Proteasome-Associated Deubiquitinating Enzymes in Fission Yeast

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Declaration

I declare that :

a. this thesis was composed by myself

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c. this work has not been submitted for any other degree or professional qualification.

Miranda Stone

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Abstract

Ubiquitination is a well characterised modification that has a role in the regulation of diverse cellular processes such as proteasome mediated proteolysis, ribosomal function and postreplicative DNA repair (Pickart, 2001). The deubiquitinating enzymes (DUBs) are required in the cell to recycle ubiquitin, process ubiquitin precursor proteins and possibly to edit ubiquitin chains (Chung and Baek, 1999; Wilkinson, 2000). Sequence homology searches have revealed that there are 18 predicted DUBs in the *Schizosaccharomyces pombe* genome (C. Semple, M. Stone and C. Gordon, unpublished).

Studies using deletion strains for each of the *Saccharomyces cerevisiae* DUBs have shown that none is essential and that there is a high level of redundancy (Amerik et al., 2000). Therefore, it is proposed that the functional specificity of the DUBs may be determined by their cellular localisation. Regulation of 26S proteasome-mediated degradation is a major function of ubiquitination, implying that there might be a subset of redundant DUBs localised at the proteasome.

Two putative proteasome-associated DUBs were identified in *S. pombe*. Uch2 had previously been suggested to copurify and colocalise with the proteasome (Li et al., 2000). A second DUB, Ubp6, was identified in a sequence homology search for *S. pombe* proteins containing a ubiquitin-like (UBL) domain (C. Semple and C. Gordon, unpublished). This domain has been shown to mediate interactions with the proteasome, implying that Ubp6 might also be proteasome-associated (Wilkinson et al., 2001).

In the first part of this study, both Uch2 and Ubp6 are shown to be proteasome-associated DUBs. The *S. pombe* proteasome is purified and Uch2 and Ubp6 are both demonstrated to be present. In support of this finding, immunofluorescence microscopy reveals that Uch2 and Ubp6 colocalise with the proteasome at the nuclear periphery (Wilkinson et al., 1998).

Construction of *uch2* and *ubp6* null mutants and the *uch2ubp6* double mutant is described. These mutants do not have any obvious phenotype, suggesting that other redundant DUBs may be present at the proteasome. *ubp6* is shown to be synthetically lethal with the *mts1*, *mts2* and *mts3* proteasome mutants, however *uch2* is not synthetically lethal with any proteasome mutant tested (Gordon et al., 1993; Gordon et al., 1996; Penney et al., 1998; Wilkinson et al., 1997; Wilkinson et al., 2000, C. Gordon, unpublished).

The DUBs function to cleave the αNH-peptide and the εNH-isopeptide bonds between ubiquitin and other species (Chung and Baek, 1999; Wilkinson, 2000). Purified 26S proteasomes and recombinant Uch2 are shown to cleave peptide linked ubiquitin using an in vitro assay and Uch2 is identified as the major ubiquitin hydrolase of the proteasome. Both Uch2 and Ubp6 are demonstrated to be capable of cleaving isopeptide-linked ubiquitin in vitro.

Abbreviations

Ala	alanine
AMC	7-amino-4-methylcoumarin
APC	anaphase promoting complex
ATP	adenosine triphosphate
BSA	bovine serum albumin
CDK	cyclin dependent kinase
C-terminal	carboxyl-terminal
Cys	cysteine
DAPI	4,6-diamidino-2-phenylindole
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DUB	deubiquitinating enzyme
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid disodium salt
EM	electron microscopy
EMM	Edinburgh minimal medium
EMS	ethyl methanesulfonate
ER	endoplasmic reticulum
GFP	green fluorescent protein
Gly	glycine

GST	glutathione S transferase
HA	haemaglutinin
His	histidine
HRP	horseradish peroxidase
IFN-γ	interferon-γ
IκB	inhibitor of NFκB
IPTG	isopropylthio-β-D-thioglactopyranosidase
kb	kilobase pairs
kD	kilodaltons
Leu	leucine
Lys	lysine
MBC	methyl benzimidazole-2-yl carbamylate
ME	malt extract
MAPK	mitogen-activated protein kinase
Met	methionine
$MgCl_2$	magnesium chloride
min	minutes
mRNA	messenger RNA
mts	MBC-resistant temperature sensitive
NEM	N-ethylmaleimide
NFκB	nuclear factor κB
NMJ	neuromuscular junction
NMR	nuclear magnetic resonance
nmt	no message thiamine
N-terminal	amino-terminal

OD	optical density
oligo	oligonucleotide
PA	protein A
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	PBS Tween
PCR	polymerase chain reaction
PEG	polyethylene glycol
Phe	phenylalanine
Plk	polo-like kinase
RNA	ribonucleic acid
rpm	revolutions per minute
Rpn	regulatory particle nonATPase
Rpt	regulatory particle ATPase
rt	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
Ser	serine
SPB	spindle pole body
S. pombe	*Schizosaccharomyces pombe*
TBS	Tris buffered saline
TBST	TBS Tween
Thr	threonine
tRNA	transfer ribonucleic acid
Tyr	tyrosine
ts	temperature sensitive

Ub	ubiquitin
UBA domain	ubiquitin-associated domain
Ubal	ubiquitin aldehyde
UBC	ubiquitin conjugating enzyme
UBL domain	ubiquitin-like domain
U-box	Ufd2-box
UBP	ubiquitin-specific processing protease
UBS	UBL-binding sequence
UCH	ubiquitin C-terminal hydrolase
UDP	ubiquitin-like domain protein
UIM	ubiquitin interaction motif
ura	uracil
UV	ultraviolet
YES	yeast extract supplemented medium

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Chapter 1 Introduction

1.1 Ubiquitination

1.1.1 Ubiquitin

Ubiquitin is a small protein of 76 amino acids, which is remarkably conserved throughout eukaryotes (Weissman, 2001; Ozkaynak et al., 1984). Multiple mRNAs encode ubiquitin and in each case the protein is synthesised as a precursor of either tandem copies of ubiquitin or ubiquitin fused to ribosomal proteins (Finley et al., 1989; Lund et al.1985; Ozkaynak et al., 1987). The crystal structures of ubiquitin from several eukaryotes have been solved, showing a highly conserved, compact structure comprising a five stranded β-sheet and an α-helical section (Vijay-Kumar et al., 1987b; Vijay-Kumar et al., 1987a). The C-terminus of the ubiquitin protein protrudes from the core structure, probably to expose the C-terminal Gly residue for conjugate formation (Vijay-Kumar et al., 1987a).

1.1.2 Functions of Ubiquitination

In eukaryotic cells, a vast array of proteins is modified by the covalent conjugation of ubiquitin, or ubiquitination. Therefore, this modification has a role in diverse cellular processes such as proteasome mediated proteolysis, endocytosis and postreplicative DNA repair (Pickart, 2001; Weissman, 2001). Proteins may be modified by either a single ubiquitin or a chain of isopeptide linked ubiquitin monomers and in almost all cases the C-terminal Gly of ubiquitin is ligated to a Lys residue in the target protein. Monoubiquitination has been found to have several functions including histone regulation, viral budding and the internalisation of plasma membrane proteins (Hicke, 2001). Ubiquitin chains are formed by the covalent attachment of ubiquitin monomers through internal Lys residues and of the seven Lys residues found in ubiquitin, four (Lys11, Lys29, Lys48 and Lys63) are

able to mediate chain formation in vivo (Dubiel and Gordon, 1999). Mutation of each of the Lys residues in budding yeast ubiquitin demonstrated that only Lys48 was essential; cells expressing ubiquitin K48R arrested at G2-M and were deficient in the degradation of canavanine-containing proteins, indicating a defect in proteolysis (Finley et al., 1994; Spence et al., 1995). The role of Lys48 linked ubiquitin chains in targeting proteins for degradation by the proteasome has been well documented and a chain of four ubiquitins is the minimal targeting signal (Thrower et al., 2000). Mutation of any of the other Lys residues in ubiquitin was not lethal, however, the ubiquitin K63R mutant was found to be defective in DNA repair (Spence et al., 1995). Further studies of Lys63 linked ubiquitin chains have confirmed that they are involved in regulating of the DNA repair response and also demonstrated that they function in endocytosis and targeting yeast transporters for vacuolar degradation (Weissman, 2001; Hicke, 2001).

1.1.3 The Process of Ubiquitination

Ubiquitination is catalysed by a cascade of three enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase (Figure 1.1). Several rounds of catalysis by these enzymes can result in the formation of a ubiquitin chain. The specificity of ubiquitination increases through the pathway with few E1s, a number of E2s and many E3s. The activities, structures and specificities of each of these families of enzymes will be discussed below.

1.1.4 E1 Ubiquitin-Activating Enzymes

The first step in ubiquitination is the activation of ubiquitin by an E1 ubiquitin-activating enzyme. Initially, a ubiquitin adenylate intermediate is formed by the sequential binding of MgATP and ubiquitin to the E1. The ubiquitin adenylate is then converted to a ubiquitin thiolester and a second ubiquitin is activated as a ubiquitin adenylate. Therefore, the process results in the formation of a ternary

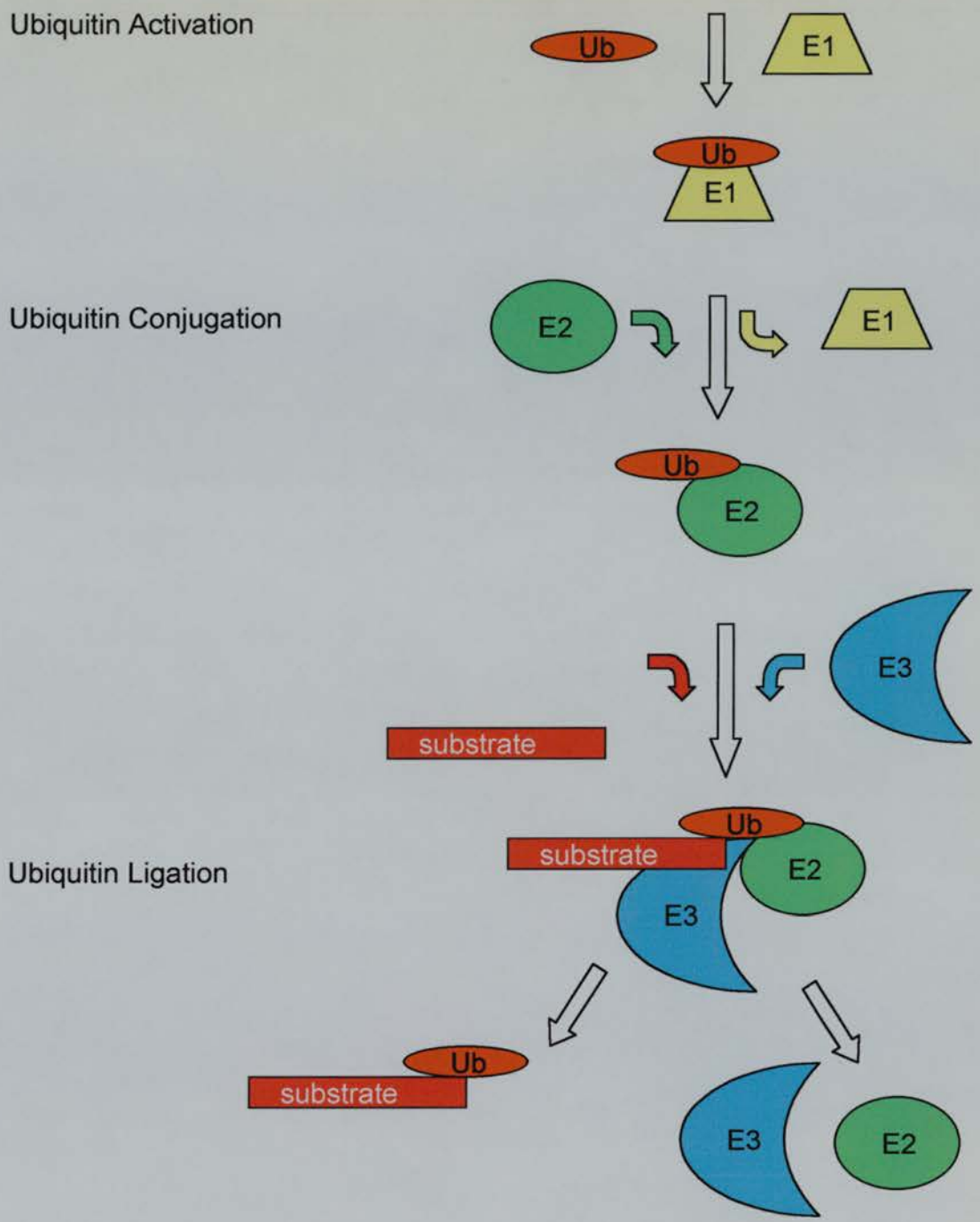

Figure 1.1: The Ubiquitin Pathway
Ubiquitin is covalently attached to a substrate protein by the sequential action of three enzymes. The E1 Enzyme activates ubiquitin as a thiol ester, the activated ubiquitin is then carried by the E2 ubiquitin conjugating enzyme to the E3 enzyme, which facilitates the ligation of ubiquitin to the substrate. The ubiquitinated substrate, the E2 and E3 are released and further rounds of conjugation may be used to create a multiubiquitin chain.

complex of E1-Ub-AMP-Ub and involves the hydrolysis of two ATP molecules. The thiol ubiquitin in this complex is activated, ready to be received by the E2 enzyme (Haas and Rose, 1982).

The ubiquitin activation reaction is carried out by a single E1 in most species. In *S. cerevisiae* and *S. pombe* the E1 enzymes are encoded by the UBA1 and *ptr3*$^{+}$ genes respectively and both have been shown to be essential for viability. Ptr3 is 59% homologous to Uba1 and the human E1 and all three proteins contain a conserved glycine rich motif. Both Ptr3 and Uba1 are localised in both the nucleus and the cytoplasm (McGrath et al., 1991; Azad et al., 1997). In human cells, two isoforms of the E1 ubiquitin-activating enzyme exist: E1a and E1b. The larger E1a isoform is phosphorylated and is found only in the nucleus, whereas E1b is found predominantly in the cytoplasm (Handley-Gearhart et al., 1994; Stephen et al., 1997).

1.1.5 E2 Ubiquitin-Conjugating Enzymes

The function of the E2 enzymes is to carry ubiquitin between the E1 and E3 enzymes as an active thiol ester. There are 11 E2s in the *S. cerevisiae* genome and 14 are predicted to be present in *S. pombe*. There is considerable homology between E2s, with all containing a conserved region of approximately 150 amino acids, which includes the active site cysteine. A number of the E2s also contain N- or C-terminal extensions that have been suggested to confer specificity (Jentsch, 1992).

X-ray crystal structures of the *A. thaliana* UBC1, *S. cerevisiae* Ubc4 and clam E2-C show that the E2 core structure of four α-helices and a four stranded β-sheet is highly conserved. All major secondary structures align well between E2s but the highest conservation of tertiary structure is seen in a group of buried hydrophobic residues and in the surface around the ubiquitin-accepting Cys (Cook et al., 1992; Cook et al., 1993; Jiang and Basavappa, 1999). NMR studies of the human E2, HsUbc2b, demonstrated that ubiquitin interacts with an area on this surface via its C-terminal amino acids 70-76, Lys48 and Gln49 (Miura et al., 1999).

The roles of E2s are diverse, with some having a general role in proteolysis of short-lived and misfolded proteins and others being involved in more specific processes such as cell cycle progression, development and spermatogenesis (Hershko and Ciechanover, 1998). Two conserved E2s that have been characterised in *S. pombe* are UbcP4 and Rhp6. UbcP4 is a mitotic E2, which is required for G2-M progression and the metaphase to anaphase transition (Osaka et al., 1997). Rhp6 and its homologue in *S. cerevisiae*, Rad6, are both involved in regulation of DNA repair, UV mutagenesis and sporulation (Reynolds et al., 1990).

Many reports have focused on the interactions of E2s with their cognate E3s (Pickart, 2001). These studies have shown that a single E3 may interact with several different E2s, for example, the E6AP hect domain E3 can interact with both UbcH7 and UbcH8 E2 (Kumar et al., 1997). Conversely, a particular E2 may bind a number of E3s, as is the case for UbcH7, which can bind both HHARI and H7-AP1 E3s (Moynihan et al., 1999). The specific nature of the E2-E3 association have been investigated using binding assays and x-ray crystallography (Pickart, 2001). Using chimaeric E2s, the region of UbcH5 that includes the active site Cys and a conserved Phe have been shown to be crucial for its interaction with hect E3s (see section 1.1.6.1; Nuber and Scheffner, 1999). The crystal structure of the SCF and E6AP-UbcH7 complexes have been solved, providing insights into the mechanism of action (sections 1.1.6.1 and 1.1.6.2.1; Nuber and Scheffner, 1999; Zheng et al., 2002).

1.1.6 E3 Ubiquitin Ligases

The E3s are the largest family of proteins in the E1-E2-E3 cascade and they facilitate the transfer of ubiquitin from E2 to substrate, and bring specificity to the system. The two major classes of E3s that have been characterised are the hect domain E3s and the RING domain containing E3s and until recently, it was thought that almost all E3s belonged to one of these groups. However, further study has identified a novel

family of E3s named the U-box proteins, which contain RING-like domains. Each of these three groups of E3s will be discussed below.

1.1.6.1 hect domain E3s

Numerous hect E3s have been identified in the available genome databases, however, the first hect domain protein to be characterised was E6AP from oncogenic human papilloma virus (Pickart, 2001; Scheffner et al., 1993) . E6AP was found to act in a complex with E6 to ubiquitinate p53 and while E6 was shown to provide specificity for p53, E6AP was demonstrated to be the active ubiquitin ligase (Scheffner et al., 1993). Further studies identified several proteins that showed similarity to a domain of approximately 350 amino acids found at the C-terminus of E6AP, named the hect domain. In each case this domain was found at the C-terminus, with the proteins being divergent at their N-termini. A number of the hect domains were shown to form thioester bonds with ubiquitin and a conserved Cys residue within the domain was found to be critical for this activity (Huibregtse et al., 1995). Therefore, it was suggested that all hect domains would function as ubiquitin ligases using a similar mechanism, a proposal that has been supported by subsequent work (Pickart, 2001). The crystal structure of E6AP bound to the UbcH7 E2 has been solved and shows that the hect domain is composed of two lobes joined by a hinge. These two lobes make a U-shaped structure with the E2. The active site Cys of E6AP is located at the junction between the two lobes; however, this is not close enough to the E2 active site for nucleophilic attack to mediate the transfer of the ubiquitin thioester. Therefore, the mechanism of hect E3 activity remains undetermined (Nuber and Scheffner, 1999). As the C-termini of hect domain E3s provide the E2 binding site and the catalytic activity, it is the N-termini that bring substrate specificity, for example, Rsp5 hect E3 binds its substrate Rbp1 (the large subunit of RNA polymerase II) via a WW domain located N-terminally of the hect domain (Wang et al., 1999).

1.1.6.2 RING E3s

The RING family of E3 ubiquitin ligases all contain a protein with a RING (Really Interesting New Gene) finger domain, which has a structure containing conserved Cys and His residues that allow the incorporation of two zinc ions (Pickart, 2001). The function of RING E3s appears to be as a molecular scaffold that brings the E2 linked ubiquitin thioester and the substrate protein into close proximity. Unlike the hect E3s, there is no evidence that RING E3s can form a ubiquitin thioester (Joazeiro and Weissman, 2000; Pickart, 2001). RING E3s may be a single protein, for example, c-Cbl, which is involved in the down-regulation of receptor protein tyrosine kinases and Mdm2, which regulates p53 turnover. Other RING E3s are multisubunit complexes, where just one subunit contains a RING finger domain (Joazeiro and Weissman, 2000; Pickart, 2001). Examples of these multisubunit E3s are the well-characterised SCF and APC complexes, both of which are discussed in further detail below (Tyers and Jorgensen, 2000).

1.1.6.2.1 SCF

The SCF complex has been well defined as a highly conserved multisubunit E3 ubiquitin ligase, which has a wide range of substrates including many cell cycle regulators. The SCF complex is made up of a core complex containing the Rbx1 RING finger protein, Skp1 protein, a cullin and an E2. An additional variable subunit, an F-box protein, associates with the core and provides the substrate specificity of the SCF (Pickart, 2001; Tyers and Jorgensen, 2000) .

Rbx1 is a small, highly conserved protein containing a RING finger, which was first identified in the VHL ubiquitin ligase complex, but is also found at the heart of the SCF complex (Kamura et al., 1999a). Rbx1 brings the subunits of the E3 together, as it can interact with cullins and promotes association of cullin with E2. The importance of this function is indicated by the fact that Rbx1 is essential in budding

yeast and that its assembly into the SCF complex results in potent activation of ubiquitin ligase activity (Kamura et al., 1999a; Skowyra et al., 1999).

A variety of F-box proteins associate with the SCF to act as receptors for specific substrates, for example, the F-box protein, Cdc4, recognises phosphorylated Sic1 as a substrate and Grr1 recognises Cln1 (Feldman et al., 1997; Skowyra et al., 1997). The phosphorylation state of many SCF substrates is controlled in a cell cycle dependent manner, and provides a mechanism for cell-cycle stage dependent ubiquitination and degradation of regulatory factors (Laney and Hochstrasser, 1999; Pickart, 2001). The interaction of the F-box proteins with the SCF is via the Skp1 subunit, which recognises the F-box motif found in all F-box proteins. As well as regulation of substrate specificity by the F-box proteins, the SCF is also regulated by modification with the small ubiquitin-like modifier, Rub1/Nedd8. The ubiquitin ligase activity of the SCF is promoted by conjugation of Rub1/Nedd8 to the SCF cullin subunit (see section 1.1.8.1.2).

The crystal structure of the Cul1-Rbx1-Skp1-F-box^{Skp2} SCF complex has recently been solved. The structure reveals that Cul1 acts as a scaffold and has an elongated structure with its N-terminus binding to Skp1-F-box^{Skp2} and the C-terminus binding Rbx1. The Cul1 C-terminal domain is made up of a five-stranded β-sheet, with the second strand provided by the conserved Rbx1 N-terminus, indicating that Rbx1 binds Cul1 via strand insertion. When combined with data from other E2-E3 structures, it is predicted that the E2 and substrate will be positioned on the same side of the complex, thus allowing ligation of the activated ubiquitin (Zheng et al., 2002).

1.1.6.2.2 APC

The APC is a second type of multisubunit RING E3, but with at least 12 subunits in budding yeast, it is a much larger complex than the SCF. The APC is similar to the SCF in that it contains a cullin, Apc2, and a RING finger protein, Apc11. Both of these subunits are essential in budding yeast indicating that they are likely to be at

the core of the complex, as for the SCF. Apc11 is crucial for the function of the APC as it is the only APC subunit that is able to show ubiquitin ligase function in vitro (Gmachl et al., 2000). The function of the APC as an E3 is of particular importance in cell cycle regulation, as it is required at anaphase, mitotic exit and to maintain G1 (Page and Hieter, 1999; Zachariae and Nasmyth, 1999).

Regulatory subunits of the APC are required for specific activation of APC mediated ubiquitination of cell cycle components. Genetic studies in yeast and *Drosophila* have shown that APC dependent degradation is regulated by Cdc20/Fizzy/Slp1 and Cdh1/Fizzy-related/Srw1/Ste9. These proteins both contain WD repeats, which are implicated as substrate specific binding sites (Zachariae and Nasmyth, 1999). The association of Cdc20 and Cdh1 with the APC is dependent on their phosphorylation state and both are phosphorylated in vitro by Cdc2-Cyclin B. Cdc20 must be phosphorylated to activate the APC but conversely, Cdh1 must be in its unphosphorylated form for activation (Kotani et al., 1999). The result of Cdc20 and Cdh1 mediated activation is a cell cycle stage specific regulation of the APC, with Cdc20 required at mitosis and Cdh1 at G1 (Zachariae and Nasmyth, 1999). Therefore, ubiquitination by both the SCF and APC is regulated by cell cycle dependent phosphorylation. However, an important difference is that the SCF is constitutively active and it is the substrates that are phosphorylated, whereas for the APC it is the activity of the E3 complex itself that is regulated by phosphorylation.

Polo-like kinases also play a role in the regulation of the APC, as they are able to phosphorylate three APC subunits. There is considerable evidence that in yeast, *Xenopus* and mammalian cells Plks act as positive regulators of the APC (Nigg, 1998). Plx activity is required in *Xenopus* for cyclin degradation and Cdk inactivation, to allow exit from mitosis: expression of a dominant negative inactive version of Plx prevents mitotic exit and depletion of Plk from *Xenopus* oocyte extracts results in inhibition of degradation of known APC substrates (Descombes and Nigg, 1998). The budding yeast Polo-like kinase, Cdc5p is a positive regulator of APC dependent cyclin ubiquitination and Cdc5p is also a substrate of the APC, permitting coordinated cell cycle stage specific regulation of APC activity (Charles

et al., 1998; Nigg, 1998). The physiological relevance of Plk activation of the APC may be to provide further substrate specificity, as Plk was found to activate mammalian APC specifically for ubiquitination of cyclin B but not Cut2 (Charles et al., 1998; Kotani et al., 1999).

1.1.6.3 U-Box Proteins

The U-Box is a domain that resembles the RING finger domain, except that it uses salt-bridges to stabilise its structure as it lacks the zinc binding motifs (Cyr et al., 2002). UFD2 was the first protein to be found to contain a U-box domain and a role for this domain in ubiquitination was suggested by the fact that UFD2 was found to act as a ubiquitin chain elongation factor. It promoted the extension of ubiquitin chains, but required the presence of an E3 for this activity, as it was unable to interact with E2s directly. UFD2 was therefore originally termed an E4, rather than an E3 (Koegl et al., 1999). However, further studies with the mammalian homologue of UFD2 and other U-box proteins suggest that the U-box acts in a similar way to the RING finger, as an E3 ubiquitin ligase (Cyr et al., 2002). A possible role for the U-box in promoting the ubiquitination and degradation of misfolded proteins has been proposed (Cyr et al., 2002). The U-box protein CHIP was found to ubiquitinate unfolded proteins with a requirement for the U-box domain. CHIP can interact directly with the chaperone proteins Hsp90 and Hsp70 and can thus ubiquitinate chaperone-bound misfolded substrates (Murata et al., 2001). Binding of the BAG-1 chaperone cofactor, which contains a ubiquitin-like domain, is thought to target CHIP to the proteasome (Demand et al., 2001 and see section 1.1.9.2). Therefore, the U-box proteins are emerging as a new family of E3 ubiquitin ligases that may act specifically in the ubiquitination of misfolded proteins.

1.1.7 Targeting Proteins for Ubiquitination

A wide range of proteins are targeted for ubiquitination and the modification may be of a specific Lys, any one of the target's Lys residues, or in many cases several Lys residues (Weissman, 2001). Proteins that are destined for proteolysis by the 26S proteasome are marked for ubiquitination by a number of sequence motifs and modifications, which may be recognised by specific E3 ubiquitin ligases. The simplest of ubiquitination signals is the N-terminal residue of the protein. In a process named the N-end rule, proteins where the N-terminal residue is either a basic (Arg, His, Lys) or hydrophobic (Phe, Leu, Tyr, Trp, Ile) are recognised by the N-end rule E3, Ubr1 (Xie and Varshavsky, 2000; Pickart, 2001). Ubiquitination of substrates of the APC, including many cyclins, depends on a nine residue motif called the destruction box, or D-box that contains key Arg and Lys residues (Koepp et al., 1999; Laney and Hochstrasser, 1999). An alternative signal is the PEST region, which lacks a consensus sequence but is enriched for Proline (P), Glutamic Acid (E), Serine S and Threonine (T). This motif is found to target G1 cyclins and IκBα for ubiquitin dependent proteolysis, but its mechanism of action remains ambiguous (Rechsteiner and Rogers, 1996). In the case of the SCF, many substrates, such as the Sic1 Cdk inhibitor and the G1 cyclins, bind to the F-box subunits in a phosphorylation-dependent manner (Laney and Hochstrasser, 1999; Pickart, 2001). Finally, for the mating type transcription factor α2, a 19 amino acid hydrophobic region is required for ubiquitination (Laney and Hochstrasser, 1999). These examples indicate the diversity of signals for ubiquitination and show a further level of complexity in the ubiquitin pathway.

1.1.8 Ubiquitin-like Proteins

There are a number of proteins that have significant sequence homology to ubiquitin, and are thus called ubiquitin-like proteins. These can be divided into two subclasses, first, there are the ubiquitin-like modifiers, which show homology to ubiquitin

throughout all or most of their sequence and may be covalently attached to other proteins in the same manner as ubiquitin. The second class comprises the ubiquitin-domain proteins, which have just one domain with homology to ubiquitin and are not conjugated to other proteins (Jentsch and Pyrowolakis, 2000).

1.1.8.1 Ubiquitin-like Modifiers

In addition to ubiquitin, a number of small ubiquitin-like modifying proteins have been identified. Some of these proteins, such as SUMO and Rub1/Nedd8 are related in sequence and structure to ubiquitin but others such as Apg12 show no obvious homology (Jentsch and Pyrowolakis, 2000). However, generally they are related to ubiquitin in the mechanism by which they modify other proteins: they bind to Lys residues in their target proteins and use enzymes that resemble E1s, E2s and E3s (Hochstrasser, 2000; Jentsch and Pyrowolakis, 2000; Yeh et al., 2000). In the case of SUMO, and possibly for Rub1/Nedd8, deconjugating enzymes have also been identified (see section 1.2.5). The ubiquitin-like modifiers are involved in diverse cellular processes including regulation of localisation, ubiquitination, E3 ubiquitin ligase activity, autophagy and polarised morphogenesis (Jentsch and Pyrowolakis, 2000; Dittmar et al., 2002). As SUMO and Rub1/Nedd8 have each been described in number of studies, they are discussed further below.

1.1.8.1.1 SUMO

The best characterised of the ubiquitin-like modifiers is SUMO (also named PIC1, UBL1, GMP1, sentrin, Pmt3, SMT3C and DAP1), which is a highly conserved eukaryotic protein. SUMO is about 18% homologous to ubiquitin and has a short N-terminal extension (Jentsch and Pyrowolakis, 2000; Muller et al., 2001). Despite this relatively low homology, analysis by NMR shows that the SUMO structure contains the ubiquitin fold and is closely related to the ubiquitin structure (Bayer et al., 1998). SUMO is essential in *S. cerevisiae*, although in *S. pombe* cells lacking SUMO are

viable, but are slow growing and stress sensitive (Jentsch and Pyrowolakis, 2000; Tanaka et al., 1999).

Several proteins that are modified by covalent attachment of SUMO have now been identified and the evidence so far is that the function of SUMO conjugation or sumoylation may be to regulate the localisation or ubiquitination of its targets (Muller et al., 2001). The first sumoylated protein to be described was RanGAP1, the GTPase–activating protein for the Ran GTPase. Sumoylation of RanGAP1 was shown to cause it to redistribute from the cytoplasm to the nuclear pore complex (Matunis et al., 1996). The cellular localisation of the RING finger protein, PML was also found to be affected by SUMO conjugation. In this case, the modified form was shown to localise to the PML nuclear bodies, whereas the unmodified form was distributed in the nucleoplasm. The sumoylation of PML was found to be dependent on its phosphorylation state, with hyperphosphorylated PML being resistant to SUMO attachment (Muller et al., 1998). The effect of phosphorylation on sumoylation is reminiscent of the relationship between phosphorylation and ubiquitination (see section 1.1.7). The sumoylation of another substrate, IκBα has also been shown to be regulated by phosphorylation. However, for this target protein, the effect of sumoylation is on its ubiquitination state. IκBα can be modified by either SUMO or ubiquitin, but as both attach via the same Lys residue, this binding is competitive and phosphorylated form of IκBα that can be ubiquitinated, is not sumoylated. Ubiquitination of IκBα leads to its degradation, so that its binding partner NF-κB is released can translocate into the nucleus, where it functions as a transcription factor. Therefore, the sumoylation of IκBα prevents its ubiquitination and therefore inhibits NF-κB mediated transcription (Desterro et al., 1998). This competition between ubiquitination and sumoylation has also been observed for Mdm2, which is the E3 ubiquitin ligase for p53 (Buschmann et al., 2000).

1.1.8.1.2 Rub1/ Nedd8

The ubiquitin-like modifier, Rub1/Nedd8 shows greater homology to ubiquitin than SUMO, with approximately 50% sequence identity and a greater similarity in structure (Jentsch and Pyrowolakis, 2000; Yeh et al., 2000). Unlike SUMO, Rub1/Nedd8 has a narrow target specificity, as it appears to exclusively modify members of the cullin family, which are components of the multisubunit RING finger E3 ubiquitin ligases (see section 1.1.6.2). Rub1/Nedd8 modification of cullins promotes ubiquitination of substrates by the E3 complex, for example, the $SCF^{\beta Trcp}$ cullin, Cul1, is modified by Nedd8, which promotes its association with the substrates IκBα and β-catenin (Read et al., 2000). In *S. cerevisiae*, modification of the cullin component of SCF^{Skp2} by the Nedd8 homologue, Rub1 is required for the ubiquitination of the cyclin dependent kinase inhibitor $p27^{Kip1}$ (Kamura et al., 1999b; Podust et al., 2000).

