin chains containing K48, K63 and K11 linkages. Mass spectrometry confirmed a definite increase in unanchored polyubiquitin chains containing K48 and K63 linkages but change in level of K11 chains was inconclusive. Also, considering the lower abundance of K11-linked polyubiquitin (when compared to K48 and

K63 linkages) in cells [42], the mass spectrometers used lacks the sensitivity to probe finer changes to K11 linked unanchored polyubiquitin pool.

7.4.2.2. KNOCKDOWN OF UBE3C

UBE3C was an interesting target to probe for its possible role as a contributor to cellular unanchored polyubiquitin pool. As mentioned previously, UBE3C is a proteasome associated E3 ligase capable of ubiquitin chain elongation and is integral to proteasomal processing. The possibility of its role in synthesising unanchored polyubiquitin chains *in vivo* has been postulated but not yet proven. Hence we decided to use our *in house* protocol to test for changes in purified endogenous unanchored chains post knockdown of the enzyme.

As was the case with IsoT, we (post optimisation) used an siRNA pool to knockdown UBE3C in HEK293T cells. However, only two of the replicates of the experiment were possible due to shortage of reagents required for siRNA transfection. Western blots of the affinity purified samples were visually assessed and did not show significant variation in unanchored polyubiquitin chain post UBE3C knockdown. Similar results were observed in yeast strains containing a deletion of the *HUL5* gene [151]. Unfortunately, due to failure of the LC system on the mass spectrometer, data generated from only one (out of three) repeats could be analysed. All three linkages analysed (K48, K63 and K11) showed a decrease upon UBE3C knockdown. This is what one would expect if indeed UBE3C were involved in generation of unanchored polyubiquitin chains, but this cannot be confirmed.

7.4.3. RESPONSE TO STRESS

The involvement of ubiquitin in cellular processes concerned with overcoming effects of a stresses (heat shock, DNA damage, hypoxia), has been extensively documented [42, 151, 168, 216]. By characterising cellular unanchored polyubiquitin pool, we have attempted to determine the possible role of this faction in eliciting such a response.

7.4.3.1. HEAT SHOCK

Unlike previous conditions HEK293T cells were subject to, which required modulation of levels of an enzyme or activation of a pathway, unanchored polyubiquitin chains were affinity purified from cells incubated at 42^{o}C for duration of 1 hour (while compared with those that were left to incubate at 37^{o}C). Subjecting mammalian cell lines to such a temperature stress is by no means uncommon (or lethal) with several studies documenting a recovery of cells upon withdrawal of stress and return to basal growth conditions [42, 159, 168]. As we were interested in the immediate consequence of such a stress, cells were homogenised and unanchored polyubiquitin chains were affinity purified immediately after the duration of the shock. Western blotting did not however provide evidence of change in total unanchored polyubiquitome upon heat shock treatment (Section 6.3.1.1). However, unanchored K48 linked chains showed a consistent decrease in chains thought to contain between 9-14 ubiquitin moieties. A possible explanation could be the increased K48 linked ubiquitination of substrates observed upon heat shock [42] could be formed by attachment of existing pool of unanchored K48 linked chains, thereby acting as a reservoir if required [174].

Analysis of purified unanchored chains following heat shock using mass spectrometry (although not conclusively) did reveal an increase in K48 chains while K63 and K11 chains were seen to decrease. Two discrepancies were encountered here. Firstly and importantly, the variation of K48 linked chains as observed by mass spectrometry did not tally with what was seen using western blotting. Possible repeat of the mass spectrometry analysis may confirm this. Also, the conjugated K11 and K63 chains were observed to increase in response to heat shock (in HEK293T cells) [42], hence there may be a possibility where variations in unanchored may not match substrate bound ubiquitin chains.

7.4.3.2. MG132 TREATMENT

The response of the UPS to pharmacological proteasomal inhibition has been extensively studied, with respect to total ubiquitin levels in cells and downstream effects. However, aside from a study previously published by our group [172], not much else has been noted about the involvement of unanchored polyubiquitin in cellular machinery active in recovering from such a proteolytic stress. MG132 has previously been seen to drive an increase in total ubiquitin levels and polyubiquitin chains conjugated to protein in cell lines (including HEK293), it did cause a decrease in "free" monoubiquitin levels [16, 78]. The only other studies which can draw a link between proteasomal inhibition and unanchored polyubiquitin levels were to do with aggresome formation. Aggregated proteins which are ubiquitinated are transported to aggresomes by HDAC6, making use of the latter's Znf-UBP

domain to bind unanchored polyubiquitin chains exposed by action of DUB ataxin-3 (for recruiting proteins to aggresome) and proteasomal DUB POH-1 (for clearance) [133, 134]. Also, proteasomal inhibition by MG132 leads to increased aggresome formation and clearance [129, 134].

Western blotting of unanchored polyubiquitin chains from HEK293T cells (MG132 treated, Section 6.3.2.1) with ubiquitin specific antibody surprisingly did not support what was previously observed with a similar treatment to HeLa and U2OS cell lines [172]. A possible explanation to this discrepancy was the loss of cell extract proteins due to initiation of apoptosis as a result of MG132 treatment. Protein content has routinely been normalised with their respective controls, but due to such a large difference in protein levels (up to 1 mg/ml lost in MG132 treated sample), error during sampling may be responsible for the use of decreased protein content in MG132 treated sample. However, the lanes (of gel, and subsequently the western blot) containing sample loads showed a remarkable increase in ubiquitin positive material, indicating increase in total ubiquitin levels upon proteasomal inhibition. Immunoblots probed with K48 specific antibody though showed an increase in unanchored polyubiquitin chains of this linkage.

