Proteomic and Molecular Genetic Investigation of Deubiquitinating Enzymes in the Budding Yeast *Saccharomyces cerevisiae*

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy in the Graduate Department of Molecular Genetics University of Toronto

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Abstract

Protein ubiquitination is essential for the proper functioning of many eukaryotic cellular processes. The cleavage of ubiquitin chains from ubiquitinated proteins is performed by deubiquitinating enzymes, of which there are 16 in the Ubp (ubiquitin specific protease) group in the budding yeast *Saccharomyces cerevisiae*. The goal of my thesis has been to examine the biological roles and molecular functions of these enzymes using a combination of proteomic and molecular genetic approaches.

As part of a large collaborative effort, interacting protein partners of the Ubps were isolated through affinity purification of tagged proteins, followed by protein identification by mass spectrometry. Purification of tagged Ubp6 led to the identification of the 19S proteasome complex, along with a novel subunit, Sem1. As the human homologue of Sem1 was previously identified as being associated with a protein involved in the repair of DNA double-strand breaks, I examined the possible role of Sem1 in DNA damage repair. A deletion of Sem1 and other 19S subunits resulted in hypersensitivity to various DNA damaging drugs, implicating the 19S complex in the process of DNA repair.
I also found that purified Ubp2 interacted stably with the ubiquitin ligase Rsp5 and the protein Rup1. UBP2 interacts genetically with RSP5, indicating a functional relationship, while Rup1 facilitates the physical tethering of Ubp2 to Rsp5. Using the uracil permease Fur4, a Rsp5 substrate, as a model reporter, I found that ubp2Δ cells exhibited a temporal stabilization of Fur4 at the plasma membrane following the induction of endocytosis, implicating Ubp2 in protein sorting, specifically at the multivesicular body. In order to understand the role of Ubp2, I examined the effect of Ubp2 on Rsp5 function. I found that Rsp5, similar to its mammalian homologues, is auto-ubiquitinated in vivo, and that Ubp2 is able to directly deubiquitinate Rsp5 in vitro. Moreover, the presence of a substrate or Rup1 both resulted in increased auto-ubiquitination, implying an auto-inhibitory mechanism of Rsp5 regulation. Taken together, the data presented in this thesis implicate deubiquitinating enzymes in interesting and varied roles in the cell, and suggest a novel mechanism for the modulation of Rsp5-dependent trafficking processes.
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List of Abbreviations

APC: anaphase promoting complex
APMS: affinity purification and mass spectrometry
ATP: adenosine triphosphate
CTD: carboxy-terminal domain
Cvt sorting: cytoplasm to vacuole sorting
DNA: deoxyribonucleic acid
DSB: DNA double-strand breaks
DUB: deubiquitinating enzyme

E. coli: Escherichia coli

EGF: epidermal growth factor
ER: endoplasmic reticulum
ESCRT: endosomal sorting complex required for transport
HECT: homologous to the E6-AP carboxyl terminus
HR: homologous recombination
HU: hydroxyurea
HIS: histidine
IgG: immunoglobulin G
kDa: kilodalton

LC-MS/MS: liquid chromatography tandem mass spectrometry

JAMM: JAB1/MPN/Mov34 metallo-enzymes

MJD: Josephin domain enzymes

MALDI-ToF MS: matrix assisted laser desorption/ionization time of flight mass spectrometry

MMS: methylmethane sulfonate

MVB: multivesicular bodies

NHEJ: non-homologous end joining

OUT: ovarian tumour domain cysteine protease family

PCA: protein-fragment complementation assay

PCNA: proliferating cell nuclear antigen

RNA: ribonucleic acid

RNAPII: RNA polymerase II

RING: really interesting new gene

*S. cerevisiae*: Saccharomyces cerevisiae

SGA: synthetic genetic array

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TEV: tobacco etch virus protease

TAP: tandem affinity purification
TRP: tryptophan

Ub: ubiquitin

Ubp: ubiquitin specific protease

UBL: ubiquitin-like domain

UBA: ubiquitin associated domain

UIM: ubiquitin interacting motif

UCH: ubiquitin C-terminal hydrolases

URA: uracil

VHL: Von Hippel-Lindau

Y2H: yeast two-hybrid
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Chapter 1

Introduction
1. Introduction

1.1 Protein regulation by post-translational modification

The regulation of cellular processes at the levels of transcription and translation has been well studied for many decades. Many intricate mechanistic details and critical effector proteins mediating the well coordinated steps of these core control systems have been elucidated (Chapter 3; Alberts 2002). Pathways selectively targeting proteins for post-translational modification have also been discovered that are likewise central to the regulation of protein abundance and activity. Protein substrates are regulated through the covalent attachment and removal of small molecular tags, such as acetylation or phosphorylation, lipids, such as palmitoylation and prenylation, or peptides, such as ubiquitination and sumoylation, which modulate the folding, macromolecular associations, and stability of select proteins.

Protein ubiquitination, a particularly widespread and well conserved modification, is particularly important for controlling numerous processes at the levels of protein abundance, the physical interactions between proteins, and intracellular trafficking and localization within different subcellular compartments. Systematic large-scale proteomic, functional genomic, and other phenotypic screens of the ubiquitination machinery in various model systems have emerged as a major interest and have revealed many substrates, regulatory pathways and components, and functions of ubiquitin as a common, vital protein modification in eukaryotes (Gupta 2007; Liu 2009; Xu 2009). Other smaller-scale studies have identified the complex mechanisms underlying ubiquitination pathways (Bruce 2008; Gallagher 2006), described the fates of proteins which become ubiquitinated (Galan 1996; Maki 1996), and have identified the specific roles of novel players in the regulation of the ubiquitination process (Li 2002a; Papa 1993). Taken together, these studies show that protein ubiquitination plays essential and often highly conserved roles in the regulation of virtually all cellular processes. Ubiquitination is reversible, and many details of deubiquitination, the major focus of my thesis, are also just now emerging, including the specific enzymes involved, their physical and functional interaction partners, the various modes of regulation, and the identities of the specific substrates and processes which are targeted.
The unicellular budding yeast *Saccharomyces cerevisiae* has been useful as a model eukaryote in studying the molecular underpinnings of many different core processes in the eukaryotic cell. The genome contains approximately 6,000 genes (Goffeau 1996), many of which have homologues in higher eukaryotes. Yeast can exist in the haploid state, have a short generation time in culture, and are relatively simple to manipulate genetically (e.g. gene deletion). Many useful pioneering methods to investigate molecular relationships, including gene expression profiling, protein affinity tagging, and genetic interaction screens, have been devised over the past few decades through the work of the yeast biology community. More importantly, many of the components and pathways needed for the basic function of eukaryotic ubiquitin and deubiquitination machineries are present in yeast, and therefore many conclusions about this system can be extrapolated to other organisms, including humans. For this reason, most of my work has involved experimentation using yeast laboratory strains.

1.2 Ubiquitination

Ubiquitin (Ub) is an abundant, highly conserved 8 kDa polypeptide which is found in all eukaryotes (Figure 1-1). It was first purified and characterized in a series of groundbreaking studies in the late 1970s and early 1980s as an important component of ATP-dependent proteolysis in rabbit reticulocytes (Ciechanover 1980a; Ciechanover 1980b; Ciehanover 1978; Wilkinson 1980). Since its initial discovery, ubiquitin has since been found to be involved as a co-factor or regulator in many essential and important processes. At first, ubiquitin was thought simply to be a signal to trigger degradation of proteins by the 26S proteasome, a large multi-protein complex responsible for the regulation of cellular levels of many proteins through ubiquitin-dependent degradation (Finley 2009), but many other non-proteolytic functions linked to the modification of proteins by ubiquitin have since been discovered (reviewed in Bergink 2009; Horak 2003). These include control of core pathways linked to cell growth and proliferation through events including cell cycle progression, mitosis, chromosomal segregation, DNA damage repair, signal transduction, and of particular importance to this thesis, receptor-mediated endocytosis and intracellular trafficking.
Figure 1-1: The amino acid sequence and structure of ubiquitin. (A) The primary amino acid sequence of ubiquitin. The polypeptide contains seven highly conserved lysines (highlighted in red). All of these residues are potential sites at which poly-ubiquitin chains are conjugated. (B) The tertiary structure of ubiquitin (modified from Komander 2009). Poly-ubiquitin conjugation sites, at various residues along the surface of ubiquitin, are indicated with arrows. Conjugation of additional ubiquitin molecules to form poly-ubiquitin chains at these different lysines can lead to different protein fates. For example, and of particular importance to this thesis, K48-linked chains lead to proteasomal degradation, while K63-linked chains confer a non-proteolytic fate on the substrate (Finley 1994; Spence 1995).
Ubiquitin is conjugated covalently to a protein substrate as a single polypeptide tag, as a chain of many covalently attached ubiquitins (poly-ubiquitin), or as multiple single ubiquitins or ubiquitin chains on different sites (multi-ubiquitin). The conjugation of ubiquitin or a ubiquitin chain onto a protein substrate occurs through a cascade of three conserved and well characterized steps (Figure 1-2); (reviewed in Pickart 2004)). In the initial ATP-dependent step, ubiquitin is conjugated to the E1 activating enzyme, encoded by a single gene in most eukaryotes, through the formation of an Ub-adenylate intermediate. A thioester bond is then formed between a sulfur group present on the perfectly conserved cysteine in the enzyme pocket to the carboxy group on the C-terminal glycine residue of Ub. The activated ubiquitin is then passed by an ATP-independent transthioleation reaction onto a cysteine residue in the E2 conjugating enzyme, of which a broad family of 11 enzymes is known in yeast. After E2 conjugation, ubiquitin is then transferred onto a lysine residue on the substrate either directly with the help of an E3 ligase, which number over 20 in yeast, or passed onto the E3 ligase itself first, from which the Ub is then covalently attached to the substrate. While all E2 conjugating enzymes possess a common conserved catalytic domain, a number of E2s have specificity for a single E3, whereas many other E2s have been reported to work with multiple E3s (reviewed in Pickart 2001). For example, the yeast E2 Ubc2 (Rad6) transfers Ub to both Ubr1 (reviewed in Varshavsky 1996) in the N-end rule pathway and Rad18, an E3 ligase involved in post-replicational DNA repair (Hoege 2002).

Through the elucidation of the components and enzymatic steps of cellular pathways mediating ubiquitination, the ubiquitination process itself has been revealed to be tightly regulated. Regulation occurs at the substrate conjugation step, in terms of different chain compositions and lengths, in the recognition and selection of suitable substrates through the use of different targeting cofactors or substrate targeting signals, or, as explored in this thesis, at the level of the enzyme mediated removal of ubiquitin or poly-ubiquitin chains on proteins by a large group of deubiquitinating enzymes (commonly referred to as DUBs). While each of these regulatory steps have pointed to complicated and important roles that the ubiquitin machinery plays in the cell, deubiquitination in particular plays an additional, and sometimes crucial role in the regulation of these processes. The mechanisms governing the regulation of E3 ligase activity, substrate specificity, and DUB regulation are discussed in sections below.
Figure 1-2: The ubiquitination machinery. Proteins are ubiquitinated through three different enzymatic steps. First, in an ATP-dependent reaction, ubiquitin is activated by an E1 ubiquitin activating enzyme (1 in yeast). Ubiquitin is then passed onto an E2 ubiquitin conjugating enzyme (of which there are 11 in yeast), followed by covalent transfer of ubiquitin to a substrate protein recruited by an E3 ligase enzyme (20 of which are predicted in yeast). Ubiquitination of a protein can lead to its targeting to the 26S proteasome, which consists of a 19S cap and a 20S core. The 19S cap itself can be further divided into lid and base subcomplexes. Adaptor proteins, along with various subunits of the proteasome can bind ubiquitinated proteins, recruiting substrates to the proteasome for degradation. (Figure prepared with assistance from M. Davey)
1.2.1 *E3 Ubiquitin ligases and Rsp5*

E3 ligases are split into two major classes. The first is the RING (really interesting new gene) ligases, which contain a RING-finger domain and require the presence of an E2 conjugating enzyme to be able to conjugate ubiquitin. RING ligases recruit substrates to activated E2 enzymes, which transfer Ub directly to the substrate. The yeast E3 Ubr1, involved in N-end rule proteolysis by which proteins possessing certain amino-terminal destabilizing residues are targeted for proteasomal degradation, is a well known example of a RING ligase. RING ligases can occur as a single subunit, such as Ubr1, or as a large multi-subunit complex such as the anaphase promoting complex (APC). The second class of ligases is the HECT (homologous to the E6-AP carboxyl terminus) type ligases, which acquire Ub from an E2 first, then conjugate it onto a substrate by catalyzing the formation of an isopeptide bond between the ε-amino-group of a lysine residue on the target protein and the carboxy group of the last glycine residue on ubiquitin.

One of the largest and most highly conserved families of HECT ligases is the Nedd4 family, represented by one member, Rsp5, in yeast, and nine members in humans (Rotin 2009). Nedd4 ligases are characterized by the presence of an N-terminal ‘C2’ calcium dependent phospholipid binding domain (reviewed in Rizo 1998) which is important for their membrane localization. In mammals, the C2 domain has been linked to the recruitment of Nedd4 to lipid rafts in the apical membrane of polarized cells through binding to the adaptor annexin XIIIb (Plant 2000; Plant 1997). Other functional domains in Nedd4 ligases are two to four WW protein binding domains in the central region and the catalytic HECT domain on the C-terminal part of the protein (reviewed in Ingham 2004).

Of particular interest to this thesis, the yeast Nedd4 member Rsp5 is involved in many important cell functions, many of which are regulated by its various protein domains (Figure 1-3). For example, the C2 domain is necessary for proper localization of the enzyme to endosomal membranes, leading to ubiquitination and the proper protein sorting of newly synthesized cargo into multivesicular bodies (MVB). Interestingly, this domain is not required for Rsp5 dependent sorting of cargo from the plasma membrane at the MVB (Dunn 2004). In addition, the C2 domain of Rsp5 is not required for the ubiquitination of receptors and transporters at the plasma
Figure 1-3: Domain architecture of Rsp5. Rsp5, the yeast member of the widespread Nedd4 HECT ubiquitin ligase family, has the domain arrangement typical of all Nedd4 ligases: an N-terminal C2 lipid binding domain, three WW protein binding domains, and a C-terminal HECT catalytic domain. Numbers indicate the amino acid residue flanking each domain (UniProtKB protein knowledgebase; www.uniprot.org). The location of the temperature sensitive \textit{rsp5-1} hypomorphic point mutant is shown.
membrane, but it is required, through an unknown mechanism, for efficient internalization of certain proteins (Springael 1999a; Wang 2001).