1.1.9 Ubiquitin-Domain Proteins (UDPs)

The ubiquitin-domain proteins (UDPs) each contain a characteristic domain that has homology to ubiquitin. This domain has largely been characterised through studies of two of the UDPs that are multiubiquitin binding proteins, Dph1/Dsk2/HPLIC and Rph23/Rad23. Invesigation of these proteins has shown UBL domain is similar to ubiquitin both in sequence and in structure: the UBL domains found in Dsk2 and Rad23 are 36% and 22% homologous to ubiquitin and the NMR structure of the hPLIC-2 UBL domain reveals that it closely resembles ubiquitin (Watkins et al., 1993; Biggins et al., 1996; Walters et al., 2002). In the case of Rad23, deletion of the UBL domain results in UV-sensitivity. However, substitution of the UBL domain with ubiquitin is sufficient to rescue this phenotype, indicating the close homology between ubiquitin and the UBL domain (Watkins et al., 1993). There is gathering evidence from a number of studies that the UBL domain may function as a proteasome-binding domain and Mts4/Rpn1 has been identified as a potential receptor for these domains within the proteasome (R. Hartmann-Petersen and M.

Seeger, unpublished). In *S. pombe*, there are 11 predicted UDPs and in human there are homologues for most of these proteins as well as other unique UDPs (Table 1.1; C. Semple and C. Gordon, unpublished; Jentsch and Pyrowolakis, 2000). The discussion here will focus on those conserved UDPs that have been characterised in *S. pombe*.

1.1.9.1 Multiubiquitin Binding UDPs

Two of the UDPs, Dph1/Dsk2/HPLIC and Rhp23/Rad23, function in the recognition and delivery of multiubiquitinated substrates for the proteasome. These proteins have similar domain structure as each contains not only a UBL domain, but also at least one multiubiquitin-binding UBA domain (Hofmann and Bucher, 1996; Wilkinson et al., 2001). The UBL domains mediate association with the proteasome and UBA domains are responsible for binding to multiubiquitin chains. Mutation of the UBL domain and the UBA domain of Rhp23 indicated that both are required for its in vivo function (Wilkinson et al., 2001). The binding of the UBL domains to the proteasome has been shown to be via a direct interaction with the Mts4/Rpn1 19S subunit (R. Hartmann-Petersen and M. Seeger, unpublished). The observation that Dph1 and Rhp23 could bind both the proteasome and multiubiquitin suggested that they might act in a similar way to Pus1/Rpn10, which shares these binding properties (see section 1.3.2.2.1). Genetic experiments in *S. pombe* using null mutants for each of these proteins showed that although the single null mutants *dph1Δ*, *rhp23Δ* and *pus1Δ* were able to grow, the triple mutant *dph1Δrhp23Δpus1Δ* was not viable. This implied that these three proteins act in parallel, redundant pathways to target proteins to the proteasome (Wilkinson et al., 2001). Further support for this model came from studies in *S. cerevisiae*, where *dsk2* and *dsk2rad23* mutants were found to be defective in the degradation proteasome substrates (Rao and Sastry, 2002; Funakoshi et al., 2002). A more recent study has supported this work by showing that the redundancy observed in *S. pombe* is conserved in budding yeast, where the *dsk2rad23rpn10* triple mutant is temperature sensitive, with significant enhancement of the double mutant phenotypes and accumulation of large amounts of

UDP	Pompep ID	Alternative Names	Proposed Function
Udp1	SPBC2D10.12	Rhp23, Rad23, hhR23	Delivery of multiubiquitinated proteasome substrates
Udp2	SPAC26A3.16	Dph1, Dsk2, HPLIC, SonA	Delivery of multiubiquitinated proteasome substrates
Udp3	SPBC16G5.11C	Bag1	Chaperone cofactor
Udp4	SPAC6G9.08	Ubp6	Deubiquitinating Enzyme
Udp5	SPBC1E8.02	Bat3, Bag6, Scythe	Chaperone cofactor
Udp6	SPAC4G9.01	Alp11, TFC-B	Tubulin folding
Udp7	SPCC1442.07C	Wss1	SUMO pathway?
Udp8	SPBC1271.05C	-	DNA binding
Udp9	SPBC646.07C	TSC13	Lipid biosynthesis
Udp10	SPBC1921.02	-	?
Udp11	SPBC16H5.03C	E1, Uba2	Ubiquitin activation

Table 1.1: The Ubiquitin-like Domain Proteins of *S. pombe*
Eleven UDPs have been identified in the *S. pombe* genome and many have been assigned functions in diverse cellular processes – see text for details.

multiubiquitinated proteins (Saeki et al., 2002). A further UBA domain containing UDP, Ddi1 has been identified in *S. cerevisiae*, however its homologue in *S. pombe*, Mud1, lacks the UBL domain. The function of Ddi1/Mud1 remains to be determined, but if it is involved in the recognition and delivery of proteins, Mud1 is likely to interact with other proteins to bind the proteasome.

1.1.9.2 Chaperone Co-Factor UDPs

Another UDP that has been widely investigated is the chaperone regulatory cofactor, BAG-1, which was first identified due to its anti-apoptotic function. It has been shown to bind the Hsp70/Hsc70 chaperones via its BAG domain and to act as a nucleotide exchange factor, promoting the release of substrates (Takayama and Reed, 2001). Studies of human BAG-1 have shown it is able to interact with the proteasome and more recently this has also been demonstrated for S. *pombe* BAG-1 (Luders et al., 2000; M.Stone and C.Gordon, unpublished). As was the case for Dph1 and Rhp23, the N-terminal region of human BAG-1, containing the UBL domain, was found to be required for interaction with the proteasome (Luders et al., 2000). In addition to binding chaperones and the proteasome, BAG-1 is also able to interact with the E3s, CHIP and Siah-1A (Matsuzawa et al., 1998; Demand et al., 2001). It appears that BAG-1 acts at the interface between chaperones, the proteasome and ubiquitination, perhaps to coordinate ubiquitination of misfolded proteins bound to Hsp70/Hsc70 and their release at the proteasome prior to degradation. BAG-1 is not essential in *S. pombe*, therefore other proteins may act in a redundant manner or its function may only be important for specific cellular conditions, such as stress (C.Gordon, unpublished). Two other UDPs have been shown to bind chaperone proteins: human Dsk2 can associate with Hsp70 and Bat3/Scythe binds to and inhibits Hsp70 (Kaye et al., 2000; Thress et al., 2001). The significance of these interactions remains to be determined, but it appears that a subset of the UDPs may act as coupling factors between chaperones and the proteasome.

1.1.9.3 Ubiquitin Pathway Enzyme UDPs

One of the UBP family deubiquitinating enzymes, Ubp6, was identified in *S. cerevisiae* and was shown to have deubiquitinating activity against artificial substrates (Park et al., 1997). Subsequently, a UBL domain, named the SUb domain, was identified at its N-terminus and was found to be conserved in all Ubp6 homologues. Although the human Ubp6 UBL domain was found to be only 20% similar to ubiquitin, structure prediction programs indicated that it was likely to form a ubiquitin-like fold, as was observed for the hPLIC-2 UBL domain crystal structure (Wyndham et al., 1999; Walters et al., 2002). In addition, the E1 ubiquitin-activating enzyme from *S. pombe* contains a putative UBL domain and the human E3 ubiquitin ligases Parkin and VCB are members of the UDP family (Jentsch and Pyrowolakis, 2000).

1.1.9.4 Other UDPs

As well as the proteins already discussed, several other UDPs have been characterised to a lesser extent. The first of these is Wss1, which was identified as a high copy suppressor of a *S. cerevisiae* Smt1 (SUMO) mutant. Deletion of Wss1 did not result in any significant phenotype, apart from mild cold sensitivity and it is unclear whether the genetic interaction with Smt1 is due to Wss1 being a substrate, a regulator or a protein with overlapping function (Biggins et al., 2001). Other UDPs include TSC13, which is an enoyl reductase involved in very long chain fatty acid biosynthesis and Alp11, which has a role in tubulin folding (Kohlwein et al., 2001; Radcliffe and Toda, 2000). Therefore, it appears that UDPs participate in a variety of cellular processes, through domains other than their UBL domains.

1.2 Deubiquitination

Ubiquitination is a dynamic process and therefore there is a requirement for enzymes to catalyse not only the ligation of ubiquitin but also its removal from proteins and multiubiquitin chains. This activity is also required in the cell to release free ubiquitin by processing of the ubiquitin precursor proteins (Figure 1.2). The cleavage of ubiquitin, or deubiquitination, is carried out by a family of enzymes called the deubiquitinating enzymes (DUBs).

1.2.1 Classification of DUBs

Based on their sequence homology, the DUBs can be divided into two classes: the Ubiquitin C-terminal Hydrolases (UCHs) and the Ubiquitin Specific Processing Proteases (UBPs) (Wilkinson, 2000; Chung and Baek, 1999). Both types of DUB are members of the cysteine protease family, which has members in all organisms from bacteria and archaea to human. Based on their evolutionary similarity to other cysteine proteases, both families of DUBs have been assigned to Clan CA, which contains all papain-like proteases (Barrett and Rawlings, 2001). The UCHs are more closely related to papain than the UBPs and their active site contains one Cys and one His that are highly conserved (see section 1.2.2.1). There is significant homology between members of the UCH family and they are all relatively small proteins, usually of less than 40kD (Chung and Baek, 1999; Wilkinson and Hochstrasser, 1998). Conversely, the UBPs are generally large proteins of between 50 and 250kD (Wilkinson and Hochstrasser, 1998). There is considerable diversity within the UBP family, with the exception of two conserved regions that contain catalytically important residues: the Cys box contains the active site Cys and the His box contains two conserved His residues (Wilkinson and Hochstrasser, 1998). There is no apparent similarity between the regions of the UCHs and the UBPs surrounding the Cys and His residues (Wilkinson and Hochstrasser, 1998; Chung and Baek, 1999). Evidence so far is that the UCHs are a much smaller family of proteins compared to the UBPs with only two UCHs and 16 UBPs in *S. pombe* and one UCH

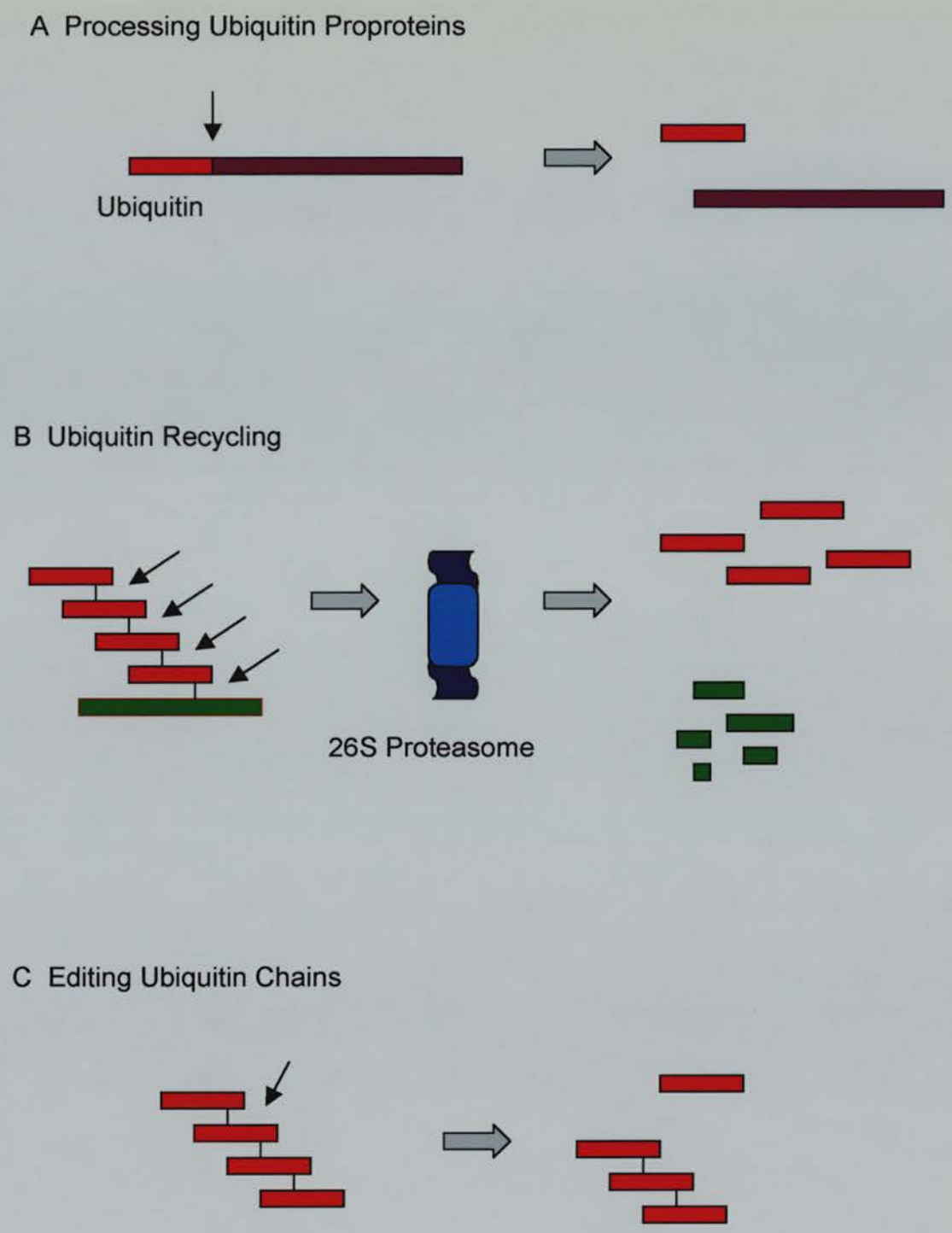

Figure 1.2: The Functions of Deubiquitination
The DUBs have several cellular functions including: cleaving ubiquitin from the precursor proteins (A); recycling ubiquitin (B), for example from proteins that are destined for degradation by the proteasome; and by editing ubiquitin chains (C). The arrows indicate cleavage sites.

and 16 UBPs in *S. cerevisiae* (Table 1.2) (Amerik et al., 2000; C. Semple and M. Stone, unpublished).

1.2.2 Biochemical Activities of the DUBs

1.2.2.1 Activity of the UCHs

The catalytic mechanism of the papain-like cysteine proteases has been well described and based on their homology it is thought that the DUBs operate in a similar manner (Figure 1.3). Cleavage of a peptide bond begins with formation of a tetrahedral intermediate by the nucleophilic attack of the substrate carbon and by the active site Cys. This is converted to an acyl-enzyme intermediate as the C-terminal peptide leaving group is released. A deacylation reaction then takes place with a water molecule acting as a nucleophile to form a second tetrahedral intermediate. Finally, the ubiquitin is released and the Cys thiol sidechain is restored. The role of the active site His imidazole group is to enhance the nucleophilicity of the catalytic Cys (Storer and Menard, 1994; Johnston et al., 1999). Support for this mechanism is provided by the crystal structure of UCH Yuh1 bound to Ubal, which shows the formation of a tetrahedral structure analogous to the tetrahedral intermediate (Johnston et al., 1999).

The activity of several UCH proteins has been well described. The conserved active site Cys and His residues of UCH-L1 were identified and mutation of the Cys abolished activity and mutation of the His residue resulted in little or no activity (Larsen et al., 1996). Ubiquitin fusions appear to be the major substrate for the UCHs as none of those tested are able to cleave isopeptide linked ubiquitin chains. Human UCH-L1 and UCH-L3 and *S. cerevisiae* Yuh1 were all shown to prefer to cleave ubiquitin from short fusions or small adducts (Baker et al., 1992; Larsen et al., 1998). There is some degree of specificity within the UCH family; UCH-L1 and UCH-L3 showed different substrate preferences in the cleavage of ubiquitin

S. pombe DUB	Pompep ID	*S. cerevisiae*	*D. melanogaster*	*H. sapiens*
Uch1	UCH1,SPAC27F1.03C	UBL1_YEAST P35127	UBL_DROME P35122	UBL3_HUMAN P15374*
Uch2	UCH2,SPBC409.06		Q9VYQ3 CG1950	UBL5_HUMAN Q9Y5K5*
Ubp1	SPCC16A11.12C			
Ubp2	SPAC328.06	UBP2_YEAST Q01476*		
Ubp3	SPBP8B7.21	UBP3_YEAST Q01477	Q9W0L5 CG13903	UBPA_HUMAN Q14694*
Ubp4	SPBC18H10.08C	UBP4_YEAST P32571*		
Ubp5	SPCC188.08C			
Ubp6	SPAC6G9.08	UBP6_YEAST P43593*	Q9VKZ8 CG5384	TGT_HUMAN P54578
Ubp7	SPAC23G3.08C		Q9W4C3 CG4165	UBPG_HUMAN Q9Y5T5*
Ubp8	SPAC13A11.04C	UBP8_YEAST P50102*	Q9VVR1 NOT PROTEIN	UBP3_HUMAN Q9Y6I4
Ubp9	SPBC1703.12	UBP9_YEAST P39967	Q9VCT9 CG7023	UBPC_HUMAN O75317*
Ubp10	SPBC577.07	YFH5_YEAST P43589	Q9VWP1 CG7288	AAK49524 U4/U6.U5*
Ubp11	SPBC19C2.04C			
Ubp12	SPCC1494.05C	UBPC_YEAST P39538*	Q9W117 CG387	Q9UNP0
Ubp13	SPAC22G7.04	PAN2_YEAST P53010	Q9V505 CG8232*	O75189 KIAA0710
Ubp14	SPBC6B1.06C	UBPE_YEAST P38237	Q9VZU7 CG12082	UBP5_HUMAN P45974*
Ubp15	UBPD,SPBC713.02C	UBPF_YEAST P50101*	Q9VYQ8 CG1490	UBP7_HUMAN Q93009
Ubp16	SPCC1682.12C		Q9VRP5 CG5505	Q9H9C5 FLJ12851*

Table 1.2: The *S. pombe* Deubiquitinating Enzymes
Putative *S. pombe* DUBs were identified by a bioinformatics search for the UCH and UBP domains. Candidate orthologues in *S. cerevsiae*, *D. melanogaster* and *H. sapiens* were predicted using BLAST searches. Asterices indicate the proteins showing the highest similarity to *S. pombe* homologues (C. Semple, unpublished).

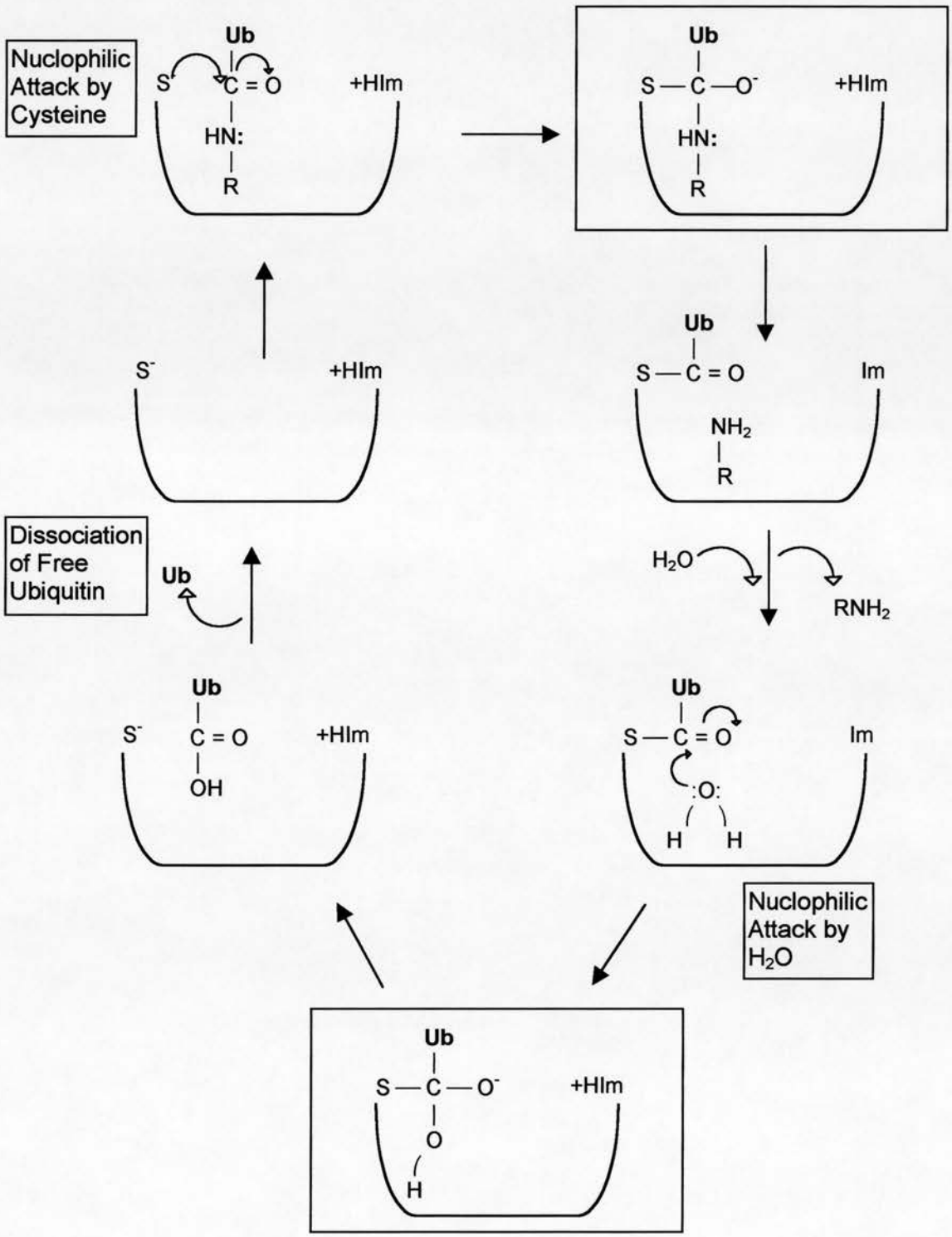

Figure 1.3: Catalysis by Ubiquitin C-terminal Hydrolases
The cleavage of ubiquitin (Ub) from a peptide (R) begins with the nuclophilic attack by the active site Cys. A tetrahedral intermediate is formed and subsequently the peptide is released. A water molecule then acts as a nuclophile to form a second tetrahedral intermediate before release of the ubiquitin molecule. The active site His imidazole group (Im) enhances the nucleophilicity of the active site Cys thiol group. The high energy tetrahedral intermediates are indicated by the boxes.

precursors (Larsen et al., 1998). In addition, there is evidence that precursors are cotranslationally processed, as a substantial increase in activity is observed when enzyme and substrate are coexpressed (Larsen et al., 1998). In general, it is accepted that the in vivo function of UCHs is to process ubiquitin precursors and remove small adducts from ubiquitin (Wilkinson, 2000; Chung and Baek, 1999).

1.2.2.2 Activity of the UBPs

A large number of UBPs have been assayed for ubiquitin cleavage activity, and a range of activities and specificities has been observed. The conserved Cys and His residues have been mutated in many UBPs including those from mouse (Unp), *Drosophila* (Faf) and *S. pombe* (Ubp21) (Gilchrist and Baker, 2000; Huang et al., 1995; Richert et al., 2002). In each case, mutation resulted in loss of activity. As with the UCHs, cotranslational processing of ubiquitin precursors has been proposed, as *S. cerevisiae* Ubp1 and Ubp2 are unable to process the polyubiquitin precursor unless coexpressed with the substrate (Baker et al., 1992).

The UBPs are able to cleave most ubiquitin substrates with peptide or isopeptide linkages and small or large leaving groups. However, there is often preference for particular substrates, for example, *S. cerevisiae* Ubp6 is most active against ubiquitin fused to small extensions with charged amino acids although it does have some activity in cleaving isopeptide-linked ubiquitin and larger ubiquitin fusions (Park et al., 1997). Other UBPs that show substrate preference are the testis specific UBPs, which have high activity against isopeptide linked ubiquitin and linear ubiquitin-protein fusions but low activity for linear diubiquitin (Lin et al., 2001). Isopeptidase T and its homologues are unusual in the UBP family in having a specific role in the cleavage of ubiquitin in Lys48 linked chains. Thus, their cellular function is to recycle ubiquitin from multiubiquitin chains. (Hadari et al., 1992; Amerik et al., 1997; Doelling et al., 2001). It therefore appears that there are subtle variations in

activity between the UBPs and that only by further analysis in vivo will their true specificities be determined.

1.2.2.3 Inhibition of DUB activity

Cysteine protease activities, including those of the DUBs, can often be characterised by their sensitivity to the alkylating agents iodoacetamide and N-ethylmaleimide (NEM), which block the active site cysteine by binding to the thiol group. Another valuable tool in the study of DUB activity is the inhibitor Ubiquitin-Aldehyde (Ubal). This was originally identified as a potent inhibitor for several purified UCH activities. These activities were also inhibited by iodoacetamide and manganese, suggesting that they were thiol proteases and that the Ubal would bind as a tetrahedral intermediate (Mayer and Wilkinson, 1989). This model was confirmed by kinetic and structural studies, which showed the formation of an extremely stable tetrahedral Enzyme-Ubal complex (Dang et al., 1998; Johnston et al., 1999).

1.2.3 Structure of the DUBs

Structural characterisation of the DUBs has so far been restricted to members of the UCH family with no structure for a UBP yet determined (Barrett and Rawlings, 2001). The crystal structures of two UCHs have been described: the human UCH-L3 and the budding yeast Yuh1 bound to Ubal. Although they are only 33% identical, both proteins share a conserved overall structure of an anti-parallel β-sheet surrounded by α-helices, which is similar to that of the papain-like proteases (Johnston et al., 1997; Johnston et al., 1999). UCH-L3 shows greatest general structural similarity to cathepsin B with the positions of the Cys, His and Asp active site residues also conserved (Johnston et al., 1997). The active sites of the UCHs are buried in between the two lobes of the proteins, making them relatively inaccessible to substrates. Comparison of the UCH-L3 and Ubal bound Yuh1 structures reveals

that the UCH undergoes a conformational change on binding to its substrate, with the N-terminal residues that block the UCH-L3 active site being moved on a hinge to allow substrate binding. Therefore, it appears that ubiquitin binding is coupled to opening of the active site chamber (Johnston et al., 1997) (Johnston et al., 1999). The Yuh1-Ubal structure also explains the specificity for the C-terminal di-Gly of ubiquitin as conserved UCH residues posses side chains that block the access of residues larger than Gly (Johnston et al., 1999).

1.2.4 DUB Function

1.2.4.1 Cellular Roles of the DUBs

The roles of the UBPs on a cellular level are proposed to be in processing ubiquitin precursor proteins, recycling conjugated ubiquitin and editing of multiubiquitin chains (Figure 1.2). Understanding of the specificity and control of these functions within the cell has been advanced by studies in yeast and using cell culture systems.

Regulation of proteasome mediated proteolysis is a major function of ubiquitination, suggesting that DUBs must be involved in this process. One indicator of a defect in proteolysis is sensitivity to the arginine analogue, canavanine. Mutation of several DUBs in *S. cerevisiae* and *A. thaliana* has been shown to result in canavanine sensitivity, implying that these DUBs promote proteolysis (Amerik et al., 2000; Yan et al., 2000). However, overexpression of the *S. cerevisiae* DUB Ubp2 was found to confer sensitivity to canavanine, implying that it may act in the opposite manner to restrict proteolysis (Gilchrist and Baker, 2000). Another DUB that has been shown to be involved in proteasome dependent proteolysis is Isopeptidase T, which is proposed to regulate proteolysis by recycling ubiquitin from the multiubiquitin chains of proteolytic substrates (Hadari et al., 1992; Amerik et al., 1997; Doelling et al., 2001). Genetic studies in *S. cerevisiae* have implicated the Doa4 UBP in controlling proteasome directed proteolysis via ubiquitin homeostasis. The *doa4* null

mutant has a pleiotropic phenotype, including accumulation of low molecular weight ubiquitin conjugates, compromised degradation of 26S proteasome substrates, defective DNA repair and temperature sensitivity (Papa and Hochstrasser, 1993; Amerik et al., 2000a). This suggests that Doa4 functions in a number of cellular processes, an observation that is supported by the finding that *doa4* shows genetic interactions with mutants of both the proteasome and vacuolar degradation pathways. (Papa and Hochstrasser, 1993; Amerik et al., 2000a; Dupre and Haguenauer-Tsapis, 2001). Silencing is another process that has been proposed to be regulated by DUB activity. In *S. cerevisiae*, Ubp3 was identified due to a direct physical interaction with the Sir4 silencing protein. Deletion of *UBP3* was demonstrated to increase silencing at the telomeres and mating type loci, indicating a function for Ubp3 in the positive regulation of silencing. Although the mechanism by which Ubp3 regulates silencing is unknown, the most favourable model is that it promotes the degradation of a negative regulator of silencing, which would provide a means to regulate silencing in response to a change in environmental conditions, such as the carbon source (Moazed and Johnson, 1996). Ubp3 has also been implicated in the control of the cell cycle in response to the environment, via the regulation of the pheromone response pathway. Cells deleted for *UBP3* were found to accumulate multiubiquitinated Ste7 MAPK kinase as well as unconjugated multiubiquitin chains. The ubiquitination of Ste7 is stimulated by pheromone and its deubiquitination and degradation are promoted either directly or indirectly by Ubp3. It is proposed that, by this mechanism, Ubp3 acts to restrict the MAPK pathway, as *ubp3* null mutants show enhanced signalling in response to pheromone compared to wild type cells. (Wang and Dohlman, 2002).

In higher eukaryotes, a number of DUBs have been shown to be regulated by external stimuli. Investigation of human UBPY in fibroblasts showed that it is upregulated in response to serum stimulation of and downregulated once the cells became confluent. In these cells it was found to be required for entry into S phase. However in transformed cells levels of UBPY remained high and cells lacking UBPY arrested in S phase. Taken together, these data indicating that UBPY has a role in growth regulated cell cycle control (Naviglio et al., 1998). A subset of

mammalian DUBs, including mouse DUB-2, have been suggested to function in the regulation of cell growth and differentiation as they are immediate-early genes induced by cytokines (Zhu et al., 1997; Baek et al., 2001). Another DUB that is involved in controlling cell growth and differentiation is encoded by the tre-2 oncogene, which was originally identified as a DUB based on its homology to Doa4 (Papa and Hochstrasser, 1993). Tre-2 protein was found to have transforming activity in 3T3 fibroblasts and its murine homologue, Unp, has been demonstrated to be tumourigenic in transgenic mice (Gupta et al., 1993; Gupta et al., 1994; Nakamura et al., 1992).

1.2.4.2 Roles in Multicellular organisms

Two of the best described DUBs in higher eukaryotes are the *Drosophila* Fat facets (Faf) protein and its murine homologue Fam. Faf is expressed widely in the CNS, regulates synaptic development at the neuromuscular junction and is required for normal eye development (Huang et al., 1995; DiAntonio et al., 2001). Using genetic analysis, Liquid facets, a homologue of the endocytic protein epsin, was identified as a substrate of Faf, suggesting that Faf mediates its developmental function via the regulation of endocytosis (Cadavid et al., 2000; Chen et al., 2002). In mouse, Fam is expressed in expanding cell populations at gastrulation and neurulation, in apoptotic regions between the digits, and like Faf, in the CNS. There is evidence that the cell-cell adhesion protein, AF-6, which is a target of Ras, is a Fam substrate, indicating a link between Ras signaling and ubiquitination in the control of cell growth and differentiation (Taya et al., 1998). There are many other examples of DUBs that are involved in developmental processes in a range of organisms. In A. *thaliana*, Ubp14 (a homologue of isopeptidase T) is required for early embryo development as homozygous mutants fail to produce viable mature seeds (Doelling et al., 2001). The temporal and spatial regulation of development in *Dictyostelium* is partly controlled by the MEK kinase MEKKα, which is also regulated by ubiquitination. A DUB named UbpB has been identified as a negative regulator of MEKKα; it is proposed to

promote MEKKα degradation and therefore allow prespore cell differentiation (Chung et al., 1998). Another DUB that, like Faf, functions in synaptic control, is Aplysia Ap-Uch, which is required for long term memory storage (Hegde et al., 1997). In addition, two murine DUBs of the UCH family have been found to function in the CNS. Mice that are homozygous mutants for Uch-L1 and Uch-L3 show axonal degeneration in the medulla and dorsal root ganglia, with the corresponding phenotypes of posterior paralysis and dysphagia (Kurihara et al., 2001). The observation of neurodegeneration in these mutants is in keeping with the finding that a missense mutation in Uch-L1 is linked to familial Parkinson's disease (Leroy et al., 1998). Although only a small fraction of the known DUBs have been characterised, it appears that their key functions include regulation of MAP kinase signaling pathways and roles in the nervous system.

1.2.5 UDP Deconjugating Enzymes

Like ubiquitination, the conjugation of ubiquitin-like modifiers is a reversible process and several enzymes that catalyse the deconjugation of SUMO have been identified. A Nedd8 specific deconjugating enzyme has yet to be identified, but deneddylating activity has been found for a subset of DUBs and also associated with the COP9/signalosome (Muller et al., 2001; Yeh et al., 2000).