Results obtained by analysing data derived by mass spectrometry however (Section 6.3.2.2), was much more on the anticipated lines. The levels of endogenous unanchored polyubiquitin containing K48 and K11 linkages showed a remarkable increase in abundance. Even K63 linked chains consistently appeared to increase upon proteasomal inhibition. All but K63

linked polyubiquitin (of total cellular ubiquitome) have been observed to increase in response to proteasomal inhibition [82]. Hence, this could translate to increased assembly of aggresomes for clearance of such aggregates [129], which would require recruitment of such aggregates by HDAC6 with the help of ataxin-3 [133], thereby producing unanchored polyubiquitin chains in the process. Production of unanchored K63 chains (by POH-1) would then be necessary for clearance of such aggregates thus explaining the observations made in our experiments.

7.5. FUTURE WORK

The basis of the study undertaken was to enable direct inspection of and explore the biological significance of unanchored polyubiquitin. While the use of AQUA ubiquitin standards for accurate estimation of ubiquitin linkages by LC-MRM-MS/MS has been widely published, we have encountered numerous challenges in the process of developing a platform that would ensure self-reliance. The caveats of the study have been discussed and must be addressed while addressing similar work in the future. One of the factors we would look into is the introduction (spiking) of AQUA standards into the sample before analysis, instead of relying on standard curves. While one of the main challenges is to try and reduce the amount of monoubiquitin being purified with our setup, an alternate means is already being developed. The other challenge is to minimise the presence of detergent remnants in affinity purified samples, which have in the past compromised the results obtained from such analysis. Finally, if possible we would look to repeat the LC-MRM-MS/MS analysis of the cellular affinity purified samples generated in response to conditions described in Chapter 6. The number of SRM transitions per experiment could also be reduced and limited to the readily detectable relatively abundant isopeptide linkages. Further access to the LTQ-Orbitrap-Velos through our collaboration with the University of Leicester could provide a much more sensitive and reliable tool to further study such low-abundance modifications.

REFERENCES

1. Xia, Z.P., et al., *Direct activation of protein kinases by unanchored polyubiquitin chains.* Nature, 2009. **461**(7260): p. 114-9.
2. Ciehanover, A., Y. Hod, and A. Hershko, *A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes.* Biochem Biophys Res Commun, 1978. **81**(4): p. 1100-5.
3. Wilkinson, K.D., M.K. Urban, and A.L. Haas, *Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes.* J Biol Chem, 1980. **255**(16): p. 7529-32.
4. Goldstein, G., et al., *Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells.* Proc Natl Acad Sci U S A, 1975. **72**(1): p. 11-5.
5. Schlesinger, D.H., G. Goldstein, and H.D. Niall, *The complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells.* Biochemistry, 1975. **14**(10): p. 2214-8.
6. Hershko, A., et al., *Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis.* Proc Natl Acad Sci U S A, 1980. **77**(4): p. 1783-6.
7. Ciechanover, A., et al., *ATP-dependent conjugation of reticulocyte proteins with the polypeptide required for protein degradation.* Proc Natl Acad Sci U S A, 1980. **77**(3): p. 1365-8.

8. Etlinger, J.D. and A.L. Goldberg, *A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes.* Proc Natl Acad Sci U S A, 1977. **74**(1): p. 54-8.

9. Hough, R., G. Pratt, and M. Rechsteiner, *Purification of two high molecular weight proteases from rabbit reticulocyte lysate.* J Biol Chem, 1987. **262**(17): p. 8303-13.

10. Arrigo, A.P., et al., *Identity of the 19S 'prosome' particle with the large multifunctional protease complex of mammalian cells (the proteasome).* Nature, 1988. **331**(6152): p. 192-4.

11. Vierstra, R.D., S.M. Langan, and G.E. Schaller, *Complete Amino-Acid-Sequence of Ubiquitin from the Higher-Plant Avena-Sativa.* Biochemistry, 1986. **25**(11): p. 3105-3108.

12. Wilkinson, K.D., et al., *Structure and activities of a variant ubiquitin sequence from bakers' yeast.* Biochemistry, 1986. **25**(18): p. 4999-5004.

13. Vijay-Kumar, S., C.E. Bugg, and W.J. Cook, *Structure of ubiquitin refined at 1.8 A resolution.* J Mol Biol, 1987. **194**(3): p. 531-44.

14. Lenkinski, R.E., et al., *Nuclear magnetic resonance studies of the denaturation of ubiquitin.* Biochim Biophys Acta, 1977. **494**(1): p. 126-30.

15. Takada, K., et al., *Ability of ubiquitin radioimmunoassay to discriminate between monoubiquitin and multi-ubiquitin chains.* Biochim Biophys Acta, 1996. **1290**(3): p. 282-8.

16. Ryu, K.Y., R.T. Baker, and R.R. Kopito, *Ubiquitin-specific protease 2 as a tool for quantification of total ubiquitin levels in biological specimens.* Anal Biochem, 2006. **353**(1): p. 153-5.

17. Ohtani-Kaneko, R., et al., *Nerve growth factor (NGF) induces increase in multi-ubiquitin chains and concomitant decrease in free ubiquitin in nuclei of PC12h.* Neurosci Res, 1996. **26**(4): p. 349-55.

18. Kimura, Y. and K. Tanaka, *Regulatory mechanisms involved in the control of ubiquitin homeostasis.* J Biochem, 2010. **147**(6): p. 793-8.

19. Wiborg, O., et al., *The human ubiquitin multigene family: some genes contain multiple directly repeated ubiquitin coding sequences.* EMBO J, 1985. **4**(3): p. 755-9.

20. Ryu, K.Y., et al., *The mouse polyubiquitin gene UbC is essential for fetal liver development, cell-cycle progression and stress tolerance.* EMBO J, 2007. **26**(11): p. 2693-706.

21. Goldknopf, I.L., et al., *Isolation and characterization of protein A24, a "histone-like" non-histone chromosomal protein.* J Biol Chem, 1975. **250**(18): p. 7182-7.