Apart from roles in intracellular trafficking (reviewed in further detail in sections below), Rsp5 has been implicated in various nuclear events. For example, Rsp5 is involved in the ubiquitination and subsequent degradation of the large subunit of RNA polymerase II (RNAPII) after transcriptional arrest due to DNA damage or other means, resulting in the clearing of stalled RNAPII complexes from DNA (Beaudenon 1999; Somesh 2005). Rsp5 has been shown, in both *in vitro* and *in vivo* experiments, to add a non-degradative K63-linked poly-ubiquitin chain to RNAPII in response to DNA damage, which can then be trimmed down to a mono-ubiquitin by the DUB Ubp2 (Harreman 2009). Subsequently, another E3 complex containing Elc1 and Cul3 subunits forms an extended K48-linked poly-ubiquitin chain, which results in the degradation of RNAPII (Harreman 2009; Ribar 2007). The regulation of fatty acid levels reflects the essential function of *RSP5*, as oleic acid can restore the growth of *RSP5* mutants (Hoppe 2000). Rsp5 is involved in the ubiquitination and proteasome-dependent cleavage of the functionally redundant ER bound transcription factors Spt23 and Mga2 (Hoppe 2000; Shcherbik 2003). These regulators are synthesized as ER-localized precursors, which are released by proteolytic cleavage allowing for their translocation into the nucleus to activate the expression of *OLE1*, which codes for an enzyme essential in the oleic acid synthesis pathway (Hoppe 2000; Shcherbik 2003). Interestingly, Spt23 processing may be regulated by the lipid composition of cells, as the addition of palmitoleic acid and linoleic acid to growth media results in a decrease in Spt23 processing, although the molecular mechanism for this is unclear (Hoppe 2000). In addition, mass spectrometric analysis suggests that Rsp5-mediated ubiquitination of Mga2 occurs through K63-linked chains, illustrating a rare 26S proteasome-related function for this chain type (Saeki 2009).

### 1.2.2 Poly-ubiquitin chains

Proteins can be modified by a single ubiquitin (mono-ubiquitination) or a poly-ubiquitin chain. There are seven lysines among the 76 amino acids in ubiquitin, all of which are targets for the formation of different poly-ubiquitin chains through a covalent link to the C-terminal end of
another ubiquitin (Figure 1-1). Poly-ubiquitin chains joined at lysine positions 6, 11, 27, 29, 33, 48, and 63 have been reported in the literature (Xu 2009). A recent quantitative proteomics study examining the prevalence of different types of Ub chains in yeast of various growth densities (Xu 2009) found that the abundance of certain Ub species ranged from 3.2% of total Ub chains for K29-linked chains to as much as 29% for K48-linked chains (Xu 2009). By far, the best studied types of poly-ubiquitin chains involve K48 and K63 type chains. Together, these two chain types comprise of almost half of the Ub chain population (Xu 2009).

Different types of ubiquitin chains confer alternative fates upon their substrate proteins. Generally, K48 poly-ubiquitin chains target protein substrates to the 26S proteasome (Finley 1994), while K63-linked chains result in various non-proteolytic fates for the substrate proteins (Spence 1995). Most of the remaining types of Ub chains accumulate after treatment of the cells with a proteasome inhibitor (Xu 2009), suggesting that they participate in proteasomal degradation. Although only a small number of studies on ubiquitin chains other than K48 and K63 have been performed, these alternate linkage chain types have been implicated in some non-proteasome related roles. For example, K29-type chains are involved in lysosomal degradation, as the degradation of Deltex, a K29-linked Ub chain substrate, is sensitive to lysosomal protease inhibitors (Chastagner 2006). The biological significance of many other alternate poly-ubiquitin chain types remains mostly unknown.

Members of downstream effector pathways must recognize and differentiate between different chain types. It is thought that chains with various lysine linkages result in different structural conformations, which are in turn recognized as signals for various downstream processing pathways. Structural studies comparing K48 and K63 chains have determined that they indeed assume very different conformations (Phillips 2001; Tenno 2004). For example, K48 tetra-ubiquitin chains can have a few different conformations, including a very closed, globular conformation (Phillips 2001), while K63 tetra-ubiquitin chains exhibit an extended conformation (Tenno 2004). The conformations of other chain types are currently unknown.
1.3 Ubiquitination in proteasomal protein degradation

As mentioned above, attachment of poly-ubiquitin chains to a protein often leads to targeting and degradation of the substrate by the 26S proteasome (reviewed in Finley 2009). This large complex consists of a large barrel shaped core proteolytic structure termed the 20S core that is capped at both ends with a multisubunit 19S regulatory ‘cap’ or ‘particle’. The 19S cap consists of linked ‘lid’ and ‘base’ subcomplexes (Figure 1-2). The 20S core has four stacked rings, with each ring containing 7 subunits. The proteolytic sites of the proteasome are present in the internal channel formed by the stacked rings, thereby rendering it difficult for folded substrates to enter the narrow opening and be degraded unless the polypeptides are first unfolded by the 19S cap. The 19S complex provides critical functions in the recognition, binding, deubiquitination, unfolding, and translocation of ubiquitinated proteins into the 20S core (reviewed in Finley 1994). For binding by the proteasome, a tetra-ubiquitin K48-linked chain is the minimum signal for recognition in vitro (Thrower 2000). As the 19S cap is important for the regulatory functions of the proteasome, I review below the activities associated with its various subunits.

Of the 19S subunits (Rpn3, 5-9, 11, and 12 in the lid and Rpt1-6, Rpn1, 2, 10, and 13 in the base subunit) annotated in yeast, Rpn10 and Rpn13 play complementary roles in the binding of ubiquitinated substrates via the direct recognition of ubiquitin (Deveraux 1994; Husnjak 2008). Rpn10 binds ubiquitinated substrates through an ubiquitin interacting motif (UIM) (Elsasser 2004), while Rpn13 binds ubiquitin through an N-terminal domain (Husnjak 2008). The six ATPase proteins (Rpt subunits in yeast) in the base subcomplex are thought to be involved in the unfolding and translocation of the substrate protein to the 20S. This was shown by various methods including the demonstration that the archaeabacterial complex PAN, which is homologous to the six ATPases in eukaryotes, has the ability unfold a substrate protein in vitro (Navon 2001). The 19S particle also displays deubiquitination activity, which is associated both with the Zn-dependent metalloprotease Rpn11 located in the lid (Guterman 2004), and the proteasome-associated DUB Ubp6 in the base subcomplex (Guterman 2004). In addition to cleaving off ubiquitin for recycling before the entry of polypeptide substrates into the 20S particle, deubiquitination has a critical function in modulating substrate degradation, as cells deleted for Rpn11 have a defect in proteasome-mediated proteolysis (Verma 2002). Verma et al. (2002) hypothesized that deubiquitination may facilitate substrate entry into the 20s proteolytic
core by removing the bulky ubiquitin chain. Although both are part of the 19S particle, Ubp6 and Rpn11 likely have both shared and distinct roles within the proteasome. This is evidenced through the identification of RPN11 as an essential gene, while UBP6 is non-essential under standard culture conditions; together, the two genes display synthetic lethal genetic interactions when mutated (Guterman 2004). Specific details on the roles of both these, and other deubiquitinating enzymes, in regulating protein degradation by the 26S proteasome remain to be elucidated. Of critical importance to my thesis, most of the DUBs annotated to date in yeast and other organisms have been shown to function separately from the 26S proteasome complex itself.

1.4 Non-proteasomal functions of ubiquitin

Other than proteasomal protein degradation, many non-26S proteasome-related roles for ubiquitination and deubiquitination have been described. For example, studies in budding yeast have implicated ubiquitination in the cellular response to DNA damage, as DNA damage induces modification of components of the DNA repair and replication machinery. One example is the DNA sliding clamp PCNA (reviewed in Bergink 2009). Mono-ubiquitination of PCNA by the ubiquitin ligase Rad18 (due to stalled replication forks resulting from DNA damage) serves as a signal for DNA damage repair by the error-prone translesion synthesis pathway. Extension of this initial Ub by another ligase, Rad5, to form a K63-linked poly-ubiquitin chain, results in a switch to a recombination-dependent error-free DNA repair pathway. Moreover, the ubiquitin modification of substrates such as PCNA is usually dynamic as Ub chains can be removed by deubiquitinating enzymes. For instance, the human DUB Usp1 deubiquitinates mono-ubiquitinated PCNA. Ultraviolet radiation can specifically induce downregulation of Usp1 by an auto-cleavage mechanism, leading to preferential repair of DNA damage by the translesion synthesis pathway (Huang 2006). The function of ubiquitin, and in particular the identities of other DUBs participating in DNA repair remain active avenues of exploration.

In addition to DNA damage response, by far the best characterized non-proteasomal functions of ubiquitination and deubiquitination are in membrane protein trafficking. Such functions include participation in the initial internalization step during endocytosis of plasma membrane associated proteins, the subsequent sorting of these cargo proteins into endosomes with multiple internal vesicles termed multivesicular bodies (MVBs), and finally, the sorting of
these cargoes or newly synthesized proteins to the lysosome (or vacuole in yeast) for non-proteasomal degradation (reviewed in Horak 2003). Below, I describe the role of ubiquitin and deubiquitinating enzymes in subcellular protein trafficking, as it is directly relevant to my work described in Chapter 3.

1.4.1 The role of ubiquitination in subcellular protein trafficking

Many plasma membrane-bound proteins require ubiquitination for internalization (reviewed in Horak 2003). In almost all cases of cell surface transporters and receptors examined in the budding yeast, including proteins with varied numbers of transmembrane domains and G protein-coupled receptors, ubiquitination is mediated through the E3 ubiquitin ligase Rsp5, a conserved member of the Nedd4 family of ubiquitin ligases (Horak 2003). Rsp5 is also involved in other steps in vesicle trafficking, including cargo sorting into the MVB pathway (Morvan 2004), and sorting of newly synthesized proteins from the Golgi to the vacuole (Blondel 2004).

The ubiquitination events underlying trafficking of the uracil permease Fur4 have been particularly well characterized. As illustrated schematically in Figure 1-4, the binding of its natural substrate uracil, or various stress conditions such as nutrient starvation, heat shock or chemical inhibition of protein synthesis, triggers phosphorylation of Fur4 by the Yck1 and Yck2 kinases. This in turn drives the subsequent recognition and Lys63-linked di-ubiquitination of Fur4 by Rsp5 on two specific lysine residues, which were mapped by monitoring Fur4 ubiquitination in cells with various lysine sites mutated (Galan 1997; Marchal 1998, 2000; Seron 1999; Volland 1994). Ubiquitination of Fur4 results in its complete clearance from the plasma membrane into early endosomes within 60 minutes. Numerous studies of the roles of various members of the endocytic machinery, including some conserved and some yeast-specific components involved in the reorganization of the actin cytoskeleton such as End3 and Sla1, clathrin-binding proteins such as the epsin homologues Ent1 and Ent2 and myosins Myo3 and Myo5, which mediate the invagination of the plasma membrane and membrane fission, have revealed tight coordination underlying the formation of the internalized endosome (reviewed in Dupre 2004).
Figure 1-4: Internalization pathway of the model substrate Fur4. The schematic shows key steps in endocytosis as elucidated for the plasma membrane permease Fur4 in yeast. Upon engagement by its ligand uracil, Fur4 is phosphorylated by two kinases, Yck1 and Yck2. Rsp5, an HECT family E3 ubiquitin ligase, is then recruited, and ubiquitinates Fur4 with K63-linked di-ubiquitin chains on two lysine residues. Ubiquitination results in Fur4 internalization into an endosome, followed by sorting via the multivesicular body (MVB) and eventual degradation in the vacuole by vacuolar proteases. Deubiquitination of Fur4 by the DUB Doa4 occurs immediately before MVB internalization. (Figure prepared with assistance from T. Yang)
After internalization, ubiquitination by Rsp5, is again required for the proper entry of cargo such as Fur4 into the MVB (Morvan 2004). Co-immunoprecipitation assays implicated adaptor proteins, such as Ear1, in binding to both Rsp5 and various cargo substrates (Leon 2008). Ear1 and its redundant paralog Ssh4 are important for MVB sorting, as *ear1Δ ssh4Δ* double mutants missort cargo onto the vacuolar membrane (Leon 2008), a phenotype consistent with the inability of cargo to enter the internal MVB vesicles before fusion with the vacuole. However, a Ub-cargo chimera was able to bypass the need for Ear1, implicating Ear1 in Rsp5-mediated substrate ubiquitination (Leon 2008).

Ubiquitinated cargo is then sorted by ‘class E’ proteins into MVBs. Cargo is recognized sequentially by the multiprotein ESCRT (endosomal sorting complex required for transport)-I, II, III complexes and other class E proteins (reviewed in Katzmann 2002). First, the Vps27-Hse1 complex, along with the ESCRT-I complex, is thought to bind ubiquitinated cargo, and then recruit the ESCRT-II and –III complexes. Many members of these ESCRT complexes have ubiquitin binding domains, which are thought to bind either ubiquitinated cargo or ubiquitinated components of the MVB machinery itself. Other accessory proteins are recruited by the ESCRT-III complex, including the deubiquitinating enzyme Doa4 (reviewed below), and the ATPase Vps4, which functions in the release of all three ESCRT complexes enabling the internalization of cargo into the MVB. After sorting into the MVB, the MVB can fuse with the vacuole, and cargo enters into the vacuolar lumen where it is degraded by vacuolar proteases (Volland 1994).

Deubiquitinating enzymes have been implicated in various steps in the trafficking pathway. One example is the yeast DUB Doa4/Ubp4. Dupre *et al.* reported a marked accumulation of internalized vacuolar ubiquitinated Fur4 in the absence of *DOA4*, implicating Doa4 in the deubiquitination of Fur4 and other membrane cargo prior to MVB internalization (Dupre 2001). Doa4 was initially thought to serve a role only in the recycling of ubiquitin, as sorting defects in *doa4Δ* cells can be overcome with the overexpression of ubiquitin (Dupre 2001; Springael 1999b). Interestingly, a recent study reported that *doa4Δ* cells with normal levels of free ubiquitin still exhibited a MVB sorting defect, implicating Doa4 in a currently unclear but more direct role in MVB sorting in addition to mediating free ubiquitin levels (Nikko 2007). In agreement with a more direct role in MVB sorting, other studies have found that Doa4 localizes to the late endosome via interaction with Bro1, a class E protein recruited by the ESCRTIII.
complex (Luhtala 2004). Although the exact details of the role(s) of Doa4 at the MVB are still unclear, Doa4 appears to be required both for the general maintenance of ubiquitin levels in the cell and for MVB sorting.

In addition to plasma membrane to vacuole trafficking, ubiquitin is also involved in other areas of intracellular sorting. For example, in the presence of its substrate uracil, newly synthesized Fur4 is diverted to the endosomal system directly from the Golgi, where it undergoes Rsp5-dependent ubiquitination, followed by internalization into MVBs and degradation in the vacuole (Blondel 2004). Although the mechanism regulating this diversion is unclear, uracil binding is an important requirement, as newly synthesized mutant Fur4 with a decreased affinity for uracil is not directed efficiently to the vacuole (Blondel 2004). As with receptor internalization, ubiquitination appears to be the trigger as Ub-Fur4 chimeras can be sorted to the vacuole, albeit inefficiently, in the absence of uracil (Blondel 2004).

