Investigation of diverse polyubiquitin chains in the mouse brain using ubiquitin binding domains

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Abstract

Ubiquitination is a post-translational modification of protein by ubiquitin (Ub) and plays a vital role in the regulation of a number of cellular functions, including protein degradation *via* the ubiquitin proteasome system (UPS). These functions require recognition of specific ubiquitinated substrates by ubiquitin binding proteins (also known as ubiquitin receptors), which possess ubiquitin binding domains (UBDs) that interact directly with monoubiquitin and/or polyubiquitin chains. There are at least 16 different UBDs to date of which the ubiquitin associated domain (UBA) is an example. Unanchored polyubiquitin chains are a relatively new phenomenon. Studies suggest that these may be involved in the regulation of innate immunity, stress response and aggresome formation and disassembly. The level of unanchored polyubiquitin chains may be controlled by the action of specific ubiquitin ligases that synthesise them, e.g. E2-25K, or deubiquitinating enzymes that release unanchored polyubiquitin chains from polyubiquitinated substrate proteins, e.g. Isopeptidase T (IsoT). Major neurodegenerative diseases such as Alzheimer's and Parkinson's diseases are characterized by selective neurodegeneration and the formation of protein inclusions containing misfolded and aberrant proteins, and ubiquitin. UPS impairment has been implicated in the cause or progression of neurodegenerative disease.

This thesis investigates the interaction of five different UBDs to synthetic ubiquitin; UBA of p62 and ubiqulin1 (UQ1), coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) of Vsp9, ubiquitin binding to ABIN and NEMO (UBAN) of NEMO and zinc finger domain (ZnFUBP) of IsoT. These studies are followed by investigating a mouse model of neurodegeneration caused by conditional genetic 26S proteasomal deletion in mouse forebrain neurons that shows accumulation of ubiquitin.

I show in this thesis that the UBDs have different ubiquitin-binding properties. The p62 UBA and Vps9 CUE domains have similar binding affinity to ubiquitin; binding Lys48- and Lys63-linked, and linear polyubiquitin chains, but not to monoubiquitin. The UBAN domain of NEMO binds only to linear polyubiquitin chains. The IsoT ZnFUBP domain, which interacts with the C-terminus of proximal ubiquitin, only binds to unanchored/free ubiquitin. UQ1 did not show differential binding and bound to all species of ubiquitin investigated. Given the limited studies investigating linear and free chains *in vivo*, I used the UBAN domain of NEMO and the ZnFUBP domain of IsoT to investigate the abundance of linear and

unanchored polyubiquitin chains respectively in the mouse brain cortex of control and 26S proteasome-deleted mice.

Although I did not detect the presence of linear polyubiquitin in my studies, I demonstrate accumulation of unanchored polyubiquitin chains in the cortex and cortical mitochondria of 26S proteasome-depleted mice. I suggest that the accumulation of unanchored polyubiquitin chains in this mouse model may be due to increased de novo synthesis, disassembly of polyubiquitin chains from polyubiquitinated proteins by the action of deubiquitinating enzymes or inhibition of their degradation by the 26S proteasome. Further investigations of IsoT/USP5 and E2-25 levels did not show any significant differences that may explain the accumulation of unanchored polyubiquitin chains following 26S proteasome impairment. However, I show significantly decreased levels of p-TAK1 in the 26S proteasome-depleted mice compared to controls that will be further investigated in the future.

Acknowledgement

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Abbreviations

ABIN	A20-binding inhibitor of NF-kB
AD	Alzheimer's disease
aPKCs	Atypical protein kinase Cs
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
BAFF	β-cell-activated factor
BRAP2	BRCA1-associated protein 2
CaMKIIα	Calcium calmodulin-dependent protein kinase IIα promoter
CARD	Caspase activation and recruitment domains
CNBr	Cyanogen bromide activated-Sepharose-4B
CP	Core particle
CoxIV	Cytochrome c oxidase IV
CUE	Coupling of ubiquitin to endoplasmic reticulum degradation
DNA	Deoxyribonucleic acid
Dsk2p	Ubiquitin-binding protein from Saccharomyces Cerevisiae
DTT	Dithiotheritol
DUBs	Deubiquitinating enzymes
E1	Ubiquitin-activating enzymes
E2	Ubiquitin-conjugating enzymes
E3	Ubiquitin ligase enzymes
ER	Endoplasmic reticulum
GAPs	GTPase activating proteins
GEF	Guanine nucleotide exchange factor
GDP	Guanosine diphosphate
GTPase	Guanosine triphosphate hydrolyse
GTP	Guanosine triphosphate
GSH	Glutathione

GST	Glutathione-S-transferase
JAMM	JAB1/MPN/Mov34 is a zinc-dependent metalloprotase
HDAC6	Histone deacetylase 6
HCl	Hydrochloric acid
HECT	Homologous to the E6-AP
hHR23A	Human homolog of Saccharomyces Cerevisiae Rad23
HRP	Horseradish Peroxidase
IkBs	Inhibitor proteins of kB family
IKK	IkB kinase complex
IKKα	IKK alpha
IKKβ	IKK beta
IKKγ	IKK gamma
IL-1	Interleukin-1
IPTG	Isopropyl-β-thiogalactopyrinosid
IsoT /USP5	IsopeptidaseT/Ubiquitin specific protease 5
LB	Luria broth
LUBCA	Linear ubiquitin chain assembly complex
Met 1	Methionine
MG-132	Carbobenzoxy-Leu-Leu-leucinal (proteasome inhibitor)
MJD	Josephin domain
monoUb	Mono ubiquitin
MS	Mass-spectrometry
Mud1	Ortholog of the Saccharomyces Cerevisiae DNA-damage respons protein Ddi1
N27	Rat mesencephalic dopaminergic neurons
NEM	N-Ethylmaleimide
NEMO	NF-kB essential modulator
NF-kB	Nuclear factor kappa B
NIK	NF-kB inducing kinase

NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
Rad23	Radiation sensitivity abnormal 23
RHD	Rel-homology domain
RIP1	Receptor-Interacting Protein 1
RIG-1	Retinoid-inducible gene 1
RING	Really interesting new gene
RP	Regulatory particles
Rpn	Regulatory particle non-ATPase
PAZ	PolyUb associated zinc finger domain
PBI	Acid interaction motif
PD	Parkinson's disease
PEST	Proline, Glutamate, Serine and Threonine
PIs	Protease inhibitor cocktail
PMSF	Phenylmethylsulfonyl fluoride
p-TAK1	Phosphate TAK1
PolyUb	Polyubiquitin
PSMC1	Proteasome 26S subunit ATPsae 1
OTU	Ovarian tumour domain
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SH2	src homology 2
TAB2/3	TAK1 binding protein 2/3
TAK1	Transforming growth factor β-activated kinase-1
TCB	Thrombin cleavage buffer
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor alfa
TNFR	TNF receptors
TRAF6	TNF receptor associated factor 6
TUBE	Tandem ubiquitin-binding entities

UBA	Ubiquitin-associated domain
UBAN	Ubiquitin binding in ABIN and NEMO
UBA6	Ubiquitin-like modifier activating enzyme 6
UBE1	Ubiquitin activating enzyme 1
Ub	Ubiquitin
UBC	Ubiquitin conjugating domain
UBDs	Ubiquitin binding domains
UBPs	Ubiquitin binding proteins
Ubl	Ubiquitin-like domain
UCH	Ubiquitin c-terminal hydrolase
UIM	Ubiquitin interacting motif
UPS	Ubiquitin-Proteasome System
USP/UBP	Ubiquitin specific processing protease
USP2	Ubiquitin specific peptidase2
UQ1	Ubiquilin1
Vsp9	Vacuolar protein sorting 9
XIAP	X-linked inhibitor of apoptosis
ZF	Zinc finger
ZnFUBP	Zinc finger domain
Zz	Zinc finger

Chapter 1 Introduction

1.1 Introduction

The most common features of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, are the aggregation of ubiquitin and abnormal proteins and the progressive loss of specific neurons (Ardley & Robinson, 2004; Q. A. Huang & Figueiredo-Pereira, 2010). Defects in the ubiquitin-proteasome pathway (UPS) and impairment in mitochondrial function have been implicated in the pathogenesis and progression of neurodegenerative diseases (Ross & Pickart, 2004). The UPS is one of the major routes for the degradation of unwanted proteins, preventing their accumulation and has recently been associated with mitochondrial homeostasis (Takalo et al., 2013).

The number of investigations into the mechanisms of neurodegenerative disease is growing, using human, animal and cellular studies. Bedford and colleagues developed a mouse model of neurodegenerative disease, showing that proteasomal dysfunction is involved in disease pathogenesis (Bedford et al., 2008).

Unanchored polyubiquitin (polyUb) chains are a relatively novel phenomenon and their function is unclear. Studies have suggested they may be involved in the activation of innate immunity and aggresome formation (Xia et al., 2009; Zeng et al., 2010; Ouyang et al., 2012).

This thesis is divided into two main areas of study: (1) investigations into the binding interactions of ubiquitin binding domains (chapter 3) and (2) investigations into the expression of unanchored polyUb chains in the 26S proteasome-depleted mouse brain (chapter 4).

1.2.Ubiquitin

Ubiquitin (Ub) is a small (76 amino acid residues), highly conserved protein with a molecular weight of approximately 8.5 kDa (Figure 1.1). Ub is a heat stable protein and produced by cleavage of either ubiquitin-ribosomal fusion protein or Ub chains (polyubiquitin). Deubiquitinating (DUB) enzymes cleave Ub from its fusion protein or polyUb chain to produce monoUb (Reyes-Turcu et al., 2009). Ub can exist either in a free form (unanchored Ub) or conjugated to substrate proteins.

Ub plays an important role in the regulation of a number of cellular functions, including degradation of unwanted proteins via the ubiquitin-proteasome pathway, cell division, regulation of transcription, the stress response, intracellular signalling, immunity, embryonic development and DNA repair (Groothuis et al., 2006; Y. Kang et al., 2006; Jadhav & Wooten, 2009).

These processes require recognition of specific ubiquitinated substrates by ubiquitin binding proteins (UBPs; also known as ubiquitin receptors), which possess ubiquitin binding domains (UBDs) that interact directly with monoUb and/or polyUb chains. In addition to the UBDs, UBPs contain various structural features and have diverse functions, such as p62 and ubiquilin1 (Seibenhener et al., 2004; Hicke et al., 2005; Zhang et al., 2008).

Figure 1.1 from Spasser *et al*. (2012), represent the Ub structure in ribbon, shown all the 7 lysine residues, C-termini glycine (Gly-76) and N-termini methionine (Met 1), and the hydrophobic patch (Leu8, Ile44, Val70). {Spasser, 2012 #960}

Figure 1.1

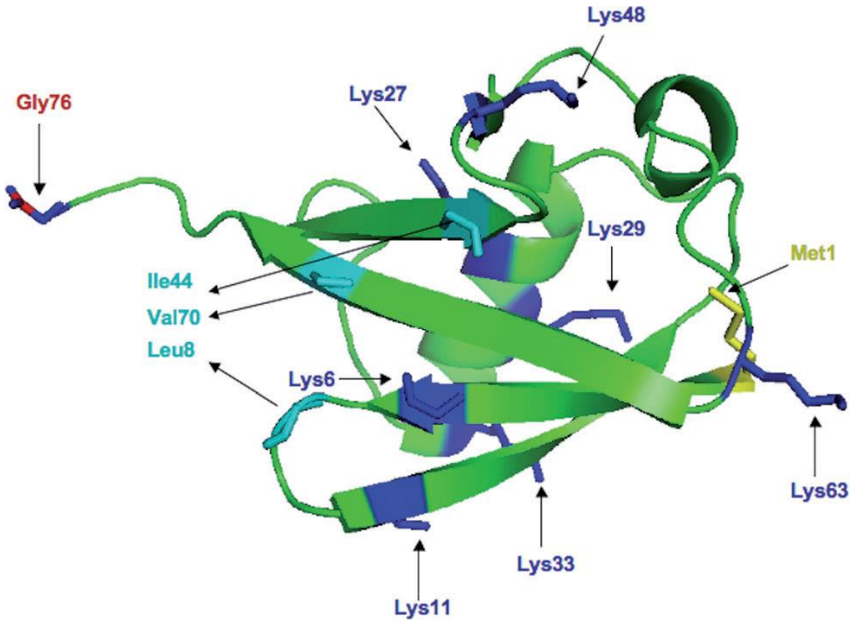


Figure1.1 Schematics represent the Ubiquitin ribbon structure. Taken from Spasser *et al*. (2012). Shown the all seven Lysine residues, N-terminal Methionine, C-terminal Glycine and the hydrophobic patch (Leucine 8, Isoleucine44 , Valine 70).

1.3 Ubiquitination

Ubiquitination is a post-translation modification involving the attachment of Ub to a target protein or itself by an isopeptide or peptide bond between the C-terminal carboxy group of Ub and a lysine or the methionine of the substrate (Figure 1.2). This modification has an important role in the regulation of various cellular functions, including protein degradation, receptor endocytosis, cell cycle control, DNA repair, transcription, intracellular signalling and the stress response (Groothuis et al., 2006; Kang et al., 2006; Jadhav & Wooten, 2009; Komander, 2009).

Ub conjugation or ubiquitination is carried out by a cascade of three enzymatic reactions. Ubiquitin-activating enzymes (E1) activate Ub using ATP for the formation of a ubiquitin-adenylate intermediate. The activated Ub is conjugated by ubiquitin-conjugating enzymes (E2). In the final step, Ub is bound by its C-terminus in an amide isopeptide link to an amino group of a lysine residue in the target protein by ubiquitin ligase enzymes (E3) (see Figure 1.2) (Pickart, 2001; Komander, 2009;Husnjak & Dikic, 2012).

The addition of Ub molecules to Ub forms polyUb chains. The terminal carboxy residue of Ub is attached to the ϵ-amino group of a lysine residue in the next Ub to form a polyUb chain. Ub contains seven lysine residues that can be involved in ubiquitination (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) (Peng et al., 2003). Linear polyUb can also be formed using the N-terminal methionine of Ub (M1) (Kirisako et al., 2006; Komander, 2009).

Modification of substrate proteins can be achieved by a single Ub (mono-ubiquitination), by several Ubs at different sites (multiple mono-ubiquitination) or by polyUb chains (Miranda & Sorkin, 2007). An overview of Ub modifications: monoubiquitination, multi-monoubiquitination and polyubiquitination described here are presented in Figure 1.3. Figure 1.4 shows the crystal structure for most of these chains has been solved (Husnjak & Dikic, 2012).

MonoUb has been shown to play an important role in endocytic degradation, membrane trafficking, gene transcription and DNA repair (Chen & Sun, 2009; Sun et al., 2011). Specific Ub-linkages may also be associated with specific cellular functions. For example, Lys6-linked polyUb is involved in DNA repair; Lys11-linked polyUb plays an important role in protein degradation via endoplasmic reticulum associated degradation and in the regulation of cell cycle; Lys27-linked polyUb and Lys29-linked polyUb in ubiquitin fusion and lysosomal protein degradation respectively. Lys29- and Lys33-linked polyUb have been shown to play a role in kinase modification (Husnjak & Dikic, 2012). Lys48-linked polyUb chains, consisting of a minimum of four Ub moieties, are an important signal for targeting ubiquitinated proteins for degradation by the 26S proteasome. Studies suggest Lys63-linked polyUb chains are involved in the regulation of DNA repair, innate and adaptive immunity (Pickart & Fushman, 2004). Met1-linear polyUb activates NF-kB pathways (Husnjak & DiKic, 2012.). Several studies have indicated that all Lys polyUb chains are involved in the protein degradation (Jacobson et al., 2009; Saeki et al., 2009).

Ub signals are recognised by UBPs (also known as Ub receptors). Ub receptors have one or more UBDs that interact with ubiquitinated substrates through binding to monoUb and/or polyUb chains. To date there are approximately 20 families of UBDs and the UBA (ubiquitin associated domain) domain is one of the most common (Fushman & Wilkinson, 2011; Spasser & Brik, 2012).

Deubiquitinating enzymes (DUBs) reverse ubiquitination; these are cysteine proteases that hydrolyze the isopeptide or peptide bond between Ub and the

target protein or between Ubs in a polyUb chain (Kim et al., 2003; Komander et al., 2009a).

1.3.1 Ubiquitin activating enzyme (E1)

The human genome encodes only two ubiquitin activating enzymes: ubiquitin activating enzyme 1 (UBE1) and ubiquitin-like modifier activating enzyme 6 (UBA6) also known as UBE1 L2 and E1-L2. E1-L2 is about 42% identical to UBE1 (Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007; Groettrup et al., 2008). Ub activation occurs in two steps and is ATP-dependent. First an acyl phosphoanhydride bond between the C-terminal glycine of Ub and the AMP moiety of ATP is formed. Then the activated Ub is bound to the cysteine residue in the E1 active site by a thio-ester linkage with the release of AMP (Haas et al., 1982; Ciechanover et al., 1991; Lee & Schindelin, 2008; Schulman & Harper, 2009).

1.3.2 Ubiquitin conjugated enzyme (E2)

In humans there are at least 38 E2 enzymes encoded in the genome. E2 ubiquitin conjugating enzymes work as Ub transporters. E2s facilitate the transfer of activated Ub to the target substrate via direct interaction with an E3 enzyme. E2 enzymes have a ubiquitin conjugating domain (UBC) that accommodates the activated Ub. The E2 determines the Ub linkage in polyUb chains (Windheim et al., 2008; Ye & Rape, 2009; van Wijk & Timmers, 2010).

1.3.3 Ubiquitin ligase (E3)

The human genome encodes approximately 1000 ubiquitin protein ligases (E3) (Ramanathan & Ye, 2012). E3s interact with the ubiquitin conjugating enzyme (E2) and transfer Ub to the substrate. E3 ubiquitin protein ligase enzymes (E3) are responsible for substrate selection (Deshaies & Joazeiro, 2009). There are two main classes of E3 ligases; RING (really interesting new gene) and HECT (homologous to the E6-AP C terminus) ligases. RING E3 enzymes deliver activated E2-Ub to the target protein and the Ub molecule is transferred directly from the E2 to the target protein substrate. HECT E3 enzymes bind activated Ub before Ub is transferred to the target substrate (Furukawa et al., 2005; Hoeller et al., 2007). Furthermore, HECT ligases are also responsible for chain linkage specificity, while for RING ligases it is the E2 that determines linkage specificity. The linear Ub chain assembly complex (LUBCA) is a RING ubiquitin protein ligase that has the ability to generate only linear Ub chains showing linkage specificity independent of E2 (Stieglitz et al, 2012).

Figure 1.2

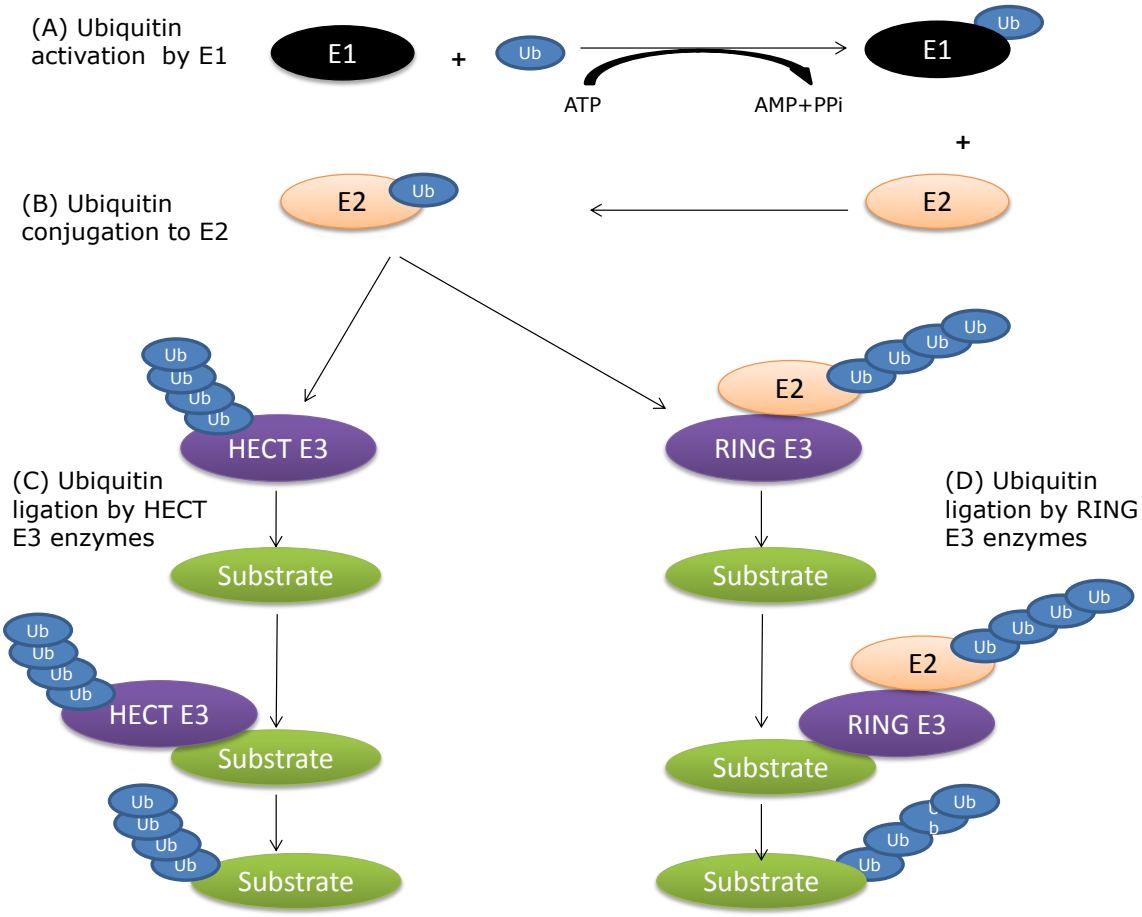


Figure 1.2 Schematic representation of the ubiquitination cascade. The first step in ubiquitination involves the ATP-dependent activation of ubiquitin (Ub) by ubiquitin activating enzyme (E1; section 1.3.1) (A). Ubiquitin is transferred from the E1 to a ubiquitin conjugating enzyme (E2; section 1.3.2) (B). Ubiquitin protein ligase (E3) either facilitate (RING E3) the transfer of ubiquitin from the E2 to the substrate protein (C) or directly catalyse (HECT E3) the transfer of ubiquitin to the substrate (D) (section 1.3.3).

Figure 1.3

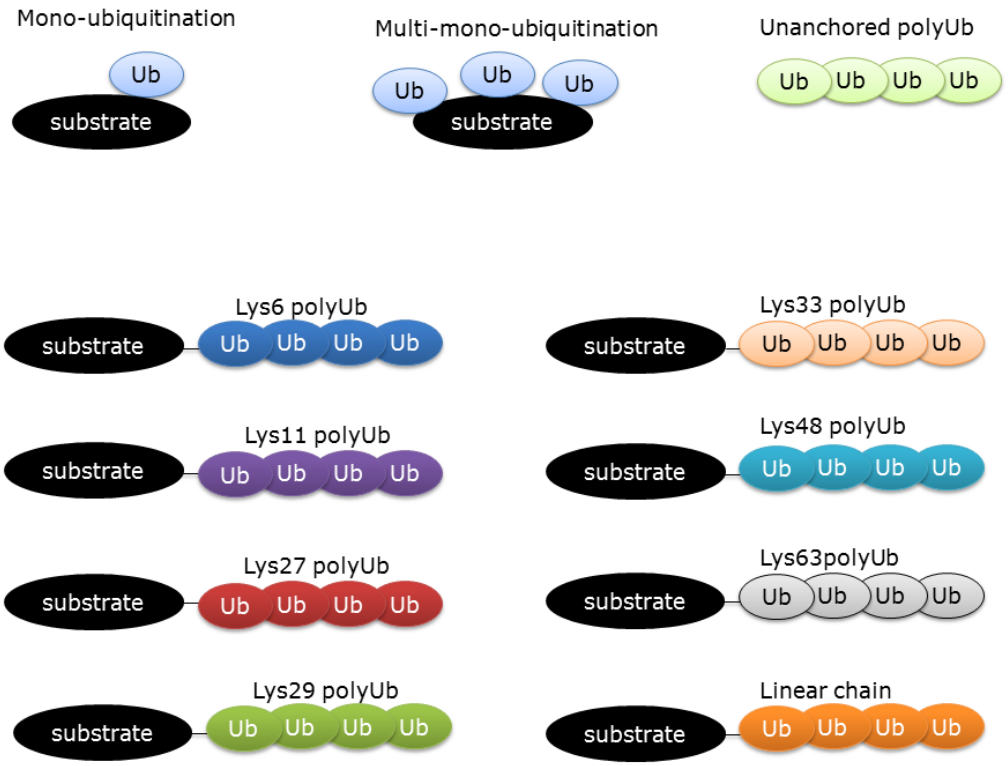


Figure 1.3 Representaion of ubiquitin (Ub) modifications: monoubiquitination, multi-monoubiquitination and polyubiquitination. polyUb chains can be conjugated to a substrate protein or Ub through one of seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63). There are also linear and unanchored ubiquitin chains using the C-terminus glycine of Ub with methione of another Ub or not conjugated to the substrate respectively.

Figure 1.4

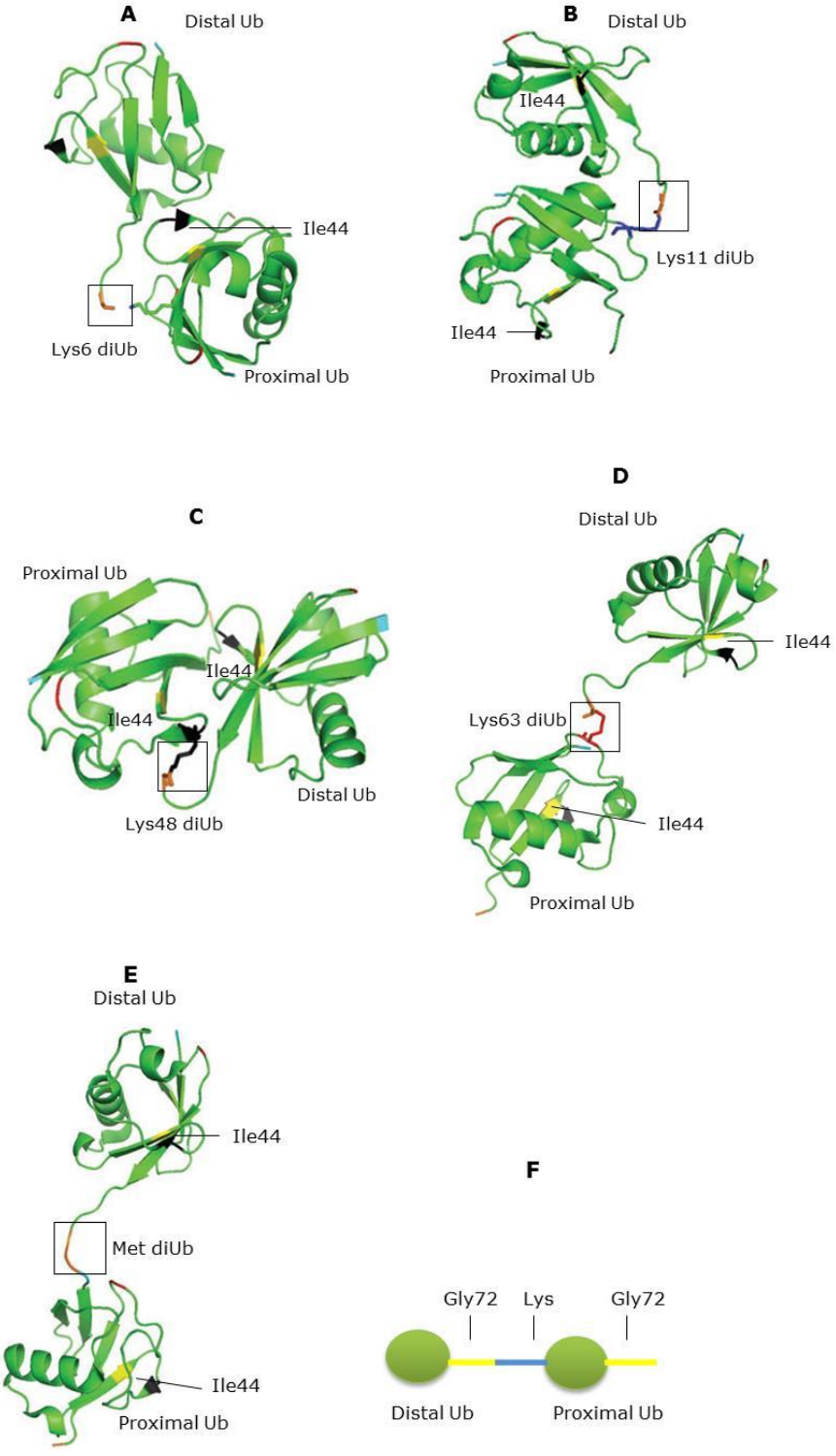


Figure 1.4 Ubiquitin chains can have different linkages; the crystal structure for most of these chains has been solved. A) Lys6-linked diUb and B) Lys11-linked diUb adopt a compact conformation. C) Lys48-linked diUb adopts a closed conformation. D) Lys63-linked diUb and E) Met1-linked diUb form an extended conformation. F) Schematic picture representing diUb showing the distal and proximal Ub. Adapted from Husnjak& Dikic, 2012.

1.4 Deubiquitination

Deubiquitination is the reverse of ubiquitination. Deubiquitinating enzymes (DUBs) are proteases that hydrolyse the isopeptide/peptide bond between Ub and substrate or Ub and Ub in polyUb chains (Figure 1.4) (Ventii & Wilkinson, 2008; Komander, et al., 2009a; Reyes-Turcu et al., 2009). The human genome encodes approximately 100 DUBs (Amerik & Hochstrasser, 2004; Nijman et al., 2005). These are classified into five groups; four are cysteine proteases [ubiquitin c-terminal hydrolase (UCH), ubiquitin specific protease (USP/UBP), ovarian tumour domain (OTU), and Josephin domain (MJD)] and the fifth group is a zinc-dependent metalloprotase [JAB1/MPN/Mov34 (JAMM)] (Amerik & Hochstrasser, 2004; Reyes-Turcu et al., 2009).

DUB enzymes have an important role in the regulation of the Ub pathway, including the generation of mono-Ub moieties from newly expressed Ub fusion proteins or polyUb chains (Figure 1.5A) as well as the cleavage of Ub bound to substrate protein (Figure 1.5B) (Kim et al., 2003; Komander et al., 2009a; Reyes-Turcu et al., 2009). Deubiquitination may negatively regulate protein degradation by removing Ub from substrates before delivery to the 26S proteasome.

Figure 1.5

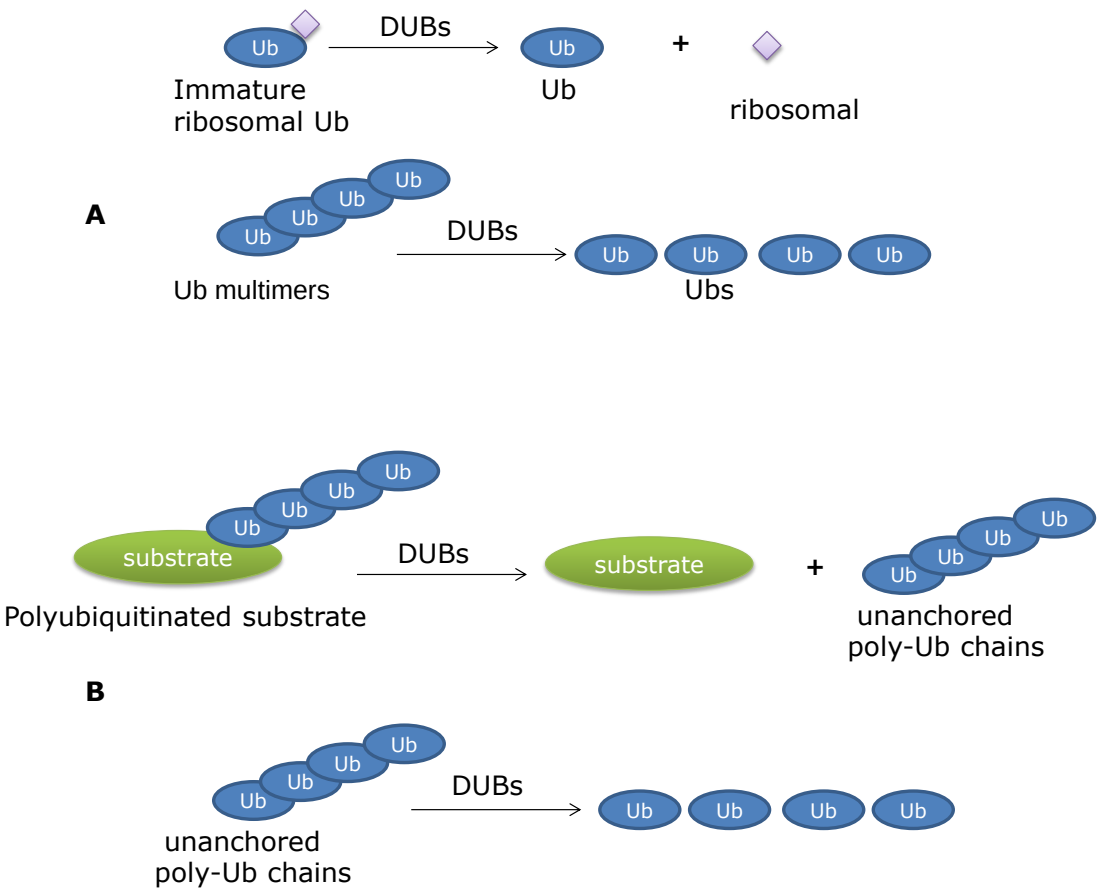


Figure 1.5 Schematic representation of deubiquitination. (A) Generation of mono-Ub moieties from newly expressed immature Ub fusion proteins or polyUb chains. (B) Cleavage of Ub bound to substrate protein and unanchored polyUb chains.

1.5 Protein degradation

It is well-known that proteins have different half-lives and there is a balance between protein synthesis and degradation (Martinez-Vicente et al, 2005). Intracellular protein degradation plays an essential role in numerous cellular pathways, including the cell cycle, immune and inflammatory responses, and removal of unwanted and abnormal proteins. There are two major protein degradation pathways; the autophagy-lysosome pathway (ALP) and ubiquitin proteasome system (UPS) (Champe *et al.,* 2008).

1.5.1 Autophagy-lysosome pathway

Autophagy is restricted to the cytoplasm and is capable of degrading a wide range of substrates, including cellular organelles, but also functional and misfolded soluble proteins, protein complexes and aggregates that are poor substrates for proteasomal degradation. There are three different types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) (Blommaart et al, 1997; Settember et al, 2013). Macroautophagy involves expansion of a phagophore membrane which engulfs cytoplasmic material as it closes to form a double membrane vesicle; the autophagosome. The autophagosome goes on to fuse with the lysosome. CMA involves the direct import of cytosolic proteins that contain a pentapeptide motif recognised by a chaperone leading to a receptor-mediated translocation into lysosomes. Microautophagy involves direct uptake of cytoplasmic components by invagination of the lysosomal membrane.

Macroautophagy is a catabolic mechanism for the cellular degradation of proteins and organelles (Figure1.6). In the first step, long-lived proteins and organelles are sequestered inside double-membraned phagophores. Then, the phagophore expands to produce an autophagosome. Finally, the autophagosome fuses with the lysosome to form the autophagolysosome where the contents are exposed to hydrolytic enzymes that degrade the proteins and organelles (Bergamini et al., 2004; Knaevelsrud & Simonsen, 2010; Kraft et al., 2010 ; Benbrook & Long, 2012).

Figure 1.6

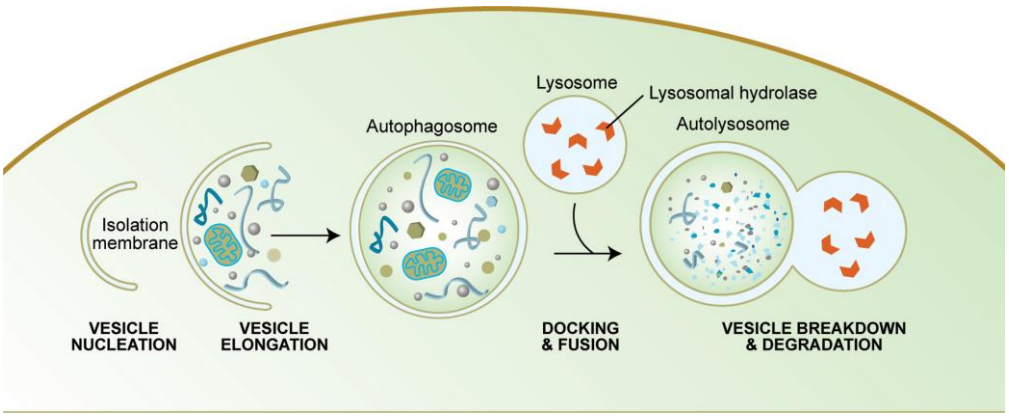


Figure 1.6 Macroautophagy taken from (Melendez & Levine, 2009).

1.5.3 The ubiquitin proteasome system

Ubiquitin proteasome system (UPS) play a vital role in variety of cellular function, including cell signalling and immune response (Ben-Neriah, 2002; Ciechanover, 1998). In the cell, the major regulatory protein degradation pathway is UPS (Du & Mei, 2013).

Protein degradation by the UPS requires two steps. First step is protein polyubiquitination by the action of three cascade enzymatic reaction, activation of Ub by E1, conjugation by E2 and ligation by E3 (section 1.3) (Lys48-linked polyUb chain of at least Ub_4 is the classical signal for protein degradation). Second step is polyubiquitinated proteins degradation by the 26S proteasome (Ciechanover, 1998). Polyubiquitinated proteins are then recognised by subunits associated with the base of 19S such as Rpn10/S5a (Hartmann-Petersen et al., 2003; Kirkin & Dikic, 2007).

In addition to degradation of short lived protein and regulatory proteins UPS play an important role in the degradation of abnormal proteins, including misfolded proteins, and prevent their accumulation and aggregation which causes intracellular inclusion formation (Cook et al., 2012).

The 26S proteasome is multicatalytic protease which recognises and degrades polyubiquitinated proteins into peptides and free Ubs are liberated (section 1.5) (Coux et al., 1996; Hartmann-Petersen & Gordon, 2004). See also Figure 1.8 for a schematic representation of protein degradation via the UPS.

1.5.3.1 The 26S Proteasome

The 26S proteasome is a multimeric protease which plays an important role in the degradation of small unwanted intracellular proteins (Coux et al., 1996; Hartmann-Petersen & Gordon, 2004). The 26S proteasome is composed of a 20S catalytic core particle (CP), which is proteolytically active, and one or two 19S regulatory particles (RP). The 19S RP functions is recognition of ubiquitinated proteins, deubiquitination and substrate translocation into the core particle (Coux et al., 1996; Glickman et al., 1998 ; Ferrell et al., 2000).

1.5.3.1.1 The 20S catalytic core particle (CP)

The 20S catalytic core particle is composed of four stacked rings; two outer α rings and two inner β rings. Each outer ring is made up of seven different α subunits and each inner ring consists of seven different β subunits (Figure 1.7) (Lowe et al., 1995; Ciechanover, 2005).

The crystal structure of the 20S CP showed three internal chambers (Lowe et al., 1995; Unno et al., 2002). In the central chamber there are at least three active sites (β1, β2 and β5), which hydrolyse the majority of peptides bonds. β1 cleaves on the C-terminal of acidic residues (caspase-like activity), β2 cleaves after tryptic residues (trypsin-like activity) and β5 cleaves after hydrophobic residues (chymotrypsin-like activity) (Borissenko & Groll, 2007).

The alpha subunits of the CP form a gate to restrict entry of substrates into the 20S and bind to ATPase subunits in the base of the RP. Ubiquitinated substrates need to pass through this gate in order to enter the catalytic chamber of the CP for degradation (Lam et al., 2002; Smith et al., 2007).

1.5.3.1.2 The 19S regulatory particle (RP)

The 19S regulatory particle (RP) consists of two sub-complexes; the lid and the base (Figure 1.5). The 19S RP binds to the 20S particle to make up the 26S proteasome complex (Glickman et al., 1998).

The base is composed of 10 subunits. Six AAA ATPases [Rpt1/S7, Rpt2/S4/PAMC1, Rpt3/S6b, Rpt4/S10b, Rpt5/S6a and Rpt6/S8] form a hexameric ring and interact with the outer ring of the 20S CP (Hartmann-Petersen & Gordon, 2004). Rpt2/S4/PSMC1 has regulatory function by opening the entry gate by the α-subunit of the of the 20S control particle, which allow polyubiquitinated proteins to get into the proteolytic channel for degradation (Kohler et al., 2001; Smith et al., 2005). The remaining non-ATPase subunits are termed regulatory particle

non-ATPase (Rpn); Rpn1/S2, Rpn2/S1, Rpn13/hRpn13 and Rpn14 (Glickman et al., 1998; Chen et al., 2008; Hendil et al., 2009; Kaneko et al., 2009).

The function of the lid subcomplex is to recognize and unfold ubiquitinated protein substrates. The lid of the RP contains eight non-ATPase (Rpn) subunits; Rpn3/S3, Rpn5/p55, Rpn6/S9, Rpn7/S10a, Rpn8/S12, Rpn9/S11, S5a/Rpn10, Rpn11/S13 and Rpn12/S14 (Glickman et al., 1998; Verma et al., 2000; Chen et al., 2008). Rpn11/S13 is involved in substrate deubquitination, releasing Ub from the delivered protein substrate (Verma et al., 2002). S5a (Rpn10/pus1) recognizes and binds polyubiquitinated substrates. This subunit is a ubiquitin binding protein containing a UBD named the ubiquitin interacting motif (UIM) (Hartmann-Petersen et al., 2003; Wang et al., 2005; Kirkin & Dikic, 2007).

Figure 1.7

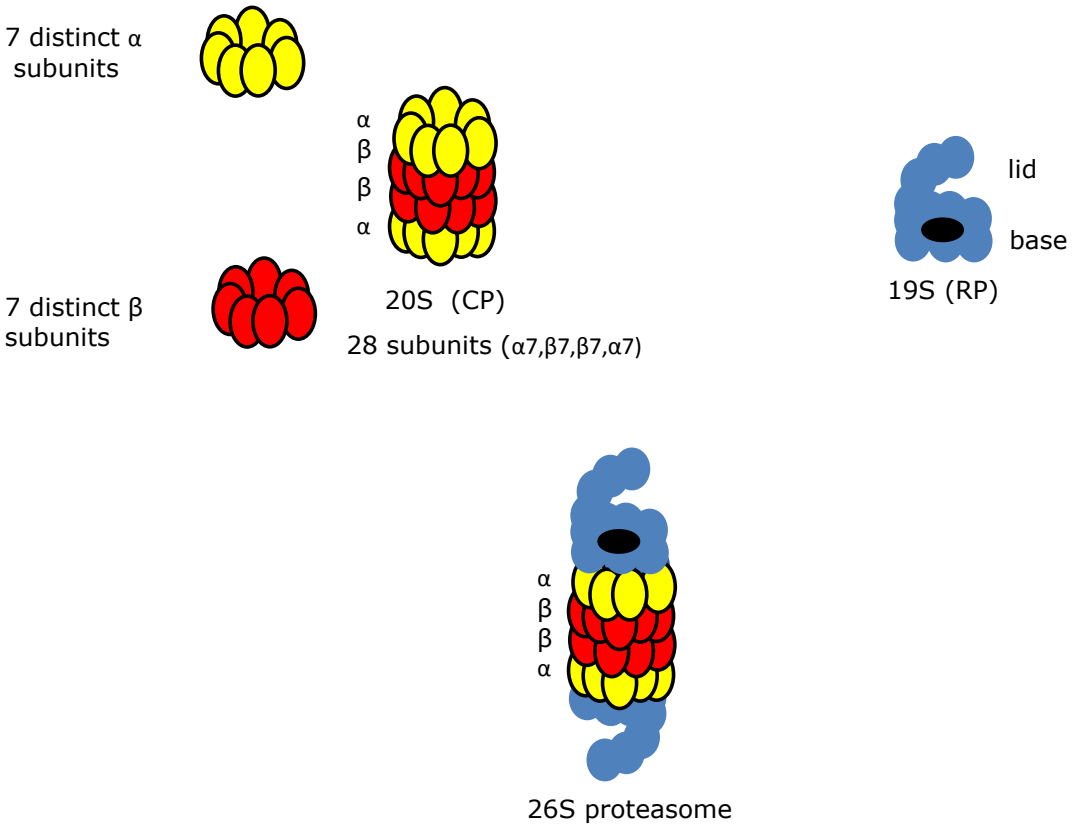


Figure 1.7 Schematic representation of the 26S proteasome. The 26S proteasome complex is composed of 20S core particle and 19S regulatory particle.

Figure 1.8

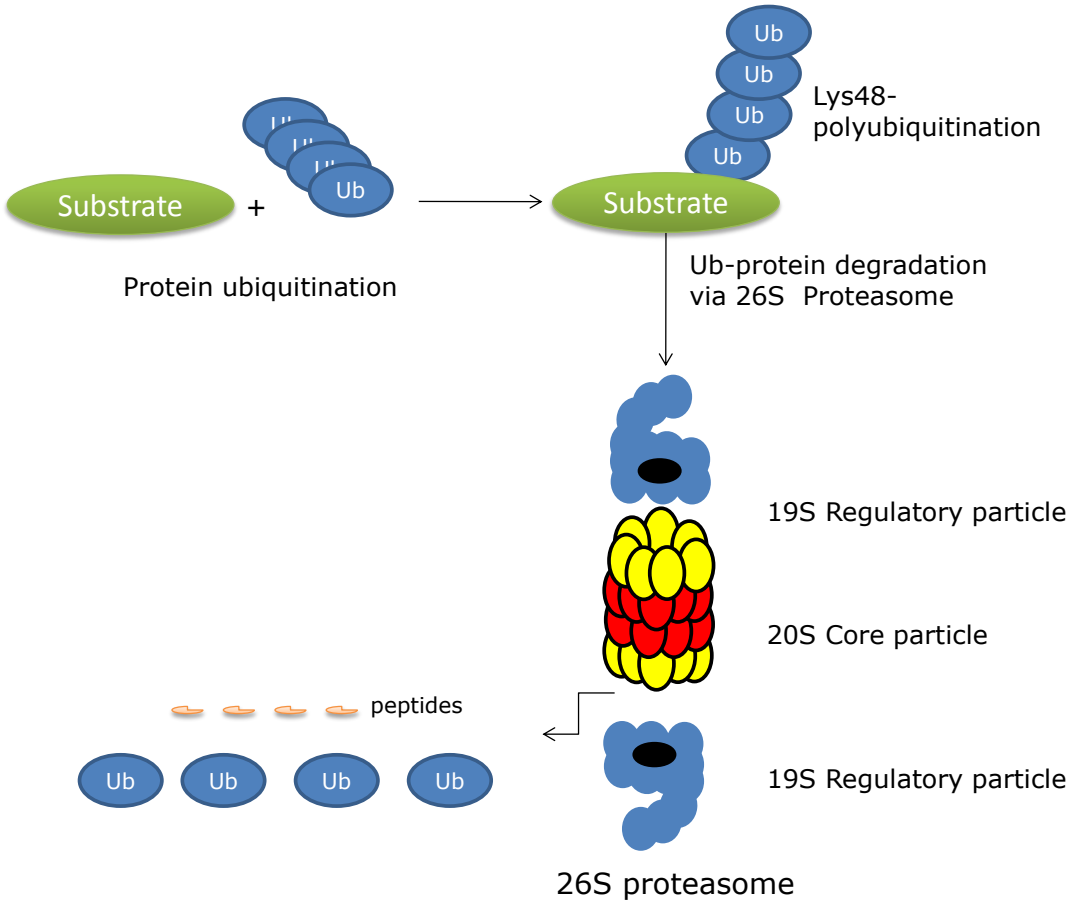


Figure 1.8 Cartoon representation of the ubiquitin proteasome system (UPS).

1.5.2 Endoplasmic reticulum-associated degradation

The UPS takes part in protein quality control in association with the endoplasmic reticulum-associated degradation pathway (ERAD). The ERAD pathway directs ubiquitin-mediated degradation of misfolded proteins and most proteins of secretory pathway in the endoplasmic reticulum via the UPS. ERAD machinery selects proteins for dislocation across the ER membrane and delivers them to the UPS in the cytoplasm. ERAD involves four distinct steps; (1) substrate recognition, (2) substrate dislocation across the ER membrane to the cytoplasm, (3) addition of Ub/polyUb chains to the substrate and (4) finally proteins are degraded by the UPS (Figure1.9) (Hampton, 2002; Goder, 2012; Guerriero & Brodsky, 2012 ; Olzmann et al., 2013).

Figure 1.9

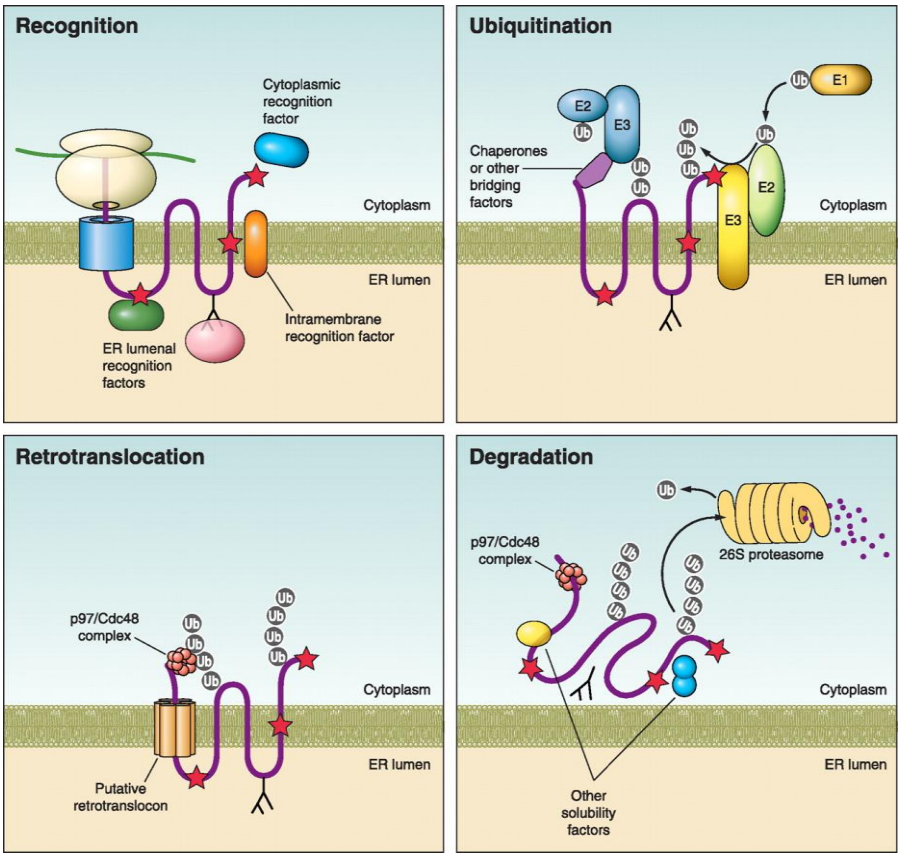


Figure 1.9 Schematic diagram of the steps involved in ERAD; recognition, ubiquitination, retrotranslocation and degradation. Cytosolic chaperones recognise misfolded or damaged proteins that contain a recognition region (red star). Ubiquitination occurs either directly within the ER membrane or by interactions with chaperones in the cytosol. Retrotranslocation is mediated via a channel or retrotranslocon. This process requires AAA ATPase p97 complex to provide the energy for moving the substrate. Degradation, the protein substrate is delivered to the 26S proteasome for degradation. Taken from (Guerriero & Brodsky, 2012).