22. Goldknopf, I.L. and H. Busch, *Isopeptide linkage between nonhistone and histone 2A polypeptides of chromosomal conjugate-protein A24.* Proc Natl Acad Sci U S A, 1977. **74**(3): p. 864-8.

23. Chau, V., et al., *A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein.* Science, 1989. **243**(4898): p. 1576-83.

24. Hershko, A. and H. Heller, *Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates.* Biochem Biophys Res Commun, 1985. **128**(3): p. 1079-86.

25. Hershko, A., et al., *Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown.* J Biol Chem, 1983. **258**(13): p. 8206-14.

26. Hershko, A. and A. Ciechanover, *The ubiquitin system.* Annu Rev Biochem, 1998. **67**: p. 425-79.

27. Lorenz, S., et al., *Macromolecular juggling by ubiquitylation enzymes.* BMC Biol, 2013. **11**: p. 65.

28. Chen, Z. and C.M. Pickart, *A 25-kilodalton ubiquitin carrier protein (E2) catalyzes multi-ubiquitin chain synthesis via lysine 48 of ubiquitin.* J Biol Chem, 1990. **265**(35): p. 21835-42.

29. van Nocker, S. and R.D. Vierstra, *Multiubiquitin chains linked through lysine 48 are abundant in vivo and are competent intermediates in the ubiquitin proteolytic pathway.* J Biol Chem, 1993. **268**(33): p. 24766-73.

30. Hochstrasser, M., *Ubiquitin-dependent protein degradation.* Annu Rev Genet, 1996. **30**: p. 405-39.

31. Komander, D., M.J. Clague, and S. Urbe, *Breaking the chains: structure and function of the deubiquitinases.* Nature Reviews Molecular Cell Biology, 2009. **10**(8): p. 550-563.

32. Reyes-Turcu, F.E., K.H. Ventii, and K.D. Wilkinson, *Regulation and Cellular Roles of Ubiquitin-Specific Deubiquitinating Enzymes.* Annual Review of Biochemistry, 2009. **78**: p. 363-397.

33. Clague, M.J., et al., *Deubiquitylases from genes to organism.* Physiol Rev, 2013. **93**(3): p. 1289-315.

34. Wolberger, C., *Mechanisms for regulating deubiquitinating enzymes.* Protein Sci, 2014. **23**(4): p. 344-53.

35. Wilkinson, K.D., *Regulation of ubiquitin-dependent processes by deubiquitinating enzymes.* FASEB J, 1997. **11**(14): p. 1245-56.

36. Arnason, T. and M.J. Ellison, *Stress resistance in Saccharomyces cerevisiae is strongly correlated with assembly of a novel type of multiubiquitin chain.* Mol Cell Biol, 1994. **14**(12): p. 7876-83.

37. Finley, D., et al., *Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant.* Mol Cell Biol, 1994. **14**(8): p. 5501-9.

38. Spence, J., et al., *A ubiquitin mutant with specific defects in DNA repair and multiubiquitination.* Mol Cell Biol, 1995. **15**(3): p. 1265-73.

39. Baboshina, O.V. and A.L. Haas, *Novel multiubiquitin chain linkages catalyzed by the conjugating enzymes E2EPF and RAD6 are recognized by 26 S proteasome subunit 5.* J Biol Chem, 1996. **271**(5): p. 2823-31.

40. Peng, J., et al., *A proteomics approach to understanding protein ubiquitination.* Nat Biotechnol, 2003. **21**(8): p. 921-6.

41. Xu, P., et al., *A proteomic strategy for quantifying polyubiquitin chain topologies.* Israel Journal of Chemistry, 2006. **46**(2): p. 171-182.

42. Dammer, E.B., et al., *Polyubiquitin linkage profiles in three models of proteolytic stress suggest the etiology of Alzheimer disease.* J Biol Chem, 2011. **286**(12): p. 10457-65.

43. Kirisako, T., et al., *A ubiquitin ligase complex assembles linear polyubiquitin chains.* EMBO J, 2006. **25**(20): p. 4877-87.

44. Tokunaga, F., et al., *SHARPIN is a component of the NF-kappaB-activating linear ubiquitin chain assembly complex.* Nature, 2011. **471**(7340): p. 633-6.

45. Kim, H.T., et al., *Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages.* J Biol Chem, 2007. **282**(24): p. 17375-86.

46. Xu, P. and J. Peng, *Characterization of polyubiquitin chain structure by middle-down mass spectrometry.* Anal Chem, 2008. **80**(9): p. 3438-44.

47. Dikic, I., S. Wakatsuki, and K.J. Walters, *Ubiquitin-binding domains - from structures to functions.* Nat Rev Mol Cell Biol, 2009. **10**(10): p. 659-71.

48. Husnjak, K. and I. Dikic, *Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions.* Annu Rev Biochem, 2012. **81**: p. 291-322.

49. Hicke, L., H.L. Schubert, and C.P. Hill, *Ubiquitin-binding domains.* Nat Rev Mol Cell Biol, 2005. **6**(8): p. 610-21.

50. Ikeda, F. and I. Dikic, *Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series.* EMBO Rep, 2008. **9**(6): p. 536-42.

51. Hofmann, K. and P. Bucher, *The UBA domain: a sequence motif present in multiple enzyme classes of the ubiquitination pathway.* Trends Biochem Sci, 1996. **21**(5): p. 172-3.

52. Hurley, J.H., S. Lee, and G. Prag, *Ubiquitin-binding domains.* Biochem J, 2006. **399**(3): p. 361-72.

53. Beal, R., et al., *Surface hydrophobic residues of multiubiquitin chains essential for proteolytic targeting.* Proc Natl Acad Sci U S A, 1996. **93**(2): p. 861-6.

54. Swanson, K.A., et al., *Solution structure of Vps27 UIM-ubiquitin complex important for endosomal sorting and receptor downregulation.* EMBO J, 2003. **22**(18): p. 4597-606.