S. cerevisiae has at least two SUMO specific proteases, Ulp1 and Ulp2, and both have conserved homologues in *S. pombe* (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000; Taylor et al., 2002). Ulp1 was the first SUMO protease to be characterised, and was shown to be specific for SUMO as it was unable to cleave ubiquitin and its activity was unaffected by Ubal. Deletion of Ulp1 is lethal, and its activity was shown to be required for cell cycle progression from G2-M (Li and Hochstrasser, 1999). The second SUMO protease, Ulp2 is not essential, although the deletion strain has pleiotropic defects including enhanced chromosome loss and sensitivity to HU and DNA damage (Li and Hochstrasser, 2000). Both Ulp1 and Ulp2 are able to cleave peptide and isopeptide linked SUMO. However, as the

phenotype, including the pattern of SUMO conjugate accumulation differs between the two mutant strains, indicating that they have different in vivo specificities (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000). In *S. pombe*, a Ulp1 homologue has been characterised, however, unlike in *S. cerevisiae*, deletion of *ulp1*$^{+}$ is not lethal. This probably reflects the fact that SUMO is essential in *S. cerevisiae* but not in *S. pombe* (Jentsch and Pyrowolakis, 2000; Tanaka et al., 1999). The phenotype of *ulp1* null mutant cells suggests that its major role is in processing of the SUMO precursor, to maintain cellular levels of SUMO. However, *ulp1* cells are sensitive to UV and are slow growing and neither of these defects can be rescued by the expression of mature SUMO. Therefore, Ulp1 is also likely to function in the deconjugation of SUMO from specific substrates involved in cell cycle regulation and the DNA damage response (Taylor et al., 2002). The Ulps show no sequence or structural similarity to the DUBs except that they are both members of the cysteine protease family (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000; Mossessova and Lima, 2000). Comparison of the Ulp1-SUMO and Yuh1-Ubal crystal structures shows that the mechanism of substrate recognition by the two enzymes is largely different, although both recognise the substrate diGly via van der Waals contacts (Mossessova and Lima, 2000). Surprisingly, Ulp1 and Ulp2 show greatest similarity with adenoviral proteases (Li and Hochstrasser, 1999; Mossessova and Lima, 2000). There are at least seven SUMO specific proteases in human but only one, SENP1, has been characterised. SENP1 is only able to cleave SUMO conjugates and not ubiquitin or Nedd8 conjugates and shows substrate specificity as it could cleave SUMO-PML but not SUMO-RanGAP1 (Yeh et al., 2000). The conservation between the SUMO specific proteases is limited to a region of about 200 amino acids, including the active site residues, in a manner which is reminiscent of the UBP family of DUBs (Li and Hochstrasser, 2000; Yeh et al., 2000).

Studies to date suggest that unlike deconjugation of SUMO, the deconjugation of Nedd8 may overlap with deubiquitination. The UCH-L3 ubiquitin C-terminal hydrolase has been shown to have Nedd8 cleaving activity and a human ubiquitin-specific processing protease, UBP21 is able to deconjugate ubiquitin and Nedd8, but not SUMO (Wada et al., 1998; Gong et al., 2000). These observations are perhaps to

be expected, as the homology between Nedd8 and ubiquitin is much higher than between SUMO and ubiquitin. However, as these experiments have been conducted in vitro, it is unclear whether the deneddylating activity of the DUBs will be relevant under physiological conditions. A series of reports have identified the COP9/signalosome complex as a positive regulator of deneddylation (Lyapina et al., 2001; Zhou et al., 2001; Yang et al., 2002). The COP9/signalosome complex has homology to the 19S regulator lid and although its function is unclear, it has been shown to regulate a number of cellular processes including signal transduction (section 1.3.2.1.1; Wei and Deng, 1999). The cullin component of the human SCF complex was found to interact with the COP9/signalosome, and this association was conserved in *S. pombe* (Lyapina et al., 2001). Furthermore, the *S. pombe* COP9/signalosome was able to promote cleavage of Nedd8 from the Pcu1 cullin and COP9/signalosome mutants accumulated Nedd8 conjugated cullins (Lyapina et al., 2001). A second study showed that Nedd8 conjugated to the Pcu3 cullin, which is found in a stress specific ubiquitin ligase, was also removed in the presence of the COP9/signalosome. In addition, its ubiquitin ligase activity was increased in COP9/signalosome mutants (Zhou et al., 2001). Finally, degradation of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor, a known proteasome substrate, has been found to be regulated by the COP9/signalosome. Nedd8 that is conjugated to the SCF is removed in a COP9/signalosome dependent manner, reducing the SCF ubiquitin ligase activity against $p27^{Kip1}$, and therefore decreasing $p27^{Kip1}$ degradation (Yang et al., 2002). The proposed Nedd8 deconjugating activity of the COP9/signalosome has not yet been attributed to any subunit; the Csn5 subunit has homology to the cysteine proteases but mutation of the putative active site cysteine had no effect on promotion of Nedd8 deconjugation by the COP9/signalosome (Lyapina et al., 2001). Further studies will be required to identify the active subunit or associated factor and to determine whether the deubiquitinating activity found in the 19S complex and the Nedd8 deconjugating activity associated with the COP9/signalosome are mediated by homologous subunits (see section 1.3.2.1.1).

1.3 26S Proteasome

One of the major functions of ubiquitination is to target proteins for 26S proteasome-mediated degradation. The 26S proteasome is a multisubunit complex that is composed of two 19S regulatory complexes positioned at either end of a 20S catalytic core complex (Figure 1.4). The 19S complexes function to activate the proteasome for the degradation of ubiquitinated proteins (Voges et al., 1999). A second regulator of the 20S proteasome, the 11S (or PA28) complex, is found only in higher eukaryotes and is thought to be involved in antigen presentation and the immune response (Kloetzel, 2001). However, as the focus here is on ubiquitin dependent proteolysis, it is the 20S and 19S complexes, which make up the 26S proteasome, that will be described in detail below.

1.3.1 20S Core

1.3.1.1 Structure of the 20S Core

The 20S core of the 26S proteasome resembles proteasome complexes found in archaea. Studies of these complexes have given an insight into the structure of the eukaryotic complex with the first X-ray crystal structure of a 20S complex coming from the archaea, *Thermoplasma acidophilum*. This structure revealed that two types of subunit, α and β, make up the 20S core and that these subunits are arranged in stacked, seven-membered rings, in a pattern of 7α,7β,7β,7α (Lowe et al., 1995). Using electron microscopy, these stacked rings can be seen to form a barrel shaped structure (Figure 1.4) (Walz et al., 1998). The 20S complex contains three chambers and the β subunit rings form the central chamber, which is the site of proteolysis (Lowe et al., 1995; Groll et al., 1997). The archaeal 20S complex is a simplified version of that found in eukaryotes. The eukaryotic 20S complex is made up of fourteen different subunits and, based on their homology to the archaeal subunits, seven of these have been classed as α-subunits and seven as β-subunits. The crystal

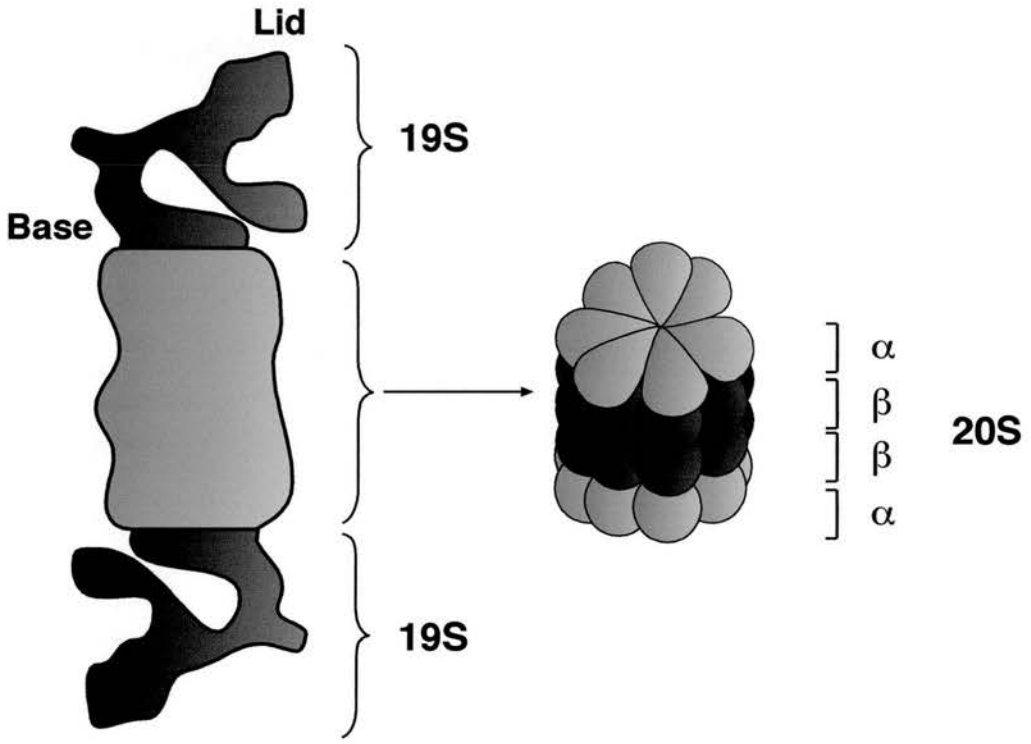

Figure 1.4: The Structure of the 26S Proteasome
The 26S proteasome comprises a barrel-shaped 20S catalytic core with a 19S regulatory complex bound at either end. The 20S core is made up of α and β type subunits, which are arranged in stacked rings.

structure of the budding yeast 20S complex has been solved and although there are seven distinct subunits of both the α and β types, the overall eukaryotic 20S structure was found to very similar to that of the archaeal complex (Groll et al., 1997). A major difference between the two structures is that the channel into the archaeal 20S complex is constitutively open whereas entry into the eukaryotic 20S complex appears to be controlled by a gating mechanism. The N-terminal 13 residues of the α3 subunit, which are disordered in the *T. acidophilum* structure, were found to have an ordered structure covering the opening into the budding yeast 20S core. Analysis of proteasomes purified from a strain deleted for the α3 N-terminal tail using x-ray crystallography demonstrated that the channel was opened and biochemical assays revealed a corresponding increase in protease activity. As with most other subunits of the proteasome, deletion of the entire α3 subunit is lethal. However, a strain deleted for only the N-terminal tail of the α3 subunit was viable, indicating that the gating function of α3 is not essential (Groll et al., 2000). This is in keeping with the proposal that the gate may be opened in vivo by the binding of the 19S complex to the α-subunit rings.

1.3.1.2 Activity of the 20S Core

The catalytic function of the 26S proteasome is provided by the 20S core complex, which has been shown to function as an N-terminal nucleophile hydrolase (Voges et al., 1999). Mutation and structural studies of the *T. acidophilum* proteasome have demonstrated that the β-subunits act as threonine proteases to provide the catalytic activity of the 20S complex (Wlodawer, 1995; Seemuller et al., 1995). At least five proteolytic activities have been assigned to the 26S proteasome: chymotrypsin-like, trypsin-like and peptidylglutamyl activities have widely been described but in addition cleavage between small neutral amino acids and at the C-side of branched chain amino acids has been reported (Orlowski et al., 1993; Voges et al., 1999). It has been proposed that the size of products released from the proteasome might be determined by the distance between active sites in a mechanism described as a

molecular ruler (Wenzel et al., 1994). However, several studies have provided evidence that this is not the case, for example, both mammalian and archaeal proteasomes have been shown to produce a similar distribution of product sizes in spite of the fact that the archaeal proteasomes contain many more active sites (Kisselev et al., 1999b). Protein degradation is observed to be processive, without release longer breakdown intermediates and the oligopeptides generated by the proteasome are in the range of 3-30 amino acids long with an average size between 6-9 residues (Akopian et al., 1997; Nussbaum et al., 1998; Kisselev et al., 1999b; Kisselev et al., 1999a).

1.3.2 The 19S Regulator

In eukaryotes, the 26S proteasome complex is formed by the association of two 19S complexes with the 20S core, one at either end. This structure has been observed using three-dimensional electron microscopy, which revealed that the 20S complex is connected by a flexible linkage to the 19S regulators (Walz et al., 1998). The 19S regulator (or 19S Cap, PA700) provides the specificity of the 26S complex for ubiquitinated substrates and activates the 20S complex for degradation in an ATP dependent manner. The specific functions of the 19S complex are the recognition of ubiquitinated substrates; unfolding of substrates by chaperone activity; opening of the gate into the 20S core to allow substrate entry and recycling of ubiquitin by a deubiquitinating activity. Each of these functions will be discussed further below; however, first, the structure of the 19S complex will be described.

1.3.2.1 The Structure of the 19S Complex

It is generally agreed that 17 subunits make up the 19S complex and these can be divided into two groups, the ATPases and the nonATPases (Voges et al., 1999; Holzl et al., 2000; Li et al., 2000). In certain species, additional 19S subunits have been

proposed, however, as these are not conserved members of the 19S complex they are unlikely to be essential for proteasome function (Voges et al., 1999). In addition, the sum of the masses of the 18 conserved subunits is in close agreement with the experimentally determined mass of the 19S complex (Holzl et al., 2000). The subunits of the 19S complex have been assigned unique names in several different organisms. Here, the subunits will be referred to using both the original *S. pombe* nomenclature and the unified nomenclature of RPT (Regulatory Particle ATPase) and RPN (Regulatory Particle Non-ATPase) that was proposed by Finley et al (1998).

The 19S complex is highly conserved in terms of subunit composition. It also shows functional conservation, as is demonstrated by the fact that 19S complexes from human erythrocytes are able to activate the *S. pombe* 20S complex (Seeger et al., 1996). Studies using a *S. cerevisiae* strain deleted for the Rpn10 19S complex subunit, showed that the 19S complex can be dissociated into two subcomplexes: the base and the lid (Figure 1.4; Glickman et al., 1998). Ubiquitin dependent proteolysis requires the presence of both of these subcomplexes (Braun et al., 1999)

1.3.2.1.1 The 19S Base

The base of the 19S Regulator is composed of all six ATPases, of the AAA ATPase family, and three non-ATPases (Glickman et al., 1998). Several other AAA ATPases including katanin, dynein and those of the proteasome-activating nucleotidase (PAN) archaeal complex have been shown to adopt a hexameric ring structure (Neuwald et al., 1999; Zwickl and Baumeister, 1999). Therefore, it has been proposed that the ATPases of the base also form a heterohexameric ring. Two of the non-ATPases of the base are the two largest subunits of the complex, Mts4/Rpn1 and Rpn2, which are related in sequence, but of unknown function. The third nonATPase in the base is the multiubiquitin binding protein Pus1/Rpn10 (see section 1.3.2.2.1) (Glickman et al., 1998; Voges et al., 1999). The base alone is

sufficient to activate the 20S complex for peptide degradation, but not multiubiquitinated proteins (Braun et al., 1999).

1.3.2.1.1 The 19S Lid

The lid subcomplex is made up of eight non-ATPases and its function remains elusive. However, there is considerable sequence homology between subunits of the lid and those of the COP9/signalosome and eIF3 translation initiator complex. Indeed, two structural motifs, the PINT/PCI domain and the MPN domain are found uniquely in subunits of these complexes (Glickman et al., 1998). The similarity of the lid and the COP9/signalosome is also reflected on a structural level (Kapelari et al., 2000; Henke et al., 1999). The significance of these homologies remains unclear, however, it has been suggested that it may reflect a common evolutionary ancestor for the complexes (Glickman et al., 1998; Braun et al., 1999).

1.3.2.2 Functions of the 19S Complex

1.3.2.2.1 Recognition of Multiubiquitinated Proteins

The 19S complex is required for the degradation of ubiquitinated substrates implying that it must contain multiubiquitin recognition factors. The first of these to be identified was the Pus1/Rpn10 subunit, which contains a multiubiquitin-binding domain called the UIM (ubiquitin-interacting motif) (Deveraux et al., 1995; van Nocker et al., 1996; Wilkinson et al., 2000; Hofmann and Falquet, 2001). However, as Pus1/Rpn10 was found to be non-essential, it was suggested that other multiubiquitin binding proteins must exist (van Nocker et al., 1996; Wilkinson et al., 2000). Further studies have revealed that this redundant function is provided not by proteasome subunits but by two proteasome-interacting, multiubiquitin-binding proteins, Rhp23 and Dph1 (Wilkinson et al., 2001 and see section 1.1.9.1). These proteins both contain a UBL domain and bind to the Mts4/Rpn1 subunit of the 19S

regulator to deliver multiubiquitated substrates. A third UBL domain protein, BAG-1 is also proposed to be involved in delivery of substrates to the proteasome (see section 1.1.9.2). Finally, the Cdc48/Valosin-Containing Protein (VCP), which, like Rhp23 and Dph1, is able to bind multiubiquitin chains and associate with the proteasome, has also been implicated in the recognition and delivery of proteasome substrates. However, in this case the interaction with the proteasome has not been shown to be direct, so other accessory proteins may be involved (Dai et al., 1998; Verma et al., 2000).

In addition to these proteins, the base of the 19S regulator has also been recently implicated in the binding of multiubiquitin. This interaction was shown to be ATP dependent and the Rpt5 ATPase subunit was found specifically to crosslink to multiubiquitin chains (Lam et al., 2002). These findings were based on in vitro studies, and therefore require verification in vivo. However, one possible model is that substrates may be selectively delivered to the proteasome by Pus1, Dph1, Rph23 and possibly BAG-1 and Cdc48/VCP then subsequently bound to Rpt5.

1.3.2.2.2 Deubiquitinating Activity

Two studies have described intrinsic deubiquitinating activity for the 26S proteasome (Eytan et al., 1993; Lam et al., 1997). The first used 26S proteasomes from rabbit reticulocytes and identified an activity capable of releasing free ubiquitin from ubiquitin-protein conjugates or ubiquitin fusions. This activity was found to be insensitive to the ubiquitin C-terminal hydrolase inhibitor, ubiquitin aldehyde suggesting that it may not be a thiol protease (Eytan et al., 1993). A later study using bovine 19S complexes identified a ubiquitin aldehyde sensitive isopeptidase likely to be a thiol protease, which was capable of editing multiubiquitin chains by removing ubiquitin sequentially from the end of the chain (Lam et al., 1997). Therefore, it appeared that the 26S proteasome might contain two distinct deubiquitinating activities, however, the subunits responsible for this activity have yet to be identified.

1.3.2.2.3 Chaperone Activity

It has been proposed that 19S regulator chaperone activity may be necessary for the unfolding of substrates prior to degradation, as the opening into the 20S catalytic core is too small to allow the entry of folded proteins (Groll et al., 1997). This activity has been observed in proteasomes from human erythrocytes, which can reactivate denatured citrate synthase and inhibit its aggregation. The presence of AAA ATPases in the base of the 19S regulator suggests that this may be the site of chaperone activity as many members of this family are known chaperones (Neuwald et al., 1999; Zwickl and Baumeister, 1999). In support of this observation, a binding site for unfolded proteins has been localised to the base subcomplex (Braun et al., 1999).

Understanding of the chaperone activity of the 26S proteasome has been advanced by studies using the PAN complex, which contains AAA ATPases homologous to those in the 19S base. PAN can catalyse protein refolding or unfolding and promotes degradation by 20S proteasomes (Benaroudj et al., 2001). The hexameric ring of the AAA ATPases is proposed to mediate unfolding of proteasome substrates via coordinated conformation changes coupled to ATP hydrolysis (Neuwald et al., 1999; Zwickl and Baumeister, 1999).

1.3.2.2.4 Regulation of Entry into the 20S Channel

Opening of the gated channel into the 20S core is thought to be mediated by the binding of the 19S complex to the 20S α-subunit rings (Groll et al., 2000). The 19S regulator base subunit Rpt2 has been proposed to be crucial for this function. Mutations in the ATPase domain of Rpt2 decrease the activity of proteasomes but when combined with an open channel mutant, activity is restored. Therefore, it is suggested that Rpt2 acts in an ATP dependent manner to regulate gating of the 20S core (Groll et al., 2000).

1.3.3 Identification of Genes Encoding 26S Proteasome Subunits in *S. pombe*

Several genes encoding subunits of the 26S proteasome in *S. pombe* were identified in a screen to isolate mutants that were resistant to the microtubule destabilising drug, MBC (methylbenzylcarbamylate) and temperature sensitive for growth. Five different genes were found to be mutated and these were named mts (for MBC resistant and temperature sensitive). Subsequent work revealed that each of the mts genes encoded a subunit of the 19S regulator: *mts1*$^+$ (Rpn9), *mts3*$^+$ (Rpn12), *mts4*$^+$ (Rpn1) and *mts5*$^+$ (*pad1*$^+$, Rpn11) encode non-ATPase subunits and *mts2*$^+$ (Rpt2) encodes an ATPase subunit (Gordon et al., 1993; Gordon et al., 1996; Wilkinson et al., 1997; Penney et al., 1998; C. Gordon unpublished).

The *mts* mutations are all loss of function mutations as deletion of any of the *mts* genes is lethal (Gordon et al., 1993; Gordon et al., 1996; Wilkinson et al., 1997; Penney et al., 1998; C. Gordon unpublished). Each of the *mts* mutants has a similar phenotype, with cells at the restrictive temperature arresting during mitosis at metaphase indicating a defect in the metaphase to anaphase transition. The arrest at metaphase probably reflects the requirement for ubiquitin dependent proteasome mediated degradation of Cut2 for sister-chromatid separation and thus the progression from metaphase to anaphase (Funabiki et al., 1996).

Analysis of extracts from mutant cells has indicated an accumulation of high molecular weight ubiquitin conjugates at the restrictive temperature for *mts2*, *mts3* and *pad1*, providing evidence that Mts2, Mts3 and Pad1 function in the degradation of multiubiquitinated proteins in vivo (Gordon et al., 1993; Seeger et al., 1996). In support of this observation, proteasomes purified from the *mts2* and *mts3* mutants were found to be defective in degradation of ubiquitin ^{125}I Lysozyme conjugates in vitro at the restrictive temperature (Seeger et al., 1996).

1.3.4 Localisation of the Proteasome

The localisation of the proteasome has been investigated in many cell types from yeast to human, using a variety of cytological and biochemical methods. There appears to be a clear difference between the cellular localisation of proteasomes in yeast and higher eukaryotes, therefore each will be discussed in turn.

1.3.4.1 Proteasome Localisation in Yeast

Complementary studies in both fission and budding yeast have demonstrated that the proteasome is primarily localised to the nuclear periphery in a punctate pattern (Figure 1.5). The localisation of the proteasome in *S. pombe* was first investigated using immunofluorescence microscopy, which revealed punctate spots of staining at the nuclear periphery (Wilkinson et al., 1998). More detailed examination using immunogold electron microscopy confirmed that the precise localisation of the proteasomes was inside the nucleus (Wilkinson et al., 1998). In *S. cerevisiae*, the use of a GFP tagged proteasome subunit demonstrated that proteasomes have a punctate localisation at the nuclear periphery and colocalise with Kar2/Bip, a marker for the nuclear envelope-ER network. Confirmation of these findings was provided by biochemical fractionation experiments showing that the proteasome is enriched in the nuclear envelope-ER network (Enenkel et al., 1998). Although alternative nuclear localisation has been proposed, the accumulated evidence suggests that the nuclear periphery is the major site of proteasome localisation in vegatively growing yeast cells (Wilkinson et al., 1998; Enenkel et al., 1998; Russell et al., 1999).

The localisation of the proteasome during mitosis and meiosis has been investigated in live cells using a *S. pombe* strain in which the 19S subunit Pad1 has been tagged with GFP. In mitosis the proteasomes remained localised at the nuclear periphery, except that a spot of staining was observed between the separating nuclei at anaphase. In meiosis, a change in distribution of the proteasome was seen firstly

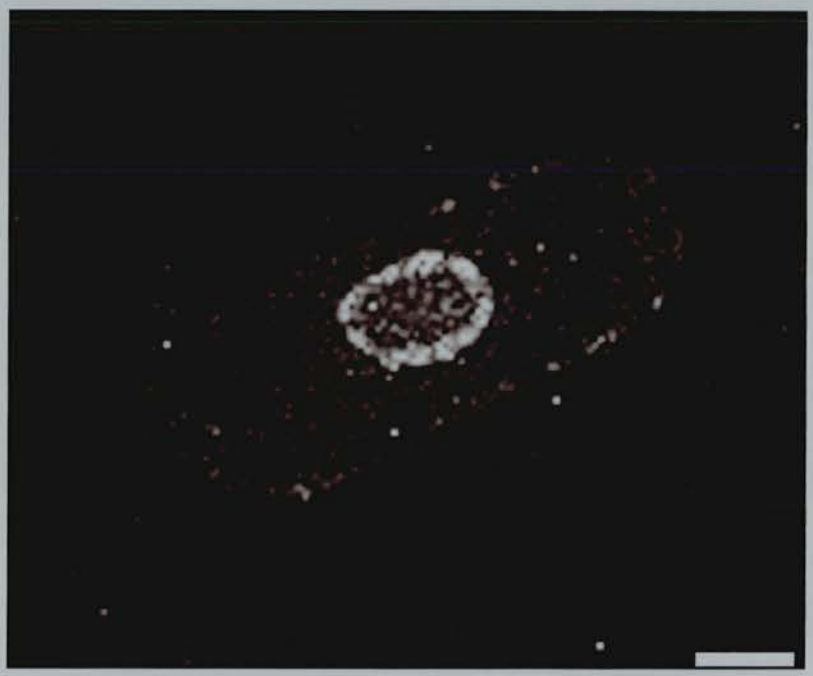

Figure 1.5: Localisation of the Proteasome in *S. pombe*
Using a strain that has the Pad1 19S subunit tagged with GFP, the proteasome can be seen to localise at the nuclear periphery in a puncate pattern (Wilkinson et al, 1998).

during karyogamy and horsetail movement. Proteasome staining was found throughout the nucleus with the exception of the nucleolus and a distinct spot was also observed. Although the localisation in meiosis I resembled that of mitosis, in meiosis II, the proteasomes were dramatically relocalised to a concentrated spot at the centre of the nucleus between the separating DNA. This signal dispersed at the end of meiosis II and relocalisation to the nuclear periphery was observed once spores had formed. It therefore appears that events in spore formation may require a specific localisation of proteasomes (Wilkinson et al., 1998).

The localisation of the proteasome at the nuclear periphery may be maintained due to interactions with other factors. Recently, Cut8 has emerged as a possible proteasome tethering protein; Cut8 localises in a proteasome-like manner and proteasomes in a *cut8* null mutant are delocalised. The degradation of multiubiquitinated proteasome substrates was delayed in *cut8* mutants, indicating that the Cut8-dependent localisation of proteasomes has a functional significance (Tatebe and Yanagida, 2000).

1.3.4.2 Proteasome Localisation in Mammalian Cells

Although there are many studies that have examined the localisation of proteasomes, a clear consensus on their distribution in mammalian cells has yet to be reached. What is evident, is that in most cell types proteasomes are found both in the nucleus and the cytosol and are often associated with the ER (Rivett, 1998; Hirsch and Ploegh, 2000). The presence of variant proteasome types in mammalian cells further complicates the issue of proteasome distribution. Immunoproteasomes, which contain specialised 20S subunits, have been reported to be concentrated in the ER , although the regulatory 11S complexes could not be detected (Brooks et al., 2000). However, a second study using several cell types showed that in response to IFNγ, immunoproteasomes and 11S regulatory complexes are both directed to PML (promyelocytic leukaemia oncoprotein) bodies. This is in keeping with previous

indications that PML bodies have a role in regulating the immune response (Fabunmi et al., 2000).

A number of studies have revealed a distinctive localisation of proteasomes with microtubule dependent protein aggregates called aggresomes (Kopito, 2000). These structures were first identified in a study examining the degradation of mutant forms of the cystic fibrosis transmembrane conductance regulator (CFTR) that is inefficiently folded, and thus degraded in a ubiquitin dependent manner by the proteasome. In conditions where the mutant CFTR was able to accumulate, it was found to aggregate at a perinuclear structure, which was named the aggresome (Johnston et al., 1998). Further investigation has demonstrated that this structure is composed of aggregated protein surrounded by a cage of vimentin filaments (Johnston et al., 1998; Garcia-Mata et al., 1999). The 20S, 11S and 19S complexes have all been detected in the aggresome, and it has been found to be active in the degradation of proteins (Garcia-Mata et al., 1999; Wigley et al., 1999). In addition, molecular chaperones such as Hsp70 have also been identified in aggresomes (Wigley et al., 1999). Both cytosolic and membrane proteins can incorporate into aggresomes and it has been shown that they first form aggregates throughout the cell which are then relocalised to the centrosome region in a microtubule dependent manner, possibly through the action of dynein/dynactin motor proteins (Garcia-Mata et al., 1999). Although the microtubule network is required for the redistribution of protein aggregates it is not necessary for the transfer of proteasomes to the aggresome (Garcia-Mata et al., 1999; Wigley et al., 1999). In terms of cellular function, aggregation has been shown to interfere with the process of ubiquitin dependent proteolysis. Therefore, it has been suggested that aggresomes may acquire a large number of proteasomes, leaving a reduced number for normal cellular functions (Bence et al., 2001). This loss of normal activity, may contribute to the cell death seen in diseases which are characterised by protein aggregation, such as Alzheimer's disease and Huntington's disease (Bence et al., 2001; Hirsch and Ploegh, 2000).

1.4 Project Aims

Studies using deletion strains for each of the *S. cerevisiae* DUBs have shown that none is essential and that there is a high level of redundancy. Indeed, cells in which up to five DUBs had been deleted were found to be viable. (Amerik et al., 2000). Therefore, it is proposed that the functional specificity of the DUBs may be determined by their cellular localisation. Regulation of 26S proteasome-mediated degradation is a major function of ubiquitination, implying that there might be a subset of redundant DUBs localised at the proteasome.

Two putative proteasome-associated DUBs have been identified in *S. pombe*. Uch2 had previously been suggested to copurify and colocalise with the proteasome (Li et al., 2000). A second DUB, Ubp6, was identified in a sequence homology search for *S. pombe* proteins containing a ubiquitin-like (UBL) domain (C. Semple and C. Gordon, unpublished). This domain has been shown to mediate interactions with the proteasome, implying that Ubp6 might also be proteasome-associated (Wilkinson et al., 2001).

Both Uch2 and Ubp6 are highly conserved proteins with homologues in higher eukaryotes (see Figures 1.6 and 1.7). *S. cerevisiae*, has a Ubp6 homologue but no Uch2 homologue is found. Therefore, in this respect, *S. pombe* appears to be more similar to higher eukaryotes than *S. cerevisiae*, making it a good model system for the investigation of proteasome-associated DUBs.

The aims of this study are firstly to determine whether Uch2 and Ubp6 are associated with the proteasome. If this was found to be the case, genetic experiments would be undertaken to investigate the possibility that Uch2 and Ubp6 have redundant functions. Finally, the biochemical activities of both DUBs would be analysed to provide information about their substrates.

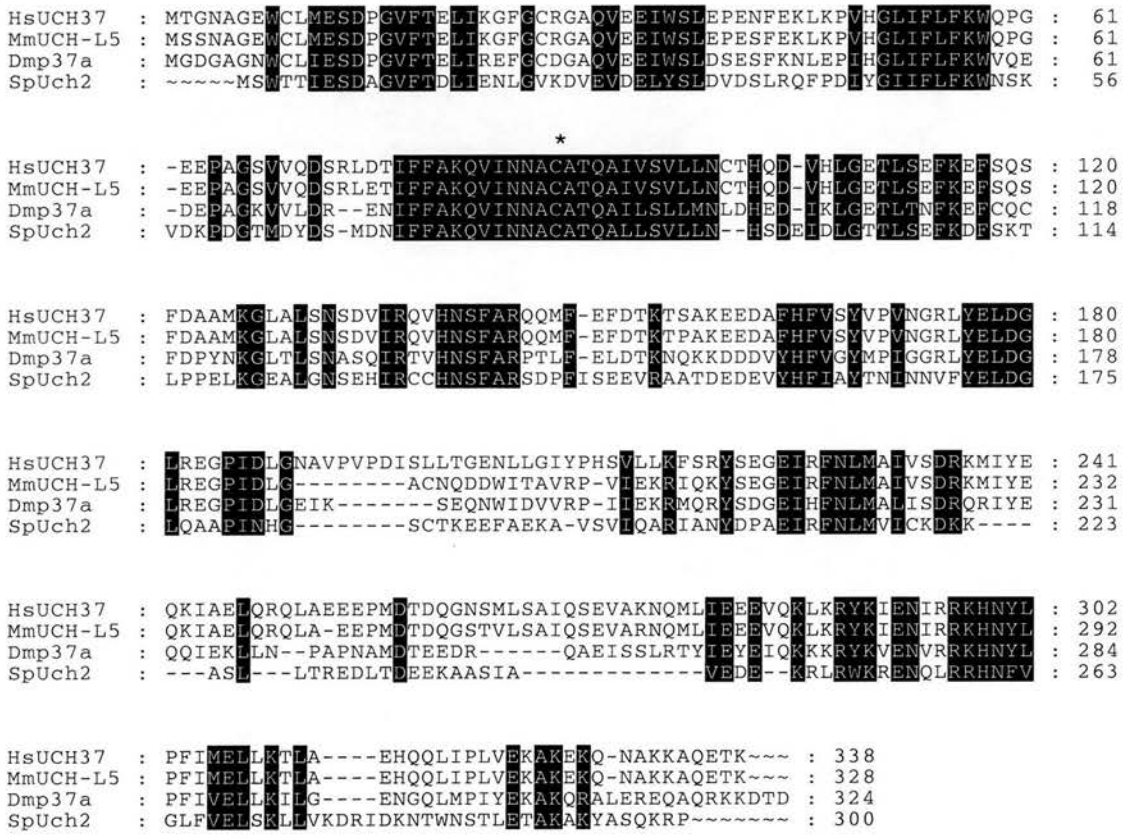

Figure 1.6: The Uch2 Protein Sequence is Conserved
Alignment of the protein sequence of *S. pombe* Uch2 (SpUch2 – SPBC409.06) with those of its homologues in Human (HsUCH37 –Q9Y5K5), Mouse (MmUCH-L5 –AF175903) and *Drosophila* (Dmp37a –CG1950). Conserved amino acids are highlighted and the proposed active site Cys residue is marked (*).