1.6 Unanchored ubiquitin

Not all ubiquitin is conjugated to a target protein; mono-ubiquitin is free single ubiquitin and unanchored polyubiquitin chains can be produced either from de novo synthesis or as a product of the deubiquitination of ubiquitinated proteins (Dayal et al., 2009). The level of unanchored polyubiquitin chains is controlled by the action of Isopeptidase T (IsoT) (Reyes-Turcu et al., 2009).

Depletion of the Ubp14 gene responsible for the disassembly of unanchored polyUb chains in yeast causes the accumulation of unanchored polyUb chains (Amerik et al., 1997). In human cells suppression of IsoT; the deubiquitinating enzyme that breaks down unanchored polyUb chains, causes an increase in the abundance of free polyUb chains (Dayal et al., 2009). Proteasome function is inhibited by the accumulation of unanchored polyUb chains which compete with polyUb substrates in the proteasome binding sites (Dayal et al., 2009; Piotrowski et al., 1997).

Recent work has proposed that unanchored polyUb chains may serve as a reservoir of Ub (Kimura et al., 2009). Unanchored Lys63 polyUb chains synthesized by TNF receptor-associated factor 6 (TRAF6) E3 ligase can directly activate protein kinase complexes (Xia et al., 2009). Furthermore, unanchored Lys63 polyUb chains activate RIG-I, a signaling protein that plays an important role in the immune response (Zeng et al., 2010). More recently, unanchored polyUb chains have been proposed to play a regulatory role in aggresome formation (Ouyang et al., 2012; Hao et al., 2013) and in the stress response (Braten et al., 2012).

1.7 Neurodegenerative diseases

Neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD) are one of the growing problems in developed countries because of the cost of medical care. There is a steady increase in the number of people suffering from neurodegenerative diseases because of the improvement of life style, which allows us to live longer (Landrigan et al., 2005; Skovronsky et al., 2006).

In the non-familial form of the neurodegenerative diseases, the causes of the disease are unclear. Several reports have indicated that some factors may involve in the cause of neurodegenerative diseases, including aging, genetic mutation, UPS dysfunction, mitochondrial impairment and environmental agents (Mizuno et al., 2001; Ross & Pickart, 2004; Bedford et al., 2008; Domingues et al., 2008; Farooqui & Farooqui, 2009; Cannon & Greenamyre, 2011). The role of genetic mutation and environmental factors in the development of neurodegenerative disorders have been debated (Przedborski, & Jackson-Lewis, 2003).

Neurodegenerative diseases are characterised by progressive loss of selective neurons in the brain and aggregation of abnormal proteins, including disease-specific proteins, in surviving neurons, causing impairment of nervous system function (Soto, 2003; Ardley & Robinson, 2004). A common neuropathological feature of most of the major human neurodegenerative diseases is that their protein inclusions contain ubiquitinated proteins suggesting an imbalance between protein synthesis and degradation that may lead to the accumulation of proteins which aggregate and form inclusions, e.g. Lewy bodies (LB) (Caughey & Lansbury, 2003; Ardley & Robinson, 2004).

1.7.1 The role of the ubiquitin proteasome system in neurodegenerative diseases

The UPS plays an important role in the regulation of intracellular protein homeostasis by degrading short-lived proteins and removing aberrant proteins that accumulated in neurodegeneration (section 1.5.3). Ubiquitinated proteins and Ub are associated with inclusion bodies present in surviving neurons in the brains of patients with most of the chronic human neurodegenerative diseases, including AD and PD (Alves-Rodrigues & Figueiredo-Pereira, 1998). Accumulation of ubiquitinated proteins in neurodegenerative diseases may indicate a role for the UPS in the pathogenesis of disease (Wooten et al., 2006).

The UPS is involved in the development and survival of neuron, synaptic function and plasticity (Tai & Schuman, 2008; Yi & Ehlers, 2007). Genetic mutation of some component of the UPS is associated with different forms of familial neurodegenerative diseases including AD and PD (Ciechanover & Brundin, 2003).

1.7.1.1 Parkinson's disease and the UPS

Parkinson's disease (PD) is a neurodegenerative movement disorder characterised by selective loss of nigral dopaminergic neurons. Insoluble and ubiquitinated proteins accumulate in inclusions known as Lewy bodies (LB). The major component of LB is α-synuclein. Increasing evidence suggests that defects in protein degradation via the UPS, mitochondrial dysfunction and oxidative stress have a role in the pathogenesis of PD (Domingues et al., 2008; Henchcliffe & Beal, 2008).

Dysfunction of the UPS has been connected to PD from neuropathology, genetics and research. Mutations in E3 ubiquitin ligase Parkin and neuron-specific deubiquitinating enzyme ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) are associated with familial PD. The presence of ubiquitinated proteins and components of the UPS in LBs also supports dysfunction of this protein degradation in PD patients (McNaught et al., 2001; Domingues et al., 2008).

1.7.1.2 Alzheimer's disease and the UPS

AD is associated with progressive loss of neurones in the hippocampus and cortex of the brain and is characterised by gradual memory loss, cognitive impairment and behavioural dysfunction (Takalo et al., 2013; Jackson, 2014). The cause of the disease remains unclear, but there are genetic and non-genetic factors involved (DeVrij *et al* 2001). Intraneuronal accumulation of phosphorylated tau and ubiquitinated proteins, which form neurofibrillary tangles (NFT), and extracellular deposits of amyloid peptide (Aβ), processed from the amyloid precursor protein (APP) are the pathological hallmarks of AD (Sisodia & Price, 1995; Braak et al., 1998).

Impairment of the UPS has been associated with the pathology of AD (Upadhya & Hegde, 2007). Studies have shown lower proteasome activity and higher levels of Ub in brain homogenates of Alzheimer's patients compared to age-matched controls (Kudo et al., 1994; Keller et al., 2000). Ubiquitination enzymes, including E1 and E2 enzymes, are down-regulated in Alzheimer's patients (Lopez et al., 2000; Loring, Wen et al., 2001; Lu et al., 2004). In addition, the protein

inclusions in Alzheimer's brains contain ubiquitinated proteins (Ciechanover & Brundin, 2003).

Interestingly, a mutant form of ubiquitin-B^1 ($UBB^{1)}$ has been demonstrated in AD brain (van Leeuwen et al., 1998). UBB^1 lacks a functional Gly76 residue, which is essential in conjugating to lysine of the protein substrate (De Vrij et al., 2001). De Vrij and colleagues have shown that UBB^1 is unable to be degraded via the UPS in reticulocyte lysate and neurones (De Vrij et al., 2001) .

1.7.2 Animal models of neurodegenerative disease

Animal models have been widely used to investigate neurodegenerative disease development, progression and treatment. This progress has been supported by the identification of genetic mutations linked to familial forms of these diseases. In animals such as rat, mouse, monkey, Drosophila and C.elgans genetic depletion or overexpression can be created. However, none of the animal models have completely recapitulated the clinical and pathological changes of disease.

1.7.2.1 Conditional genetic mouse model of neuronal 26S proteasomal depletion; $Psmc1^{fl/fl}$;CaMKIIα-Cre

A mouse model has been created to investigate the contribution of the UPS to neurodegeneration and protein inclusion formation, involving conditional genetic depletion of the 26S proteasome in mouse brain neurones. The model was created using Cre recombinase/loxP conditional gene targeting, inactivating an essential subunit of the 19 RP, ATPase *Psmc1*, leading to depletion of 26S proteasomes (see section 2.1.1) (Bedford et al., 2008). PSMC1 has a regulatory function in the 19RP, opening the entry gate formed by the α subunits of the 20S CP and provides energy for unfolding protein substrates (Kohler et al., 2001; Smith et al., 2005). To achieve conditional deletion of *Psmc1* in forebrain neurones, floxed Psmc1 mice were crossed with calcium calmodulin-dependent protein kinase IIα promoter-driven Cre recombinase mice ($Psmc1^{fl/fl}$;CaMKIIα-Cre). Deletion of PSMC1 did not affect the formation and activity of the 20S proteasome; ubiquitin-independent protein degradation.

26S proteasomal depletion in forebrain neurones causes the formation of intraneuronal inclusions resembling Lewy bodies and extensive neurodegeneration (Bedford et al., 2008). CaMKIIα-Cre is expressed from approximately postnatal week 2. In 3 week-old mice expression of 26S complexes is lower in proteasome-depleted mouse brain compared to controls and this is increasingly clear at 4 and 6 weeks of age (Bedford et al., 2008) .In this study 4

week-old mice were used which demonstrated significant neuropathological changes, including ubiquitin accumulation.

Inflammatory processes induce oxidative stress and reduce cellular antioxidant capacity. After activation, cells of the innate immune system secrete proinflammatory cytokines and chemokines that induce reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Khansari et al., 2009). In 26S proteasome-depleted mouse brain, immunohistochemical investigations showed extensive gliosis using glial fibrillary acidic protein (GFAP) (Bedford et al., 2008). An increase in p53 expression was also observed. These finding suggest that 26S proteasome depletion causes inflammation in the brain (Bedford et al., 2008). Recent work in our laboratory has shown that the levels of ROS in 26S-depleted mouse brain cortex were significantly increased when compared to control cortices. Furthermore, peroxiredoxin 6 (PRDX6), a bifunctional enzyme with antioxidant peroxidase and phospholipase A2 (PLA2) activities, was increased in the 26S proteasome-depleted mouse cortex and shown to provide an antioxidant response in this mouse model (Elkharaz et al., 2013). These observations indicate that inflammation and oxidative stress, involving astrocytes play a role in the neuropathology following 26S proteasomal depletion in forebrain neurones (Bedford et al., 2008; Elkharaz et al., 2013).

1.8 Nuclear factor kappa B (NF-kB) pathway

The nuclear factor kappa B (NF-kB) pathway is a stress response that activates the NF-kB family of transcription factors, which play important roles in a number of physiological and pathological mechanisms, including immunity, inflammation, and cell survival (Husnjak & Dikic, 2012).

NF-kB is composed of homodimers and heterodimers of five Rel family members, including p105/p50, p100/p52, p65(RelA), c-Rel and RelB. Rel members contain a conserved N-terminal domain called the Rel-homology domain (RHD). The DNA-binding domain and nuclear localization signal (NLS) lie in the N-terminal and C-terminal parts of the RHD respectively. The NLS is crucial for the transportation of activated NF-kB complex into the nucleus (Hayden & Ghosh, 2004; Habelhah, 2010; Sun, 2012).

NF-kB dimers are sequestered in the cytosol by binding to the inhibitor proteins of kB family (IkBs). The binding of IkBs to NF-kB prevents the transcription factor from entering the nucleus, keeping them in the cytoplasm in an inactive form.

The IkBs bind NF-kB by ankyrin repeats and block the NLS domain thereby inhibiting NF-kBs nuclear translocation activity. The p105 and p100 Rel family members have IkBs-like ankyrin repeat domains at the C-terminal, which undergo degradation to produce the mature Rel subunits (Hayden & Ghosh, 2004; Sun, 2012; Chen & Chen, 2013).

NF-kB is classified into two main pathways; canonical and non-canonical. These pathways lead to activation of the IkB kinase (IKK) complex, which consists of two kinase subunits (IKKα and IKKβ) and NF-kB essential modulator (NEMO) (Gilmore, 2006; Perkins, 2007; Habelhah, 2010; Oeckinghaus et al., 2011).

1.8.1 The canonical NF-kB pathway

Stimuli such as tumor necrosis factor alfa (TNFα), interleukin-1 (IL-1) and Toll-like receptors (TLRs) activate the canonical NF-kB pathway. These stimuli are transduced via their specific receptors and converge on the IKK complex. Activated IKK phosphorylates IkBs leading to K48 polyubiquitination by the Skp1-Cullin-F-Box E3 ubiquitin ligase and subsequent 26S proteasomal degradation. This liberates the NF-kB dimer p50/p65, which translocates into the nucleus and increases expression of target genes (Hayden & Ghosh, 2004; Brasier, 2006; Perkins, 2007; Sun, 2012).

NF-KB also up-regulates IkBs, which enter the nucleus, bind to the NF-kB dimer and the NF-kB-IkBs complex returns back to the cytoplasm, leading to inactivation of NF-kB (Skaug et al., 2009). An overview of the canonical NF-kB signalling events described here are presented in Figure 1.10.

1.8.2 The noncanonical NF-kB pathway

The noncanonical NF-kB pathway is activated in response to stimulation of a group of TNF receptors (TNFR) family members, including CD40, β-cell-activated factor (BAFF) and lymphotoxin α/β(LTα/β) (Sun, 2012).

In contrast to the canonical pathway, the noncanonical pathway causes partial degradation of some Rel family members, including p100 and p105. This leads to liberation and nuclear translocation of NF-kB dimers. p100 and p105 contain an ankyrin repeat domain, which blocks the NLS domain preventing NF-kB from entering the nucleus and thereby keeping the transcriptional factors in an inactive state (Brasier, 2006; Sun, 2012).

TNFRs stimulators cause the activation of NF-kB inducing kinase (NIK). The activated NIK then activates the IKKα subunit, which in turn phoshorylates p100. The phosphorylated form of p100 is recongized by the Skp1-Cullin-F-Box E3 ubiquitin ligase leading to its Lys48-linked polyubiquitination. Lys48-linked polyubiquitination of p100 triggers degradation of only the ankyrin repeat domain by the 26S proteasome, releasing p52. p52 forms a dimer with RelB and the RelB/p52 dimer moves from the cytosol into the nucleus to activate the expression of specific genes (see Figure 1.11) (Hayden & Ghosh, 2004; Gilmore, 2006; Perkins, 2007; Wertz & Dixit, 2010; Habelhah, 2010; Sun, 2011) .

1.8.3 Role of ubiquitin in NF-kB activation

Ubiquitination plays an important role in the regulation and activation of NF-kB pathways. The important step in the NF-kB activation is the degradation of IkB proteins or the processing of p100 and p105 precursors to produce active p52 and p50 respectively (Brasier, 2006; Sun, 2012).

1.8.3.1 Role of Lys48-linked polyubiquitin chains

Lys48-linked polyUb chains are the classical 26S proteasomal degradation signal and target IKBs proteins to the 26S proteasome for degradation, which in turn activates the NF-kB pathways (Hayden & Ghosh, 2004; Brasier, 2006; Perkins, 2007; Habelhah, 2010; Wertz & Dixit, 2010) (sections 1.6.1 and 1.6.2).

1.8.3.2 Role of Lys63-linked polyubiquitin chains

TNF receptor associated factor 6 (TRAF6) is a ubiquitin protein ligase known to generate Lys63-linked polyUb chains. IL1 and/or TLR stimuli cause TRAF6 to synthesis Lys63-linked polyUb chains. Lys63-linked polyubiquitination has been shown to lead to IKK activation, which in turn activates the NF-kB pathways (Conze et al., 2008).

1.8.3.3 Role of linear ubiquitin chains

Linear ubiquitination is via a peptide bond between Gly76 and Met1. The only known E3 that can synthesis linear polyUb chains is linear Ub chain assembly complex (LUBAC). LUBAC activates NF-kB by linear ubiquitination of NEMO, which in turn leads to the activation of IKK (Stieglitz et al., 2012).

1.8.3.4 Role of unanchored ubiquitin chains

Unanchored polyUb chains can play a role in the activation of NF-κB. Unanchored Lys63-linked polyUb chains have been shown to be involved in the activation of protein kinases and innate immune response (Xia et al., 2009; Zeng et al., 2010).

Unanchored Lys63-linked polyUb chains synthesized by TRAF6/ubc13 in response to IL-1/TLR bind to TAB2/3 (TAK1 binding protein 2/3). This binding leads to the phoshorylation of TAK1 (transforming growth factor β-activated kinase-1; p-TAK1). p-TAK1 in turn phosphorylates and activates IKK and the subsequent NF-kB pathway (Xia et al., 2009).

Figure 1.10

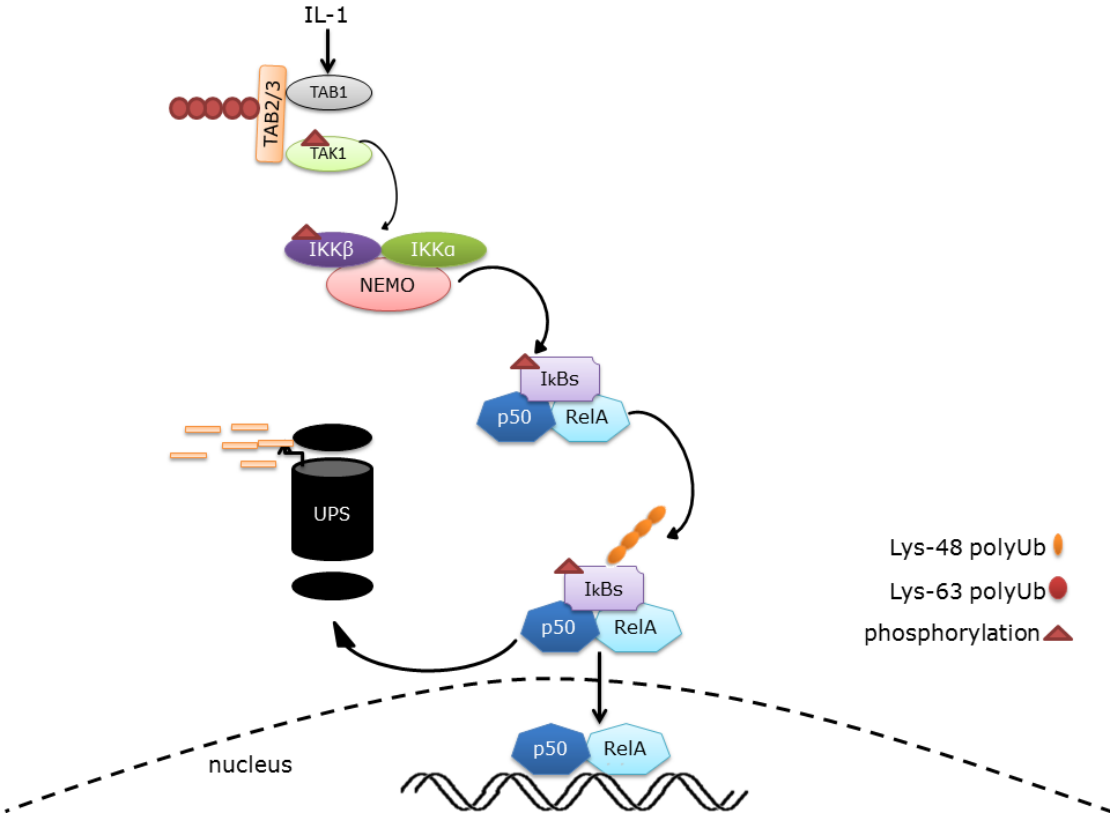


Figure 1.10 Cartoon representing the canonical NF-kB pathway. The pathway may be stimulated by IL-1 which induces synthesis of Lys63-linked polyUb chains. PolyUb chains bind to TAK1 through TAB2/TAB3 subunits, leading to phosphorylation of IKK. IKK then phoshorylates IkBs, which lead to Lys48-linked polyubiquitination and subsequent degradation by the UPS. The NF-kB dimer p50/p65(RelA) translocates into the nucleus to mediate gene transcriptoon.

Figure 1.11

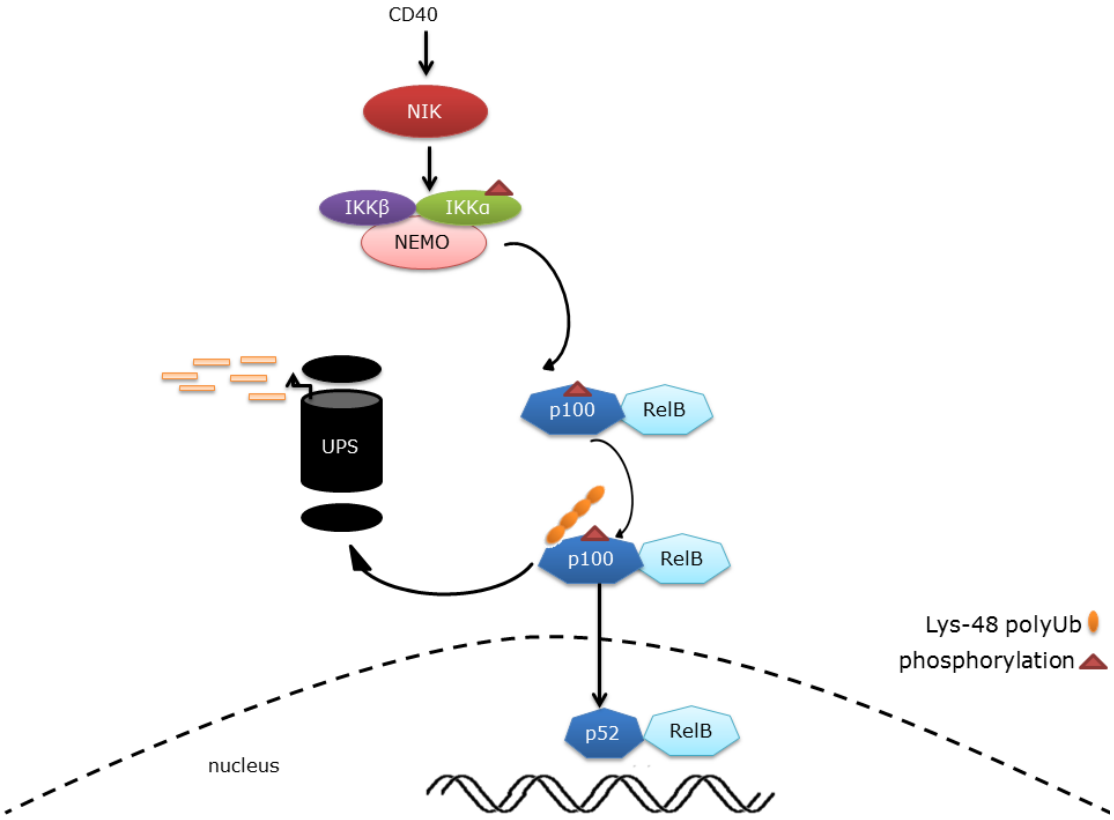


Figure 1.11 Cartoon represention of the non-canonical NF-kB pathway. This pathway may be stimulated by CD40 and induces NIK activation, which in turn phosphorylates the p100 precursor, leading to its Lys48-linked polyubiquitination and then degradation of its ankyrin repeat domain to produce p52. The NF-kB dimer p52/RelB can translocate into the nucleus to activate gene transcription.

1.9 Ubiquitin binding proteins (ubiquitin receptors)

Ubiquitin binding proteins (UBPs) interact and bind with ubiquitinated substrates. UBPs have one or more ubiquitin binding domains (UBDs) which are responsible for binding directly with Ub (Hoeller et al., 2006; Schnell & Hicke, 2003). These are involved in the regulation of diverse cellular functions, such as endocytosis, signal transduction and transcription (Figure 1.12) (Di Fiore, 2003; Hicke et al., 2005).

Figure 1.12

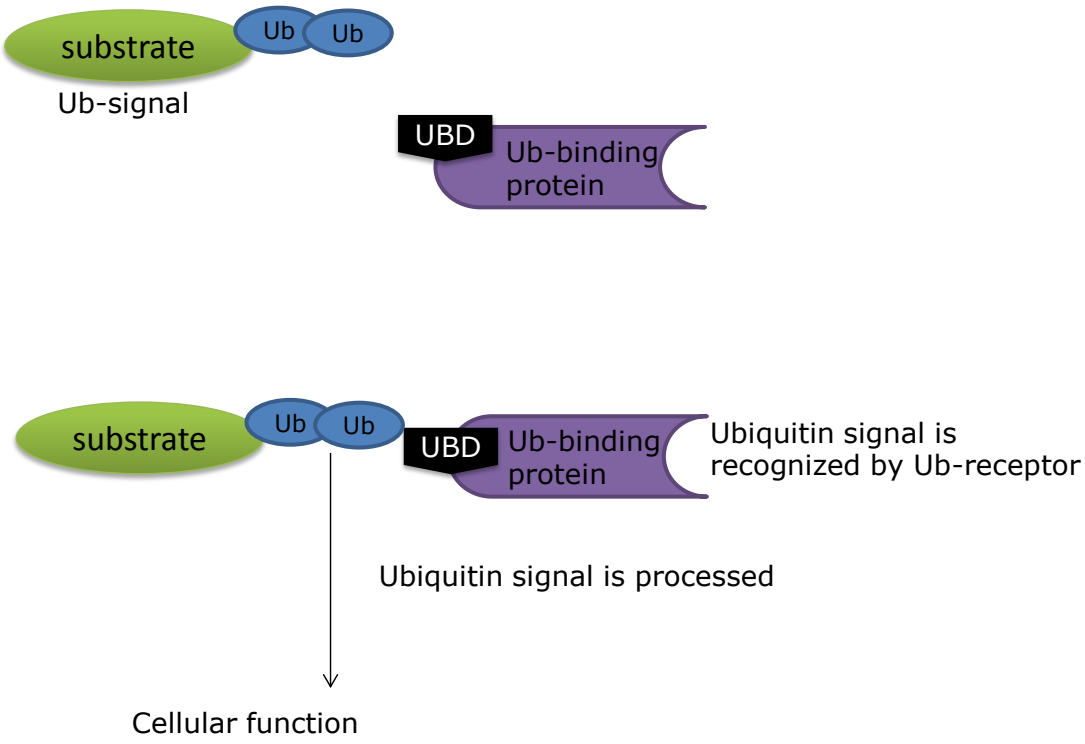


Figure 1.12 Schematic representation of Ubiquitin binding proteins decode ubiquitin-mediated cellular functions.

1.9.1 p62/sequestosome1 (SQSTM1)

p62 is a multi-modular protein which was identified as a phosphotyrosine-independent ligand of the src homology 2 (SH2) domain of p56. It has a role in various cellular functions such as NF-ĸB signalling, TNFα activation and proteasomal degradation (Ciani et al., 2003; Seibenhener et al., 2004; Babu et al., 2005; Moscat et al., 2007; Pankiv et al., 2010).

p62 is a UBP, has several domains, including a ubiquitin-associated domain (UBA) which binds to Ub at its C-terminus. p62 also has a PB1/AID (Phox and Bem p1) domain that has structural homology to the Ubl domain. p62 binds to polyubiquitinated proteins via its UBA domain and to the ubiquitin interacting motif (UIM) of S5a/Rpn10 by its PB1 domain, shuttling polyubiquitinated proteins to the proteasome for degradation (Seibenhener et al., 2004). p62 also acts as an autophagic cargo receptor, which may target ubiquitinated proteins in aggresomes to selective autophagy. The UBA domain of p62 binds to the ubiquitinated proteins and its LC3-interacting region (LIR) binds to the LC3 autophagic effector protein to facility their degradation by autophagy (Pankiv et al., 2007; Komatsu & Ichimura, 2010). AID (acid interacting domain) binds to atypical protein kinase Cs (aPKCs) involved in the activation of tumour necrosis factor α (TNFα) and interleukin-1 (IL-1) receptor signalling complexes (Puls et al., 1997; Sanchez et al., 1998). p62 also has a zinc finger (zz) that can bind to the RING (really interesting new gene) finger protein TRAF6 that activates the NF-kB pathway (Geetha & Wooten, 2002). Finally, p62 has two PEST domains (Proline, Glutamate, Serine and Threonine) known as a degradative signal, indicating that p62 has a short half-live, a characteristic feature of regulatory proteins (Geetha & Wooten, 2002a). Mutation of the p62 UBA domain causes Paget's disease of bone (Laurin et al., 2002; Layfield et al., 2006; Morissette et al., 2006). Interestingly, the expression of p62 protein has been shown to be increased in several stress conditions, including proteasome inhibition and oxidative stress (Kuusisto et al., 2001; Nakaso et al., 2004).

1.9.2 Ubiquilin1

Ubiquilin1 (UQ1)/presenilin-interacting protein(hPLIC1) (UQ1) is also known as presenilin-interacting protein and contains a UBA at its C-terminus and a ubiquitin-like domain (Ubl) at its N-terminus. UQ1 has a role in the regulation of protein degradation via the UPS (Zhang et al., 2008). Proteins containing UBL-UBA domains act as ubiquitin binding protein for the proteasome, interacting with the proteasome via the UBL domain and with the ubiquitinated protein via UBA domains. These proteins deliver ubiquitinated proteins to the proteasome for degradation (Kang et al., 2003; Elsasser & Finley, 2005). UQ1 also contains two domains that have molecular chaperone activity and may be a molecular chaperone for the amyloid precursor protein (Stieren et al., 2011).

1.9.3 Vacuolar protein sorting 9 (Vsp9)

Vacuolar protein sorting 9 (Vps9) contains a CUE (conjugating of Ub to endoplasmic-reticulum degradation) and GEF (guanine nucleotide exchange factor) domain. The CUE domain binds to ubiquitinated cargo and is required for Vps9 ubiquitination. Vps9 GEF domain activates the Vps21 protein. Vps21 is a Rab protein, which is involved in the endocytic pathway and cell signalling (Davies et al., 2003; Hislop et al., 2004; Carney et al.,2006). Rab proteins play a role in integrating intracellular protein trafficking. Localization and activity of Rab proteins is regulated by three proteins; GTPase-activating proteins (GAPs), guanine-nucleotide dissociation inhibitors (GDIs), and guanine-nucleotide exchange factors (GEFs). GEFs activate Rab5 by enhancin GTP binding, which is required to complete vesicle targeting and fusion events (Carney et al., 2006; Keren-Kaplan et al., 2012 ; Shih et al., 2003).

1.9.4 NF-kB essential modulator (NEMO)

NF-kB essential modulator (NEMO) is the regulatory subunit of the IKK complex. The IKK complex is composed of two kinase subunits, IKKα and IKKβ, and a regulatory subunit, NEMO/IKKγ (Cordier et al., 2009; Laplantine et al., 2009). NEMO plays a role in the activation of the canonical NF-kB pathway. NEMO is an adaptor that links stimulatory signals such as TNFα to activation of the IkB kinase complex. NEMO is a UBP , containing two UBDs; ubiquitin binding in ABIN and NEMO (UBAN), which binds exclusively to linear Ub chains (Kang et al., 2003; Elsasser & Finley, 2005; Laplantine et al., 2009; Rahighi et al., 2009), and a zinc finger (ZF) that binds Lys63-linked polyUb chains (Cordier et al., 2009; Yoshikawa et al., 2009).

1.9.5 IsopeptidaseT (IsoT)

Isopeptidase T (IsoT/USP5) is a deubiquitinating enzyme that preferably cleaves unanchored polyUb chains. IsoT has four UBDs, a ubiquitin-specific processing protease (UBP), two UBA, and a zinc finger domain (ZnFUBP) (figure1.13) (Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008a). Recently, a new ZnFUBP domain in Isopeptidase T was discovered (Avvakumov et al., 2012). ZnFUBP recognizes the intact C-terminal glycine of proximal Ub. ZnFUBP has a specific binding pocket where glycines 75 and 76 of Ub are inserted. This binding pocket explains the specificity of ZnFUBP to free Ub (Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008). The UBP domain of IsoT is the active site of the enzyme, containing Cys and His boxes. The ZnFUBP domain recognition of unanchored polyUb chains causes conformational changes which activate cleavage of isopeptide/peptide bond to the proximal Ub (unanchored Ub) (Reyes-Turcu & Wilkinson, 2009).

Figure 1.13

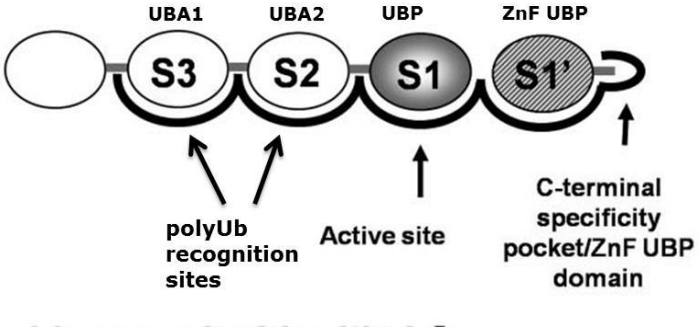


Figure 1.13 Schematic representation of the IsoT domains .The S1` ZnFUBP domain is occupied by proximal Ub. S1 formed by UBP is the active site. S2 and S3 sites are UBA2 and UBA1 domains respectively and are involved in polyUb recognition. Adapted from (Reyes-Turcu et al., 2008).

1.10 Ubiquitin binding domains (UBDs)

UBDs are motifs found in proteins that are capable of binding non-covalently to Ub. They are usually small, ranging from 20-150 amino acid residues, and adopt specific structures. UBDs are found in UBPs and in enzymes that catalyze ubiquitination or deubiquitination (Hicke et al., 2005; Husnjak & Dikic, 2012). There are at least 16 different UBDs to date; UIM (ubiquitin interact motif), UBA and coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) are examples of these proteins (Hurley et al., 2006).

Their binding affinity for Ub varies from moderate to weak. Some UBDs do not interact with Ub and their function is unclear. UBDs differ in their chemical properties and their ability bind to Ub (Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008). There are four sites in Ub that can be recognized by UBDs; patch Ile44 (Leu8, Ile44 and Val70), patch Ile36 (Ile36, Leu71 and Leu73), patch Phe4 (Gln2, Phe4 and Thr12) and the TEK-box (Thr12, Thr14, Glu34, Lys6, and Lys11) (FIGURE 1.14). Most of the UBDs have an α helical structure which binds to the canonical hydrophobic patch around Ile44 in the β-sheet of Ub, including the UBA, UIM and CUE domains (Hurley et al., 2006). The Ile36 hydrophobic patch is involved in the recognition and binding of Ub/E2 (Ub conjugated to E2) by E3 ligase enzymes. The ZnFUBP domain also interacts with the Ile36 patch; the glycine free C-terminal penetrates into the hydrophobic pocket of the ZnFUBP domain. UBAN also binds to the Ile36 patch and to the Phe4 and TEK-box of Ub (Hurley et al., 2006; Husnjak & Dikic, 2012; Komander & Rape, 2012). Figure 1.13 shows binding of different UBDs to different Ub sites.

Figure 1.14

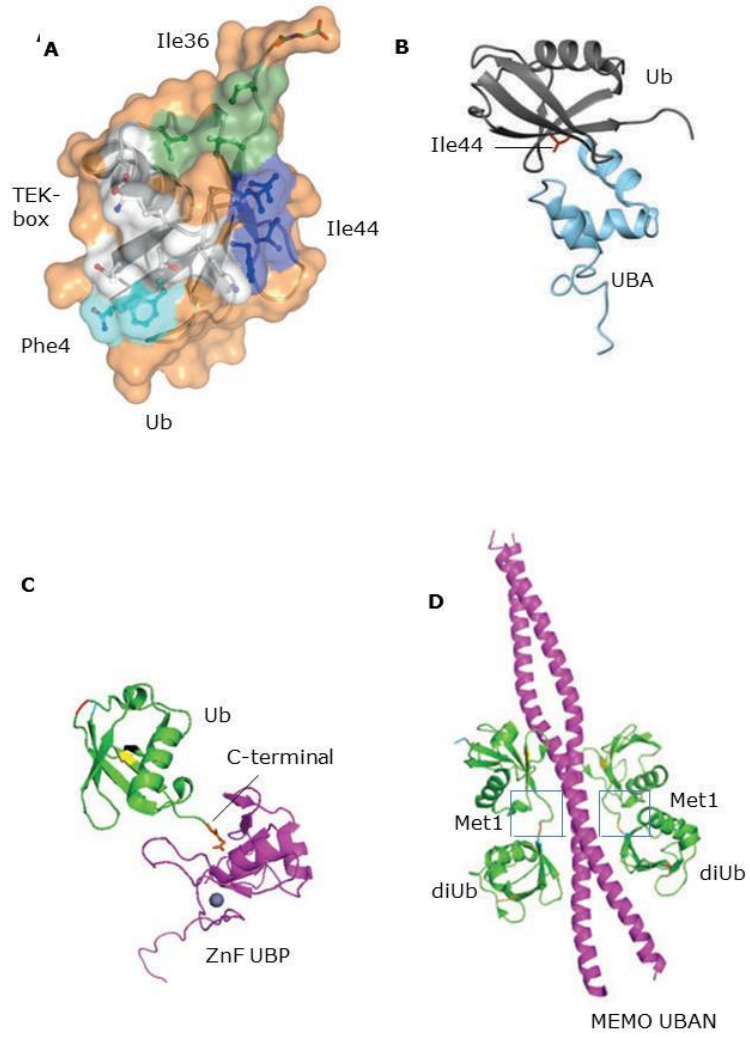


Figure 1.14 A) Ubiquitin binding patches Ile44, Ile36, Phe4 and TEK-box. Different UBDs bind to different Ub sites: B) The UBA domain binds to the Ile44 patch of Ub. C) The ZnFUBP domain binds to the C-terminal of Ub using the Ile36 site. D) NEMO UBAN binds to the Ile36 and Phe4 patches of Ub. Adapted from (Husnjak & Dikic, 2012).

1.10.1 Ubiquitin associated domain (UBA)

The UBA domain was the first ubiquitin binding domain to be described. UBA is a small peptide sequence containing approximately 40-50 amino acids. The UBA domain is present in some proteins involved in the recognition and degradation of proteins via the UPS (Wilkinson et al., 2001).Proteins containing UBA domain possess extra domains involved in a number of cellular functions like endocytosis and apoptosis (Hurley et al., 2006; Dikic et al., 2009). The UBA domain structure composed of three helix bundle; with a hydrophobic patch which known to bind a hydrophobic sheet of the ubiquitin (Layfield et al., 2004).

1.10.2 Coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE)

CUE was first recognized by a bioinformatics analysis. The structure of the CUE domain is similar to that of the UBA domain. It binds Ub through hydrophobic residues at its C-terminus. The CUE domain has been shown to recognise monoUb and polyUb (Shih et al., 2002; Davies et al., 2003; Hurley et al., 2006). CUE domains bind to monoUb with different affinities; the cue1 CUE shows weak interaction with monoUb, whereas the Vps9 CUE interacts with monoUb strongly (Shih et al., 2003). The CUE domain has been found in proteins that play a role in cellular functions.

1.10.3 Ubiquitin binding in ABIN and NEMO (UBAN)

The UBAN domain is present in proteins involved in the NF-kB pathway, including A20-binding inhibitor of NF-kB (ABIN) and NF-kB essential modulator (NEMO) (Ikeda & Dikic, 2008; Dikic et al., 2009). NEMO UBAN domain binds linear di-Ub 100 times more strongly than Lys63-linked polyUb (Lo et al., 2009).

1.10.4 ZnFUBP

ZnFUBP is a zinc-finger UBD; also known as polyUb associated zinc finger domain (PAZ). The ZnFUBP domain is present in several proteins, such as E3 ligase BRAP2 (BRCA1-associated protein 2 also known as IMP), cytoplasmic deacetylase HDAC6 and a group of deubiquitinating enzymes, including IsoT (USP5) (Pai et al., 2007; Hard et al., 2010).

ZnFUBP contains a binding pocket where the glycine 75 and 76 of Ub interact, which explains the specificity of ZnFUBP to unanchored Ub (Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008; Ouyang et al., 2012; Strachan et al., 2012).

1.11 Methods used for the investigation of ubiquitinated proteins

Cellular protein modifications are extremely regulated pathways; ubiquitination of proteins is one of the major intracellular modifications and is a key to a number of cellular functions, including the activation of NF-kB pathway or the degradation of ubiquitinated proteins via UPS. Defect in the UPS causes accumulation of Ub and ubiquitinated proteins. There is a need for reliable methods to purify, detect and identify protein modifications via ubiquitination to obtain proteomic data related to this modification (Tomlinson et al., 2007; Hjerpe & Rodriguez, 2008). Different techniques have been used to detect and purified Ub and ubiquitinated proteins. Some of these techniques will be discussed here.

1.11.1 The use of mass spectrometry to identify Ub and ubiquitinated proteins

Mass spectrometry (MS) is a tool used to detect and map protein modifications and quantifying their changes. Recent developments in MS technologies have been used to characterize native Ub and ubiquitinated proteins (Peng, 2008). Bedford and colleagues (2011) detected five linked polyUb chains in mouse brain homogenate using quantitative MS; Lys6, Lys11, Lys29, Lys48 and Lys 63. They were unable to detect Lys27 and Lys33 in the mouse brain, which may due to the low abundance of these chains in the mouse brain homogenate (Bedford et al., 2011). Recently, the use of advanced mass spectrometry techniques has lead to the discovery of increased numbers of ubiquitination sites on proteins (Danielsen et al., 2011.).

1.11.2 The use of protein microarray to identify Ub and ubiquitinated proteins

Protein microarray procedures could be used to identify Ub-modified proteins. Merbl and Kirschner (2009) used a protein microarray strategy to identify a number of regulated polyubiquitination proteins involved in the mitotic checkpoint and anaphase in cell extracts (Merbl & Kirschner, 2009).

1.11.3 The use of ubiquitin binding proteins to identify ubiquitinated proteins

Proteins containing UBDs, such as the S5a subunit of the 26S proteasome, have high affinity binding for ubiquitinated proteins. Therefore, GST-S5a-coupled Sepharose has been effectively used to purify polyubiquitinated substrates from protein homogenates, e.g. pig brain, and studies have confirmed that the UIM domain of S5a could bind Lys48- or Lys63-linked polyubiquitinated substrates (Tomlinson et al., 2007; Wang et al., 2005).

1.11.4 The use of isolated ubiquitin binding domains to identify ubiquitinated proteins

The specificity of UBDs for Ub can be used directly to purify ubiquitinated proteins *in vitro* and *in vivo*. The different domains may show differential interaction properties for monoUb and diverse polyUb chains (Tomlinson et al., 2007; Long et al., 2008). Tandem ubiquitin-binding entities (TUBE), the use of multiple UBDs, might increase the binding avidity to Ub and ubiquitinated substrates (Hjerpe et al., 2009; Aillet et al., 2012; Lopitz-Otsoa et al., 2012).

Some of UBDs shows selectivity to specific polyUb linkages or to unanchored polyUb chains. These domains can be used to purified and detect specific Ub linkages or unanchored Ub (Reyes-Turcu et al., 2008; Rahighi et al., 2009; Strachan et al., 2012). Non-specific binding of proteins to beads presents the most difficulties in the pull-down assay using beads and to prevent this binding washing steps with detergents and salts is necessary, these chemical may reduce the binding affinity of the UBDs (Nakayasu et al., 2013). Also to overcome of the non-specific binding of beads the use of control beads was introduced.

1.12 Aims

Increased studies are started to take interest in unanchored polyUb chains. Unanchored polyUb chains may play a regulatory role in the activation of innate immunity, in the aggresome formation. On the other hand, their accumulation causes protein degradation inhibition by the UPS. Some UBDs have shown linkage specificity and others bind only to unanchored polyUb chains.

The aim of this study were

(1) To investigate the binding specificity of several UBDs as detailed in Table 1 using affinity chromatography to the commercial available different Ub length and linkages. Previous studies suggest that the UBDs in this study show different binding to different Ub linkages and length. The UBA domain of p62 binds Ub in a linkage independent manner, but prefers Lys63 polyUb chains to Lys48. The UBA domain of UQ1 shows strong Ub binding and binds to Ub of all topology. Vsp9 CUE binds monoUb in preference to polyUb chains. The NEMO UBAN domain binds to Lys63 and linear polyUb chains. IsoT ZnFUBP is selective to unanchored monoUb and polyUb chains. Taking these studies into consideration, I chose to further investigate the Ub binding of these UBDs with a more comprehensive series of synthetic ubiquitin species that have not previously been completely investigated for these domains, including monoUb, Lys48 and Lys63 polyUb chains (Ub2-7), linear polyUb chains (Ub2, Ub3, Ub4) and $Ub5^{+1}$, which is a frame-shift mutant Ub that represents anchored polyUb chains. The aim was to identify UBDs that preferentially bind different Ub signals to increase our understanding of Ub signalling. Also, this work will lead to the development of sensitive and reliable methods to investigate specific Ub signalling in a cellular environment, identifying proteins modified by diverse Ub signals.

(2) To detect the abundance of unanchored polyUb chains in the cortex, cerebellum and cortex mitochondria in the conditional 26S proteasome knockout mouse ($Psmc1^{fl/fl}$, CaMKIIα-Cre) (26S KO), and compared with the control mouse (Bedford et al., 2008).

(3) To investigate the expression of several enzymes that may contribute to the unanchored polyUb chain formation, function, and disassembly. These enzymes are E2-25K, p-TAK1 and IsoT/USP5.

Table.1 The structure and function of UBDs used in this study

Domain	Protein	Amino acid	Protein source	Function	Molecular weight kDa
UBA	p62	387-436	Human	Proteasome degradation & Autophagy	Less than 10 kDa
UBA	UQ1	517-588	Human	Proteasome degradation & autophagy	Less than 10 kDa
CUE	VPS9	394-451	Yeast	Endoplasmic trafficking	Less than 10 kDa
UBAN	NEMO	290-330	Human	Regulation of NF-kB signalling pathway.	Less than 10 kDa
ZnFUBP	IsoT	263-291	Human	Deubiquitinating enzyme, disassembly of unanchored polyubiquitin chains	Less than 17 kDa

CHAPTER 2 Material and methods

2.1 Experimental animals

2.1.1 Generation of Psmc1$^{fl/fl}$; CaMKIIα-Cre mice

Conditional Cre/loxP gene targeting was used to generate the experimental animals. ATPase *Psmc1* gene, a key subunit of the 26S proteasome, was inactivated by breeding mice expressing Cre recombinase from the calcium calmodulin-dependent protein kinase IIα promoter (CaMKIIα-Cre) with floxed *Psmc1* mice (Psmc1$^{fl/fl)}$ (Bedford et al 2008). This leads to inactivation of *Psmc1* in forebrain neurons (Psmc1$^{fl/fl}$, CaMKIIα-Cre). Mice of Psmc1$^{fl/fl}$; CaMKIIα-Wt and Psmc1$^{fl/Wt}$, CaMKIIα-Wt were used as controls. Mice were created by Dr Bedford prior to the commencement of this study.

2.2 Preparation of affinity chromatography matrices

2.2.1 Preparation of *E.coli* glycerol stocks

Bacteria on LB-agar plates can be stored for a few weeks at 4°C. For longer periods, glycerol stocks are prepared and stored at -80°C.

2.2.1.1 Reagents

1) XL10-Gold® Escherichia coli: transformed with pGEX-4T-1 plasmid containing GST-p62 UBA, GST-UQ1 UBA, GST-Vsp9 CUE, GST-NEMO UBAN or GST-IsoT ZnFUBP and a β-Lactam antibiotic selection cassette. All domains were kindly provided by Dr R. Layfield, University of Nottingham.
2) Luria Broth (LB, 20g/L).
3) 80% (v/v) glycerol.
4) 2 ml cryogenic tubes.

2.2.1.2 Protocol

1.5 ml of an overnight culture of *E.Coli* transformed with the appropriate GST-UBD (Appendix I and Table1) was transferred to a micorcentrfuge tube and centrifuged at 3300 xg for 10 minutes at 4°C. The supernatant was discarded. The cell pellets were re-suspended in 425 µl LB plus 75 µl 80% (v/v) glycerol, transferred to a cryogenic tube and snapped frozen in liquid nitrogen. Glycerol stocks were kept at -80°C.

2.2.2 Plasmid DNA purification

2.2.2.1 Reagents

1) 5 ml overnight culture of GST-UBD.
2) QIA prep Spin Mini-Prep kit for plasmid purification (Qiagen®).

2.2.2.2 Protocol

Overnight cultures were centrifuged at 15,200 xg for 10 minutes. The supernatant was removed and bacterial pellet re-suspended in 250 µl of buffer P1 (including RNase). The cell suspension was lysed by adding 250 µl of buffer P2 and mixing. 350 µl of N3 buffer was then added and the eppendorf tube mixed until the solution became cloudy. Tubes were centrifuged for 10 minutes at15, 200 x g. The supernatant was transferred to a QIAprep column and centrifuged for 1 minute at 15,200 x g. The flow-through was discarded and the column washed by adding 500 µl of buffer PB, followed by centrifugation at 15,200 x g for 1 minute. The flow-through was again discarded and columns washed with 750 µl of buffer PE, followed by centrifugation for 1 minute at15, 200 x g. The flow-through was discarded and columns were centrifuged for 1 minute to remove any residual buffer. The plasmid DNA was collected into a clean eppendorf tube by adding 50 µl of buffer EB to the column and centrifugation for 1 minute at15, 200 x g . The purified plasmid DNA was stored at -20°C. The concentration of plasmid DNA was measured using a Nano Drop (ND 1000) spectrophotometer.

2.2.3 Preparation of overnight *E.*Coli culture

2.2.3.1 Reagents

1) Luria-Bertani (LB) broth (20 g/L).
2) Antibiotic stock solution (80 mg/ml ampicillin plus 20 mg/ml oxacillin).
3) Bacto-agar (15 g/L).

2.2.3.2 Protocol

LB-agar was prepared from the LB broth plus Bacto-agar reagents and autoclaved for sterilization. After the solution cooled to less than 50°C, antibiotic was added to a final concentration of 80 µg/ml ampicillin plus 20 µg/ml oxacillin and the LB-

agar plates poured. Bacterial cells from the glycerol stocks (section 2.2.1) were streaked onto LB-agar plates and incubated overnight at 37°C. A single colony from an LB-agar plate was inoculated into 20 ml LB culture containing antibiotics at a final concentration of 80 µg/ml ampicillin plus 20 µg/ml oxacillin. The culture was grown overnight at 37ºC in a shaking incubator.

2.2.4 Bacterial growth and induction of GST-fusion protein expression

2.2.4.1 Reagents

1) LB (20 g/L)
2) Antibiotics stock solution (80 mg/ml ampicillin plus 20 mg/ml oxacillin)
3) 200 mM isopropyl-β-thiogalactopyrinosid stock (200 mM IPTG).
4) Tris-buffered saline with Triton-X100 pH 7.5 (10 mM Tris, 150 mM sodium chloride and 0.1% (v/v) Triton-X100).
5) Photometer (BioPhotometer-eppendrof®).

2.2.4.2 Protocol

500 ml of LB containing antibiotics was inoculated with the 20 ml overnight cultures (section 2.2.3) and incubated at 37ºC in a shaking incubator until the OD600 reached 0.6-0.8 (approximately 3 hours). Expression of the GST-fusion protein was induced by addition of IPTG to a final concentration of 200 µM IPTG. The bacterial culture was then incubated at 37ºC for a further 3 hours in a shaking incubator. Bacterial cultures were then centrifuged at 4,000 xg for 30 minutes at 4°C in a CELLSEP 6/720R centrifuge. The supernatant was discarded. The bacterial pellet was re-suspended in 5 ml of TBS/Triton-X100 and stored at -20°C until required for purification of GST-fusion protein.

2.2.5 Purification and thrombin cleavage of GST-fusion proteins

2.2.5.1 Reagents

1) Glutathione Sepharose 4B (GSH) beads (GE Healthcare).
2) Tris-buffered saline with 0.1% Triton-X100 pH 7.5 (TBST): (10 mM Tris, 150 mM sodium chloride and 0.1% (v/v) Triton-X100).
3) 0.5 U/µl thrombin (Sigma).

4) Thrombin Cleavage Buffer (TCB); 20 mM Tris, 150 mM sodium chloride and 2.5 mM calcium chloride dehydrate pH 8.4.