55. Bosanac, I., et al., *Ubiquitin binding to A20 ZnF4 is required for modulation of NF-kappaB signaling.* Mol Cell, 2010. **40**(4): p. 548-57.

56. Lee, S., et al., *Structural basis for ubiquitin recognition and autoubiquitination by Rabex-5.* Nat Struct Mol Biol, 2006. **13**(3): p. 264-71.

57. Reyes-Turcu, F.E., et al., *The ubiquitin binding domain ZnFUBP recognizes the C-terminal diglycine motif of unanchored ubiquitin.* Cell, 2006. **124**(6): p. 1197-1208.

58. Sloper-Mould, K.E., et al., *Distinct functional surface regions on ubiquitin.* J Biol Chem, 2001. **276**(32): p. 30483-9.

59. Rahighi, S., et al., *Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation.* Cell, 2009. **136**(6): p. 1098-109.

60. Sato, Y., et al., *Specific recognition of linear ubiquitin chains by the Npl4 zinc finger (NZF) domain of the HOIL-1L subunit of the linear ubiquitin chain assembly complex.* Proc Natl Acad Sci U S A, 2011. **108**(51): p. 20520-5.

61. Hospenthal, M.K., S.M. Freund, and D. Komander, *Assembly, analysis and architecture of atypical ubiquitin chains.* Nat Struct Mol Biol, 2013. **20**(5): p. 555-65.

62. Kamadurai, H.B., et al., *Insights into Ubiauitin Transfer Cascades from a Structure of a UbcH5B similar to Ubiauitin-HECTNEDD4L Complex.* Molecular Cell, 2009. **36**(6): p. 1095-1102.

63. Jin, L.Y., et al., *Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex.* Cell, 2008. **133**(4): p. 653-665.

64. Wickliffe, K.E., et al., *The Mechanism of Linkage-Specific Ubiquitin Chain Elongation by a Single-Subunit E2.* Cell, 2011. **144**(5): p. 769-781.

65. Kulathu, Y. and D. Komander, *Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages.* Nat Rev Mol Cell Biol, 2012. **13**(8): p. 508-23.

66. Ye, Y., et al., *Ubiquitin chain conformation regulates recognition and activity of interacting proteins.* Nature, 2012. **492**(7428): p. 266-70.

67. Tenno, T., et al., *Structural basis for distinct roles of Lys63- and Lys48-linked polyubiquitin chains (vol 9, pg 865, 2004).* Genes to Cells, 2004. **9**(11): p. 1137-1137.

68. Varadan, R., et al., *Structural properties of polyubiquitin chains in solution.* J Mol Biol, 2002. **324**(4): p. 637-47.

69. Bremm, A., S.M. Freund, and D. Komander, *Lys11-linked ubiquitin chains adopt compact conformations and are preferentially hydrolyzed by the deubiquitinase Cezanne.* Nat Struct Mol Biol, 2010. **17**(8): p. 939-47.

70. Virdee, S., et al., *Engineered diubiquitin synthesis reveals Lys29-isopeptide specificity of an OTU deubiquitinase.* Nat Chem Biol, 2010. **6**(10): p. 750-7.

71. Michel, M.A., et al., *Assembly and specific recognition of k29- and k33-linked polyubiquitin.* Mol Cell, 2015. **58**(1): p. 95-109.

72. Komander, D., et al., *Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains.* EMBO Rep, 2009. **10**(5): p. 466-73.

73. Datta, A.B., G.L. Hura, and C. Wolberger, *The structure and conformation of Lys63-linked tetraubiquitin.* J Mol Biol, 2009. **392**(5): p. 1117-24.

74. Rohaim, A., et al., *Structure of a compact conformation of linear diubiquitin.* Acta Crystallogr D Biol Crystallogr, 2012. **68**(Pt 2): p. 102-8.

75. Komander, D. and M. Rape, *The ubiquitin code.* Annu Rev Biochem, 2012. **81**: p. 203-29.

76. Wilkinson, K.D., et al., *Recognition of polyubiquitin isoforms by the multiple ubiquitin binding modules of isopeptidase T.* Journal of Biological Chemistry, 2008. **283**(28): p. 19581-19592.

77. Sims, J.J. and R.E. Cohen, *Linkage-specific avidity defines the lysine 63-linked polyubiquitin-binding preference of rap80.* Mol Cell, 2009. **33**(6): p. 775-83.

78. Kaiser, S.E., et al., *Protein standard absolute quantification (PSAQ) method for the measurement of cellular ubiquitin pools.* Nat Methods, 2011. **8**(8): p. 691-6.

79. Yang, X., et al., *Absolute quantification of E1, ubiquitin-like proteins and Nedd8-MLN4924 adduct by mass spectrometry.* Cell Biochem Biophys, 2013. **67**(1): p. 139-47.

80. Xu, G., J.S. Paige, and S.R. Jaffrey, *Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling.* Nat Biotechnol, 2010. **28**(8): p. 868-73.

81. Kim, W., et al., *Systematic and quantitative assessment of the ubiquitin-modified proteome.* Mol Cell, 2011. **44**(2): p. 325-40.

82. Xu, P., et al., *Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation.* Cell, 2009. **137**(1): p. 133-45.

83. Ziv, I., et al., *A perturbed ubiquitin landscape distinguishes between ubiquitin in trafficking and in proteolysis.* Mol Cell Proteomics, 2011. **10**(5): p. M111 009753.

84. Thrower, J.S., et al., *Recognition of the polyubiquitin proteolytic signal.* EMBO J, 2000. **19**(1): p. 94-102.

85. Bedford, L., et al., *Diverse polyubiquitin chains accumulate following 26S proteasomal dysfunction in mammalian neurones.* Neurosci Lett, 2011. **491**(1): p. 44-7.