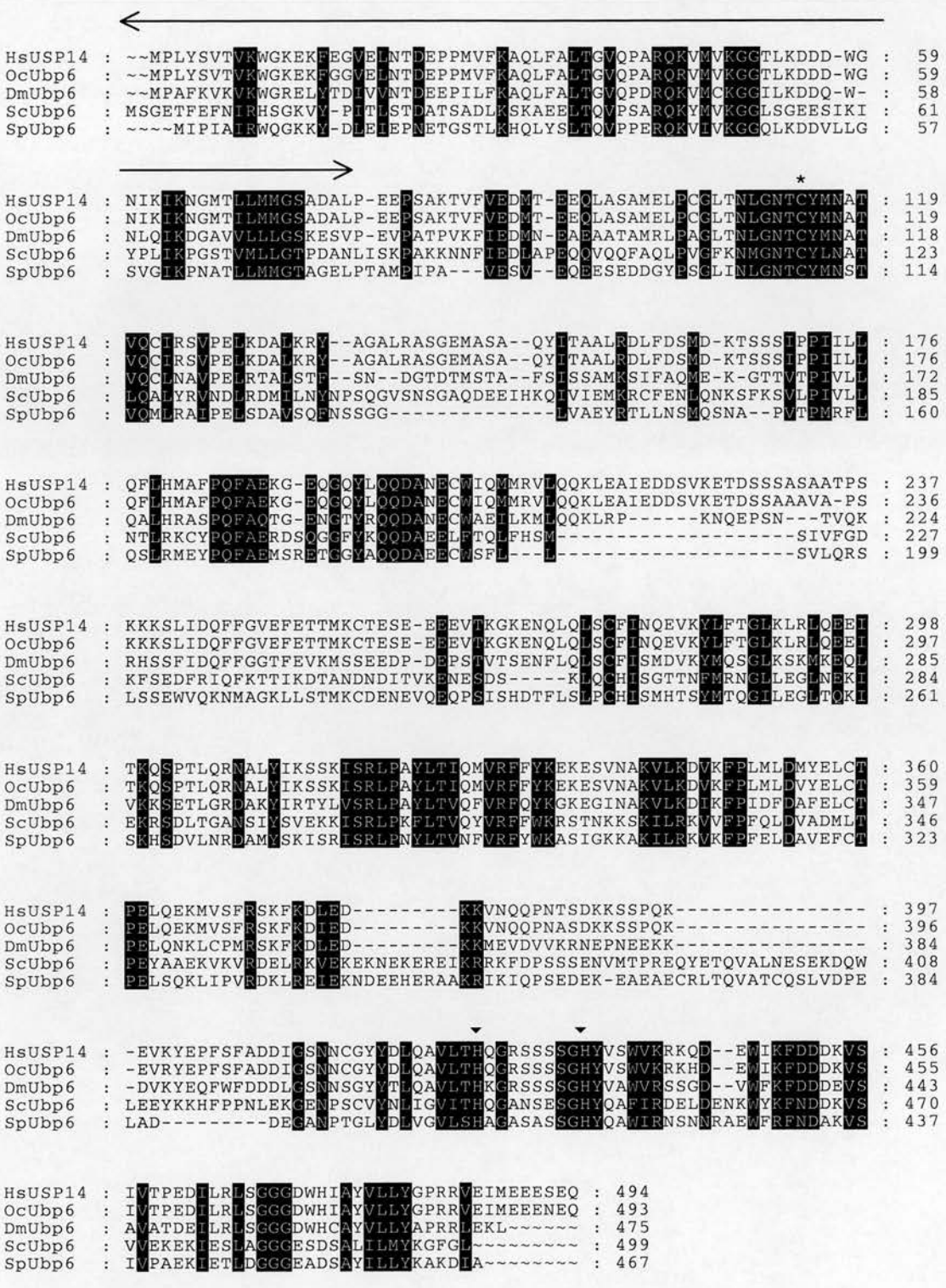

Figure 1.7: The Ubp6 Protein Sequence is Conserved
Alignment of the protein sequence of *S. pombe* Ubp6 (SpUbp6 – SPAC6G9.08) with those of its homologues in Human (HsUSP14 – P54578), Rabbit (OcUbp6 – L37420), *Drosophila* (DmUbp6 – CG5505) and budding yeast (ScUbp6 – NC_00138). Conserved amino acids are highlighted, the ubiquitin-like domain is indicated by the arrows and the proposed active site Cys (*) and His (▼)residues are marked.

Chapter 2 Materials and Methods

Unless otherwise stated all chemicals were supplied by Sigma and all molecular biology reagents by Roche.

2.1 Fission Yeast Methods

2.1.1 Fission Yeast Strains

The strains used in this study were derived from the $972h^{+S}$ and $975h^{-L}$ strains (Leupold, 1950). The standard background of strains was: *leu1-32, ura4-D18, his3-D1, arg3-D4 and either ade6-M210 or ade6-M216.*

2.1.2 Growth Conditions and Media

2.1.2.1 Growth of Fission Yeast

Fission yeast was routinely grown on or in YES media at 25°C. Strains on agar plates were kept for up to 2 months at 4°C. Strains were frozen for long term storage at -70°C in 30% (v/v) Glycerol. Frozen strains were reisolated by streaking a loopful of the frozen culture onto a YES plate. The plate was then incubated at 25°C until colonies had grown.

2.1.2.2 Fully Supplemented Medium

YES

Bacto™ Yeast Extract, Technical (Difco)	5.0g
Glucose	30.0g
Adenine	0.2g
Arginine	0.2g
Histidine	0.2g
Leucine	0.2g
Lysine	0.2g
Uracil	0.2g
H_2O	to 1L

For growth of fission yeast on solid medium, YES Agar was made by adding 20g/L Micro Agar (Duchefa) to YES.

2.1.2.3 Minimal Media

For selection of nutritional prototrophs either EMM (Moreno et al., 1991) or PMG was used.

EMM

Glucose	20.0g
NH_4Cl	5.0g
KH phthalate	3.0g
Na_2HPO_4	1.8g
Salts (50x stock)	20ml
Vitamins (1000x stock)	1ml
Minerals (10 000x stock)	0.1ml
H_2O	to 1L

PMG was made up as EMM, except that 3.75g Glutamic acid was used in place of the 5.0g of NH_4Cl.

Additional growth supplements, such as leucine or uracil, were added as required, before autoclaving to give a final concentration of 0.1g/L.
For repression of the *nmt1* promoter, thiamine was made up as a 100mM stock, filter sterilised and stored at 4°C. It was added after autoclaving to give a final concentration of 2μM.

For growth of on solid medium, EMM or PMG Agar was made by adding 20g/L Micro Agar to YES.

Salts (50x Stock)

MgCl2.6H2O	53.5g
CaCl2.6H2O	1.0g
KCl	50.0g
Na2SO4	2.0g
H2O	to 1L

Vitamins (1000x Stock)

Inositol	10g
Nicotinic acid	10g
Calcium pantathenate	1g
Biotin	10mg
H2O	to 1L

Minerals (10 000x Stock)

H_3BO_3	5.0g
$MnSO_4.4H_2O$	5.2g
$ZnSO_4.7H_2O$	4.0g
$FeCl_3.6H_2O$	2.0g
H_2MoO_4	1.44g
$CuSO_4.5H_2O$	0.4g
Citric acid	10.0g
KI	0.1g
H_2O	to 1L

2.1.2.4 Malt Extract Agar

Malt Extract Agar was used to induce sporulation and meiosis.

Malt Extract Broth (Oxoid)	30g
Micro Agar	20g
H2O	to 1L

2.1.3 Genetic Manipulation of Fission Yeast

2.1.3.1 Crosses

Crosses were set up by mixing a loopful h^+ cells and loopful of h^- cells with approximately 15µl of water on a ME. The crosses were checked for the formation of asci after 2-3 days.

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2.1.3.2 Tetrad Analysis

A sample from a 2-3 day old cross was streaked onto a YES plate and using a Singer Micromanipulator, asci were picked and placed on a grid system on the plate. The plate was left at 20°C overnight or 25-30°C for 8h. Each ascus was then dissected and the four spores arranged on the grid. The spores were then left for 3-5 days at 25°C until colonies had formed.

2.1.3.3 Random Spore Analysis

A sample from a 2-3 day old cross was checked under the microscope to confirm the presence of asci. A loopful of the cross was then resuspended in 1ml H_2O containing 2µl glusculase (*Helix pomantia* juice). The cells were incubated overnight at 37°C. The cells were then washed once in 30% ethanol and once in water. The pellet was resuspended in 1ml H_2O and 5µl, 20µl, 100µl and 875µl samples plated on selective plates. The plates were then incubated at 25°C until colonies had formed.

2.1.3.4 Lithium Acetate Transformation of S. pombe

The lithium acetate method for transformation of *S. pombe* was routinely used (Moreno et al 1991). A 100ml culture of cells was grown until an OD_{600} of 0.5-1.0 ($1x10^7 - 2x10^7$ cells/ml) was reached. The cells were spun down at 2000rpm for 2 minutes and washed once in H_2O and once in 0.1M Lithium Acetate pH4.9. The pellet was resuspended in 0.1M Lithium Acetate pH4.9 to give a final concentration of $2x10^7$ cells/ml. Approximately 1µg DNA, 5µl 10mg/ml transfer RNA and 370µl 50% polyethylene glycol 3350 was added to a 100µl aliquot of the cell suspension.

This was then incubated at 25°C for 45-60 minutes, and then heat shocked at 46°C for 20 minutes. The cells were spun out at 13 000rpm for 1 minute and the supernatant removed. The pellet was resuspended in 1ml YES and incubated at 25°C for 1-3 hours. The cells were plated on selective media and left at 25°C until colonies had formed. For selection of G418 resistance, cells were plated on YES plates, incubated for 24 hours at 25°C and then replica plated onto YES plates containing 100μg/ml geneticin.

2.1.3.5 Plasmid Vectors for Expression in S. pombe

pREP1/41/81 based vectors were used to express a given gene in *S. pombe*. The promoters in these vectors are based on the *nmt1* thiamine repressible promoter from *S. pombe* (Maundrell, 1990). These vectors also contain the *S. cerevisiae* LEU2 gene for selection in fission yeast and the *S. pombe ars* origin of replication. The REP family of vectors is made up of shuttle vectors that contain an Amp^R cassette for selection in *E. coli.*

The *nmt* promoter is fully derepressed in the absence of thiamine, allowing high level expression of the cloned gene. The relative strengths of the three nmt promoters found in REP1, pREP41 and pREP81 are 80, 12 and 1 respectively. The addition of thiamine, which is present in fully supplemented media or added to minimal media, results in repression of the *nmt* promoter leaving only a small residual level of promoter activity (Tommasino and Maundrell, 1991; Maundrell, 1993).

2.1.3.6 Gene Disruption by PCR

Oligonucleotide primers were designed with 60-80bp homology to either end of the gene and 20bp homology to the selection cassette to be used (Bahler et al., 1998).

Ten PCRs were carried out as described in section 2.2.2.2.1 and the total DNA was pooled and ethanol precipitated for one transformation, using the Lithium Acetate Method (see section 2.1.3.4) into the desired strain.

2.1.4 Cytological Methods

2.1.4.1 Paraformaldehyde Fixation of Fission Yeast

A 100ml culture of cells was grown in YES at 25°C, to an OD of less than 1 at 595nm. 30ml of 2.4M Sorbitol in 2xYES was added to 30ml of cells. 6.6ml of 38% parformaldehyde fixation stock solution were added and the cells were incubated at 25°C for between 5-30 minutes depending on the antibody to be used. After incubation, the cells were spun out at 2000 rpm and washed with 20ml of PEMS. The cells were spun out, 5ml of PEMS + 0.25mg/ml Zymolyase T100 (ICN Biomedicals Ltd) was added and the suspension was incubated for 30 minutes at 37°C. The cells were spun out and resuspended in 1ml of PEMS and transferred to an eppendorf. Using a benchtop centrifuge the cells were spun out. The cell pellet was resuspended in 1ml PEMS + 1% Triton X100 (Sigma) and incubated for 5 minutes at room temperature. The cells were spun out and washed three times with 1ml of PEM. The cell pellet was resuspended in 1ml PEMBAL, incubated for 30 minutes at room temperature and then stored at 4°C.

2.1.4.2 Antibody Staining of Paraformaldehyde Fixed Cells

A 10-100µl sample of fixed cells in PEMBAL was spun down and resuspended in 100µl of PEMBAL containing the primary antibody. The cells were incubated at

4°C on a rotating wheel overnight. The cells were spun out in a benchtop centrifuge and the supernatant carefully removed. The cells were then washed three times with 1ml PEM. 100µl of PEM containing the secondary antibody was added to the cell pellet, which was then incubated at 4°C on a rotating wheel overnight. The cells were washed three times in PEM, resuspended in PEMBAL and stored at 4°C.

2.1.4.3 Mounting, DAPI Staining and Analysis of Antibody Stained Paraformaldehyde Fixed Cells

Coverslips and slides were washed with detergent and cleaned with 70% ethanol before use. Coverslips were prepared for mounting by coating in Poly-L-Lysine Solution (0.1% w/v in water, Sigma). 10µl of stained cells were spread on a coverslip and left to air dry. 30-50µl of DAPI in Vectashield Mounting Medium (Vector Laboratories Inc) was added to the coverslip, which was then covered with a microscope slide and blotted dry. Slides were stored at 4°C in the dark. The cells were viewed using a Zeiss Axoiplan2 fluorescence microscope, digital images were captured with a Princeton Instruments digital camera and analysed using in house scripts for the IPLab Spectrum 3.2 software (Scanalytics).

2.2 Molecular Biology

2.2.1 *E. coli* Methods

2.2.1.1 E. coli Strains

Subcloning Efficiency™ DH5α™ Competent Cells (Gibco BRL or Invitrogen Life Technologies) were used for routine plasmid preparation.

2.2.1.2 Media and Growth Conditions

E. coli were grown in Luria-Bertani Broth (LB) or on LB Agar plates.

LB Broth

	per L
Tryptone	10g
Yeast Extract	5g
NaCl	10g

For growth of bacteria on solid medium, LB Agar was made by adding 15g/L Agar to LB. Bacteria were grown at 37°C using an incubator for growth on plates and in an orbital shaker for growth in liquid media.

2.2.1.3 Antibiotic Selection

All antibiotics were stored at -20°C and added to autoclaved media. Stock solutions were made up as follows: a 2000x stock of 100mg/ml of ampicillin in dH2O, a 1000x stock of 34mg/ml chloramphenicol dissolved in ethanol and a 500x stock of 5mg/ml tetracycline dissolved in ethanol.

E. coli were stored on plates at 4°C for up to one month or at -70°C in LB containing 25% glycerol for longer term storage. Strains were reisolated from stocks by spreading a loopful of frozen cells onto a LB plate and incubating at 37°C overnight.

2.2.1.4 Transformation of DNA into E. coli

Bacterial transformations were carried out using commercially produced chemically competent cells: Subcloning Efficiency™ DH5α™ Competent Cells (Gibco BRL or Invitrogen Life Technologies). 1-10μl DNA was transformed into the competent cells according to manufacturer's instructions.

2.2.1.5 Preparation of plasmid DNA from E. coli

Minipreps of plasmid DNA from *E. coli* were carried out using one of the two following methods:

1. Qiaprep Spin Miniprep Kit (Qiagen Ltd) was used as manufacturer's instructions using 1-5ml cultures.
2. For DNA extraction from a large number of colonies, 96 well plates were used to grow overnight cultures which were then processed by the MRC Human Genetics Unit Technical Services, using the BioMek R 2000 Workstation Robot according to the manufacturers instructions.

2.2.2 DNA Manipulation

2.2.2.1 Agarose Gel Electrophoresis of DNA

DNA fragments were separated by electrophoresis through an agarose gel. The agarose was made up at 1% w/v in TBE buffer and heated to dissolve. Ethidium bromide solution (Sigma) was added to cooled agarose to give a final concentration of 100ng/ml. Loading buffer was added to each of the DNA samples and they were

loaded onto the agarose gel once it had set. The gel was run in TBE buffer for 15-120 minutes, depending on the size of the DNA fragments. 1Kb DNA Ladder size markers (Invitrogen) were run routinely. DNA was visualised using a UV source.

TBE (20x stock)

1.78M Tris base
1.78M Boric acid
20mM EDTA

DNA Loading Buffer (10x stock)

40% Glycerol
1% (w/v) Orange G
10mM EDTA

2.2.2.2 PCR

2.2.2.2.1 Standard PCR

PCR was carried out using AmpliTaq™ DNA Polymerase and supplied buffers and $MgCl_2$ (Roche). A PCR mix was made up as below. 10-100ng template DNA was used and negative control reaction without DNA was run for each reaction. Oligos were supplied by Genosys or MWG Biotech.

	Volume in µl
DNA template	1.0-5.0
10X PCR Buffer II	10.0
25mM $MgCl_2$	6.0
1:1:1:1 dNTPs 100mM	0.4
Forward Oligo 100ng/µl	1.0
Reverse Oligo 100ng/µl	1.0
AmpliTaq™ DNA Polymerase	0.5
dH_2O	to 100.0

The following program was then run using either a Hybaid Omnigene, in which case each reaction was overlayed with two drops of mineral oil, or a Dyad™ DNA Engine Thermal Cycler, which has a heated lid.

94°C	2 minutes	
50°C [1]	45 seconds	}
72°C	1 minute [2]	}35Cycles
94°C	30 seconds	}
50°C	45 seconds	
72°C	10 minutes	

2.2.2.2.2 High Fidelity PCR

For increased efficiency PCR, the Expand High Fidelity PCR System (Roche) was used. Reactions were set up as follows using the supplied buffer. DNA and oligos were used as for Amplitaq™ PCR.

	Volume in µl
DNA template	1.0-5.0
10X PCR Buffer with $MgCl_2$	5.0
1:1:1:1 dNTPs 100mM	0.4
Forward Oligo 100ng/µl	1.0
Reverse Oligo 100ng/µl	1.0
Expand DNA Polymerase	0.75
dH_2O	to 50.0

The following program was run using the same thermal cyclers as for Amplitaq PCR:

94°C	2 minutes	
50°C [1]	1 minute	}
68°C	1 minute [2]	}35 cycles
94°C	30 seconds	}
50°C	1 minute	
68°C	10 minutes	

[1] Annealing temperature was decreased to 45°C or 40°C if increased yield was required.

[2] The elongation time was varied depending on the length of the PCR product, allowing a time of 1 minute of elongation per 1kb of PCR product.

2.2.2.2.3 Colony PCR from *S. pombe*

For PCR from *S. pombe* colonies, the PCR mix was made up as for standard PCR except that the polymerase was omitted. *S. pombe* cells were then added until the

mixture became cloudy, and this suspension was boiled for 5 minutes. The mixture was spun for 2 minutes at 5000rpm to pellet the cells. 0.5µl of AmpliTaq™ DNA Polymerase was then added to each tube and the PCR reaction was run using the standard program.

2.2.2.3 Restriction Enzyme Digestion of DNA

DNA was cut using restriction enzymes from Roche or New England Biolabs according to the manufacturer's instructions. Digests were typically carried out using 1-5µg DNA in a final volume of 20-100µl, at 37 °C, for 1-20 hours.

2.2.2.4 Ligation of DNA

Following digestion with restriction enzymes, reactions were performed to ligate DNA fragments with compatible cohesive ends using T4 DNA ligase (Roche) with the supplied reaction buffer. A total volume of 20µl was used, including 1µl T4 DNA ligase, 2µl T4 DNA Ligase buffer and 10-1000ng DNA. Vector and insert DNA was used in a ratio of approximately 1:3. Reactions were routinely carried out for 16 hours at 20°C.

2.2.2.5 Purification of DNA

PCR DNA was purified using the Qiaquick PCR Purification Kit (Qiagen Ltd) and DNA from restriction digests or alkaline phosphatase treatment was purified using the Qiaquick Nucleotide Removal Kit (Qiagen Ltd). Both kits were used according to manufacturer's instructions.

To gel purify DNA, it was first run on a 1% agarose gel. The DNA band was cut from the gel and the DNA extracted using the Qiaquick Gel Extraction Kit (Qiagen Ltd) according to manufacturer's instructions.

2.2.2.6 Site-Directed Mutagenesis

Site-Directed Mutagenesis was carried out using the Quickchange™ kit (Strategene) according to the manufacturer's instructions.

2.2.2.7 DNA Sequencing

Sequencing reactions were set up using 500ng plasmid or 200ng PCR product, with the Big Dye Sequencing Kit (Applied Biosystems) according to manufacturers instructions. The following PCR program was used:

96°C	30 seconds	}
50°C	15 seconds	} 25 cycles
60°C	4 minutes	}

DNA was then ethanol precipitated by adding 1/10 volume NaOAC (pH?) and two volumes of absolute ethanol. The samples were spun for 10 minutes at 14 000rpm, at 4°C in a refrigerated benchtop centrifuge. The ethanol was then removed and one volume of 70% ethanol was added to wash the DNA pellet. The samples were spun as before but for two minutes. The 70% ethanol wash was removed and the DNA pellets were air dried before sequencing.

Sequencing was carried out by the MRC Human Genetics Unit Technical Services. DNA pellets were resuspended in 2-4µl loading dye (PE Applied Biosystems) and

heated for 2 minutes at 90°C. Samples were incubated at 4°C and then loaded onto an Applied Biosystems DNA sequencer (Model 373A or 377) according to manufacturers instructions. Raw sequence data was processed using the Sequencing Analysis Version 3.0 program (PE Applied Biosystems).

Sequence data was analysed using the Sequencer Version 3.0.1 software (Gene Codes Corp.).

2.3 Protein Purification and Manipulation

2.3.1 Preparation of Total Protein Extracts from *S. pombe*

A 100ml culture was grown in YES at 25°C until mid-exponential phase with an OD_{600} of 0.5-1.0. The cells were spun down for 2 minutes at 2000rpm and washed once in H_2O. The cell pellet was resuspended in 1ml of Lysis Buffer and transferred to 2ml screw capped tubes. This cell suspension was centrifuged for 2 minutes at 13000rpm and the pellet resuspended in 100µl Lysis Buffer. 300µl of Braun Glass Beads were added and the sample cooled on ice for 10 minutes. The cells were then ribolysed three times for 10 seconds in a Hybaid RiboLyser at a speed of 6.5, with 2 minutes incubation on ice between each burst. A further 250µl Lysis Buffer was added and the sample ribolysed once for 1-10 seconds at a speed of 6.5. The beads were spun down and the supernatant removed to a fresh eppendorf tube. The supernatant was then spun at room temperature for 10 minutes to clear cell debris. The supernatant was removed to a fresh tube.

Lysis Buffer

50mM Tris pH8
10% Glycerol
50mM NaCl
0.1% Triton

Immediately before use, Complete™ Protease Inhibitor Cocktail Tablets (1 tablet in 50ml) and PMSF (100 µg/ml) were added.

2.3.2 Purification of 26S proteasomes from *S. pombe*

2.3.2.1 Immunoprecipitation of 26S Proteasomes

A 200ml culture of a *pad1*$^+$ or *pus1*$^+$ Protein A tagged strain was grown in YES, at 25°C, to late logarithmic or stationary phase. Cells were harvested by centrifugation at 2000rpm for 1 minute and washed once with 20ml dH_2O and once with 1-3ml 26S Binding buffer. The final pellet was resuspended in 1 ml of 26S Binding buffer and transferred to 2ml screwcap tube. The tube was then stored on ice between each of the following steps and all centrifugation steps were carried out at 4°C. The cells were spun down in a microcentrifuge at 14 000rpm for 1 minute. The cell pellet was resuspended in 100 µl 26S Binding Buffer and approximately 1ml of ice-cold glass beads (Braun). The sample was ribolysed using a Hybaid RiboLyser three times for 10 seconds at a speed of 6.5, with 2 minutes incubation on ice between each burst. The 2ml tubes were then filled completely with 26S Binding buffer and ribolysed once for 2-10 seconds at 6.5. The glass beads were spun down in a microcentrifuge at 14 000rpm for 1 minute and the supernatant was transferred to a fresh eppendorf tube. The cell debris was spun down by centrifuging the sample twice at 14 000rpm for 30 minutes, each time removing the supernatant to a fresh tube. The final volume of the sample was adjusted to 1ml with 26S Binding buffer. 40-60 µL IgG

Sepharose beads were added to the cleared lysate, which was then incubated on a wheel overnight at 4°C. The beads were washed twice with 26S Binding buffer and resuspended in 26S Binding buffer.

For SDS-PAGE, the final pellet was resuspended in 40 µL 2x SDS sample buffer and boiled for 5 minutes.

26S-binding buffer

Tris-base	25 mM
NaCl	50 mM
$MgCl_2$	10 mM
Glycerol	20 (v/v) %
DTT	1 mM
ATP	5 mM
Triton-X 100	0.1 (v/v) %

The pH was adjusted to pH to 7.2 and, immediately before use, Compete™ Protease Inhibitor Cocktail Tablets (1 tablet in 50ml) and PMSF (100 µg/ml) were added.

2.3.2.2 TEV Protease Cleavage of Proteasomes bound to IgG Beads

As the Protein A cassette contains a TEV protease cleavage site, proteasomes were cleaved from the IgG beads using rTEV protease (Gibco BRL) according to the manufacturers instructions.

2.3.3 Expression and Purification of GST-fusion Proteins in *E. coli*

2.3.3.1 Induction of GST-fusion protein Expression

Expression of recombinant proteins was carried out in the BL21 pLysS strain: pLysS:F', *ompT*, *hsdS*$_B$ (r_B-, m_B-), *dcm*, *gal*, pLysS, Cm^r

The cDNA for the gene of interest was subcloned into the appropriate expression vector: pGEX-KG (GST tag) (Guan and Dixon, 1991) and pQE or pET (6 His tag) (supplier). This plasmid was then transformed into BL21 pLysS. Cells were plated on LB AC (LB containing 50μg/ml ampicillin and 68μg/ml chloramphenicol).

An overnight culture of the strain was grown in 20ml LB AC at 37°C. This was used to inoculate a 400ml culture, which was grown at 20-25°C until an OD_{600} of 0.4-0.8 was reached. A 1ml sample was then taken as a negative control for non-induced cells. Expression of the recombinant protein was induced by the addition of 0.5mM IPTG. The cultures were then grown for a further 3-3.5h at 20-25°C and then a 1ml sample was taken to check for induction. The 1ml cell samples taken before and after induction were spun down and the negative control sample was resuspended in 250μl Cracking Buffer and the induced sample in 500μl Cracking Buffer, to allow for the increase in cell number during induction. A 10μl sample of each was run on a 12% SDS-PAGE gel to check for induction of the recombinant protein.

Cracking Buffer

10mM Sodium Pyrophosphate

1% SDS

8M Urea

0.1% Triton

0.1% Bromophenol Blue

50μl β-mercaptoethanol was added to 950μl cracking buffer before use.

2.3.3.2 Purification of Recombinant GST Fusion Proteins

The 400ml induced culture was spun down at 5000rpm for 10 minutes. The cell pellet was resuspended in 20ml GST Binding Buffer containing protease inhibitors, frozen on dry ice and stored at -20°C. The samples were defrosted at room temperature and kept on ice during the following purification stages. The cell suspension was sonicated three times for 10 seconds at power 18-20 and then mixed on a roller for 1h at 4°C. The insoluble proteins were spun out by centrifugation at 12500rpm for 30 minutes at 4°C. The supernatant was removed to a fresh tube and incubated with 400µl TBS washed Glutathione Sepharose™ 4B (Amersham) on a roller at 4°C for 1h.

The samples were centrifuged at 3000rpm for 5 minutes, the supernatant was removed and the Glutathione Sepharose beads resuspended in 2ml GST Binding Buffer with protease inhibitors. To confirm that the recombinant protein had been purified, 5µl of the beads were added to 5µl SDS Loading Buffer and analysed by SDS-PAGE and Coomassie staining.

2.3.3.3 Thrombin Cleavage of Recombinant GST-Fusion Proteins

A sample of recombinant protein bound to Glutathione Sepharose beads was washed four times in GST Binding buffer to remove the protease inhibitors. The beads were then resuspended in 400µl GST binding buffer containing 20 units of Thrombin Protease (Amersham) and incubated on a wheel overnight at room temperature. The beads were spun out and the supernatant containing the cleaved recombinant protein was removed to a fresh tube.

GST Binding Buffer

50mM Tris pH8.0
100mM NaCl
10% Glycerol
1% Triton

Immediately before use, Complete™ Protease Inhibitor Cocktail Tablets (1 tablet in 50ml) and PMSF (100 µg/ml) were added.

2.3.4 SDS-PAGE Protein Analysis

2.3.4.1 SDS-PAGE

Proteins were separated under denaturing conditions using discontinuous gels (Laemmli, 1970). The BioRad Mini-Protean II dual slab cell apparatus for miniature polyacrylamide gels was used to pour the gels.

The lower resolving gel solution was poured first, leaving about 2cm empty at the top of the apparatus. This was then overlayed with H_2O and left for 30 minutes to polymerise. The H_2O was removed and the remaining space filled with the upper stacking gel solution. A comb was immediately inserted into the upper gel and the acrylamide was left for 30 minutes to polymerise. The polymerised gel was then fitted into the buffer tank, which was filled with Tris-Glycine buffer.

Before loading the protein samples were denatured by boiling for 2-5 minutes in SDS-Loading Buffer. The samples were loaded onto the gel and 7µl Prestained Protein Marker Broad Range (New England Biolabs) was run for molecular weight

size markers. Gels were run for 45-60 minutes at 200V. Following electrophoresis, gels were removed for staining or Western blotting.

Resolving Gel Monomer Solutions

	12%	15%
1.5M Tris.Cl pH8.8	2.5ml	2.5ml
10% SDS	0.1ml	0.1ml
30/0.8% Acrylamide	4.0ml	5.0ml
H_2O	3.35ml	2.25ml

Immediately before pouring, 100µl 10% APS and 10µl TEMED were added to the solution.

Stacking Gel Monomer Solution

	4%
0.5M Tris.Cl pH6.8	2.5ml
10% SDS	0.1ml
30/0.8% Acrylamide	1.3ml
H_2O	6.1ml

Immediately before pouring, 100µl 10% APS and 10µl TEMED were added to the solution.

Tris Glycine Buffer, pH8.3

25mM Tris
250mM Glycine
0.1% SDS

2x SDS Loading Buffer

100mM Tris pH6.8
200mM Dithiothreitol
4% SDS
0.2 % Bromophenol blue
20% Glycerol

2.3.4.2 Coomassie Staining

Coomassie staining of SDS-PAGE gels was routinely carried out using Biosafe™ Coomassie Stain II (BioRad) or GelCode® Blue Stain Reagent (Pierce) according to the manufacturer's instructions. Stained gels were dried onto 3MM Whatman paper using a vacuum drier at 80°C.

2.3.5 Western Blot Analysis

2.3.5.1Transfer

Western blots were routinely carried out using the Millipore Graphite Electroblotter System Type II apparatus. Seven sheets of Whatman 3MM paper, a sheet of nitrocellulose membrane and the SDS-PAGE gel containing the proteins to be blotted were soaked in Cathode buffer. They were then assembled in the following order from negative to positive: three sheets of Whatman 3MM paper, nitrocellulose membrane, SDS-PAGE gel, four sheets of Whatman paper. A current of 25mA/cm^2 was applied and run for 40 minutes to 2h.

Cathode Buffer:

25mM Tris pH9.4
20% Methanol
40mM Glycine

For analysis of high molecular weight Ubiquitin conjugates gels were blotted using cathode buffer containing 10% methanol and a current of 40mA/cm^2.

2.3.5.2 Probing and Developing of Western Blots

Ponceau Stain was used to visualize proteins on nitrocellulose following Western blotting.

Blots for Western analysis were blocked by immersion in a blocking solution of 5% Sainsbury's dried skimmed Milk powder in PBST for 1h.