2.2.5.2 Purification protocol

The bacterial pellets prepared in section 2.2.4 were thawed and lysed by sonication 7 times; each time for 15 seconds on ice at 6-7 micron and left for 15 seconds on ice after each sonication. The lysate was centrifuged at 40,000 xg for 30 minutes at 4°C (Avanti JA 25.5). The soluble protein supernatant 1 was transferred to a clean tube. An aliquot from the supernatant 1 was kept for SDS-PAGE analysis to visualize the expression of GST-UBDs. 0.5 ml of ethanol pre-soaked GSH beads (GE Healthcare Bio-Sciences AB) were transferred to a 15 ml tube. The GSH beads were pelleted by centrifugation and the ethanol storage buffer discarded. The beads were washed 3 times with 5 ml of TBS/Triton X-100, pelleting by centrifugation at 400 x g for 2 minutes after each wash and discarding the TBS/Triton X-100 buffer. Supernatant 1 was then added to the GSH beads and incubated for 3 hours at 4ºC on a rotating mixer. The beads were then centrifuged and the supernatant discarded. Aliquots of the supernatant were kept for SDS-PAGE analysis. The beads were washed 3 times with 5 ml TBS/Triton X-100 as described above. A sample of the beads was kept for SDS-PAGE analysis.

2.2.5.3 Thrombin cleavage protocol

The beads from section 2.2.5.2 were washed twice with 5 ml of thrombin cleavage buffer (TCB) as described above and transferred to eppendorff tubes in 1 ml TCB and pelleted. 1 ml of elution solution (3 units of thrombin in TCB) was added and incubated overnight at 4ºC on a rotating mixer. The beads were pelleted by centrifugation at 400 xg for 2 minutes and the supernatant 2 collected. An aliquot of supernatant 2 was kept for SDS-PAGE analysis to confirm successful cleavage of the UBD from the GST tag.

2.2.6 Purification of GST-fusion proteins from bacteria

2.2.6.1 Reagents

1) Glutathione Sepharose 4B (GSH) beads (GE Healthcare).

2) Tris-buffered saline with 0.1% Triton-X100 (TBST): (10 mM Tris, 150 mM sodium chloride and 0.1% (v/v) Triton-X100, pH7.5).

3) 20 mM reduced glutathione.

4) Tris-buffered saline: 10 mM Tris, 150 mM sodium chloride, pH7.5.

2.2.6.2 Protocol

The GST-fusion proteins were prepared as described in section 2.2.5.2. The GSH-GST-fusion protein coupled beads were then incubated with 5 ml of 20 mM reduced glutathione in TBS 4°C on a rotating mixer. The beads were pelleted by centrifugation at 400 xg for 2 minutes and the supernatant collected, which contains the eluted GST or GST-IsoT ZnFUBP proteins. GST and GST-IsoT ZnFUBP proteins were subjected to overnight dialysis in 5 litres of TBS and then collected and the protein concentration measured by Bradford. The GST-fusion proteins were kept at -20°C until needed.

2.2.7 Coupling of the ubiquitin binding domain to Sepharose Beads

2.2.7.1 Reagents

1) Cyanogen bromide activated-Sepharose-4B (CNBr) (GE Healthcare).
2) 1 mM hydrochloric acid (HCl).
3) Coupling buffer: 100 mM sodium hydrogen carbonate, 500 mM sodium chloride, pH 8.3.
4) 1 M ethanolamine pH 8.0.
5) Washing buffer 1: 100 mM sodium acetate, 500 mM sodium chloride, pH 4.0.
6) Washing buffer 2: 100 mM Tris-HCl, 500 mM sodium chloride, pH 8.0.
7) Storage buffer: 50% TBS, 50% glycerol, 1 mM DTT, 1 mM sodium azide, pH 7.4.

2.2.7.2 Protocol

Cyanogen bromide-activated Sepharose-4B (CnBr) beads were hydrated by incubation in 1mM hydrochloric acid (HCl) for 15 minutes on a rocking platform at 4°C. The Sepharose beads-HCl suspension was transferred into a 15 ml tube. Beads were pelleted by centrifugation at 400 xg for 2 minutes and the HCl discarded. The beads were then washed twice with 5 volumes of coupling buffer. Supernatant 2 (section 2.2.5.3) was added to the 15 ml tube containing CnBr beads. Only coupling buffer was added to prepare control beads. The beads were incubated for 3 hours at 4ºC on a rotating mixer. The CNBr beads were the pelleted by centrifugation and the supernatant discarded. The beads were washed twice with 5 ml of 1 M ethanolamine and then incubated overnight at 4ºC in ethanolamine on a rotating mixer. Following centrifugation the ethanolamine was discarded. The CNBr-UBD-coupled beads were then washed 3 times with 5 ml of coupling buffer and four times with 5 ml alternating between washing buffer 1 and 2 each time for 5 minutes. The beads were washed twice with 5 ml of storage buffer and following the final wash; the beads were re-suspended in storage buffer and kept at 4ºC. Investigations into the coupling efficiency of UBDs to Sepharose 4B are shown in 3.2.1. Due to the dilution used for coupling the UBDs it was difficult to measure protein concentration after coupling. Therefore, this step was analysed by SDS-PAGE analysis followed by Coomassie staining.

2.2.8 Capture of ubiquitinated proteins and unanchored polyubiquitin chains from mouse brain

2.2.8.1 Reagents

1) TBS-NP40 buffer: 50 mM Tris, 150 mM sodium chloride, Nonidet p-40, 1mM (DTT), pH 7.4.
2) Protease inhibitor cocktail (PIs) (Sigma).
3) N-ethylmaleimide (NEM).
4) MG-132.
5) Phenylmethane sulfonyl fluoride (PMSF).
6) Homogenizing buffer: TBS-NP40 buffer, PIs (1:100), 5 mM NEM, MG-132 2:1000, 1 mM PMSF, pH 7.4.
7) 1 M dithiothreitol (DTT).
8) Tris-DDT buffer: 50 mM Tris and 1 mM DTT, pH 7.4.

2.2.8.2 Protocol

Mouse brain cortices were homogenized using a Dounce glass homogenizer in 1 ml of cold homogenizing buffer. The homogenate was transferred to an eppendorff tube and centrifuged for 15 minutes at 16,100 xg to produce a cell debris pellet (insoluble fraction) and supernatant (soluble fraction). The supernatant was quantified using a Bradford assay. DTT was added to the supernatant to a final concentration of 10 mM and incubated for 15 minutes at 4 ºC on a rotating mixer, to remove NEM in the homogenizing buffer.

50 or 100 µl of UBD or control CNBr beads were added to eppendorff tubes. The beads were pelleted by centrifugation at 400 xg for 2 minutes and the storage buffer was discarded. After washing the beads 3 times with 1 ml of TBS-NP40 buffer, 1 or 2 mg of homogenate was incubated with the beads for 3 hours at 4ºC on a rotating mixer. The beads were then pelleted by centrifugation and the flow collected in a new tube. 40 µl of the flow (8% of the total input) was prepared for SDS-PAGE analysis and the remaining flow stored at -80ºC. The beads were washed twice with 1 ml of washing buffer and then once with 1 ml of Tris-DDT buffer. After the final wash, the beads were pelleted by centrifugation, the buffer solution discarded and 50 µl of gel application buffer added.

2.2.9 Capture of polyubiquitin chains

The methods detailed in section 2.2.8 were used, but instead of brain homogenate, synthetic polyUb chains were incubated with the UBD or control CNBr beads. 1 µl (1 µg) of synthetic polyUb chains or monoUb were diluted with 148 µl Tris/DTT buffer (Appendix III).

2.2.10 Cellular fractionation of mouse brain cortex

2.2.10.1 Reagents

1) Solution A: 0.32 M sucrose, 1 mM $NaHCO_3$,1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM PMSF, PIs.
2) Solution B: 0.32 M sucrose, 1 mM $NaHCO_3$.
3) 0.85 M, 1.0 M, and 1.2 M sucrose.

2.2.10.2 Protocol

Brain cortices were rapidly dissected and placed in 8 ml of ice-cold Solution A per 1 g wet weight cortex. Cortices were dounce homogenised and the homogenate diluted to 5% with Solution A. The homogenate was centrifuged at 1,400 xg for 10 minutes at 4°. The supernatant (S1) was retained and the pellet (P1) re-homogenised in a similar volume of Solution A and centrifuged at 800 xg for 10 minutes at 4°C. The resulting supernatant was pooled with S1 and subjected to further centrifugation at 800 xg for 10 minutes. The combined supernatant was transferred to a new centrifuge tube and centrifuged at 30,000 xg for 15 minutes. The supernatant (S2) was discarded, with a small volume (10 ml) kept as a loading control, and the pellet (P2) re-suspended in 3 ml of Solution B per 1 g of original starting material. The P2 suspension was layered on top of a discontinuous 3 step sucrose gradient: 2.5 ml of 0.85 M, 1.0 M and 1.2 M sucrose; and centrifuged at 82,500 xg for 3 hours. The fractions at each interface were collected and transferred into eppendorff tubes (Figure 2.1). The fraction was diluted 1:4 with Solution B, centrifuged at 9.300 xg for 20 minutes and the supernatant discarded. Pellets were re-suspended in 200 µl or 400 µl of Solution B. The protein concentration of each fraction was estimated by Bradford. Sub-cellular fractions were kept at -80°C until required.

2.3 Standard Protein Techniques

2.3.1 Bradford Assay

2.3.1.1 Reagents

1) Bradford reagent: 0.05% (w/v) Coomassie Brilliant Blue G250, 25% (v/v) ethanol, 50% (v/v) perchloric acid.
2) Bovine serum albumin (BSA): 1 mg/ml.

2.3.1.2 Protocol

Using a 96-well plate, BSA (Bovine serum albumin) protein standards were set up in 50 µl in duplicate for 0, 5, 10, 15, 20, 30, 40 and 50 µg of BSA. Protein samples were also added to the plate in duplicate in a final volume of 50 µl with distilled water. Bradford reagent was diluted 1:12 in distilled water and 150 µl added to each well. The optical density was measured at 560 nm and a protein standard curve plotted using the average values for each of the BSA standards. The values of the unknown samples were extrapolated from this standard curve.

2.3.2.1 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): large 5-15% gradient gel

2.3.2.1.1 Reagents

1) Acrylamide/bis-acylamide 30% solution (Sigma) with 0.1% SDS.
2) Buffer A: 1.1 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 30% (v/v) Glycerol.
3) Buffer B: 1.1 M Tris-HCl pH 8.8, 0.1% (w/v) SDS.
4) 0.1% SDS (w/v) deionised water.
5) N-tetramethylethylenediamine (TEMED).
6) 10% (w/v) ammonium persulphate solution (AMPS).
7) Stacking buffer: 1.25 M Tris-HCl pH 6.8, 0.1% SDS.
8) Electrode buffer: 25 mM Tris-HCl pH 8.3, 200 mM Glycine, 0.1% (w/v) SDS.
9) Gel Application Buffer: 150 mM Tris-HCl pH 6.8, 8 M urea, 2.5% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 3 %(w/v) DTT, 0.1% (w/v) bromophenol blue.
10) Spectra Multicolor Low Range Protein Ladder (1.7 – 40 kDa)

2.3.2.1.2 Protocol

Protein samples were generally separated on 5-15% gradient SDS-PAGE gels. The 15% acrylamide solution contained 5.0 ml of 30% acrylamide solution, 1.67 ml of 0.1% SDS (w/v) and 3.3 ml of Buffer A. The 5 % acrylamide solution contained 1.67 ml of 30% acrylamide solution, 5.0 ml of 0.1% SDS (w/v) and 3.3 ml of Buffer B. 10 µl of TEMED and 100 µl of 10% AMPS was added prior to pouring the gel. The solutions were poured using a gradient gel mixer and overlaid with water-saturated butanol to ensure an even surface. The stacking gel contained 3.5 ml of stacking buffer, 0.7 ml of 30% acrylamide solution, 5 µl TEMED and 100 µl AMPS. Protein samples were mixed with gel application buffer and boiled at 100ºC for 5 minutes. Spectra Multicolor Low Range Protein Ladder was used as a protein standard size marker and the gel was run at 40 mA. Following separation, the gel was either stained with Coomassie blue stain or processed for Western blotting.

2.3.2.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): mini 12% gel

2.3.2.2.1 Reagents

1) Acrylamide/bis-acylamide 30% solution (Sigma).
2) 1.1 M Tris pH 8.8.
3) 10% (w/v) SDS.
4) Deionized water.

2.3.2.2.2 Protocol

A 12% acrylamide solution contained 2 ml of 30% acrylamide solution, 1.6 ml of deionised water, 1.3 ml of 1.1 M Tris, 50 µl of 10% SDS, 50 µl of 10% AMPS, and 5 µl of TEMED. The stacking gel solution was prepared using 700 µl of deionised water, 170 µl of 30% acrylamide, 130 µl of 1.0 M Tris pH 8.8, 10 µl of 10% SDS, 10 µl of 10% AMPS and 2 µl of TEMED. The separation was carried out at 200 mA using a mini-protein (Bio-Rad) system. Following separation, the gel was either stained with Coomassie blue stain or processed for Western blotting.

2.3.3 Staining of SDS-PAGE gels with Coomassie blue stain G250

2.3.3.1 Reagents

1) Coomassie blue staining solution: 50% (v/v) methanol, 20% (w/v) glacial acetic acid and 0.12% (w/v) Coomassie Brilliant Blue G250
2) Destaining solution: 10% (v/v) methanol, 10% (v/v) glacial acetic acid.

2.3.3.2 Protocol

The SDS-PAGE gel was placed in a container and overlaid with Coomassie blue staining solution. The container was then placed on a gently rocking platform for approximately 1 hour. The stain was then discarded and the gel overlaid with destaining solution on a gently rocking platform until the appropriate protein staining was achieved.

2.3.4 Western Blotting Analysis

2.3.4.1 Reagents

1) Nitrocellulose membrane (Amersham Biosciences).
2) Transfer Buffer: 0.3% (w/v) Tris, 7.2% (w/v) glycine, 20% (v/v) methanol, and 0.1% (w/v) SDS.
3) Electrophoresis transfer apparatus (Bio-Rad).
4) TBST: Tris buffered saline, 0.1% (v/v) Tween^{20}.
5) 4% (w/v) non-fat dry milk (Marvel®) in TBST.
6) 1% bovine serum albumin in TBST.
7) Primary antibody and secondary antibody conjugated to Horseradish Peroxidase (HRP).
8) Chemiluminescent substrate (ECL; Thermo Scientific).
9) X-Ray film: Lumi-Film chemiluminescent detection film (Amersham Healthcare).
10) Developing solution (Calumet Photograph LTD 757314).
11) Fixative solution (Calumet Photograph LTD 2000RT).

2.3.4.2 Western blotting

Proteins were transferred from the SDS-PAGE gel following separation onto nitrocellulose membrane (Amersham Biosciences) using transfer buffer and a constant current of 60 mA overnight. Following transfer, all the remaining protein binding sites on the membrane were blocked with blocking solution: 5% (w/v)

Marvel in TBST for one hour at room temperature on a gently rocking platform. The membrane was then incubated with primary antibody in blocking solution at room temperature for one hour or overnight at 4°C (Appendix II). The membrane was washed 3 times in TBST for 10 minutes each and then incubated with 1:3000 of the appropriate secondary antibody in blocking solution for 1 hour at room temperature. The membrane was washed three times in TBST for 10 minutes each and then in TBS. To detect antibody binding, the membrane was incubated with ECL for 2 minutes at room temperature and exposed to ECL Hyperfilm for the appropriate time. The film was then developed using standard procedures. For densitometry analyses of band signal intensity, the X-ray film was scanned using ScnImage software. Immunoreactivity for most blots was normalized to β-actin as a protein loading control.

2.3.4.3 Far-Western blotting

Far-Western blotting followed the same protocol as section 2.3.4.2, but the membrane was incubated after the blocking step with 5 µg/ml of either GST or GST-IsoT ZnFUBP proteins for 1 hour at 4°C and a further 1 hour at room temperature. The membrane was washed 3 times in TBST for 10 minutes each and then incubated with 1:3000 anti-GST antibody in blocking solution for 1 hour at room temperature. The membrane was washed 3 times in TBST for 10 minutes each and then incubated with 1:3000 secondary antibody in blocking solution for 1 hour at room temperature. Following incubation with the secondary antibody, the membrane was washed three times in TBST and once in TBS for 10 minutes each time. To detect antibody binding, the membrane was incubated with ECL for 2 minutes at room temperature and exposed to ECL Hyperfilm for the appropriate time. The film was then developed using standard procedures.

CHAPTER 3 Investigations into the binding interactions of ubiquitin binding domains

3.1 Introduction

Ubiquitin-regulated cellular processes require recognition of specific ubiquitinated substrates by ubiquitin binding proteins (UBPs; also known as ubiquitin receptors), which possess ubiquitin binding domains (UBDs) that interact non-covalently with mono and/or polyUb chains. UBPs are involved in the regulation of diverse cellular functions, such as endocytosis, signal transduction and transcription as described in section 1.7 (Di Fiore et al., 2003; Hicke et al., 2005).

UBDs are usually 20-150 amino acid residues and differ in their structure, chemical properties and ability bind to Ub at its hydrophobic patch (Dikic et al., 2009; Hicke et al., 2005). Most of the UBDs have an α helical structure, which binds to a hydrophobic patch in the β-sheet of Ub. There are at least 16 different UBDs known (described in section 1.8).

Dysfunction of the UPS has been implicated in many diseases, including cancer and neurodegenerative diseases (Nalepa et al., 2006; Dahlmann, 2007) . Ub and ubiquitinated proteins aggregate inside neurones in the major human neurodegenerative diseases (Ross & Poirier, 2004; Reinstein & Ciechanover, 2006). There is a need for reliable methods to purify, detect and identify ubiquitinated proteins as well as unanchored (free) Ub to investigate the role of Ub *in vivo*. The specificity of UBDs for Ub can be used to directly purify ubiquitinated proteins *in vitro* and *in vivo*. Also, different domains show differential interaction properties for monoUb and polyUb chains (Tomlinson et al., 2007).

UBPs, containing UBDs, such as the S5a subunit of the 26S proteasome, have been shown to bind ubiquitinated proteins. Glutathione-S-transferase (GST)-S5a-coupled Sepharose (affinity chromatography) has been effectively used to purify polyubiquitinated substrates from pig brain homogenate. It was also confirmed that the UIM domain of S5a could bind Lys48- or Lys63-linked polyubiquitinated proteins (Wang et al., 2005).

The results described in this chapter investigate the differential binding affinity of five isolated UBDs for synthetic unanchoured (free) Ub, including monoUb, Lys48- and Lys63-linked polyUb chains and linear polyUb chains (di-Ub, tri-Ub and tetra-Ub). I also investigate binding to a model of substrate-conjugated Ub, where the free C-terminal G76 involved in ubiquitination is unavailable ($Ub5^{+1}$).

p62 polymerises via its N-terminal PB1 domain and increases its ability to bind polyUb chains by its C-terminal UBA domain (Bjorkoy et al., 2005). The Vps9 CUE domain forms a domain dimer, which makes extended contact with a large area on the surface of Ub. This dimerization increases its binding affinity to ubiquitin more than any other CUE domain (Prag et al., 2003).

Research has demonstrated that that GST-fusion UBA domains tend to form dimers, which increase their binding to Ub and Ub linkage binding preference compared to GST-fusion free sample (Kirkin et al., 2009; Sims et al., 2009). The GST-fused NBR1 UBA domain showed higher binding affinity to Lys63-linked diUb than monoUb (Kirkin, et al., 2009). Walinda and co-workers have prepared a purified NBR1 UBA domain free of GST and examined its binding affinity with monoUb, Lys48-linked and Lys63-linked diUb. The free NBR1 UBA domain showed no Ub linkage specificity and no preference for Lys48 or Lys63-linked over monoUb (Walinda et al, 2014).

3.2 Results

3.2.1 Preparation of UBD affinity matrices for the capture of ubiquitinated proteins

Cyanogen bromide activated sepharose beads (CNBr beads) were used to immobilize purified UBDs to provide a chromatography stationary phase (affinity matrices) for the binding investigations with synthetic and native ubiquitin topology. CNBr beads have an imido-carbonate group which binds the UBD recombinant protein.

Figure 3.1 shows the binding of purified recombinant UBD protein with CNBr beads. To determine the optimal concentration of UBDs for preparing affinity matrices, different concentrations of protein were investigated: IsoT ZnFUBP protein (10 mg protein: 1 ml CNBr and 15mg protein: 1mg CNBr) and NEMO UBAN (5 mg protein:1 ml CNBr). The results show that all the ratios investigated resulted in immobilizing UBDs because there is no or less protein in the 'after' column. I decided to use 10 mg protein for IsoT ZnFUBP and 5 mg protein for other UBDs for the preparation of affinity matrices.

Figure 3.1

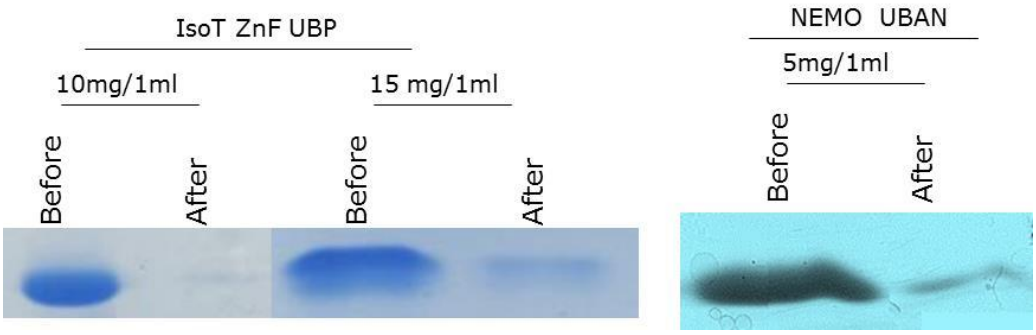


Figure 3.1 Binding of UBD proteins to CNBr activated Sepharose beads. A Coomassie Blue-stained gel showing before (Before) and after (After) binding using different concentrations of UBDs. 15% (w/v) acrylamide gel.

3.2.2.1 Expression, purification and cleavage of GST-p62 UBA fusion protein

A single colony of *E.coli* containing recombinant GST-p62 UBA fusion protein was inoculated into 20 ml Luria broth (LB) medium containig ampicillin (80 µg/ml) and oxacillin (20 µg/ml). This was grown overnight at 37ºC in a shaking incubator then diluted in 500 ml of LB containing antibiotics and incubated at 37ºC in a shaking incubator until the OD600 reached 0.6-0.8. Expression of GST-p62 UBA fusion protein was induced by addition of 200 µM isopropyl-β-D-thiogalactopyrinosid. The resulting protein was purified from the *E.coli* using glutathione (GSH) affinity chromatography (GSH beads). Samples were electrophoresed on a 5-15% (w/v) acrylamide gradient gel and visualised by Coomassie blue staining. Figure 3.1 shows the GSTp62 UBA fusion protein captured on GSH beads (~28 kDa; before cleavage). After incubation with thrombin, which cleaves the GST fusion protein, a ~23 kDa band (GST) is the GST on GSH beads (Figure 3.2; after cleavage) and a ~10 kDa band (p62 UBA) is the p62 UBA protein (Figure 3.2; eluted fraction).

Figure 3.2

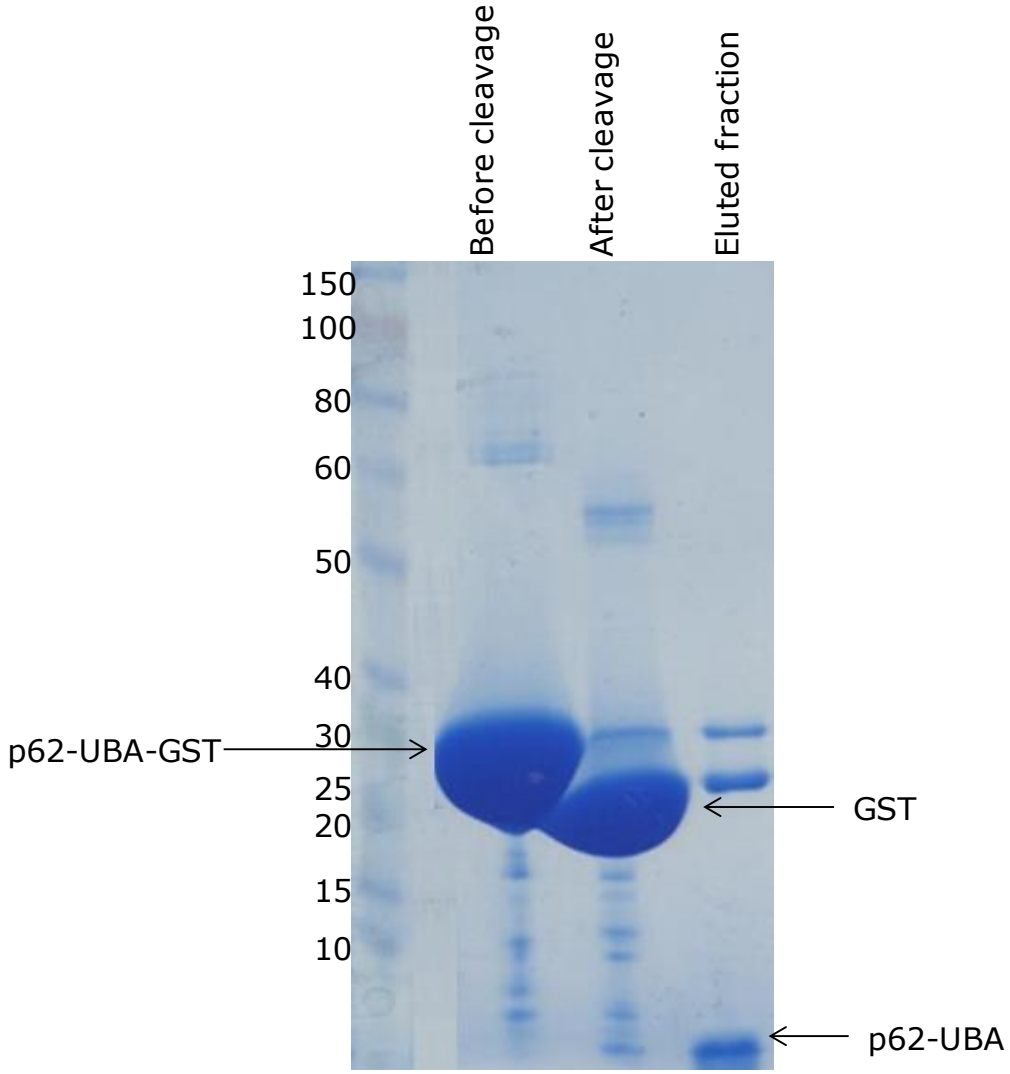


Figure 3.2 Coomassie blue stained gel showing purification and cleavage of the GST-p62 UBA fusion protein. Before cleavage = GST-p62 UBA fusion protein captured on GSH beads (GST-p62 UBA; ~28 kDa). After cleavage = GST protein on GSH beads after incubation of p62-UBA-GST-bound GSH beads with thrombin (GST; ~23 kDa). Eluted fraction = buffer after incubation of GST-p62 UBA-bound GSH beads with thrombin (p62 UBA; ~10 kDa). 5-20% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.2.2 Thrombin cleavage of GST-UBDs

The UBDs were expressed as described 3.2.1.1. I investigated the amount of thrombin required for efficient cleavage of the GST-UBDs using GST-p62 UBA fusion protein as a model. I tried three different concentrations of thrombin (3, 5 and 8 units) overnight at 4°C. Figure 3.3 shows that 3U of thrombin was sufficient to cleave the GST-p62 UBA fusion protein.

Figure 3.3

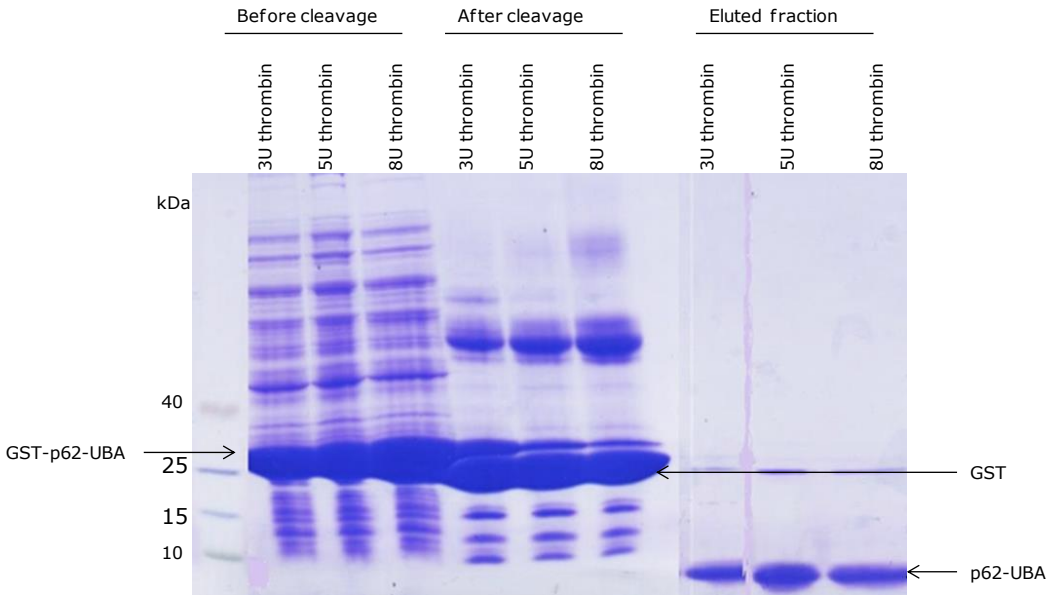


Figure 3.3 GST-p62 UBA fusion protein was efficiently cleaved using 3 units (U) of thrombin. Before cleavage = GST-p62 UBA fusion protein captured by GSH beads (GST-p62 UBA; ~28 kDa). After cleavage = GST protein on GSH beads after incubation of GST-p62 UBA-bound GSH beads with thrombin (GST; ~23 kDa). Eluted fraction = buffer after incubation of GST-p62 UBA-bound GSH beads with thrombin (p62 UBA; ~10 kDa). 5-20% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.2.3 Binding interactions of p62 UBA with unanchored monoUb, Lys48- and Lys63-linked polyUb chains

The cleaved p62 UBA domain was immobilised on cyanogen bromide-activated Sepharose 4B (CNBr) beads (p62 UBA beads). As a control for non-specific binding, CNBr only beads (control beads) were included in all experiments, where no protein was immobilised on the CNBr beads. p62 UBA and control beads were incubated with 1 µg of commercial monoUb, Lys48- or Lys63-linked polyUb chains. Buffer containing unbound Ub was collected and the CNBr beads washed before eluting bound Ub with gel application buffer. Samples were electrophoresed on a 5-15% (w/v) acrylamide gradient gel and visualised by Ub Western blotting.

Figure 3.4A shows that p62 UBA does not bind to monoUb. This result is consistent with the study of Ciani and colleagues (2003), who showed that the UBA domain of p62 bound to Lys48-linked polyUb, but not monoUb (Ciani et al., 2003). However, my findings are in contrast to several previous reports that show this UBA domain and full-length p62 bind mono-Ub (Wilkinson et al., 2001; Long et al., 2008). There may be several reasons for these inconsistent results. Full-length protein interaction with Ub may be different than that of the isolated domain. Also, the methods for the capture of Ub differ between studies. Alternatively, a study has indicated that the UBA domains have a low binding affinity to monoUb (Long et al., 2008).

As one of the proposed functions of the p62 UBA domain is to shuttle polyUb proteins to the autophagy pathway, I examined p62 UBA binding to Lys48- and Lys63-linked polyUb chains. Figure 3.4B and C show binding of p62 UBA to polyUb chains of two or more Ubs. As described above, the p62 UBA did not bind to monoUb (ub1) in these experiments. The UBA domain of p62 has previously been shown to bind Lys63-linked polyUb chains, supporting my observations (Ciani et al., 2003).

Figure 3.4

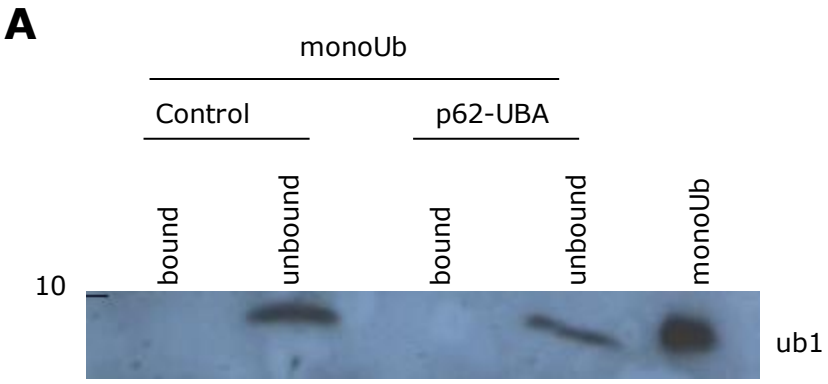


Figure 3.4

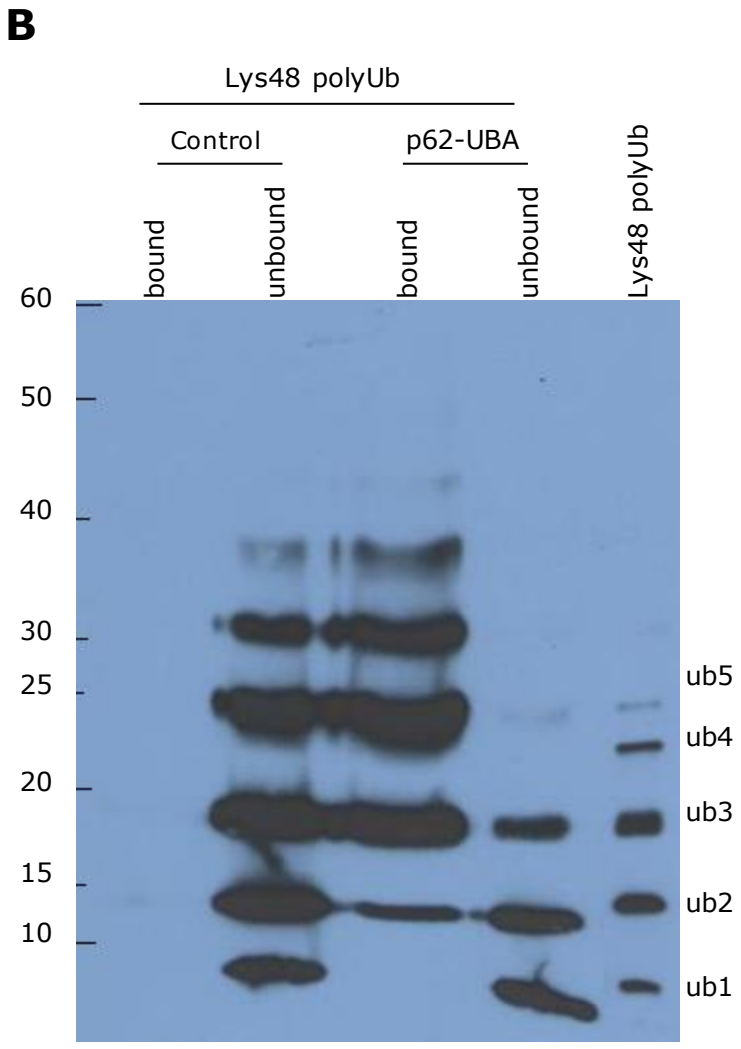


Figure 3.4

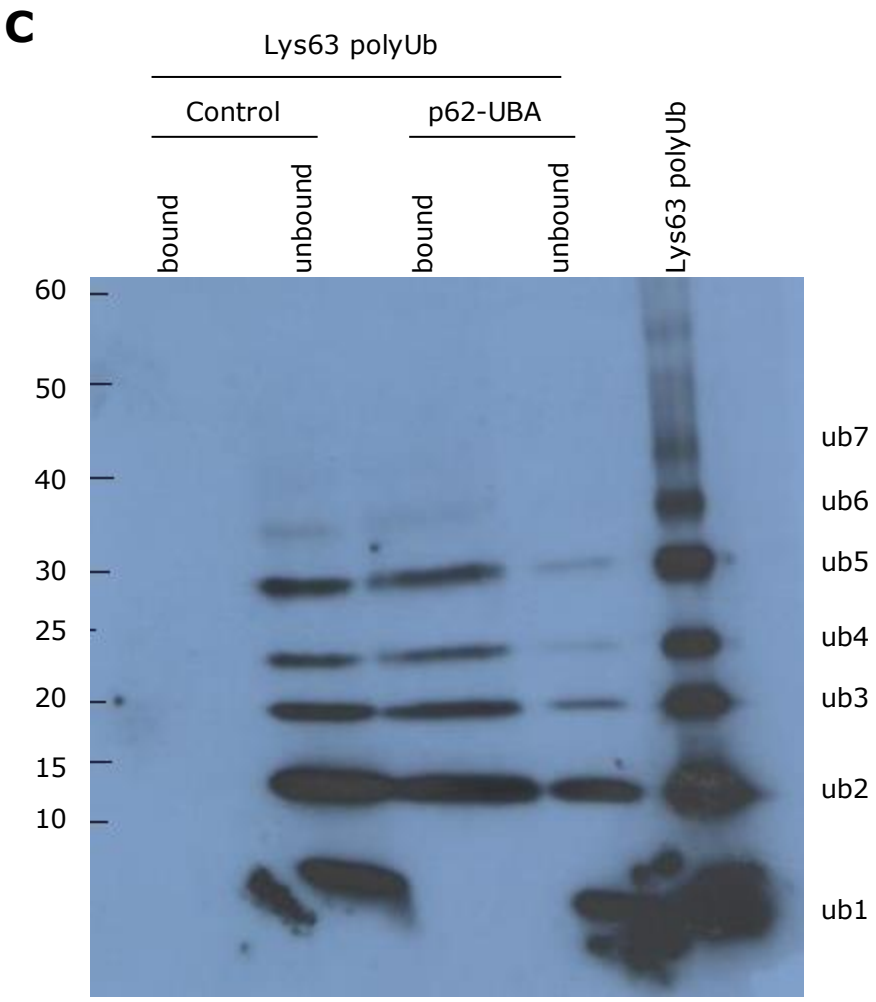


Figure 3.4 p62 UBA binds Lys48- and Lys63-linked polyUb chains, but not monoUb. Anti-ubiquitin immunoblots investigating p62 UBA binding to monoUb (A), Lys48- (B) and Lys63-linked (C) polyUb. Bound = Ub captured by control or p62 UBA beads. Unbound = buffer collected following incubation of control or p62 UBA beads with Ub. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.2.4 Binding interactions of p62 UBA with linear di-Ub, tri-Ub and tetra-Ub polyUb

Linear polyUb chains are involved in the regulation of the NF-kB pathway (Sanz et al., 2000). p62 may play an important role in activation of the NF-kB pathway via binding to TRAF6 and facility its Lys63-linked polyubiquitination (Sanz et al., 2000; Wooten et al., 2005). Therefore, I investigated the binding of p62 UBA to linear polyubiquitin chains.

p62 UBA and control beads were incubated with 1 µg of commercial linear di-Ub, tri-Ub and tetra-Ub. After incubation, buffer containing any unbound Ub was collected and the CNBr beads washed before eluting bound Ub with gel application buffer. Samples were electrophoresed on a 5-15% (w/v) acrylamide gradient gel and visualised by Ub Western blotting. Figure 3.5 show that p62 UBA binds to linear di-, tri- and tetra-Ub polyubiquitin chains. To my knowledge, this is the first report of p62 UBA binding to linear polyUb. Previous work has shown that the UBA domain of XIAP (X-linked inhibitor of apoptosis) binds to linear di-Ub chains (Tse et al., 2011).

Figure 3.5

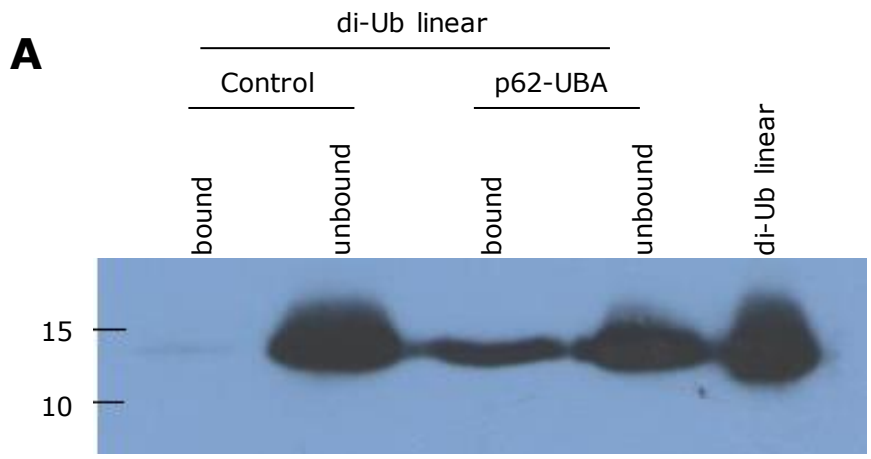


Figure 3.5

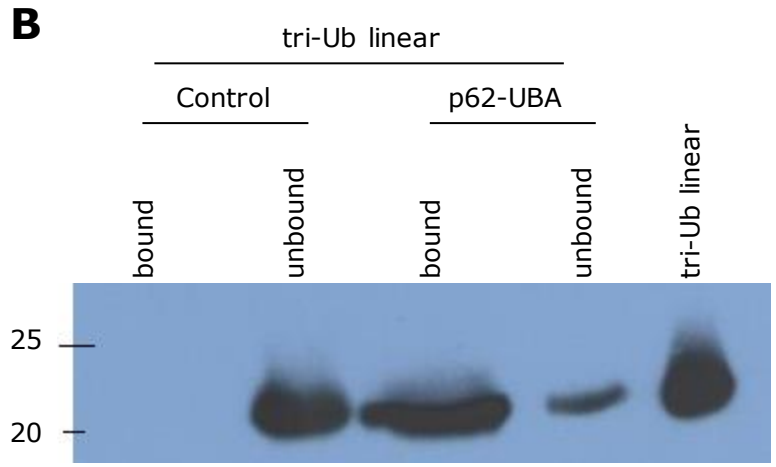


Figure 3.5

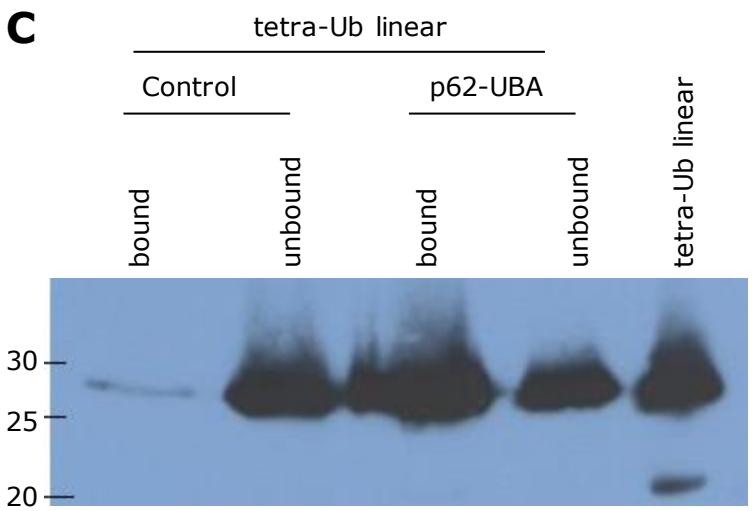


Figure 3.5 p62 UBA binds linear polyUb chains. Anti-ubiquitin immunoblots showing p62-UBA binding to linear di- (A), tri- (B) and tetra-Ub (C). Bound = Ub captured by control or p62 UBA beads. Unbound = buffer collected following incubation of control or p62 UBA beads with Ub. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.2.5 Binding interaction of p62 UBA with $Ub5^{+1}$ polyubiquitin chains

Sections 3.2.1.3 and 3.2.1.4 have investigated the binding of different unanchored (free) Ub species to the UBA domain of p62. To further examine the binding of p62 UBA to polyUb chains I used $Ub5^{+1}$. $Ub5^{+1}$ is a Ub polymer consisting of a Lys48-linked chain of four Ub molecules (Ub4) conjugated to Ub^{+1}, which is a frame-shift mutant Ub. In Ub^{+1}, the C-terminal G76 of Ub, important in Ub conjugation, is replaced with a 20 amino acid ($^{+1}$) nonsense sequence extension, i.e. the free C-terminal G76 is lost. This is a model of protein-conjugated Ub. Therefore, interactions with $Ub5^{+1}$ examine the binding of p62-UBA to ubiquitinated proteins, i.e. anchored Ub.

p62 UBA and control beads were incubated with 0.5 µg of commercial $Ub5^{+1}$. After incubation, buffer containing any unbound Ub was collected and the CNBr beads washed before eluting bound Ub with gel application buffer. Samples were electrophoresed on a 5-15% (w/v) acrylamide gradient gel and visualised by Ub Western blotting. Figure 3.6 shows that p62 UBA binds to $Ub5^{+1}$ chain, suggesting that the UBA domain of p62 can bind ubiquitinated proteins.

This result is consistent with previous studies demonstrating the binding of p62 UBA to polyubiquitinated proteins (Pridgeon et al., 2003; Seibenhener et al., 2004). The UBA domain of p62 was shown to bind Lys63-linked polyubiquitinated substrates and in another study used to identify ubiquitinated proteins in rabbit reticulocyte lysate following the induction of *in vitro* ubiquitination (Pridgeon et al., 2003; Seibenhener et al., 2004).

Figure 3.6

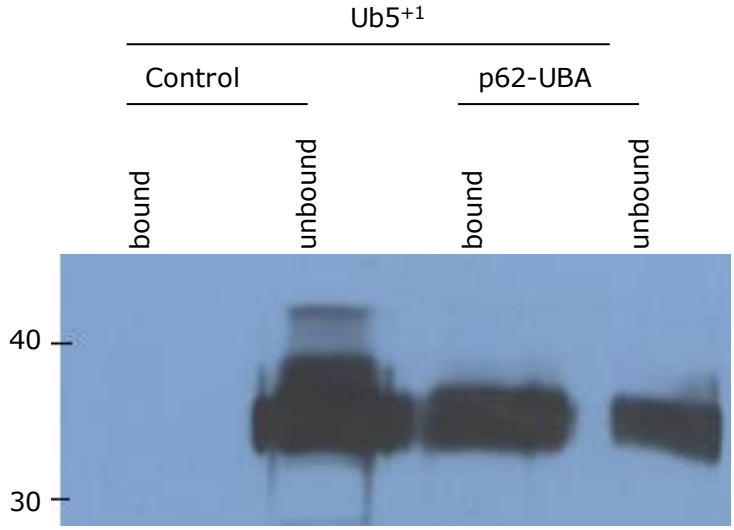


Figure 3.6 p62 UBA binds $Ub5^{+1}$ polyUb chains. Anti-ubiquitin immunoblot showing p62 UBA binding to $Ub5^{+1}$ polyUb. Bound = $Ub5^{+1}$ polyUb captured by control or p62 UBA beads. Unbound = buffer collected following incubation of control or p62 UBA beads with Ub. 15% (w/v) acrylamide gel. This is representative of 3 independent experiments showing the same result.

3.2.2.6 p62 UBA summary

In section 3.2.1 I have shown that p62 UBA can bind to unanchored Lys48- and Lys63-linked, and linear-polyUb chains, but not monoUb, suggesting that the UBA domain of p62 binds to polyUb in a linkage-independent manner. p62 UBA also bound to a model of ubiquitinated proteins, $Ub5^{+1}$.

3.2.3.1 Expression, purification and cleavage of GST-UQ1 UBA fusion protein

GST-UQ1 UBA fusion protein was expressed, purified and cleaved as described in section 3.2.1.1 for the GST-p62 UBA fusion protein. Figure 3.7 shows the GST-UQ1 UBA fusion protein captured on GSH beads (~28 kDa; before cleavage). After incubation with thrombin, a ~23 kDa band represents the cleaved GST on GSH beads (Figure 3.7; after cleavage) and a band < 10 kDa band (UQ1 UBA) is the UQ1 UBA protein (Figure 3.6; eluted fraction).

Figure 3.7

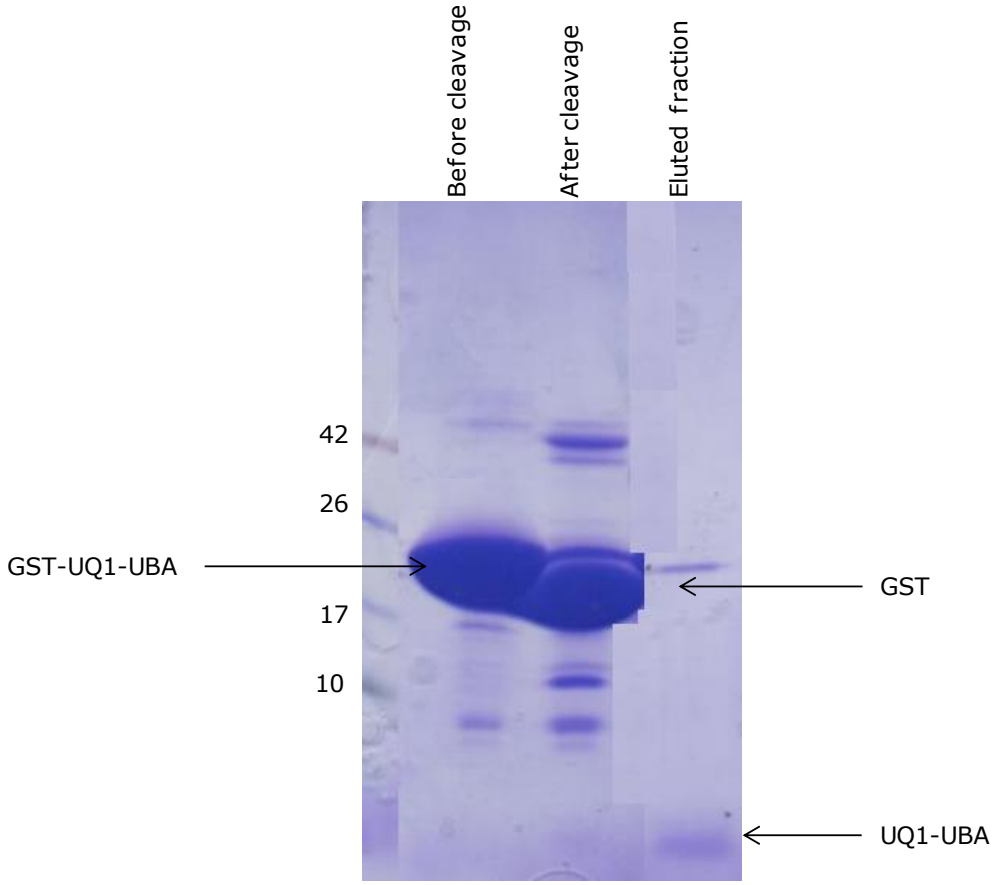


Figure 3.7 Coomassie blue stained gel showing purification and cleavage of the GST-UQ1 UBA fusion protein. Before cleavage = GST-UQ1 UBA fusion protein captured on GSH beads (GST-UQ1 UBA; ~28 kDa). After cleavage = GST protein on GSH beads after incubation of GST-UQ1 UBA-bound GSH beads with thrombin (GST; ~23 kDa). Eluted fraction = buffer after incubation of GST-UQ1 UBA-bound GSH beads with thrombin (UQ1 UBA; < 10 kDa). 5-20% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.3.2 Binding interactions of UQ1 UBA with unanchored monoUb, Lys48- and Lys63-linked polyUb chains

The cleaved UQ1 UBA domain (section 3.2.2.1) was immobilised on CNBr beads to investigate its binding to monoUb, Lys48- and Lys63-linked polyUb chains as described in section 3.2.1.3. UQ1 UBA and control beads were incubated with 1 µg of commercial monoUb, Lys48- or Lys63-linked polyUb chains. Buffer containing unbound Ub was collected and the UQ1 UBA beads were washed before eluting bound protein with gel application buffer. Samples were electrophoresed on a 5-15% (w/v) acrylamide gradient gel and visualised by Ub Western blotting.

Figure 3.8A shows UQ1 UBA binds to monoUb. This is consistent with the findings of Zhang and colleagues (2008) demonstrating that the UQ1 UBA domain bound to monoUb (Zhang et al., 2008). The UBA domain of Dsk2p (ubiquitin-binding protein from Saccharomyces Cerevisiae), has also previously been shown to bind monoUb (Ohno et al., 2005).