Another sorting event is cargo recycling. Recycling of endocytosed cargo back to the plasma membrane can occur in wildtype cells, for example, in the case of the yeast a-factor receptor Ste3 (Chen 2000), or in cells with defects in MVB sorting. Internalized cargo is recycled in cells with conditional class E/ESCRT mutations (Bugnicourt 2004), but the role of the ubiquitination/deubiquitination machinery here is currently unclear. Although deubiquitination by Doa4 is not required for recycling of Fur4 (Bugnicourt 2004), recently some evidence has implicated another DUB, Ubp1, in this process in yeast. Overexpression of Ubp1 resulted in a deubiquitination-dependant stabilization of Ste6, which functions in pheromone export, at the plasma membrane, but ubiquitination of Ste6 itself was seemingly not affected (Schmitz 2005). Therefore, Ubp1 may play an as yet unknown role in the direct regulation of members of the endocytic machinery or in the recycling of Ste6.

Given the increasing prominence of deubiquitinating enzymes in intracellular trafficking, a potential role for other DUBs in sorting pathways was a possibility when I commenced my thesis studies, and indeed my work provided evidence for such a role (Chapter 3).
1.5 Targeting ubiquitin ligase activity

Regulation of substrate ubiquitination specificity is generally dictated by the E3 ligases. Large-scale proteomic studies (Gupta 2007; Kus 2005) have revealed an ever increasing number of proteins that are modified by specific E3 ligases, and have established critical determinants of substrate recognition. As it is directly relevant to my own work (Chapter 4), I review various examples below of the regulation of ubiquitination, focusing on regulation at the level of substrate selection, and also regulation via members of the ubiquitination enzymatic machinery.

Substrate specificity of E3 ligases is usually dictated through the recognition of specific target sequences or post-translational modifications (e.g. phosphorylation) on the substrate protein, or by a specific substrate conformation. For example, certain substrates of the APC, a large ubiquitin E3 ligase assembly important for mitotic cell division (reviewed in Tyers 2000), contain a putative ‘destruction box’ motif. This motif is recognized and bound by one of the members of the APC, Cdc20, which mediates substrate ubiquitination and degradation by the ligase during the transition between metaphase to anaphase in mitosis (Hilioti 2001). One such substrate is the anaphase inhibitor Pds1 in yeast (Hilioti 2001). A possible role for deubiquitination to reverse Pds1 ubiquitination has not yet been reported.

Other unstable proteins, governed by the N-end rule pathway, have single specific destabilizing residues, such as arginine and lysine, exposed at their amino-terminus (reviewed in Varshavsky 1996). In yeast, substrates bearing destabilizing N-end rule residues are recognized and bound by the E3 ligase Ubr1, which then ubiquitinates these proteins with a K48-linked poly-ubiquitin chain leading to their proteasomal degradation (reviewed in Varshavsky 1996). An example of a putative substrate of the N-end rule pathway in yeast is the cleavage product of the cohesin subunit Scc1. At the end of metaphase, cohesin, which functions to keep sister chromatids joined after DNA replication, must be cleaved for chromosomal segregation to proceed during anaphase. After degradation of Pds1 by the APC, separase, the target of Pds1 inhibition, is free to cleave Scc1, resulting in a Scc1 carboxy-terminal fragment which contains residues which are destabilizing under the N-end rule (Rao 2001). Although Ubr1 ubiquitination of this fragment has not yet been directly assayed, degradation of this Scc1 fragment is dependent on Ubr1 expression and is important for chromosome stability by an unknown mechanism (Rao 2001).
Protein-protein interaction domains are also frequently used in the recognition of substrates by E3 ligases. For example, proline rich motifs, such as PY motifs, are recognized by WW domains in many Nedd4 HECT family E3 ligases (reviewed in Ingham 2004). The yeast membrane protein Sna3, which was recently implicated in the trafficking of the tryptophan permease Tat2 (Hiraki 2010), is a known interactor of Rsp5. Sna3 has a PY motif in its cytosolic tail which was shown by co-immunoprecipitation assays to interact with the WW domains of Rsp5 (Stawiecka-Mirota 2007). Binding to these domains of Rsp5 is in turn important for the correct sorting of Sna3 into the vacuolar lumen, as a mutation in one of the WW domains of Rsp5 or the PY motif in Sna3 results in the missorting of Sna3 (Stawiecka-Mirota 2007). This is dependent on the poly-ubiquitination of Sna3 through a K63-linked chain, as mutants of Sna3 that cannot be ubiquitinated are missorted (Stawiecka-Mirota 2007).

Many membrane-associated proteins, such as cell surface transporters or receptors, lack obvious motifs for direct recognition or binding to E3 ligases. In these cases, the ubiquitination apparatus is thought to recognize these substrates through either phosphorylation sites, or a global change in the conformation of the substrate induced by post-translational modifications. For example, ubiquitination of Fur4 by Rsp5 is dependent upon phosphorylation of serine residues at its N-terminus, even though Rsp5 has not been reported to bind phosphorylated residues directly. Although Fur4 contains a destruction box-like sequence, mutation of this sequence only partially protects the protein from degradation (Galan 1994), implying that there are other mechanisms mediating Fur4 recognition by Rsp5. Although evidence is still lacking, Marchal et al. (1998) hypothesized that phosphorylation of Fur4 may lead to a conformational change in the protein that is recognized by Rsp5 through the exposure of a new sequence or protein surface. In another example, the mammalian E3 ligase Nedd4 has been recently reported to bind, through its WW domains, to a phosphorylated region on Sprouty2, a protein involved in the inhibition of the fibroblast growth factor receptor (FGF) pathway (Edwin 2010). Binding is required for Sprouty ubiquitination and destabilization, and silencing of Nedd4 resulted in an increase of Sprouty2 levels and a concomitant decrease in FGF function (Edwin 2010). Likewise, a decrease in Sprouty2 phosphorylation through the addition of kinase inhibitors or the lowering of kinase levels with short interfering RNA resulted in a significant decrease in Nedd4 binding.
Another way for E3 ligases to bind to their substrates is indirectly through adaptor proteins, which recognize both the ligase and the substrate. PY motif-containing adaptors for Rsp5 include Ear1, which is necessary for Rsp5-dependent ubiquitination at the multivesicular body (Leon 2008), and the cargo-specific adaptor Cvs7 (Lin 2008) at the plasma membrane. Moreover, a recent study (Nikko 2009) showed that proteins in the arrestin family may function as adaptors for Fur4 recruitment and ubiquitination by Rsp5 at the plasma membrane. Although binding to phosphorylated Fur4 was not examined directly, deletion of these adaptor candidates both individually and in combination resulted in a slight defect in Fur4 internalization (Nikko 2009).

1.5.1 Regulation of RING E3 ligases

The ubiquitin machinery itself is highly regulated at each of the three enzymatic reactions (E1, E2, E3) required for ubiquitination. For example, the expression of individual E2 transcripts rises and falls during different phases of cell differentiation (Wefes 1995). Another major form of regulation, particularly relevant to this thesis, is at the level of the E3 ligase activity. The methods of ligase regulation are often complex, however, and include mechanisms ranging from differential binding of regulatory cofactors to post-translational modifications, including phosphorylation (Gallagher 2006; Helliwell 2001). Of particular importance to my work is E3 regulation via reversible auto-ubiquitination, which is emphasized in following sections.

One particularly well studied example of ligase regulation is the mammalian RING E3 Mdm2 (reviewed in Michael 2003), which ubiquitinates the tumour suppressor protein p53 as its principal substrate in unstressed cells, resulting in proteasomal degradation of p53. Two proteins regulating this process are the tumour suppressor p19 or ARF (alternative reading frame of the INK4a locus), the loss of which leads to the rapid development of multiple tumours in mice by two months of age (Kamijo 1997), and a DUB that is physically associated with Mdm2, HAUSP (also known as Usp7). ARF binds to and sequesters Mdm2 within the nucleolus when the oncoprotein Myc is induced (Weber 1999), therefore stabilizing p53. ARF is also able to directly inhibit the ubiquitin ligase activity of Mdm2 in vitro (Honda 1999), although it is not clear whether direct inhibition of Mdm2 activity by ARF is physiologically relevant. Most relevant to the work described in this thesis, Mdm2 can be reversibly ubiquitinated, either by itself (i.e.
auto-ubiquitination) (Fang 2000), or by another ubiquitin ligase (Linares 2007), which serves as a mechanism for down-regulation of enzyme levels and therefore E3 activity. Although the relative contribution of these two modes of ubiquitination is currently unknown, auto-ubiquitinated Mdm2 is rapidly targeted to and degraded by the 26S proteasome (Fang 2000). Further discussed in a later section dedicated to deubiquitinating enzymes, deubiquitination by HAUSP regulates Mdm2 levels by stabilizing it (Li 2004). Taken together, these studies suggest that E3 ligases can be activated and inhibited through dynamic auto-ubiquitination and regulated deubiquitination events.

1.5.2 Regulation of the HECT E3 ligases

Members of the HECT family of ubiquitinating enzymes have interesting and varied mechanisms of regulation. Unlike RING type E3 ligases, HECT ligases have the ability to directly catalyze ubiquitin conjugation to their substrates. A prime example is the Nedd4 family E3 ligase Itch, which is involved in the mammalian immune response via regulation of T-cell differentiation, intercellular signalling through the ubiquitination of the Notch receptor, and apoptosis through the maintenance of transcription factor levels in T lymphocytes (reviewed in Melino 2008). Itch is regulated by at least two different forms of post-translational modification. When T-cells are stimulated, Itch is phosphorylated in a central region by Jun N-terminal Kinase 1 (JNK1), which results in a marked enhancement of its catalytic activity and auto-ubiquitination both in vitro and in vivo (Gallagher 2006; Gao 2004). In vitro binding assays detected an intramolecular physical interaction between the catalytic HECT domain and the WW domains of Itch (Gallagher 2006). Phosphorylation by JNK1 upon T-cell activation disrupts this intramolecular binding, purportedly resulting in a conformation change to an ‘active’ configuration due to dissociation of the WW domain from the HECT region. Although this model has not been directly proven, protease protection patterns of Itch were found to change after JNK1 mediated phosphorylation, implying a gross structural change in the E3 ligase (Gallagher 2006).

Particularly important with respect to Chapter 4 in this thesis, Itch is reversibly auto-ubiquitinated both in vitro and in vivo with K63-linked ubiquitin chains (Mouchantaf 2006; Scialpi 2008). Itch ubiquitination is thought to occur through an intermolecular (i.e. in trans)
auto-ubiquitination mechanism as: (i) recombinant Itch purified from *E. coli* displayed auto-
ubiquitination activity *in vitro* (Scialpi 2008), (ii) Itch is able to form dimers *in vivo* (Scialpi
2008), (iii) an Itch catalytic mutant is modified in the presence of endogenous wildtype Itch
(Oberst 2007), and (iv) an Itch catalytic mutant is not ubiquitinated in a wildtype Itch-depleted
cell extract (Mouchantaf 2006). In addition to auto-ubiquitination, Itch has been suggested to be
ubiquitinated by another, currently unknown E3 ligase(s) (Scialpi 2008). Ubiquitinated Itch
seems to have both non-proteolytic (Scialpi 2008) and proteolytic fates (Mouchantaf 2006),
although it is currently unclear which type of ubiquitination and which ligase(s) are responsible
for these different outcomes.

Deubiquitination is also important in controlling the cellular levels of Itch. At least one
deubiquitinating enzyme, USP9X, has been implicated, although indirectly, in the
deubiquitination of Itch (Mouchantaf 2006). USP9X physically interacts with Itch through one or
more of the Itch WW domains both *in vitro* and *in vivo*. In addition to stabilizing Itch against
proteasomal degradation *in vivo*, over-expression of USP9X results in a significant decrease of
ubiquitinated forms of Itch (Mouchantaf 2006). A recent study (Azakir 2009) has shed some
light on the physiological role of this effect. Epidermal growth factor (EGF) stimulation of cells
increases Itch activity by JNK1 phosphorylation, and, at the same time, increases the formation
of Itch-USP9X complexes as measured by co-immunoprecipitation (Azakir 2009). Therefore, the
overall effect of EGF signaling on Itch is an increase in ligase stability and activity towards its
substrates, through phosphorylation and DUB mediated protection from auto-ubiquitination and
degradation.

Taken together, these studies show that the regulation of a HECT E3 ligase can occur
through modifications such as phosphorylation, ubiquitination, and deubiquitination by a
physically associated DUB. Additionally, while the exact cellular role of non-degradative Itch
ubiquitination is currently unclear, ubiquitin modifications on HECT E3 ligases can exert both
degradative and non-degradative roles.
1.5.2.1 Regulation of mammalian Nedd4-2

The mammalian E3 ligase Nedd4-2, responsible for sorting of transmembrane proteins in a manner analogous to yeast Rsp5, is regulated in a similar manner to that of the E3 Itch, but with some notable differences. The most well known and well characterized substrate of Nedd4-2 is the epithelial sodium channel (ENaC). Co-immunoprecipitation experiments and ENaC activity assays on mammalian Nedd4-2 showed that the WW domains of Nedd4-2 interact with ENaC through its PY motif (Kamynina 2001). Once Nedd4-2 ubiquitinates ENaC, the substrate is internalized and degraded (reviewed in Rotin 2008). The regulation of Nedd4-2 occurs through different routes, including phosphorylation of the ligase (Bhalla 2005). Phosphorylation of Nedd4-2 in response to extracellular signals occurs through various kinases, including the aldosterone related kinase SGK (Snyder 2002) and the inflammation-related kinase IKKβ (Edinger 2009). In contrast to the positive regulation of Itch by JNK1 described above, where the phosphorylation of Itch induces enzyme activation, phosphorylation of Nedd4-2 induces binding by 14-3-3 phosphoserine binding proteins, which block the interaction of Nedd4-2 with substrates such as ENaC (Bhalla 2005; Nagaki 2006). As a consequence, ENaC is not ubiquitinated or internalized (Bhalla 2005).

Auto-ubiquitination of Nedd4-2, leading to its degradation by the 26S proteasome, appears to be a major mechanism by which levels of the ligase are controlled (Bruce 2008). This ubiquitination is likely intramolecular (i.e. in cis), as a catalytic mutant is not ubiquitinated in vitro or in vivo, even in the presence of wildtype Nedd4-2 (Bruce 2008). A recent study on Nedd4-2 domains suggests that, similar to the model of Itch regulation, the activity of the ligase is controlled by physical interactions between different domains on Nedd4-2 (Bruce 2008). First, recombinant forms of individual WW domains of the ligase are capable of binding to the HECT catalytic domain in vitro. In contrast to Itch, a specific PY motif on Nedd4-2, present in the HECT domain, is necessary for binding, as mutation of the tyrosine residue in the PY motif abrogated binding to the WW domains (Bruce 2008). Binding affinity between the HECT and WW domains was lower than for a Nedd4-2 substrate, suggesting a preference for Nedd4-2 substrates in a physiological context. Binding between HECT and WW seemingly inhibited Nedd4-2, as an increase in concentration of WW domains in the reaction resulted in a decrease in Nedd4-2 activity, as measured by auto-ubiquitination levels (Bruce 2008). Accordingly, a PY mutant form of Nedd4-2 was able to self ubiquitinate in vitro, and showed reduced stability in
cells as compared to wildtype in a pulse-chase Western blot. Whether the binding between HECT and WW occurs in \textit{cis} or \textit{trans} (i.e. intra- vs inter-molecularly) is not yet known, and whether Nedd4-2 is capable of dimerizing \textit{in vivo} is also unknown.