86. Wang, C., et al., *TAK1 is a ubiquitin-dependent kinase of MKK and IKK.* Nature, 2001. **412**(6844): p. 346-51.

87. Chen, Z.J. and L.J. Sun, *Nonproteolytic functions of ubiquitin in cell signaling.* Mol Cell, 2009. **33**(3): p. 275-86.

88. Lauwers, E., C. Jacob, and B. Andre, *K63-linked ubiquitin chains as a specific signal for protein sorting into the multivesicular body pathway.* J Cell Biol, 2009. **185**(3): p. 493-502.

89. Wu-Baer, F., et al., *The BRCA1/BARD1 heterodimer assembles polyubiquitin chains through an unconventional linkage involving lysine residue K6 of ubiquitin.* J Biol Chem, 2003. **278**(37): p. 34743-6.

90. Nishikawa, H., et al., *Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase.* J Biol Chem, 2004. **279**(6): p. 3916-24.

91. Sobhian, B., et al., *RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites.* Science, 2007. **316**(5828): p. 1198-202.

92. Jin, L., et al., *Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex.* Cell, 2008. **133**(4): p. 653-65.

93. Matsumoto, M.L., et al., *K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody.* Mol Cell, 2010. **39**(3): p. 477-84.

94. Dynek, J.N., et al., *c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling.* EMBO J, 2010. **29**(24): p. 4198-209.

95. Boname, J.M., et al., *Efficient internalization of MHC I requires lysine-11 and lysine-63 mixed linkage polyubiquitin chains.* Traffic, 2010. **11**(2): p. 210-20.

96. Goto, E., et al., *Contribution of lysine 11-linked ubiquitination to MIR2-mediated major histocompatibility complex class I internalization.* J Biol Chem, 2010. **285**(46): p. 35311-9.

97. Geisler, S., et al., *PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1.* Nat Cell Biol, 2010. **12**(2): p. 119-31.

98. Glauser, L., et al., *Parkin promotes the ubiquitination and degradation of the mitochondrial fusion factor mitofusin 1.* J Neurochem, 2011. **118**(4): p. 636-45.

99. Licchesi, J.D., et al., *An ankyrin-repeat ubiquitin-binding domain determines TRABID's specificity for atypical ubiquitin chains.* Nat Struct Mol Biol, 2012. **19**(1): p. 62-71.

100. Shabek, N., Y. Herman-Bachinsky, and A. Ciechanover, *Ubiquitin degradation with its substrate, or as a monomer in a ubiquitination-independent mode, provides clues to proteasome regulation.* Proc Natl Acad Sci U S A, 2009. **106**(29): p. 11907-12.

101. Kravtsova-Ivantsiv, Y., S. Cohen, and A. Ciechanover, *Modification by single ubiquitin moieties rather than polyubiquitination is sufficient for proteasomal processing of the p105 NF-kappaB precursor.* Mol Cell, 2009. **33**(4): p. 496-504.

102. Shih, S.C., K.E. Sloper-Mould, and L. Hicke, *Monoubiquitin carries a novel internalization signal that is appended to activated receptors.* EMBO J, 2000. **19**(2): p. 187-98.

103. Terrell, J., et al., *A function for monoubiquitination in the internalization of a G protein-coupled receptor.* Mol Cell, 1998. **1**(2): p. 193-202.

104. Henry, K.W., et al., *Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8.* Genes Dev, 2003. **17**(21): p. 2648-63.

105. Hoege, C., et al., *RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO.* Nature, 2002. **419**(6903): p. 135-41.

106. Haglund, K., et al., *Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation.* Nat Cell Biol, 2003. **5**(5): p. 461-6.

107. Amerik, A., et al., *In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome.* EMBO J, 1997. **16**(16): p. 4826-38.

108. Piotrowski, J., et al., *Inhibition of the 26 S proteasome by polyubiquitin chains synthesized to have defined lengths.* J Biol Chem, 1997. **272**(38): p. 23712-21.

109. Dayal, S., et al., *Suppression of the deubiquitinating enzyme USP5 causes the accumulation of unanchored polyubiquitin and the activation of p53.* J Biol Chem, 2009. **284**(8): p. 5030-41.

110. Kimura, Y., et al., *An inhibitor of a deubiquitinating enzyme regulates ubiquitin homeostasis.* Cell, 2009. **137**(3): p. 549-59.

111. Cao, Z., et al., *TRAF6 is a signal transducer for interleukin-1.* Nature, 1996. **383**(6599): p. 443-6.

112. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.

113. Chen, Z.J., *Ubiquitin signalling in the NF-kappaB pathway.* Nat Cell Biol, 2005. **7**(8): p. 758-65.

114. Inoue, J., J. Gohda, and T. Akiyama, *Characteristics and biological functions of TRAF6.* Adv Exp Med Biol, 2007. **597**: p. 72-9.

115. Deng, L., et al., *Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain.* Cell, 2000. **103**(2): p. 351-61.

116. Adhikari, A., M. Xu, and Z.J. Chen, *Ubiquitin-mediated activation of TAK1 and IKK.* Oncogene, 2007. **26**(22): p. 3214-26.

117. Kanayama, A., et al., *TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains.* Mol Cell, 2004. **15**(4): p. 535-48.

118. Scherer, D.C., et al., *Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination.* Proc Natl Acad Sci U S A, 1995. **92**(24): p. 11259-63.

119. Walsh, M.C., et al., *TRAF6 autoubiquitination-independent activation of the NFkappaB and MAPK pathways in response to IL-1 and RANKL.* PLoS One, 2008. **3**(12): p. e4064.

120. Chen, Z.J., *Ubiquitination in signaling to and activation of IKK.* Immunol Rev, 2012. **246**(1): p. 95-106.

121. Kawai, T., et al., *IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction.* Nat Immunol, 2005. **6**(10): p. 981-8.