Figures 3.8B and C show the binding of UQ1 UBA to unanchored Lys48- and Lys63-linked polyUb chains respectively. Previous work has shown that the UQ1-UBA domain binds to Lys48- and Lys63- tetra Ub, (Raasi et al., 2005).

Figure 3.8

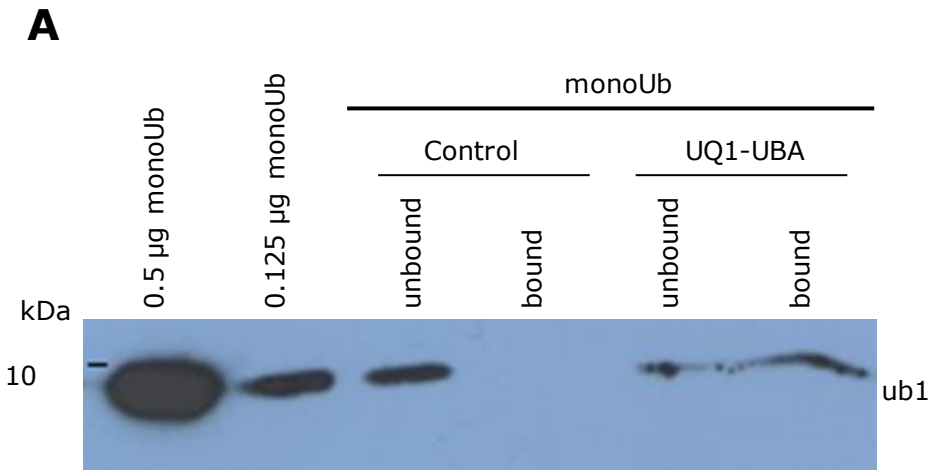


Figure 3.8

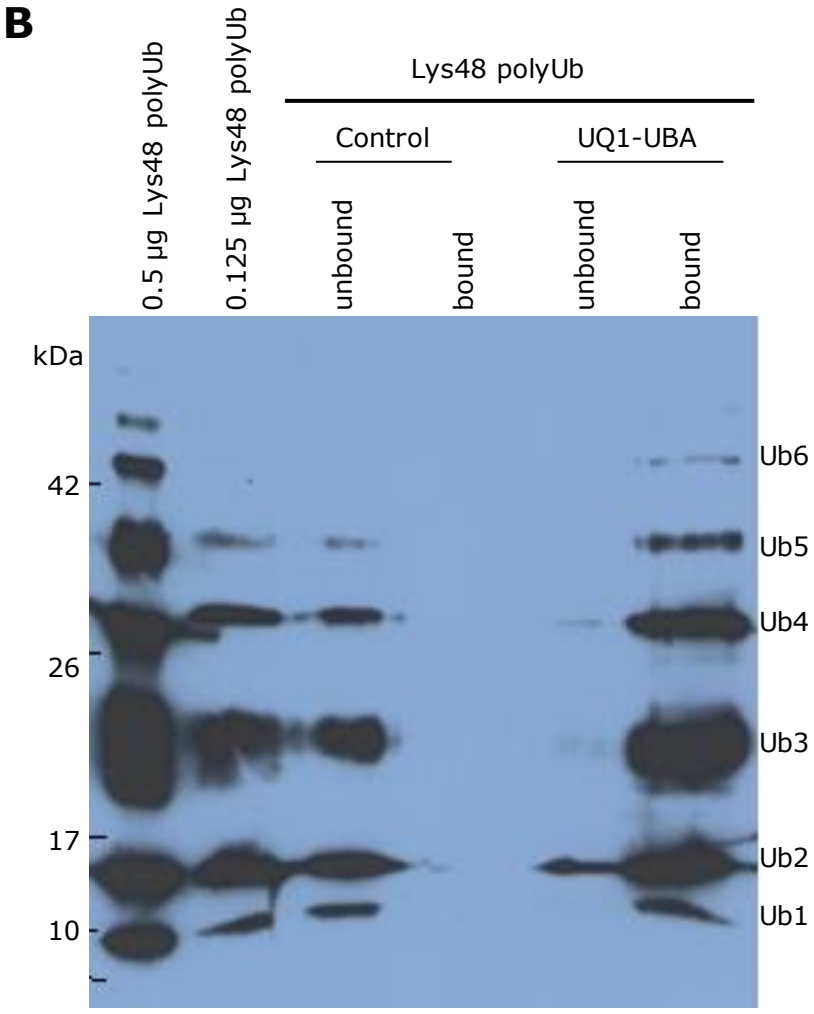


Figure 3.8

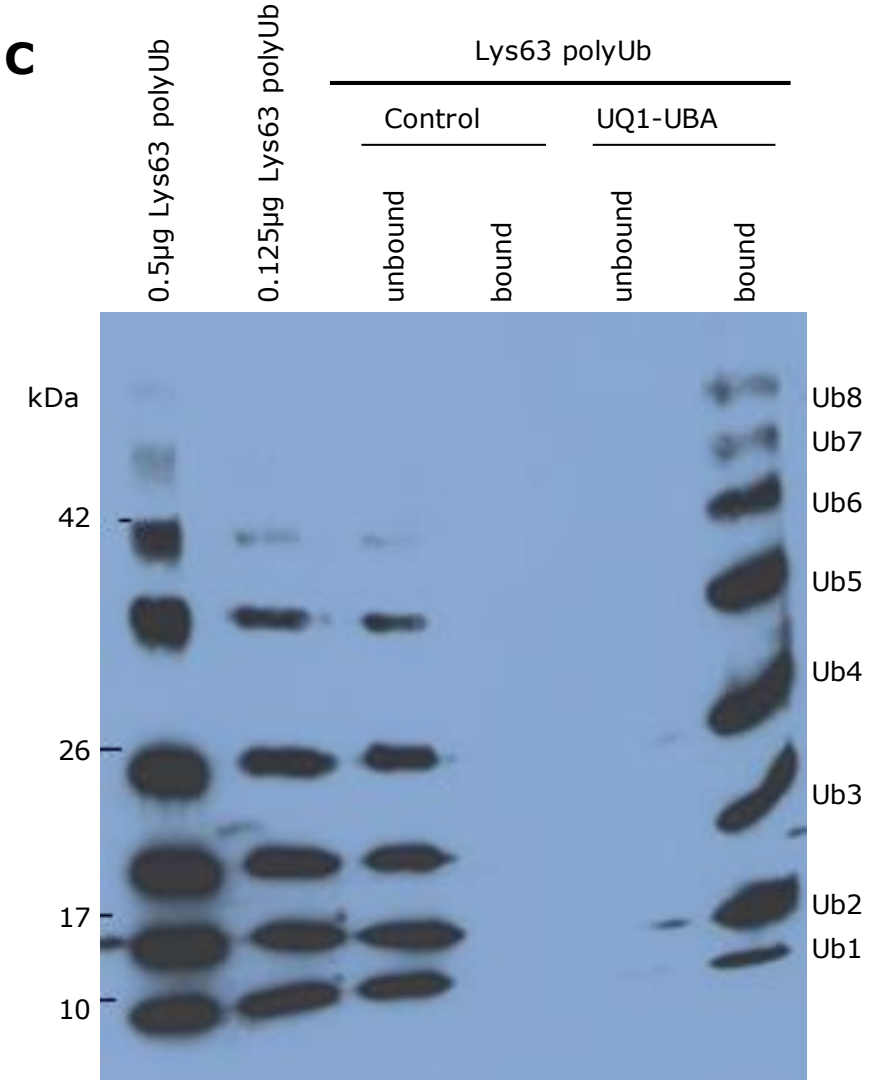


Figure 3.8 UQ1 UBA binds monoUb, Lys48- and Lys63-linked polyUb chains. Anti-ubiquitin immunoblots showing UQ1 UBA binding to monoUb (A), Lys48- (B) and Lys63-linked (C) polyUb. Bound = Ub captured by control or UQ1 UBA beads. Unbound = buffer collected following incubation of control or UQ1 UBA beads with Ub. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.3.3 Binding interactions of UQ1 UBA with linear di-Ub, tri-Ub and tetra-Ub polyUb

As described in section 3.2.1.4, I investigated binding of the UQ1 UBA domain with 1 µg of commercial linear di-Ub, tri-Ub and tetra-Ub. Figure 3.9 shows that the UQ1 UBA binds to linear polyUb chains. There was evidence of control beads binding tetra-Ub (Figure 3.9C), but this was a relatively low level compared to UQ1 UBA beads, suggesting that it is non-specific. This is the first report of UQ1 UBA binding to linear polyUb.

Figure 3.9

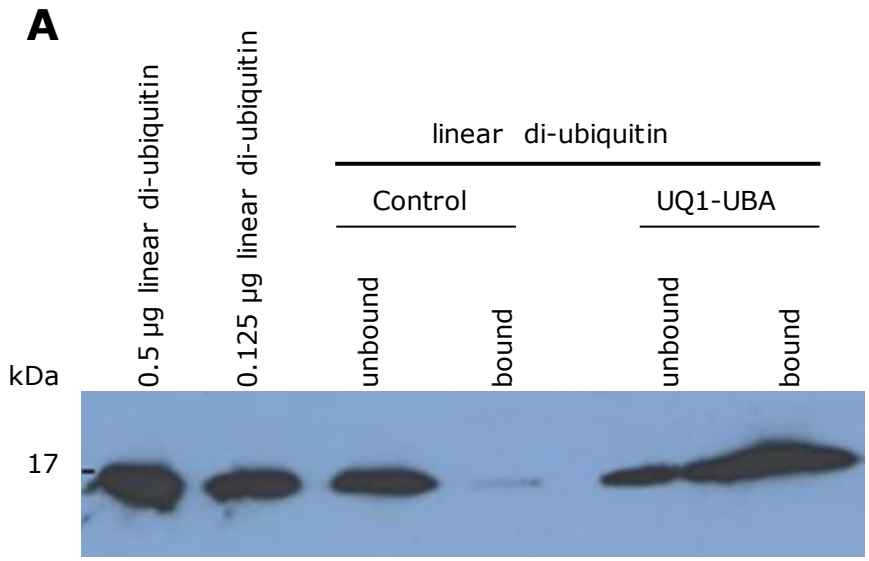


Figure 3.9

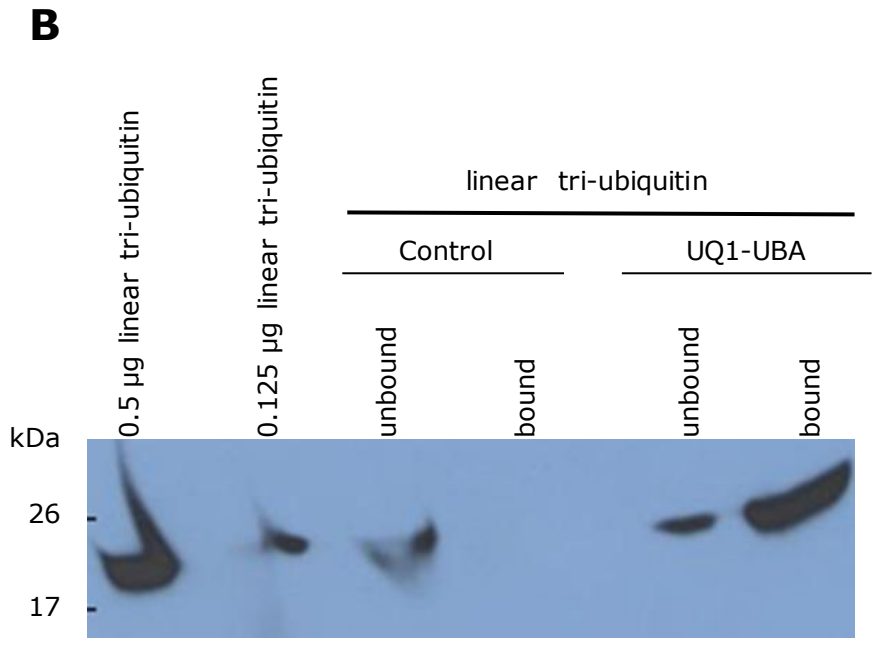


Figure 3.9

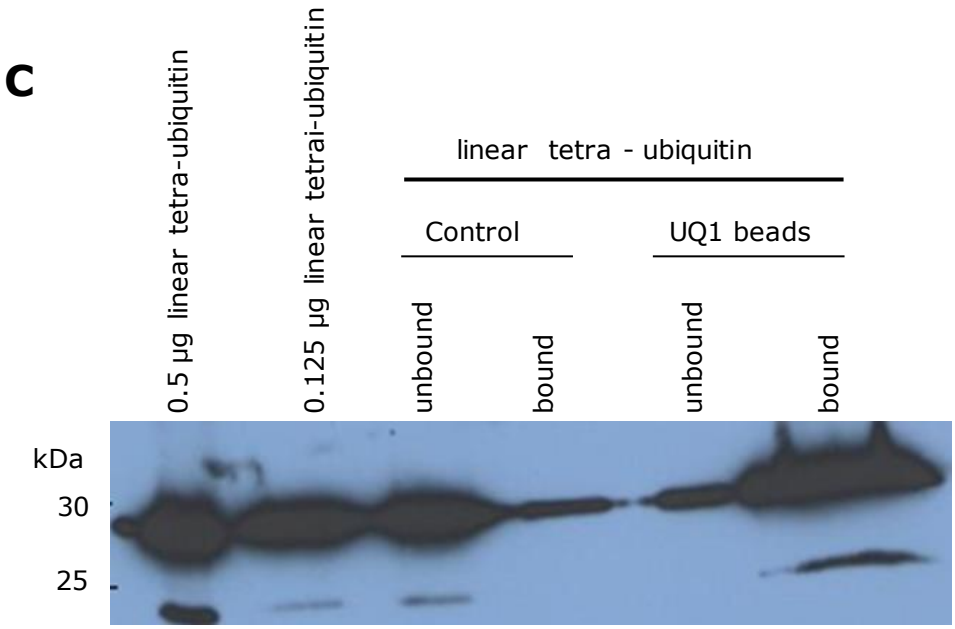


Figure 3.9 UQ1 UBA binds linear polyUb chains. Anti-ubiquitin immunoblots showing UQ1 UBA binding to linear di- (A), tri- (B) and tetra-Ub (C). Bound = Ub captured by control or UQ1 UBA beads. Unbound = buffer collected following incubation of control or UQ1 UBA beads with Ub. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.3.4 Binding interaction of UQ1 UBA with $Ub5^{+1}$ polyubiquitin chains

To investigate the interaction of UQ1 UBA with substrate-conjugated Ub (anchored), the UQ1 UBA and control beads were incubated with 0.5 µg of commercial $Ub5^{+1}$ as detailed in section 3.2.1.5. Figure 3.10 shows that the UBA domain of UQ1 binds to $Ub5^{+1}$. Therefore, this domain has the ability to bind ubiquitinated substrates as well as unanchored (free) Ub as shown in sections 3.2.2.2 and 3.2.2.3. The result shown in Figure 3.10 is consistent with a recent study by Nakayasu et al. (2013) demonstrating that the UQ1 UBA domain bound tightly to ubiquitinated proteins from RAW 264.7 murine macrophages cell line and detected endogenous Lys48-linked polyubiquitinated proteins (Nakayasu et al., 2013).

Figure 3.1o

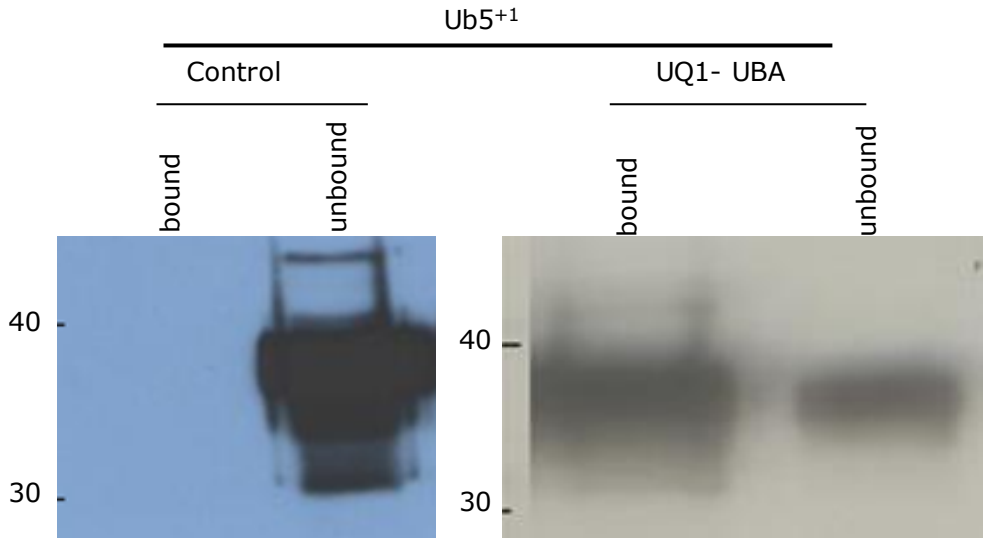


Figure 3.10 UQ1 UBA binds $Ub5^{+1}$ polyUb chains. Anti-ubiquitin immunoblot showing UQ1 UBA binding to $Ub5^{+1}$ polyUb. Bound = $Ub5^{+1}$ polyUb captured by control or UQ1 UBA beads. Unbound = buffer collected following incubation of control or UQ1 UBA beads with Ub. 15% (w/v) acrylamide gel. This is representative of 3 independent experiments showing the same result.

3.2.3.5 UQ1 UBA summary

In section 3.2.2 I have shown that the UBA domain of UQ1 can bind to unanchored monoUb, Lys48- and Lys63-linked, and linear-polyUb chains, as well as $Ub5^{+1}$. Therefore, the UQ1 UBA does not show specific Ub-binding interactions.

3.2.4.1 Expression, purification and cleavage of GST-Vps9 CUE fusion protein

Expression, purification and cleavage of GST-Vps9 CUE fusion protein was carried out as described for the GST-p62 UBA fusion protein in 3.2.1.1. Figure 3.10 shows the GST-Vps9 CUE fusion protein captured on GSH beads (~28 kDa; before cleavage). After incubation with thrombin, ~23 kDa band (GST) represents the cleaved GST on GSH beads (Figure 3.11; after cleavage) and a band < 10 kDa band is the Vps9 CUE protein (Figure 3.11; eluted fraction).

Figure 3.11

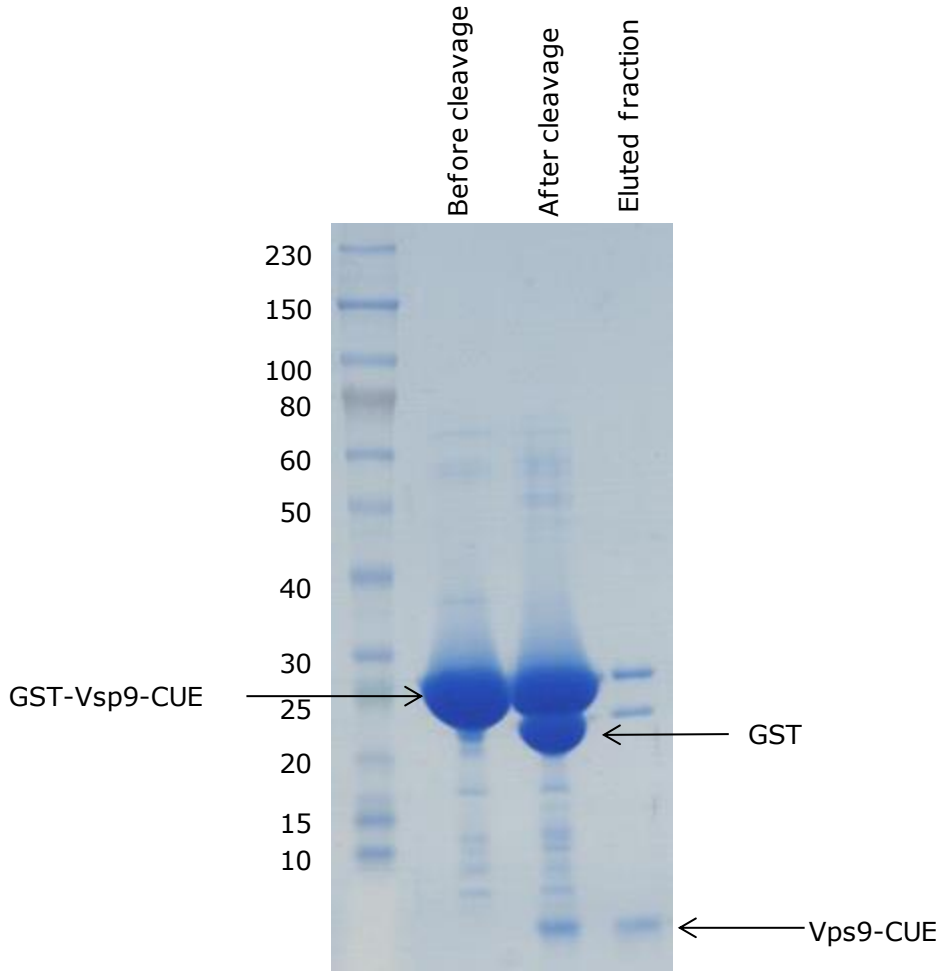


Figure 3.11 Coomassie blue stained gel showing purification and cleavage of the GST-Vps9 CUE fusion protein. Before cleavage = GST-Vps9 CUE fusion protein captured on GSH beads (GST-Vps9 CUE; ~28 kDa). After cleavage = GST protein on GSH beads after incubation of GST-Vps9 CUE-bound GSH beads with thrombin (GST; ~23 kDa). Eluted fraction = buffer after incubation of GST-Vps9 CUE-bound GSH beads with thrombin (Vsp9 CUE; < 10 kDa). 5-20% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.4.2 Binding interactions of Vsp9 CUE with unanchored monoUb, Lys48- and Lys63-linked polyUb chains

The cleaved Vsp9 CUE domain (section 3.2.3.1) was immobilised on CNBr beads to investigate its binding to monoUb, Lys48- and Lys63-linked polyUb chains as described in section 3.2.1.3. Vsp9 CUE and control beads were incubated with 1 µg of commercial monoUb, Lys48- or Lys63-linked polyUb chains. Buffer containing unbound Ub was collected and the bound proteins were eluted with gel application buffer. Samples were electrophoresed on a 5-15% (w/v) acrylamide gradient gel and visualised by Ub Western blotting.

Although previous studies have shown that the CUE domain of Vsp9 binds monoUb, I did not observe an interaction between Vsp9 CUE and monoUb in our studies (Figure 3.12A) (Shih et al., 2002; Davies et al., 2003; Donaldson et al., 2003; R. S. Kang et al., 2003). Figures 3.12B and C show that the CUE domain of Vsp9 bound to Lys48- and Lys63-linked polyUb chains respectively, consistent with previous *in vitro* studies of this domain (Donaldson et al., 2003; Shih et al., 2003). Interestingly, a recent study showed that the CUE domain of Cue1 had higher affinity for longer Lys48-linked polyUb chains (Bagola et al., 2013).

Figure 3.12

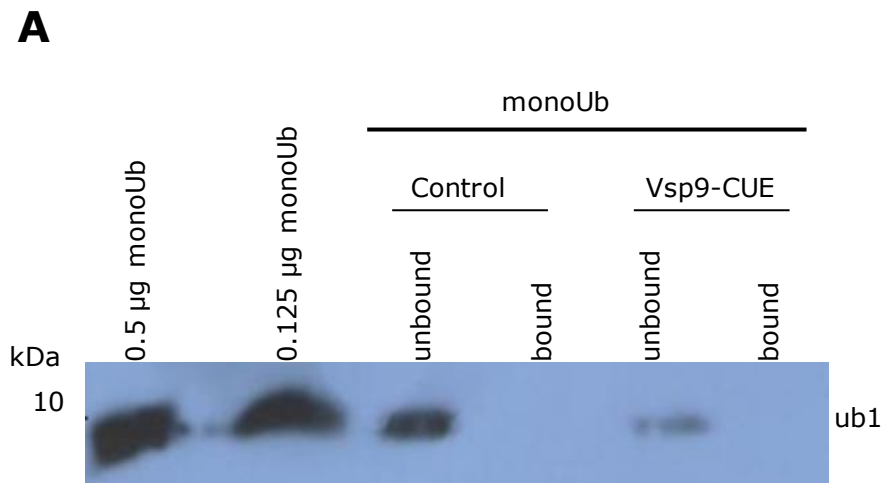


Figure 3.12

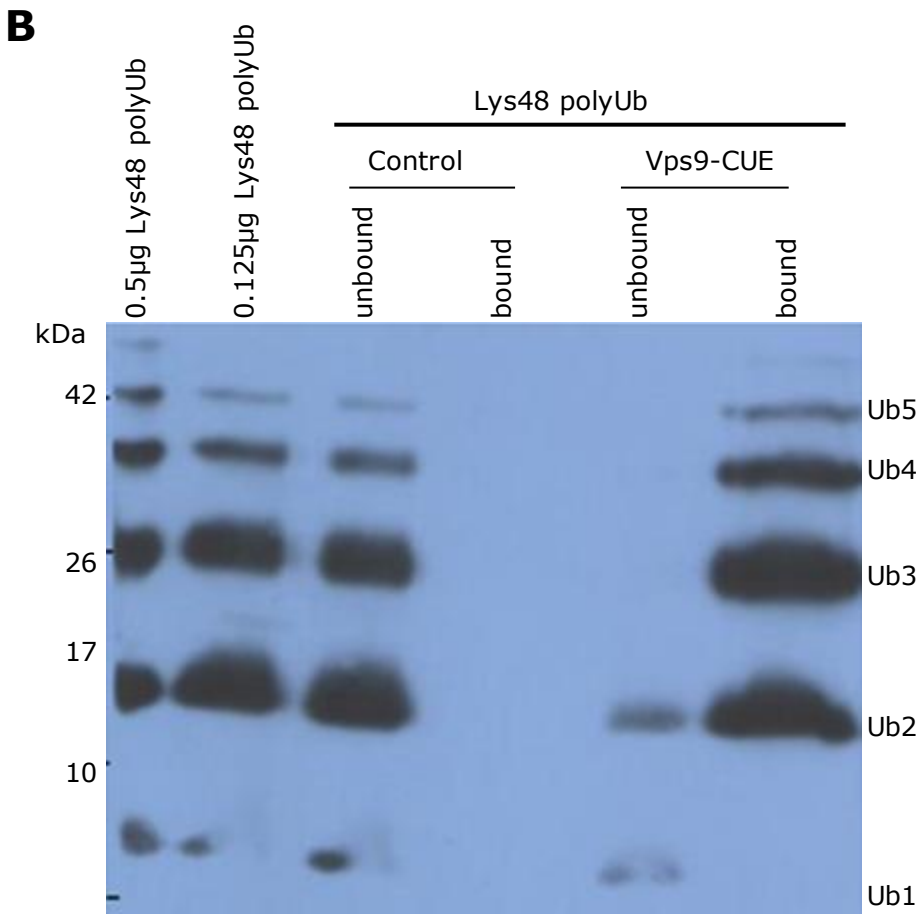


Figure 3.12

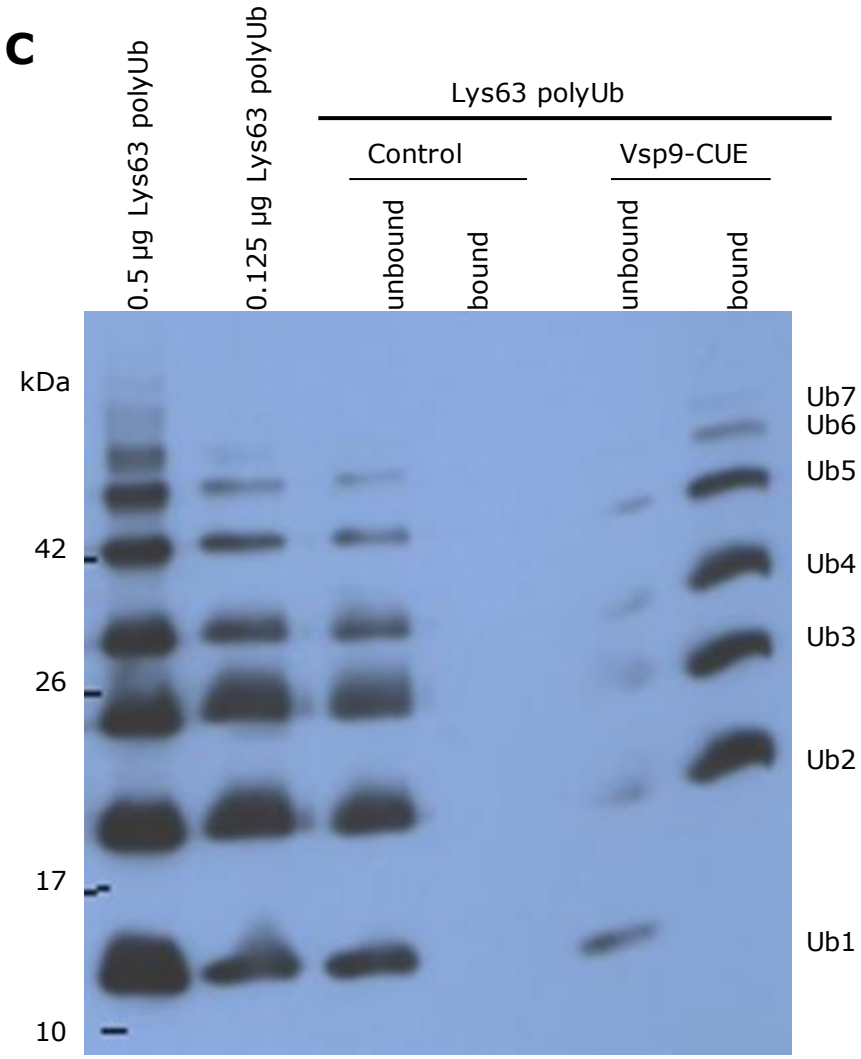


Figure 3.12 Vsp9 CUE binds Lys48- and Lys63-linked polyUb chains, but not monoUb. Anti-ubiquitin immunoblots investigating Vsp9 CUE binding to monoUb (A), Lys48- (B) and Lys63-linked (C) polyUb. Bound = Ub captured by control beads or Vsp9 CUE beads. Unbound = buffer collected following incubation of control or Vsp9 CUE beads with Ub. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.4.3 Binding interactions of Vsp9 CUE with linear di-Ub, tri-Ub and tetra-Ub polyUb

Similar to section 3.2.1.4, I investigated binding of the Vsp9 CUE domain immobilised on CNBr with 1 µg of commercial linear di-Ub, tri-Ub and tetra-Ub. Figure 3.13 shows that Vsp9 CUE binds to linear di- (A), tri- (B) and tetra- (C) polyUb chains. Binding of tri-Ub to control beads (Figure 3.13B) was detected, but this was very low compared to Vsp9 CUE binding. The binding of Vsp9 CUE to linear Ub chains is a novel finding.

Figure 3.13

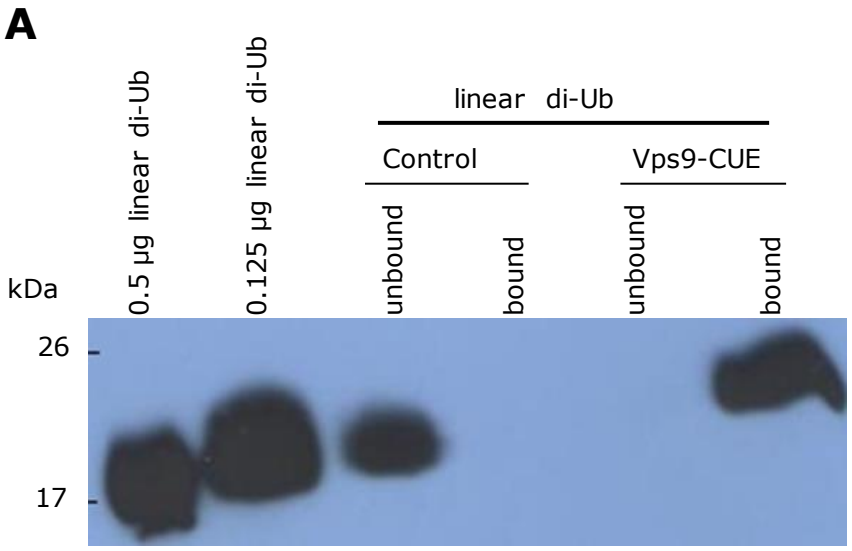


Figure 3.13

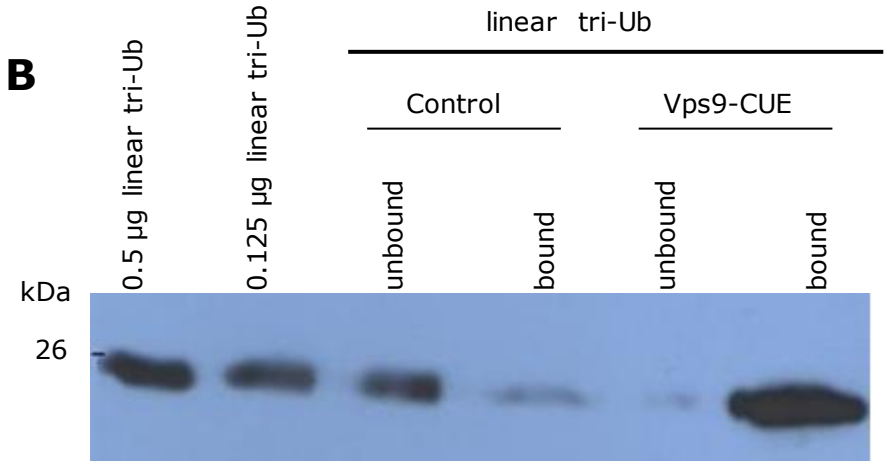


Figure 3.13

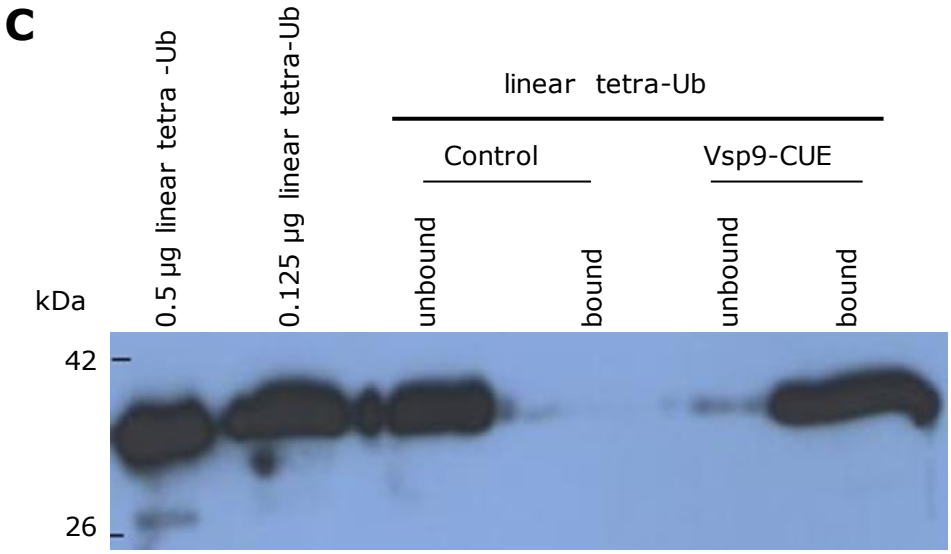


Figure 3.13 Vsp9 CUE binds linear polyUb chains. Anti-ubiquitin immunoblots showing Vsp9 CUE binding to linear di- (A), tri- (B) and tetra-Ub (C). Bound = Ub captured by control or Vsp9 CUE beads. Unbound = buffer collected following incubation of control or Vsp9 CUE beads with Ub. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.4.4 Binding interaction of Vsp9 CUE with $Ub5^{+1}$ polyubiquitin chains

To investigate the ability of Vsp9 CUE to bind with substrate-conjugated Ub (anchored), Vsp9 CUE and control CNBr beads were incubated with 0.5 µg of commercial $Ub5^{+1}$ as described in section 3.2.1.5. Figure 3.14 shows that Vsp9 CUE binds to $Ub5^{+1}$, suggesting Vsp9 will bind to ubiquitinated proteins.

Figure 3.14

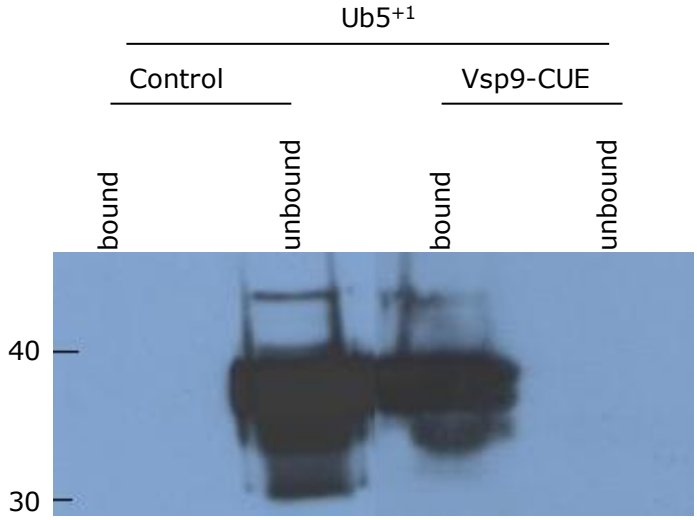


Figure 3.14 Vsp9 CUE binds $Ub5^{+1}$ polyUb chains. Anti-ubiquitin immunoblot showing Vsp9 CUE binding to $Ub5^{+1}$ polyUb. Bound = $Ub5^{+1}$ polyUb captured by control or Vsp9 CUE beads. Unbound = buffer collected following incubation of control or Vsp9 CUE beads with Ub. 15% (w/v) acrylamide gel. This is representative of 3 independent experiments showing the same result.

3.2.4.5 Vsp9 CUE summary

In section 3.2.3, I have shown that Vsp9 CUE binds Lys48- and Lys63-linked, and linear polyUb chains as well as a model of ubiquitinated proteins. The CUE domain of Vsp9 did not interact with monoUb.

3.2.5.1 Expression, purification and cleavage of GST NEMO UBAN fusion protein

The NEMO UBAN domain was expressed, purified and cleaved as described in 3.2.1.1 for the GST-p62 UBA fusion protein. Figure 3.15 shows the GST-NEMO UBAN fusion protein captured on GSH beads (~28 kDa; before cleavage). After incubation with thrombin, the ~23 kDa band (GST) is the cleaved GST on GSH beads (Figure 3.15; after cleavage) and a band < 10 kDa band (NEMO UBAN) is the NEMO UBAN protein (Figure 3.15; eluted fraction).

Figure 3.15

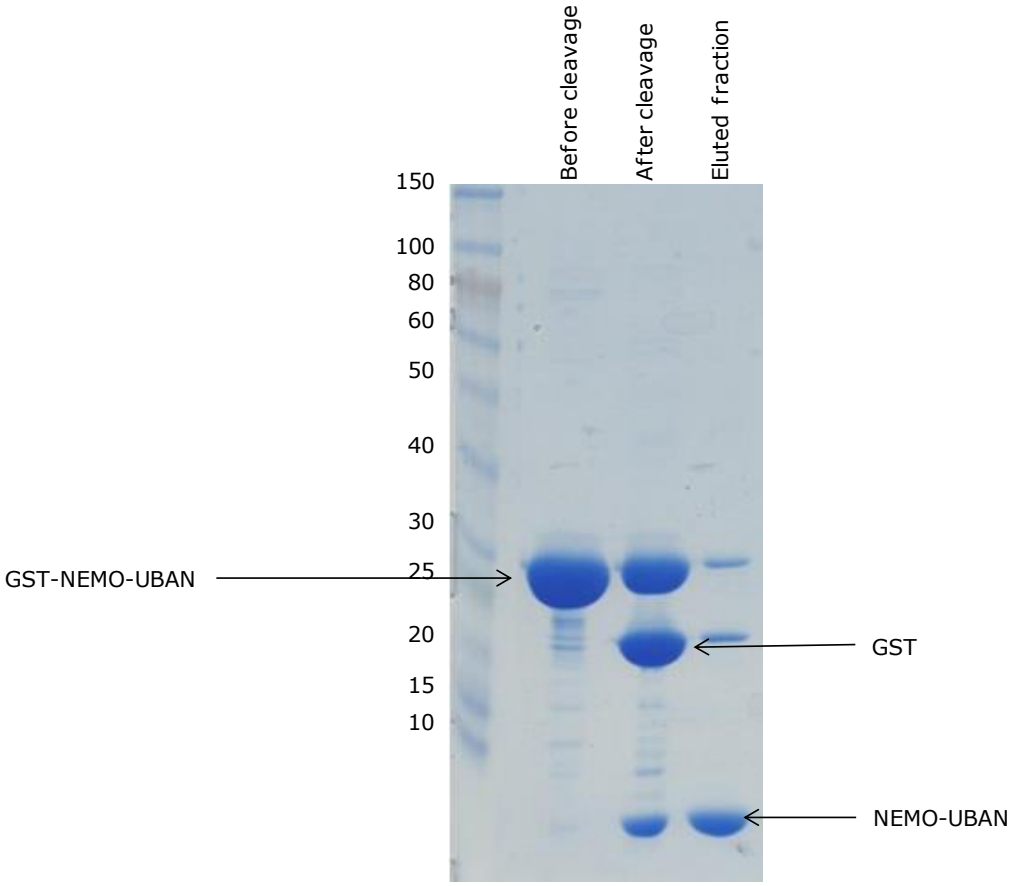


Figure 3.15 Coomassie blue stained gel showing purification and cleavage of the GST-NEMO UBAN fusion protein. Before cleavage = GST-NEMO UBAN fusion protein captured on GSH beads (GST-NEMO UBAN; ~28 kDa). After cleavage = GST protein on GSH beads after incubation of GST-NEMO UBAN-bound GSH beads with thrombin (GST; ~23 kDa). Eluted fraction = buffer after incubation of GST- NEMO UBAN-bound GSH beads with thrombin (NEMO UBAN; < 10 kDa). 5-20% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.5.2. Binding interactions of NEMO UBAN with unanchored monoUb, Lys48- and Lys63-linked polyUb chains

The cleaved NEMO UBAN domain (section 3.2.5.1) was immobilised on CNBr beads to investigate its binding to monoUb, Lys48- and Lys63-linked polyUb chains as described in section 3.2.1.3. NEMO UBAN and control beads were incubated with 1 µg of commercial monoUb, Lys48- or Lys63-linked polyUb chains. Buffer containing unbound Ub was collected and the bound proteins were eluted with gel application buffer. Samples were electrophoresed on a 5-15% (w/v) acrylamide gradient gel and visualised by Ub Western blotting.

Figure 3.16A shows that the UBAN domain of NEMO does not interact with monoUb. This is consistent with previous studies that have shown monoUb does not bind to the UBAN domain (Hadian et al.; Ivins et al., 2009; Lo et al., 2009). Further work using isothermal titration calorimetry (ICT) also did not detect an interaction between monoUb and UBAN of NEMO (Ivins et al., 2009; Lo et al., 2009a). Similar to the ICT study, time-resolved fluorescence analysis using Europium (Eu)-labelled with anti-His antibody indicated that the NEMO-UBAN-ZF domain did not interact with monoUb (Hadian et al., 2011).

In contrast to my findings, a GST pull-down study showed NEMO weakly interacted with GST-monoUb (Ea et al., 2006). Furthermore, Bloor et al. (2008) showed that NEMO and the UBAN (known as CoZi) domain bind monoUb (Bloor et al., 2008).

Several reports have shown that the UBAN domain of NEMO interacts with Lys63-linked polyUb chains (Wu et al., 2006;Laplantine et al., 2009; Lo et al., 2009; Yoshikawa et al., 2009). Specifically the UBAN domain of NEMO was shown to be involved in binding of GST-NEMO to Lys63-, but not Lys48-linked, di-Ub (Wu et al., 2006). Also, ICT and crystal structure studies of the UBAN domain showed binding to Lys63 di-Ub (Lo et al., 2009; Yoshikawa et al., 2009). Interestingly, in contrast to these studies, I found that NEMO UBAN did not bind Lys48- or Lys63-linked polyUb (Figures 3.16B and C).

Figure 3.16

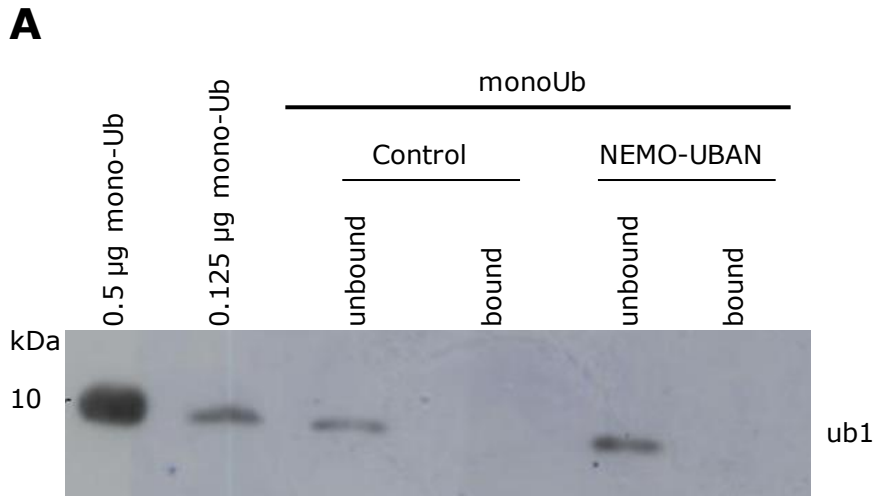


Figure 3.16

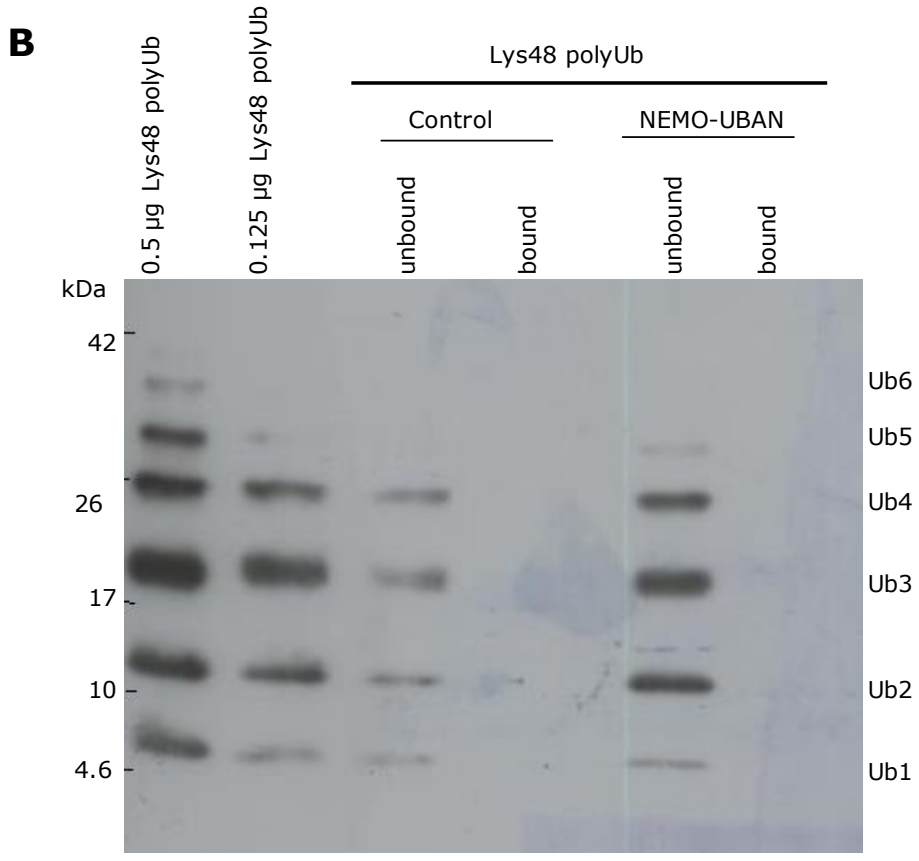


Figure 3.16

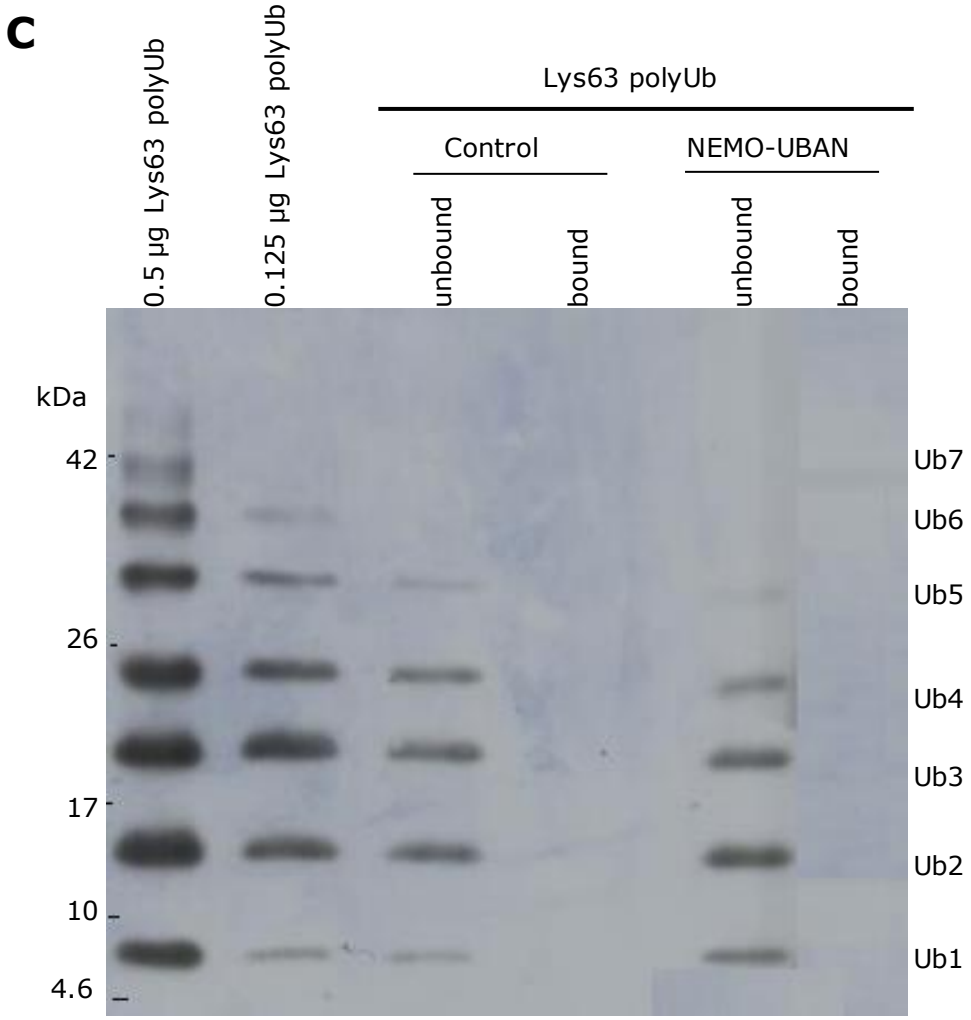


Figure 3.16 NEMO UBAN does not bind monoUb, Lys48- or Lys63-linked polyUb chains. Anti-ubiquitin immunoblots investigating NEMO UBAN binding to monoUb (A), Lys48- (B) and Lys63-linked (C) polyUb. Bound = Ub captured by control beads or NEMO UBAN beads. Unbound = buffer collected following incubation of control or NEMO UBAN beads with Ub. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.5.3 Binding interactions of NEMO UBAN with linear di-Ub, tri-Ub and tetra-Ub polyUb

Previous work has shown that the UBAN domain of NEMO can bind to linear polyUb chains and this may play an important role in activation of NF-kB(Stieglitz et al., 2012) . I investigated binding of my NEMO UBAN domain immobilised on CNBr with 1 µg of commercial linear di-Ub, tri-Ub and tetra-Ub. Figure 3.17 shows that NEMO UBAN binds selectively to linear polyUb chains as shown in previous studies (Rahighi et al., 2009; Hadian et al., 2011). Similar pull-down assays with Lys48- and Lys63-linked, and linear di- and tetra-Ub chains have shown that NEMO UBAN binds selectively to linear Ub chains (Rahighi et al., 2009; Komander et al., 2009a). Hadian and colleagues (2011) demonstrated that UBAN-ZF interacts with linear tetra-Ub, but not Lys63-linked tetra-Ub (Hadian et al., 2011).