In contrast to Itch, however, phosphorylation has not been shown to regulate the folding of Nedd4-2. Rather, a conformational change in Nedd4-2 has been suggested to occur upon the interaction of the ligase with its substrates, as auto-ubiquitination of Nedd4-2 increases \textit{in vivo} when ENaC is co-expressed (Bruce 2008). One model suggests that the ligase is folded and inactive in the absence of a substrate (such as ENaC), whereas binding of a substrate could result in a conformational change leading to activation of Nedd4-2 by alleviating the steric inhibition that prevented access of a substrate to, or function of, the HECT catalytic domain. Since the affinity of the Nedd4-2 WW domains for substrates such as ENaC is higher than for its own HECT domain PY motif, the Nedd4-2 WW domain would then prefer to bind substrates when they are presented. This could result in an active ligase, allowing for the ubiquitination of both the substrate and the ligase itself. Auto-ubiquitination would then lead to downregulation of the E3 through degradation by the 26S proteasome, completing the cycle. Although the participation of a DUB (as with Itch) in countering Nedd4-2 ubiquitination has not been reported, a similar mode of reversible regulation as found for Itch may exist for Nedd4-2 and more generally with other HECT family ligases.

\section*{1.5.2.2 Regulation of the yeast HECT E3 ligase Rsp5}

When I commenced my thesis research, Rsp5 was known to be modulated in several ways. A major form of regulation appears to be at the level of Rsp5 substrate recognition. Since few documented Rsp5 substrates have an apparent WW domain binding motif, a general requirement for adaptor proteins to mediate proper targeting seemed likely. In various protein trafficking steps such as endocytosis at the plasma membrane and during cargo transport into the MVB, Rsp5 adaptor proteins, such as Cvs7/Art1 (Lin 2008) and Ear1 (Leon 2008) play an important role in the tethering of diverse substrates to Rsp5. Cvs7/Art1, for example, serves as a cargo-specific adaptor for a couple of transmembrane proteins, such as the arginine transporter Can1, in the initial internalization step at the plasma membrane (Lin 2008). Although the exact mechanism by which Cvs7 serves as an adaptor for Rsp5 is still unclear, Cvs7 contains two PY
motifs which can bind Rsp5 WW domains in in vitro binding assays, and appears to mediate Rsp5 recruitment and the proper internalization of Can1 (Lin 2008). Cvs7 translocates to the plasma membrane in response to conditions which induce Can1 internalization, such as cell stress by cycloheximide (Lin 2008), a response that is dependent upon mono-ubiquitination by Rsp5 (Lin 2008). A computer-predicted, but as yet experimentally unconfirmed, structure for Cvs7 suggested the existence of a structural domain which is similar to the arrestin fold, present in the mammalian arrestin protein family (Lin 2008). These proteins, among other roles, function as adaptors for mammalian RING and HECT E3 ligases.

Other well known Rsp5 cofactors are Bul1 and its redundant paralog Bul2. A Rsp5-Bul1/2 complex, first detected through yeast two-hybrid (Y2H) screens and confirmed through co-immunoprecipitation experiments (Yashiroda 1998; Yashiroda 1996), has been implicated in the regulation of Rsp5-dependent ubiquitination at different steps in the vesicle trafficking cascade. The regulation of ubiquitin K63 chain extension by this complex has been demonstrated in the case of the amino acid permease Gap1, another well studied model membrane substrate. Bul1/2-mediated Rsp5-catalyzed poly-ubiquitination of Gap1 is important for correct sorting of newly synthesized Gap1 to the vacuole from the Golgi in the presence of a rich nitrogen source such as glutamate, as import of extracellular amino acids is no longer needed. Under these conditions, mutant cells with deletions in BUL1 and BUL2 exhibited decreased Gap1 poly-ubiquitination and increased sorting of Gap1 to the plasma membrane (Helliwell 2001). In addition to targeting poly-ubiquitination, Bul1/2 is important for the mono- or di-ubiquitination of Gap1 and its subsequent endocytosis at the plasma membrane, as deletions in genes encoding both of these proteins result in defective Gap1 clearance from the plasma membrane (Soetens 2001). Recently, Bul1 was found to contain an arrestin-like domain, and was implicated in the internalization step of Fur4 (Nikko 2009). Therefore, whether Bul1/2 function solely in the determination of Rsp5-catalyzed ubiquitin chain length or additionally as an Rsp5 adaptor remains to be elucidated.

Unlike the aforementioned Nedd4 ligases Itch and Nedd4-2, Rsp5 has not previously been reported to be ubiquitinated in vivo. While Rsp5 can auto-ubiquitinate under certain conditions in vitro (Huibregtse 1995), the physiological significance of this modification has not been demonstrated. In addition, at the start of my thesis project, the role of DUBs in modulating Rsp5-related functions was not studied in detail. Although DUBs such as Doa4 have been
implicated in the deubiquitination of Rsp5 substrates such as Fur4 at the MVB (Dupre 2001; Nikko 2007), the role of deubiquitination in regulating Rsp5 substrates was unclear. Therefore, whether deubiquitination serves to regulate Rsp5 substrates or the activity and levels of the ligase itself was largely unexplored when I started the work reported in Chapters 3 and 4.

1.5.3 Deubiquitinating enzymes (DUBs)

As mentioned previously, the cleavage of ubiquitin chains from ubiquitinated proteins is performed by a group of proteases termed the deubiquitinating enzymes. There are three general functions which DUBs are thought to perform in the cell (reviewed in Wilkinson 1997). First, DUBs play a basic role in ubiquitin maturation. In both yeast and mammalian cells, precursor ubiquitin moieties are produced as fusion proteins, as either linear fusions of multiple ubiquitins, or in association with ribosomal proteins. These precursors need to be post-translationally cleaved to maintain cellular Ub pools. A second, routine role of DUBs is in the recycling of ubiquitin from substrate proteins targeted and degraded by the 26S proteasome or vacuole. Cleavage by DUBs of poly-ubiquitin chains, either during, or post degradation serves to replenish cellular Ub stores. For example, in yeast, one of the functions of the DUB Doa4/Ubp4 is to cleave ubiquitin from proteins bound for degradation at the proteasome and at the vacuole, allowing released Ub to be reused (Swaminathan 1999). A deletion in DOA4 results in a cell-wide depletion in free ubiquitin levels, leading to a growth defect evident under certain conditions (Swaminathan 1999). Lastly, a subset of DUBs appear to reverse E3 ubiquitin ligase catalyzed attachment of ubiquitin, thereby potentially modifying the function and/or regulating the degradation of these target proteins. This latter function is the focus of this thesis, and is therefore reviewed in greater detail in the following sections.

1.5.3.1 Families of deubiquitinating enzymes

To date, five major groups of deubiquitinating enzymes have been characterized. As the roles of these DUBs have been increasingly studied, they have proven to provide an unanticipated and interesting regulatory aspect to the ubiquitin machinery. The first group of enzymes consists of the ubiquitin C-terminal hydrolases (UCH), which number four in humans
and only one in yeast (Yuh1). UCHs are able to remove small peptides and molecular adducts conjugated to ubiquitin, but are generally not able to remove ubiquitin from large proteins (Larsen 1998). Therefore, they are thought to participate in the “cleaning up” of small ubiquitin conjugates produced upon 26S proteolysis, thereby re-generating free ubiquitin. In general, however, the physiological roles and specific substrates, if any, of these DUBs, including yeast Yuh1, are largely unknown.

The second group of DUBs belongs to the ovarian tumour domain cysteine protease (OTU) family, whose members share homology to the Drosophila Ovarian Tumor gene. Their deubiquitination activity was identified by their ability to bind to the DUB inhibitor ubiquitin-aldehyde (Balakirev 2003). There are 14 OTUs in humans, but only 2 in yeast. Although some members of this group have shown an ability to cleave ubiquitin chains *in vitro* and *in vivo* (Balakirev 2003; Evans 2003), the role of and substrates of these enzymes in ubiquitin-related pathways has not been extensively characterized. A recent study has implicated the human OTU, OTUB1, in the deubiquitination of the estrogen receptor α transcription factor both *in vitro* and *in vivo* (Stanisic 2009). Over-expression of OTUB1 resulted in suppression of estrogen receptor-linked transcriptional activity (Stanisic 2009). However, considerable work remains to be done to characterize the physiological roles of the other OTU enzymes.

Two other groups of DUBs are the Josephin domain enzymes and JAB1/MPN/Mov34 metallo-enzymes (JAMM), each consisting of only a few annotated members to date. The best characterized member of the Josephin domain group is the human protein Ataxin-3, which preferentially cleaves K63-linked chains *in vitro* through an N-terminal Josephin domain (Winborn 2008), although the physiological significance of this activity is unknown. The yeast DUB Rpn11 is a member of the JAMM deubiquitinating enzyme group. Rpn11 is a highly conserved proteasome-lid associated enzyme, and as mentioned in above sections has been implicated in the deubiquitination of substrates at the proteasome (Guterman 2004). A zinc ion, bound to the catalytic site, is required for Rpn11 activity (Berndt 2002).

By far the largest and most highly characterized group of DUBs is the ubiquitin specific protease (USP) family. USPs are the most widespread group of DUBs evident across evolution, numbering over 50 members in human cells and 16 in budding yeast (Ubp1-16) (Reyes-Turcu 2009). Although USPs are very diverse in sequence and size, all possess conserved catalytic
domains termed the Cys and His boxes (reviewed in Wilkinson 1997). In addition to the catalytic core which does not require ATP for catalytic activity, the USP enzymes generally have large N- and C-terminal extensions. Located in these regions are other domains such as protein binding domains which are involved in substrate or co-factor interactions (reviewed in Wilkinson 1997). USPs are thought to generally cleave ubiquitin conjugated to protein substrates and large polypeptides, and are mostly predicted to be substrate specific (reviewed in Reyes-Turcu 2009). Since they are particularly relevant to my own work, I review the biological roles and molecular functions of select USPs, where known, as examples in detail below.

1.5.3.2 The functions of representative ubiquitin-specific proteases (USPs)

USPs have been implicated in many diverse cellular pathways such as those in development and diseases such as cancer. For example, in the fruit fly *D. melanogaster*, the USP Fat facets (Faf) plays an important role in the patterning of the developing compound eye. A substrate of Faf, Liquid facets (Lqf), was first uncovered through a genetic screen for enhancers of the mutant eye phenotype seen in flies carrying a Faf hypomorphic allele (Fischer 1997). Lqf is similar in sequence to epsin, a protein involved in endocytosis (Cadavid 2000). Hypomorphic alleles of genes critical for endocytosis were found to enhance the phenotypes resulting from hypomorphic alleles of both Lqf and Faf, implying that both these genes were involved in trafficking (Cadavid 2000). The endocytic roles of Faf and Lqf were confirmed in another study, in which these proteins were implicated in the endocytosis of the Delta ligand during Notch signalling, a highly conserved cell-cell signalling pathway important in metazoan development (Overstreet 2004). In addition, both Faf and Lqf were found to physically interact, with Faf stabilizing Lqf protein levels (Chen 2002b). Although a direct deubiquitination assay has not been done to determine if Faf deubiquitinates Lqf, higher molecular weight ubiquitinated forms of Lqf were stabilized in a Faf catalytic site mutant (Chen 2002b). In eye disc cells expressing Lqf and Faf hypomorphic alleles, Delta localizes in a pattern which was different to that of wildtype discs (Overstreet 2004). Although the mechanism of Lqf function is unclear, one working model is that Lqf engages Neur, an E3 ligase targeting Delta, thereby stimulating endocytosis, while Faf is able to deubiquitinate and stabilize Lqf, leading to efficient Delta internalization, signaling, and proper Drosophila eye development.
Other deubiquitinating enzymes have been linked to a human cancer syndrome, Von Hippel-Lindau (VHL) disease. The USP deubiquitinating enzymes VDU1 and VDU2 have been implicated in the disease through their physical association with the tumour suppressor protein VHL, a RING E3 ubiquitin ligase (Li 2002b; Li 2002c). VHL targets HIF-1α (hypoxia-inducible factor 1-α) (Li 2005), a protein involved in the regulation of hypoxia inducible genes which is frequently overexpressed in cancers (reviewed in Harris 2002). As shown via both in vitro and in vivo biochemical experiments, VHL physically interacts with and ubiquitinates VDU1 and VDU2, targeting them for proteasomal degradation (Li 2002b; Li 2002c). Mutations in VHL that prevent its interaction with these DUBs are present in many cases of VHL disease (Li 2002b). In addition, VDU2 deubiquitinates the VHL substrate HIF-1α in vitro (Li 2005). VDU2 has a stabilizing effect on HIF-1α, as HIF-1α protein levels increase upon over-expression of VDU2 in vivo (Li 2005). The interaction between VDU1/2 and VHL is one example, out of many, in which DUBs interact with, regulate, or are regulated by, an E3 ubiquitin ligase. In this case, the interaction of a USP with an E3 ligase results in the destabilization of a substrate, as the DUB is itself ubiquitinated and degraded rendering it unable to deubiquitinate and stabilize the substrate.

Another notable example, mentioned briefly before, is in the well characterized relationship between the USP Hausp and the E3 ligase Mdm2. As noted above, Mdm2 is involved in the regulation, through ubiquitination and proteasomal degradation, of the levels of the tumour suppressor protein p53 (reviewed in Michael 2003). In addition to ubiquitination, deubiquitination by Hausp also plays a role in the regulation of p53 levels. Hausp physically interacts with both p53 and Mdm2 both in an in vitro affinity pulldown assay and in an in vivo assay in which either tagged forms or endogenous proteins are studied (Li 2004; Li 2002a). Hausp also deubiquitinates p53 both in vivo and directly in vitro, as Hausp over-expression decreased the ubiquitinated p53 species, and increased unmodified p53 protein levels (Li 2002a). Interestingly, Hausp also deubiquitinates the ligase Mdm2 (Li 2004), which has been reported to be ubiquitinated and degraded (Fang 2000; Linares 2007). Deubiquitination of Mdm2 in vivo depends on Hausp, while a Hausp catalytic mutant is unable to deubiquitinate Mdm2 in vitro (Li 2004). Mdm2 becomes unstable in cells depleted for Hausp, which leads to an increase in p53 levels (Li 2004).

A series of follow up studies showed that, under normal growth conditions, Hausp binds to and deubiquitinates its preferred substrate Mdm2 (Hu 2006), increasing E3 activity and
leading to low levels of p53. However, when the cell is subjected to genotoxic stress, such as DNA damage, Mdm2 is destabilized immediately in a manner dependent on DNA-damage induced kinases; p53 is therefore stabilized to preserve genomic integrity (Stommel 2004). Interestingly, over-expression of Hausp does not restore Mdm2 levels after DNA damage because it is unable to bind to Mdm2, as shown by in vivo immunoprecipitation of Hausp (Meulmeester 2005). The binding of Mdm2 with HAUSP was found, in a later study, to be regulated by the death-domain-associated protein Daxx. Although the exact mechanism for this is not yet clear, some evidence indicate that DNA damage signaling dissociates Daxx (along with Hausp) from Mdm2 in a ATM kinase dependent manner (Tang 2006). Hausp, therefore, is free to exclusively bind with and directly stabilize p53, while at the same time, Mdm2 is auto-ubiquitinated and degraded. These events then lead to an increase in p53 protein levels in the cell, activating p53 dependent events such as apoptosis. This paradigm provides an interesting mechanistic model into the dynamic regulatory interplay between DUB and E3 activities.