The filter was incubated in the antibody at 4°C or room temperature, overnight, with agitation in a tube containing the primary antibody diluted in blocking solution. To remover the primary antibody, the filter was washed three times for 10 minutes in TBST. The secondary horseradish peroxidase (HRP) conjugated antibody was then diluted in TBST and the filter was incubated with this solution for 1h at 4°C or room temperature. The filter was again washed three times for 10 minutes in TBST. The filter was developed using the ECL+ Plus Western Blotting Detection System (Amersham Pharmacia Biotech AB) according to the manufacturer's instructions.

2.4 Biochemical Assays

2.4.1 In vitro Binding Assays

To prepare the extract for in vitro binding assays, the desired protein was expressed in *E. coli* as described (section 2.3.3). The pellet from 400ml of cells was resuspended in 10ml GST binding buffer containing protease inhibitors, frozen on dry ice and stored at -20°C. The samples were defrosted at room temperature and kept on ice during the following purification stages. The cell suspension was sonicated three times for 10 seconds at power 18-20 and then mixed on a roller for 1h at 4°C. The insoluble proteins were spun out by centrifugation at 12500rpm for 30 minutes at 4°C. The extracts were then filtered through a 0.22 μm filter and stored in 1 ml aliquots at -20°C. One aliquot was used for each binding assay. Protein concentration of the extracts varied between 10 and 50 mg/ml depending on the expression level of the protein.

For each assay, 0.5 ml of the cleared extract were mixed with 2-30 μl of beads bound to GST or GST fusion protein. The relative amount of each protein bound to the beads was assessed by Coomassie stain, and the beads volume adjusted to ensure equal amounts of each protein were used. The extract was then incubated with the beads for 2-16 hours at 4°C. The bead volumes were made up to 30μl to make them clearly visible during washes. The beads were washed 5 times in GST binding buffer and resuspended in 30μl 2xSDS sample buffer. 5-10μl of the samples were separated by 12 % SDS PAGE and subjected to Western blot analysis.

For in vitro binding assays using total *S. pombe* protein, extract was prepared from 20ml cells as described in section 2.3.1. The binding assay was then carried out with beads bound to GST or GST fusion proteins as for binding assays using *E. coli* extract.

2.4.2 Assays for DUB Activity

2.4.2.1 Ubiquitin Hydrolase Assay

Ubiquitin hydrolase peptidase activity was assayed using the fluorogenic substrate Ubiquitin-AMC (Ubiquitin-7-amino-4-methylcoumarin) (Affiniti Research Products Ltd). This substrate is ubiquitin fused to an AMC. The release of the fluorescent AMC from the ubiquitin can be measured using Luminescence Spectrometer LS30 (Perkin Elmer) with 360nm excitation and 460nm emission wavelengths.

For assays with recombinant GST-fusion proteins, the proteins were prepared as described in section 2.3.3. A sample of each of the proteins was analysed by SDS-PAGE and Coomassie staining in order to determine the relative amount of each protein. The volume of beads used in the assay was then adjusted to ensure equal amounts of each protein were used. Assays using purified 26S proteasomes were carried out using proteasomes that were either bound to IgG beads or in solution, cut from the beads using TEV protease. The amount of 26S in was assessed using either a Western blot or a Coomassie stained SDS-PAGE gel.

2.4.2.2 Tetraubiquitin Cleavage Assay

To assay ubiquitin isopeptidase activity proteins were expressed as GST fusions in *E. coli* and extracts were prepared as described in section 2.3.3. 10µl of the extract was then incubated with 1µg Tetraubiquitin (Affiniti Research Products Ltd), in a total volume of 20µl of GST binding buffer, at 30°C, overnight. Cleavage of tetraubiquitin to monoubiquitin was assessed by Western blot analysis using the anti-ubiquitin antibody.

2.5 Antibodies

2.5.1 Antibodies used in this study

Each of the antibodies used in this study is listed in Table 2.1, together with its conjugate, source and concentration used for Western and cytological analysis.

2.5.2 Affinity Purification of Antibodies

A 10% SDS-PAGE gel was run with all the wells containing the protein to which the antibody was made. The gel was western blotted and the filter Ponceau S stained and the protein strip was cut out. This strip was then blocked in PBS containing 0.1% Tween-20 (PBST) and 5% Sainsburys Dried Skimmed Milk Powder. The filter was cut into tiny pieces using a scalpel and transferred to an eppendorf tube containing 800µl TBS and 200µl of the antisera. This was incubated for 3h to overnight on a wheel at room temperature. The supernatant was then removed and the filter rinsed three times in PBST. The supernatant was removed and the filter was washed three times in PBST with incubation on a wheel for 30 minutes for the first wash and for 10 minutes for each of the final two washes.

The filter was then incubated with 200µl 200mM Glycine pH2.5 containing 1.1% BSA for 20 minutes on a wheel. The supernatant was removed and transferred to a fresh tube. The filter was incubated twice with 50µl Glycine/BSA on a wheel for 10 minutes and the supernatants added to the original 200µl. The supernatant was neutralised to approximately pH 7.0 by the addition of up to 30µl 1M Tris, with the pH tested by spotting onto pH paper. The filters were stored in TBST containing 0.02% Sodium Azide for reuse several times. The neutralised supernatant was dialysed using a 0.025µm filter in a petri dish containing PBS. The antibody was then used for Western blotting and immunofluorescence.

Antibody	Conjugate	Supplier	Concentration for Western Analysis	Concentration for Cytology
Anti-Mts4		in house	1:1000	1:100
Anti-Uch2		in house	1:1000	1:100
Anti-Uch3		in house	1:2000	1:100
Anti-HA		in house		1:100
Anti-Mts3		in house	1:1000	
Anti-Ubiquitin		Dako Ltd UK	1:2000	
Anti-Sheep	Alexa Fluor 488	Molecular Probes		1:10 000
Anti-Sheep	Alexa Fluor 594	Molecular Probes		1:10 000
Anti-Mouse	Alexa Fluor 488	Molecular Probes		1:10 000
Anti-Mouse	Alexa Fluor 594	Molecular Probes		1:10 000
Anti-Rabbit	Alexa Fluor 488	Molecular Probes		1:10 000
Anti-Rabbit	Alexa Fluor 594	Molecular Probes		1:10 000
Anti-Sheep	Horse Radish Peroxidase (HRP)	TCS Biologicals Ltd	1:10 000	
Anti-Mouse	HRP	Amersham Pharmacia Biotech AB	1:1000	
Anti-Rabbit	HRP	Amersham Pharmacia Biotech AB	1:1000	

Table 2.1: Antibodies Used in this Study
Each of the antibodies used in this study is listed, together with its conjugate, source and concentration used for Western and cytological analysis.

Chapter 3 Uch2 and Ubp6 are Associated with the 26S Proteasome

3.1 Introduction

The DUBs are a large family of conserved proteins with 18 putative members in the *S. pombe* genome (C. Semple and M. Stone, unpublished). Studies in *S. cerevisiae*, where 19 DUBs have been identified, show that there is a high level of redundancy within the family. Deletion strains were created for 17 of the DUBs; however, it was found that none was essential for cell viability. These mutants were then crossed, but in the resulting double mutants no synthetic enhancement of the single mutant phenotypes was observed. Indeed, when up to five DUBs were deleted in one strain, the cells remained viable (Amerik et al., 2000). This result was surprising as DUB activity is presumed to be essential for editing ubiquitin precursors and recycling ubiquitin.

Although there appears to be a high level of redundancy amongst the DUBs, the fact that they form such a large family suggests that specificity is likely to exist. One aim of this study was to consider the hypothesis that the specificity of the DUBs may be determined by their cellular location. DUB activity is required to remove multiubiquitin chains from 26S proteasome substrates prior to degradation and to recycle ubiquitin from multiubiquitin chains for reuse. Therefore, it seems likely that a subset of redundant DUBs may be localised at the proteasome to perform these functions. In *S. pombe*, Uch2 and Ubp6 were identified as candidates for proteasome associated DUBs.

Uch2 was recently identified in *S. pombe* as a Ubiquitin C-terminal Hydrolase due to its homology to mouse UCH-L5. The Uch2 protein was tagged with GFP and fluorescence microscopy revealed that it was localised in a punctate pattern at the nuclear periphery (Li et al., 2000). The localisation was proposed to reflect a

proteasome-like location for Uch2, as several subunits of the *S. pombe* proteasome have previously been shown to localise in this manner (Li et al., 2000; Wilkinson et al., 1998; Wilkinson, et al 2000). As the localisation studies suggested that Uch2 might be proteasome associated, fractions from glycerol gradient centrifugation were analysed. This demonstrated that Uch2 cofractionated with 26S proteasome subunits, providing further evidence of its association with the proteasome. This association was shown to be mediated by the C terminus of Uch2, which contains KEKE motifs that may function in protein-protein interactions with large protein complexes including the proteasome (Li et al., 2000).

Ubp6 was the second protein to be identified as a potential proteasome-interacting DUB. It is a member of the UBP family and was identified in a sequence homology search for proteins in *S. pombe* that contain a UBL domain (C. Semple and C. Gordon, unpublished). The UBL domain was first shown to mediate an interaction with the proteasome in the *S. cerevisiae* protein, Rad23 (Schauber et al., 1998). Its homologue in *S. pombe*, Rhp23, and the Dph1 protein have both been shown to associate with the proteasome via UBL domains (Wilkinson et al., 2001). It was therefore proposed that Ubp6 might associate with the 26S proteasome via its UBL domain.

This chapter describes experiments to verify the proteasome association of Uch2 and to establish Ubp6 as a proteasome-associated DUB. This was carried out using two approaches: biochemically by copurification and in vivo by immunofluorescence. Firstly, in order to facilitate these studies, antibodies were produced to Uch2 and Ubp6. Association with the proteasome was then investigated using affinity purified, tagged 26S proteasomes, followed by Western blot analysis to determine whether Uch2 and Ubp6 were present. To examine the proteasome-association in vivo, immunofluorescence microscopy was used to confirm the Uch2 localisation observed by GFP, to determine the Ubp6 localisation and to ascertain whether Uch2 and Ubp6 colocalise with the proteasome.

3.2 Association of Uch2 with the 26S Proteasome

3.2.1 Antibodies to Uch2

Antibodies to Uch2 were made in order to facilitate experiments to determine whether Uch2 could coimmunoprecipitate and colocalise with the proteasome.

To obtain Uch2 protein to raise an antibody, Uch2 was overexpressed in *E. coli* as a recombinant GST-fusion. Uch2 was first subcloned into the pGEX-KG expression vector and the resulting plasmid was transformed into BL21 pLysS cells. Expression of the GST-Uch2 fusion was induced by the addition of IPTG. Samples were taken before and after induction and analysed by SDS-PAGE and Coomassie staining. This showed expression of a protein of 60kD, the expected size for the fusion protein (Figure 3.1A). The GST-Uch2 protein was purified using glutathione sepharose beads and Uch2 was cleaved from the GST tag using thrombin. The purified and cleaved proteins were observed by SDS-PAGE and Coomassie staining and were found to be 60kD and 34kD as expected (Figure 3.1B). The amount of purified Uch2 protein was determined using the BioRad Protein Assay. For production of the antibody, 700µg of protein was used. The antibody was raised in rabbit, by Diagnostics Scotland.

Before use, the Uch2 antibody was affinity purified using GST-Uch2 bound to nitrocellulose membrane. Three bleeds of the anti-Uch2 serum were supplied, and the terminal bleed was used, as it was likely this would contain the highest titre of anti-Uch2 antibodies. To test that the antibody was specific, a Western blot was prepared using total protein extracts from *S. pombe* wild type and *uch2* deletion strains (see section 4.2). The blot was probed using the affinity purified terminal bleed anti-Uch2 with anti-Mts4 as a loading control. A band of 34kD, the predicted size for Uch2, was observed in the wild type extract, but was absent in the *uch2*Δ extract (Figure 3.2). This showed that the antibody was able to detect Uch2 and was specific for Uch2.

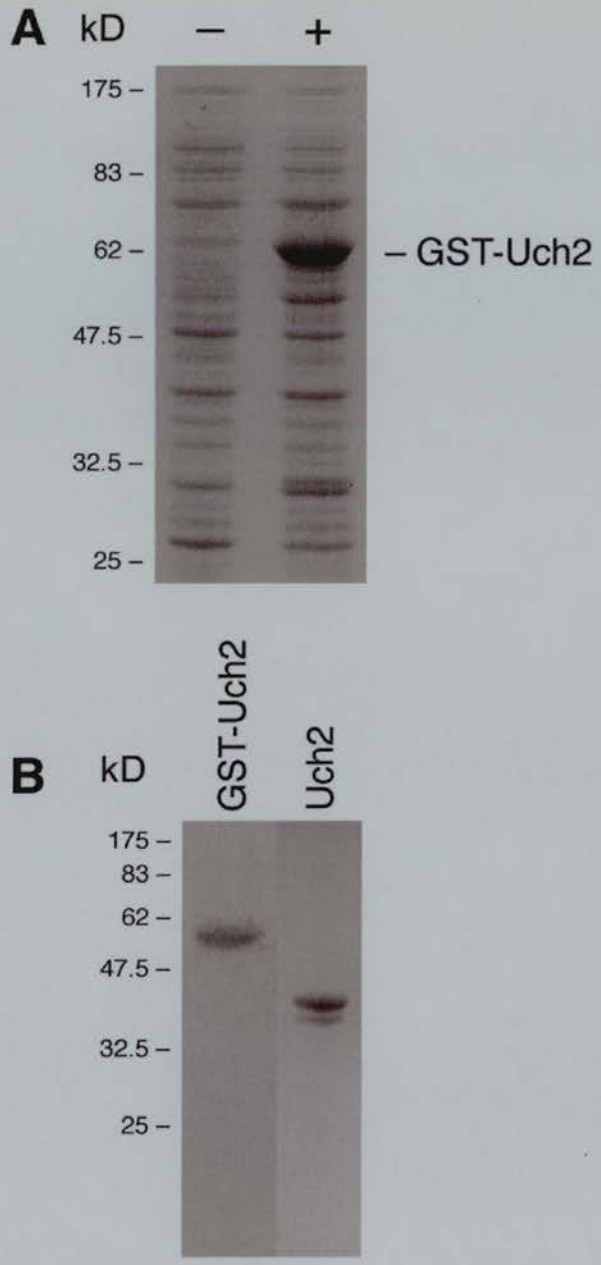

Figure 3.1: Expression and Purification of Uch2
A. GST-Uch2 expression from the pGEX-KG plasmid was induced by IPTG. SDS-PAGE and Coomassie staining of samples taken before (-) and after (+) induction show the expression of GST-Uch2.
B. SDS-PAGE and Coomassie staining shows that GST-Uch2 was purified by binding to glutathione sepharose (GST-Uch2). Uch2 protein was cleaved from the GST tag using thrombin (Uch2).

Figure 3.2: Anti-Uch2 is Specific for Uch2
Western blot analysis using anti-Uch2 on total protein extract from wild type (wt) and *uch2* deletion (Δ*uch2*) strains. A band of 34kD is detected in the wild type extract only, indicating that the anti-Uch2 antibody is specific for Uch2. Anti-Mts4 was used as a loading control.

3.2.2 Proteasome Association of Uch2 by Copurification

3.2.2.1 Affinity Purification of the 26S Proteasome

In order to determine whether Uch2 was proteasome associated, the first approach used was to purify the 26S proteasome and to examine whether this proteins was present in the purified complex. Purification of the 26S proteasome was carried out using a strain tagged at the *pus1*$^{+}$ genomic locus with a Protein A (PA) tag containing a TEV cleavage site. This tag allows efficient immunoprecipitation of the proteasome using IgG sepharose. The resulting proteasomes can be observed by SDS-PAGE followed by Coomassie staining; the larger 19S subunits and smaller 20S subunits can be seen clearly (Figure 3.3A). The purified proteasomes were released from the IgG sepharose using TEV protease, which cleaves between the PA tag and the Pus1 protein. Removal of the protein A tag is useful prior to Western blot analysis; protein A is detected by any antibody, resulting in the appearance of a band which may obscure the protein of interest. In addition, release of the proteasomes into solution allows for easier quantification using the BioRad Protein Assay. The presence of proteasomes was confirmed by Western blotting of the SDS-PAGE gel and staining with Ponceau stain. Further Western blot analysis confirmed the presence of the known subunits Mts4 and Mts3 (Figure 3.3B).

3.2.2.2 Uch2 copurifies with the 26S proteasome

26S proteasomes purified using the *pus1*:PA strain were run on a SDS-PAGE gel and Western blotted. The membrane was Ponceau stained to confirm the presence of proteasomes (data not shown) and then probed using anti-Uch2 and an anti-Mts4 loading control. The blot shows that Uch2 is present in the purified proteasomes as a band of the expected size of 34kD is detected using anti-Uch2 (Figure 3.4).

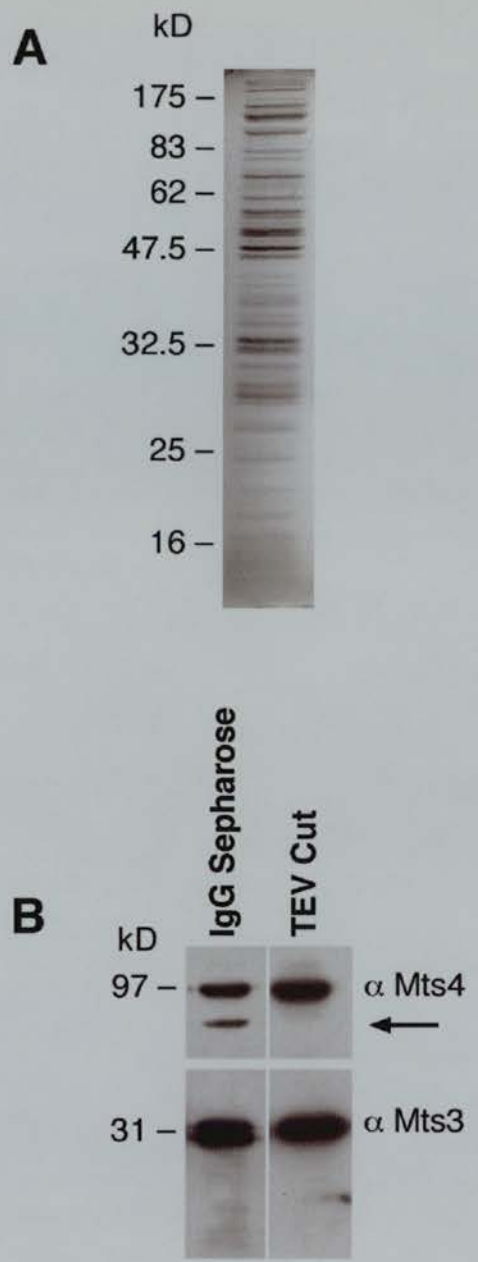

Figure 3.3: Purification of the 26S Proteasome

A. Coomassie stained SDS-PAGE of the 26S proteasome purified from a pus1:PA tagged strain using IgG sepharose.

B. Western blot of purified 26S proteasome bound to IgG sepharose confirms the presence of Mts3 and Mts4. The PA tagged Pus1 subunit is also detected, as indicated by the arrow. Treatment of the IgG sepharose bound proteasomes with TEV protease cleaves between the PA tag and Pus1, releasing proteasomes into the supernatant so that the Pus1-PA band is no longer detected.

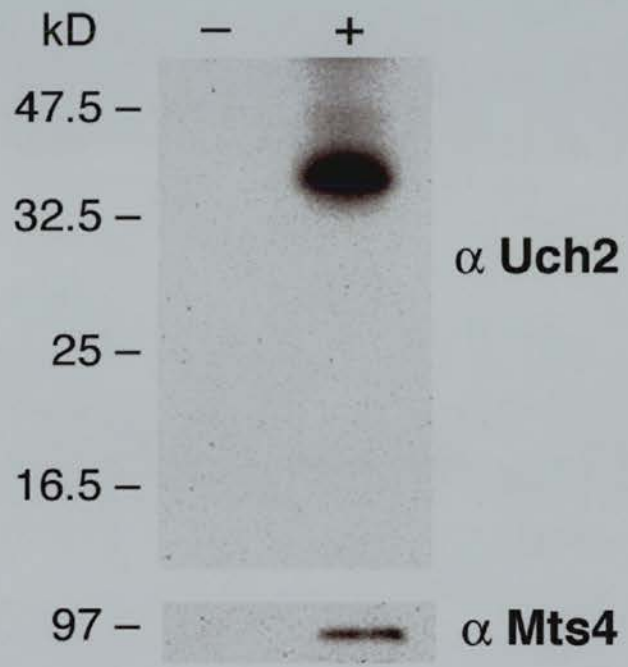

Figure 3.4: Uch2 is Present in Purified 26S Proteasomes
Western blot analysis using anti-Uch2 reveals the presence of Uch2 in purified proteasomes from a pus1:PA tagged strain (+) but not in a control immunoprecipitation using an untagged strain (-). Anti-Mts4 was used to indicate the presence of proteasomes.

3.2.3 Localisation of Uch2

Uch2 had previously been tagged with GFP and observed to localise in a punctate pattern at the nuclear periphery, in a manner reminiscent of that seen for 26S proteasome subunits. In order to determine whether this localisation pattern reflected a colocalisation of Uch2 with the proteasome, immunofluorescence microscopy was performed.

Cells tagged at the endogenous *pad1*$^{+}$ locus with HA were fixed in paraformaldehyde for 10, 20 and 30 minutes at 25°C. Each sample of cells was then stained with anti-Uch2 and an Alexa Fluor 594 anti-rabbit secondary antibody. In addition, DAPI was used to stain the nuclear DNA. Microscopic analysis showed that Uch2 was localised at the nuclear periphery, with the clearest signal obtained using cells fixed for 10 minutes. The confirmed the localisation that had been observed for GFP-Uch2.

The same cells were then stained using anti-HA and an Alexa Fluor 488 anti-mouse secondary antibody to detect Pad1:HA. Microscopic analysis revealed that the Uch2 and Pad1:HA proteins localised at the nuclear periphery with punctate staining (Figure 3.5). When the red anti-Uch2 signal and the green anti-HA signal were merged, yellow overlapping signal was observed (Figure 3.5). Therefore, it was clear that Uch2 was colocalising with the 26S proteasome at the nuclear periphery. These results showed that the GFP localisation previously observed for Uch2 was the same as that of the endogenous protein, and suggested that Uch2 is closely associated with the proteasome, possibly as a subunit of the proteasome.

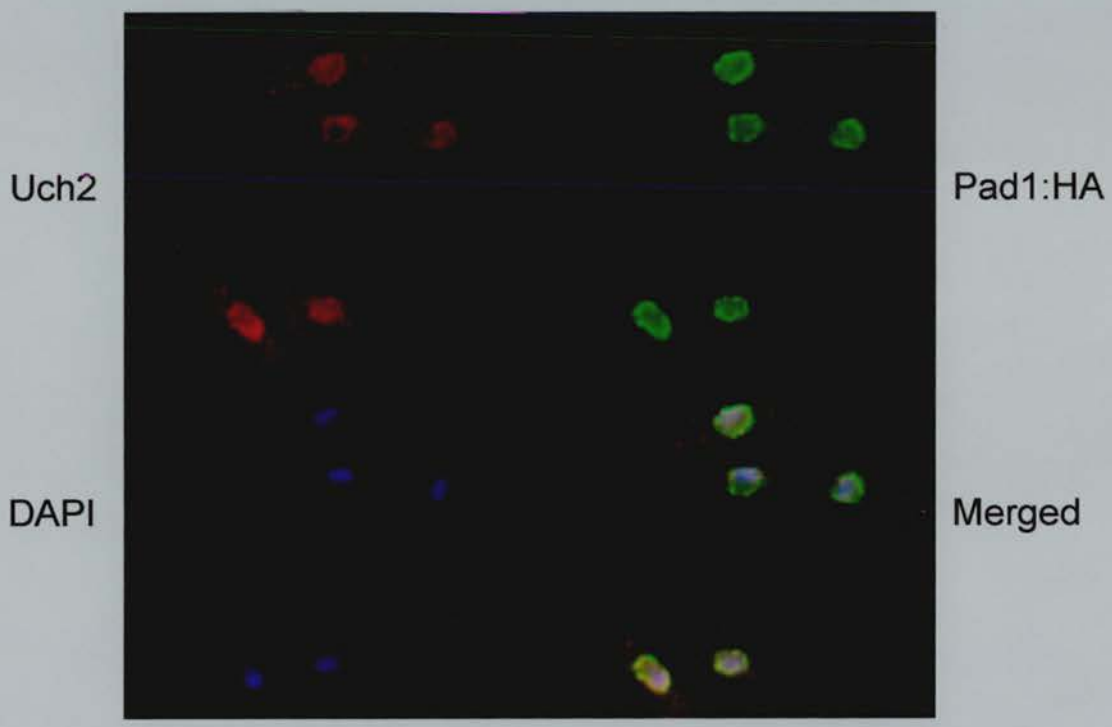

Figure 3.5: Cellular Localisation of Uch2
Immunofluorescence microscopy of paraformaldehyde fixed pad1:HA cells using anti-Uch2 and anti-HA shows that Uch2 and Pad1 localise in a punctate pattern at the nuclear periphery. DAPI staining was used to detect the nucleus. The merged Uch2 and pad1:HA signals reveal that Uch2 and Pad1 colocalise.

3.3 Association of Ubp6 with the 26S Proteasome

3.3.1 Antibody to Ubp6

For examining the association of Ubp6 with the proteasome, an anti-Ubp6 antibody was made as for anti-Uch2, except that it was raised in sheep. Expression and purification were assessed by Coomassie stained SDS-PAGE gels (Figure 3.6).

The terminal bleed of the Ubp6 antibody was affinity purified using GST-Ubp6 bound to nitrocellulose membrane. Western blot analysis was used to test the specificity of the antibody using total protein extracts from a wild type strain and a *ubp6*$^{+}$ deletion strain (see section 4.3). The blot was probed using the affinity purified terminal bleed anti-Ubp6 with anti-Mts4 as a loading control. A doublet at about 52kD, the predicted size for Ubp6, was observed only in the wild type extract (Figure 3.7). This showed that the antibody was able to specifically detect Ubp6.

3.3.2 Ubp6 copurifies with the 26S proteasome

To determine whether Ubp6 could associate with the 26S proteasome, the *pus1*:PA strain was used to purify proteasomes. The purified proteasomes were run on a SDS-PAGE gel and Western blotted. To confirm the presence of proteasomes, the membrane was stained using Ponceau Stain (data not shown). It was then probed using anti-Ubp6 and anti-Mts4 to confirm the presence of proteasomes and as a loading control. The blot shows that Ubp6 is present in the purified proteasomes as a band of the correct size, 52kD, is detected using anti-Ubp6 (Figure 3.8).

Figure 3.6: Expression and Purification of Ubp6

A. GST-Ubp6 expression from the pGEX-KG plasmid was induced by IPTG. SDS-PAGE and Coomassie staining of samples taken before (-) and after (+) induction show the expression of GST-Ubp6.

B. GST-Uch2 was purified by binding to glutathione sepharose (GST-Ubp6). Ubp6 protein was cleaved from the GST tag using thrombin (Ubp6).

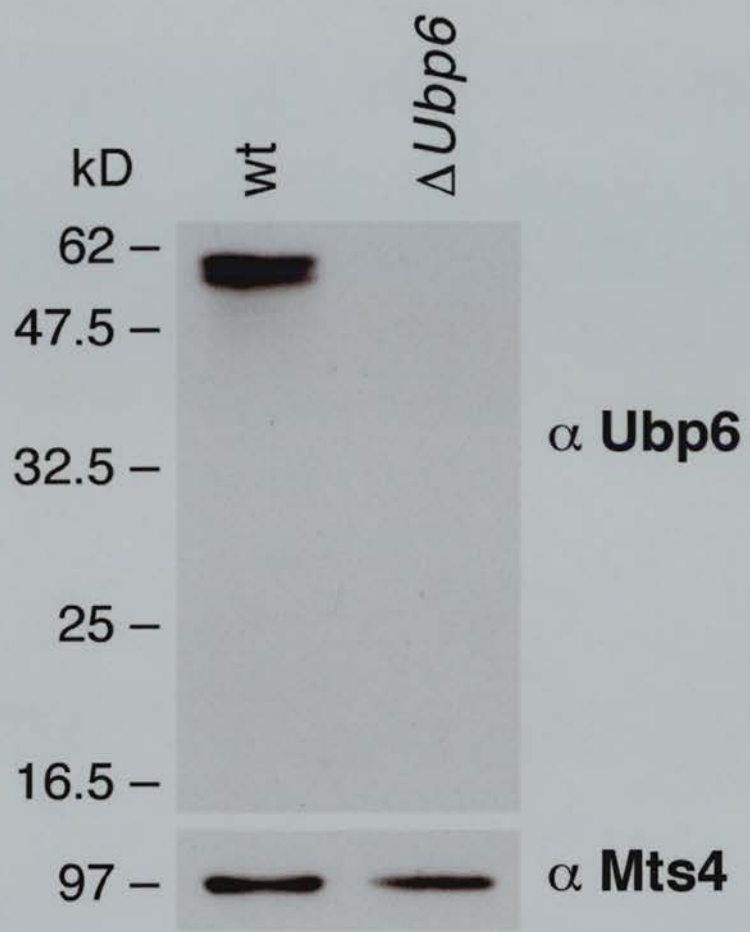

Figure 3.7: Anti-Ubp6 is specific for Ubp6
Western blot analysis using anti-Ubp6 on total protein extract from wild type (wt) and *ubp6* deletion (Δ*ubp6*) strains. A band of 52kD is detected in the wild type extract only, indicating that the anti-Ubp6 antibody is specific for Ubp6. Anti-Mts4 was used as a loading control.

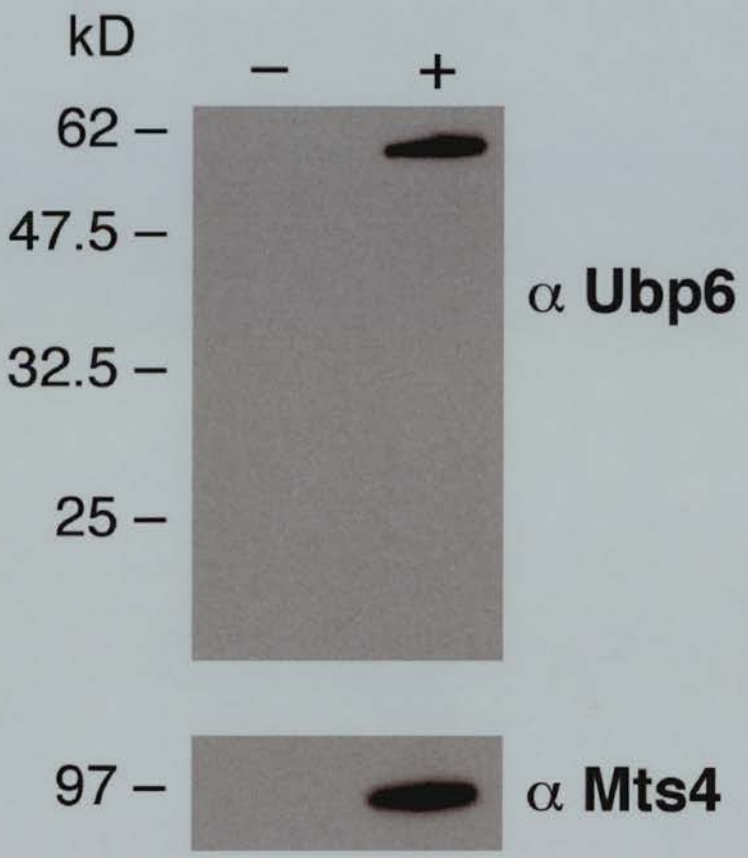

Figure 3.8: Ubp6 is Present in Purified 26S Proteasomes
Western blot analysis using anti-Ubp6 reveals the presence of Ubp6 in purified proteasomes from a *pus1*:PA tagged strain (+) but not in a control immunoprecipitation using an untagged strain (-). Anti-Mts4 was used to indicate the presence of proteasomes.

3.3.3 Localisation of Ubp6

As Ubp6 had been detected in the 26S proteasome pulldown, it was then important to show that this was reflected in its cellular localisation. Immunofluorescence microscopy was used to determine whether Ubp6 could associate with the 26S proteasome in vivo.

Wild type cells were paraformaldehyde fixed for 10, 20 and 30 minutes at 25°C. A sample of cells from each time point was stained with anti-Ubp6 and an Alexa Fluor 594 anti-sheep antibody. The cells were observed under a microscope and the clearest signal was obtained using the cells fixed for 30 minutes. The red signal indicating Ubp6 was seen as a punctate pattern at the nuclear periphery. This indicated that Ubp6 localised in a proteasome-like pattern.

Cells stained for Ubp6 were washed and stained using antibodies to the proteasome subunit Mts4 and Alexa Fluor 488 anti-rabbit secondary antibody. In addition, DAPI was used to stain the nuclear DNA. Microscopic analysis revealed that both Ubp6 and Mts4 proteins localised at the nuclear periphery with punctate staining. When the red Ubp6 and green Mts4 signals were merged they were found to overlap, indicated by the appearance of yellow patches of signal (Figure 3.9). Therefore, Ubp6 colocalises with the 26S proteasome at the nuclear periphery.