Furthermore, Yoshikawa et al. (2009) showed high binding affinity of the UBAN domain of NEMO with linear di-Ub compared to Lys63-linked polyUb (Yoshikawa et al., 2009). In a competitive binding study using linear and Lys63-linked polyUb, NEMO was found to prefer linear Ub even when increased concentrations or length of Lys63-linked polyUb was used (Kensche et al., 2012).

Figure 3.17

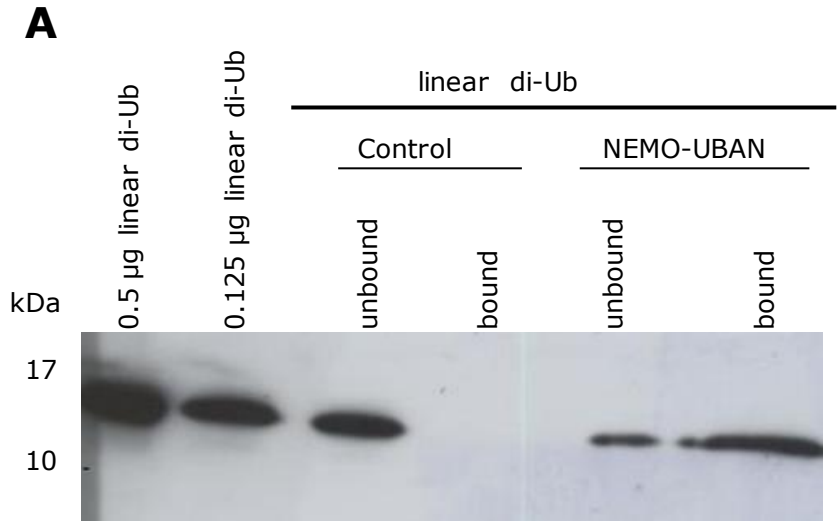


Figure 3.17

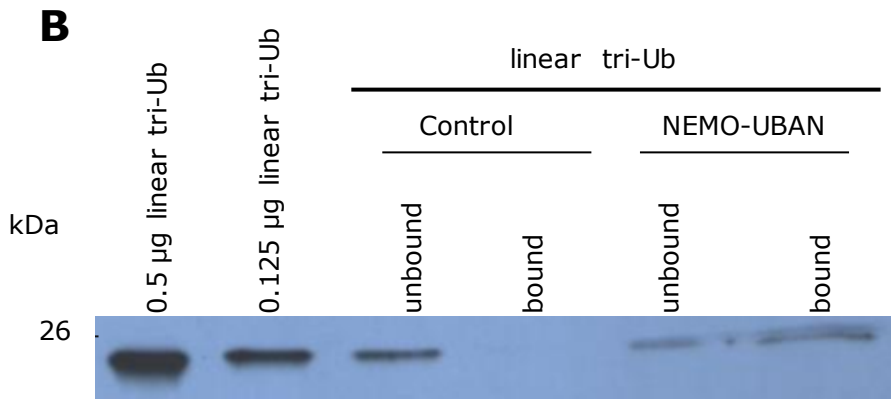


Figure 3.17

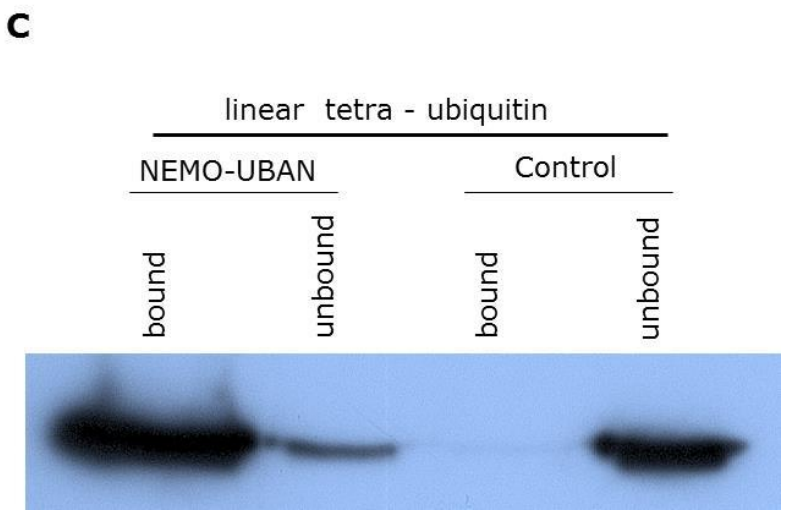


Figure 3.17 NEMO UBAN binds linear polyUb chains. Anti-ubiquitin immunoblots showing NEMO UBAN binding to linear di- (A), tri- (B) and tetra-Ub (C). Bound = Ub captured by control or NEMO UBAN beads. Unbound = buffer collected following incubation of control or NEMO UBAN beads with Ub. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.5.4 Binding interaction of NEMO UBAN with $Ub5^{+1}$ polyubiquitin chains

To investigate the binding of NEMO UBAN to substrate-conjugated Ub (anchored), NEMO UBAN and control CNBr beads were incubated with 0.5 µg of commercial $Ub5^{+1}$ as described in section 3.2.1.5. Figure 3.18 shows that the UBAN domain of NEMO does bind to $Ub5^{+1}$. This result confirms my previous data which demonstrated that NEMO UBAN domain interacts only with linear polyUb chains. shows a. A previous study has suggested NEMO binds to polyubiquitinated RIP1 (Ea et al., 2006).

Figure 3.18

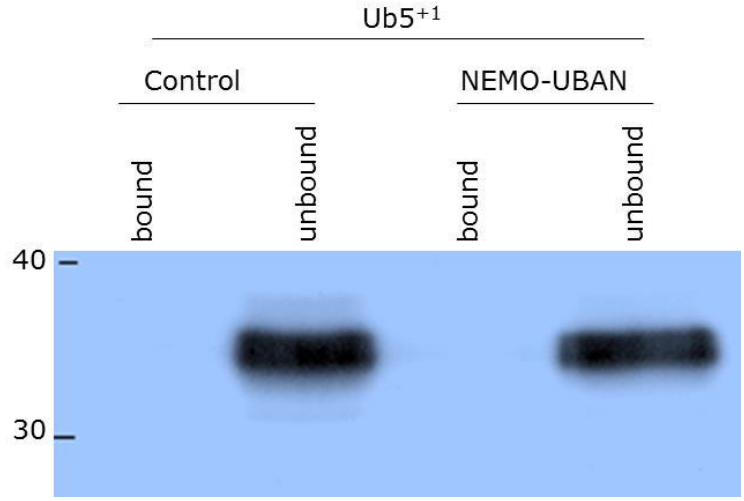


Figure 3.18 NEMO UBAN does not bind $Ub5^{+1}$ polyUb chains. Anti-ubiquitin immunoblot investigating NEMO UBAN binding to $Ub5^{+1}$ polyUb. Bound = $Ub5^{+1}$ polyUb captured by control or NEMO UBAN beads. Unbound = buffer collected following incubation of control or NEMO UBAN beads with Ub.15% (w/v) acrylamide gel. This is representative of 3 independent experiments showing the same result.

3.2.5.5 NEMO UBAN summary

In section 3.2.5 I have shown that the UBAN domain of NEMO is selective for linear Ub chains.

3.2.6.1 Expression, purification and cleavage of GST-IsoT ZnFUBP fusion protein

GST-IsoT ZnFUBP fusion protein was expressed, purified and cleaved as described for the GST-p62-UBA fusion protein in section 3.2.1.1. Figure 3.19 shows the expressed GST-IsoT ZnFUBP fusion protein captured on GSH beads (~40 kDa; before cleavage). After incubation with thrombin, the cleaved GST (~23 kDa) is on GSH beads (Figure 3.19; after cleavage) and a band ~17 kDa band is the IsoT ZnFUBP protein (Figure 3.19; eluted fraction).

Figure 3.19

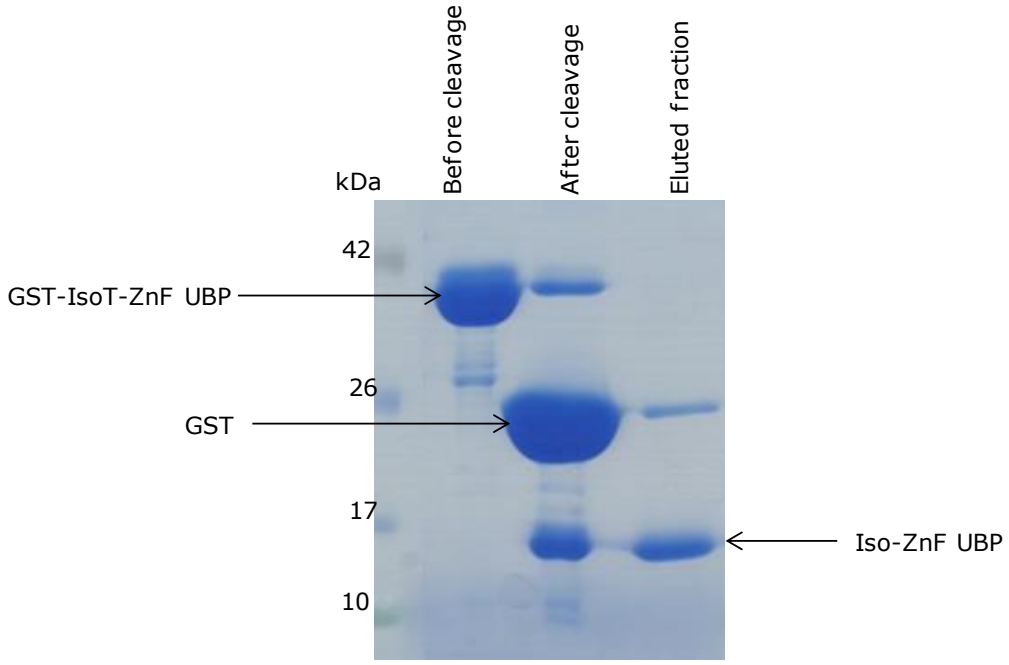


Figure 3.19 Coomassie blue stained gel showing purification and cleavage of the GST-IsoT ZnFUBP fusion protein. Before cleavage = GST-IsoT ZnFUBP fusion protein captured on GSH beads (GST-IsoT ZnFUBP; ~28 kDa). After cleavage = GST protein on GSH beads after incubation of GST-IsoT ZnFUBP-bound GSH beads with thrombin (GST; ~23 kDa). Eluted fraction = buffer after incubation of GST-IsoT ZnFUBP-bound GSH beads with thrombin (IsoT ZnFUBP; < 17 kDa). 5-20% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.6.2. Binding interactions of IsoT ZnFUBP with unanchored monoUb, Lys48- and Lys63-linked polyUb chains

IsoT ZnFUBP protein domain (section 3.2.6.1) was immobilised on CNBr beads to investigate its binding to monoUb, Lys48- and Lys63-linked polyUb chains as described in section 3.2.1.3. IsoT ZnFUBP and control beads were incubated with 1 µg of commercial monoUb, Lys48- or Lys63-linked polyUb chains. Buffer containing unbound Ub was collected and the bound proteins were eluted with gel application buffer. Samples were electrophoresed on a 5-15% (w/v) acrylamide gradient gel and visualised by Ub Western blotting. Figure 3.20 shows the interaction of IsoT ZnFUBP with unanchored (free) monoUb, Lys48- and Lys63-linked polyUb chains.

In vitro binding studies with ZnFUBP domains from different UBPs, including IsoT and HDAC6, have shown that ZnFUBP binds selectively to unanchored monoUb and polyUb chains using the free C-terminal residues of Ub (Reyes-Turcu et al., 2006;Pai et al., 2007; Ouyang et al., 2012; Strachan et al., 2012). In a study using the ZnFUBP domain and Ub labelled with a thio-reactive fluorescent dye (IA-Ub), the authors were able to show that monoUb, but not mutant Ub lacking the C-terminal di-glycine, competed with IA-Ub for ZnFUBP binding (Reyes-Turcu et al., 2006).

Figure 3.20

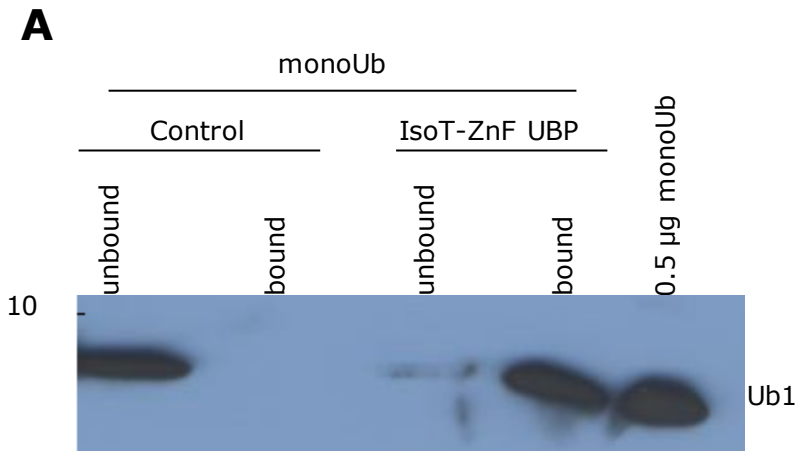


Figure 3.20

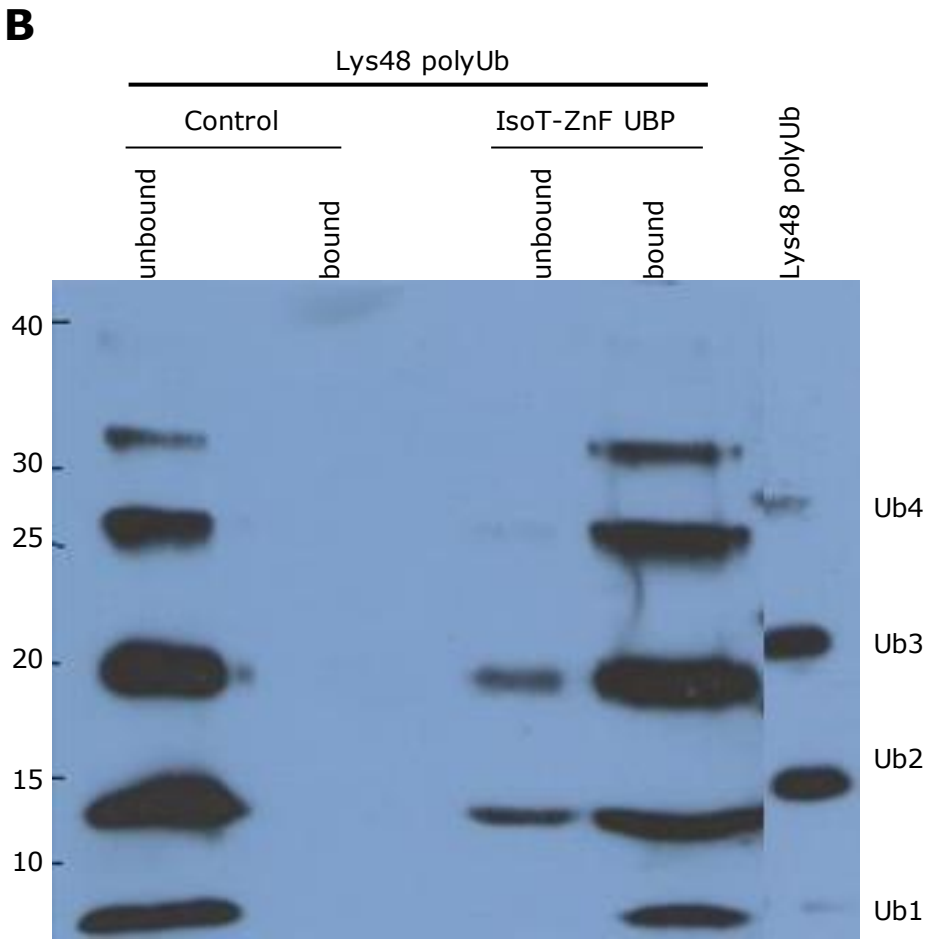


Figure 3.20

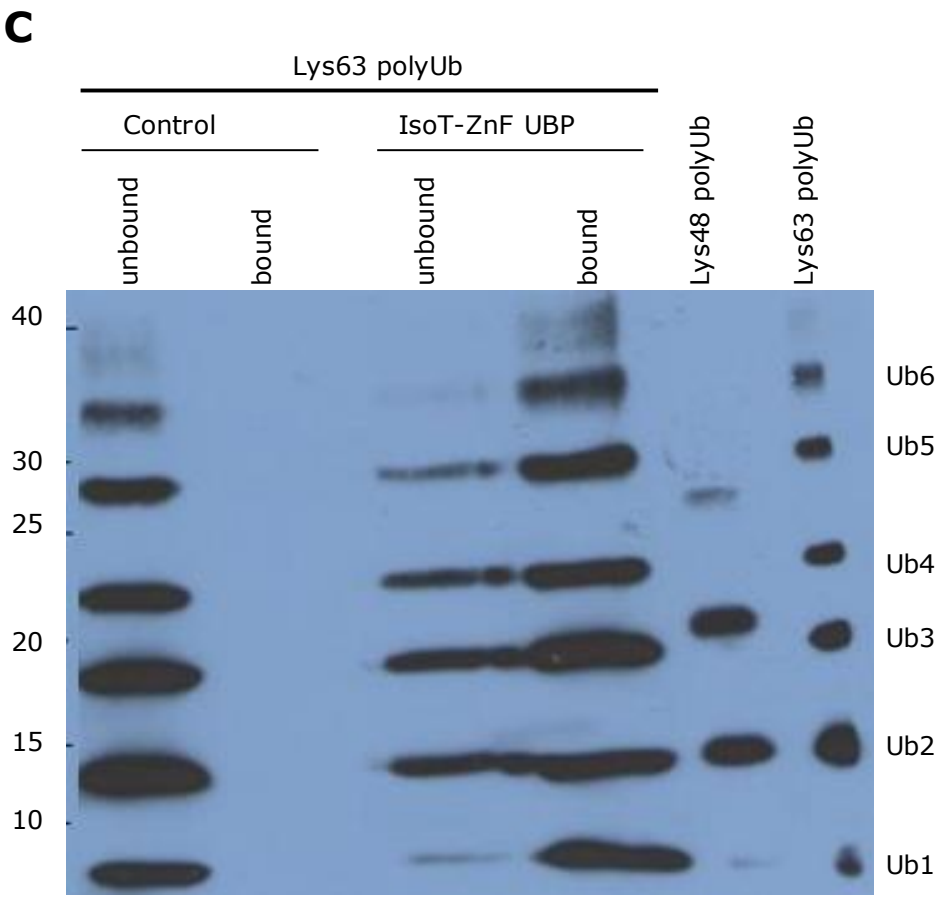


Figure 3.20 IsoT ZnFUBP binds monoUb, Lys48- and Lys63-linked polyUb chains. Anti-ubiquitin immunoblots investigating IsoT ZnFUBP binding to monoUb (A), Lys48- (B) and Lys63-linked (C) polyUb. Bound = Ub captured by control beads or IsoT ZnFUBP beads. Unbound = buffer collected following incubation of control or IsoT ZnFUBP beads with Ub. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.6.3 Binding interactions of IsoT ZnFUBP with linear di-Ub, tri-Ub and tetra-Ub polyUb

I investigated the binding of IsoT ZnFUBP to 1 µg of commercial linear di-, tri- or tetra-polyUb chains. As expected, IsoT ZnFUBP bound to linear polyUb chains, which contain free C-terminal Ub residues (Figure 3.21). Recently Kasier and colleagues (2011) used the IsoT ZnFUBP domain to capture endogenous unanchored Ub (Kaiser et al., 2011).

Figure 3.21

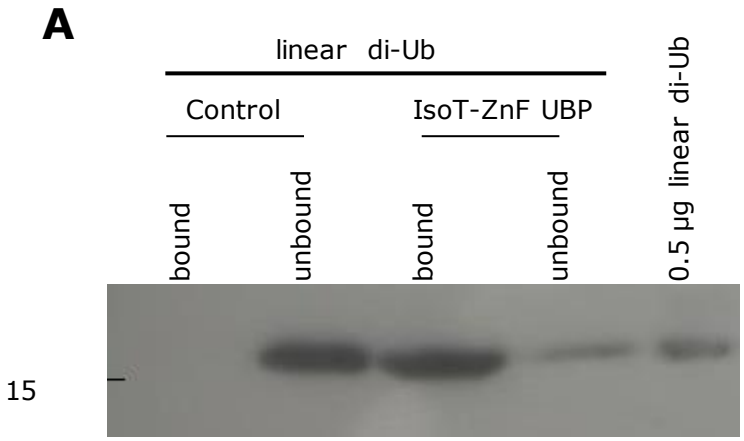


Figure 3.21

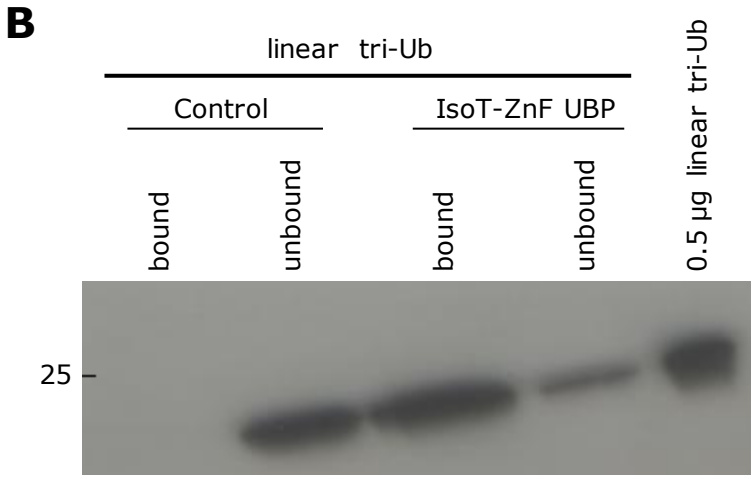


Figure 3.21

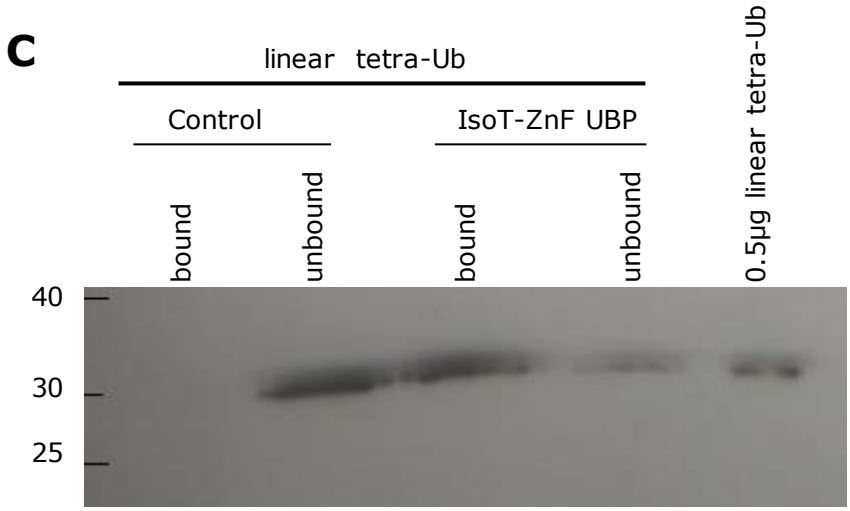


Figure 3.21 IsoT ZnFUBP binds linear polyUb chains. Anti-ubiquitin immunoblots showing IsoT ZnFUBP binding to linear di- (A), tri- (B) and tetra-Ub (C). Bound = Ub captured by control or IsoT ZnFUBP beads. Unbound = buffer collected following incubation of control or IsoT ZnFUBP beads with Ub. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

3.2.6.4 Binding interaction of IsoT ZnFUBP with $Ub5^{+1}$ polyubiquitin chains

To investigate the binding of IsoT ZnFUBP to anchored Ub, IsoT ZnFUBP and control CNBr beads were incubated with 0.5 µg of commercial $Ub5^{+1}$ as described in section 3.2.1.5. Ub immunoblotting showed that the IsoT ZnFUBP domain did not bind to $Ub5^{+1}$ (Figure 3.22). This is consistent with previous studies showing that the ZnFUBP domain of IsoT requires the free C-terminal residues of Ub for interaction (Reyes-Turcu et al., 2006; Pai et al., 2007; Ouyang et al., 2012; Strachan et al., 2012). This result indicates that the IsoT ZnFUBP domain does not interact with mono- or poly-ubiquitinated proteins, i.e. substrate conjugated Ub.

Using fluorescence anisotropy experiments, Reyes-Turcu et al. (2006) also demonstrated that Ub lacking the free C-terminal end did not bind to IsoT ZnFUBP (Reyes-Turcu et al., 2006). The ZnFUBP domain from HDAC lost its binding interactions with Ub when the C-terminal of Ub was mutated (Pai et al., 2007).

Figure 3.21

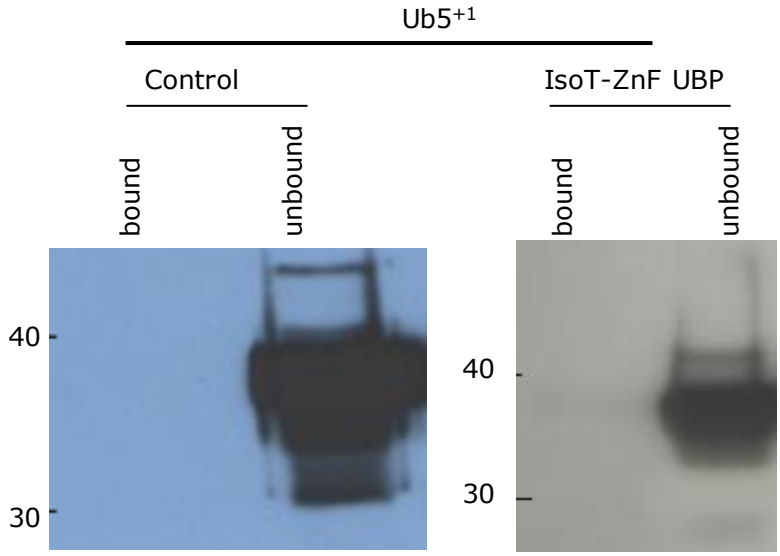


Figure 3.21 IsoT ZnFUBP does not bind $Ub5^{+1}$ polyUb chains. Anti-ubiquitin immunoblot investigating IsoT ZnFUBP binding to $Ub5^{+1}$ polyUb. Bound = $Ub5^{+1}$ polyUb captured by control or IsoT ZnFUBP beads. Unbound = buffer collected following incubation of control or IsoT ZnFUBP beads with Ub. 15% (w/v) acrylamide gel. This is representative of 3 independent experiments showing the same result.

3.2.6.5 IsoT ZnFUBP summary

As described in previous studies, the IsoT ZnFUBP domain specifically interacts with the C-terminal end of Ub. Therefore, this domain is only able to bind unanchored (free) mono- or poly-Ub of different linkages.

3.3 Discussion

Table 2. Summary of the binding specificity of UBDs to synthetic free monoUb, unanchored and anchored polyUb chains.

Domains	Mono-Ub	Lys48-polyUb (2-7)	Lys63-polyUb (2-7)	Linear -Ub_2	Linear -Ub_3	Linear -Ub_4	$Ub5^{+1}$	Binding specificity
p62 UBA	-Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	Linkage independent
UQ1 UBA	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	Linkage independent
Vsp9 CUE	-Ve	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	Linkage independent
NEMO UBAN	-Ve	-Ve	-Ve	+Ve	+Ve	+Ve	-Ve	Only binds linear polyUb
IsoT ZnFUBP	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve	-Ve	Only binds unanchored Ub

UBDs that show binding specificity to ubiquitin linkages or unanchored ubiquitin represent a promising tool for investigating Ub and ubiquitinated proteins in ubiquitome analyses. To date, more than 16 families of UBDs have been described with a diversity of functions and structures. First, I compared Ub binding characteristics of the five UBDs listed in Table 2 to different lengths and linkages of Ub. Interestingly, all of the UBDs were capable of binding linear polyUb chains under the experimental conditions employed for this study, whereas only UQ1 UBA and IsoT ZnFUBP domains bound monoUb.

The UBDs domains used in this study can be sorted into three classes based on their ability to bind structurally distinct unanchored and anchored Ub. First class; p62 UBA, UQ1 UBA and Vps9 CUE domains bind non-selectively to unanchored and anchored polyUb chains. The second and third classes; NEMO UBAN and IsoT ZnFUBP domains bind selectively to linear polyUb chains and unanchored Ub respectively.

3.3.1 p62 UBA Ub binding interactions

The p62 protein was identified as phosphotyrosine-independent ligand of the src homology 2 (SH2) domain of p56. p62 is known to play a role in several cellular functions, including gene transcription and protein degradation (Babu et al., 2005; Ciani et al., 2003; Moscat, Diaz-Meco, & Wooten, 2007b; Pankiv et al., 2010).

Studies have shown that p62 is involved in inclusion body formation and clearance of ubiquitinated substrates via autophagy (Kirkin, et al., 2009; Johansen & Lamark, 2011). The p62 UBA domain not only recognizes Lys63 polyubiquitinated proteins targeted for autophagic degradation, but also Lys48 polyubiquitin chains associated with proteasomal degradation, suggesting that p62 can deliver ubiquitinated proteins to both major pathways of protein degradation (Figure 3.22) (Benbrook & Long, 2012). Studies suggest that through these mechanisms p62 may compensate for impaired proteasome function.

Figure 3.22

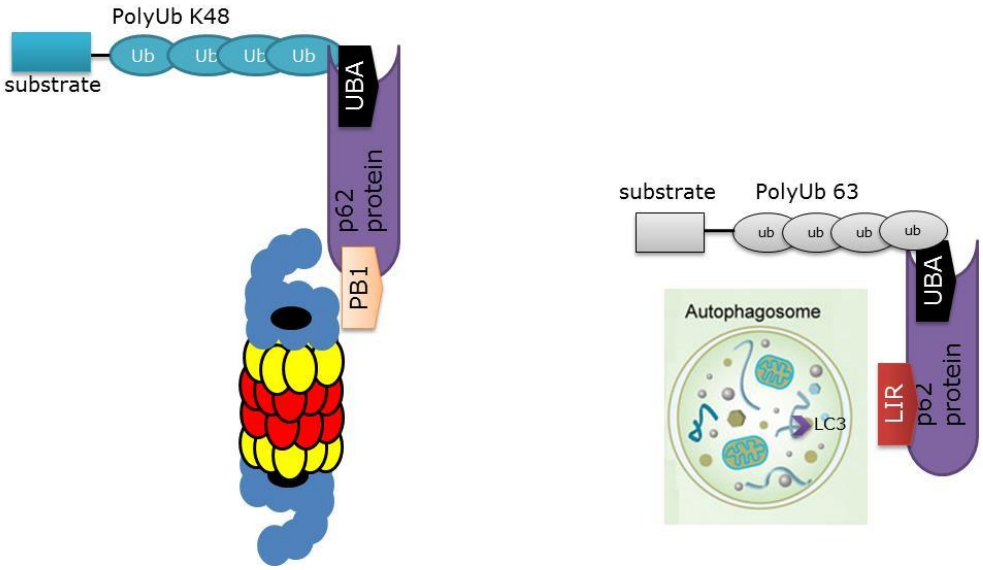


Figure 3.22 p62 can shuttle ubiquitinated proteins to both the UPS and autophagy systems via binding to Lys48- or Lys63-polyubiquinated proteins respectively.

The p62 protein contains a number of domains involved in protein-protein interactions; an acid interaction motif (PBI) binds to atypical protein kinase Cs (aPKCs); zz a zinc finger can bind to the RING (really interesting new gene) finger protein TRAF6; two PEST (Proline, Glutamate, Serine and Threonine) sequences and the UBA domain (Geetha & Wooten, 2002; Seibenhener et al., 2004; Wooten et al., 2005).

The Ub-binding UBA domain of p62 is at the C-terminal between residues 387-436 (Vadlamudi et al, 1996). NMR chemical shift mapping shows binding between a conserved hydrophobic epitope (mainly Met404, Gly405, Phe406 and Ser407) within the p62 UBA domain and the hydrophobic patch (mainly Leu8, Ile44 and Val70) on the β-sheet of Ub (Long et al., 2008).

Here I have shown that the UBA domain of p62 binds diverse polyUb chains, but not monoUb. This is consistent with previous studies showing that the p62 UBA domain does not bind or has low binding affinity to monoUb compared to polyUb (Wilkinson et al., 2001; Ciani et al., 2003; Raasi et al., 2004; Cavey et al., 2005; Long et al., 2008). Variations in the binding interactions of p62 UBA with Ub may be explained by different methods used, i.e. affinity chromatography compared to NMR spectroscopy. A study investigating the Ub-UBA binding interactions of Mud1(Mud1 is an ortholog of the Saccharomyces Cerevisiae DNA-damage response protein Ddi1) showed that the Mud1 UBA bound 300 times stronger to tetra-polyUb than monoUb, suggesting that this domain does not bind monoUb (Wilkinson et al., 2001).

I have shown that the p62 UBA domain binds unanchored (free) and anchored polyUb chains. This is supported by previous studies showing binding of the p62-UBA domain to Lys48- and Lys63-linked polyUb (Seibenhener et al., 2004; Long et al., 2008; Tan et al., 2008). In addition, several studies have shown that the p62 UBA domain has higher affinity for Lys63-linked polyUb (Seibenhener et al., 2004; Long et al., 2008; Tan et al., 2008). In contrast, the UBA1 domain of hHR23A (human homolog of Saccharomyces Cerevisiae Rad23) shows preferential binding to Lys48-linked than Lys63-linked polyUb chains (Raasi et al., 2004).

Although a recent study has shown that the UBA domain of XIAB protein binds linear di-Ub chains (Tse et al, 2011), my results are the first demonstration that the p62 UBA binds with diverse linear polyUb chains. I also showed that the UBA domain of p62 has the ability to bind anchored ubiquitin using the $Ub5^{+1}$ model, supported by previous studies (Pridgeon et al., 2003; Seibenhener et al., 2004).

The p62 UBA domain was used to bind and identify ubiquitinated proteins from rabbit reticulocyte lysate following induction of *in vitro* ubiquitination (Pridgeon et al., 2003). An *in vivo* investigation of the binding of full-length p62 protein and p62 minus the UBA domain demonstrated that this domain was essential for the interaction of p62 with polyUb (Seibenhener et al., 2004).

I conclude that the UBA domain of p62 binds polyUb chains in a non-selective manner; binding Lys-, linear- and anchored-linked polyUb. This is in agreement with a study by Raasi and colleagues (2005) showing p62 UBA binds non-selectively to polyUb (Raasi et al., 2005).

3.3.2 UQ1 UBA Ub binding interactions

The results show that the UQ1 UBA domain binds non-selectively to Ub, including monoUb, free Lys- and linear-polyUb chains, and a model of substrate-conjugated polyUb ($Ub5^{+1}$).

The UBA domains of UQ1, IsoT and Dsk2 have shown high binding affinity with monoUb previously (Raasi et al., 2005). The UQ1 UBA domain binds both mono- and poly-Ub, and is considered to be one of the strongest UBDs (Hjerpe & Rodriguez, 2008; Zhang et al., 2008).

UBA domains had been classified into four classes according to their binding affinity to polyUb chains; the first class interacts with Lys48 tetra-Ub (hHR23A UBA2); the second class with Lys63 tetra-Ub (E2-25K UBA and Drm2 UBA); the third class is UBA domains that do not bind any polyUb chains tested with (IsoT UBA and c-Cbl UBA) and the fourth class interacts with all polyUb chains tested (IsoT UBA2, cbl-b UBA and UQ1 UBA) (Raasi et al., 2005).

I have shown that the UBA domains of p62 and UQ1 bind non-selectively with unanchored and anchored polyUb chains. The UQ1 UBA is also interacts with monoUb, unlike p62 UBA. Therefore, the p62 UBA domain could be used as a tool for the capture of endogenous poly-Ub (> Ub2), whereas UQ1 UBA would interact with any Ub topology.

3.3.3 Vsp9 CUE Ub binding interactions

CUE domains have been found in a number of yeast and mammalian proteins, e.g. the yeast Vsp9 protein (Polo et al., 2002; Shih et al., 2003). Vsp9 is involved in the regulation of signal transduction pathways and receptors endocytosis (Davies et al., 2003; Donaldson et al., 2003).

Structure analysis of co-crystallized Vsp9 CUE domain with Ub showed similarities to the UBA domain, as well as the structure of the CUE-Ub complex (Kang et al., 2003; Prag et al., 2003).

In my studies, I found that the Vsp9 CUE domain interacts with synthetic unanchored and anchored polyUb chains, but not monoUb.

Earlier reports have shown that CUE domains containing the MFP (Methionine, Phenylalanine, and Proline) sequence bind well to mono- and poly-Ub, including the CUE domain of Vsp9, which interacted with monoUb and polyUb *in vitro* (Davies et al., 2003; Shih et al., 2003). The Cue2 protein has two independent Ub-binding CUE domains; both domains contain the MF sequence and can interact with monoUb (Kang et al., 2003). CUE domains that lack the MF sequence, e.g. Cue1 CUE, bind weakly with monoUb (Shih et al., 2003). In contrast to these studies, I did not detect binding of monoUb with Vsp9 CUE, containing the MF sequence. This may be explained by different methods that have been used for the study of Ub binding with the CUE domain of Vsp9, e.g. the use of GST-Ub (Kang et al., 2003).

3.3.4 NEMO UBAN shows specificity for linear Ub chains

The NF-kB pathway plays an important role in the regulation of gene expression during inflammatory and immune responses. Nuclear factors are retained in the cytosol in an inactive form by binding to NF-kB inhibitor (IkB). In response to stimulation, the IkB kinase (IKK) complex phosphorylates IkB (Hayden & Ghosh, 2008; Wertz & Dixit, 2010; Kensche et al., 2012). Phosphorylation of IkB leads to its Lys48-linked polyUb and degradation via the UPS. The IKK complex is composed of two catalytic subunits, IKKα and IKKβ, and one regulatory subunit, IKKγ or NF-kB essential modulator (NEMO) (Hayden & Ghosh, 2008; Kensche et al., 2012). NEMO contains two UBDs; UBAN (ubiquitin binding domain in ABIN and NEMO) and ZF (Laplantine et al., 2009). PolyUb chain binding by NEMO is important for the activation of the IKK complex in response to TNFα stimulation (Kensche et al., 2012).

NEMO is an X-linked gene and mutations in this gene have been associated with severe immunodeficiency caused by inappropriate activation of NF-kB (Makris et al,, 2002; Hubeau et al., 2011).

UBAN bound the distal Ub via its hydrophobic patch Ile44, whereas the proximal Ub uses residues located adjacent to the Ile44 patch. In addition to these hydrophobic interactions, hydrogen bonds and salt bridges contribute to the binding. An Asp304 residue of NEMO forms a salt bridge with Ub. Leu73 and Leu74 of the distal Ub formed a hydrogen bond. (Ivins et al., 2009; Lo et al., 2009; Hadian et al., 2011).

I did not detect an interaction between NEMO UBAN and monoUb in my studies. This is consistent with previous studies, which used different methods to investigate Ub-UBAN binding (Ivins et al., 2009; Lo et al., 2009; Hadian et al., 2011). However, Bloor and colleagues (2008) showed that the UBAN domain of NEMO interacted with monoUb using GST-Ub and Coomassie blue staining (Bloor et al., 2008). My methods may be more specific because I used antiubiquitin antibody.

Studies have suggested that the UBAN domain of NEMO binds Lys63-linked polyUb chains (Wu et al., 2006; Lo et al., 2009; Yoshikawa et al., 2009). Using crystallization, Yoshikawa and colleagues (2009) demonstrated binding between the UBAN domain and Lys63-Ub2 (Yoshikawa et al., 2009). A study comparing

I did not detect any interaction of NEMO UBAN with synthetic Lys63-linked polyUb. I also showed NEMO UBAN did not interact with Lys48-linked polyUb, supporting earlier work (Komander et al., 2009a; Rahighi et al., 2009).

I found that the UBAN domain of NEMO shows specificity for linear polyUb chains. This is in agreement with a study by Rahighi et al. (2009). Using a pull-down assay they showed the UBAN domain interacted with linear di-Ub, but not Lys48- and Lys64-linked di-Ub, and this was supported by surface plasmon resonance results. However, incubation of NEMO with high molecular weight Lys63-linked polyUb showed binding, but it was unclear whether this was a specific or non-specific interaction. The length and concentration of Lys63-linked polyUb chains involved in binding studies may affect the binding to UBDs (Rahighi et al., 2009).

3.3.5 IsoT ZnF UBP shows specificity for free Ub

ZnFUBP domains have been found in a number of cellular proteins, including Isopeptidase T (IsoT) and histone deacetylase 6 (HDAC6) (Ouyang et al., 2012). IsoT is also known as ubiquitin-specific peptidase 5 (USP5) and is a deubiquitinating enzyme that specifically cleaves unanchored (free) polyUb chains (Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008).

The ZnFUBP domain of IsoT recognizes the intact C-terminus of mono- or the proximal-Ub in a chain, and for that reason explains the specificity of IsoT to unanchored Ub (Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008).

Disassembly of unanchored polyUb is an essential process, preventing their accumulation, which may interfere with the degradation of ubiquitinated proteins via the UPS (Dayal et al., 2009) . IsoT has four UBDs; a ZnFUBP; a UBP, which forms the active site; and two UBA domains, whose functions are unknown (Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008). A recent report has suggested that IsoT has two ZnFUBP domains (Avvakumov et al., 2012).

Inhibition of IsoT leads to the accumulation of unanchored polyUb chains and activation of p53 (Dayal et al., 2009). Studies have suggested that the ZnFUBP domain is one of the strongest UBDs (Grabbe & Dikic, 2009).

A crystal structure study of ZnFUBP binding with Ub demonstrated that the classical hydrophobic patch of Ub (residues I44 and V70) does not interact with the ZnFUBP domain. The ZnFUBP N-terminal residues (173-196) are involved in the interaction with the C-terminal of Ub (L71 to G76), which accommodates the ZnFUBP hydrophobic pocket (Reyes-Turcu et al., 2006).

My work supports earlier studies of the ZnFUBP domain showing binding interactions with diverse types of free Ub, including synthetic monoUb, Lys48-, Lys63- and linear-linked polyUb chains, but not $Ub5^{+1}$, a C-terminal mutant Ub which models ubiquitinated proteins.

Binding interactions between IsoT ZnFUBP and free monoUb have been described in earlier studies (Reyes-Turcu et al., 2006; Pai et al., 2007; Ouyang et al., 2012; Strachan et al., 2012). Also, the ZnFUBP domain of HDAC6 binds monoUb (Pai et al., 2007; Ouyang et al., 2012). Two groups have shown that IsoT ZnFUBP interacts with polyUb chains, and this domain has recently been used to pull-down native unanchored polyUb (Pai et al., 2007; Strachan et al., 2012).

The inability of IsoT ZnFUBP to capture $Ub5^{+1}$ confirms that the domain interacts with the free C-terminal of Ub. Further work has demonstrated that ZnFUBP

domains bind only to the unanchored C-terminal end of Ub using Ub mutants with modified C-terminals (Reyes-Turcu et al., 2006; Pai et al., 2007; Ouyang et al., 2012; Strachan et al., 2012). Similar studies investigating the Ub-binding of HDAC6 ZnFUBP using Ub mutant proteins; G76A and G75A_G76A mutations or deletion of one or two C-terminal glycines, showed that the HDAC6 ZnFUBP also binds only to free Ub (Ouyang et al., 2012).

In conclusion, ZnFUBP domains show high binding affinity to all unanchored Ub topologies, including monoUb, Lys- and linear-polyUb chains. This domain will not interact with ubiquitinated proteins where the C-terminal residues are involved in binding to the protein substrate.

Summary

Several groups have suggested that UBDs may have Ub linkage selectivity, e.g. the UBA domain of Mud 1 binds Lys48-, but not Lys63- and linear-linked chains (Komanderet al., 2009a); and Lys63-linked polyUb chain specificity of the UBA domain of E2-25K (Raasi et al., 2005).

In this study, p62 UBA, Vps9 CUE and NEMO UNAN domains showed no binding to monoUb, whereas UQ1 UBA and IsoT ZnFUBP domains bound to monoUb. I also found that the UBDs in this study showed some differences in their binding selectivity to Ub. The UQ1 UBA domain bound to various linkages and lengths of Ub, including monoUb. This domain is known to be one of the highest affinity UBDs, but it lacks linkage selectivity. p62 UBA and Vsp9 CUE domains shared common binding characteristics; binding various Ub linkages, but not monoUb.

The UBAN of NEMO and ZnFUBP of IsoT domains showed some Ub binding selectivity. NEMO UBAN binds only to linear polyUb chains. The IsoT ZnFUBP domain only bound to unanchored (free) Ub, including monoUb and polyUb chains.

These results suggest that the p62 and UQ1 UBA domains and the Vps9 CUE domain sharing the same binding characteristics to polyUb chains. This suggests that these domains would be useful tools for pulling down all polyUb proteins in tissue homogenates when investigating the *in vivo* ubiquitome. The IsoT ZnFUBP and NEMO UBAN domains can be used to capture native unanchored Ub and linear polyUb chains respectively. These results indicate UBDs may be used to capture and purified diverse or specific Ub topology from tissue. The NEMO UBAN and IsoT ZnFUBP domains will be used as tools to investigate the abundance of

endogenous linear and unanchored Ub, respectively, in the mouse brain *in vivo* in the next chapter.

CHAPTER 4

Expression of unanchored polyubiquitin chains in the mouse brain

4.1 Introduction

Aggregation of abnormal proteins is a common feature of several neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (Huang & Figueiredo-Pereira, 2010). The UPS plays an important role in preventing the accumulation of these proteins. UPS dysfunction may lead to the accumulation of Ub and ubiquitinated proteins (Ciechanover & Brundin, 2003).

Increasing studies are taking an interest in unanchored polyubiquitin chains. These chains could be potential regulators of ubiquitin-signaling processes. Recently, two groups have shown that unanchored polyUb chains can directly activate innate immunity (Xia et al., 2009; Zeng et al., 2010). In a cell there is free ubiquitin that is not conjugated to a protein substrate; monoUb is single unanchored Ub and unanchored polyUb chains can be synthesized or released from polyUb proteins by the action of deubiquitinating enzymes (Dayal et al., 2009). IsoT plays an important role in unanchored polyUb chains levels (Reyes-Turcu et al., 2009). A number of studies; have shown that accumulation of unanchored polyUb chains competes with polyUb proteins for binding to the proteasome, which causes inhibition of polyUb proteins degradation (Amerik et al., 1997; Piotrowski et al., 1997; Dayal et al., 2009). Therefore, unanchored polyUb chains may act as a negative regulator of proteolysis by compete with polyubiquitinated proteins for binding to the proteasome (Piotrowski et al., 1997).Further studies are needed to investigate whether unanchored polyUb chains are involved in the regulation of other physiological functions.

In vitro experiments have indicated that the Ub conjugating enzyme E2-25K plays an important role in the synthesis of Lys48-linked unanchored polyUb chains (Chen & Pickart, 1990; Chen et al.,1991).

The level of unanchored polyUb chains is controlled by the action of Isopeptidase T (IsoT/USP5), a DUB enzyme that preferably cleaves unanchored polyUb chains (Reyes-Turcu et al., 2009). IsoT has four UBDs, two UBA domains, a UBP domain and ZnFUBP domain. The ZnFUBP domain recognizes the intact C-terminus of proximal Ub, and for that reason it can explain the specificity of IsoT to unanchored chains (Reyes-Turcu et al., 2006; Reyes-Turcu et al., 2008).

Previous work in our laboratory demonstrated significantly increased ubiquitinated proteins in the cortex of the Psmc1$^{fl/fl}$, CaMKIIα-Cre mice compared to control mice (Bedford et al., 2008). It is possible that accumulation of unanchored polyUb chains following 26S proteasome function in this mouse model further inhibits proteasome activity in the brain.

The aim of this chapter was to investigate the expression of unanchored polyUb chains in different sub-cellular fractions of the mouse brain using the IsoT ZnFUBP affinity matrices in Ub pull-down assays, which was shown to be selective for unanchored chains in Chapter 3. The expression of unanchored Ub chains in the control and conditional 26S proteasome knockout mouse will be investigated (Psmc1$^{fl/fl}$;CaMKIIα-Cre or 26S KO) (Bedford et al., 2008). The expression of several enzymes, namely E2-25K, involved in unanchored Ub synthesis, IsoT involved in deubiquitination, and p-TAK1, which is involved in the activation of NF-$_k$B by unanchored polyUb chains, will be investigated.

4.2 Results

4.2.1 Solubility of ubiquitin

Because the aim of this work was to investigate Ub topology in the mouse brain, I first examined the solubility of Ub and ubiquitinated proteins in the control and Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mouse cortex using different homogenizing buffers (Figure 4.1). Cortices from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mice were homogenized in ice-cold homogenizing buffer containing NP-40, SDS or Urea (see section 2.2.8.2). The proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and subjected to Ub Western blotting. Figure 4.1 shows the solubility of ubiquitinated proteins in different buffers. Most ubiquitinated proteins were observed using buffer containing SDS, then urea and then NP40. As expected, there were increased levels of ubiquitinated proteins in the Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortex compared to the control. Figure 4.1 also shows potential unanchored polyUb chains in the mouse cortex (below 60 kDa), which are increased in the Psmc1$^{fl/fl}$, CaMKIIα-Cre compared to the control. Binding experiments used cortex homogenate in SDS buffer failed to bind to the UBDs beads and this due the inhibitory effect of SDS, while the cortex homogenate in NP-40 buffer bound to UBDs. For that reason the NP-40 homogenate buffer were used.