1.5.3.3 Ubiquitin specific proteases in yeast

Based on sequence analysis of their catalytic domains, S. cerevisiae encodes 16 USP family deubiquitinating enzymes (Ubp1 through 16; Figure 1-5), most of which have been confirmed to have DUB activity when expressed in E. coli (Amerik 2000). Strikingly, these proteins form the largest family of enzymes in the yeast ubiquitin system (reviewed in Wilkinson 1997). However, little information was available concerning the substrates, interaction partners, or functions of this broad class of DUBs at the commencement of my thesis research. Deletion studies, however, had been performed on all 16 of the Ubps, and none was found to be essential for cell viability under standard growth conditions, possibly due to functional redundancy (Amerik 2000). Indeed, a ubp1Δ, ubp2Δ, ubp3Δ, ubp7Δ, ubp8Δ quintuple mutant did not show an aggravated growth defect phenotype (Amerik 2000).

The first well characterized Ubp was Doa4/Ubp4. Doa4 was originally identified genetically in a screen for factors regulating the degradation of the yeast mating type transcriptional repressor MATα2 (Papa 1993). Subsequently, Doa4 was found to have a
Figure 1-5: Ubiquitin specific proteases encoded in *S. cerevisiae*. By sequence analysis, there are 16 Ubps in yeast (Amerik 2000), which are named Ubp1 through 16. They all contain conserved Cys and His box sequences, which contain the residues needed for catalysis. These enzymes all have unique N- and C-terminal extensions, which are thought to be involved in their specificity in various cell processes.
generalized deubiquitination activity both in vivo and in vitro: when co-expressed in E. coli with a chimeric Ub-β-galactosidase reporter substrate, and in an in vitro deubiquitination assay with a K48 conjugated di-ubiquitin substrate (Papa 1993). The physiological role of Doa4 was then examined in various follow-up studies. For example, Doa4 was found to associate physically with the 26S proteasome (Papa 1999), where it was implicated in the removal of ubiquitin from substrates shortly before they undergo proteolysis (Swaminathan 1999). Shortly before the start of my thesis research, Doa4 was also found to be involved in the deubiquitination of membrane-bound substrates undergoing intracellular trafficking (Dupre 2001), as described in previous sections.

Other DUBs with characterized functions in yeast include Ubp3, Ubp8 and Ubp10. Both Ubp8 and Ubp10 have been implicated in the targeted deubiquitination of histones, specifically histone H2B (Emre 2005; Henry 2003). Ubp8, in addition, was characterized as an important component of the SAGA histone acetyltransferase complex (Henry 2003), establishing that transcription elongation by RNA polymerase II is regulated by coupled patterns of histone ubiquitination and acetylation. More relevant to my thesis, Ubp3 has been implicated in intracellular trafficking. A null mutant lacking UBP3 displays a partial defect in the trafficking of proteins from the ER to the Golgi, which is linked to the deubiquitination of Sec23, a general transport machinery component (Cohen 2003a). Deubiquitination is important for trafficking as mono-ubiquitinated Sec23 is unable to engage with other members of the transport machinery, and is eventually poly-ubiquitinated and degraded (Cohen 2003a). Ubp3 mutants are also impaired in cytoplasm to vacuole (Cvt) sorting of vacuolar lumen proteins (Baxter 2005). One component of this pathway, Atg19, appears to be deubiquitinated in vivo by Ubp3 (Baxter 2005). Although the exact role of the ubiquitination and deubiquitination of Atg19 in this pathway is still unclear, cells lacking Ubp3 have a defect in the maturation of a vacuolar protease, implying a defect in Cvt transport (Baxter 2005). Intriguingly, Ubp3 and its cofactor Bre5 was also implicated in a role in the reverse pathway of retrograde trafficking from the Golgi to the ER (Cohen 2003b). Most recently, through a genetic screen, Ubp3 was implicated in ribophagy, a process by which mature ribosomes are targeted to the vacuole for degradation upon nutrient starvation (Kraft 2008). Cells lacking Ubp3 have a defect in the accumulation of ribosomal subunits in the vacuole (Kraft 2008). Upon nutrient starvation, ubp3Δ cells show increased levels of the 60S ribosome compared to wildtype, and ribosome subunits were found to be
ubiquitinated, implying a novel role for reversible ubiquitination as a signal for ribophagy (Kraft 2008).

1.6 Thesis rationale and outline

The focus of this thesis is an examination of the biological roles and molecular functions of the Ubp group of proteins of S. cerevisiae using a combination of proteomic, biochemical and molecular genetics approaches.

In Chapter 2, I describe my contributions to a collaborative large-scale proteomic project aimed at purifying and characterizing all stable protein complexes from yeast. I present data showing successful affinity-tagging, purification and identification by mass spectrometry of putative protein interactors for 9 Ubps, which linked several previously unstudied DUBs to proteins with well characterized functions. In the second half of this Chapter, I present data focusing on the characterization of Ubp6 in association with the 19S proteasome regulatory complex, and describe follow-up data linking this enzyme, together with another novel 19S component, to conserved DNA damage repair pathways.

In Chapter 3, I present data showing the physical and functional interactions of Ubp2 with the HECT ubiquitin E3 ligase Rsp5 and a (ubiquitin-associated) UBA domain containing protein which I discovered and termed Rup1 (Rsp5 Ubp2 interacting protein 1). I show that UBP2 interacts genetically with RSP5, while Rup1 facilitates the tethering of Ubp2 to Rsp5 via a PPSPSY motif. I also show that Ubp2 has a previously overlooked role in the regulation of Rsp5 mediated protein trafficking, using the uracil permease Fur4 as a model reporter system. Similar to hypomorphic rsp5 alleles, cells deleted for UBP2 exhibited a temporal stabilization of Fur4 at the plasma membrane which was ubiquitin dependent, indicative of perturbed protein trafficking. Moreover, the defect was bypassed in conditions where recycling was absent, implicating Ubp2 in sorting at the multivesicular body.

In Chapter 4, I expand on the direct regulatory relationship between Ubp2 and Rsp5. In order to understand the mechanism of the Rsp5-dependent role of Ubp2, I looked for a possible presence of Rsp5 auto-ubiquitination. I found that, Rsp5, similar to its mammalian homologues,
is auto-ubiquitinated \textit{in vivo}, and that the presence of a substrate or an interacting co-factor such as Rup1 resulted in increased auto-ubiquitination, implying an auto-inhibitory mechanism of Rsp5 regulation common in other E3 ligases. Furthermore, I found that Ubp2 was able to deubiquitinate Rsp5 \textit{in vitro}, implicating Ubp2 in the regulation of Rsp5 and in the modulation of Rsp5-dependent processes. I end with a putative model describing a novel mode of regulation of Rsp5 that may mirror the regulation of its mammalian counterparts and in Chapter 5 I propose a mechanistic model that integrates all of the available data.

In the Appendix, I describe research avenues that I think would be particularly productive for further investigation, including a novel screen for substrates of deubiquitinating enzymes that I have devised, along with some genetic interaction data that implicate Ubp2 and Rup1 in other important functions in the cell.

The data presented in this thesis provide evidence for the functions of a small subset of the Ubps, and my findings implicate deubiquitination as important regulatory counterpart in diverse cellular pathways to the better studied process of ubiquitination.
Chapter 2

Proteomic Characterization of Deubiquitinating Enzyme Protein-Protein Interactions in S. cerevisiae, and Functional Assessment of a Novel Ubp6-Sem1-19S Proteasome Complex involved in DNA Repair

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In Figure 2-3, A. Emili supervised the experimentation, and I performed all experiments and analyses. In Figures 2-4 to 2-6, A. Emili and J. Greenblatt supervised the experimentation, and I, along with a senior graduate student from the Greenblatt lab, N. Krogan, performed all relevant experiments and analysis with assistance from other co-authors as listed in (Krogan 2006). In Figures 2-7 to 2-10, A. Emili and J. Greenblatt supervised the experimentation. I performed all experimentation and analysis in these figures, apart from some tests for genetic interactions in Figure 2-9 and the lower panel of Figure 2-10 which was performed by N. Krogan, with the construction of some yeast mutant strains performed by me with technical assistance from A. Lam and M. Raghavan under my supervision.
2. Proteomic Characterization of Deubiquitinating Enzyme Protein-Protein Interactions in S. cerevisiae, and Functional Assessment of a Novel Ubp6-Sem1-19S Proteasome Complex involved in DNA Repair

2.1 Introduction

In the last decade, proteomics has emerged as an important tool to study the molecular components underlying diverse cellular processes. Various types of physical interaction studies, including the identification of protein complexes through the systematic purification of affinity tagged proteins (Gavin 2002; Ho 2002), large-scale yeast two hybrid assays (Ito 2001; Uetz 2000), and protein microarray assays (Gupta 2007), have been helpful in the characterization of binding partners, and hence probable functional roles, of many proteins of previously unknown function. The budding yeast Saccharomyces cerevisiae has served as a popular organism for large scale proteomic surveys. It is an excellent model in which to perform genome-wide interaction mapping, as the genome is fairly easy to manipulate genetically (e.g. targeted integration of ectopic tagging sequences in haploid and diploid states), as large cell volumes can be grown effortlessly in standard culture conditions, and as it shares many functional attributes and gene homologues with other species including mammalian species.

The elucidation of protein-protein interactions is often an informative way of investigating the role of a protein, as the interaction of a protein with ‘prey’ proteins of known function can give clues as to the function of the ‘bait’ protein. The tandem affinity purification (TAP) method (Rigaut 1999) is an ideal technique for the isolation of stable protein complexes. In this method, a PCR cassette encoding the dual affinity TAP tag and a selectable marker is integrated via homologous recombination at the endogenous yeast locus of the corresponding target gene, resulting in a TAP fusion protein containing the tag at the carboxy-terminus which is expressed at near-endogenous levels. Avoidance of overexpression (e.g. via use of the native regulatory context rather than a heterologous promoter) results in the isolation and identification of protein complexes under conditions that are more physiologically relevant. In addition, compared with a simple immunoprecipitation, the two-step TAP purification protocol itself results in highly purified protein preparations largely free of non-specific contaminants. Although TAP tagging is an excellent technique for finding protein-protein interactions, it has a
few limitations. It is possible, as the tag is fused to the protein, that the tag itself may interfere with the function or physical interactions of the protein, by impeding the natural folding of the protein or obscuring a function or region in the fused region. In addition, the tagged protein itself may not be expressed at suitable levels, or the tag may not be exposed at the protein surface to permit purification. Therefore, it is essential that any putative protein-protein interactions are confirmed through other biochemical or molecular biological methods, or through reciprocal tagging of a prey protein.

The ubiquitin-proteasome pathway has a crucial role in the control of most major eukaryotic cell functions. Covalent modification of proteins with ubiquitin is important for the proper execution of various pathways regulating the abundance, half-lives and activity of diverse proteins, the trafficking of proteins between different intracellular compartments, and the physical interactions between different proteins to form macromolecular complexes. A critically important and well characterized fate of ubiquitinated proteins is degradation by the 26S proteasome complex. Ubiquitinated protein substrates, generally bearing K48-linked poly-ubiquitin chains of at least four coupled ubiquitin moieties (Thrower 2000), are specifically recognized and recruited to the proteasome for unfolding and rapid proteolysis. The proteasome in turn consists of two major sub-structures, the 20S proteolytic core, and the 19S regulatory cap (reviewed in Finley 2009). The 20S contains the proteolytic sites of the proteasome, while the 19S cap serves a mostly regulatory role. In budding yeast, the 19S consists of a lid complex (Rpn3, 5-9, 11, 12) and a base complex (Rpt1-6, Rpn1, Rpn2, Rpn10, Rpn13), which have demonstrated roles in the recognition, binding, unfolding, and translocation of ubiquitinated proteins into the 20S (reviewed in Finley 2009).

Deubiquitination has emerged as an equally important regulator of ubiquitin regulated cellular processes. For example, deubiquitinating activity is associated with the 19S particle. The Zn-dependent metalloprotease Rpn11 (Guterman 2004), and Ubp6 (Guterman 2004) are both important in ubiquitin recycling through the cleavage of ubiquitin chains from newly recruited substrates immediately prior to substrate degradation. Deubiquitination, at least by Rpn11, is important for the translocation of substrates into the 20S proteasome (Verma 2002). However, a role for other DUBs in this process was unclear at the time I commenced my studies.
By sequence analysis, yeast encodes 16 DUBs within the ubiquitin-specific protease family (reviewed in Wilkinson 1997), only two of which (Ubp6 and Doa4/Ubp4) were previously shown to have proteasome-associated roles in the turnover of certain substrates (Guterman 2004; Swaminathan 1999). Alternative functions have been identified for several other Ubps (reviewed in Wilkinson 1997). This includes post-translational cleavage of newly synthesized ubiquitin precursor polypeptides, and the partial or complete reversal of ubiquitination on select substrates prior to degradation, resulting in a change in the stability and cellular fate of proteins.

The regulation of substrate fate by the Ubps represented a particularly exciting avenue for research when I commenced my thesis research, since relatively little was known regarding the non-proteasomal associations of Ubps in yeast, or the regulatory functions of their orthologues in other organisms. At the start of my thesis studies, the functions of most of the Ubps, and DUBs in general, were uncharacterized, as relatively limited functional studies had been performed on this class of enzymes. What was apparent was that certain DUBs were likely involved in crucial regulatory decisions in various processes, ranging from roles in development (Cadavid 2000), to proper execution of human disease-linked pathways (Li 2002a; Li 2002b; Li 2002c). Particularly noteworthy studies had implicated Doa4 in the deubiquitination of internalized membrane cargo proteins at the multivesicular body (Dupre 2001; Nikko 2007; Springael 1999b). However, the roles for many of the other Ubps remained a mystery at the start of my thesis work.