3.3.4 Interaction of Ubp6 and the 26S Proteasome

3.3.4.1 Ubp6 Associates with the Proteasome via its UBL domain

Ubp6 was first identified as a candidate for a proteasome associated DUB due to the presence of a UBL domain at its N terminus. Other proteins containing the UBL domain, most notably Rhp23, have been shown to interact with the proteasome via

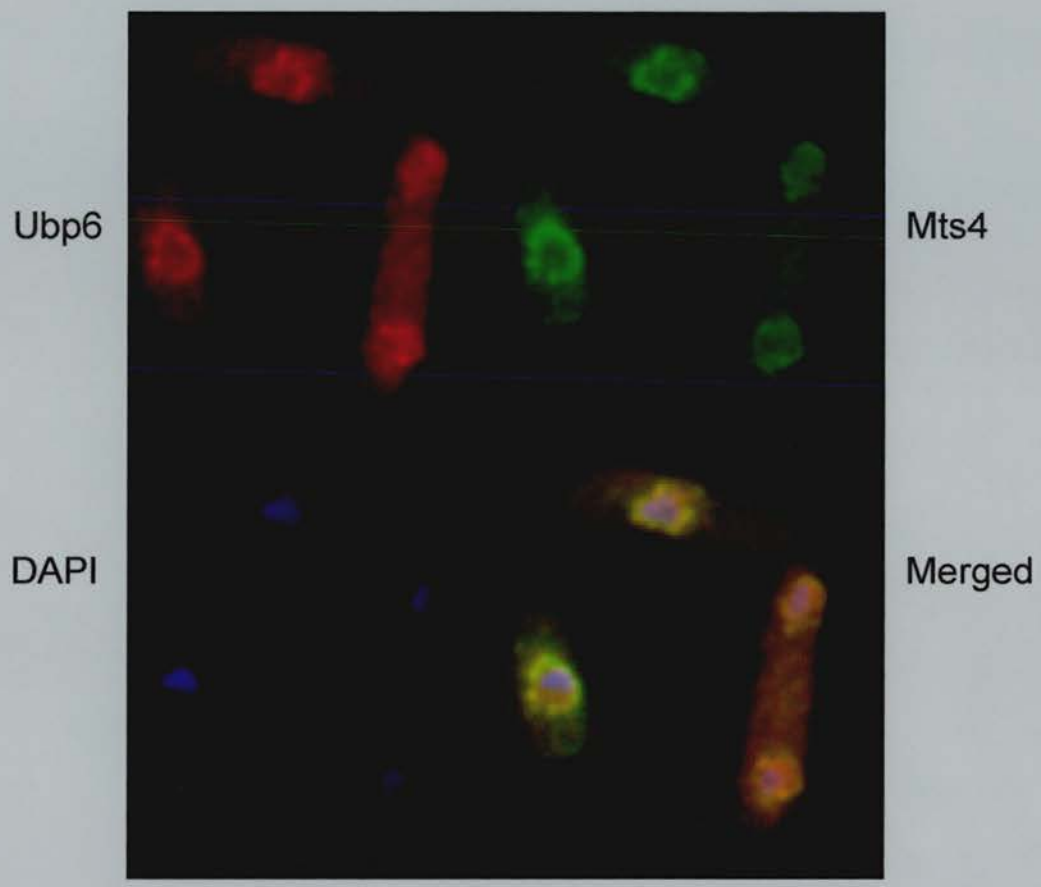

Figure 3.9: Cellular Localisation of Ubp6
Immunofluorescence microscopy of paraformaldehyde fixed wild type cells using anti-Ubp6 and anti-Mts4 shows that Ubp6 and Mts4 localise in a punctate pattern at the nuclear periphery. DAPI staining was used to detect the nucleus. The merged Ubp6 and Mts4 signals reveal that Ubp6 and Mts4 colocalise.

this domain. Experiments were therefore undertaken to determine whether the UBL domain of Ubp6 was mediating its interaction with the proteasome.

Firstly, two truncations of Ubp6 were created in the pGEX-KG plasmid to allow their expression in *E. coli*. The first truncation, NΔ1, removed the 76 N-terminal amino acids that make up the UBL domain and the second truncation, CΔ1, consisted of only the UBL domain (Figure 3.10A). GST-Ubp6, GST-Ubp6NΔ1, GST-Ubp6CΔ1 and GST were expressed in *E. coli* and bound to glutathione sepharose beads. The amount of each protein bound to the beads was determined using a Coomassie stained SDS-PAGE gel and the volume of beads was adjusted to give an equal amount of protein in each assay (Figure 3.10B). These beads were then used to carry out in vitro binding assays using wild type total *S. pombe* protein extract.

A sample of each pulldown assay was analysed by SDS-PAGE followed by Western blotting. To detect the presence of proteasomes bound to the GST fusion proteins, the membrane from the Western blot was divided into two and the high molecular weight half probed using anti-Mts4 and the lower molecular weight half using anti-Mts2 (Figure 3.10C).

Mts4 and Mts2 proteins were detected in the GST-Ubp6 and GST-Ubp6CΔ1 pulldowns indicating that full length Ubp6 and the UBL domain of Ubp6 are able to bind the proteasome in vitro. Neither proteasome subunit was detected in the pulldowns with GST-Ubp6NΔ1, which lacks the UBL domain, and the GST negative control. Therefore, this demonstrates that Ubp6 binds the proteasome in vitro and that its UBL domain is both necessary and sufficient for this interaction.

3.3.4.2 Ubp6 has a direct physical interaction with Mts4

In order to determine whether the association of Ubp6 and the proteasome is the result of a direct interaction with a proteasome subunit, further binding assays were

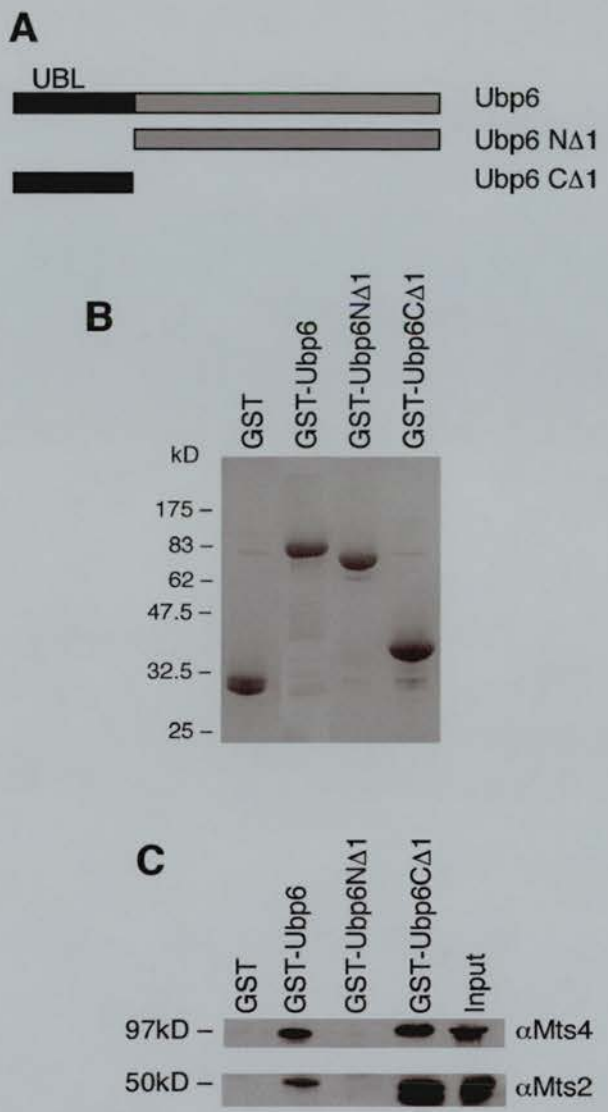

Figure 3.10: The UBL Domain of Ubp6 Binds to the 26S Proteasome

A. Truncations of Ubp6 were constructed to delete the UBL domain (Ubp6NΔ1) or to include only the UBL domain (Ubp6CΔ1).

B. A Coomassie stained SDS-PAGE gel shows that equal amounts of GST-Ubp6, GST-Ubp6NΔ1, GST-Ubp6CΔ1 and the control GST were used in in vitro pull-down assays.

C. GST-Ubp6, GST-Ubp6NΔ1, GST-Ubp6CΔ1 and GST were assayed for their ability to bind the 26S proteasome from fission yeast total protein extract. Western blot analysis of the assays using antibodies to the Mts2 and Mts4 proteasome subunits indicates that only GST-Ubp6 and GST-Ubp6CΔ1 were able to bind the 26S proteasome.

carried out. As the UBL domain of Rhp23 interacts with Mts4, this was the best candidate for the Ubp6 binding subunit. His tagged Mts4 was expressed from the pET plasmid in *E. coli* and extracts were prepared. Binding assays were then carried out using GST and GST-Ubp6 bound to glutathione sepharose beads with the Mts4 extract. A sample of each of pulldown was run on a SDS-PAGE gel and Western blotted. The blot was then probed with anti-Mts4. Mts4 protein was detected in the GST-Ubp6 pulldown but not the GST control pulldown (Figure 3.11). This indicates that Ubp6 can bind directly to Mts4 in vitro (R. Hartmann-Petersen and M. Stone).

3.5 Discussion

The first aim of this study was to verify the proteasome association of two DUBs, Uch2 and Ubp6. Antibodies were made to these proteins using recombinant proteins expressed in *E. coli*. These antibodies were affinity purified and shown to be specific for the desired protein by probing Western blots of protein extracts from wild type and deletion strains.

Uch2 was found to coimmunoprecipitate with the 26S proteasome and to colocalise with the known 19S subunit, Pad1, in a punctate pattern at the nuclear periphery (Penney et al., 1998; Wilkinson et al., 1998; Li et al., 2000). This reinforced evidence from a previous study that showed that Uch2 was localised in a proteasome-like manner and cofractionated in glycerol gradients with the 26S proteasome. The C-terminus of Uch2 was found to be essential for this association, which is not surprising as the N-terminus contains the catalytic domain (Li et al., 2000). Therefore, it appears that Uch2 is a novel proteasome subunit. Recently, during biochemical characterisation of *Drosophila melanogaster* 19S complexes by 2D gel electrophoresis, a homologue of Uch2, p37a, was identified. This 37kD protein is 43% identical to Uch2 and was found to have a homologue in human (UCH37) and mouse (Figure 1.6; Li et al., 2000). The position of p37a in the 26S

Figure 3.11: Ubp6 Interacts Directly with Mts4
GST-Ubp6 was tested for its ability to bind Mts4 from *E. coli* extracts expressing His-tagged Mts4. Western blot analysis using anti-Mts4 shows that GST-Ubp6 is able to bind Mts4. The control GST shows no binding to Mts4.

complex was investigated by scanning transmission electron microscopy, using Ubiquitin aldehyde, a potent UCH inhibitor, conjugated to colloidal gold particles. This revealed that p37a is located at the interface of the base and lid subcomplexes, in a similar location to Rpn10. It is possible that this position allows p37a to remove ubiquitin from proteasome substrates prior to their degradation (Holzl et al., 2000).

Ubp6 was proposed as a proteasome associated DUB because of the UBL domain found at its N terminus. Immunoprecipitation of the proteasome in *S. pombe* revealed that Ubp6 coimmunoprecipitated with the proteasome. Mass spectrometry of whole proteasomes has since identified the *S. cerevisiae* homologue of Ubp6, as a proteasome interacting protein. This interaction was confirmed by coimmunoprecipitation of proteasome subunits with Ubp6 (Verma et al., 2000). In support of these findings, the human homologue of Ubp6, USP14, is detected in Superose 6 fractions containing the 26S proteasome and in immunoprecipitated proteasomes. The activity of USP14 was proposed to be functionally coupled to proteasome activity, as binding of an active site directed probe was increased 15 fold when proteasome activity was inhibited (Borodovsky et al., 2001). *S. pombe* Ubp6 was found to have a proteasome-like localisation at the nuclear periphery and was shown to colocalise with the Mts4 proteasome subunit (Wilkinson et al., 1998). This makes Ubp6 the first of the proteasome-associated ubiquitin-like domain proteins (UDPs) to be observed to colocalise with the proteasome in *S. pombe*, as Dph1 and Rhp23 do not show a proteasome-like localisation (C. Gordon, unpublished). Although this may indicate that Ubp6 is more abundant at the proteasome than the other proteasome associated UDPs, the Dph1 homologue hPLIC-2 has been found to partially colocalise with the proteasome at the cytoskeleton and in the cytosol of differentiated human keratinocytes (Kleijnen et al., 2000). Both Dph1 and Rhp23 are proposed to shuttle between the proteasome and other cellular locations. Therefore, it would be useful to examine fractions from glycerol gradient centrifugation to ascertain whether free Ubp6 is present in addition to the proteasome associated form and, if appropriate, to assess the relative amounts and activities of each form (Wilkinson et al., 2001). Western blot analysis revealed there are two bands

corresponding to Ubp6 in *S. pombe* extracts, suggesting that it may be posttranslationally modified. The band detected in the purified proteasomes does not appear to be a doublet, which implies that only one form associates with the proteasome, and that the posttranslational modification may regulate the interaction of Ubp6 with the proteasome. In order to characterise this modification, Ubp6 could be tagged and purified from *S. pombe* and subjected to mass spectrometric analysis. As phosphorylation is a major mechanism of posttranslational modification, Ubp6 could also be treated with phosphatase to determine whether phosphorylation is responsible for the change in mobility.

Several UDPs have been found to be proteasome-associated, therefore, it was proposed that Ubp6 would also interact with the proteasome via its UBL domain. In pulldown experiments, GST-Ubp6 was found to bind the 26S proteasome from *S. pombe* total protein extracts, as shown by the presence of Mts2 and Mts4 subunits. Using truncated versions of Ubp6 it was demonstrated that the UBL domain is necessary and sufficient to mediate this interaction. A similar association has been found with the *S. pombe* UCPs Rhp23 and Dph1, which both interact with the proteasome in vitro in a UBL domain dependent manner (Wilkinson et al., 2001). The Rhp23 homologues in *S. cerevisiae* (Rad23) and human (HHR23-B) have also been shown to interact with the proteasome via their UBL domains (Schauber et al., 1998). In addition, Dph1 homologues in budding yeast (Dsk2) and human (hPLIC-1 and hPLIC-2) were found to associate with the proteasome (Funakoshi et al., 2002; Walters et al., 2002). Mutation of three potential proteasome interacting sites of the UBL domain of hPLIC-2, was found to abolish binding (Walters et al., 2002). Another UDP which has also been reported to interact with the proteasome is BAG-1, a Hsp70 regulator (Luders et al., 2000). Therefore, the UBL domain may have a general function in targeting of proteins to the proteasome. In the case of Rhp23, the role of the UBL domain in mediating the interaction with the proteasome has been demonstrated to have functional significance for its roles in the DNA repair response pathway and in the recognition and targetting of proteasome substrates. A truncated form of Rhp23 that lacks the UBL domain is unable to rescue the growth defect of a

pus1rhp23 double mutant and deletion of the UBL domain of *S. cerevisiae* Rad23 results in sensitivity to UV (Watkins et al., 1993; Wilkinson et al., 2001). Further experiments will elucidate the significance of the UBL domain for the function of Ubp6.

As Ubp6 was proved to be proteasome associated, it was of interest to determine whether this reflected a direct physical interaction with a proteasome subunit. GST pulldown experiments were able to show that Ubp6 could bind to the Mts4 subunit, an interaction that was predicted as Rhp23 had previously been shown to bind to Mts4 (R. Hartmann-Petersen and M. Seeger, unpublished). In the case of Rhp23, the UBL domain was found to mediate the interaction with Mts4, therefore, future experiments will use the Ubp6 truncations to examine whether the UBL domain of Ubp6 interacts with Mts4 (R. Hartmann-Petersen and M. Seeger, unpublished). The region of Mts4 that interacts with the UBL domain of Rhp23 has been narrowed down by the use of truncated forms of Mts4 in in vitro binding assays (R. Hartmann-Petersen and M. Seeger, unpublished). Sequence homology analysis has identified a putative domain, named the UBL-Binding Sequence (UBS) in the region of Mts4 that interacts with Rhp23. The UBS is found only in the Mts4 proteasome subunit in *S. pombe*, between residues 180-352. Significantly, the UBS is highly conserved amongst Mts4 homologues from a number of species, indicating that it is likely to be a true, functional domain (M. Taylor and C. Gordon, unpublished). It will be important to determine whether the UBL domains of Ubp6 and Dph1 also bind to this domain and whether there is competition between the UBL proteins for binding to the proteasome.

In summary, biochemical and cytological analysis has revealed that Uch2 and Ubp6 are proteasome associated DUBs. Other DUBs have previously been suggested to be associated with the proteasome, such as the *S. cerevisiae* Doa4 and Aplysia ApUbp (Hegde et al., 1997; Papa et al., 1999). However, Doa4 is not conserved in any other system that has been studied, and no homologue of ApUbp has been found in

purified 26S proteasomes from other species (Voges et al., 1999; Verma et al., 2000). Therefore, it is significant that both Uch2 and Ubp6 are highly conserved proteins as this suggests that they may act as proteasome associated DUBS in pathways that are conserved from yeast to humans (Wyndham et al., 1999; Li et al., 2000). The discovery of DUBs localised at the proteasome is in keeping with emerging data that components of the ubiquitin pathway may be associated with the proteasome. Two studies in *S. cerevisiae* have found that E3s are able to interact with the proteasome: Ubr1p and Ufd4 were both shown to interact directly with proteasome subunits and several SCF components were identified when purified proteasomes were analysed by mass spectrometry (Verma et al., 2000; Xie and Varshavsky, 2000). The proteasome has also been demonstrated to have E2 activity; four *S. cerevisiae* E2s have been shown to associate with the proteasome, although it is unclear whether this reflects a direct physical interaction with proteasome subunits (Tongaonkar et al., 2000). Therefore, it appears that the ubiquitin pathway and proteasome mediated degradation may be more intricately coupled than was previously assumed.

Chapter 4 Genetic Interactions of Uch2 and Ubp6

4.1 Introduction

It has been shown in *S. cerevisiae* that the DUBs have high level of redundancy, and as yet no feature of the DUBs has been linked to specificity (Amerik et al., 2000). One aim of this study was to ascertain whether DUB specificities are determined by cellular localisation. The first part of this study established that Uch2 and Ubp6 share a similar cellular localisation, therefore, it was proposed that they might also share functions within the cell. In addition, the colocalisation of Uch2 and Ubp6 with the 26S proteasome suggested that they might have a role in the regulation of ubiquitin dependent proteolysis. To investigate these hypotheses, a series of genetic experiments were undertaken.

The first step in the genetic analysis of $uch2^{+}$ and $ubp6^{+}$ was to determine whether there were conditions under which either gene was essential. Uch2 had previously been deleted from *S. pombe*, with no obvious phenotype, suggesting that another DUB may act in a redundant manner (Li et al., 2000). This chapter describes experiments designed to address the question of whether Ubp6 provides redundant function with Uch2. This involved construction of *uch2* and *ubp6* null mutants and the *uch2 ubp6* double mutant followed by examination of the mutant phenotypes to determine whether there was any synthetic interaction that would suggest redundancy.

Regulation of proteolysis by the 26S proteasome is one of the major functions of ubiquitination, and it would therefore be expected that certain DUBs would have specific functions in this pathway. In *S. cerevisiae*, two DUBs have been found to show genetic interactions with proteasome mutants. The phenotypes of *ubp14* (Isopeptidase T) and *doa4* mutants increase in severity when combined with mutations in 26S proteasome subunits (Amerik et al., 1997; Papa et al., 1999). In

this study genetic crosses were undertaken in order to investigate whether the association of Uch2 and Ubp6 with the proteasome reflects a role in ubiquitin-dependent proteolysis. There are several well characterised 26S proteasome mutants available in *S. pombe* (see section 1.3.3) and each of these was crossed to the *uch2* and *ubp6* null mutants. In addition, the deletion strain for the *S. pombe* Ubp14 homologue, Ubp14, was crossed to each of the single and double mutants to examine whether Uch2, Ubp6 and Ubp14 share functions in the regulation of proteolysis.

4.2 Deletion of uch2⁺

To further examine the role of Uch2 in the cell, the *uch2*$^+$ gene was deleted from *S. pombe* strain carrying the following nutritional markers: *leu1-32, ura4-D18, his3-D1, arg3-D4* and *ade6-210*. The deletion was carried out using the PCR-based method to insert a *ura4*$^+$ gene to replace the *uch2*$^+$ open reading frame (Bahler et al., 1998). PCR primers to the inserted gene and a region upstream of *uch2*$^+$ were used to confirm that the deletions were successful (Figure 4.1A). In each case the appearance of a band in a subset of colonies that were positively selected for the inserted marker gene indicated correct insertion of the marker gene and therefore deletion of *uch2*$^+$. The successful deletion of the *uch2*$^+$ gene was supported when the anti-Uch2 antibody was used on extracts prepared from this strain. This showed that the Uch2 protein was absent in these *uch2*Δ extracts (figure 3.2). The *uch2*Δ strain was streaked out at 18°C, 25°C and 36°C and was found to have no obvious phenotype, as it grew as the wild type strain at these temperatures on minimal and complete media. To give greater flexibility for selection when crossing to other *ura4*$^+$ deletion strains, the *ura4*$^+$ inserted into *uch2*$^+$ was replaced by the G418^R cassette to give a *uch2*::G418^R strain. Colonies were selected on plates containing geneticin and then replica plated to FOA to select for loss of the *ura4*$^+$ gene. The replacement was then confirmed using a PCR test (Figure 4.1B).

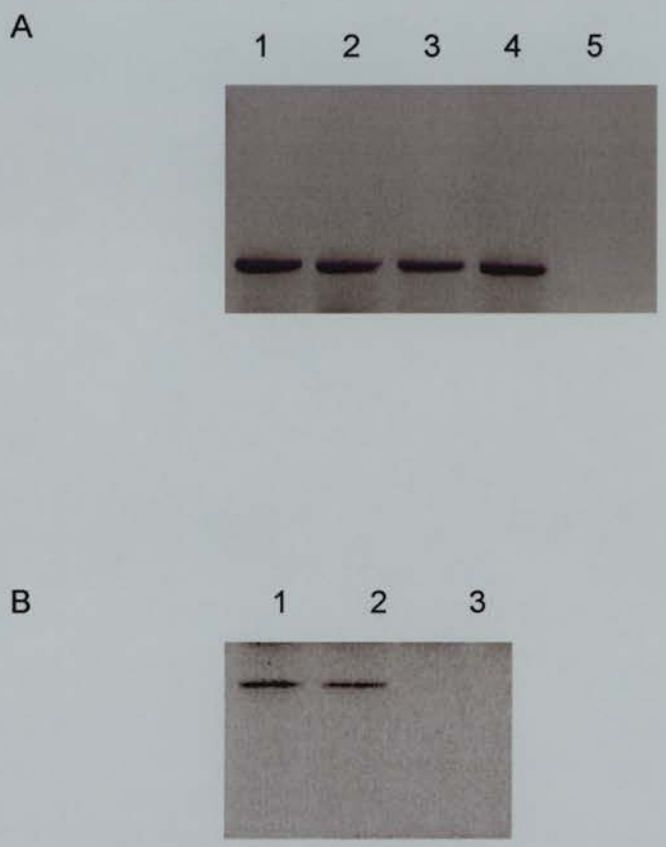

Figure 4.1: PCR Test of *uch2*$^+$ Deletion Strains

A Following transformation with the *uch2*$^+$ knockout construct containing the *ura4*$^+$ gene, ura4$^+$ transformants were selected. These were then tested by colony PCR to ensure that the *ura4*$^+$ had inserted into the *uch2*$^+$ locus. Lanes 1-4 show the postive colonies and lane 5 is the control PCR reaction using a wild type strain.

B The *uch2::ura4*$^+$ strain was transformed with a construct to replace the *ura4*$^+$ marker with a G418^R cassette. Colonies that were G418^R were tested by colony PCR to confirm that the *ura4*$^+$ had been replaced by the G418^R cassette. Lanes 1 and 2 are the positive strains and lane 3 is a wild type control.

4.3 Deletion of ubp6⁺

The *ubp6*$^+$ gene had not previously been deleted in *S. pombe*, so in order to determine the function of Ubp6, the *ubp6*$^+$ open reading frame was replaced using the *arg3*$^+$ gene in the *leu1-32 ura4-D18 arg3-D4 his3-D1 ade6-210.* strain. The deletion was carried out and confirmed by PCR and using the anti-Ubp6 antibody as for *uch2*$^+$ (Figure 4.2 and Figure 3.7)(Bahler et al., 1998). The *ubp6* deletion strain showed no obvious phenotype and grew as the wild type strain at 18°C, 25°C and 36°C on minimal or complete media.

4.4 uch2Δ ubp6Δ Double Mutant

As Uch2 and Ubp6 had both been shown to be proteasome associated, it was proposed that they might have redundant functions. It was therefore predicted that they might show a genetic interaction and that deletion of both *uch2*$^+$ and *ubp6*$^+$ in the same cell would be lethal or would result in a growth defect. To test this hypothesis, the *uch2Δ* and *ubp6Δ* strains were crossed together to create a *uch2Δubp6Δ* double mutant strain.

The double mutant strain was isolated by tetrad analysis and was found to be viable with no obvious phenotype. The strain was streaked out at 18°C, 25°C and 36°C but was found to grow as the wild type strain at each temperature on both minimal and complete media.

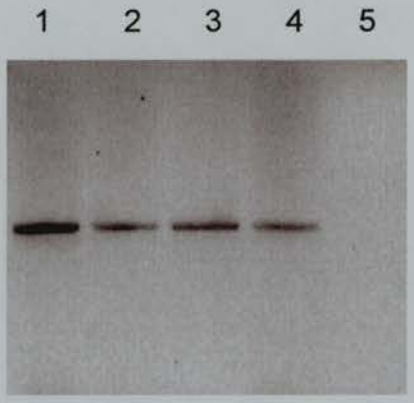

Figure 4.2: PCR Test of *ubp6*$^{+}$ Deletion Strain

A Following transformation with the *ubp6*$^{+}$ knockout construct containing the *arg3*$^{+}$ gene, arg3^{+} transformants were selected. These were then tested by colony PCR to ensure that the *arg3*$^{+}$ had inserted into the *ubp6*$^{+}$ locus. Lanes 1-4 show the postive colonies and lane 5 is the control PCR reaction using a wild type strain.

4.4 Crosses of uch2Δ *and* ubp6Δ *with* ubp14Δ

The deletions strains for *uch2* and *ubp6* were both crossed to the only other available DUB mutant in *S. pombe*, *ubp14*Δ. *ubp14*$^{+}$ encodes the *S. pombe* homolgue of Isopeptidase T (Hadari et al., 1992; Amerik et al., 1997; C. Semple, unpublished). The double mutants resulting from these crosses, *uch2*Δ *ubp14*Δ and *ubp6*Δ*ubp14*Δ were both viable. These two strains were then crossed to create the triple mutant *uch2*Δ *ubp6*Δ *ubp14*Δ, which was also viable. The double and triple mutant strains were tested for viability at 18°C, 25°C and 36°C and were found to grow as wild type cells at each temperature (Table 4.1).

4.5 Crosses of uch2Δ *and* ubp6Δ *Deletion Mutants with Proteasome Subunit Mutants*

*uch2*Δ and *ubp6*Δ were each crossed to mutants for several proteasome subunits: Mts1, Mts3, Mts4, Pad1 and Pus1, which are non-ATPase subunits of the 19S regulator; Mts2, which is an ATPase subunit of the 19S regulator and Mts8, which is a β-subunit of the 20S core (Gordon et al., 1993; Gordon et al., 1996; Wilkinson et al., 1997; Wilkinson et al., 2000; Penney et al., 1998; C. Gordon, unpublished). For each cross approximately 20 tetrads were analysed (see table 4.1 for summary). The double mutants resulting from the *uch2*Δ crosses were all found to be viable and showed no enhancement of the single mutant phenotypes.

Tetrad analysis revealed that *ubp6*Δ was synthetically lethal with three of the mts mutants, *mts1-46*, *mts2-1* and *mts3-1*. *ubp6*Δ*mts1-46*, *ubp6*Δ*mts2-1* and *ubp6*Δ*mts3-1* had similar phenotypes: in some cases spores did not germinate and cells from those that did germinate divided a maximum of three times and were therefore never found to grow to colonies. The double mutant cells were swollen and in some cases elongated, a morphology which is reminiscent of the phenotypes of the *mts* mutants

	DUB Mutant		
	uch2Δ	*ubp6Δ*	*uch2Δubp6Δ*
Proteasome Mutant			
19S lid			
mts1-46	**x**	**SL**	**-**
mts3-1	**x**	**SL**	**-**
pad1-1	**x**	**x**	**x**
pus1Δ	**x**	**x**	**x**
19S base			
mts2-1	**x**	**SL**	**-**
mts4-3	**x**	**x**	**n**
mts4-17	**x**	**x**	**n**
20S core			
mts8	**x**	**x**	**n**

Table 4.1: Crosses of the *uch2Δ* and *ubp6Δ* strains with Proteasome Mutant Strains

The *uch2Δ* and *ubp6Δ* single mutants and the *uch2Δ ubp6Δ* strains were crossed to strains containing mutations in proteasome subunits in the 19S lid, 19S base or the 20S core. **SL** indicates that the two mutations are synthetically lethal, **x** indicates that there is no difference between the double/triple mutant and the single/double mutants used for the cross, **n** indicates that the strain has not yet been constructed and – indicates that the triple mutant cannot be made as one of the double mutants is not viable.

at the restrictive temperature (Gordon et al., 1993; Gordon et al., 1996; Wilkinson et al., 1997; Penney et al., 1998).

The *ubp6Δmts4-3*, *ubp6Δmts4-17*, *ubp6Δpad1-1*, *ubp6Δpus1Δ* and *ubp6Δmts8Δ* strains were all viable and demonstrated no enhancement of the single mutant phenotypes.

4.6 Overexpression of uch2$^+$ *and* ubp6$^+$

Overexpression of a given gene can often provide insights into its function. In order to observe the phenotype of cells overexpressing *uch2*$^+$ or *ubp6*$^+$, both genes were subcloned into the pREP1 *S. pombe* expression vector. The pREP1-Uch2 and pREP1-Ubp6, and empty pREP1 plasmids were used to transform a wild type *S. pombe* strain, and leu$^+$ colonies were selected. Analysis of the strains overexpressing *uch2*$^+$ or *ubp6*$^+$ revealed that there was no effect on growth at 18°C, 25°C and 36°C.

4.7 Discussion

In order to investigate the function of the proteasome-associated DUBs, *uch2* and *ubp6* null strains were constructed. As previously reported, deletion of *uch2Δ* was not found to have any noticeable effect on cells. The *ubp6Δ* strain also grew as wild type, which was not surprising as its *S. cerevisiae* homologue has been deleted and found have no phenotype under normal growth conditions (Amerik et al., 2000). The *uch2Δ* and *ubp6Δ* strains both grew as wild type at 20°C, 25°C and 36°C. To assess whether Uch2 and Ubp6 have redundant functions, the two single mutant strains were crossed to give the *uch2Δ ubp6Δ* double mutant. It was expected that deletion of both proteasome-associated DUBs might have a noticeable effect, as deubiquitination at the proteasome is presumed to be required for normal growth.

However, the *uch2Δubp6Δ* strain grew as wild type and was not cold or temperature sensitive. This indicates that if the deubiquitinating activity of the proteasome is essential, there must be other DUBs that can act at the proteasome.

To further examine the cellular roles of Uch2 and Ubp6, the deletion strains *uch2Δ* and *ubp6Δ* were crossed to a series of proteasome mutant strains (Table 4.1). If Uch2 and Ubp6 have a function in regulating 26S proteasome-mediated proteolysis, then it would be expected that they would show genetic interactions with these mutants. A synthetic lethal phenotype was found for three of the resulting strains, namely *ubp6Δmts1-46*, *ubp6Δmts2-1* and *ubp6Δmts3-1*. It was surprising that loss of Ubp6 was only synthetically lethal with a subset of the proteasome mutants tested, as all the proteasome mutants apart from *pus1Δ* show a very similar metaphase arrest phenotype (Gordon et al., 1993; Gordon et al., 1996; Wilkinson et al., 1997; Wilkinson et al., 2000; Penney et al., 1998; C. Gordon, unpublished). It appears unlikely that these synthetic interactions are due to a physical interaction of Ubp6 with these subunits as Mts2 is found in the base of the complex and Mts1 and Mts3 in the lid (see section 1.3.2.1). Overexpression of $mts3^{+}$ has been found to rescue the *mts1* mutation and in vitro binding assays show that these two subunits interact directly, taken together, these results suggest that Mts1 and Mts3 will interact in vivo. However, neither Mts1 nor Mts3 has been shown to bind to Mts2 (R. Hartmann-Petersen, G. McGurk and C. Gordon, unpublished). If these three subunits are not clustered in the proteasome complex, it is not expected that Ubp6 would be able to interact with all of them. This evidence suggests that the synthetic lethality is not caused by a physical interaction between Ubp6 and the proteasome. In support of this finding, the *ubp6Δmts4* mutants were found to be viable despite the fact that Ubp6 has been shown to interact directly with Mts4. If a physical interaction is not responsible for the synthetic lethality, there must be subtle differences between the phenotypes of the *mts* mutants that result in the varied phenotypes when combined with *ubp6Δ*.