Figure 4.1

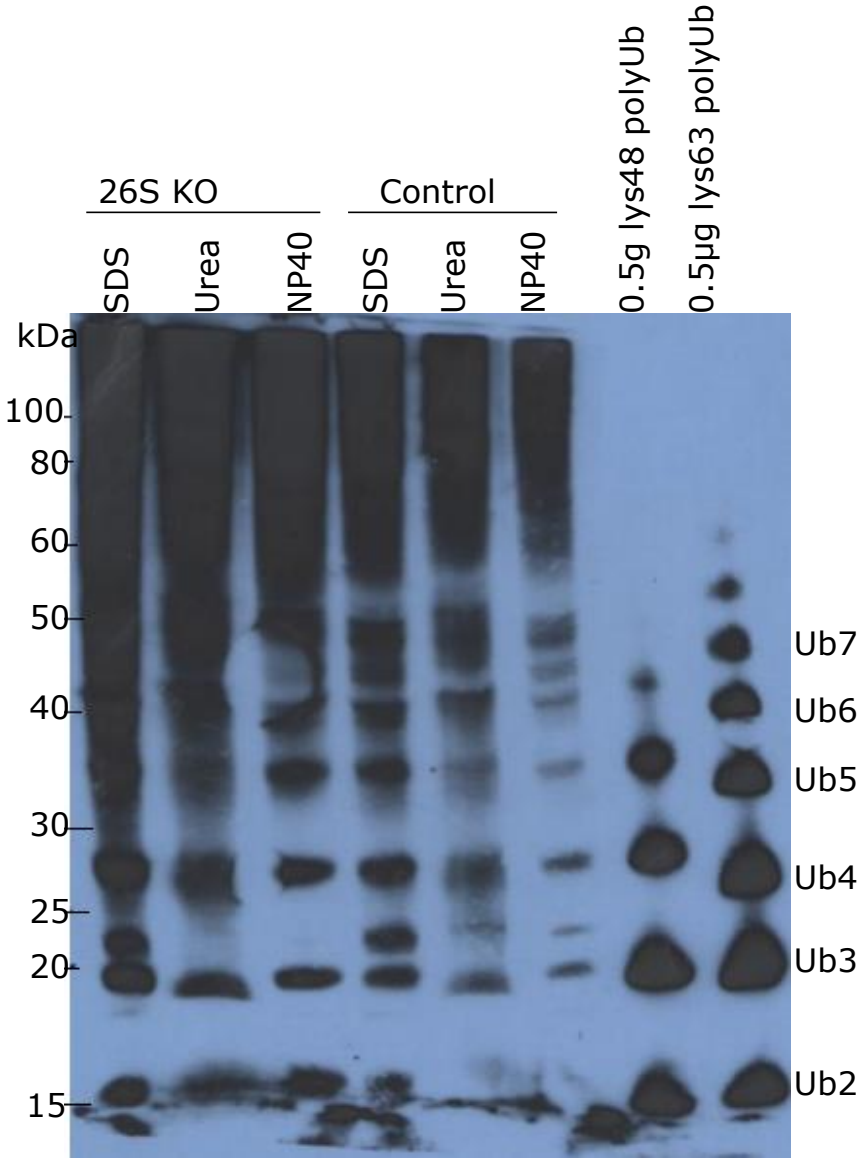


Figure 4.1 Differential solubility of ubiquitinated proteins in mouse cortex. Anti-ubiquitin immunoblot investigating the solubility of ubiquitinated proteins in different buffers containing SDS, urea and NP-40. The highest levels of ubiquitinated proteins were observed using buffer containing SDS. Increased levels of ubiquitin topology were evident in the Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mouse cortex compared to the control. 5-20% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.2 Pull-down of endogenous ubiquitin by UBDs

Control mouse cortices were homogenised in TBS-NP40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP40) containing protease inhibitors and incubated with the different UBDs as indicated in Figure 4.2 and investigated in Chapter 3. Binding was visualized by Ub Western blotting.

I previously showed that the Vsp9 CUE domain and UBA domains of p62 and UQ1 bound to anchored polyUb and polyUb chains (section 3.2). Figure 4.2 shows that the p62 UBA, UQ1 UBA and Vsp9 CUE domains also bind endogenous ubiquitinated substrates and polyUb chains, but not monoUb from the mouse cortex (Figure 4.2A-C). In section 3.2.5 I showed that the NEMO UBAN domain recognized only linear polyUb chains. I did not detect any linear polyUb chains in the control mouse cortex using this domain (Figure 4.2D). This may be due to their relatively low abundance *in vivo*.

The IsoT ZnFUBP domain recognizes only unanchored Ub (section 3.2.6). Figure 4.2E shows that the ZnFUBP domain of IsoT binds endogenous monoUb and polyUb chains. This result indicates that endogenous unanchored polyUb chains are found in the mouse brain cortex.

Figure 4.2

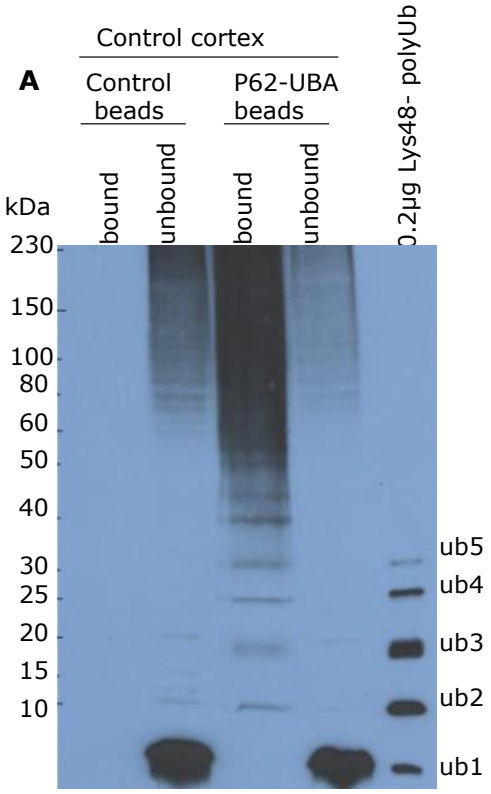


Figure 4.2

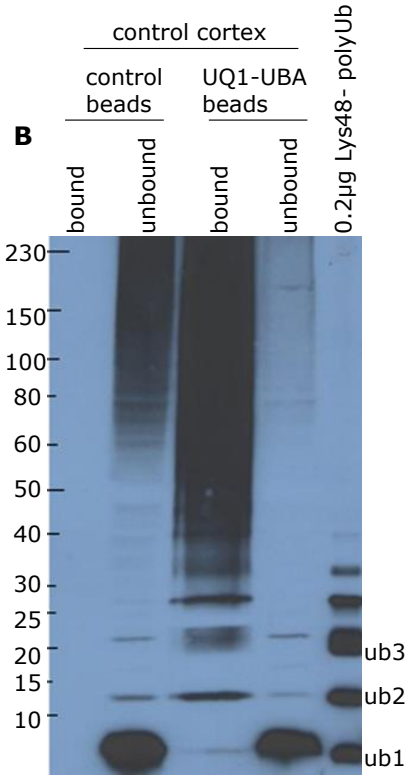


Figure 4.2

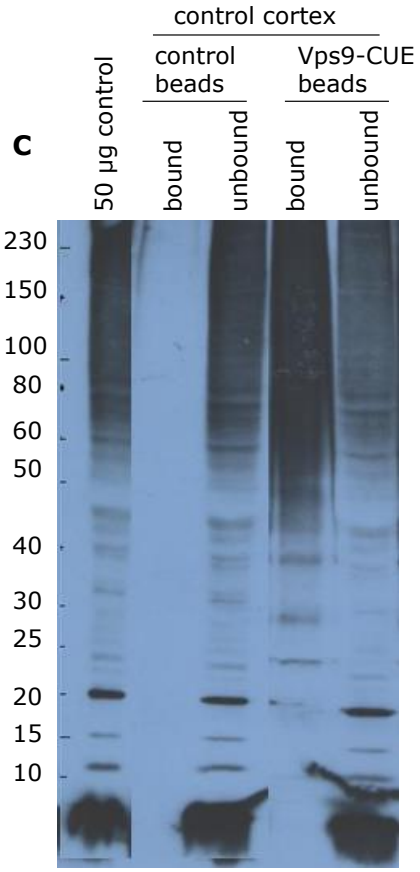


Figure 4.2

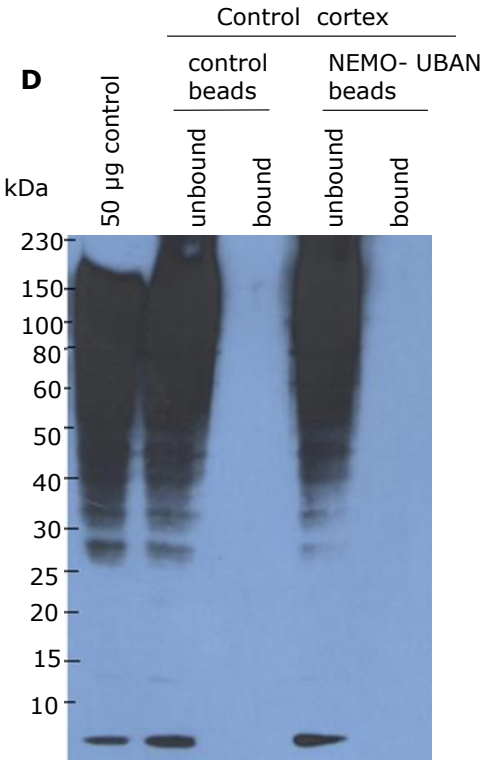


Figure 4.2

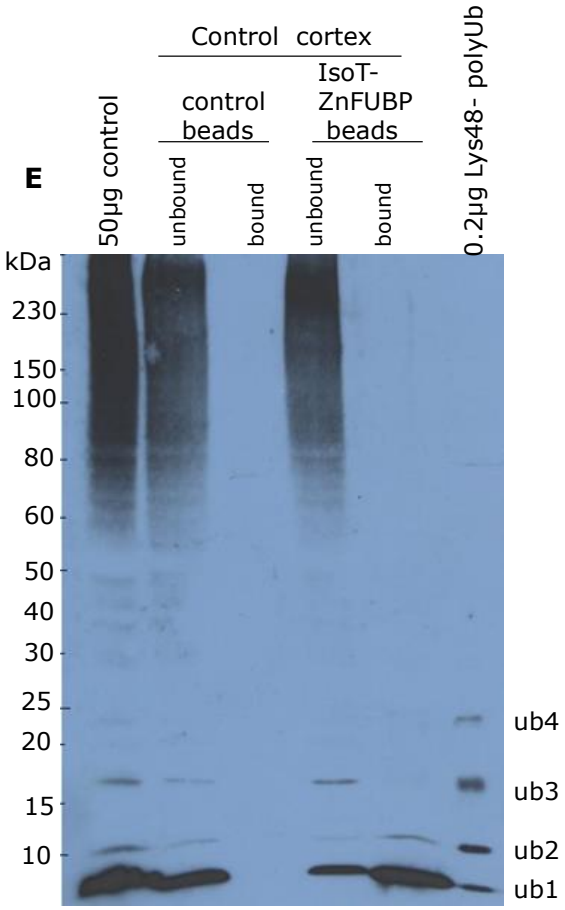


Figure 4.2 Anti-ubiquitin immunoblots investigating p62 UBA (A), UQ1 UBA (B), Vsp9 CUE (C), NEMO UBAN (D) and IsoT ZnFUBP (E) binding to endogenous ubiquitin from the mouse brain cortex. There was no binding with control beads. p62 UBA, UQ1 UBA and Vsp9 CUE bind to diverse ubiquitin species, but not monoUb. NEMO UBAN did not detect endogenous linear polyUb chains. IsoT ZnFUBP detected unanchored polyUb (Ub2) and monoUb (Ub1) in the mouse cortex. Bound = Ub captured by beads. Unbound = buffer collected following incubation of beads with mouse cortices. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.3 Optimisation for capture of endogenous unanchored ubiquitin by IsoT ZnFUBP

4.2.3.1 Increasing the input

Here I investigated the binding capacity of IsoT ZnFUBP beads using a constant amount of IsoT ZnFUBP beads (200 µl of 50% slurry) with increasing amounts of protein homogenate from mouse cortex (0.5 - 2.0 mg protein).

Anti-ubiquitin Western blotting showed that the control beads did not bind to Ub in mouse cortex homogenate (Figure 4.3A). Figure 4.3B and C show binding of the IsoT ZnFUBP domain to unanchored Ub, including monoUb and polyUb chains, and that binding increases with increasing protein input (compare Figure 4.3B and C; control cortex). In additon, I note there are more unanchored Ub chains in the Psmc1$^{fl/fl}$, CaMKIIα-Cre cortex (26S KO) (Figure 4.3B).

Figure 4.3

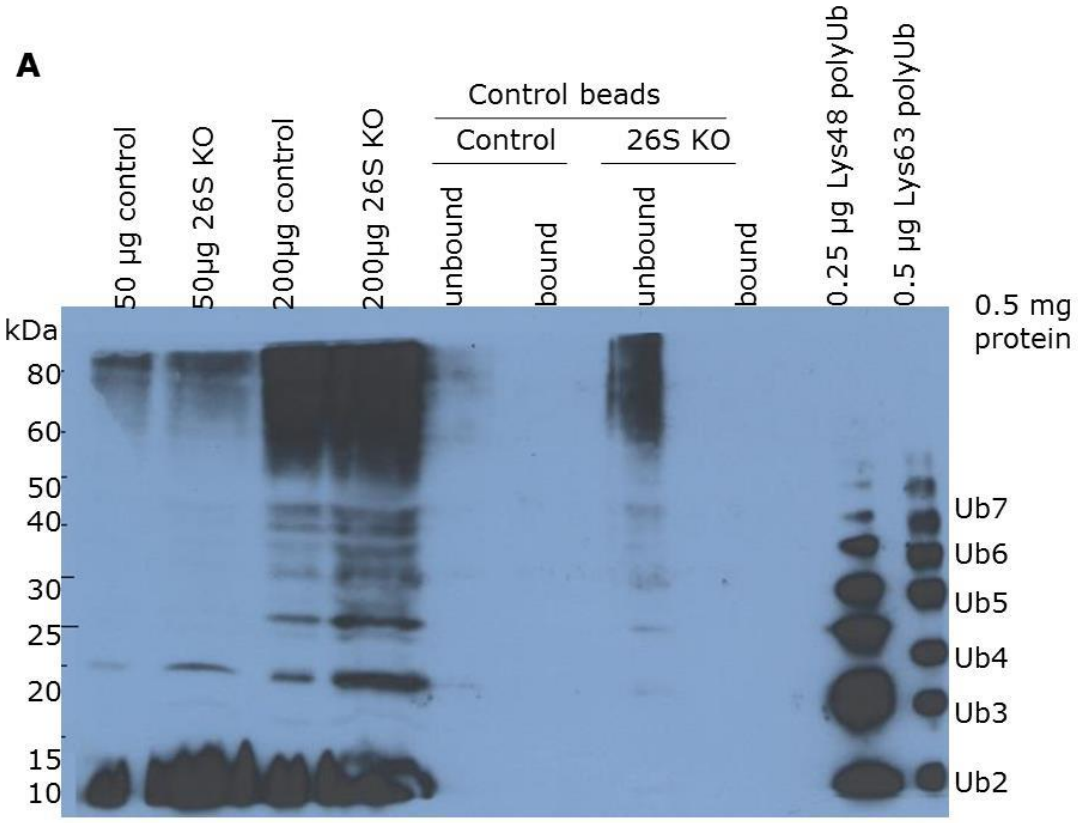


Figure 4.3

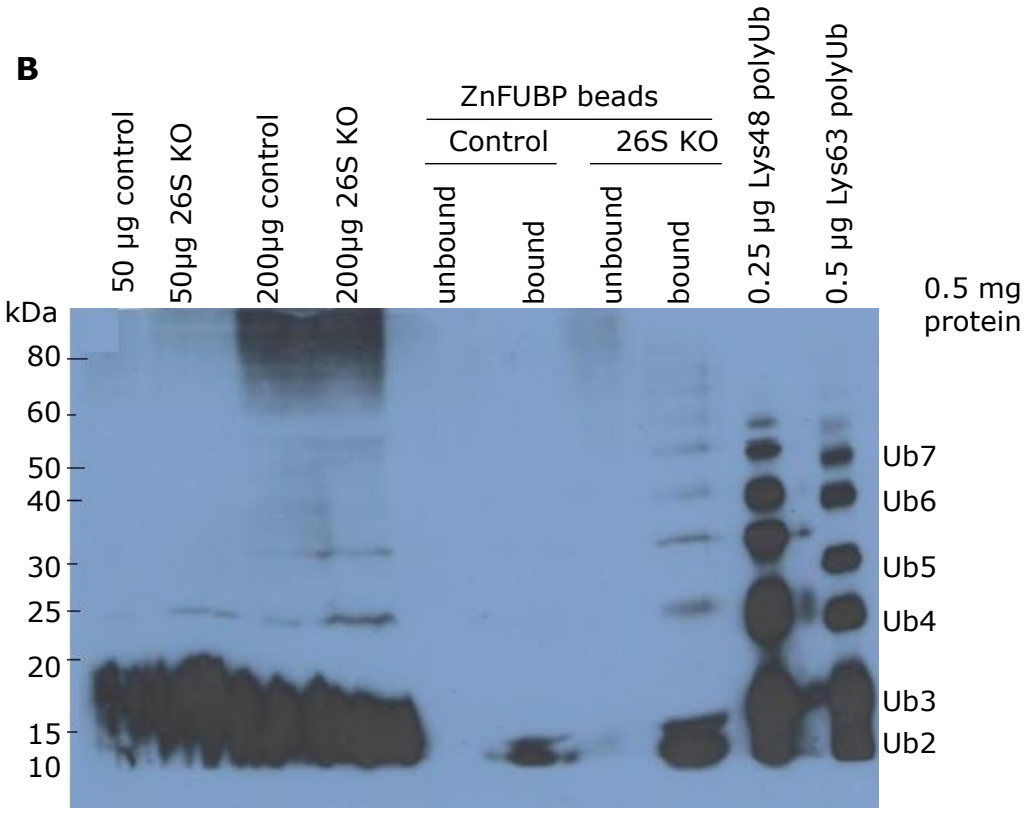


Figure 4.3

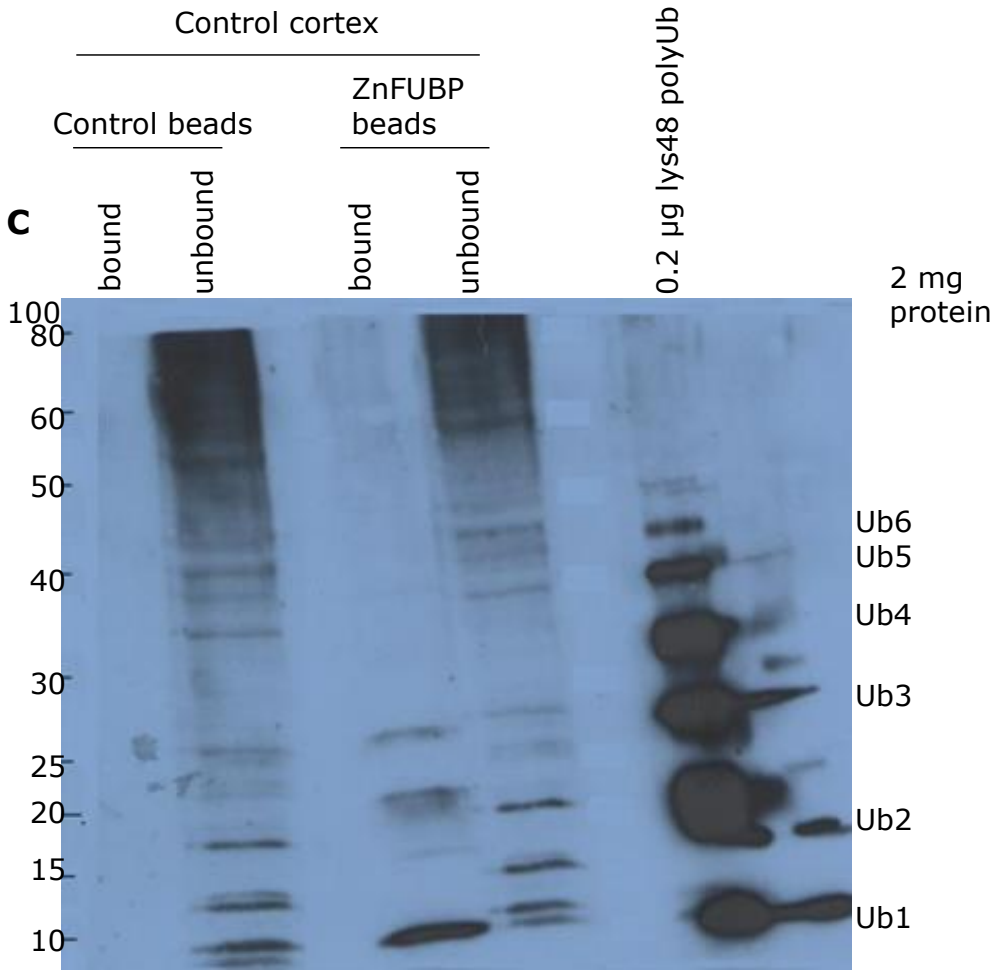


Figure 4.3 Anti-ubiquitin Western blots showing binding of the IsoT ZnFUBP domain to endogenous unanchored ubiquitin from the control and Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mouse cortices. A and B use 0.5 mg protein homogenate; C uses 2 mg protein homogenate. Bound = Ub captured by beads. Unbound = buffer collected following incubation of beads. 5-20% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.3.2 Preliminary removal of monoUb did not improve subsequent binding of higher molecular weight polyUb to IsoT ZnFUBP beads

High levels of endogenous monoUb may compete with and therefore interfere with the binding of higher molecular weight unanchored polyUb chains to IsoT ZnFUBP. Therefore, to reduce the availability of monoUb in the homogenate I first incubated 2 mg of brain cortex protein with the IsoT ZnFUBP beads on a rotating mixer for 3 hours at 4^{o}C. The beads were then centrifuged and the unbound fraction collected and incubated with new IsoT ZnFUBP beads overnight at 4^{o}C. The binding was then investigated by anti-ubiquitin Western blotting as previously described. Figure 4.4 show that the preliminary 3 hour step did not improve the binding of IsoT ZnFUBP domain to higher molecular weight polyUb. There was a decrease in the presence of unanchored Ub following the overnight binding. I conclude that a preliminary binding step to remove monoUb does not improve the binding of unanchored polyUb chains.

Figure 4.4

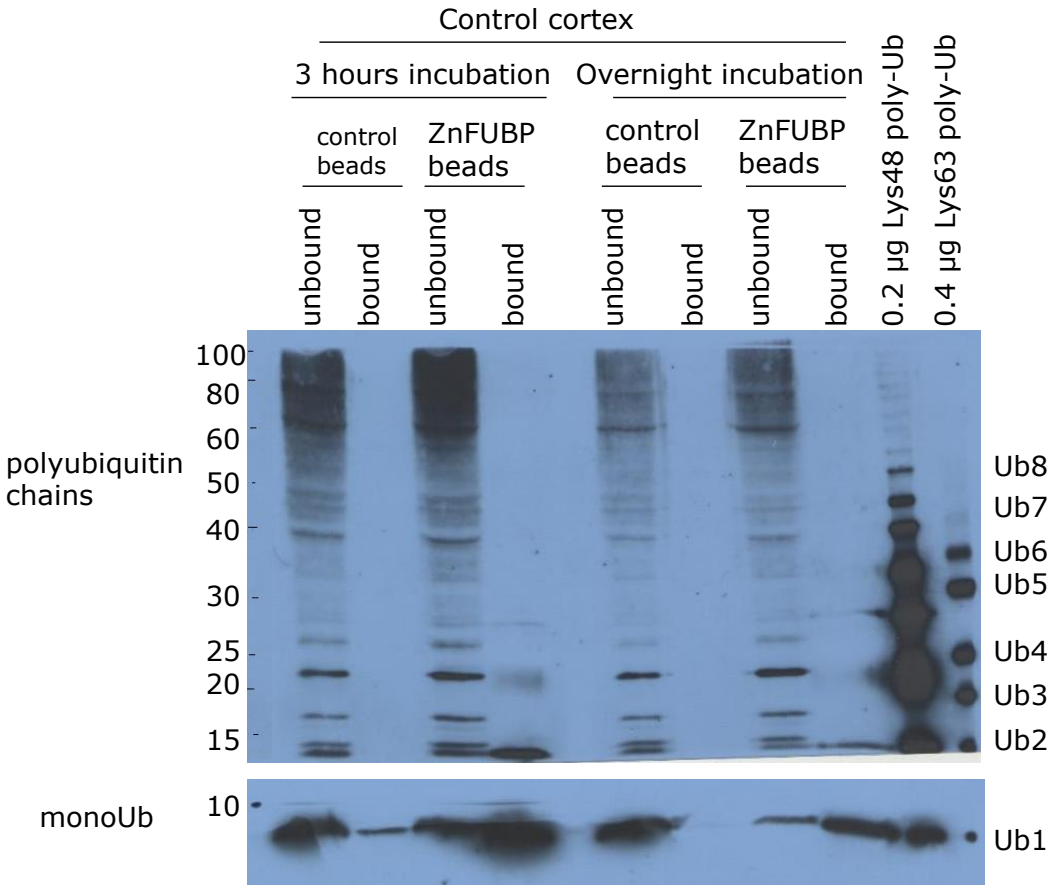


Figure 4.4 Preliminary binding of protein homogenate with IsoT ZnFUBP beads does not improve the binding to high molecular weight unanchored polyUb chains. Bound = Ub captured by beads. Unbound = buffer collected following incubation of beads. 5-20% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

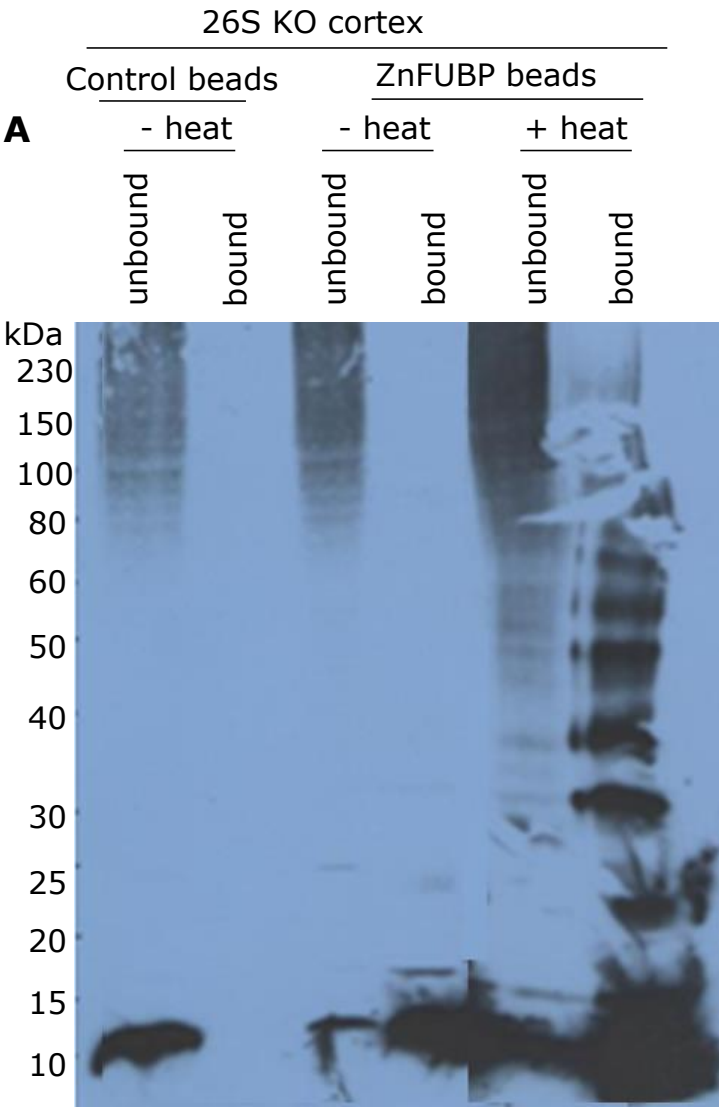
4.2.3.3 Heating

Ub is a highly heat stable protein. Two groups have demonstrated that heating of samples for 5 minutes at 70°C will lead to the denaturation of ubiquitin receptors,

i.e. retinoid-inducible gene 1 (RIG-1), but not the heat stable unanchored polyUb chains (Zeng et al., 2010; Strachan et al., 2012). Ubiquitin receptors in the protein homogenate would compete with the IsoT ZnFUBP domain for binding to the unanchored polyUb chains. Therefore, I investigated this heating step before binding of the cortical homogenate to IsoT ZNFUBP beads. The cortex homogenate was heated at 70°C for 20 minutes and I compared heated with non-heated cortex samples (Figure 4.5). Figure 4.5A shows that heating signifcantly increased the availability and hence binding of unanchored Ub to IsoT ZnFUBP beads.

Anti-Lys48 immunoblotting suggests that some of the unanchored Ub captured by IsoT ZnFUBP is Lys48-linked and that these Ub species are tetraUb and higher molecular weight (Figure 4.5B).

Figure 4.5


A
26S KO cortex
Control beads
ZnFUBP beads
- heat
- heat
+ heat
unbound
bound
unbound
bound
unbound
bound
kDa
230
150
100
80
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25
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Figure 4.5

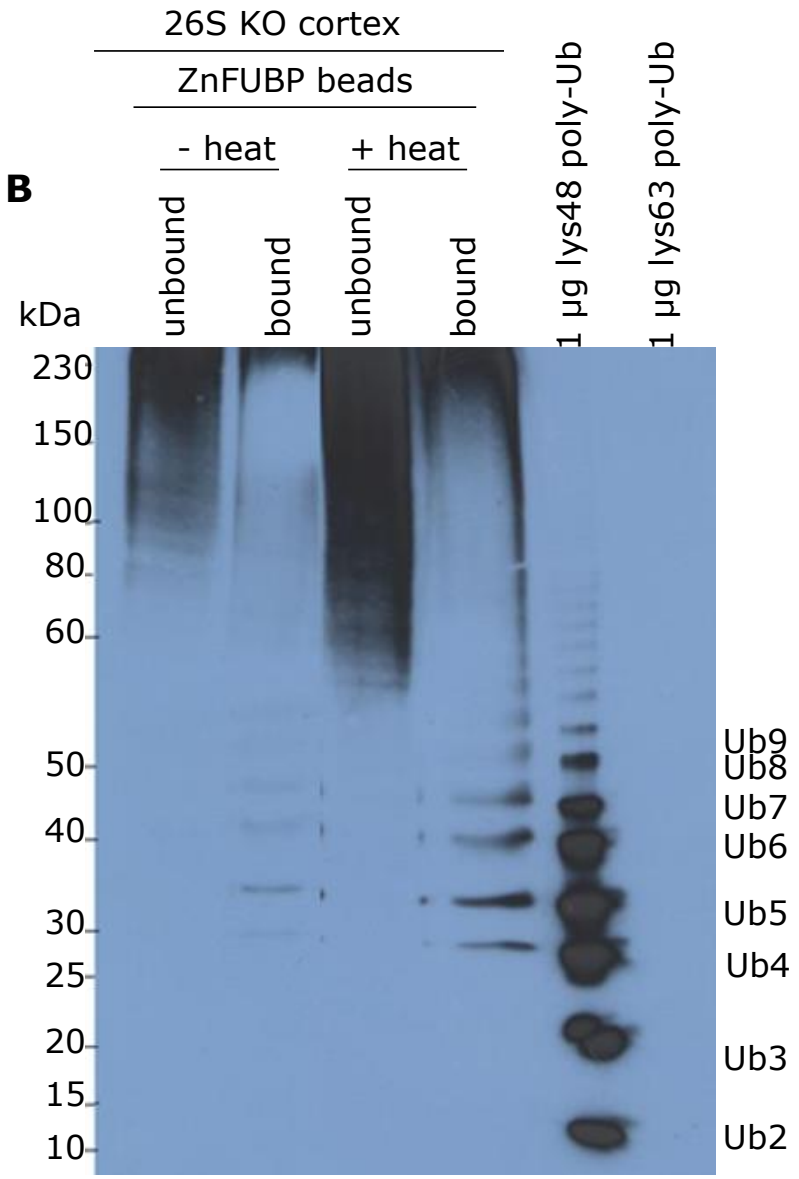

Figure 4.5 IsoT ZnFUBP binding to unanchored ubiquitin was optimised by introducing a heating step. Ubiquitin and Lys48-specific ubiquitin Western blotting. Bound = Ub captured by beads. Unbound = buffer collected following incubation of beads. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.4 Accumulation of unanchored polyUb chains in the Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortex

Unanchored polyUb chains are still a relatively novel phenomenon and their function is unclear. Several studies have suggested they may be involved in the activation of innate immunity (Xia et al., 2009; Zeng et al., 2010). The IsoT ZnFUBP domain was recently used for the capture of endogenous unanchored polyUb chains from rat skeletal muscle homogenates (Strachan et al., 2012). Figure 4.6 is a cartoon representing the detection of unanchored polyUb chains by the IsoT ZnFUBP domain immobilised on CNBr beads.

The next step was to investigate and compare the presence of unanchored polyUb chains in control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse brain cortices using affinity purification with the IsoT ZnFUBP domain. 2 mg of cortical protein homogenate from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mice were incubated with IsoT ZnFUBP or control beads and investigated by Ub Western blotting as previously described using all-ubiquitin, Lys48- and Lys63-specific antibodies.

Figure 4.7A shows the capture of unanchored polyUb chains from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mice brain cortices. The Psmc1$^{fl/fl}$, CaMKIIα-Cre cortex has significantly higher levels of unanchored Ub compared to the control. Very low binding was evident with the control beads and considered non-specific.

Western blotting with the Lys48-specific ubiquitin antibody (Millipore) showed the presence of free Lys48-linked polyUb chains in the mouse cortex (Figure 4.7B). There was a significant accumulation of unanchored Lys48-linked polyUb chains in the Psmc1$^{fl/fl}$, CaMKIIα-Cre cortex compared to the control. These were detected at Ub4 and higher.

Figure 4.7C shows Ub western blotting using a Lys63-specific antibody (Enzo; HRP-conjugate). There was no significant difference in the capture of Lys63-specific Ub between the cortex of control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mice. Using MS approaches in our laboratory we have previously showed that there was no accumulation of Lys63 Ub linkages following deletion of the 26S proteasome in mouse cortex, supporting my observations here (Bedford et al., 2011). The pattern of Lys63 immunostaining and the detection of a band below 10 kDa, which might be monoUb (Figure 4.7C; arrow), I decided to further investigate the specificity of the Lys63 antibody section 4.2.6.

In this study, the accumulation of unanchored Ub in Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse brain cortex may be explained by either the over-expression of unanchored polyUb chains or the deubiquitination from ubiquitinated substrates. Interestingly, early work has shown that accumulation of unanchored polyUb chains competitively inhibited protein degradation via the proteasome pathway (Amerik et al., 1997). Here I have shown an accumulation of unanchored polyUb chains as a result of proteasome impairment in the mouse brain.

Figure 4.6

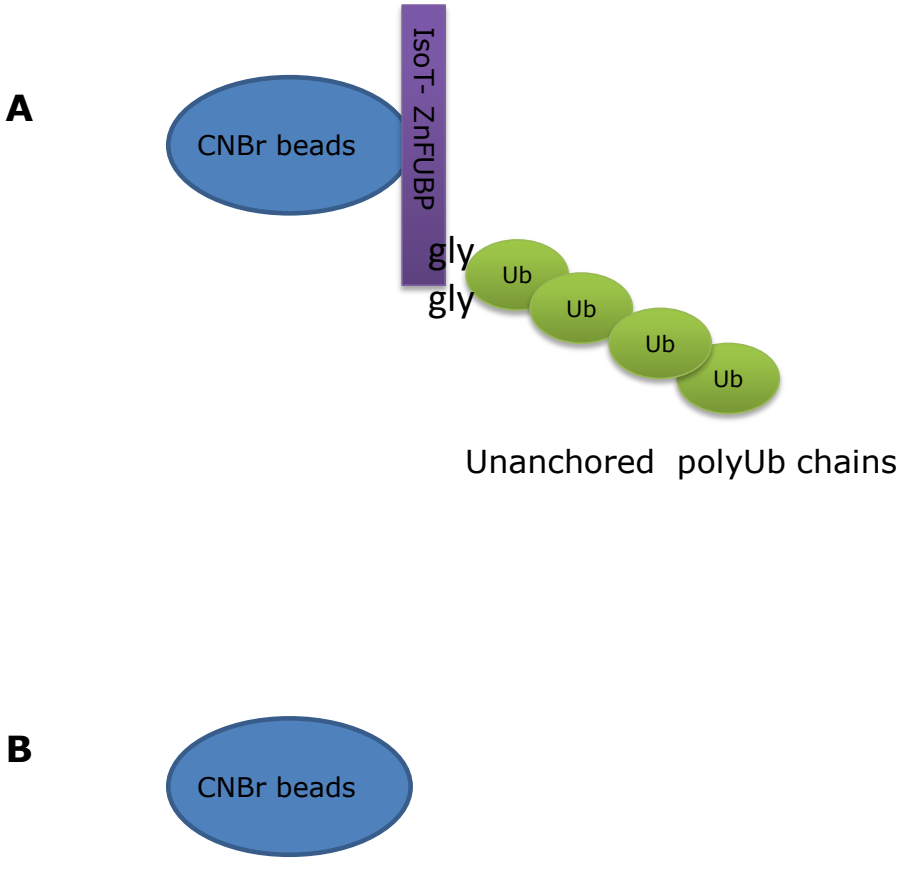

Figure 4.6 Schematic represenation of pull-down of unanchored polyUb chains using IsoT ZnFUBP domain. Purified IsoT ZnFUBP domain (A) was coupled to CNBr beads and only CNBr beads (B) were used as control. IsoT ZnFUBP and control beads were incubated with mouse brain cortex or cerebellum homogenate.

Figure 4.7

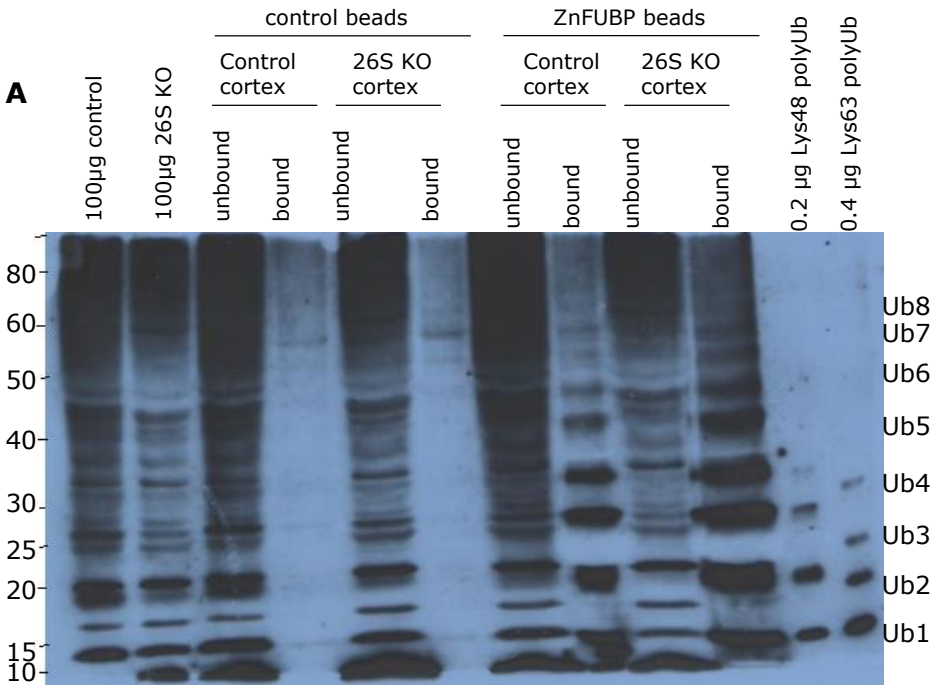

Figure 4.7

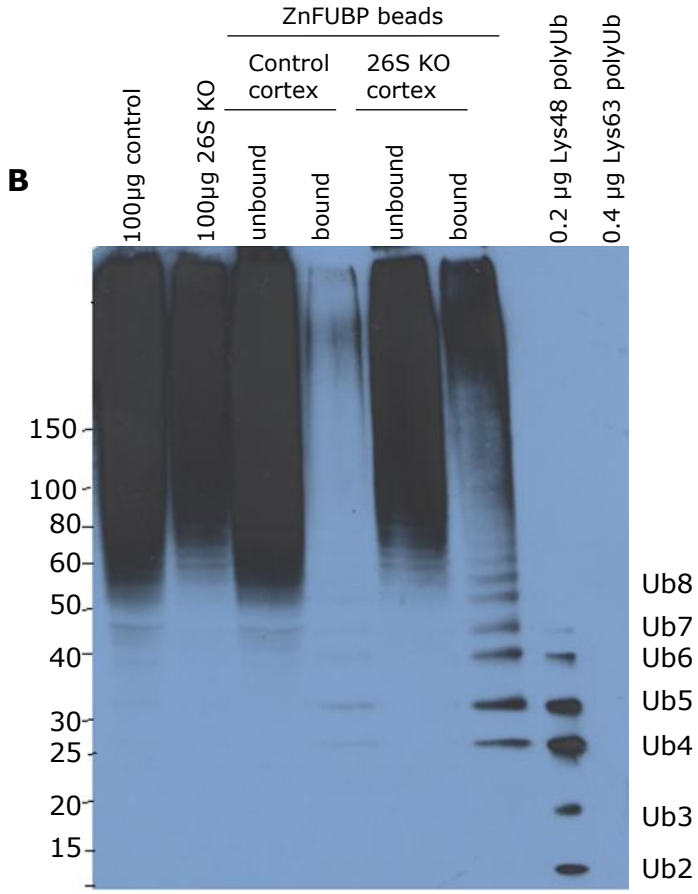

Figure 4.7

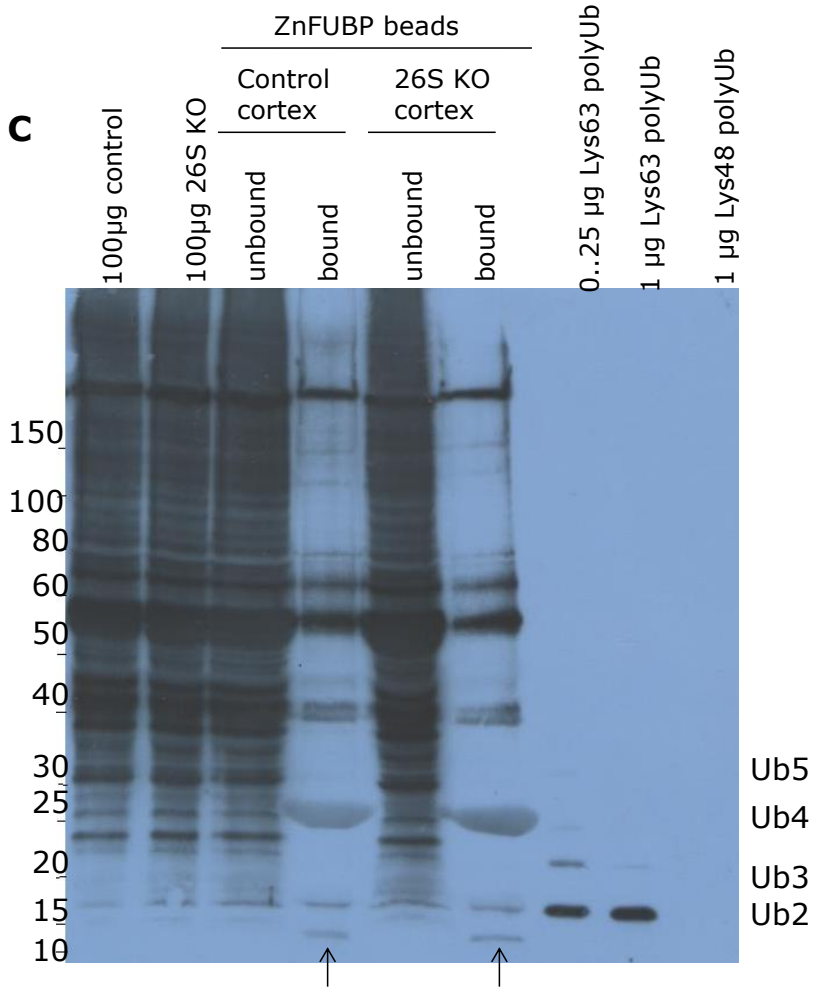

Figure 4.7 Unanchored polyUb chains accumulate in the cortex of Psmc1$^{fl/fl}$, CaMKIIα-Cre mice. Anti-ubiquitin immunoblotting investigating the capture of unanchored ubiquitin from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mouse cortex by IsoT ZnFUBP. All-ubiquitin (A) and Lys48-specific ubiquitin (B) antibodies show significant accumulation of unanchored polyUb chains in the Psmc1$^{fl/fl}$, CaMKIIα-Cre cortex compared to the control. There was no different in the binding of ubiquitin using a Lys63-specific ubiquitin antibody (C). Arrow indicates a band below 10 kDa. Bound = Ub captured by beads. Unbound = buffer collected following incubation of beads. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.5 Detection of unanchored polyubiquitin chains in mouse brain cerebellum

Next, I examined the presence and abundance of unanchored polyUb chains in the cerebellum of control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mice.

Cerebellum tissue from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mice was investigated in a similar manner to that described for the cortex in section 4.2.4. Figure 4.8A shows the presence of unanchored polyUb chains in the cerebellum of the control and the Psmc1$^{fl/fl}$, CaMKIIα-Cre mice. There is no significant accumulation of unanchored polyUb chains in the cerebellum of Psmc1$^{fl/fl}$, CaMKIIα-Cre mice compared to the control. The number of CaMKIIα-expressing neurons in the cerebellum is low, which means that the proteasome is not significantly impaired in cerebellum tissue (Bedford et al., 2011). This serves a control for observations made in the cortex where the number of CaMKIIα-expressing neurons is high. Interestingly, there was no evidence of unanchored Lys48-linked polyUb chains in the cerebellum of Psmc1$^{fl/fl}$, CaMKIIα-Cre and control mice (Figure 4.8B). This may be explained by the levels of this Ub in the cerebellum and/or the sensitivity of the Lys48-specific antibody. A similar pattern of bands were observed following Lys63-specific Western blotting to that observed in the cortex and again I noted the presence of a band below 10 kDa which may be a monoUb (Figure 4.8C). Investigation of the Lys63-specific antibody is described in section 4.2.6.

Figure 4.8

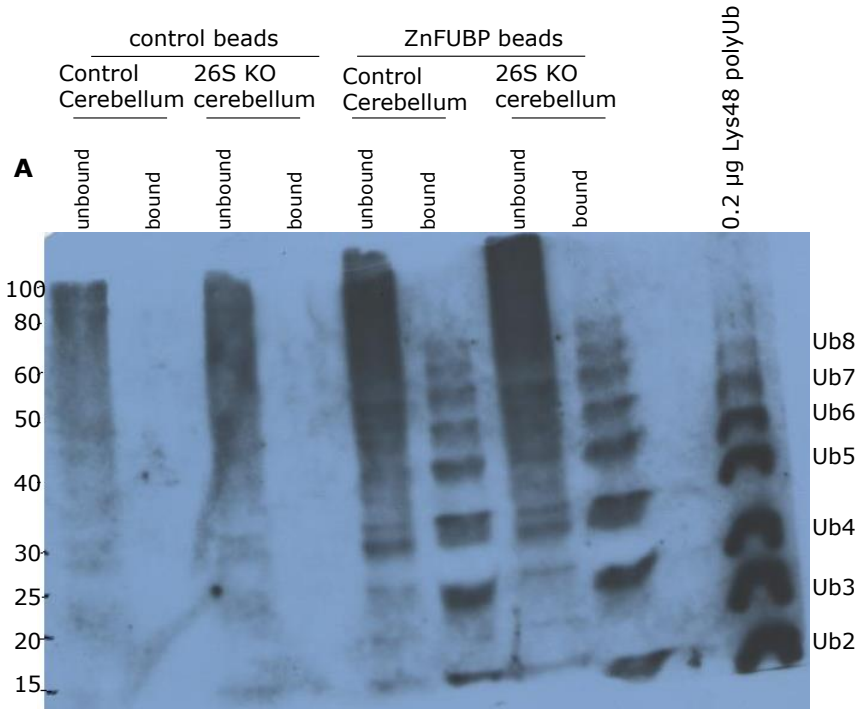

Figure 4.8

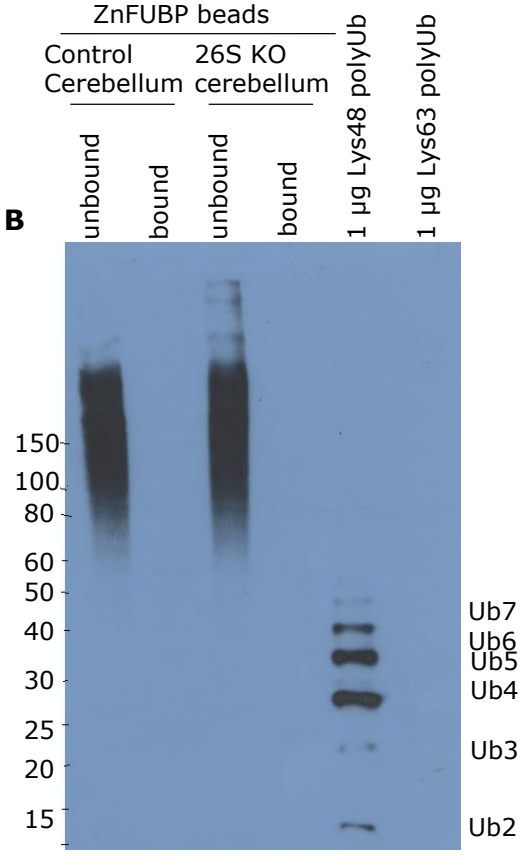

Figure 4.8

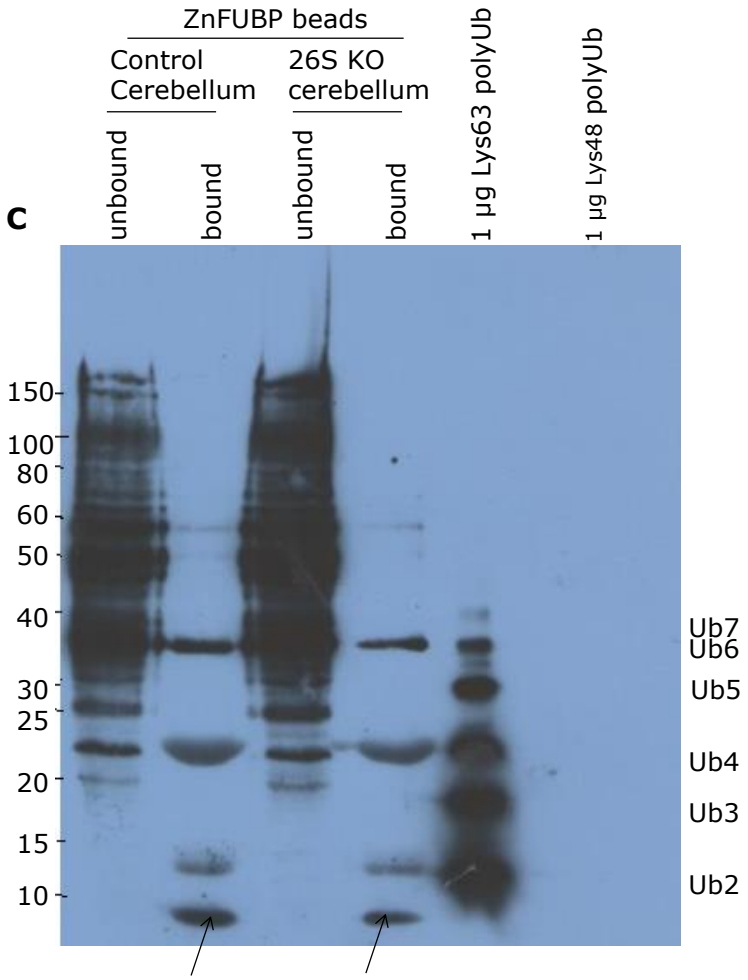

Figure 4.8 Unanchored polyUb chains do not accumulate in the cerebellum of Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mice. Anti-ubiquitin immunoblotting investigating the capture of unanchored ubiquitin from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cerebellum by IsoT ZnFUBP. All-ubiquitin (A), Lys48- (B) and Lys63-specific (C) ubiquitin antibodies. Arrow indicates a band below 10 kDa. Bound = Ub captured by beads. Unbound = buffer collected following incubation of beads. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.6 Investigations of the ubiquitin linkage-specific antibodies

As mentioned previously, the pattern of Lys63-specific immunoblotting and the presence of a band below 10 kDa suggested that the Lys63-specific Ub antibody may not be specific. The antibody detects linkages, therefore should not detect monoUb. In this section I used the deubiquitinating enzyme USP2 with beads or homogenate to examine the Lys63-specfic linkage antibody. I also included N-Ethylmaleimide (NEM) in the homogenization buffer, which inhibits deubiquitinating enzymes. NEM is a thiol-reactive compound that irreversibly modifies cysteine residues in proteins. Homogenates were subsequently treated with Dithiotheritol (DTT) to stop the action of NEM.