2.1.1  Project rationale

As the ubiquitination of proteins has been shown to serve as an important regulatory switch for many pathways, the reverse process, deubiquitination, provides an additional and potentially equally important means of regulating cellular protein stability and activity. Although this has been shown as the case for certain DUBs in mammalian systems (Chen 2002a; Li 2002a; Li 2002b; Li 2002c), studies on the yeast Ubps had been limited when I joined the Emili laboratory. More specifically, even though the Ubp group of DUBs make up the largest family of enzymes annotated in the ubiquitin system in budding yeast, the functions and molecular binding partners of very few of these proteins had been elucidated.
The study of protein-protein interactions has proven to be extremely useful in the study of protein function and regulation. The roles of many gene products with previously unknown biological roles have been illuminated by the identification of specific linkages of these proteins to other proteins whose functions were already well characterized. As our group, in collaboration with the laboratory of Jack Greenblatt at the University of Toronto, had developed a productive experimental pipeline and had embarked on a large scale proteomic assessment of protein complexes isolated using a sensitive and reliable protein affinity purification and mass spectrometry (APMS) screening procedure (Krogan 2001; Krogan 2002), I proceeded with a comprehensive proteomics investigation of each of the deubiquitinating enzymes in the budding yeast *S. cerevisiae*. In the first section of this Chapter, I present the results of our systematic mapping of putative physical interaction networks associated with 9 of the 16 Ubps (Krogan 2006). Of particular interest, and as outlined in detail below, we identified many previously unknown protein-protein interactors, many of which participate in well characterized and conserved cellular processes and pathways. Since our identification of these physical interactions, the functional significance of many of these protein interactions and Ubp complexes has been confirmed either by myself (Krogan 2004; Lam 2009), or by other groups (Cohen 2003a; Henry 2003).

In the second half of the Chapter, I show both biochemical and genetic data from a focused study on the interactors of Ubp6, which led to the discovery of a small, novel, and conserved subunit of the 19S proteasome, Sem1. I also present follow-up experiments which showed that Sem1, Ubp6, and the proteasome are directly involved in the DNA damage repair response in yeast.

Collectively, this work shows that many of the Ubps are likely involved in important cell functions, and that proteomic investigations of their protein-protein interactions could lead to informative mechanistic insights and hypotheses regarding DUB functions.
2.2 Materials and methods

2.2.1 Strains, transformations, and growth media

Yeast strains are of the BY4741 (MATa \textit{his3Δ1 leu2Δ0 ura3Δ0 met15Δ0}) background unless otherwise indicated. All transformations of yeast strains were done using a standard protocol (Gietz 1992; Soni 1993), and strains were grown in YPD (yeast extract, peptone, and 2% glucose) at 30°C unless otherwise specified.

2.2.2 Construction of TAP-tagged strains

TAP tagged strains were as described in (Krogan 2006), with a HIS selection marker, or constructed with a TRP selection marker as described in (Puig 2001). The TAP-TRP cassette was amplified by PCR from the plasmid pBS1479 with primer sequences complementary to base pairs immediately upstream of each \textit{UBP} ORF stop codon and sequences with homology immediately downstream of the stop codon (Figure 2-2). The sizes of the PCR products were first checked by gel electrophoresis, and the PCR products then transformed into the tryptophan auxotroph haploid W303 strain (\textit{MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100}) of \textit{S. cerevisiae} and integrated into a haploid auxotrophic cell by homologous recombination. Expression of the tagged protein in transformants was confirmed by Western blotting against the TAP tag.

2.2.3 Cell Lysis, minigel electrophoresis and Western blotting

Cells were harvested and lysed by glass bead beating in YEB (Yeast Extraction Buffer: 245mM KCl, 1mM EDTA, 5mM EGTA, 50mM Hepes-KOH pH 8.0, 10% glycerol, 2mM DTT) containing an EDTA-free protease inhibitor cocktail (Complete; Roche) and centrifuged at 14 krpm at 4°C for 10 minutes to pellet insoluble debris. Approximately 6mg of total soluble protein extract (~10mg/ml) was then incubated for 1 hour at 4°C with IgG Sepharose 6 Fast Flow beads (Amersham Pharmacia). The beads were washed extensively, and tightly bound proteins were eluted with 15ul of SDS sample buffer by heating at 90°C for 5 minutes. The samples were separated by electrophoresis on a 4-12% Bis-Tris polyacrylamide gel (Invitrogen), and the
proteins transferred from the gel onto nitrocellulose membrane (Trans-Blot Transfer Medium; BioRad). The membranes were then extensively pre-blocked with a 5% milk protein solution, followed by overnight incubation with anti-TAP antibody. Detection was performed by ECL (SuperSignal West Pico, Pierce) using suitable secondary antibodies conjugated to horseradish peroxidase.

2.2.4  

*TAP tag purifications*

Large scale late log-phase cell cultures were grown in YPD medium at 30°C to an OD_{600} of 1.0-1.5. Cells were pelleted at 4100 rpm, and washed with chilled ddH_{2}O. The pellet was frozen at −80°C in a 50ml falcon tube until needed. Proteins were extracted and purified as described in (Krogan 2002). The frozen pellet was ground along with pellets of dry ice in a prechilled coffee grinder until powder-like. The powder was put into a beaker, and an equal volume of YEB added and the mixture was mixed and thawed. The cell extract was then centrifuged at 34,000 rpm at 4°C for 2 hours to pellet the insoluble cell debris. Soluble protein extract was then dialyzed for 1 hour at 4°C against 2L of IPP buffer (10mM Tris pH 7.9, 100mM NaCl, 0.1% Triton X-100, 0.5mM DTT, 0.2mM EDTA, and 20% glycerol). Protein extracts were then incubated with IgG Sepharose 6 Fast Flow beads (Amersham Pharmacia) for 3 hours at 4°C for binding to the protein-A portion of the TAP-tag. After incubation, the beads were briefly centrifuged and washed with IPP buffer, followed by TEV protease cleavage buffer (50mM Tris-Cl pH 7.9, 1mM DTT, 0.1% Triton X-100, and 100mM NaCl). The bound proteins were then eluted from the IgG beads by overnight incubation at 4°C with 100U of recombinant Tobacco Etch Virus protease (TEV; Invitrogen) in TEV protease cleavage buffer. The next day, the eluate was removed, and incubated with calmodulin beads (Amersham Pharmacia) in calmodulin binding buffer (10mM Tris-Cl pH 7.9, 10mM β-mercaptoethanol, 2mM CaCl_{2}, 0.1% Triton X-100, 100mM NaCl) for 1 hour at 4°C. The beads were washed with calmodulin binding buffer and calmodulin wash buffer (10mM Tris-Cl pH 7.9, 10mM β-mercaptoethanol, 0.1mM CaCl_{2}, 0.1% Triton X-100, 100mM NaCl) and the bound proteins were then eluted with calmodulin elution buffer (10mM Tris pH 7.9, 10mM β-mercaptoethanol, 3mM EGTA pH 8.0, 0.1% Triton X-100, 100mM NaCl) for 1 hour. Purified proteins were separated on 10% polyacrylamide gels, and silver stained using a standard protocol. Subsequent analysis of the
mixtures of purified proteins by both gel-free tandem mass spectrometry and gel-based MALDI-TOF mass spectrometry were performed essentially as described in (Krogan 2006).

2.2.5 Construction of double mutant strains

Heterozygous diploid strains containing two deletion mutations were generated using SGA technology as previously described (Tong 2001), or by manual mating of single deletion strains, followed by sporulation and tetrad dissection.

2.2.6 Serial dilutions

Strains were grown overnight to saturation in YPD, and diluted with sterile water to OD$_{600} = 0.5$. 5- or 10-fold dilutions with sterile water were then made, and spotted onto media plates containing YPD plus additional drugs as indicated. Plates were incubated for 2-3 days at 30°C and then imaged.

2.3 Results

2.3.1 Identification of protein-protein interactions for certain members of the yeast Ubp group of deubiquitinating enzymes

As a part of a collaborative proteome wide study on yeast protein-protein interactions (Krogan 2006), I attempted to TAP tag all 16 Ubp proteins (Figure 2-1), of which 12 were successfully tagged. First, the TAP cassette, along with a nutritional selective prototrophic marker (TRP or HIS) was amplified by PCR from a plasmid, and the cassette was transformed and integrated into a haploid auxotrophic cell by site-specific recombination (Figure 2-2A). Small (50ml) cultures were grown of individual clones for each of the transformants, and the expression of the tagged proteins in the strains was confirmed by Western blotting using an IgG antibody recognizing the protein A moiety of the fusion tag (Figure 2-3). Successfully tagged clones were then grown in large scale culture in rich media overnight, lysed, and subjected to the complete TAP purification protocol (Figure 2-2B). This consisted first, of the binding of the
Figure 2-1: Workflow for TAP analysis of yeast Ubp proteins. The TAP cassette, amplified by PCR using primers targeting each of the 16 Ubps in S. cerevisiae, was transformed into a haploid yeast strain. After confirmation of tagged protein expression by Western blotting, tagged Ubps (and their stably interacting protein partners) were purified by the TAP method. After large-scale purification, one portion of the samples was run on a SDS-PAGE gel and silver stained, with visible polypeptide bands excised, digested in-gel with trypsin, and the peptides subjected to mass fingerprinting by MALDI-ToF mass spectrometry, while another portion was digested with trypsin in solution and analyzed by gel-free tandem mass spectrometry.
**Figure 2-2: Tandem affinity purification (TAP).** (A) A cassette encoding the dual affinity TAP (protein A-TEV-calmodulin binding peptide) tag along with a nutritional selection marker (TAP-TRP1; Rigaut 1999) is integrated into the yeast genome by homologous recombination after PCR amplification with targeted primers. The cassette replaces the endogenous stop codon of the gene, resulting in a C-terminal protein-tag fusion protein. (B) The two-step TAP purification procedure. After cell lysis, the protein A portion of the TAP tagged bait protein is first immunoprecipitated using IgG agarose beads. Bound proteins are released with the addition of TEV protease, which cleaves at the specific TEV cleavage site between the dual affinity tags. A second round of binding follows, in which the calmodulin binding peptide (CBP) portion of the tag is captured with calmodulin agarose beads. The tagged bait protein, along with any stably bound interacting proteins, is then released with the addition of EGTA, which chelates calcium needed for calmodulin binding. Proteins are subsequently visualized by SDS-PAGE and identified by mass spectrometry.
Figure 2-3: Western blot confirming the successful tagging and expression of certain TAP tagged Ubp strains. Cultured yeast cells were harvested and lysed, and protein lysates incubated with IgG agarose beads. The beads were washed, boiled in SDS sample buffer, and electrophoresed on a 4-12% polyacrylamide gel. After transfer onto a nitrocellulose membrane, the TAP tagged proteins were visualized with an anti-TAP antibody, which binds to the protein A portion of the TAP tag. The identity of the bait proteins (*) is indicated on top, while the mobility of reference molecular weight markers is shown at the left.
tagged Ubp fusion bait protein and any interacting proteins to IgG agarose beads, which bound to the protein A portion of the TAP tag. The bound proteins were then eluted from the beads by cleavage by the recombinant Tobacco Etch Virus (TEV) protease which recognizes a unique cleavage site embedded between the two tags. The eluate from this reaction was then incubated with calmodulin beads, which bound to the calmodulin binding peptide on the TAP tag. Proteins bound to the calmodulin beads were eluted by incubation with EGTA, which chelates the Ca\(^{2+}\) ions needed for calmodulin binding, thereby releasing the protein complex. A portion of the eluate was analyzed by SDS-PAGE followed by silver staining to visualize interacting proteins, which were then identified through two complementary mass spectrometry procedures: (1) peptide mass fingerprinting using MALDI-ToF mass spectrometry of in-gel digested polypeptide bands after SDS-PAGE and silver staining; and (2) direct sequence analysis of an in solution peptide tryptic digest of a second portion of the purified protein preparation by tandem mass spectrometry (gel-free LC-MS/MS).

A protein interaction diagram (Figure 2-4) illustrates all the high scoring interactors of the Ubps reproducibly identified in this study. Some of the Ubps (Ubp4, 9, 11, 16) are not on the diagram, as they were never successfully purified. This may be due to a failure in the expression of these proteins under the rich media growth conditions. Other Ubps (Ubp1, 5, 12) were only purified as bait alone, and did not show any high scoring interacting proteins, perhaps due to unstable or transient interactions with their interacting proteins, or low abundance prey proteins that were not detected. Examination of these protein-protein interaction networks allowed me to draw two main conclusions. First, Ubps (at least the ones in the subset that was successfully purified) do not physically interact with each other. Second, the Ubps do not seem to share any common interactors. This strongly suggested that the Ubps are involved in different, non-redundant functions in the cell, and seemingly do not cooperate as stable heterodimers.

Many of the Ubps in the purified subset co-purified with many interesting interactors. For example Ubp8, which at the time did not have a characterized function, was found to interact reproducibly with nine protein components of the SAGA acetyltransferase complex, an observation that has since been validated by several other groups showing that Ubp8 is involved in histone deubiquitination (Henry 2003). The co-purification of Ubp3 with its cofactor Bre5 has also been extensively evaluated by other labs, which confirmed the interaction through co-
Figure 2-4: High confidence protein-protein interaction networks of the Ubps. Diagram includes all reproducible high scoring putative Ubp binding proteins identified through mass spectrometry (Krogan 2006). Common contaminants associated with a large percentage of TAP purifications were deleted prior to visualization of the dataset using Osprey. Edges indicate protein-protein interactions (two arrows indicate a reciprocal interaction, directed arrows point from bait proteins, and looped edges indicate recovery and identification of the TAP-tagged bait). Ubps are highlighted in blue, and the remaining node colors indicate functional annotations. No high confidence binding partners were identified for Ubp1, Ubp5, or Ubp12, while Ubp4, 9, 11, and 16 were not successfully tagged or purified.
immunoprecipitation assays and have established a physiological role for this complex in the
deubiquitination of crucial components of trafficking pathways, including ER to Golgi transport
and the cytoplasm to vacuole trafficking pathway (Baxter 2005; Cohen 2003a). Of particular
interest to me was the observation that Ubp2, previously of unknown function, stably interacted
with the well characterized E3 ubiquitin ligase Rsp5, and a protein of unknown function,
YOR138c. As mammalian E3s were just then being reported as being regulated by DUBs (Li
2004; Li 2002b), this heterotrimeric complex was selected as a major focus of my thesis work,
and my efforts in characterizing the role of these proteins are discussed in the following chapters
(Chapters 3 and 4). However, my initial validation studies were centered on elaborating a novel
(at the time) complex consisting of Ubp6 in association with 19S proteasome and (another) new
component Sem1.