In *S. cerevisiae*, two DUBs have been found to have genetic interactions with proteasome mutants, Ubp14 and Doa4 (Amerik et al., 1997). Deletion of *UBP14* does not result in any gross growth defect at 30°C or 36°C, but does confer sensitivity to canavanine and a strong sporulation defect. Examination of extracts from the *ubp14* strain reveals an accumulation of ubiquitin species, most likely to be unanchored chains. Overexpression of *UBP14* was found to inhibit the degradation of proteasome substrates in vivo. Therefore, it is possible that the role of Ubp14 is to maintain the balance between having sufficient preformed multiubiquitin chains for addition to proteasome substrates and keeping multiubiquitin levels low enough to prevent inhibition of substrate proteolysis (Amerik et al., 1997). When combined with deletion of *ubp14*, both the *doa3* and *doa5* 20S complex proteasome mutants become more sensitive to increased temperature. In addition, the *doa3ubp14* and *doa5ubp14* double mutants have a dramatically increased number of unanchored ubiquitin chains, especially tetraubiquitin. A model has been proposed where mutation of the proteasome would lead to higher levels of ubiquitin chain release as multiubiquitinated substrates are stalled at the proteasome. Ubp14 would normally process these chains, even at the increased levels, but in the absence of Ubp14 multiubiquitin chains accumulate and inhibit proteolysis (Amerik et al., 1997)

Deletion of either *S. pombe* Ubp6 or *S. cerevisiae* Ubp14 results in enhancement of proteasome mutant phenotypes. For *ubp6*Δ this interaction is seen with mutants of 19S subunits. However, for *ubp14* the synthetic phenotype was seen with 20S core subunit mutants (Amerik et al., 1997). There was no enhancement of the single mutant phenotype of the 20S core mutant *mts8* in the *ubp6*Δ*mts8* double mutant. Furthermore, neither *ubp6*Δ or *uch2*Δ showed a genetic interaction with *ubp14*Δ. This suggests that if Ubp6 and Uch2 are involved in the regulation of proteolysis, it is unlikely to be via the same mechanism as Ubp14.

A second *S. cerevisiae* DUB, Doa4, has been suggested to have a role in proteolysis by the 26S proteasome. In some cases, Doa4 appears to be associated with the proteasome, however it is not reliably found in proteasome preparations, indicating that this may be a weak or transient interaction (Papa et al., 1999; Voges et al., 1999). Furthermore, in certain mutant backgrounds, Doa4 colocalises with vacuolar-sorting pathway proteins in a late endosome-like compartment near the vacuole (Amerik et al., 2000a). Deletion of *DOA4* also results in a pleiotropic phenotype suggesting that it has multiple roles in vivo (Papa and Hochstrasser, 1993). Studies so far indicate that its major functions are the regulation of proteasome-mediated proteolysis, vacuolar proteolysis and endocytosis and that it mediates these roles by controlling ubiquitin homeostasis (Papa and Hochstrasser, 1993; Swaminathan et al., 1999; Papa et al., 1999; Dupre and Haguenauer-Tsapis, 2001; Amerik et al., 2000a).

In the *doa4* mutant, known proteasome substrates are stabilised and there is accumulation of small, ubiquitinated species, which have been proposed to be the remnants of ubiquitinated proteins that have been degraded by the proteasome (Papa and Hochstrasser, 1993). When *doa4* is crossed to the *doa3* proteasome mutant, the resulting double mutant is slower growing and the restrictive temperature is reduced compared to either single mutant (Papa et al., 1999). However, although there is synthetic enhancement of the *doa3* temperature sensitive phenotype, the accumulation of ubiquitinated proteins seen in the *doa4* single mutant is much reduced in the double mutant. In addition, ubiquitin levels in *doa4* are often reduced, but in the *doa4doa3* mutant, ubiquitin is restabilised (Papa et al., 1999). Therefore, it appears that the change in ubiquitin levels in *doa4* cells is separate from the proteolytic defect as the levels of ubiquitin and small ubiquitinated species are rescued by the *doa3* mutation, but its proteolytic defect is enhanced. Mutations in the vacuolar protein-sorting pathway suppress the accumulation of 26S proteasome substrates in *doa4*, suggesting that Doa4 may act to coordinate vacuolar and proteasome mediated protein degradation. It is unclear what mechanism Doa4 uses to carry out this function, but examination of the mutant phenotypes suggests that it is not simply by regulating ubiquitin levels in the cell (Amerik et al., 2000a).

Although, Doa4 is similar to Ubp6 in that loss of either protein is synthetically lethal with proteasome mutants, it seems unlikely that both proteins have analogous functions as the *doa4* mutant has multiple defects but the *ubp6*Δ mutant grows as wild type (Papa and Hochstrasser, 1993). Ubp6 may have a specific role in the regulation of 26S proteasome mediated proteolysis, whereas Doa4 may have a more general role in processing ubiquitinated species that may affect the degradation of proteasome substrates indirectly.

There are many possible mechanisms for the synthetic lethality of *ubp6*Δ with the proteasome mutants. The first is that there is a direct physical interaction of Ubp6 with the proteasome subunits, but as mentioned above, the observations so far are not in favour of this model. It could be further tested with in vitro pulldowns of individual proteasome subunits with Ubp6 and also genetic experiments to determine whether expression of particular domains of the proteins is able to rescue the lethality. If a physical interaction is not responsible for the lethality, it is most likely that a loss of deubiquitinating activity combined with loss of proteasome activity causes the phenotype. Three ways in which this might work are discussed below.

Synthetic lethality may indicate that two proteins work in the same or related pathways, depending on the mutant phenotypes (Guarente, 1993). One explanation for the synthetic interaction is that both Ubp6 and the proteasome are on the same pathway for the essential degradation of a proteasome substrate. The lethality in this case would be due to a decrease in flow through the pathway (Figure 4.3A). If it is assumed that the substrate must be deubiquitinated by one of several DUBs prior to degradation, then deletion of Ubp6 alone may reduce the amount of substrate that is deubiquitinated ready for the proteasome, but not to a level that has an observable effect under normal growth conditions. A defect in the proteasome would reduce degradation of the substrate sufficiently to cause the cells to be temperature sensitive.

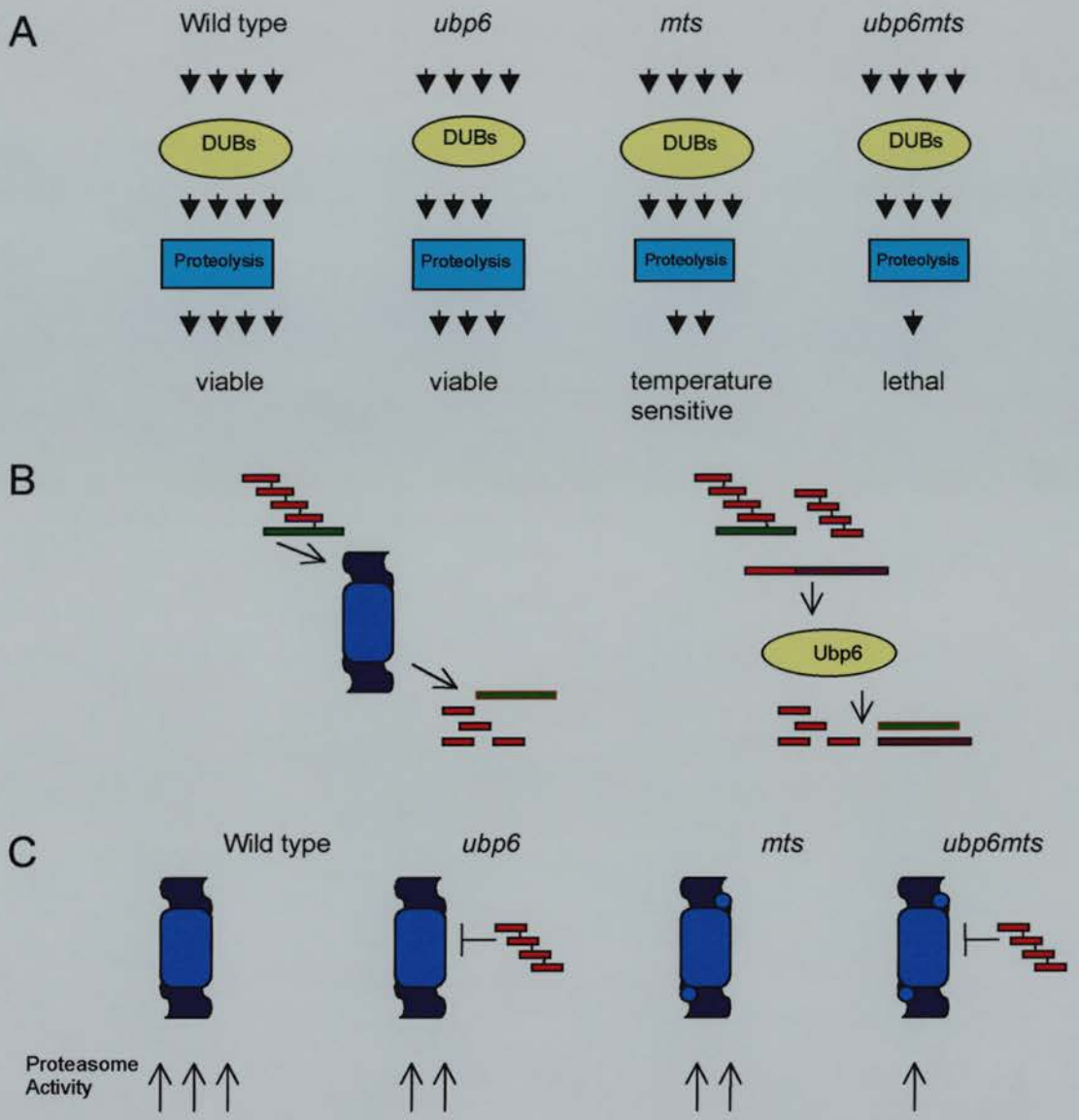

Figure 4.3: Possible Mechanisms for the Synthetic Lethality of *ubp6Δ* and *mts* Mutants

A. Ubp6 and the proteasome may be in the same pathway for the essential degradation of a substrate. Loss of Ubp6 results in a decrease in DUB activity and therefore a decrease in substrate for the proteasome. In the *mts* mutant proteasome activity is reduced. In the double mutant, flow through the pathway is too slow to retain viability.

B. Both Ubp6 and the proteasome may act to promote release of free ubiquitin – loss of both activities in the double mutant may result in a lethal reduction in free ubiquitin levels.

C. Ubp6 may act to degrade chains that can inhibit the proteasome; when combined with the proteasome defect in the *mts* mutant, proteasome activity is too low for viability.

See text for a detailed explanation of each mechanism.

However, when Ubp6 is absent and the proteasome is impaired, there is a reduced pool of deubiquitinated substrate for the proteasome and reduced proteolysis of this deubiquitinated substrate. This would result in accumulation of the substrate to lethal levels. If this model is correct, then it would be expected that the *ubp6*Δ mutant would show some stabilisation of one or more substrates and that its phenotype would resemble a milder version of the *mts* phenotype. This hypothesis could be tested by looking at the levels of multiubiquitin conjugates in the *ubp6*Δ cells, as this might indicate a defect like that seen in proteasome mutants.

A second mechanism for the synthetic lethality is that Ubp6 and the proteasome act in parallel pathways. One way in which this could happen is that both are involved in generating free ubiquitin (Figure 4.3B). It may be that deubiquitination of proteasome substrates is mediated by a different DUB rather than Ubp6 and that this deubiquitination is tightly associated with degradation. Therefore, when proteolysis is efficient, the release of free ubiquitin will be efficient. Ubp6 may also act to release free ubiquitin from chains or from other ubiquitinated proteins not destined for the proteasome. This would mean that if Ubp6 was lost and the proteasome was compromised, that two parallel pathways for the release of ubiquitin would be defective, resulting in a reduction in free ubiquitin to a level where cells are no longer viable. For this model to be true, it would be expected that both the proteasome and *ubp6*Δ mutants would have reduced ubiquitin levels and that the phenotype could be rescued by supplementation of ubiquitin.

Finally, it is possible that Ubp6 acts in a similar manner to Ubp14 in *S. cerervisiae*, in that it acts to break down ubiquitin chains that can inhibit the proteasome (Figure 4.3C). In this case the *ubp6*Δ mutant would have a slight impairment of proteolysis due to the accumulation of multiubiquitin chains, but this would not affect cell viability. However, when combined with the decrease in proteasome activity seen in the *mts* mutants, the inhibition by multiubiquitin chains may cause activity to drop to

a lethal level. To test this model, the *ubp6*Δ strain could be examined to determine whether it shows a similar chain accumulation phenotype to *S. cerevisiae ubp14*. The fact that *ubp6*Δ and *ubp14*Δ, have no genetic interaction in *S. pombe* argues against this model.

One problem with the models suggested here is that none can explain why *ubp6*Δ is only synthetically lethal with certain proteasome mutants. Therefore, it will be important to examine the levels of ubiquitin, multiubiquitin and ubiquitin conjugates in the *ubp6*Δ mutant and also in the *mts* mutants, to detect any differences in phenotype. It is known that proteasome mutants accumulate high molecular weight ubiquitin conjugates so it would be interesting to investigate whether these are also found in the *ubp6*Δ and *uch2*Δ mutants (Gordon et al., 1993; Penney et al., 1998). Another way in which the role of Ubp6 and Uch2 in proteasome dependent proteolysis could be assessed would be to look at whether *ubp6*Δ and *uch2*Δ are sensitive to canavanine, which is an arginine analogue that incorporates into proteins. Mutations in subunits of the proteasome confer sensitivity to canavanine as the defective proteasomes are unable to cope with the high levels of canavanine containing proteins to be degraded (Wilkinson et al., 2000; C. Gordon, unpublished). Therefore, sensitivity to canavanine can provide a good indicator of a defect in proteolysis. In *S. cerevisiae* and *A. thaliana*, several DUBs have been shown to be required for resistance to canavanine, including the Ubp6 homologue (Amerik et al., 2000a; Yan et al., 2000). This provides further support for the finding that *S. pombe* Ubp6 appears to be involved in proteasome mediated degradation.

One way that the involvement of Uch2 and Ubp6 in ubiquitin dependent proteolysis could be further investigated genetically would be to look at interactions of the *uch2*Δ and *ubp6*Δ mutants with other ubiquitin pathway mutants. This method has proved informative in the characterisation of the Faf UBP in *Drosophila*. Faf is known to be involved in the regulation of presynaptic growth at the neuromuscular

junction (NMJ) (DiAntonio et al., 2001). The first indication that Faf functions in ubiquitin dependent proteolysis was provided by genetic data showing that mutations in either a proteasome subunit, or an E2 were able to suppress the *faf* mutant phenotype (Huang et al., 1995; Wu et al., 1999). In addition, overexpression of *faf* causes overgrowth of the NMJs, a phenotype that is similar to *highwire* (*hiw*) loss of function mutants. This finding was significant as *hiw* encodes a protein that is though to be a ubiquitin ligase, based on its homology to known E3s (DiAntonio et al., 2001). Following characterisation of the *hiw*, *faf* and double mutant phenotypes, it was proposed that Faf and Hiw may act antagonistically to regulate synaptic development by maintaining the balance between ubiquitination and deubiquitination (DiAntonio et al., 2001). Further experiments will be required to determine whether Faf and Hiw share a common substrate or act on separate pathways. Further genetic analysis used a candidate gene approach to identify ubiquitinated Liquid Facets, an epsin, as a Faf substrate (Cadavid et al., 2000). It will be thus be interesting to see whether ubiquitination of Lqf is mediated by Hiw. These studies show that genetic analysis using ubiquitin pathway and candidate substrate mutants can reveal information about the pathways and mechanisms of the DUBs and could be used for investigation of Uch2 and Ubp6 function.

Recently, the first genetic interaction between two DUBs in *S. pombe* was described. The first of the UBPs, Ubp15 (also named Ubp21) was found as a high copy suppressor of a point mutant in the Prp4 protein kinase, which phosphorylates the Prp1 protein to control spliceosome formation (Richert et al., 2002). Prp4 protein was shown to be stabilised by Ubp15, suggesting that it works antagonistically to the proteasome, perhaps by removing the multiubiquitin chain required to target it for degradation. A second UBP, Ubp5 (or Ubp22) is very similar to Ubp15 in sequence and genetic analysis showed that *ubp15*Δ and *ubp5*Δ are synthetically lethal at 20°C and 36°C. However, the high sequence similarity between these two proteins suggests that they may be paralogues (Richert et al., 2002).

Although more experiments are required to clarify the situation, the finding from this work is that *uch2*Δ and *ubp6*Δ do not show synthetic phenotypes. Therefore, assuming that DUB activity at the proteasome is essential, these data indicate that Uch2 and Ubp6 are not the only DUBs responsible for deubiquitination at the proteasome. Uch2 does not yet show any indication that it is involved in proteasome activity, a finding which is surprising as it is a subunit of the complex. Conversely, the genetic analysis has shown that Ubp6 is likely to have a role in the regulation of proteolysis by the 26S proteasome.

Chapter 5 Biochemical Activities of Uch2 and Ubp6

5.1 Introduction

In order to cleave ubiquitin from a variety of different substrates the family of DUBs must be able to catalyze two different reactions. Firstly, there is a requirement for ubiquitin peptidase activity, cleaving at the αNH-peptide bonds between ubiquitin and other species, to process the ubiquitin proproteins and to cleave ubiquitin from small adducts such as glutathione. In addition, it is necessary for DUBs to hydrolyze the εNH-isopeptide bonds found between each ubiquitin in a multiubiquitin chain, or between ubiquitin and other proteins which, for example, may be targeted for degradation via the proteasome (Chung and Baek, 1999; Wilkinson, 2000).

Several assays have been developed to test the activity of the DUBs against various ubiquitin-containing substrates. Peptidase activity may be assayed using either an artificial substrate of ubiquitin fused to a peptide or protein sequence, such as GST or PESTc, or alternatively by using substrates such as the polyubiquitin or ubiquitin-ribosomal protein fusions that are the in vivo precursors of ubiquitin (Baker et al., 1992; Park et al., 1997; Larsen et al., 1998; Wyndham et al., 1999). This activity can also be measured in a model in vivo system where DUBs and Ubiquitin-X-β-Galactosidase are coexpressed in *E. coli* (Baker et al., 1992). However, the most sensitive and precise assay for peptidase activity of the DUBs uses Ubiquitin-AMC (7-amino-4-methylcoumarin) as a substrate; the reaction is followed by monitoring the fluorescence that accompanies the release of AMC from ubiquitin (Dang et al., 1998). Isopeptidase activity is normally measured by incubating a DUB with synthetic Lys48 linked multiubiquitin chains, which may be as short as diubiquitin (Baker et al., 1992). Synthetic substrates comprising ubiquitin linked through an isopeptide bond to another protein have also been tested. To investigate in vivo activity, some studies have examined the general level of high molecular weight

ubiquitin conjugates or the level of ubiquitin conjugates of a specific protein in DUB mutant strains or in cells overexpressing a particular DUB (Hadari et al., 1992; Amerik et al., 1997; Chen et al., 2002).

Members of both the UCH and UBP families have been shown to have isopeptidase and peptidase activities. However, the majority of studies support the general trend that UCHs prefer to cleave small leaving groups from ubiquitin and that UBPs process fusions irrespective of their size and also have activity in disassembling multiubiquitin chains (Wilkinson, 2000; Chung and Baek, 1999). In addition, deubiquitinating activity has also been found in the 26S proteasome. Studies of bovine 19S complexes lead to the identification of a deubiquitinating activity that was capable of editing multiubiquitin chains. This activity was not sensitive to Ubal, which specifically inhibits cysteine proteases by binding to the thiol side chain of the active site Cys. Therefore, this deubiquitinating activity was not thought to involve a cysteine protease (Mayer and Wilkinson, 1989; Eytan et al., 1993; Dang et al., 1998; Johnston et al., 1999). Deubiquitinating activity was also identified in 26S proteasome preparations from rabbit reticulocytes, which were found to be capable of releasing free ubiquitin from conjugates or ubiquitin-protein fusions. This activity was distinct from the previously identified isopeptidase as it was sensitive to ubiquitin aldehyde (Ubal), suggesting that it was mediated by a cysteine protease (Lam et al., 1997).

This chapter describes characterisation of the DUB activity of Uch2, Ubp6 and the 26S proteasome. Firstly, recombinant forms of Uch2 and Ubp6 proteins were tested for peptidase activity against UbAMC and isopeptidase activity against Lys48 linked tetraubiquitin. The effect of mutation of the active site Cysteine on Uch2 activity was also analysed. The DUB activity of the *S. pombe* 26S proteasome was then examined and the contribution of Uch2 and Ubp6 to this activity was investigated.

5.2 Ubiquitin Peptidase Activity of Uch2 and Ubp6

To characterise the biochemical activities of the Uch2 and Ubp6 enzymes the first step was to use the Ub-AMC assay for ubiquitin peptidase activity. To obtain purified Uch2 and Ubp6 proteins they were expressed in *E. coli* as GST fusion proteins and purified as described in sections 3.2.1 and 3.3.1. GST was also expressed from the pGEX plasmid and purified in the same manner for use as a negative control.

The amount of GST, GST-Uch2 and GST-Ubp6 protein was assessed using a Coomassie stained SDS-PAGE gel. For the assay the amount of protein used was enough to give a clear band on a Coomassie stained gel, which corresponded to approximately 10µl of beads. The volume of beads for each of the bound proteins was adjusted to ensure equal amounts of each protein were used in the assay.

The assays were set up with the GST protein bound to the sepharose beads and 1µl 50µM Ub-AMC in a total volume of 100µl GST binding buffer. A control sample containing empty glutathione sepharose beads was also set up. The reactions were incubated for 3h at 30°C, and then the ubiquitin hydrolase activity was measured using a luminescence fluorimeter. The fluorescence obtained with the glutathione sepharose only sample was used as a zero and 2-3 readings were taken for each reaction. Each assay was performed in at least three independent experiments. The resulting data subjected to statistical analysis allowing for the variance between experiments (Andrew Carothers).

Using GST a background level of fluorescence was seen, however, when GST-Uch2 was used the level of fluorescence dramatically increased indicating that Uch2 has ubiquitin peptidase activity (Figure 5.1). When GST-Ubp6 was subjected to this assay, no increase on the background level of fluorescence was seen (Figure 5.1).

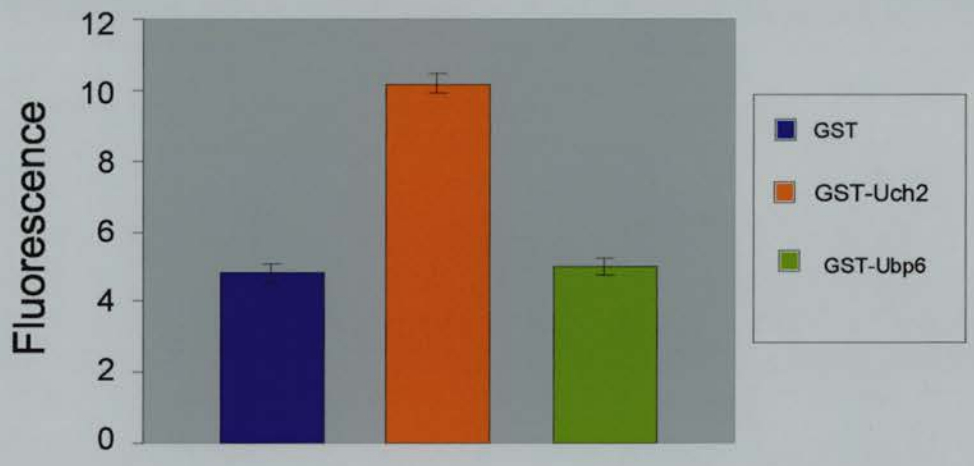

Figure 5.1: Ubiquitin Peptidase Activity of GST-Uch2 and GST-Ubp6
GST-Uch2 and GST-Ubp6 were expressed in *E. coli* and bound to glutathione sepharose. The proteins were then assayed for ubiquitin peptidase activity by measuring the change in fluorescence coupled to cleavage of ubiquitin-AMC. GST was used as a negative control to assess background fluorescence. See text for details

5.3 Mutation of the Uch2 Active Site Cysteine

Uch2 was predicted as a DUB due to its sequence homology to other known UCHs. This family of proteins have homology to other well characterised families of cysteine proteases such as papains and calpains (Rawlings and Barrett, 1994; Barrett and Rawlings, 2001). This strongly suggested that Uch2 would have a Cys residue in its active site and that mutation of this residue would result in loss of activity. Based on its homology to Mouse UCH-L5 and human UCH-L3, Cys 83 was identified as the putative active site Cys for Uch2 (Figure 1.6; Li et al., 2000). This residue was mutated to an Ala using the Stratagene site directed mutagenesis kit. The mutation was made such that a novel SacII restriction site was introduced concurrently with the Cys-Ala mutation. Following mutagenesis miniprep DNA was prepared from 12 colonies and digested with SacII. All of the plasmids cut with SacII, indicating that the mutagenesis was likely to have been successful. Four of the positive clones were then sequenced to ensure the presence of the Cys to Ala mutation and the absence of any additional mutations resulting from the mutagenesis.

To determine whether mutation of the putative active site Cys would have an effect on the activity of Uch2, the activity of the Uch2C83A protein was assayed using Ub-AMC. The Uch2C83A protein was expressed and purified as a GST fusion protein from the pGEX vector as described. It was found to be expressed normally at the expected size of 34kD. The GST-Uch2C83A protein was then subjected to the UbAMC assay using GST-Uch2 as a positive control and GST alone as a negative control. To assess the amount of each protein purified, a sample of each protein was run on an SDS-PAGE gel, which was then Coomassie stained. The amount of beads used was adjusted to give an equal amount of each protein in the assay. Readings were obtained for at least three experiments and statistical analysis was carried out as in section 5.2 (Andrew Carothers). The activity observed with GST-Uch2 was completely absent when GST-Uch2C83A was used, indicating a total loss of activity when Cys83 is mutated to Ala (Figure 5.2).

Figure 5.2: Mutation of the Uch2 Active Site Cysteine Abolishes Ubiquitin Peptidase Activity
GST-Uch2 and GST-Uch2C83A were expressed in *E. coli* and bound to glutathione sepharose. The proteins were then assayed for ubiquitin peptidase activity by measuring the change in fluorescence coupled to cleavage of ubiquitin-AMC. GST was used as a negative control to assess background fluorescence. See text for details.

5.4 Ubiquitin Isopeptidase Activity of Uch2 and Ubp6

A second assay was used to determine whether Uch2 and Ubp6 were able to cleave isopeptide bonds. In this assay, extracts from *E. coli* expressing GST-Uch2, GST Ubp6 and GST as a negative control were incubated with tetraubiquitin, which is made up of four Lys48 linked ubiquitin residues. After incubation overnight, a sample of each reaction was run on a SDS-PAGE gel and analysed by Western blot probed with an anti-ubiquitin antibody.

In the GST control reaction, diubiquitin and triubiquitin are present in addition to some residual tetraubiquitin. These probably represent breakdown products that were either contained in the original tetraubiquitin preparation or created during the incubation or the boiling step prior to SDS-PAGE. These bands are also present in the reactions with GST-Uch2 or GST-Ubp6 but significantly, a large band of about 8kD representing monoubiquitin also appears in these lanes (Figure 5.3). Therefore, GST-Uch2 and GST-Ubp6 both have ubiquitin isopeptidase activity, as they are able to hydrolyse isopeptide linked tetraubiquitin to monoubiquitin.

5.5 Ub-AMC Cleavage Activity of the 26S Proteasome

Previous studies have reported intrinsic ubiquitin hydrolase activity for the 26S proteasome in other organisms; however, this activity has not been described in *S. pombe* (Eytan et al., 1993; Lam et al., 1997). Therefore, 26S proteasomes were purified from the *pus1*:PA tagged strain as described in section 3.2.2.1 and examined for ubiquitin peptidase activity using the Ub-AMC assay. Purified proteasomes were run on a SDS-PAGE gel and the presence of proteasomes was confirmed by Coomassie staining. Assays were carried out on wild type 26S proteasomes, data was collected for three independent experiments and statistical analysis was performed to ensure that the activity observed was significantly higher than the

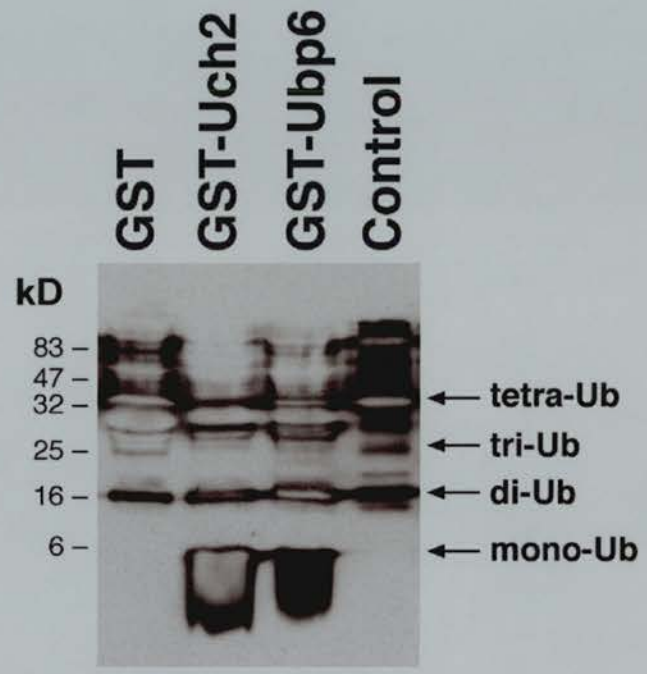

Figure 5.3: Ubiquitin Isopeptidase Activity of Uch2 and Ubp6
Extract from *E. coli* expressing GST, GST-Uch2 or GST-Ubp6 was incubated with tetraubiquitin. The samples were then analysed by SDS-PAGE and Western blot, using an anti-ubiquitin antibody. Monoubiquitin is only released when GST-Uch2 or GST-Ubp6 is present, indicating that both proteins can act as ubiquitin isopeptidases.

background level (Andrew Carothers). The 26S proteasomes showed significant activity in this ubiquitin peptidase assay, indicating that the activity observed in proteasomes from other organisms is present in *S. pombe* (Figure 5.4).

5.6 Uch2 Provides the Ubiquitin Hydrolase Activity of the 26S Proteasome

Having established that the 26S proteasome has ubiquitin hydrolase activity, it was of interest to determine whether this activity could be assigned to either of the proteasome associated DUBs, Uch2 or Ubp6. In order to answer this question, it was necessary to obtain proteasomes that were lacking Uch2 and Ubp6. To purify proteasomes from the *uch2* and *ubp6* single mutants and the *uch2ubp6* double mutant, the *pus1*:PA strain was crossed to each of the DUB mutants and tetrad analysis was carried out. The presence of the *uch2* or *ubp6* deletions was indicated by the auxotrophic markers used for the deletions and *pus1*:PA was detected by PCR. Proteasomes were then purified from each of the mutant strains as described. The amount of proteasome purified from each strain was assessed either by Coomassie stained SDS-PAGE gel or by Western blot analysis. An equal amount of proteasomes from each of the mutant strains as well as the wild type strain were then assayed for their ability to cleave Ub-AMC. Activities for proteasomes from each strain were obtained in at least three experiments and the data was subjected to statistical analysis to allow for the variance between experiments and between strains (Andrew Carothers). The proteasomes from the wild type strain showed activity as expected, however when $uch2^{+}$ was deleted almost all of this activity was abolished (Figure 5.5). Deletion of $ubp6^{+}$ had no effect on the level of activity observed in a wild type or *uch2* deletion background (Figure 5.5). Therefore, it appears that, using this assay, Uch2 is the major ubiquitin hydrolase of the 26S proteasome.

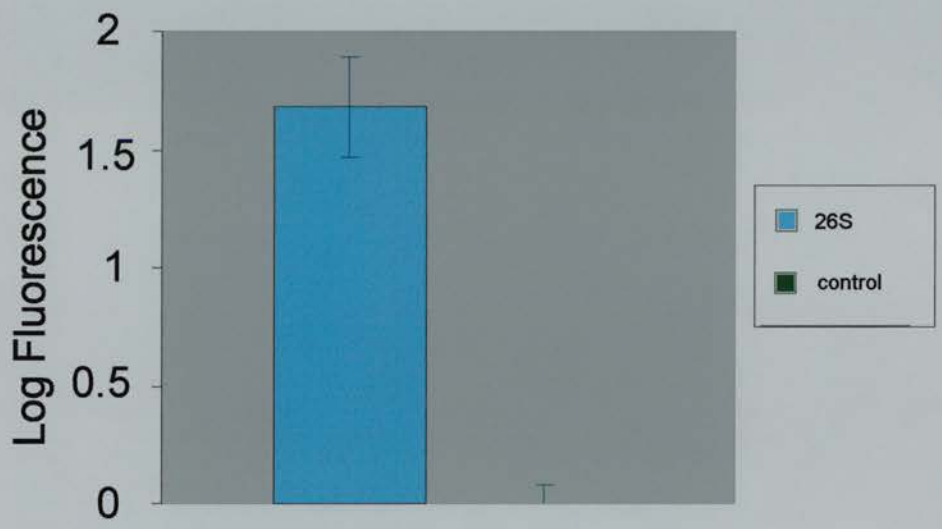

Figure 5.4: Deubiquitinating Activity of the *S. pombe* 26S Proteasome
26S proteasomes were purified and assayed for ubiquitin peptidase activity by measuring the change in fluorescence coupled to cleavage of ubiquitin-AMC.