4.2.6.1 On-bead deubiquitination

Unanchored polyUb chains from mouse cortex homogenate were captured with IsoT ZnFUBP beads. The beads were then incubated with USP2. Figure 4.9 shows a cartoon representation of the elution using USP2.

All-Ub and Lys48-specific linkage immunoblotting revealed deubiquitination of unanchored Ub that was bound to IsoT ZnFUBP following incubation with USP2 (Figure 4.10A and B). MonoUb and diUb were detected in the bound fraction after USP2 incubation (Figure 4.10A). The Lys48-specific antibody also shows deubiquitination of captured unanchored polyUb and will not detect monoUb (Figure 4.10B).

Figure 4.10C shows that the bands detected by Lys63-specific Ub immunoblotting are not affected by the incubation with USP2. This suggests that these are not polyUb chains and that the Lys63-specific antibody is non-specific. A recent report has also suggested that the senstivity of commerically available Lys63-specific linkage antibodies towards native Lys63 is weak (Lim & Lim, 2011).

Figure 4.9

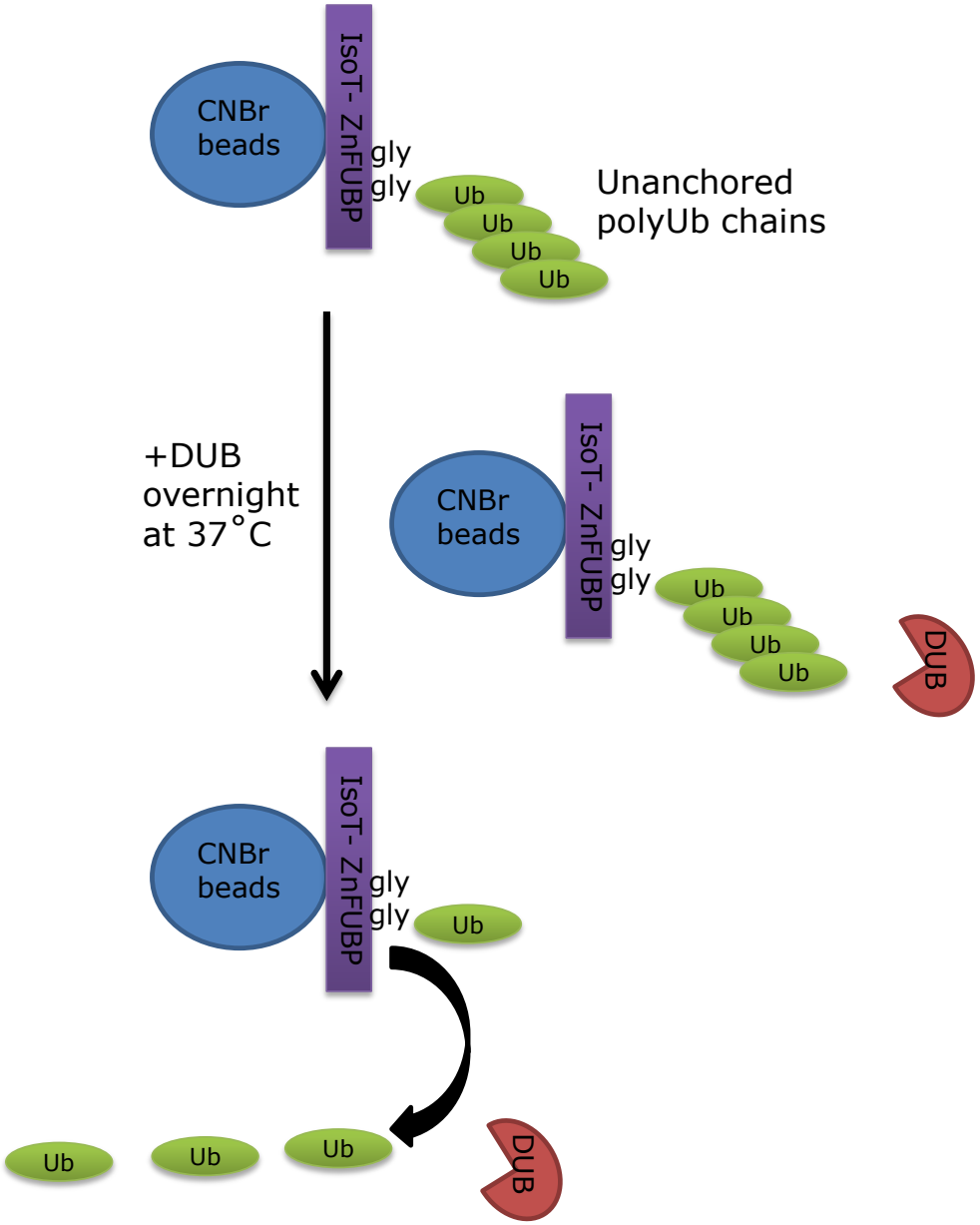

Figure 4.9 Schematic representation of the elution of IsoT ZnFUBP-Sepharos-bound unanchored polyUb chains by deubiquitinating enzyme USP2. Unanchored polyUb chains are broken down into monoUb and diUb by the action of USP2.

Figure 4.10

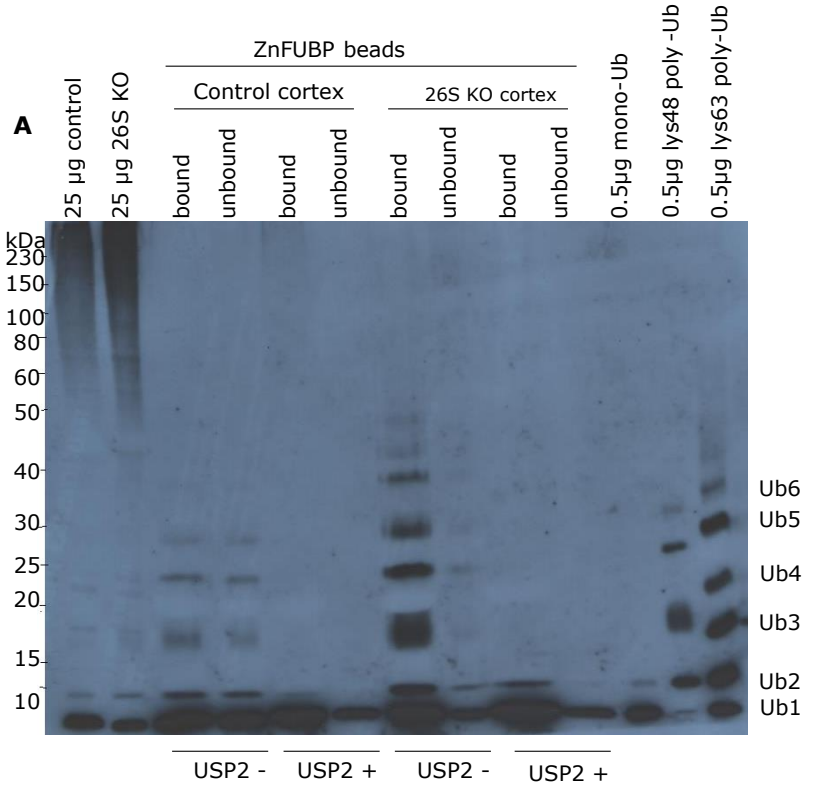

Figure 4.10

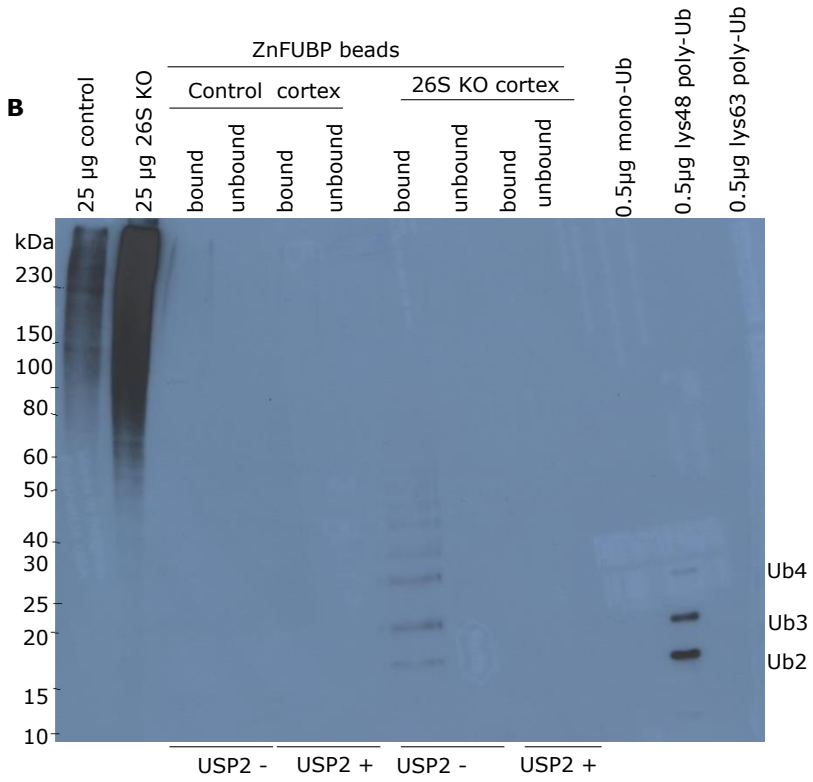

Figure 4.10

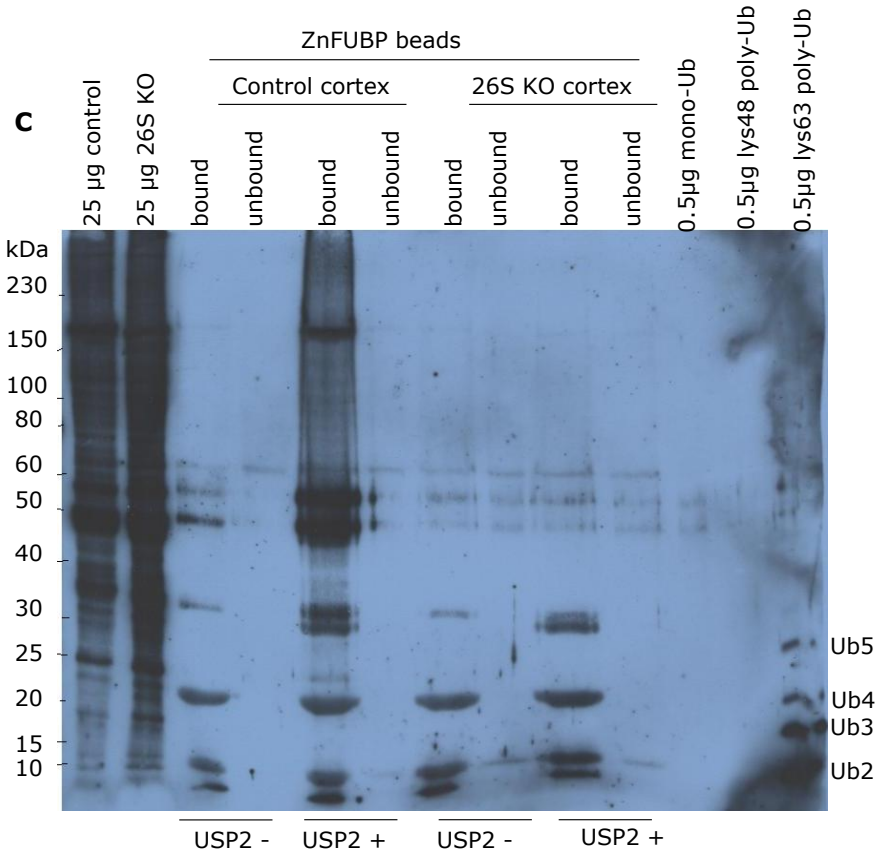

Figure 4.10 Elution of IsoT ZnFUBP-bound unanchored polyUb chains by deubiquitinating enzyme USP2. All-ubiquitin (A), Lys48- (B) and Lys63-specific (C) ubiquitin antibodies. Anti-all-ubiquitin and anti-K48-specific linkage antibodies show disassembly of unanchored polyUb chains by USP2. Anti-Lys63-specific linkage immunoblotting shows the Lys63-specific ubiquitin antibody is non-specific. Bound = Ub captured by beads. Unbound = buffer collected following incubation of beads. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.6.2 Deubiquitination of protein homogenates

The steps described in section 4.2.6.1 were repeated using mouse cortex homogenate to further investigate Ub antibody specificity. In addition, Lys48 and Lys63 synthetic ployUb chains were included in the investigations.

The anti-all-Ub and Lys48-specific linkage antibodies revealed deubiquitination of polyUb chains in cortical protein homogenate and synthetic polyUb following incubation with USP2, producing low molecular weight Ub species, predominantly monoUb (Figure 4.11A and B). However, Lys63-specific Ub immunoblotting showed that although synthetic Lys63-linked polyUb was cleaved by USP2, the bands detected in the cortical homogenates were not affected by USP2, suggesting that this is non-specific reactivity and not Lys63 (Figure 4.11C). Together with the investigations in section 4.2.6.1, I suggest that the Lys63-specifc antibody behaves non-specifically with protein homogenate, but is sensitive to synthetic Lys63-linked polyUb chains. Therefore, this antibody was not included in further investigations.

Figure 4.11

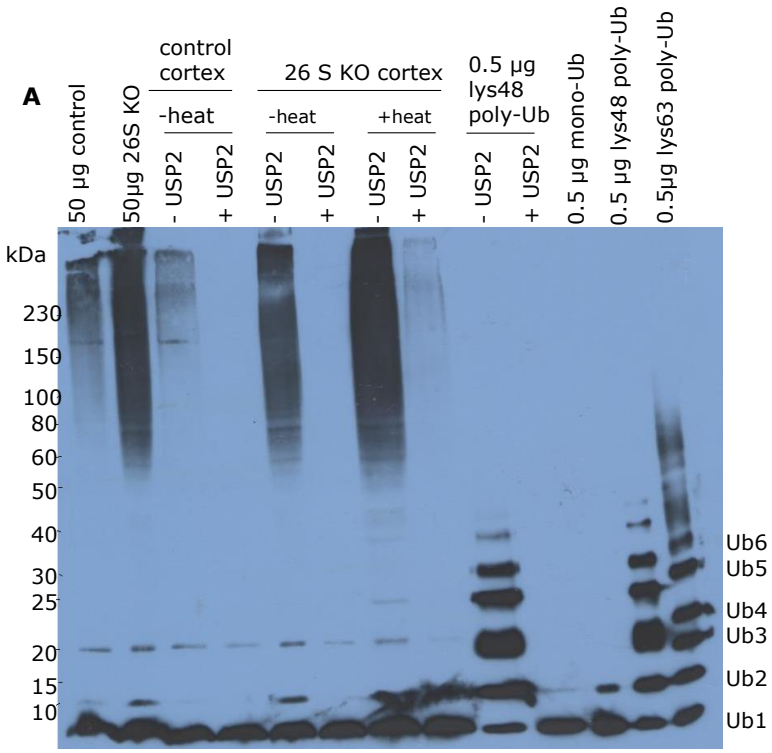

Figure 4.11

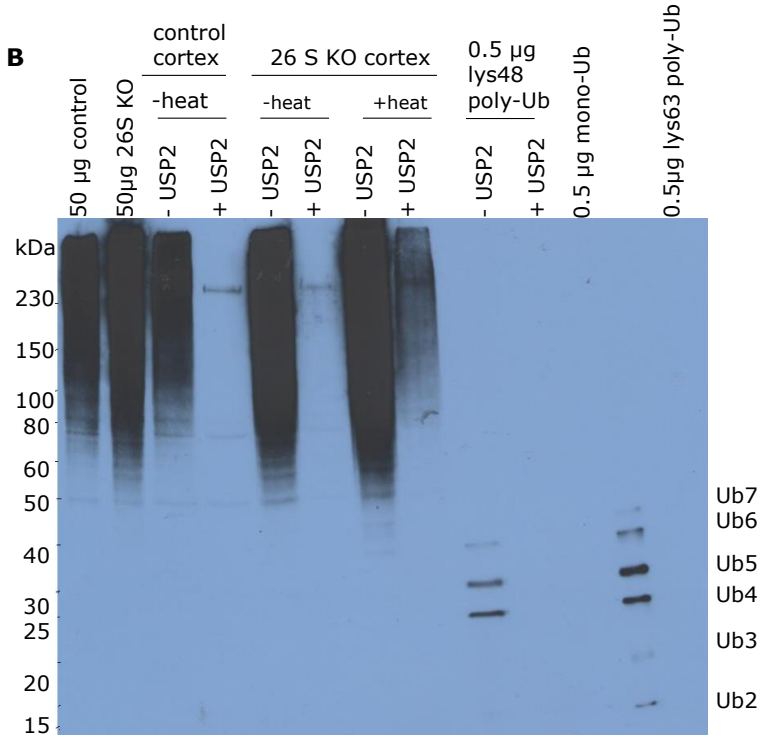

Figure 4.11

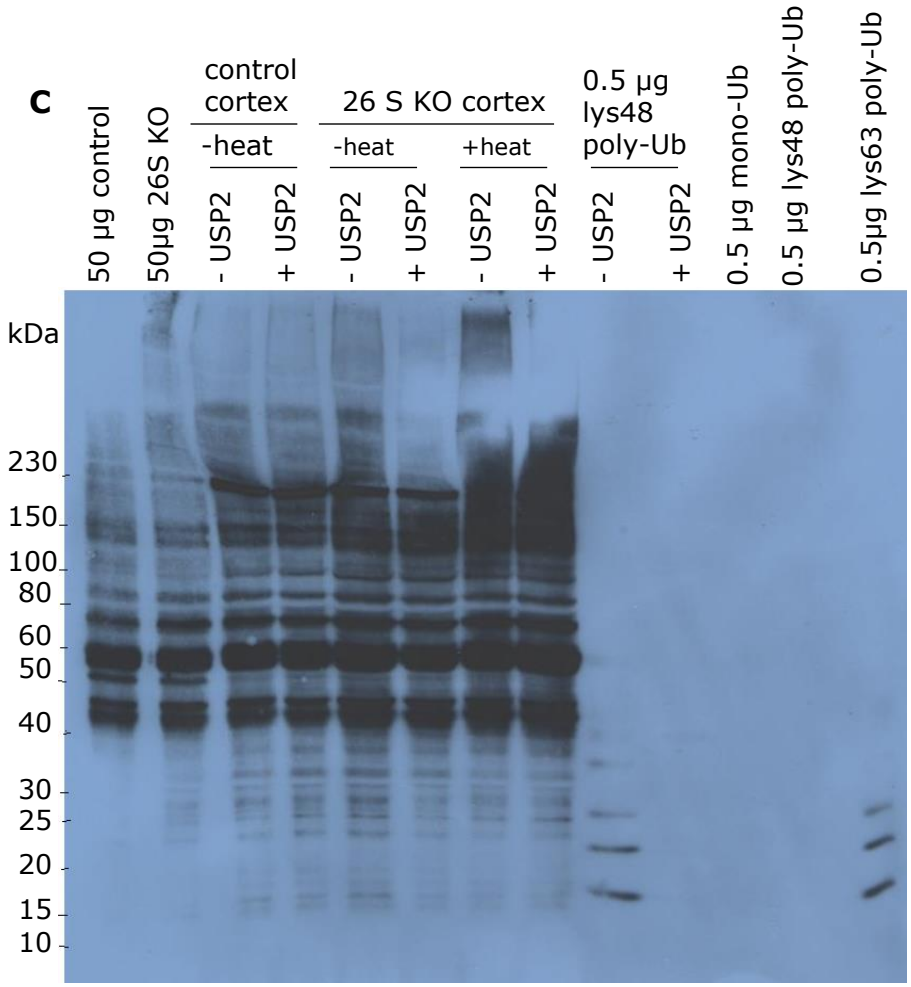

Figure 4.11 Deubiquitination of polyUb chains in mouse cortical protein homogenate by USP2. All-ubiquitin (A), Lys48- (B) and Lys63-specific (C) ubiquitin antibodies. Anti-all-ubiquitin and anti-Lys48-specific linkage antibodies show disessemmbly of polyUb chains by USP2. Anti-Lys63-specific linkage immunoblotting shows that the Lys63-specific ubiquitin antibody is not specific to endogenous Lys63-linked polyUb. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.7 Sub-cellular fractionation of the mouse brain cortex

In addition to proteasome impairment, neurodegenerative diseases are characterized by mitochondrial dysfunction (Huang et al., 2013). I chose to investigate unanchored Ub in mitochondria in control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse brain cortex following sub-cellular fractionation of mouse brain.

4.2.7.1 Sub-cellular fractionation validation

Figure 4.12 shows myelin (fraction 1), endoplasmic reticulum (fraction 2), synaptosome (fraction 3) and mitochondrial fractions (fraction 4) obtained from control mouse cortex homogenate. These sub-cellular fractions were examined using specific antibodies: ER72 (73 kDa), Synaptophysin (37 kDa) and CoxIV (17 kDa) in the endoplasmic reticulum, synaptosome and mitochondria fractions respectively. These methods do not produce 'pure' fractions, but Figure 4.13 shows that each fraction is enriched for its respective cellular component.

4.2.7.2 Detection of ubiquitin in sub-cellular fractions of mouse brain tissue

Figure 4.14 shows the differential expression of Ub in different sub-cellular fractions of mouse brain cortex. This suggests that there are higher levels of Ub and ubiquitinated proteins in the endoplasmic reticulum and synaptosomes than in mitochondria and myelin.

Figure 4.12

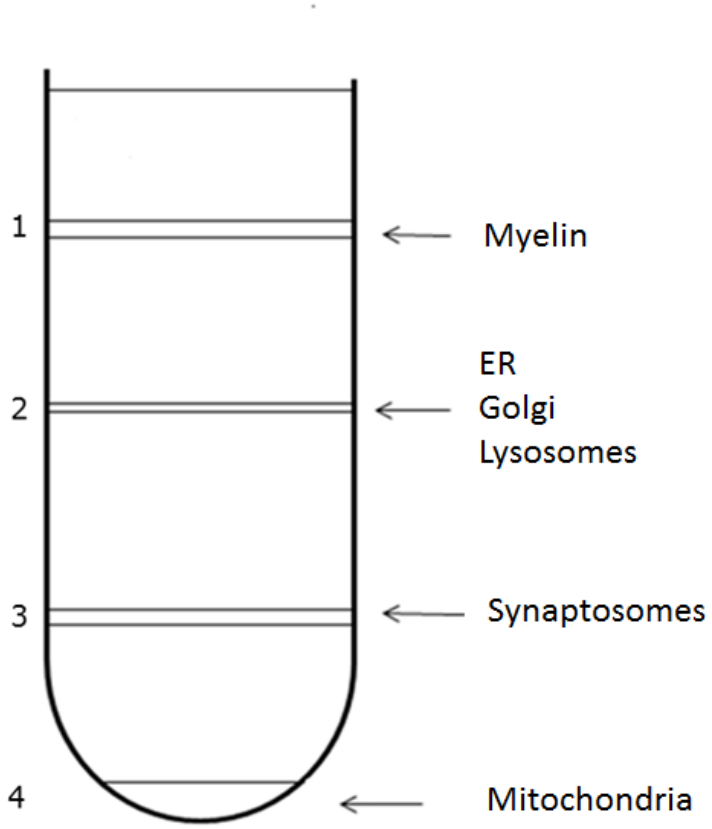

Figure 4.12 Schematic representation of sub-cellular fractionation.

Figure 4.13

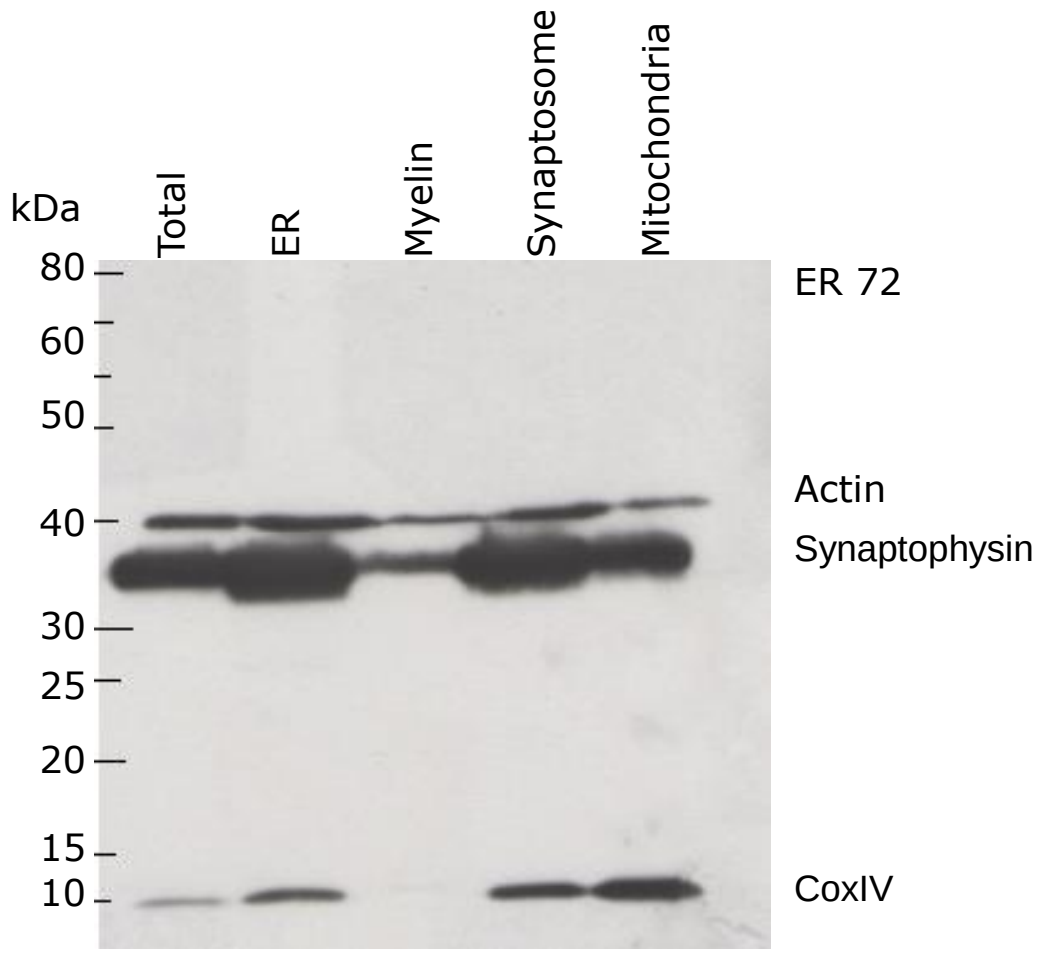

Figure 4.13 Western blots shown the antibodies subcellular fraction marker to confirm the identity of fractions in the subcellular fractionation experiments. ER72 as marker for endoplasmic reticulum, anti-Synaptophysin antibody to confirm the synaptosome fraction, anti-CoxIV antibody to confirm mitochondria fraction. Actin antibody (β-actin) was used as a loading control. 15% (w/v) acrylamide gel. This is representative of 3 independent experiments showing the same result.

Figure 4.14

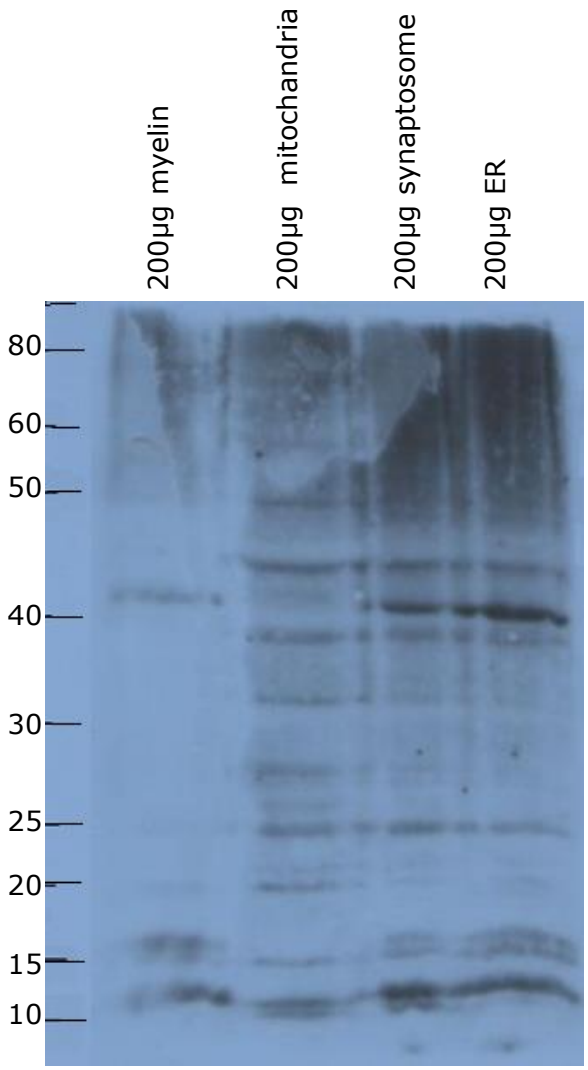

Figure 4.14 Anti-ubiquitin Western blotting of sub-cellular fractions from mouse brain cortex Ubiquitin is more abundant in the synaptosome and endoplasmic reticulum fractions than in the myelin and mitochondria. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.7.3 Mitochondrial accumulation of unanchored polyUb chains in Psmc1$^{fl/fl}$, CaMKIIα-Cre mice

Following 26S proteasomal conditional depletion (Psmc1$^{fl/fl}$, CaMKIIα-Cre) in mouse brain the Lewy-like inclusions contain numerous mitochondria, confirmed using CoxIV immunostaining (Bedford et al., 2008). Previously I showed that unanchored polyUb chains accumulated in Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortex compared to control. To determine whether 26S proteasome impairment had any effect on the homeostasis of mitochondria and presence of unanchored polyUb chains, I examined the mitochondrial fraction purified from mouse brain cortices using IsoT ZnFUBP affinity matrices. Figure 4.15A shows the presence of unanchored polyUb chains, mainly monoUb and some diUb, in the cortex mitochondria of control mice. Figure 4.15B shows the capture of unanchored polyUb chains from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse brain cortical mitochondria. There are significantly higher levels of unanchored Ub chains in the Psmc1$^{fl/fl}$, CaMKIIα-Cre compared to the control. However, no immunoreactivity was detected using the Lys48-specific antibody (Figure 4.15C). This result suggests that the chains may not be Lys48-linked or they are at a level below the detection limit of this antibody. The unbound fractions show high molecular weight Lys48-linked polyUb chains which may be substrate-conjugated and these are increased in the Psmc1$^{fl/fl}$, CaMKIIα-Cre cortex compared to the control.

Figure 4.15

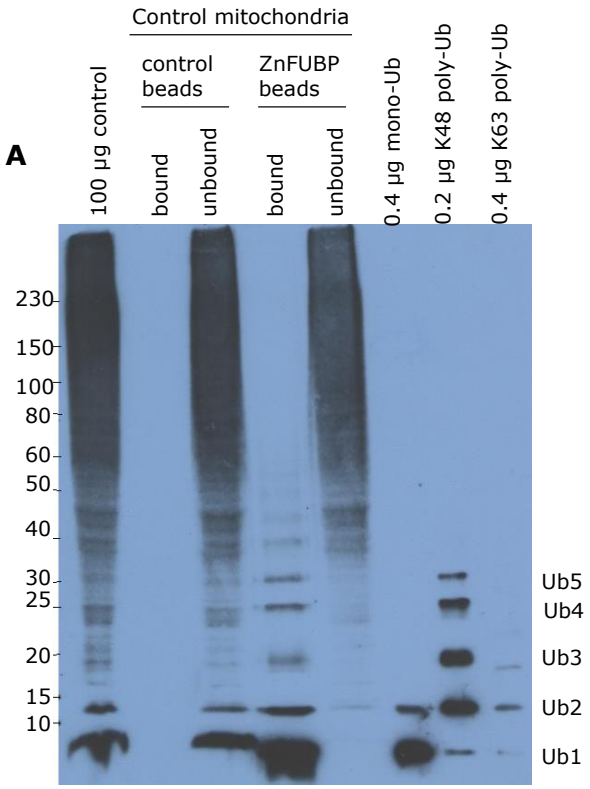

Figure 4.15

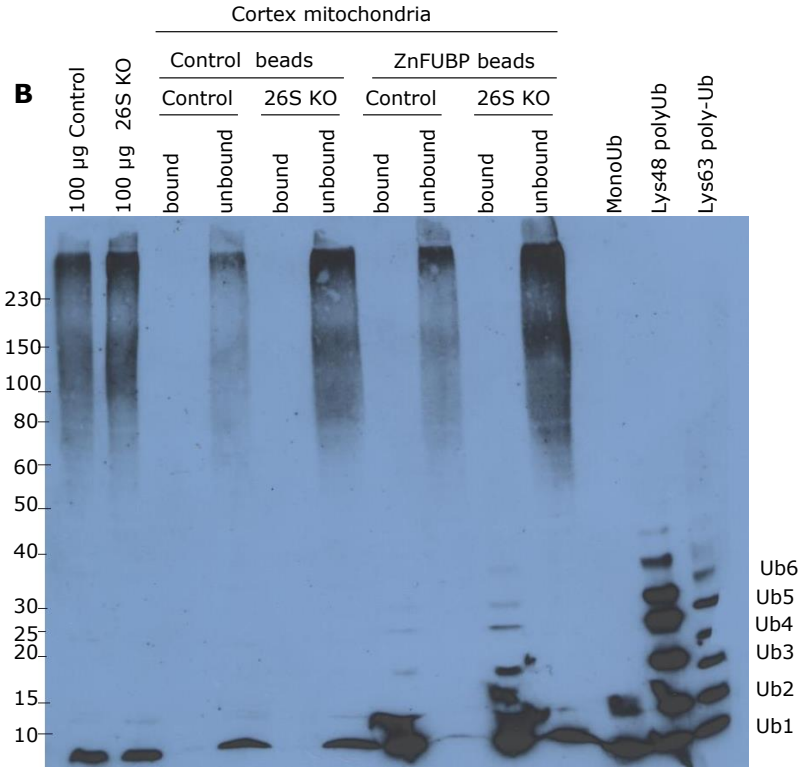

Figure 4.15

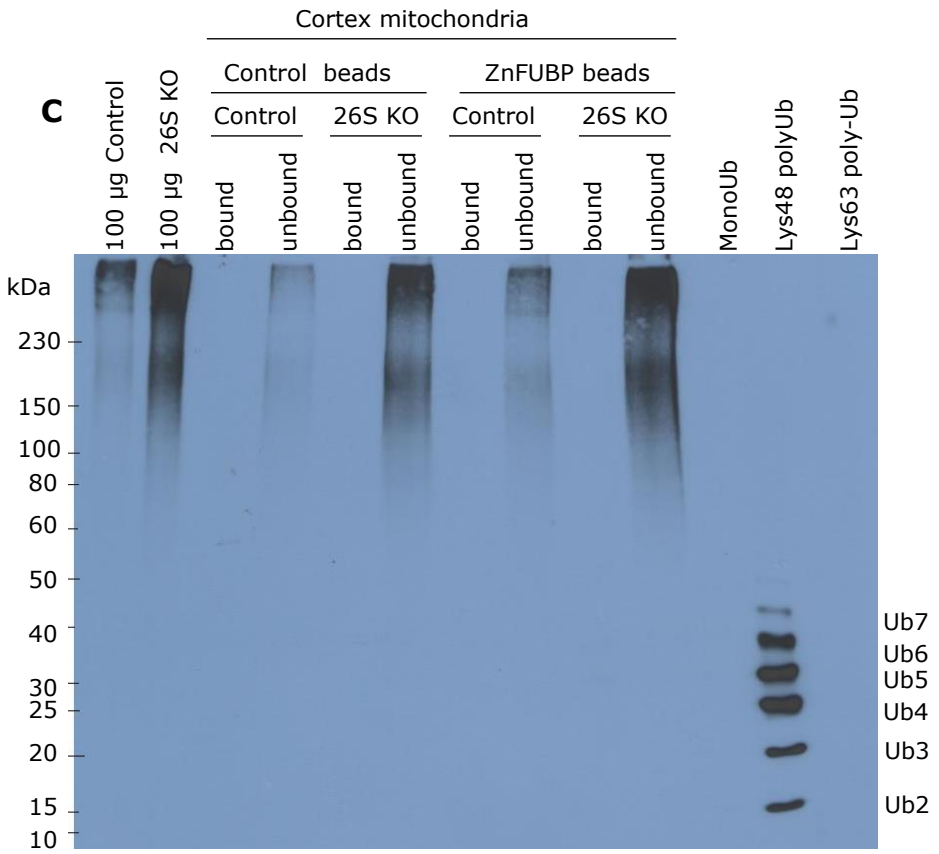

Figure 4.15 Accumulation of unanchored polyUb chains associated with cortical mitochondria from Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mice. All-ubiquitin (A and B) and Lys48-specific ubiquitin (C) antibodies. Unanchored polyUb chains are associated with mitochondria from the mouse cortex (A) and accumulate following impairment of the 26S proteasome (B). Lys48-linked polyUb was not detected (C). Bound = Ub captured by beads. Unbound = buffer collected following incubation of beads. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.8 E2-25K expression is not affected in the Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortex

Unanchored polyUb chains may be produced either by de novo synthesis or disassembly from ubiquitinated substrates as part of degradation (Reyes-Turcu et al., 2009). E2-25K is a 25 kDa unique E2 that is known to generate unanchored polyUb chains *in vitro* (Chen & Pickart, 1990; Chen et al., 1991). Upregulation of the E2-25K enzyme may cause an increase in the amount of unanchored Ub (Lee et al., 2007).

My data showed an accumlation of unanchored polyUb chains in the cortices of Psmc1$^{fl/fl}$, CaMKIIα-Cre compared to the control mice. One possible explanation for the accumulation of unanchored polyUb chains in Psmc1$^{fl/fl}$, CaMKIIα-Cre mice is an increase in the synthesis of unanchored polyUb chains. *In vitro*, E2-25K plays a role in the formation of unanchored Ub (Chen & Pickart, 1990; Chen et al., 1991). Therefore, I examined whether there were any differences in the levels of E2-25K between control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mice.

Figure 4.16 shows that there is no difference in the levels of the E2-25K protein between control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortices. This result suggests that the accumulation of unanchored polyUb chain in Psmc1$^{fl/fl}$, CaMKIIα-Cre cortex is not caused by changes in the synthesis of these chains involving E2-25K.

Figure 4.16

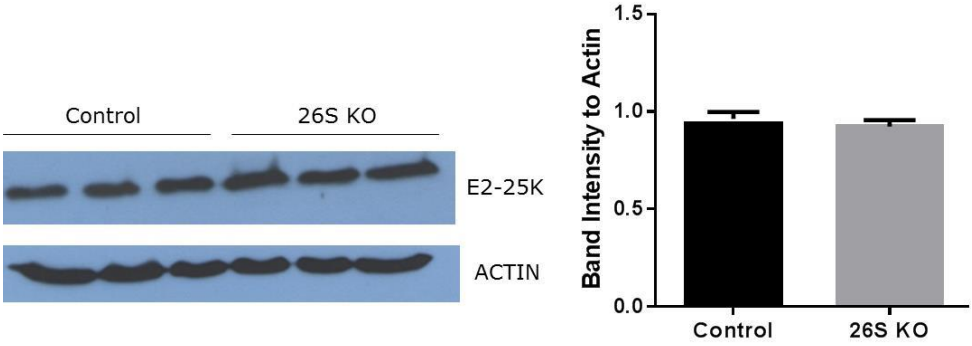

Figure 4.16 E2-25K levels are not altered in the Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mouse cortex. 15% (w/v) acrylamide gel. Representative Western blotting (left-hand-side) and densitometric (right-hand-side) analyses of cortical homogenates from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mice. Actin antibody (β-actin) was used as a loading control. Error bars indicate SEM, n=3, p was not significant, student's *t*-test.

4.2.9 IsoT/USP5 expression is not affected in the Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortex

Another possible explaination for the accumulation of unanchored poluUb chains in the Psmc1$^{fl/fl}$, CaMKIIα-Cre cortex is decreased IsoT/USP5 activity. IsoT/USP5 is a deubiquitinating enzyme that preferably cleaves unanchored polyUb chains. Dayal et al. (2009) demonstrated that unanchored polyUb chains and p53 accumulate when USP5 expression is suppressed (Dayal et al., 2009) .

Cortical homogenates were investigated with an anti-USP5 antibody. Figure 4.17 shows that there is no significnat difference in the levels of USP5 between control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mice. This result suggests changes in IsoT do not play a role in the accumulation of unanchored polyUb chains in Psmc1$^{fl/fl}$, CaMKIIα-Cre mice.

Figure 4.17

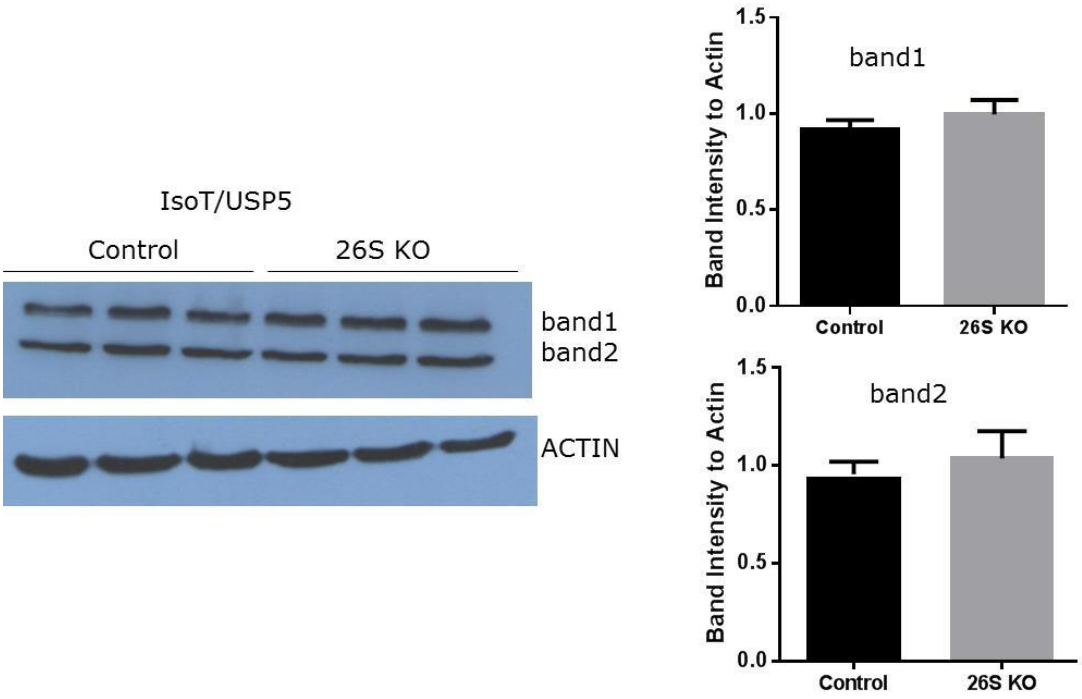

Figure 4.17 IsoT/USP5 levels are not altered in the Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortex. 15% (w/v) acrylamide gel. Representative Western blotting (left-hand-side) and densitometric (right-hand-side) analyses of cortical homogenates from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mice. Actin antibody (β-actin) was used as a loading control. Error bars indicate SEM, n=3, p was not significant, student's *t*-test.

4.2.10 p-TAK1 is down-regulated in Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortex

One of the proposed functions of unanchored polyUb chains is to activate protein kinases (Xia et al., 2009). TAK1 is transforming growth factor β-activated kinase 1. TAK1 plays an important role in the NF-kB pathway and its phosphorylation leads to changes in activity (p-TAK1) (Buglio et al., 2012). It has been demonstrated that *in vitro*-generated K63 unanchored polyUb chains have the ability to ubiquitinate TAK. TAK1 ubiquitination leads to its phosphorylation (p-TAK1) and subsequent activation (Xia et al., 2009). We therefore investigated the activation of TAK1 in Psmc1$^{fl/fl}$, CaMKIIα-Cre and control mouse cortices.

Figure 4.18 shows a significant decrease in the levels of p-TAK1 in the Psmc1$^{fl/fl}$, CaMKIIα-Cre compared to the control ($p<0.05$). This is interesting and indicates that the accumulation of unanchored polyUb chains in the mouse cortex of Psmc1$^{fl/fl}$, CaMKIIα-Cre is not involved in the activation of TAK-1. However this function of unanchored Ub may be restricted to Lys63-linked chains.

Figure 4.2.18

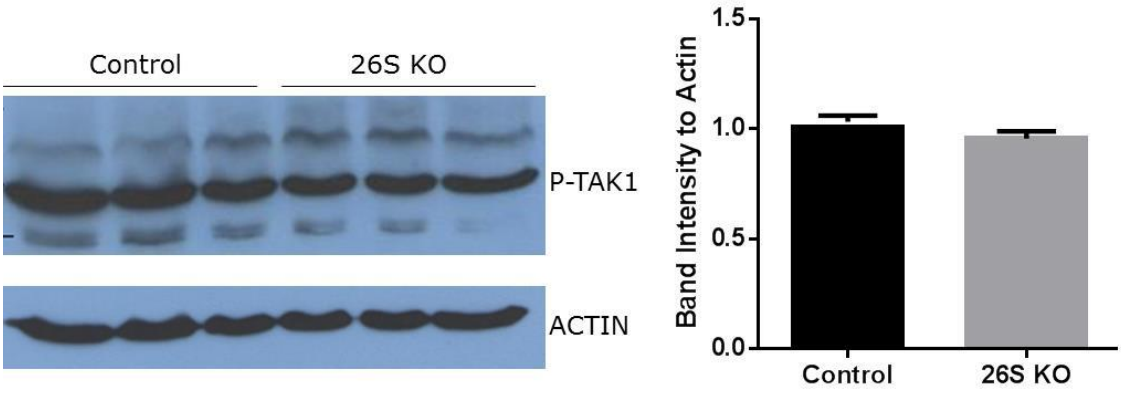

Figure 4.18 p-TAK1 levels are significantly decreased in the Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortex. 15% (w/v) acrylamide gel. Representative Western blotting (left-hand-side) and densitometric (right-hand-side) analyses of cortical homogenates from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mice. Actin antibody (β-actin) was used as a loading control. Error bars indicate SEM, n=3, $p<0.05$, student's *t*-test.

4.2.11 Far-Western blotting

Far Western blotting is used to detect protein-protein interactions. I planned to develop the Far Western approach to use the IsoT ZnFUBP domain to directly detect endogenous unanchored Ub in mouse cortical tissue sections. I optimised this technique using GST-IsoT ZnFUBP and GST only (control) proteins. These proteins are then recognized by antibody detection, e.g. anti-GST (Ohba et al., 1998; Machida & Mayer, 2009). When specific binding domains are used as probes, this techinque allows the characterization of protein-protein interactions that may involve regulation by post-translation modification such as ubiquitination.

4.2.11.1 Expression, purification and cleavage of GST and GST-IsoT ZnFUBP fusion proteins

GST and GST-IsoT ZnFUBP fusion proteins were expressed, purified and eluted using methods as described in section 3.2.1.1.

Figures 4.19A and B show the GST and GST-IsoT ZnFUBP fusion proteins captured on GSH beads. Incubation with reduced gulathione elutes the GST and GST-IsoT ZnFUBP fusion proteins.

Figure 4.19

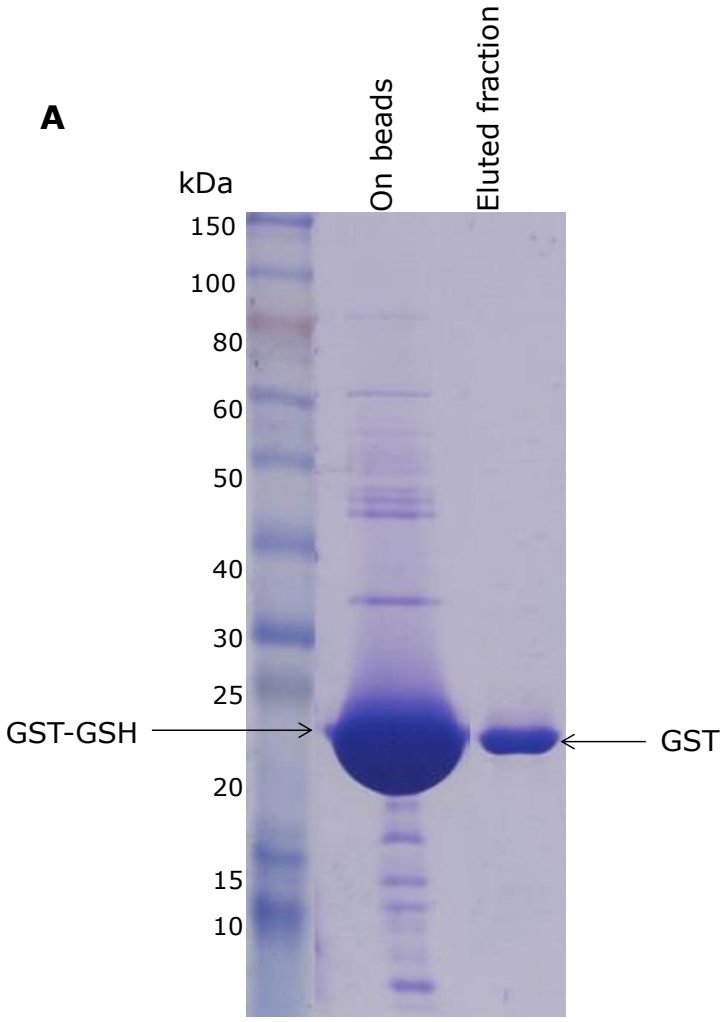
A
On beads
Eluted fraction
kDa
150
100
80
60
50
40
30
25
20
15
10
GST-GSH
GST

Figure 4.19

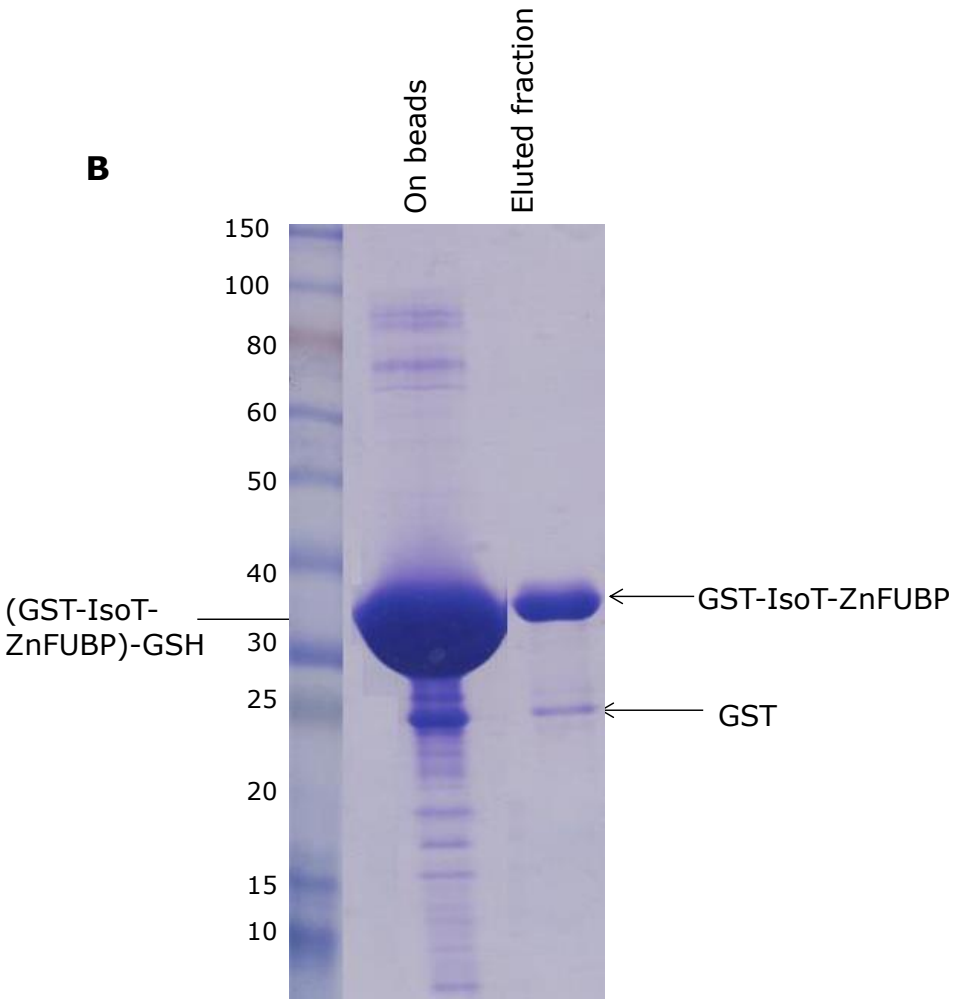

Figure 4.19 Purification of GST (A) and GST-IsoT ZnFUBP (B) proteins. On beads GST or GST-IsoT ZnFUBP fusion protein captured on GSH beads. Eluted fraction = buffer after incubation of GST or GST-IsoT ZnFUBP bound GSH beads with reduced gulthionine. 5-15% (w/v) acrylamide gradient gel. This is representative of 3 independent experiments showing the same result.