2.3.2 The Ubp2-Rsp5-Rup1 Complex

TAP-tag purification of Ubp2 revealed two proteins which reproducibly and specifically
copurify with Ubp2: the E3 ubiquitin ligase Rsp5, and an ubiquitin associated (UBA) domain
containing protein of previously unknown function, YOR138c, which I have named Rup1 (Rsp5-
Ubp2 interacting Protein 1) (Figure 2-5). Neither of these binding partners was detected in
parallel purifications from an untagged negative control strain or from several hundred other
TAP-tagged bait proteins (Krogan 2006). To further validate these results, I performed reciprocal
affinity purification using a strain expressing endogenous TAP-tagged Rup1 (a viable C-terminal
Rsp5-TAP strain could not be generated). Rup1-TAP co-purified with a seemingly identical
amount of Ubp2 but with a sub-stoichiometric level of Rsp5 (Figure 2-5). As Rsp5 is a relatively
well characterized enzyme with well known functions, specifically in intracellular protein
trafficking, I decided to proceed with follow-up studies on this complex, which I describe in
Chapters 3 and 4.
Figure 2-5: Ubp2 physically interacts with Rsp5 and Rup1. Silver-stained SDS polyacrylamide gel showing affinity purified Ubp2-TAP and Rup1-TAP. Arrows indicate proteins identified by gel band excision followed by MALDI-ToF mass spectrometry. Asterisks indicate degradation products of Ubp2 and Rup1.
2.3.3 The Ubp6-Sem1-19S complex

TAP-tagging of Ubp6 revealed components of the 19S proteasome as interacting proteins (Figure 2-6A). This confirmed previous data from other studies which characterized Ubp6 as a putative 19S subunit interactor in affinity purification assays (Verma 2000). All 19 of the previously annotated subunits of the 19S proteasome (Rpns 1-3 and 5-13, Rpts 1-6 and Ubp6) were identified with high statistical confidence. Interestingly, we also noticed a small (10 kDa, 89 amino acids) protein, Sem1, which reproducibly copurified with Ubp6 and other TAP-tagged components of the 19S lid and base subcomplexes. The identification of Sem1 as a novel subunit of a Ubp6-19S proteasome complex was only made through gel-free tandem mass spectrometry, as its small size resulted in it running off the gel during electrophoresis, and hence it failed to be detected by MALDI-ToF MS. Tandem affinity tagged Sem1 likewise co-purified with Ubp6 and all the known subunits of the 19S complex (Figure 2-6B). The finding that Sem1, like Ubp6, was a novel 19S interacting protein was confirmed in parallel studies published by two other laboratories at the same time (Funakoshi 2004; Sone 2004). The non-essential SEM1 gene was originally identified as a multi-copy suppressor of mutations affecting the secretory pathway (suppressor of exocyst mutations 1; (Jantti 1999)). However, a Sem1 GFP fusion protein is localized exclusively to the nucleus (Huh 2003), implying that its effect on exocytosis was likely indirect via perturbation of the proteasome.

2.3.4 UBP6 and SEM1 are functionally involved in the 19S proteasome

To test whether SEM1 and UBP6 behave genetically as proteasome components, I constructed double deletion mutants of these genes along with other non-essential 19S components. Following sporulation and tetrad dissection of heterozygous double-mutant diploid strains, I found that sem1Δ and ubp6Δ resulted in a synthetic growth defect when combined together or with a deletion of rpn10Δ (Figure 2-7), further implicating Sem1 and Ubp6 in the normal functioning of the 19S proteasome in vivo. As the 26S proteasome is a complex essential for cell function, the deletion of two of its non-essential members may have destabilized the complex as a whole or decreased proteasome activity, thereby resulting in a growth defect in these double mutant cells.
Figure 2-6: Ubp6 and Sem1 are stably associated with the 19S proteasome. (A) Silver-stained SDS polyacrylamide gel showing affinity purified preparations of Ubp6-TAP and Sem1-TAP. Sem1 ran off the bottom of the gel, and was only identified by gel-free LC-MS/MS. (B) Summary schematic showing the proteins identified in each of the TAP-tag purifications of the eleven putative 19S subunits. A gray box represents a protein identified in the purified material by either MALDI-ToF MS or LC-MS/MS.
Figure 2-7: *SEM1* genetically interacts with *UBP6* and other components of the 19S proteasome. Tetrad analysis of *sem1Δubp6Δ* and *sem1Δrpn10Δ* double mutants on synthetic complete medium. After mating of haploid single mutants, heterozygous double mutants were sporulated and the resulting tetrads manually dissected.
2.3.5  

A role for Ubp6, Sem1, and the 19S in yeast DNA damage repair

Identified shortly before my studies began as a component of the 19S proteasome subunit (Verma 2000), Ubp6 was thought to be involved in the cleavage and recycling of ubiquitin chains prior to the degradation of proteins by the proteasome (Leggett 2002). Sem1 was not as well characterized in yeast, but its human homologue, Dss1, originally identified as a putative candidate gene for the split hand/split foot disorder (Crackower 1996), had been shown to interact with the breast cancer related tumour suppressor protein BRCA2 (Marston 1999). BRCA2 has been implicated as having an important function in mammalian DNA double-strand break (DSB) repair through its interaction with Rad51, a key component of the homologous recombination DNA repair machinery (Marmorstein 1998). A crystal structure analysis of the BRCA2-Dss1-DNA complex revealed that highly conserved residues in Dss1 contact BRCA2, while the residues in BRCA2 that interface with Dss1 are frequently mutated in certain cancers (Yang 2002). Hence, Dss1 appeared to be functionally linked to BRCA2 DNA repair activity, and its homologue Sem1 may also participate in DNA damage repair in the yeast cell.

DNA double-strand breaks are formed occasionally during DNA replication and more frequently when cells are exposed to ionizing radiation or certain other DNA damaging agents. Eukaryotic cells repair DSBs primarily by two genetically separable pathways: non-homologous end joining (NHEJ) and homologous recombination (HR) (reviewed in Paques 1999). In S. cerevisiae, HR utilizes extensive homology (up to hundreds of base pairs) to faithfully restore the sequence at a break site by processes that involve components of the Rad52 epistasis group, including Rad51, Rad52 and Rad54. In contrast, NHEJ directly rejoins the two ends of the DNA molecule at the site of the DNA DSB without any requirement for homologous DNA sequences, and without necessarily restoring the sequence around the DSB. In yeast, the NHEJ pathway involves the Yku70-Yku80, Rad50-Mre11-Xrs2, and Dnl4-Lif1 protein complexes.

The physical interaction of the 19S, Ubp6 and Sem1 in yeast suggested that these proteins may participate in the DNA damage response. To test for a possible connection to DNA repair, I serially diluted deletion strains lacking UBP6 or SEM1 and assayed them for growth after spotting on to media containing the DNA damaging agents methylmethane sulfonate (MMS), which methylates DNA and is a known inhibitor of cells defective in certain repair pathways (Chang 2002), or hydroxyurea (HU), an inhibitor of dNTP synthesis causing DNA
replication fork collapse (Rittberg 1989). Both *ubp6Δ* and *sem1Δ* strains showed hypersensitivity to both genotoxins, especially *ubp6Δ*, which showed a significant growth defect on MMS (Figure 2-8).

To further investigate the role of Ubp6 and Sem1 and the 19S proteasome in DNA damage repair, deletions of these genes were made in combination with some genes known to be involved in DSB repair. Single haploid deletion strains were mated, and growth defects in the double mutant analyzed after tetrad dissection. Many of these mutants had synthetic growth defects. In particular, mutations in 19S components were found to cause synthetic growth defects when combined with deletions from the *RAD52* epistasis group (*RAD51*, *RAD52*, *RAD54* and *RAD55*), involved exclusively in DSB repair via HR, as well as with deletions of genes (*RAD50* and *XRS2*), encoding proteins involved both HR and NHEJ (Figure 2-9).

To determine more directly whether the 19S proteasome is involved in the repair of DNA DSBs, we examined the sensitivity of additional yeast deletion strains to hydroxyurea, MMS, and bleomycin, which generates free radicals that induce DNA lesions similar to those caused by ionizing radiation (Aouida 2004). Deletion of genes involved in DSB repair pathways confer hypersensitivity to these compounds (Aouida 2004; Chang 2002; Parsons 2004). Although strains containing single *sem1Δ*, *rpn4Δ*, *rpn10Δ* or *ubp6Δ* deletions displayed little or no added sensitivity, double mutants combining deletions in these genes exhibited marked hyper-sensitivity to all three agents (Figure 2-10). These observations further implicate the 19S proteasome complex containing Sem1 and Ubp6 in the repair of DNA DSBs.

Since components of the 19S proteasome, including *SEM1* and *UBP6*, interact genetically with genes involved in both the HR and NHEJ repair pathways, it is possible that these genes act in both systems. Consistent with this, 7 (out of 12) of the yeast 19S proteasome subunits, including Ubp6, co-purified with small amounts of DNA polymerase IV (Pol4) as judged by LC-MS/MS (Figure 2-6B). Pol4 has been linked to DSB repair via NHEJ through its interaction with the Dnl4-Lif1 complex (Tseng 2002).
Figure 2-8: *UBP6* and *SEM1* mutants are hypersensitive to DNA damaging agents. Serial dilutions of wildtype (WT), *sem1Δ*, and *ubp6Δ* strains were spotted onto YPD medium lacking (no drug) or containing the DNA damaging agents methylmethane sulfonate (MMS, 0.03%) or hydroxyurea (HU, 100mM). Strains were grown at 30°C and imaged.
**Figure 2-9: Network diagram of genetic interactions.** Deletion mutations of *UBP6, SEM1*, the non-essential 19S proteasome subunit *RPN10*, and the proteasome-related transcription factor *RPN4* show aggravating genetic interactions when combined with mutations in DNA repair factors involved in homologous recombination (*RAD51, 52, 54, 55*) and in both homologous recombination and non-homologous end joining (*RAD50* and *XRS2*). A line connecting two genes indicates a synthetic growth defect (slow growth) in the double mutant.
Figure 2-10: Double mutants in *UBP6*, *SEM1*, 19S components and DNA damage repair genes are hypersensitive to DNA damaging agents. Five-fold (ten-fold in the top panel) serial dilutions of the indicated double mutant strains were spotted onto YPD medium lacking or containing the DNA damaging agents hydroxyurea (HU, 100 or 150 mM), MMS (0.01-0.035%), or bleomycin (10 µg/ml). Strains were grown at 30°C and imaged.
2.4 Discussion

In this Chapter, I present my study on the physical binding partners of the Ubp family of deubiquitinating enzymes in budding yeast, undertaken in an attempt to characterize the functions of this incompletely understood group of enzymes. I showed the successful purification of certain Ubp-related multiprotein complexes, which in turn motivated follow up studies, both by me and other groups, which have resulted in the characterization of the functions of several of proteins in diverse cell processes. In particular, in this Chapter, I have described my own work that led me to investigate, in a productive collaboration with members of the Greenblatt laboratory, a novel role for the Ubp6-Sem1-19S proteasome complex in DNA repair. My identification of a novel Ubp2 complex is further characterized in the following chapters.

The proteasome has long been known to have a central role in the degradation of ubiquitinated proteins (reviewed in Finley 2009). The subunits which make up the proteasome are now increasingly well characterized, with specific functions elucidated for many of them (reviewed in Finley 2009). In this Chapter, I reported both physical interaction data and genetic data that implicate Ubp6 and Sem1 as subunits of the 19S proteasome. In addition, I show genetic and drug sensitivity data that implicate this complex in the repair of DNA damage. In addition to these data, my collaborators in the same study (Krogan 2004) found that the 19S proteasome is physically recruited to sites of DNA damage undergoing repair. Through in vivo crosslinking and chromatin immunoprecipitation (ChIP), Sem1 and another subunit of the 19S proteasome, Rpt6, were found to localize to a double strand DNA break induced by a site-specific endonuclease. Sem1 recruitment was lowered when members of the NHEJ or HR pathways were absent, implicating the proteasome in both types of repair pathways (Krogan 2004). Interestingly, a role for the entire 26S proteasome in DNA damage repair was also found, as a component of the 20S proteasome, Pre2, had also been found to bind to a DNA breakage site. Recruitment of Pre2 to the DSB was not dependent upon the presence of Sem1 (Krogan 2004). Therefore, it is likely that Sem1 and Ubp6 may function in the regulation of proteasome activity, and not specifically in the recruitment of the proteasome to sites of DNA damage. In agreement with this, other studies have shown that the absence of SEM1 results in the lowered activity of the proteasome (Sone 2004).
The work presented here in yeast can be extended to other organisms; through the co-immunoprecipitation of the human homologue of Sem1, DSS1, in human cell extracts, we found that DSS1 was also physically associated with 19S components (Krogan 2004), implicating Sem1/Dss1 as a conserved subunit of the 19S proteasome. Together, these data implicate the 26S proteasome in a role at sites of DNA damage, perhaps in the degradation of an unknown component of the repair machinery after repair. Protein degradation following the repair of DSBs may require poly-ubiquitination of one or more target proteins, as is perhaps consistent with the discovery that the BRCA1/BARD1 complex, important for DSB repair by HR in humans, is an E3 ubiquitin ligase (reviewed in Starita 2003).

Although the exact mechanism of the Ubp6-Sem1-19S complex in the process of DNA repair is currently unclear, recent studies by other laboratories have characterized the role of Ubp6 in the proteasome complex. For example, reports have suggested that Ubp6 may function simply to remove ubiquitin chains for reuse before substrate degradation, as cells deleted in \textit{UBP6} have a pronounced ubiquitin deficiency (Amerik 2000). More recent studies have implicated Ubp6 in a more complex role, affecting the commitment (through proteasome binding) of a ubiquitinated substrate to proteasomal degradation by trimming of its ubiquitin chain at the proteasome (Crosas 2006). In addition, Ubp6 inhibits the proteolytic function of the proteasome both \textit{in vitro} and \textit{in vivo}, as proteasomes without Ubp6 degrade various model substrates much faster than wildtype proteasomes (Hanna 2006). The inhibition occurred in a manner that is independent of its DUB activity, and has been speculated to aid in the ‘slowing-down’ of proteolysis in order for substrates to be deubiquitinated progressively first (Hanna 2006). Together, these data show that a proteasome-bound DUB, Ubp6, has a complex regulatory relationship with the proteasome and proteasomal substrates.

In addition to proteasome-related roles, recent genetic and biochemical studies on Ubp6 and Sem1 have revealed various novel, non-proteasome related roles for these proteins. A proteasome-independent role for Sem1 in mRNA export and splicing was found in one study, as defects in mRNA export are present in \textit{SEM1} mutants (Wilmes 2008). First linked through a synthetic lethality screen, Sem1 was subsequently shown through co-immunoprecipitation assays to physically interact with the Sac3-Thp1 complex that functions in mRNA export (Wilmes 2008). These interactors were not found in my TAP purifications, as the presence of the fused tag at the C-terminus of Sem1 may have blocked binding of these proteins. In agreement with this, a
tag fused to Sem1 in this region has been reported in the literature to result in a defect in mRNA export (Wilmes 2008). Sem1 was also found to interact with Csn21, a COP9 signalosome complex member, through affinity-purification assays. This complex was then implicated in mRNA splicing. A second study by another group independently confirmed the role of Sem1 in these two pathways (Faza 2009). In another study, both Ubp6 and Sem1 were implicated in a proteolysis-independent role in telomeric silencing (Qin 2009). These studies link both Sem1 and Ubp6 to multiple diverse roles in the cell both in conjunction with the proteasome and independently of it.