122. Seth, R.B., et al., *Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3.* Cell, 2005. **122**(5): p. 669-82.

123. Loo, Y.M. and M. Gale, Jr., *Immune signaling by RIG-I-like receptors.* Immunity, 2011. **34**(5): p. 680-92.

124. Bhoj, V.G. and Z.J. Chen, *Ubiquitylation in innate and adaptive immunity.* Nature, 2009. **458**(7237): p. 430-7.

125. Gack, M.U., et al., *TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity.* Nature, 2007. **446**(7138): p. 916-920.

126. Zeng, W., et al., *Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity.* Cell, 2010. **141**(2): p. 315-30.

127. Jiang, X., et al., *Ubiquitin-induced oligomerization of the RNA sensors RIG-I and MDA5 activates antiviral innate immune response.* Immunity, 2012. **36**(6): p. 959-73.

128. Pertel, T., et al., *TRIM5 is an innate immune sensor for the retrovirus capsid lattice.* Nature, 2011. **472**(7343): p. 361-5.

129. Johnston, J.A., C.L. Ward, and R.R. Kopito, *Aggresomes: a cellular response to misfolded proteins.* J Cell Biol, 1998. **143**(7): p. 1883-98.

130. Kawaguchi, Y., et al., *The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress.* Cell, 2003. **115**(6): p. 727-38.

131. Hook, S.S., et al., *Histone deacetylase 6 binds polyubiquitin through its zinc finger (PAZ domain) and copurifies with deubiquitinating enzymes.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13425-30.

132. Boyault, C., et al., *HDAC6-p97/VCP controlled polyubiquitin chain turnover.* EMBO J, 2006. **25**(14): p. 3357-66.

133. Ouyang, H., et al., *Protein aggregates are recruited to aggresome by histone deacetylase 6 via unanchored ubiquitin C termini.* J Biol Chem, 2012. **287**(4): p. 2317-27.

134. Hao, R., et al., *Proteasomes activate aggresome disassembly and clearance by producing unanchored ubiquitin chains.* Mol Cell, 2013. **51**(6): p. 819-28.

135. Wilkinson, K.D., et al., *Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T.* Biochemistry, 1995. **34**(44): p. 14535-46.

136. Raasi, S., et al., *Diverse polyubiquitin interaction properties of ubiquitin-associated domains.* Nat Struct Mol Biol, 2005. **12**(8): p. 708-14.

137. Ansari-Lari, M.A., et al., *A gene-rich cluster between the CD4 and triosephosphate isomerase genes at human chromosome 12p13.* Genome Res, 1996. **6**(4): p. 314-26.

138. Izaguirre, D.I., et al., *PTBP1-dependent regulation of USP5 alternative RNA splicing plays a role in glioblastoma tumorigenesis.* Mol Carcinog, 2012. **51**(11): p. 895-906.

139. Lindsey, D.F., et al., *A deubiquitinating enzyme that disassembles free polyubiquitin chains is required for development but not growth in Dictyostelium.* J Biol Chem, 1998. **273**(44): p. 29178-87.

140. Doelling, J.H., et al., *The ubiquitin-specific protease UBP14 is essential for early embryo development in Arabidopsis thaliana.* Plant J, 2001. **27**(5): p. 393-405.

141. Kapuria, V., et al., *Deubiquitinase inhibition by small-molecule WP1130 triggers aggresome formation and tumor cell apoptosis.* Cancer Res, 2010. **70**(22): p. 9265-76.

142. Wang, C.H., G.C. Chen, and C.T. Chien, *The deubiquitinase Leon/USP5 regulates ubiquitin homeostasis during Drosophila development.* Biochem Biophys Res Commun, 2014. **452**(3): p. 369-75.

143. You, J. and C.M. Pickart, *A HECT domain E3 enzyme assembles novel polyubiquitin chains.* J Biol Chem, 2001. **276**(23): p. 19871-8.

144. Leggett, D.S., et al., *Multiple associated proteins regulate proteasome structure and function.* Mol Cell, 2002. **10**(3): p. 495-507.

145. You, J., et al., *Proteolytic targeting of transcriptional regulator TIP120B by a HECT domain E3 ligase.* J Biol Chem, 2003. **278**(26): p. 23369-75.

146. Aviram, S. and D. Kornitzer, *The ubiquitin ligase Hul5 promotes proteasomal processivity.* Mol Cell Biol, 2010. **30**(4): p. 985-94.

147. Chu, B.W., et al., *The E3 ubiquitin ligase UBE3C enhances proteasome processivity by ubiquitinating partially proteolyzed substrates.* J Biol Chem, 2013. **288**(48): p. 34575-87.

148. Crosas, B., et al., *Ubiquitin chains are remodeled at the proteasome by opposing ubiquitin ligase and deubiquitinating activities.* Cell, 2006. **127**(7): p. 1401-13.

149. Fang, N.N., et al., *Hul5 HECT ubiquitin ligase plays a major role in the ubiquitylation and turnover of cytosolic misfolded proteins.* Nat Cell Biol, 2011. **13**(11): p. 1344-52.

150. Fang, N.N. and T. Mayor, *Hul5 ubiquitin ligase: good riddance to bad proteins.* Prion, 2012. **6**(3): p. 240-4.

151. Braten, O., et al., *Generation of free ubiquitin chains is up-regulated in stress and facilitated by the HECT domain ubiquitin ligases UFD4 and HUL5.* Biochem J, 2012. **444**(3): p. 611-7.

152. Mastrandrea, L.D., et al., *E2/E3-mediated assembly of lysine 29-linked polyubiquitin chains.* J Biol Chem, 1999. **274**(38): p. 27299-306.

153. Tissieres, A., H.K. Mitchell, and U.M. Tracy, *Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs.* J Mol Biol, 1974. **84**(3): p. 389-98.

154. Colaco, C.A., et al., *Heat shock proteins: stimulators of innate and acquired immunity.* Biomed Res Int, 2013. **2013**: p. 461230.