4.2.11.2 Detection of unanchored ubiquitin in mouse cortex using GST-IsoT ZnFUBP Far-Western blotting

Cortical homogenate from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mice, Lys48-linked polyUb chains and GST protein were separated by SDS-PAGE, transferred to nitrocellulose membrane and incubated with 3 µg/ml GST or GST-IsoT ZnFUBP protein in 5% Marvel 0.1% TBST for 2 hours. Following washing, the membrane was incubated with anti-GST antibody for 1 hour and subsequently secondary antibody for 1 hour; visualised by ECL. The GST protein was used as a control for non-specific binding and Lys48-linked unanchored polyUb chains as a positive control to show binding with GST-IsoT ZnFUBP, but not GST protein.

Far-Western blotting showed that GST bound only weakly to cortical homogenates suggesting non-specific interactions are low in this approach (Figures 4.20A, B and C; I). There is evidence of specific binding to Lys48-linked polyUb chains. The GST-IsoT ZnFUBP domain showed an increased interaction with Ub in cortical homogenates compared to the GST only protein, suggesting that the GST-IsoT ZnFUBP is binding to unanchored Ub (Figures 4.20A, B and C; II). In addition, Figure 4.20B indicates that there is a significant increase in unanchored polyUb chains in the Psmc1$^{fl/fl}$, CaMKIIα-Cre cortex compared to control mice.

When the GST and GST-IsoT ZnFUBP proteins were investigated using BSA as a blocking reagent, to enable examination of unanchored Ub chains in mouse cortical tissue sections, the binding of these proteins became non-specific (figure 4.20 D).

Figure.4.20

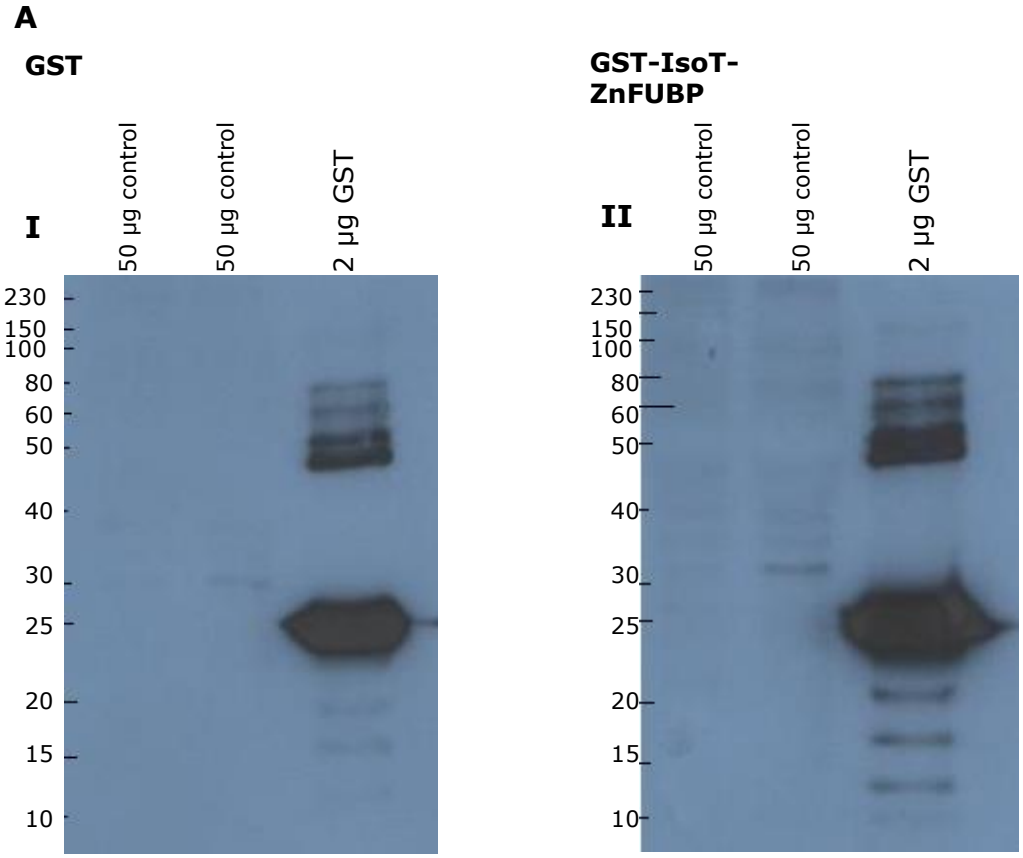

Figure 4.20

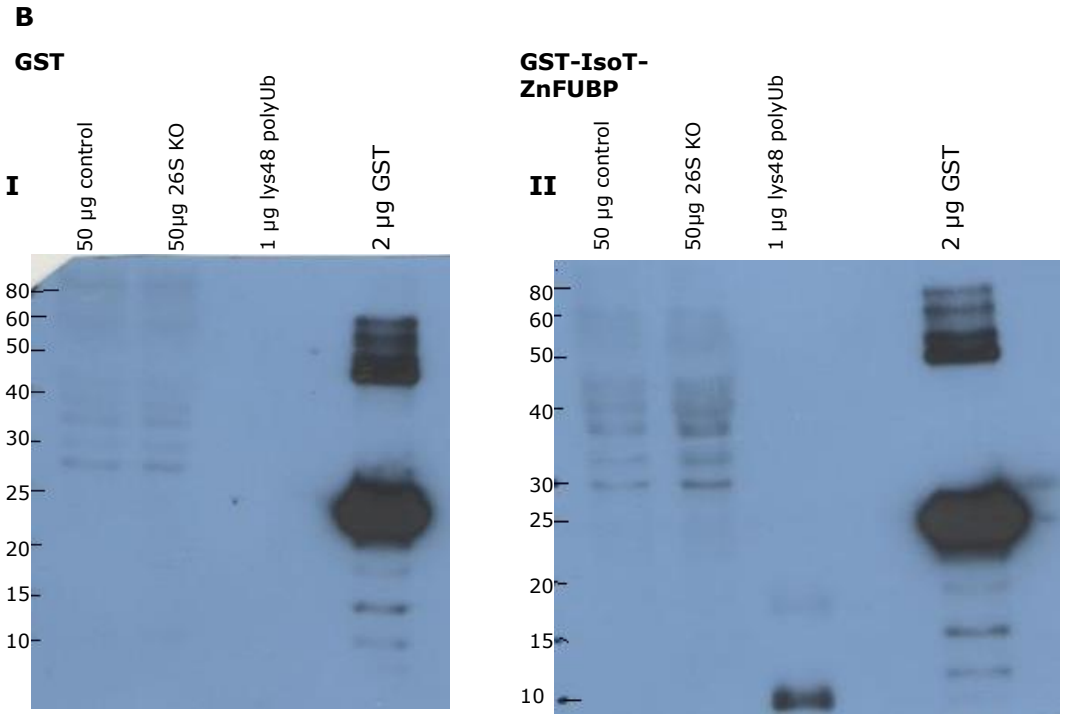

Figure 4.20

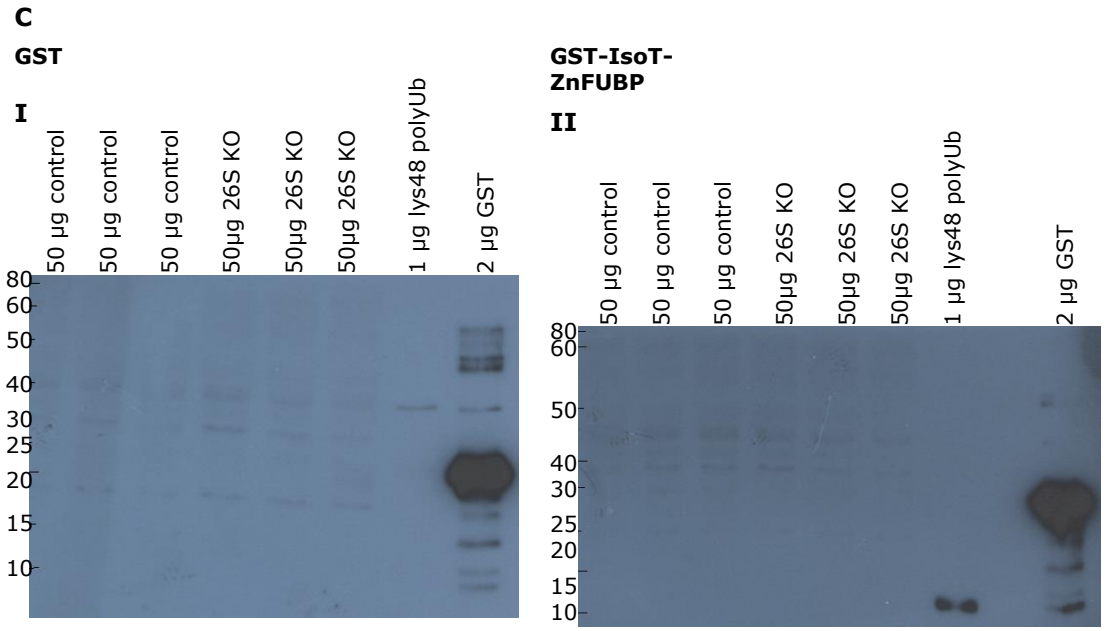

Figure 4.20

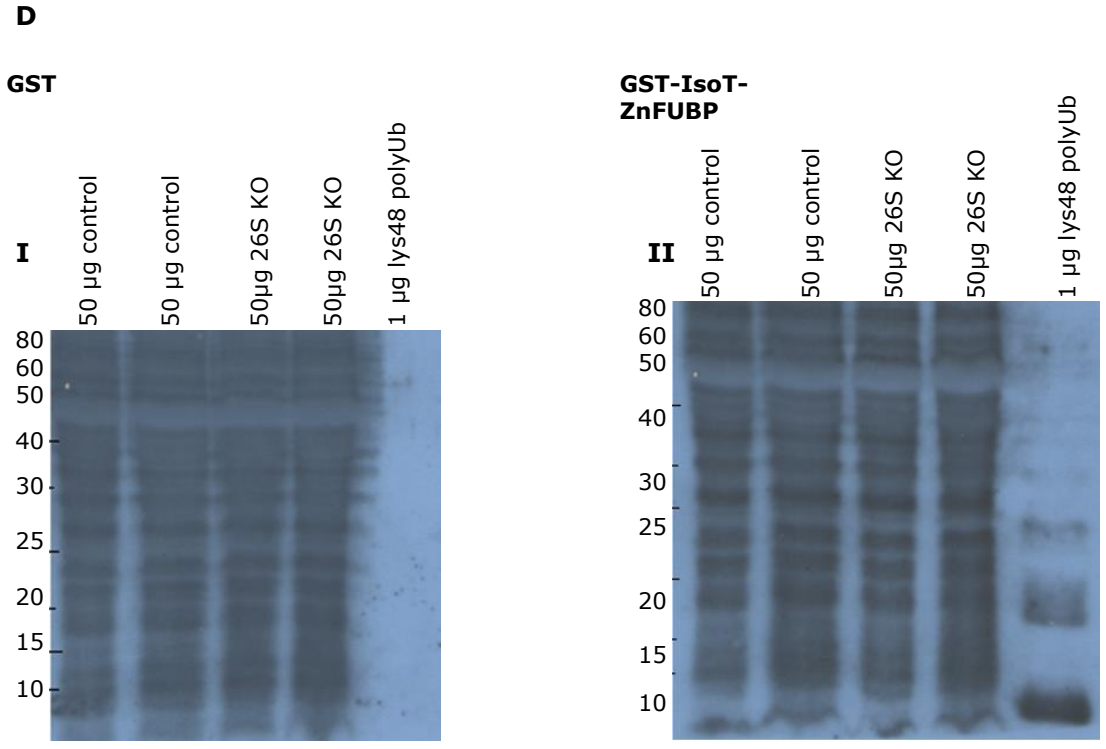

Figure 4.20 GST-IsoT ZnFUBP binds to unanchored polyUb chains in a Far-Western approach. Samples from control and Psmc1$^{fl/fl}$, CaMKIIα-Cre (26S KO) mouse cortices were examined using purified GST (I) and GST-IsoT ZnFUBP (II) proteins followed by detection with anti-GST antibody. A-D are independent experiments; GST (left-hand-side) and GST-IsoT ZnFUBP (right-hand-side). All the blots were blocked with 5% Marvel excpet D which was blocked with 1% BSA. 15% (w/v) acrylamide gel.

4.3 Discussion

4.3.1 Optimising capture of unanchored polyUb chains

Ub is a heat stable protein. Several groups have introduced a heating step to dissociate Ub from Ub receptors in the protein homogenate (Zeng et al., 2010 ; Strachan et al., 2012). Previously I showed that the UBA domain of p62 and UQ1 and the Vps9 CUE domain bound unanchored polyUb chains (section 3.3). Endogenous Ub receptors, such as p62 and UQ1 (via their UBA domains), may compete with IsoT ZnFUBP for binding to endogenous unanchored polyUb chains. Therefore, cortical homogenate was heated before incubation with the affinity matricies, which significantly improved the capture of unanchored polyUb by the IsoT ZnFUBP domain.

4.3.2 Accumulation of unanchored polyUb chains in the Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse brain

A common feature of neurodegenerative diseases is the accumulation of misfolded proteins in neurons forming inclusions, which include the ubiquitinated proteins and Ub (Ciechanover & Brundin, 2003; Wilde et al., 2011).

Recent reports have suggested that unanchored polyUb chains may be involved in the activation of protein kinases (Xia et al., 2009), activation of innate immunity (Zeng et al., 2010), aggresome formation, and clearance and degradation of aggresomes via autophagy (Ouyang et al., 2012; Hao et al., 2013) and DNA damage response (Braten et al., 2012).

Studies involving deubiquitinating enzymes specific for the disassembly of unanchored polyUb chains have shown that depletion of the activity of these enzymes leads to accumulation of unanchored polyUb chains, which competitively inhibit degradation of ubiquitinated proteins and ubiquitinated p53 (Amerik et al., 1997; Dayal et al., 2009). Suppression of IsoT/USP5 activity in mammalian cells showed an increase in unanchored polyUb chains and ubiquitinated p53 (Dayal et al., 2009) . Depletion of the UBP14 gene, the yeast homolog of IsoT, causes accumulation of unanchored polyUb chains that inhibit proteasomal degradation of polyubiquitinated substrates (Amerik et al., 1997). In addition, *in vitro* experiments using synthetic unanchored K48-linked polyUb chains showed that free chains compete with polyubiquitinated proteins for binding to the 26S proteasome and cause proteasomal dysfunction (Amerik et al., 1997; Piotrowski et al., 1997; Dayal et al., 2009).

Previous work in our laboratory reported an accumulation of polyubiquitinated proteins in the Psmc1$^{fl/fl}$, CaMKIIα-Cre compared to the control mouse cortex (Bedford et al., 2008). Furthermore, mass spectrometry analysis of the mouse cortex demonstrated the presence of five different polyUb chain linkages; Lys48, Lys63, Lys6, Lys11, and Lys29. Linear polyUb chain was not included in their study and has not yet been described *in vivo.* The study was unable to detect Lys27 and Lys33 Ub linkages, which may be due to their low abundance in the mouse brain. They found that Lys48, Lys6, Lys11, and Lys29 Ub linkages were increased in the Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortex compared to the control, but that Lys63-linked Ub did not significantly change (Bedford et al., 2011).

Here I have shown that 26S proteasomal depletion also causes accumulation of unanchored polyUb chains in the mouse brain cortex using the ZnFUBP domain of IsoT. In addition, there is accumulation of Lys48-specific linkages, consistent with Bedford (2011) published observations (Bedford et al., 2011). Due to the specificity of the commercially available Lys63-specific antibody towards endogenous Lys63-linked Ub, I was unable to make a conclusion about unanchored Lys63-linked Ub (Lim & Lim, 2011).

To validate my findings in the cortex of Psmc1$^{fl/fl}$, CaMKIIα-Cre mice I performed similar experiments using the IsoT ZnFUBP domain in the cerebellum. Given that the number of neurons expressing CaMKIIα-Cre and therefore with 26S proteasome impairment is significantly lower in the cerebellum, I did not observe an accumulation of unanchored polyUb chains (Tighilet et al., 1998; Bedford et al., 2011; Wang et al., 2013).

4.3.3 Cortical mitochondria accumulation of unanchored polyUb chains

Protein degradation via the UPS and mitochondrial dysfunction have been consistently implicated in the pathogenesis of neurodegenerative diseases (Sun et al., 2009). The UPS may also play an important role in mitochondrial protein homeostasis (Bragoszewski et al., 2013). Cortical mitochondria from the brain of Psmc1 mice were investigated for unanchored polyUb chains. The cerebellar neurons in this mouse model are not targeted and therefore mitochondria isolated from the cerebellum were used as a control.

Previous work from our laboratory demonstrated the formation of intraneuronal inclusions containing numerous mitochondria following 26S proteasome impairment in the mouse cortex (Bedford et al., 2008). I have shown here using

the IsoT ZnFUBP affinity matrices that mitochondria are associated with unanchored polyUb chains and that these accumulate following impairment of the 26S proteasome. At this stage I are unsure whether these unanchored polyUb chains are newly synthesized as a response to proteasome impairment or they may be a result of the activity of DUBs removing polyUb chains from substrate proteins that then associate with mitochondria.

Previous studies have shown that treatment of rat mesencephalic dopaminergic neurons (N27) with proteasome inhibitor induces mitochondrial dysfunction, a reduction in glutathione levels and up-regulation of free radicals (Kikuchi et al., 2003). A recent report also demonstrated the accumulation of polyubiquitinated proteins in the mitochondria of N27 cells following treatment with the proteasomal inhibitor MG-132 (Sun et al., 2009). I show accumulation of unanchored polyUb chains in cortical mitochondria from the 26S proteasome-depleted mouse. Therefore, inhibition of ubiquitinated protein degradation in this model causes accumulation of unanchored polyUb chains and polyubiquitinated proteins.

4.3.4 Reasons for the accumulation of unanchored polyUb chains in the 26S proteasome depleted mouse

Unanchored polyUb chains can be generated either from *de novo* synthesis, as a product of the deubiquitination of ubiquitinated substrates or suppression of IsoT/USP5 activity (Dayal et al., 2009). Therefore we investigated the expression of enzymes involved in the synthesis or breakdown of unanchored Ub, i.e. E2-25K and IsoT. We also analysed an enzyme that is activated by unanchored Ub; p-TAK1.

4.3.4.1 Increased synthesis of unanchored polyUb chains by E2-25

Early works have demonstrated that the ubiquitin conjugating enzyme E2-25K catalyzes the *in vitro* synthesis of unanchored Lys48-linked polyUb chains without any target substrate protein. However, the *in vivo* assembly of unanchored Lys48-specific polyUb chains by E2-25K is not clear (Chen & Pickart, 1990; Chen et al., 1991; Yao & Cohen, 2000). The expression of E2-25K is high in the brain.

In Huntington's disease (HD), the levels of E2-25K are higher in affected areas such as the frontal cortex. Ubiquitination by E2-25K augments aggregate formation of expanded polyglutamine proteins and arbitrate polyglutamine-induced cell death (de Pri et al., 2007). E2-25k has also been shown to be

involved in Aβ-mediated degeneration and may contribute to the pathogenesis of Alzheimer's disease (Song et al., 2003).

In this study I have shown accumulation of unanchored polyUb chains in the 26S proteasome depleted mouse brain cortex. There was no significnat difference in the levels of E2-25K by Western blotting in 26S proteasome-depleted and control mice. Therefore, this result suggest that the accumulation of unanchored polyUb chains may not be a consequence of increased E2-25K expression.

4.3.4.2 Decrease breakdown of unanchored polyUb chains by IsoT suppression

Unanchored polyUb chains are broken-down to produce free monoUb. IsoT/USP5 is a mammalian deubiquitinating enzyme that preferentially disassembles unanchored polyUb chains (Reyes-Turcu et al., 2008; Reyes-Turcu et al., 2009).

An *in vivo* study demonstrated that when IsoT activity was suppressed the levels of unanchored polyUb chains accumulated (Dayal et al., 2009). An earlier study in yeast, which deleted the UBP14 gene, a functional homolog of mammalian IsoT, showed accumulation of unanchored polyUb chains that inhibited proteasomal protein degradation (Amerik et al., 1997). Therefore, the accumulation of unanchored polyUb chains in the Psmc1$^{fl/fl}$, CaMKIIα-Cre cortex may be due to reduced activity of IsoT.

Immunoblotting with an anti-IsoT/USP5 antibody showed no significant difference in IsoT/USP5 levels between control and Psmc1$^{fl/fl}$, CaMKIIα-Cre mouse cortex. This finding suggests that the activity of IsoT does not play a role in the accumulation of unanchored polyUb chains in Psmc1$^{fl/fl}$, CaMKIIα-Cre mice.

4.3.4.3 Increased Unanchored polyUb chains in response to NF-kB stimulator

Protein kinases involved in the NF-kB signaling pathway may be directly activated by unanchored Lys63-linked polyUb chains. Xia and colleagues demonstrated that TRAF6 (E3) produces unanchored Lys63-linked polyUb chains, which can bind to kinases and lead to their activation, including I-kB kinase and transforming

growth factor β-activated kinase (TAK1) (Xia et al., 2009). Unanchored polyUb chains consisting of 3 or 4 Ub moieties have been shown to activate RIG-1. The unanchored Lys63-linked polyUb chains bind selectively and with high affinity to multiple CARDs (caspase activation and recruitment domains) of RIG-1. This binding of unanchored polyUb chain leads to activation of RIG-1 in the cell (Zeng et al., 2010).

Interestingly, I found that the expression of p-TAK1 was down-regulated in 26S proteasome depleted mouse cortex. I suggest that the accumulation of unanchored polyUb chains in the cortex of $Psmc1^{fl/fl}$, CaMKIIα-Cre mice may have functions other rather than the activation of protein kinases. P-TAK1 activates the IKK complex and this activation lead to degradation of IkBs via the 26S proteasome and subsequent activation of NF-kB pathways. In this model, because protein degradation via the UPS is inhibited, TAK1 may not be phosphorylated.

There are other possible explainations for the accumulation of unanchored polyUb chains in this mouse model. It is well known that the 26S proteasome is involved in the degradation of ubiquitinated substrate proteins, but unanchored polyUb chains may also be degraded via the UPS. Therefore, depletion of the 26S proteasome may simply lead to the accumulation of unanchored polyUb chains. It is also possible that specific unknown DUBs may be up-regulated during stress in this model that cleave polyUb chains from substrate proteins leading to their accumulation.

Unanchored polyUb chains may serve as a signalling mechanism in the defense against accumulation of ubiquitinated proteins following 26S proteasome impairment. Reports have shown unanchored polyUb chains in response to diffent types of stresses, such as inhibition of proteasome by MG-132 or DNA damage (Braten et al., 2012; Ouyang et al., 2012). Quyang and colleagues (2012) showed that DUB enzymes such as ataxin 3 release unanchored polyUb chains from polyubiquitinated proteins accumulated in aggregates (Ouyang et al., 2012). These unanchored polyUb chains act as a signal for aggresome formation by binding to histone deacetylase 6 (HDAC6). HDAC6 and the dynein motor are components of a protein complex involved in the recruitment and transport of polyubiquitinated proteins aggregates to the aggresome (Ouyang et al., 2012). Experiments using stress stimulators such as methylmethance sulfonate, which is known to promote DNA damage, have increased the levels of unanchored polyUb chains, but not the total Ub topology (Braten et al., 2012).

Anti-Ub immunoblotting results of longer Ub chains (Ub4 and more) bound to IsoT ZnFUBP may represent Lys48 polyUb chains by comparison to the migration of synthetic Lys48 polyUb chains. These results are supported by the anti Lys48-specific antibody. However, the anti Lys48-specific antibody failed to detect lower Ub chains, including diUb. MS analyses of the IsoT ZnFUBP pull-down will be important to identify smaller Ub chains and also the linkages of unanchored polyUb chains captured. This will highlihght the role of the chains *in vivo*, such as unanchored Lys-63 polyUb chains, which known to have a role in the innate immune response system (Xia et al., 2009; Zeng et al., 2010).

Summary

PolyUb chains act as a signal and each polyUb chain may have a number of functions. Classical degradation via the UPS is signalled by Lys48-conjugated polyUb. Lys63-linked polyUb is generally associated with non-proteasome functions such as activation of the NF-kB signalling pathway. Free or unanchored Ub may be monoUb or polyUb chains. Recently several studies have demonstrated a novel role of unanchored polyUb chains, including activation of NF-kB (Xia et al., 2009; Zeng et al., 2010), aggresome formation (Ouyang et al., 2012) and response to stress (Braten et al., 2012).

I have showed accumulation of unanchored polyUb chains following 26S proteasomal depletion in the mouse brain cortex. At present, the biological role for these accumulated unanchored polyUb chains in our model is unclear. This finding may indicate that unanchored polyUb chains are degraded by the 26S proteasome or they are generated as a signal in response to failure of the 26S proteasome. In addition, unanchored chains could be a consequence of the deubiquitination of increasing polyUb substrates by DUBs.

CHAPTER 5

General discussion

5.1 Summary

Ubiquitination is a post-translation modification involving conjugation of Ub to a target protein substrate or itself. Seven lysine residues within Ub may be involved in polyubiquitination, and linear polyubiquitination produced using the N-terminal methionine of Ub. PolyUb chains act as a signal and each polyUb chain linkage may contribute to one or more cellular functions. The most studied polyUb chains are Lys48- and Lys63-linked polyUb chains. Lys48-linked polyUb chains are considered to be the main protein degradation signal by the UPS, while Lys63-linked chains play non-degradative roles, including activation of the NF-κB signalling pathway. Unanchored Ub and polyUb chains are present in the cell. Recent reports have indicated regulatory roles of these unanchored polyUb chains in cellular functions, including activation of NF-κB, aggresome formation and response to stress.

Ubiquitin binding proteins (UBPs) recognise Ub. These UBPs have one or more ubiquitin binding domains (UBDs), which interact with Ub. Some UBDs may show Ub-linkage specificity, e.g. the MUD1 and E2-25K UBA domains prefers to bind to Lys48- and Lys63-linked polyUb chains respectively (Komander, Reyes-Turcu, et al., 2009b; Raasi et al., 2005).

In the first part of this thesis, I investigated the binding specificity of five different UBDs to Ub, and I conclude that there are differences in their binding affinity to Ub. The p62 UBA domain and CUE domain of Vsp9 shared similar binding characteristics, binding to polyUb chains independent of their linkage, but not monoUb. The UQ1 UBA bound to polyUb chains and monoUb. IsoT ZnFUBP and UBAN of NEMO showed binding specificity to free (unanchored Ub) and linear Ub respectively.

In the second part I have examined their binding affinity to native endogenous Ub. I was unable to capture endogenous linear polyUb chains by UBAN, but I captured *in vivo* unanchored polyUb chains using the ZnFUBP domain of IsoT. Furthermore, I used IsoT ZnFUBP to investigate the abundance of unanchored polyUb chains in the brain cortex of 26S proteasome-depleted mice. I demonstrated that there is an accumulation of unanchored polyUb chains in the 26S proteasome-depleted mouse brain cortex compared to controls. At the present time the function of unanchored polyUb chain accumulation in this mouse model is not clear. I suggest this may be due to an increase in synthesis in

response to the depletion of 26S proteasomes or to a defect in their degradation via proteasome. An interesting recent report suggests that free chains generated through the POH1 proteasomal subunit may be involved in aggresome disassembly (Hao et al., 2013).

5.2 The p62 UBA and Vps9 CUE domains do not interact with monoUb and show no Ub linkage specificity to polyUb chains

Section 3.2.1.6 shows that the p62 UBA domain binds polyUb chains, but not monoUb. Several reports have shown that the UBA domain of p62 has low of no binding affinity to monoUb (Cavey et al., 2005a; Ciani et al., 2003; Long et al., 2008). In addition, a study using the Mud1 UBA domain has indicated that UBA domain of Mud1 has a higher binding affinity to polyUb chain over monoUb (C. R. Wilkinson et al., 2001). My results showed that the UBA domain of p62 bound non-selectivity to Lys48-, Lys63-, linear- and substrate-linked polyUb chains as well as native endogenous polyUb chains from mouse brain (section 4.2.2). This result is in agreement with Raasi and colleagues who showed that the p62 UBA bound non-selectively to polyUb chains (Raasi et al., 2005).

The CUE domain of Vps9 demonstrated similar binding affinity characteristics to p62 UBA. In my studies, the CUE domain showed no binding affinity to monoUb. The Vsp9 CUE domain bound to Lys48- and Lys63-, linear-, substrate-linked and native polyUb chains (section 3.2.3.5 and 4.2.2). Previous work demonstrated that CUE domain of Vps9 binds to monoUb as well as to polyUb chains (Davies et al., 2003; Shih et al., 2003b) This may be explained by different methods that have been used for the study of Ub binding with the CUE domain of Vsp9 (R. S. Kang et al., 2003). This finding is in contrast to my results.

5.3 The UQ1 UBA domain binds monoUb and polyUb chains

In sections 3.2.2.5 and 4.2.2 I demonstrated that the UBA domain of UQ1 binds monoUb, Lys48- and Lys63-linked polyUb chains, linear polyUb chains, polyUb substrates and native endogenous Ub. A previous report has shown high binding affinity of the UQ1 UBA domain to monoUb (Raasi et al., 2005). The UBA domain of UQ1 is considered to be one of the strongest UBDs (Hjerpe & Rodriguez, 2008; Zhang et al., 2008).

5.4 The NEMO UBAN and IsoT ZnFUBP domains show Ub specificity

My affinity chromatography results indicated that the UBAN domain of NEMO binds exclusively to linear polyUb chains (section 3.2.5.5). The NEMO UBAN domain does not bind monoUb or Lys48- and Lys63-linked polyUb chains. This is supported by previous studies demonstrating that the UBAN domain of NEMO binds to linear ubiquitin (Laplantine et al., 2009; Rahighi et al., 2009). However, in contrast to my results several reports have shown that the NEMO UBAN domain binds to Lys63-linked polyUb chains (Lo et al., 2009b; Wu et al., 2006; Yoshikawa et al., 2009b). I was unable to detect linear Ub chains *in vivo*; this may due to their low abundance in the mouse brain or their concentration may be below the detection capacity by this method (section 4.2.2).

The IsoT ZnFUBP domain binds selectively to free Ub, i.e. monoUb and unanchored polyUb in a linkage-independent manner (section 3.2.6.5). Several papers have described the binding interaction between monoUb and the ZnFUBP domain of IsoT (Ouyang et al., 2012; Pai et al., 2007; Reyes-Turcu et al., 2006; Strachan et al., 2012). IsoT ZnFUBP did not bind to $Ub5^1$, confirming that the domain binds to unanchored Ub with the free C-terminus. Therefore, this domain has the ability to bind only unmodified monoUb and unanchored Ub, but not substrate-conjugated Ub. This finding is in agreement with several reports using different forms of mutant Ub with modified C-terminals (Ouyang et al., 2012; Pai et al., 2007; Reyes-Turcu et al., 2006; Strachan et al., 2012). My results have showed that the IsoT ZnFUBP domain captures native unanchored polyUb and monoUb from mouse brain homogenate (section 4.2.2).

5.5 Accumulation of unanchored polyUb chains following 26S proteasomal depletion in mouse brain

Several studies have shown that unanchored polyUb chains may be involved in the regulation of different cellular functions, including activation of protein kinases (Xia et al., 2009), innate immunity (Zeng et al., 2010), aggresome formation (Ouyang et al., 2012) and the stress response (Braten et al., 2012).

Inhibition of unanchored polyUb chain deubiquitinating enzymes causes accumulation of unanchored polyUb chains and inhibits protein degradation via the 26S proteasome (Amerik et al., 1997; Dayal et al., 2009).Furthermore, unanchored Lys48-linked polyUb chains compete with polyubiquitinated proteins for binding to the 26S proteasome and may lead to proteasome impairment (Piotrowski et al., 1997).

Pervious work in our laboratory showed accumulation of polyubiquitinated proteins in the 26S proteasome-depleted mouse brain (Bedford et al., 2008). I have shown here that unanchored polyUb chains accumulate in the mouse cortex following 26S proteasomal-depletion, including in the mitochondrial fraction (section 5.6). I was able to demonstrate an accumulation of unanchored Lys48-linked polyUb chains in the mice, but due to the lack of specificity of the Lys63-specific antibodies, I was unable to make a conclusion about unanchored Lys63-linked polyUb chains (section 4.2.6) (Lim & Lim, 2011).

5.6 Mitochondrial accumulation of unanchored polyUb chains

Previously I showed that mitochondria accumulate in the intraneuronal inclusions following 26S proteasome impairment in mouse brain (Bedford et al., 2008). Two studies using proteasome-inhibitor treatment of rat mescencephalic dopaminergic neurons (N27) showed mitochondrial dysfunction and accumulation of polyubiquitinated proteins (Sun et al., 2009). I showed using the IsoT ZnFUBP domain that there is a mitochondrial accumulation of unanchored polyUb chains in the 26S proteasome-depleted compared to control mouse.

5.7 Causes of the accumulation of unanchored (free) polyUb chains in 26S proteasome-depleted mice

It is possible that the observed increase in unanchored polyUb chains could be due to an increase in *de novo* synthesis of free Ub, up-regulation of the deubiquitination of polyubiquitinated proteins or suppression of IsoT/USP5 activity (Dayal et al., 2009) .

The E2-25K and IsoT/USP5 levels were not significantly different between 26S proteasome-depleted and control mice, suggesting there is no up-regulation of E2-25K or suppression of IsoT/USP5 activity in this animal model. Therefore, the accumulation of unanchored polyubiquitin chain could be by other causes.

It is known that unanchored polyUb chains may be involved in the activation of NF-kB. Phosphorylation of TAK1 (transforming growth factor β-activated kinase1) leads to its activation and subsequently activates NF-kB. I found that the levels of p-TAK1 were decreased in 26S proteasome-depleted mouse cortex compared to controls. This finding suggests that the NF-kB pathway may be affected or impaired in this model caused by 26S proteasomal depletion.

Recent research work has shown that unanchored polyUb chains are involved in the clearance of aggresome following inhibition of proteasome by MG-132. The study demonstrated that unanchored polyUb chains are produced by Poh1 (26S

proteasome DUB), which activates HDAC6 (histone deacetylase 6). Activated HDAC6 stimulates aggresome removal by actinomyosin and autophagy-dependent processes. There by, unanchored polyUb chains may act as an elimination signal for protein aggregates, and connect with the UPS and autophagy pathways (Hao et al., 2013).

Summary

I have shown accumulation of unanchored polyUb chains following 26S proteasomal depletion in the mouse cortex. The biological role of these chains is not clear at the present time. It would be interesting to speculate that this forms part of a signaling mechanism in response to the stress caused by 26S proteasome dysfunction. Signaling functions for free unanchored polyUb chains have been described in recent studies associated with diverse cellular functions (Braten et al., 2012; Hao et al., 2013; Ouyang et al., 2012; Xia et al., 2009; Zeng et al., 2010).

Future work

The use of IsoT ZnFUBP affinity matrices showed increased levels of unanchored polyUb chains in the cortex of Psmc1fl/fl;*CaMKIIa-Cre* mice. To investigate the accumulation of unanchored polyUb chains in this thesis, the expression of E2-25K, IsoT and p-TAK1 were studied. However, future work is needed to investigate the origin and reasons for accumulated unanchored polyUb chains following 26S proteasomal impairment *in vivo*.

Investigating 26S proteasome deubiquitinating enzyme activity may help to explore the causes of accumulated chains. The 26S proteasome recognizes and binds polyubiquitinated proteins through their Ub chains. This is followed by protein unfolding and translocation into the proteolytic core for protein degradation (Glickman et al., 1998; Verma et al., 2000; Chen et al., 2008). Deubiquitination of polyUb proteins at the 26S proteasome is essential for protein degradation and the Ub chain is recovered. Three DUB enzymes are present in the 26S proteasome; Poh1 (RPN11), UCH37 and USP14 (Lee et al., 2011).

Investigating the activity of HADC6 in the mouse brain may provide a function for the unanchored polyUb chains as described in the recent study by Hao et al (2013). In their study, aggresome disassembly and clearance involved producing unanchored ubiquitin chains, which activate HDAC6. Poh1 (RPN11), a

proteasomal DUB, cleaves and releases unanchored Lys63-polyUb chains from protein aggregates. These Lys63-linked unanchored polyUb chains activate HDAC6, an important component in the degradation of protein aggregates by autophagy (Hao et al., 2013).

Next step is the identification of unanchored polyUb linkages, e.g. by using AQUA (absolute quantification) mass-spectrometry. Mass spectrometry is an important procedure for analysing the different forms of Ub. Absolute quantification (AQUA) MS is used to study proteins and their modification states (Gerber et al., 2003; Phu et al., 2011). In the ubiquitin-AQUA approach, synthetic isotopically labelled internal standard peptides are used to quantify unbranched peptides and the branched -GG signature peptides generated by trypsin digestion of ubiquitin signals. Standard peptides are synthesized with stable isotopes to native peptides formed by trypsin proteolysis. Each synthetic peptide is prepared with covalent modifications, e.g. linear, Lys48- and Lys63-, that are chemically identical to naturally occurring ubiquitination modifications. Such AQUA internal standard peptides are then used to precisely and quantitatively measure the absolute levels of each Ub-linkage after proteolysis using a reaction monitoring analysis in a MS (Phu et al., 2011).

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Appendix I:

Bioinformatics analysis for DNA sequences identification

UBAN domain of NEMO

Result of Gene sequencing of plasmids isolated from bacteria used for NEMO protein expression

GGCCAGTCTCAATCGGATCTGGTTCCGCGTGGATCCGGCATGCAGCTGGAAGATCTGAGGCAGCAGCTCCAGC
AAGCTGAGGAGGCCCTGGTAGCCAAACAGGAATTGATTGATAAGCTGAAAGAGGAAGCTGAGCAGCACAAGAT
TGTCATGGAGACCGTTCCAGTCTTGAAAGCCCAGGCGGACATCTACAAGGCTGACTTCCAAGCTGAGAGGCATG
CCCGGGAGAAACTGGTGGAGAGGAAGGAGCTTTTGCAGGAGCAGTTGGAGCAGCTGCAGCGCGAGTTCAACAA
GTTGAAAGTTGGCCTCGAGCGGCCGCATCGTGACTGACTGACGATCTGCCTCGCGCGTTTCGGTGATGACGGT
GAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAG
CCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCGCAGCCATGACCCAGTCACGTAGCGATAGCG
GAGTGTATAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGG
TTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTC
AAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATT
CAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGG
TGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAG
ATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTA
TTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTTGAGT
ACTCACAGTCACAGAAAAAGCATCTTACGGATGGCATGACAGGTAAGAGAATTATGCAGTGCCTGCATAACCCA
TGAAGTGATTACACTGCCGCCACTACTCTGACACCGATTCCGGGAGGAAACGCGAT

Blast Nucleotides Analysis

Score	Expect	Identities	Gaps	Strand	Frame

241 bits(130) 2e-61() 140/145(97%) 0/145(0%) Plus/Plus

Features:

NF-kappa-B essential modulator isoform 3

NF-kappa-B essential modulator isoform 4

```
Query  1         GGCATGCAGCTGGAAGATCTGAGGCAACAGCTCCAGCAAGCTGAGGAGGCCCTGGTAGCC  60
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Sbjct  65697548  GGCATGCAGCTGGAAGATCTGAGGCAACAGCTCCAGCAAGCTGAGGAGGCCCTGGTAGCC
65697607

Query  61        AAACAGGAATTGATTGATAAGCTGAAAGAGGAAGCTGAGCAGCACAAGATTGTCATGGAG  120
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Sbjct  65697608  AAACAGGAATTGATTGATAAGCTGAAAGAGGAGGCTGAGCAGCACAAGATTGTGATGGAG
65697667

Query  121       ACCGTTCCAGTCTTGAAAGCCCAGG  145
                 || || |||||||||| |||||||
Sbjct  65697668  ACTGTGCCAGTCTTGAAGGCCCAGG  65697692
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UQ1 UBA domain

Result of Gene sequencing of plasmids isolated from bacteria used for UQ1 UBA protein expression

GTCCAATCCTCAAATCGGATCTGGTTCCGCGTGGATCCGTCAGATTTCAGCAACAACTGGAACAACTCAGTGCAA
TGGGATTTTTGAACCGTGAAGCAAACTTGCAAGCTCTAATAGCAACAGGAGGTGATATCAATGCAGCTATTGAAA
GGTTACTGGGCTCCTAACTCGAGCGGCCGCATCGTGACTGACTGACGATCTGCCTCGCGCGTTTCGGTGATGAC
GGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGAC
AAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCGCAGCCATGACCCAGTCACGTAGCGATA
GCGGAGTGTATAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAA
TGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACA
TTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGT
ATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCT
GGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTA
AGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGG
TATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAG
TACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATG
AGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAAGGACCGAAGAGCTAACCCGCTTTTTTTGCACA
ACATGGGGGGATCATGTAACTCGCCTTGATCGTTTGGGCAACGGAGCTGATTGAAAGCCATACCAACCGACGAG
GCGTGAACACCACGATTGCCTGCAGCAATGGGCAACACGGTGCGCGCAACCTATTTGAG

Blast Nucleotides Analysis

Score	Expect	Identities	Gaps	Strand

233 bits(126) 4e-59() 126/126(100%) 0/126(0%) Plus/Minus

Features:

Ubiquilin-1 isoform 2

Ubiquilin-1 isoform 1

```
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Sbjct  56102184  GTCAGATTTCAGCAACAACTGGAACAACTCAGTGCAATGGGATTTTTGAACCGTGAAGCA
56102125

Query  61        AACTTGCAAGCTCTAATAGCAACAGGAGGTGATATCAATGCAGCTATTGAAAGGTTACTG  120
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Sbjct  56102124  AACTTGCAAGCTCTAATAGCAACAGGAGGTGATATCAATGCAGCTATTGAAAGGTTACTG
56102065

Query  121       GGCTCC  126
                 ||||||
Sbjct  56102064  GGCTCC  56102059
```

IsoT ZnFUBP

Result of Gene sequencing of plasmids isolated from bacteria used for IsoT ZnFUBP protein expression

GGCCAGTCTCAATCGGATCTGGTTCCGCGTGGATCCAAGCAGGAGGTGCAGGCATGGGATGGGGAAGTACGGC
AGGTGTCTAAGCATGCCTTCAGCCTCAAGCAGTTGGACAACCCTGCTCGAATCCCTCCCTGTGGCTGGAAGTGC
TCCAAGTGTGACATGAGGGAGAACCTGTGGCTCAACCTGACTGATGGCTCCATCCTCTGTGGGCGACGCTACTT
CGATGGCAGTGGGGGCAACAACCACGCTGTGGAGCACTACCGAGAGACAGGCTACCCGTTAGCTGTCAAGCTG
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GTGACTGACTGACGATCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAG
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TCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGG
AAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCC
TGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTT
TTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGG
GTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAGATCCTTGAGAGTTTCGCCCCGAAGAACGTTT
TCCATGATGAGCACTTTTAAGTTCTGCTATGTGGCGCGTATTTATCCGTGTGACGTGCAGAGCACTCGTCGCCGC
ATACATTTTCGGAATGACCTGGTTGAGATACGTCA

Analysis

Score	Expect	Identities	Gaps	Strand
339 bits(183)	1e-90()	185/186(99%)	0/186(0%)	Plus/Plus

Features:

ubiquitin carboxyl-terminal hydrolase 5 isoform 1

ubiquitin carboxyl-terminal hydrolase 5 isoform 2

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Query  1        GTGGCTGGAAGTGCTCCAAGTGTGACATGAGGGAGAACCTGTGGCTCAACCTGACTGATG  60
                |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  6819598  GTGGCTGGAAGTGCTCCAAGTGTGACATGAGAGAGAACCTGTGGCTCAACCTGACTGATG  6819657

Query  61       GCTCCATCCTCTGTGGGCGACGCTACTTCGATGGCAGTGGGGGCAACAACCACGCTGTGG  120
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Sbjct  6819658  GCTCCATCCTCTGTGGGCGACGCTACTTCGATGGCAGTGGGGGCAACAACCACGCTGTGG  6819717

Query  121      AGCACTACCGAGAGACAGGCTACCCGTTAGCTGTCAAGCTGGGCACCATCACCCCTGATG  180
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Sbjct  6819718  AGCACTACCGAGAGACAGGCTACCCGTTAGCTGTCAAGCTGGGCACCATCACCCCTGATG  6819777

Query  181      GAGCTG  186
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Sbjct  6819778  GAGCTG  6819783
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p62/Sequestosome UBA domain

Result of Gene sequencing of plasmids isolated from bacteria used for p62 UBA protein expression

GGTTCCAGTCCTCAAATCGGATCTGGTTCCGCGTGGATCCCCGCCAGAGGCTGACCCGCGGCTGATTGAGTCCC
TCTCAAGAGGCTGTCCATGGGCTTCTCTGATGAAGGCGGCTGGCTCACCAGGCTCCTGCAGACCAAGAACTATG
ACATCGGAGCGGCTCTGGACACCATCCAGTATTCAAAGCATTGACTCTCCCCGGCCGCATCGTGACTGACTGAC
GATCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTT
GTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCACCGGGTGTTGGCGGGTGCCCGGGCG
CAACCATGACCCACTCACCTACCGTTCGCGGAGA

Analysis

Score	Expect	Identities	Gaps	Strand
252 bits(136)	1e-64()	143/146(98%)	1/146(0%)	Plus/Plus

Features:
Sequestosome-1 isoform 1
Sequestosome-1 isoform 2

```
Query  1          CAGAGGCTGACCCGCGGCTGATTGAGTCCCTCT-CAAGAGGCTGTCCATGGGCTTCTCTG  59
                  |||||||||||||||||||||||||||||||| | ||| ||||||||||||||||||||
Sbjct  173988599  CAGAGGCTGACCCGCGGCTGATTGAGTCCCTCTCCCAGATGCTGTCCATGGGCTTCTCTG
173988658

Query  60         ATGAAGGCGGCTGGCTCACCAGGCTCCTGCAGACCAAGAACTATGACATCGGAGCGGCTC  119
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  173988659  ATGAAGGCGGCTGGCTCACCAGGCTCCTGCAGACCAAGAACTATGACATCGGAGCGGCTC
173988718

Query  120        TGGACACCATCCAGTATTCAAAGCAT  145
                  |||||||||||||||||||||||||
Sbjct  173988719  TGGACACCATCCAGTATTCAAAGCAT  173988744
```

Vps9 CUE domain

Result of Gene sequencing of plasmids isolated from bacteria used for Vps9 CUE protein expression

GGCGCCATCTCAAATCGGATCTGGTTCCGCGTGGATCCGAAGAAGACGTTTCTTCGTTAATTAAGAAAATTGAAG
AGAACGAACGAAAGGACACGTTGAACACATTACAGAATATGTTTCCAGATATGGACCCAAGCCTGATAGAGGAT
GTTTGTATTGCTAAAAAATCGCGTATTGGGCCCTGTGTTGATGCTCTACTTTCTCTGTCAGAATAACTCGAGCGG
CCGCATCGTGACTGACTGACGATCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCT
CCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGG
TGTTGGCGGGTGTCGGGGCGCACCCATGACCCAGTCACGTACCGATAGAGGAGTGTATAATTCTTGAAGACGA
AAGGGCCTCTTGATACGCCTATTTTTATAGGTTAATGTCATGATCATAATGGTTTCCTAGACCTCACGTGGGTCTT
TTTG

>ref|NC_001145.3| Saccharomyces cerevisiae S288c chromosome XIII, complete sequence
Length=924431

Features in this part of subject sequence:
Vps9p

Score = 327 bits (177), Expect = 1e-89
Identities = 177/177 (100%), Gaps = 0/177 (0%)
Strand=Plus/Minus

```
Query  7
GAAGAAGACGTTTCTTCGTTAATTAAGAAAATTGAAGAGAACGAACGAAAGGACACGTTG  66

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78511
GAAGAAGACGTTTCTTCGTTAATTAAGAAAATTGAAGAGAACGAACGAAAGGACACGTTG  78452

Query  67
AACACATTACAGAATATGTTTCCAGATATGGACCCAAGCCTGATAGAGGATGTTTGTATT  126

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78451
AACACATTACAGAATATGTTTCCAGATATGGACCCAAGCCTGATAGAGGATGTTTGTATT  78392

Query  127
GCTAAAAAATCGCGTATTGGGCCCTGTGTTGATGCTCTACTTTCTCTGTCAGAATAA  183

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78391
GCTAAAAAATCGCGTATTGGGCCCTGTGTTGATGCTCTACTTTCTCTGTCAGAATAA  78335
```

Appendix II Antibodies

Antibody for indicated protein	Animal source	Concentration used mostly	Buffer used for Western blot	Manufacturing company
Ubiquitin	Rabbit	1:1000	TBST	House made
Lys48-specific	Rabbit	1:1000	TBST	Millipore-05-1307
Lys63-specific (HRP) conjugated	Mouse	1:1000	TBST	Enzo-PW0605
Actin	Rabbit/Mouse	1:1000/1:5000	TBST	Sigma –A2066
E2-25K	Rabbit	1:1000	TBST	Enzo Life Sciences (BML-UG9520)
USP5/IsoT	Rabbit	1:750	TBST	Proteintech Europe (10473-1-AP)
p-TAK1	Rabbit	1:1000	TBST	Cell Signalling Technology (#45365)
ER72	Rabbit	1:1000	TBST	GeneTex-GTX115263
Synaptophysin	Mouse	1:10000	TBST	Cell signalling technology-573822
CoxIV	Rabbit	1:1000	TBST	Calbiochem-4844
GST	Rabbit	1:5000	TBST	BETHYL-A190-122A

Appendix III ubiquitin substrates

Product name	Ub length	Manufacturing company
monoUb	Ub1	Enzo-BML-UW8610
Lys48-linked	Ub2-7, Lys48-linked	Enzo-BML-UW8860
Lys63-linked	Ub2-7, Lys63-linked	Enzo-BML-UW9570
Linear diUb	Ub2	Enzo-BML-UW0775
Linear triUb	Ub3	Enzo-BML-UW0780
Linear tetraUb	Ub4	Enzo-BML-UW0785
$Ub5^{+1}$ polyUb chains	Ub5	Enzo-BML-UW8855