Figure 5.5: Deubiquitinating Activity of 26S Proteasomes from *uch2* and *ubp6* Mutant Strains

26S proteasomes were purified from wild type, *uch2*, *ubp6* and *uch2ubp6* strains. The proteasomes were then assayed for ubiquitin peptidase activity by measuring the change in fluorescence coupled to cleavage of ubiquitin-AMC.

5.7 Discussion

The first observation from the analysis of the biochemical activity of the proteasome associated DUBs was that Uch2 has ubiquitin peptidase activity. Recombinant GST-Uch2 was able to cleave UbAMC. This observation is consistent with data showing that the *Drosophila* homologue of Uch2, p37a, has this activity (Holzl et al., 2000). Many of the UCH family of DUBs, for example human UCHL-1 and UCHL-3, have been shown to be active in removing αNH-linked fusions from ubiquitin (Larsen et al., 1998).

Having established that Uch2 has ubiquitin hydrolase activity, it was of interest to locate the active site Cys. Based on homology to other UCHs Cys83 was identified as the putative active site Cys and was mutated to an Ala residue (Li et al., 2000). Mutation of this residue resulted in complete loss of Uch2 activity in vitro, indicating that, as predicted, this Cys is required for activity. Similar loss of activity has been observed following active site mutation in other DUBs such as Isopeptidase T and Ubp15 (Amerik et al., 1997; Richert et al., 2002). Structural studies have revealed the nature of the conserved UCH active site and its interaction with substrates. The X-ray crystal structure of UCH-L3 has been determined and shows that the active site triad of Cys, His and Asp (or Asn) is structurally related to that seen for the papain family of cysteine proteases (Johnston et al., 1997). Further structural studies using *S. cerevisiae* Yuh1 bound to Ubal indicate the presence of a covalent bond between the Sγ atom of the active site Cys and the C terminal carbon of Ubal Gly76, as well as many other interactions via Hydrogen bonding and van der Waals contacts. The interactions observed between Yuh1 and its substrate were found to be in residues that are conserved between the UCHs, suggesting that a common catalytic mechanism is shared between members of the UCH family (Johnston et al., 1999). Therefore, the structural data, the observed sequence homology between UCHs and the loss of activity found by mutation of the Uch2 Cys83 imply that Uch2 functions as a cysteine protease. It will be interesting to further examine the role of Uch2 in

the cell by use of the Cys83A active site mutant in vivo. The mutated protein may act as a dominant negative and the phenotype resulting from its expression may reveal the physiological relevance of Uch2 activity.

Surprisingly, Ubp6 was not found to have activity in the Ub-AMC assay. As other UBP family members have been shown to cleave Ub-AMC, it would be expected that this assay would detect Ubp6 ubiquitin peptidase activity (Baker et al., 1992; Amerik et al., 2000). However, it remains possible that Ubp6 has a more specific peptidase activity that does not cleave the UbAMC substrate, but may cleave alternative Ub fusions. One possible reason for the lack of ubiquitin peptidase activity for this protein is that it may not have this activity, however, this seems unlikely as the *S. cerevisiae* homologue of Ubp6 shows activity in a peptidase assay (Park et al., 1997). The activity of the human homologue of Ubp6, USP14, has yet to be determined (Borodovsky et al., 2001). ScUbp6 was found to be optimally active at mildly alkaline pH of 8.5, with very little activity seen at pHs below 7 or above 9.5 (Park et al., 1997). Therefore, as it was possible that the lack of activity was due to the pH of the buffer, the effect of pH on *S. pombe* Ubp6 was tested using buffers of pH7.5, 8.5 and 9.5 (data not shown). No activity was detected using any of these buffers, suggesting that there is an alternative reason why Ubp6 is inactive in this assay. In investigating Ubp6 in *S. cerevisiae*, it was also found that it was degraded in *E. coli* extracts and that this resulted in loss of activity (Park et al., 1997). However, as the Ubp6 purified in *S. pombe* appeared stable when analysed by SDS-PAGE and GST-Ubp6 extracts were active in the isopeptidase assay degradation was unlikely to be the cause of the inactivity. Another possible reason for the lack of activity is that Ubp6 requires posttranslational modification in order to be converted to an active form and that these modifications are not carried out in *E. coli*. One of the predicted UBPs identified in *S. cerevisiae*, Ubp8, was also found to be inactive when expressed in *E. coli*, indicating that there may be other UBPs whose activities are regulated by modification (Amerik et al., 2000). In support of this hypothesis, Western blots of Ubp6 in wild type extracts show that anti-Ubp6 detects two bands in the wild type extract but not the *ubp6*Δ extract suggesting that Ubp6 may exist in

two different forms (see section 3.3.1 and Figure 3.7). Therefore, the most favourable explanation for the lack of observed Ubp6 activity is that Ubp6 requires post-translational modification.

Uch2 and Ubp6 were analysed for their ability to cleave εNH-linked ubiquitin conjugates, and both were found to have this isopeptidase activity. It is interesting that Uch2 shows this activity as several other UCH type DUBs, such as Yuh1, UCH-L1 and UCH-L3 are unable to cleave ubiquitin linked through Lys48 isopeptide bonds in similar in vitro assays (Baker et al., 1992; Larsen et al., 1998). In the case of Yuh1, structural studies have indicated that protein-ubiquitin substrates, including ubiquitin-ubiquitin, may not be cleaved by this UCH due to the presence of an 'active site crossover loop'. This loop covers the active site and would only allow the entry of substrates up to the size of a single helix. This suggested that only small adducts fused to ubiquitin, or proteins that are flexible at the Ub-protein linkage would be able to gain access to the active site cleft (Johnston et al., 1999). The loop structure was disordered in the X-ray crystal structure of UCH-L3, so it remains to be seen if it is conserved amongst other members of the UCH family (Johnston et al., 1997). It may be that Uch2 does not contain this loop structure, and may therefore process a wider range of substrates. Indeed, in the context of the proteasome it would be reasonable to expect that isopeptidase activity would be required to cleave ubiquitin chains from proteasome substrates to allow their entry into the 20S core of the proteasome. Ubiquitin isopeptidase activity has been reported for the 19S complex of proteasomes purified from bovine red blood cells and the protein responsible was of an estimated size of 37kD (Lam et al., 1997). This size matches that of the Uch2 homologue in *Drosophila*, p37a and both are Ubal sensitive, so this provides support for the suggestion that Uch2 may contribute this activity to the proteasome (Lam et al., 1997; Holzl et al., 2000).

In the case of Ubp6, it was not surprising that isopeptidase activity was found, as several members of the UBP family of DUBs have been shown to be active as

ubiquitin isopeptidases. Both Ubp1 and Ubp2 in *S. cerevisiae* were able to cleave εNH-linked di-Ub when coexpressed in *E. coli* and Isopeptidase T and its homologues in *S. cerevisiae* and *A. thaliana*, are all able to cleave K48-linked Ub-Ub bonds (Baker et al., 1992; Hadari et al., 1992; Doelling et al., 2001). In the *ubp14* mutant an increase in the level of free ubiquitin chains was also observed, which indicates that recycling of chains is likely to be its major role in vivo (Amerik et al., 1997). The activity of Ubp6 in *S. pombe* appears to be similar to that of Ubp14 as it is only able to cleave isopeptide linked ubiquitin and not a ubiquitin fusion in vitro. However, based on the data on *S. cerevisiae* Ubp6, it seems most likely that *S. pombe* Ubp6 has both these activities in vivo (Park et al., 1997). In addition, there is a Ubp14 homologue in *S. pombe*, which is likely to carry out this function (C. Gordon, unpublished). Further experiments using alternative assays will be required in order to confirm the activity of Ubp6. Firstly, an alternative ubiquitin fusion substrate could be used for the peptidase assay. It may be that Ubp6, like Ubp14, shows specific activity in cleaving of bonds between two ubiquitins, so that peptide linked Ub-Ub may be an appropriate substrate. Secondly, the GST-Ubp6 could be incubated with *S. pombe* extract or Ubp6 could be purified from a tagged form expressed in *S. pombe* in order to determine whether any modifications result in peptidase activity. Finally, the levels of any putative substrates, such as high molecular weight ubiquitin conjugates, could be measured in the *ubp6* deletion strain to reveal its role in vivo.

Further studies will be important to determine the in vivo specificities of Uch2 and Ubp6. Although it the specificity of Uch2 and Ubp6 may be determined solely by their cellular localisation, it is possible that other factors my narrow this specificity further. It has been suggested that the specificity of DUBs can be conferred by regions found either the N terminus or the C terminus. The testis specific DUBs, UBP-t1 and UBP-t2 are two very similar UBPs that share a common catalytic domain and differ only in their N terminal residues. These divergent N terminal regions have been shown to regulate the activity of the two UBPs against a variety of substrates (Lin et al., 2001). In the case of the hematopoietic specific DUBs, which

share a catalytic N terminus, sequence divergence is seen in only the C terminal regions of the proteins, suggesting that these may mediate specificity (Zhu et al., 1997; Baek et al., 2001). Isopeptidase T has been shown to have two ubiquitin binding sites: the first binds Ubal and is thus suggested to be the catalytic site, and the second does not bind Ubal, and may provide specificity for ubiquitin-only substrates by binding a second ubiquitin (Hadari et al., 1992). By further sequence analysis, in vitro assays and examination of mutant phenotypes, it may be possible to identify regions of Uch2 and Ubp6 that confer specificity. The only obvious domain, apart from the catalytic domains, found in either Uch2 or Ubp6 is the UBL domain of Ubp6. In *S. cerevisiae*, deletion of the UBL domain of Ubp6 was not found to have any effect on its activity in vitro, implying that this domain is not involved in mediating specificity by binding substrates (Wyndham et al., 1999). This is in keeping with our finding that this domain binds to the proteasome. Therefore, the UBL domain of Ubp6 may mediate specificity by targeting Ubp6 to the proteasome, whilst other domains may further restrict its substrates.

This study has shown for the first time that the 26S proteasome of *S. pombe* has intrinsic UCH activity. Previous work had reported this activity for proteasomes purified from other species, so this result demonstrates that the activity is conserved (Eytan et al., 1993; Lam et al., 1997; Holzl et al., 2000). However, the subunits of the proteasome that provide the DUB activity had not been identified. This work has revealed, through purification of proteasomes from mutant strains, that Uch2 is responsible for the majority of the UCH activity of the proteasome. As Uch2 is highly conserved amongst eukaryotes, it is likely that its role in *S. pombe* reflects the situation in higher organisms. The finding that Uch2 is the major ubiquitin peptidase for the proteasome is unexpected, as deletion of *uch2* does not result in an observable phenotype. Therefore, unless there are other DUBs providing an activity that is not detected by this assay, it must be assumed that the UCH activity of the proteasome is not essential for viability. Although Ubp6 is found associated with the proteasome, it was not found to contribute to its ubiquitin peptidase activity. This finding lends further support to the fact that Ubp6 is unable to catalyse the hydrolysis of the Ub-

AMC substrate, as was observed using recombinant GST-Ubp6. However, it is likely that Ubp6 may be present at low concentrations in the proteasome: Ubp6 and its homologues have not always been detected in purified proteasomes, and other UDPs have been suggested bind transiently with the proteasome (Wilkinson et al., 2001). Analysis of the ubiquitin peptidase and isopeptidase activity of proteasomes from wild type and mutant strains with alternative assays will clarify the situation.

Chapter 6 Discussion

In this study, the proteasome-associated DUBs, Uch2 and Ubp6, were characterised using a number of biochemical and genetic methods. This work has lead to several conclusions about the functions of these proteins in the cell. Here the implications of these findings and directions for future study are considered.

6.1 Uch2 is the Major Ubiquitin Hydrolase of the Proteasome

The first finding of this study was that Uch2 is associated with the proteasome in vivo and in vitro. Studies in *Drosophila* have since shown that this proteasome association is conserved (Holzl et al., 2000). The identification of Uch2 as a proteasome associated DUB explains the previously observed UCH activity seen at the proteasome (Lam et al., 1997). Uch2 provides the major UCH activity to the proteasome and the fact that deletion of *uch2* causes no obvious cellular defects, indicates that unexpectedly, the observed UCH activity of the proteasome is not essential for normal growth.

6.2 Alternative Mechanisms for Deubiquitination at the Proteasome

It is surprising that the UCH activity of the proteasome is not essential, as it is known that proteasome substrates must be deubiquitinated prior to degradation. One possibility is that other DUBs that are not subunits of the proteasome might associate to provide activity. As proteasomes purified from the *uch2*Δ strain do not have activity, any DUB that could substitute for Uch2 must not be present in the purified complex, suggesting that it would have a weak association with the proteasome. Ubp6 was considered as a candidate but it is present in the purified proteasomes and

appears not to contribute to the activity. In *S. cerevisiae*, the Doa4 DUB associates with the proteasome and has been proposed to remove ubiquitin from proteasome substrates (Papa et al., 1999). Doa4 is not an ideal candidate as it appears to have a general role in the regulation of ubiquitination for more than one cellular function, suggesting that its role in ubiquitin dependent proteolysis may be indirect (Papa and Hochstrasser, 1993; Papa et al., 1999; Swaminathan et al., 1999; Dupre and Haguenauer-Tsapis, 2001; Amerik et al., 2000a). Furthermore, the absence of a Uch2 homologue in *S. cerevisiae* shows that the situation is clearly different from that in *S. pombe* and higher eukaryotes. In addition, higher eukaryotes such as *Drosophila* and human do not appear to have a Doa4 homologue. Despite these observations, it would be useful to rule out the Doa4 homologue in *S. pombe* as the DUB that provides redundant function with Uch2, by creating a double knockout strain. *As S. pombe* contains only two DUBs of the UCH family (Uch1 and Uch2), it would also be of interest to determine whether Uch1, has redundant function with Uch2. The Uch1 homologue in *S. cerevisiae*, Yuh1, has been minimally characterised and does not yet show any obvious association with the proteasome, either physically or functionally (Baker et al., 1992; Amerik et al., 2000). In addition, Yuh1 is the only UCH in *S. cerevisiae* and its deletion is not lethal, suggesting that it is likely to act redundantly with members of the UBP family (Baker et al., 1992). However, construction of *uch1* and *uch1uch2* mutants in *S. pombe* would allow these questions to be addressed.

An alternative reason for the observation that deubiquitinating activity of the proteasome is dispensable may be that an activity in the proteasome that is not detected by the ubiquitin-AMC assay is responsible for this function. One piece of evidence that this may be the case, is that a second DUB activity has been detected in purified proteasomes and was found to be insensitive ubiquitin-aldehyde, suggesting that the enzyme responsible may not be a cysteine protease (Eytan et al., 1993). There is only one report of such an activity, however, in light of the observations in this study, it appears that it may warrant further investigation to identify the proteasome subunit or associated factor responsible. Firstly, the activity attributed to Uch2 could be tested to confirm that it is the Ubal sensitive, which would be

expected as a cysteine active site has been identified. Secondly, the observed Ubal insensitive activity could be tested with other inhibitors of thiol proteases such as the alkylating agents NEM and iodoacetamide. The findings from these experiments would facilitate further analysis of proteasome associated deubiquitinating activity.

6.3 The Role of Uch2 Activity in the Proteasome

Although there are alternative mechanisms for the deubiquitination of substrates at the proteasome, this still leaves the conundrum of the highly conserved yet non-essential UCH activity of the proteasome. The fact that the proteasome associated UCH activity, the presence of Uch2 in the proteasome and the active site of Uch2 all appear to be highly conserved amongst eukaryotes suggests that this activity does have a significant cellular function (Li et al., 2000; Holzl et al., 2000). One possible explanation is that the UCH activity of the proteasome is required only under certain cellular conditions, such as stress. The role of the UCH activity of the proteasome therefore remains elusive, however, the fact that the *uch2* null mutant shows no genetic interaction with proteasome mutants suggests that despite the fact that Uch2 is a proteasome subunit, it may not have a direct influence on proteasome mediated proteolysis. There are several ways in which any previously undetected involvement of Uch2 in proteolysis at the proteasome could be identified. The *uch2* mutant could be further analysed for characteristics of mutants that have a defect in proteolysis, such as canavanine sensitivity and accumulation of high molecular weight ubiquitin conjugates. In addition, mutants of other ubiquitin pathway, such as E2s or E3s, could be crossed to the *uch2* mutant to determine whether there is a genetic interaction that might indicate that they operate in the same pathway.

Although Uch2 was found to have isopeptidase and peptidase activities, the members of the UCH family are generally found to act only as ubiquitin peptidases (Chung and Baek., 1999; Wilkinson, 2000). If the role of Uch2 in vivo is to act in a similar manner this may explain why it does not have a direct function in ubiquitin

dependent proteolysis at the proteasome. Uch2 may instead regulate ubiquitin levels by removing small adducts attached to ubiquitin or by processing the ubiquitin precursor levels. However, it remains difficult to accept that the function of Uch2, a UCH that is a subunit of the proteasome, is entirely separate from the proteolytic function of the complex. Further investigation will therefore be required to identify the in vivo substrates and the role of Uch2.

6.4 Deubiquitination and Deneddylation in Protein Complexes

An interesting parallel with the DUB activity of the proteasome is the deneddylating activity of the COP9/signalosome, as the proteasome lid and the COP9/signalosome are related complexes (see sections 1.2.5 and 1.3.2.2.1). There are two possible scenarios if these activities are related: firstly, the signalosome and the proteasome may each contain the two separate activities or alternatively, the deneddylase activity of the signalosome may be analogous to the DUB activity of the proteasome. An obvious starting point to analyse this problem is to examine purified proteasomes for deneddylating activity and purified signalosomes for DUB activity. It will also be important to determine whether the activity of the COP9/signalosome can be assigned to a subunit or to an associated factor. If a subunit of either the 19S lid or COP9/signalosome is identified as having either DUB or deneddylating activity, this might suggest that the homologous subunit in the other complex also have the same activity. Therefore, studying the two systems concurrently may accelerate understanding of these complexes and activities. Two conserved proteins that found in the proteasome (Rpn11) and the COP9/signalosome (Csn5/Jab1) show homology to each other, but also to the UCH family of deubiquitinating enzymes (Penny et al., 1998; Lyapina et al., 2001). Although no activity has been detected for either protein, Rpn11 and Csn5 are candidates that will need to be investigated to assess whether they contribute to the deubiquitinating and deneddylating activities of the proteasome and COP9/signalosome complexes enzymes (Penny et al., 1998; Lyapina et al., 2001).

6.5 Mts4 – a general receptor for UDPs?

S. pombe Ubp6 was shown in this study to be associated with the proteasome, and more recently this interaction has also been observed in *S. cerevisiae* and human (Borodovsky et al., 2001; Verma et al., 2000). This interaction between Ubp6 and the proteasome was found to be mediated by the Ubp6 N-terminal UBL domain. In addition, the Mts4 proteasome subunit was demonstrated to be the Ubp6 binding proteasome subunit. This was in keeping with previous studies, which demonstrated that Rhp23, which is also a UDP, bound to Mts4 via its UBL domain (R. Hartmann-Petersen and M. Seeger unpublished). Another UDP that has been found to bind to the proteasome is BAG-1, a chaperone cofactor (Luders et al., 2000; M. Stone and C. Gordon, unpublished). Binding studies of Rhp23 with truncated versions of Mts4 and sequence homology analysis have revealed a putative domain of the Mts4 protein, named the UBS domain, that may mediate the association with the UBL domain (R. Hartmann-Petersen, M. Seeger and M. Taylor, unpublished). These observations suggest that Mts4 may act as a general UBL binding protein, bringing the UDPs and their associated functions to the proteasome (Figure 6.1). The implication of this model is that all the UDP proteins compete for binding at the Mts4 UBS domain. There are a number of experiments that would clarify the situation. Firstly, it will be important to test whether all of the UDPs can bind the Mts4 UBS domain via their UBL domains. The binding affinities of these proteins could be determined using the surface plasmon resonance analysis. This involves conjugation of a purified protein to a sensor chip and exposure to a binding partner, which allows the dissociation constants to be determined. The binding of Rhp23 to Mts4 has already been assessed using this method, and was found to have a Kd of 70nM (Rasmus Hartmann-Petersen, unpublished). In vitro competition assays could be used to test whether binding of one UDP to the UBS domain can be inhibited in the presence of other UDPs. Following these in vitro studies it would be important to show that the competition also exists in vivo. If the UDPs do compete for the binding to the UBS domain, then it might be expected that there would be genetic interactions between the UDPs. To test this hypothesis, experiments could be carried out to determine whether overexpression of a UDP may prevent other UDPs from

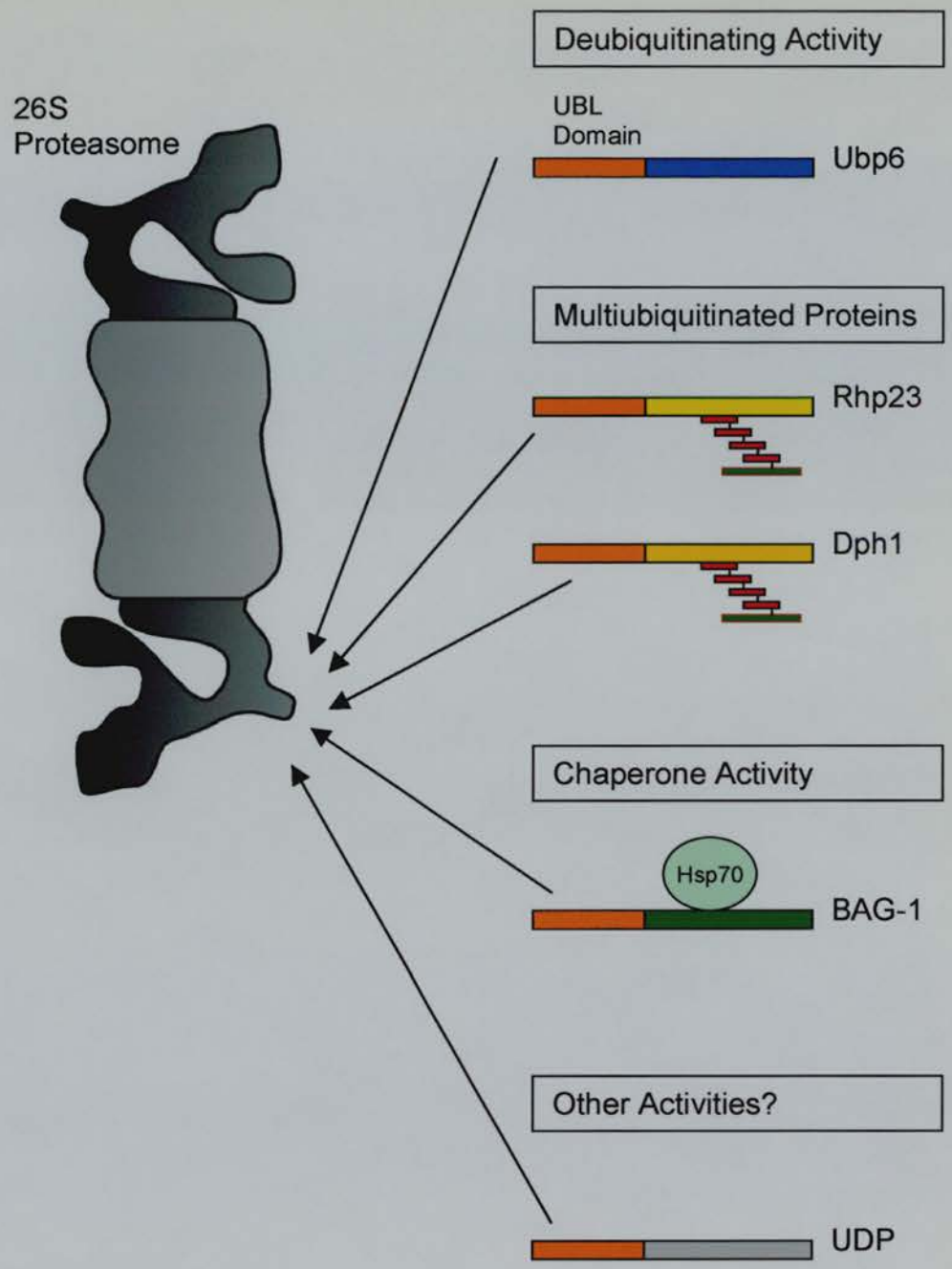

Figure 6.1: UDPs Bring Diversity to Proteasome Function
Several UDPs have been shown to interact with the proteasome via their UBL domains, suggesting that their role may be to bring other factors to the proteasome (see text for full explanation).

gaining access to the UBS site. This could be done by looking for a genetic background where a particular UDP becomes essential: for example, in the *pus1* mutant, loss of the Pus1 multiubiquitin binding protein means that either Rhp23 or Dph1 must be present (Wilkinson et al., 2001). Therefore, overexpression of another UDP, such as Ubp6, might prevent access of Rhp23 and Dph1 to the proteasome and cause the same lethal phenotype seen in the *pus1rhp23dph1* triple mutant.

6.6 The Role of Ubp6 in Proteasome-dependent Proteolysis

Although the genetic data so far does not implicate Uch2 in proteasome dependent proteolysis, the evidence for Ubp6 implies that it has a function in this pathway. This finding was surprising, as Ubp6 is thought to have a weaker association with the proteasome than Uch2. In both human and budding yeast, the Ubp6 homologues are found only associated with proteasomes and are not thought to be present in stoichiometric amounts compared to the other subunits. In contrast, *S. pombe* Uch2 is present in stoichiometric amounts and its *Drosophila* homologue, p37a, has been observed in proteasomes by electron microscopy (Li et al., 2000; Holzl et al., 2000). The use of mass spectrometry and glycerol gradients may confirm the amount of Ubp6 associated with proteasomes. An additional aspect of Ubp6 function that needs to be investigated further is the fact that exists in two forms. It would be interesting to determine whether a specific form of Ubp6 associates with the 26S proteasome and whether one or both forms are active. In addition, the nature of the modification could be studied by subjecting Ubp6 purified from *S. pombe* extracts to mass spectrometric analysis.

As *ubp6* was found to be synthetically lethal with certain proteasome subunit mutants, it is thought that it may function in promoting proteolysis. Several mechanisms for this role have been suggested, although it is unclear why only the *mts1*, *mts2* and *mts3* mutants, and not the *mts4* and *pad1* mutants, are synthetically lethal with *ubp6*. The most likely explanation is that there are subtle differences in

the phenotypes of the *mts* mutants that make *mts1*, *mts2* and *mts3* more sensitive to loss of Ubp6. Biochemical analysis of Ubp6 function revealed that it is able to act as a ubiquitin isopeptidase, suggesting that it could regulate proteasome function by degrading multiubiquitin chains, or by removing ubiquitin from conjugated proteasome substrates. In order to understand the synthetic lethality that has been observed, the *mts1*, *mts2* and *mts3* mutants could be crossed to a *ubp6* deletion strain expressing $ubp6^{+}$ under the control of a repressible promoter. This would allow viable *ubp6mts* double mutants to be obtained, and the expression of $ubp6^{+}$ could be repressed in a controlled manner to allow observation of the *ubp6mts* terminal phenotype. In addition, the use of $ubp6^{+}$ truncations and an active site mutant in this system would reveal whether it is activity or a particular domain of Ubp6 that is required for viability in the *mts* mutant background. As with *uch2*, the *ubp6* mutant could be tested for canavanine sensitivity and accumulation of high molecular weight ubiquitin conjugates, as well as being crossed to mutants of other ubiquitin pathway. These tests might clarify the role of Ubp6 in ubiquitin dependent proteolysis.

6.7 Redundancy of the DUBs

In this study, the hypothesis that DUB specificity is determined by cellular localisation was investigated using two proteasome associated DUBs, Uch2 and Ubp6. However, the genetic and biochemical experiments have not yet shown any evidence to support this hypothesis. This may be due to the presence of other, as yet uncharacterised DUBs being present at the proteasome. It is possible to identify candidates for this activity, the first example being the ubiquitin aldehyde insensitive, proteasome associated activity discussed above (section 6.2). In *S. cerevisiae*, the deletion strains for each of the DUBs have been tested for sensitivity to canavanine, which may indicate a defect in proteolysis. Several DUBs (Doa4, Ubp3, Ubp6, Ubp10, Ubp14 and Ubp15) were found to be required for resistance to canavanine, so these are good candidates for DUBs that have a role in proteolysis, possibly acting at the proteasome (Amerik et al., 2000). *S. pombe* has homologues of Doa4 (Ubp4),

Ubp3, Ubp6, Ubp14 and Ubp15, so these could be tested for redundancy with Uch2 and Ubp6 by the creation of null strains to be crossed to the *uch2* and *ubp6* mutants.

Another way in which a genetic approach could be used to identify other DUBs that are redundant with Uch2 or Ubp6 would be to use a genetic screen. In a method that has previously been described, mutants that are dependent on a high level of either DUB could be identified (Cullen et al., 2000). Firstly, a plasmid containing either $uch2^+$ or $ubp6^+$ under the control of the *nmt1* thiamine repressible promoter would be integrated into the *uch2ubp6* double mutant strain, to allow controlled overexpression of the DUB. The resulting strain could then be mutagenised, for example using EMS, and mutants that are able to grow only when $uch2^+$ or $ubp6^+$ is overexpressed could be identified by replica plating to medium containing thiamine. Those mutants that require high levels of one of the DUBs would be viable on medium lacking thiamine where $uch2^+$ or $ubp6^+$ is overexpressed, but would be unable to grow on medium containing thiamine, where $uch2^+$ or $ubp6^+$ expression would be repressed. The mutated genes could then be identified by using an *S. pombe* genomic library to rescue the lethality on medium lacking thiamine. This screen might identify other DUBs that act redundantly, as these would only be able to survive when high levels of Uch2 or Ubp6 were available to compensate for loss of their function. It would probably be most useful to carry out the screen using $ubp6^+$ overexpression, as Ubp6 has already been implicated as having a role in proteasome dependent proteolysis.

6.8 Conclusions

The study of the proteasome-associated DUBs in *S. pombe* has resulted in several significant findings in the understanding of the interaction between deubiquitination and the proteasome. Firstly, Uch2 was identified as the major ubiquitin hydrolase of the proteasome, despite its apparent lack of involvement in ubiquitin dependent proteolysis. Secondly, a function for Ubp6 in proteasome dependent proteolysis was

implied by the synthetically lethal interaction of *ubp6* with several proteasome mutants. However, the absence of any observable phenotype for the *uch2*, *ubp6* and *uch2ubp6* mutants indicated that other DUBs must be providing redundant function with Uch2 and Ubp6. Finally, the characterisation of the interaction of Ubp6 with the proteasome supported the hypothesis that UDPs function to bring diverse activities to the proteasome and indicated that the Mts4 proteasome subunit may be a general UDP binding factor. The high level of conservation of Uch2 and Ubp6 between *S. pombe* and higher eukaryotes suggests that the characterisation of these proteins will prove to be of general relevance to the study of proteasome mediated, ubiquitin dependent proteolysis.

Since this thesis was submitted for examination, three significant reports have been published regarding the deubiquitinating activity of the proteasome. Deshaies and coworkers have identified that, as proposed in this discussion, the Rpn11/Pad1 19S lid subunit is a deubiqutinating enzyme. In contrast to the classical DUBs, Rpn11/Pad1 was found to be insensitive to ubiquitin aldehyde, but instead displayed sensitivities characteristic of a metalloprotease. Rpn11/Pad1 contains a distinct conserved motif, named the JAMM (for Jab1/Pad1/MPN domain metalloenzyme) motif, which is composed of two histidines followed by an aspartate and preceded by a glutamate. Mutation of the conserved histidines to alanines was found to be lethal in *S. cerevisiae*. Furthermore, proteasomes containing this mutant Rpn11/Pad1 protein were defective in both deubiquitination and degradation of Ub-Sic1 suggesting that that Rpn11/Pad1 provides the link between these two processes (Verma et al., 2002). These findings are supported by a similar study identifying Rpn11/Pad1 as a Zn^{2+} dependent DUB (Yao and Cohen, 2002).

In complementary study, the Csn5/Jab1 subunit of the COP9/signalosome was also shown to contain the JAMM motif and mutation of residues within this motif or treatment with metal chelators abolished the COP9/signalosome dependent Nedd8 modification of Cul1 (Cope et al., 2002). Therefore, the Rpn11/Pad1 and Csn5/Jab1 isopeptidases appear to have analogous functions acting as metalloproteases, rather

that the typical Cys proteases, to carry out deubiquitination and deneddylation within multiprotein complexes.

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