155. Medicherla, B. and A.L. Goldberg, *Heat shock and oxygen radicals stimulate ubiquitin-dependent degradation mainly of newly synthesized proteins.* J Cell Biol, 2008. **182**(4): p. 663-73.

156. Ananthan, J., A.L. Goldberg, and R. Voellmy, *Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes.* Science, 1986. **232**(4749): p. 522-4.

157. Kim, H.J., N.R. Hwang, and K.J. Lee, *Heat shock responses for understanding diseases of protein denaturation.* Mol Cells, 2007. **23**(2): p. 123-31.

158. Carlson, N. and M. Rechsteiner, *Microinjection of ubiquitin: intracellular distribution and metabolism in HeLa cells maintained under normal physiological conditions.* J Cell Biol, 1987. **104**(3): p. 537-46.

159. Carlson, N., S. Rogers, and M. Rechsteiner, *Microinjection of ubiquitin: changes in protein degradation in HeLa cells subjected to heat-shock.* J Cell Biol, 1987. **104**(3): p. 547-55.

160. Bond, U., et al., *Ubiquitin in stressed chicken embryo fibroblasts.* J Biol Chem, 1988. **263**(5): p. 2384-8.

161. Gropper, R., et al., *The ubiquitin-activating enzyme, E1, is required for stress-induced lysosomal degradation of cellular proteins.* J Biol Chem, 1991. **266**(6): p. 3602-10.

162. Nanduri, P., et al., *Chaperone-mediated 26S proteasome remodeling facilitates free K63 ubiquitin chain production and aggresome clearance.* J Biol Chem, 2015. **290**(15): p. 9455-64.

163. Lecker, S.H., A.L. Goldberg, and W.E. Mitch, *Protein degradation by the ubiquitin-proteasome pathway in normal and disease states.* J Am Soc Nephrol, 2006. **17**(7): p. 1807-19.

164. Asano, S., et al., *Proteasomes. A molecular census of 26S proteasomes in intact neurons.* Science, 2015. **347**(6220): p. 439-42.

165. Tsubuki, S., et al., *Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine.* J Biochem, 1996. **119**(3): p. 572-6.

166. Palombella, V.J., et al., *The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B.* Cell, 1994. **78**(5): p. 773-85.

167. Lee, D.H. and A.L. Goldberg, *Proteasome inhibitors: valuable new tools for cell biologists.* Trends Cell Biol, 1998. **8**(10): p. 397-403.

168. Kim, H.J., et al., *Systemic analysis of heat shock response induced by heat shock and a proteasome inhibitor MG132.* PLoS One, 2011. **6**(6): p. e20252.

169. Jacobson, A.D., et al., *The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26 s proteasome*. J Biol Chem, 2009. **284**(51): p. 35485-94.

170. Hanna, J., et al., *Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasomal degradation.* Cell, 2006. **127**(1): p. 99-111.

171. Zhang, N.Y., et al., *Ubiquitin chain trimming recycles the substrate binding sites of the 26 S proteasome and promotes degradation of lysine 48-linked polyubiquitin conjugates.* J Biol Chem, 2011. **286**(29): p. 25540-6.

172. Strachan, J., et al., *Broad utility of an affinity-enrichment strategy for unanchored polyubiquitin chains.* Journal of Proteomics and Bioinformatics, 2013. **S7**.

173. Hjerpe, R., et al., *Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities.* EMBO Rep, 2009. **10**(11): p. 1250-8.

174. Strachan, J., et al., *Insights into the molecular composition of endogenous unanchored polyubiquitin chains.* J Proteome Res, 2012. **11**(3): p. 1969-80.

175. Kirkpatrick, D.S., et al., *Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology.* Nat Cell Biol, 2006. **8**(7): p. 700-10.

176. Resing, K.A. and N.G. Ahn, *Proteomics strategies for protein identification.* FEBS Lett, 2005. **579**(4): p. 885-9.

177. Gundry, R.L., et al., *Preparation of proteins and peptides for mass spectrometry analysis in a bottom-up proteomics workflow.* Curr Protoc Mol Biol, 2009. **Chapter 10**: p. Unit10 25.

178. Barker, J., *Mass Spectrometry*. Second ed. Analytical Chemistry By Open Learning, ed. D.J. Ando. 1998, Baffins Lane, Chichester, West Sussex PO19 1UD, England: John Wiley & Sons Ltd. 509.

179. Aebersold, R. and M. Mann, *Mass spectrometry-based proteomics.* Nature, 2003. **422**(6928): p. 198-207.

180. Baldwin, M.A., *Mass spectrometers for the analysis of biomolecules.* Methods Enzymol, 2005. **402**: p. 3-48.

181. Ens, W. and K.G. Standing, *Hybrid quadrupole/time-of-flight mass spectrometers for analysis of biomolecules.* Methods Enzymol, 2005. **402**: p. 49-78.

182. Liebler, D.C. and L.J. Zimmerman, *Targeted quantitation of proteins by mass spectrometry.* Biochemistry, 2013. **52**(22): p. 3797-806.

183. Kirkpatrick, D.S., S.A. Gerber, and S.P. Gygi, *The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications.* Methods, 2005. **35**(3): p. 265-73.

184. Xu, P. and J. Peng, *Dissecting the ubiquitin pathway by mass spectrometry.* Biochim Biophys Acta, 2006. **1764**(12): p. 1940-7.

185. Low, T.Y., et al., *Unraveling the ubiquitin-regulated signaling networks by mass spectrometry-based proteomics.* Proteomics, 2013. **13**(3-4): p. 526-37.

186. Leon, I.R., et al., *Quantitative assessment of in-solution digestion efficiency identifies optimal protocols for unbiased protein analysis.* Mol Cell Proteomics, 2013. **12**(10): p. 2992-3005.

187. Najat, D., et al., *Characterization of a non-UBA domain missense mutation of sequestosome 1 (SQSTM1) in Paget's disease of bone.* J Bone Miner Res, 2009. **24**(4): p. 632-42.

188. Goode, A., et al., *Paget disease of bone-associated UBA domain mutations of SQSTM1 exert distinct effects on protein structure and function.* Biochim Biophys Acta, 2014. **1842**(7): p. 992-1000.

189. Tooth, D.J., V. Gopala Krishna, and R. Layfield, *An economical high-throughput protocol for multidimensional fractionation of proteins.* Int J Proteomics, 2012. **2012**: p. 735132.

190. Baker, R.T., et al., *Using deubiquitylating enzymes as research tools.* Methods Enzymol, 2005. **398**: p. 540-54.

191. Nathan, J.A., et al., *Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes?* EMBO J, 2013. **32**(4): p. 552-65.

192. Morimoto, D., et al., *The unexpected role of polyubiquitin chains in the formation of fibrillar aggregates.* Nat Commun, 2015. **6**: p. 6116.

193. Duncan, M.W., H. Roder, and S.W. Hunsucker, *Quantitative matrix-assisted laser desorption/ionization mass spectrometry.* Brief Funct Genomic Proteomic, 2008. **7**(5): p. 355-70.

194. Lacorte, S. and A.R. Fernandez-Alba, *Time of flight mass spectrometry applied to the liquid chromatographic analysis of pesticides in water and food.* Mass Spectrom Rev, 2006. **25**(6): p. 866-80.

195. Bier, M., F.F. Nord, and L. Terminiello, *On the mechanism of enzyme action. LXI. The self digestion of trypsin, calcium-trypsin and acetyltrypsin.* Arch Biochem Biophys, 1956. **65**(1): p. 120-31.

196. Hervey, W.J.t., M.B. Strader, and G.B. Hurst, *Comparison of digestion protocols for microgram quantities of enriched protein samples.* J Proteome Res, 2007. **6**(8): p. 3054-61.

197. Strader, M.B., et al., *Efficient and specific trypsin digestion of microgram to nanogram quantities of proteins in organic-aqueous solvent systems.* Anal Chem, 2006. **78**(1): p. 125-34.

198. Hustoft, H.K., et al., *Critical assessment of accelerating trypsination methods.* J Pharm Biomed Anal, 2011. **56**(5): p. 1069-78.

199. Graciotti, M., et al., *Malaria protein kinase CK2 (PfCK2) shows novel mechanisms of regulation.* PLoS One, 2014. **9**(3): p. e85391.

200. Windheim, M., et al., *Interleukin-1 (IL-1) induces the Lys63-linked polyubiquitination of IL-1 receptor-associated kinase 1 to facilitate NEMO binding and the activation of IkappaBalpha kinase.* Mol Cell Biol, 2008. **28**(5): p. 1783-91.

201. Sen, R. and D. Baltimore, *Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism.* Cell, 1986. **47**(6): p. 921-8.

202. Sen, R. and S.T. Smale, *Selectivity of the NF-{kappa}B response.* Cold Spring Harb Perspect Biol, 2010. **2**(4): p. a000257.

203. Hayden, M.S. and S. Ghosh, *Shared principles in NF-kappaB signaling.* Cell, 2008. **132**(3): p. 344-62.

204. Chen, J. and Z.J. Chen, *Regulation of NF-kappaB by ubiquitination.* Curr Opin Immunol, 2013. **25**(1): p. 4-12.

205. Karashima, T., et al., *Nuclear factor-kappaB mediates angiogenesis and metastasis of human bladder cancer through the regulation of interleukin-8.* Clin Cancer Res, 2003. **9**(7): p. 2786-97.

206. Roebuck, K.A., *Regulation of interleukin-8 gene expression.* J Interferon Cytokine Res, 1999. **19**(5): p. 429-38.

207. McGouran, J.F., et al., *Deubiquitinating enzyme specificity for ubiquitin chain topology profiled by di-ubiquitin activity probes.* Chem Biol, 2013. **20**(12): p. 1447-55.

208. Wu, H.M., K.H. Chi, and W.W. Lin, *Proteasome inhibitors stimulate activator protein-1 pathway via reactive oxygen species production.* FEBS Lett, 2002. **526**(1-3): p. 101-5.

209. Shabek, N., et al., *The size of the proteasomal substrate determines whether its degradation will be mediated by mono- or polyubiquitylation.* Mol Cell, 2012. **48**(1): p. 87-97.

210. Shabek, N., K. Iwai, and A. Ciechanover, *Ubiquitin is degraded by the ubiquitin system as a monomer and as part of its conjugated target.* Biochem Biophys Res Commun, 2007. **363**(2): p. 425-31.

211. Wauer, T., et al., *Ubiquitin Ser65 phosphorylation affects ubiquitin structure, chain assembly and hydrolysis.* EMBO J, 2015. **34**(3): p. 307-25.

212. Tsuchiya, H., K. Tanaka, and Y. Saeki, *The parallel reaction monitoring method contributes to a highly sensitive polyubiquitin chain quantification.* Biochem Biophys Res Commun, 2013. **436**(2): p. 223-9.

213. Lazarev, A.V., et al., *Centrifugal methods and devices for rapid in-gel digestion of proteins.* Electrophoresis, 2009. **30**(6): p. 966-73.

214. Speicher, K.D., et al., *Systematic analysis of peptide recoveries from in-gel digestions for protein identifications in proteome studies.* J Biomol Tech, 2000. **11**(2): p. 74-86.

215. Yeung, Y.G., et al., *Removal of detergents from protein digests for mass spectrometry analysis.* Anal Biochem, 2008. **382**(2): p. 135-7.

216. Flick, K. and P. Kaiser, *Protein degradation and the stress response.* Semin Cell Dev Biol, 2012. **23**(5): p. 515-22.