When I joined the Emili laboratory, large-scale proteomic screens were just emerging as a promising exploratory platform to survey the biological functions of a large set of proteins. Many types of large-scale studies have been performed since then, which have both validated some of my results and provided additional putative protein interactors for the Ubps. For example, recent Y2H data (Figure 2-11; (Yu 2008)) have confirmed Rup1 as an interactor of Ubp2, along with the protein Hua1 (YGR268C) which was identified in other studies as being important in endosomal sorting (Ren 2007). Interestingly, several other proteins have been linked physically by Y2H to Ubp2, including the mitochondrial protein Acp1, and the DNA replication checkpoint protein Csm3 (YMR048W). Although these Y2H interactions will require confirmation in a biological context, they implicate Ubp2 in other cellular roles. Large scale protein-fragment complementation assays (PCA; Tarassov 2008) have also identified various physical interactions of Ubps. For example, Ubp6 was identified in this study to physically interact with a variety of proteins, specifically the mitochondria-related proteins Ggc1, Mtm1, and Tim13 (Figure 2-11), but interestingly was not found to interact with members of the proteasome. This may be due to an inability of proteasome subunit-PCA reporter fusion proteins to bind to Ubp6. In addition to proteome-wide protein-protein interaction studies, proteins involved in the ubiquitin pathway, specifically DUBs, have been the focus of at least one recent proteomic study (Sowa 2009). Affinity purification and mass spectrometric identification of protein complexes from 75 human DUBs resulted in the generation of a large human DUB interactome, which both confirmed previously known physical interactions and elucidated new functions for some of these enzymes (Sowa 2009). Therefore, proteomics studies, including my data as described in this Chapter, and other studies as discussed above, have proven useful in shedding light on the variety of pathways that these enzymes are involved in.
Figure 2-11: Protein-protein interactions of the Ubps. Ubp-related physical interactions from two recent protein-protein interaction studies are plotted using Cytoscape. A line (edge) denotes a physical interaction between two proteins (nodes), while an arrow denotes a bait to prey interaction. High confidence data from a yeast two-hybrid (Y2H) study (‘CCSB-YI1’ dataset; Yu 2008) are shown with red edges, while data from a protein-fragment complementation assay (PCA) study (PPV score> 98.2%; Tarassov 2008) are shown with blue edges.
Chapter 3

Ubp2: A Novel Regulator of Rsp5-mediated Intracellular Protein Trafficking

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In this Chapter, A. Emili, D. Urban-Grimal and R. Haguenauer-Tsapis supervised and/or advised the experimentation, and D. Urban-Grimal and I performed all relevant analysis. I also performed all of the described experimentation with the exception of Figure 3-3 and 3-6 (performed by D. Urban-Grimal), and Figure 3-5 (performed by A. Bugnicourt). I was also given technical assistance by M. Raghavan, J. Chan, O. Pogoutse and G. Guo who worked under my supervision
3. Ubp2: A Novel Regulator of Rsp5-mediated Intracellular Protein Trafficking

3.1 Introduction

Protein ubiquitination is essential for the proper functioning of many eukaryotic cellular processes. While the covalent conjugation of poly-ubiquitin chains to a protein by ubiquitin ligases often leads to subsequent targeting and degradation by the 26S proteasome (Hochstrasser 1996), ubiquitin attachment is also critical for the correct execution of non-proteasomal events during intracellular transport of membrane associated proteins (Hicke 1997). Ubiquitination serves as a key signal mediating the internalization, intracellular transport and subsequent recycling or vacuolar degradation of endocytosed plasma membrane-bound receptors and transporters (Dupre 2004). Much progress has been made in elucidating the mechanistic basis for various steps in protein endocytosis, but many aspects about the core components and enzymatic steps remain unresolved.

In the budding yeast *Saccharomyces cerevisiae*, a single essential E3 ubiquitin ligase, Rsp5, has been implicated in the internalization of most if not all endocytosed proteins (Horak 2003). The uracil permease Fur4 is an experimentally well-characterized model substrate of Rsp5. Uracil binding, or stress conditions such as nutrient starvation, heat shock, or the inhibition of protein synthesis trigger Fur4 phosphorylation, which results in the subsequent recognition and Lys63-linked di-ubiquitination of Fur4 by Rsp5 on two specific lysine residues (Galan 1997; Marchal 1998, 2000; Seron 1999; Volland 1994). Similar to other membrane proteins, the ubiquitination of Fur4 results in its rapid and complete clearance from the plasma membrane into early endosomes. This is followed by the subsequent recognition of ubiquitinated Fur4 by the ESCRT-I, II, III complexes and other class E Vps proteins, followed by sorting into multivesicular bodies (MVBs) (Katzmann 2002). When MVB sorting is inhibited in class E vps mutants, endocytosed Fur4 recycles back to the plasma membrane (Bugnicourt 2004). After sorting into the MVB, Fur4 is targeted to the vacuolar lumen where it is degraded (Volland 1994). Fur4 can also follow a different pathway when synthesized in the presence of its substrate, uracil. In this situation, newly synthesized Fur4 is diverted to the endosomal system directly from the Golgi apparatus (Blondel 2004). It then undergoes Rsp5-dependent
ubiquitination, a modification crucial for its sorting into MVBs (Morvan 2004), followed by vacuolar degradation after fusion of MVBs with the vacuole.

Although the role of ubiquitination in these trafficking processes is relatively well understood, the role of deubiquitinating enzymes in these pathways is less clear. While some enzymes in the Ubp family of DUBs have characterized functions in processes such as proteasomal degradation (Chapter 2), emerging evidence also implicates members of the vesicle-based inter-organelar protein trafficking pathway as physiologically relevant targets of several of the Ubps. These include: Doa4 (Ubp4), involved in the deubiquitination of internalized endocytic cargoes, such as Fur4, at the endosome immediately prior to their internalization into MVBs (Dupre 2001); Ubp3, implicated in the deubiquitination of regulatory proteins in both the anterograde (Cohen 2003a) and retrograde (Cohen 2003b) protein transport pathways through the endoplasmic reticulum (ER) and Golgi systems, along with a possible involvement in the cytoplasm to vacuole (Cvt) trafficking pathway (Baxter 2005); and Ubp1, which has been linked to the internalization and turnover of the ABC membrane transporter Ste6 via an as yet unknown mechanism (Schmitz 2005). As deubiquitination appears to be an important regulatory step in these intracellular protein trafficking pathways, other enzymes from the Ubp family of proteins may also play a role in these processes.

3.1.1 Project Rationale

Protein ubiquitination is essential for many events linked to intracellular protein trafficking. Parallel studies by our group (described in Chapter 2) and another laboratory (Kee 2005) have established specific physical interactions between the *S. cerevisiae* deubiquitinating enzyme Ubp2, the E3 ligase Rsp5, and a protein of previously unknown function, Rup1, which contains an ubiquitin associated (UBA) domain. Although in vitro assays reported previously (Kee 2005) have hinted at a possible role of Ubp2 in the deubiquitination of substrates of Rsp5, including the ER membrane bound transcription factor Spt23 and two other proteins, Csr2 and Ecm21, (Kee 2006), the physiological significance of Ubp2 with respect to Rsp5-mediated processes such as intracellular protein trafficking was uncertain. Hence, I sought to elucidate the possible involvement of Ubp2 in transporter and receptor trafficking. In this Chapter, I expand upon my initial observations by establishing a critical role for Ubp2 in the ubiquitin-dependent
sorting of plasma membrane proteins using Fur4 as a model substrate. In particular, I found that 
UBP2 interacts genetically with RSP5, while Rup1 facilitates the tethering of Ubp2 to Rsp5 via a 
PPPSY motif. Moreover, using the uracil permease Fur4 as a model reporter system, I have 
established a role for Ubp2 in efficient membrane protein turnover, as cells deleted for UBP2 
exhibited a temporal stabilization of Fur4 at the plasma membrane, indicative of perturbed 
protein trafficking. This defect was ubiquitin dependent, as a Fur4 N-terminal ubiquitin fusion 
construct bypassed the block and restored sorting in the mutant. In addition, the sorting defect 
could be alleviated in conditions where recycling back to the plasma membrane was suppressed, 
implicating Ubp2 in cargo sorting at the multivesicular body. Collectively, my data suggest that 
Ubp2 is not simply a negative regulator of Rsp5, but rather is engaged in a more positive and 
dynamic regulatory relationship with Rsp5.

3.2 Materials and methods

3.2.1 Strains and growth media

All transformations of yeast strains were done using a standard protocol (Gietz 1992; 
Soni 1993). All yeast strains were grown in YPD (yeast extract, peptone, and 2% glucose) and at 
30°C unless otherwise specified. TAP-tagged deletion mutant strains were constructed by direct 
transformation or by mating a relevant deletion strain with a suitable TAP tagged strain. 
Following sporulation, tetrads were dissected, and haploids containing both HIS3 (TAP) and 
NAT-resistance (deletion) markers were selected. The presence of the deletion and the 
expression of the TAP-tagged protein were confirmed by PCR and Western blotting, 
respectively. ubp2Δ::NatMX and rup1Δ::NatMX strains were constructed as described in (Tong 
2001) by transformation with EcoRI-cut p4339 into the Y3656 strain (MATα can1Δ::MFA1pr-
HIS3-MFA1pr-LEU2 his3Δ leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0) or with a PCR product from the 
NatMX cassette from p4339. Wildtype strains in Figure 3-2A and D, and rup1Δ in C, are derived 
from FY56 which is isogenic to rsp5-1 (FW1808; MATa his4-912 AR5 lys2-128Δ ura3-52 rsp5-1) 
(Wu 2001). For other figures, wildtype strains are BY4741. ubp2Δrsp5-1 and rup1Δrsp5-1 
were constructed either by direct transformation of the deletion cassette into rsp5-1, or by mating 
of haploids, tetrad dissection, and selection of haploids with the required genotype. rsp5-326 
(MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 rsp5-326) is from
3.2.2 Plasmids

Plasmids allowing for inducible overexpression of N-terminal affinity tagged proteins were constructed by recombineering essentially as previously described (Emili 2001). Briefly, full-length ORFs were amplified from yeast genomic DNA using primers similar to the Yeast Genepairs Primers (Research Genetics) bearing homology to the 5’ and 3’ ends of each ORF. The product from this first PCR reaction was re-amplified using primers which contain sequences homologous to those flanking a SmaI cloning site downstream of a galactose inducible promoter in a high copy yeast expression plasmid with a selectable URA3 marker gene (pF/H426). The PCR product was integrated into the SmaI linearized parental plasmid by cotransformation and in vivo homologous recombination. This resulted in an in-frame fusion to a triple FLAG/His\textsubscript{10}/HA\textsubscript{2} (F/H) epitope affinity tag immediately upstream of the ORF. Correct expression of the fusion protein was confirmed by Western blotting. The mutant constructs pF/H426-\textit{UBP2}-C745S and pF/H426-\textit{RUP1}-Y135F, bearing site specific point mutations, were constructed using the QuikChange II Site-Directed Mutagenesis kit (Stratagene). Mutants were confirmed by dideoxy DNA sequencing. pHA-\textit{RSP5} (p[HA-\textit{RSP5}, CEN, LEU2]) was from (Kaminska 2002). The galactose inducible expression plasmids p\textit{FUR4} (pFL38Gal\textit{FUR4}), p\textit{FUR4-GFP} (pFL38Gal\textit{FUR4-GFP}), and ubiquitin-fusion construct p\textit{Ub-FUR4-GFP} (pFL38GalUb-\textit{FUR4-GFP}; containing a variant ubiquitin with all its relevant lysine residues mutated to arginine to ensure that no additional ubiquitin can be added to the ubiquitin moiety, and glycine 76 replaced with a valine, to prevent proteolytic cleavage) were from (Blondel 2004; Marchal 2002; Seron 1999). The copper-inducible ubiquitin expression plasmid p\textit{CUP1-Ub-\textit{TRP1}} (YEp96) and the control vector (YEp46\Delta) are from (Hanna 2003).
3.2.3 Cell lysis, minigel electrophoresis and Western blotting

Cells were harvested and lysed as described in Chapter 2 by glass bead beating in YEB or YEB containing 350mM KCl to decrease non-specific binding to antibody beads (Rup1 Y135F binding experiments). For the immunoprecipitation experiments, approximately 6mg of total soluble protein extract (~10mg/ml) was incubated for 1 hour at 4°C with anti-FLAG M2 Agarose affinity beads (Sigma-Aldrich) for F/H tagged strains, or with IgG Sepharose 6 Fast Flow beads (Amersham Pharmacia) for TAP-tagged strains. Washing of, and elution from the beads were performed as described in Chapter 2. Samples were electrophoresed, transferred to a Western blot, blocked and visualized by ECL as described in Chapter 2. Primary antibodies used were: mouse monoclonal anti-HA antibody (12CA5 cell line; a kind gift from Mike Tyers, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario) to detect the TAP tag, rabbit anti-Rsp5 antibody (a kind gift from Linda Hicke; Stamenova 2004), and mouse anti-FLAG antibody (Sigma-Aldrich). For the Fur4 immunoblot, total protein extracts were prepared by the NaOH-TCA lysis technique as previously described (Volland 1994). To better detect ubiquitinated species of Fur4, membrane enriched fractions of cells producing the lower molecular weight Fur4, and not the Fur4-GFP fusion were analyzed. Membrane-enriched protein fractions were prepared as previously described (Dupre 2001) except that cells were broken in a ‘One Shot’ Cell Disrupter (Constant Systems LDT) at maximum pressure. Proteins in sample buffer were heated at 37°C, resolved by SDS-polyacrylamide gel electrophoresis in 10% acrylamide gels using tricine buffer, and transferred to nitrocellulose membranes. The membranes were probed with anti-PGK (Molecular Probes), rabbit anti-Fur4 antibody (Volland 1994), or monoclonal antibodies against porin (Molecular Probes). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G was used as the secondary antibody (Sigma) and was detected by enhanced chemiluminescence (ECL).

3.2.4 Serial dilutions

Strains were grown overnight to saturation in SC-URA or YPD media, and diluted with sterile water to OD$_{600} = 0.5$. 10-fold dilutions with sterile water were then made, and 3μl of the cell suspension spotted onto media plates containing rich (YP) or selective (SC-URA) media + 2% glucose or 2% sucrose/1% galactose. Plates were incubated for 2-4 days at 30°C or 34°C and
imaged. For 5-FU plates, cells were grown to OD$_{600}$ ~0.7. 10-fold dilutions were then made, and 20µl of the cell suspension spotted onto media plates containing SD + 5µM 5-FU (Sigma). Plates were incubated for 4 or 7 days and imaged.

3.2.5 Uracil uptake assay

Uracil uptake was measured as previously described (Volland 1994), by incubating a 1ml culture of exponentially growing cells with 5 x 10$^{-6}$ M radiolabeled uracil for 20 s at 30°C. Cells were quickly filtered through Whatman GF/C filters, which were then washed twice with ice-cold water and counted for radioactivity. Fur4 activity was measured at various times after the addition of cycloheximide (100 µg/ml; Sigma) as previously described (Volland 1994).

3.2.6 Microscopy

Cells grown to exponential growth phase in YNB medium were concentrated by a factor of ten by centrifugation. Cells were viewed immediately, without fixation, under a fluorescence microscope (type BY61, Olympus, Tokyo, Japan) and images captured with a digital camera. For Figures 3-4 and 3-7, fluorescence images of live yeast cells at a 512 x 512 pixel resolution were generated with a Leica DM IRBE confocal microscope using a 100x objective with immersion oil. Images were processed using Leica TCS software.

3.2.7 α-factor halo assay

Mat a yeast cells of the genotypes indicated from an overnight culture were added to pre-warmed (55°C) sterile 0.5% agar, vigorously mixed, and poured evenly as a thin layer onto plates containing solid YPD media. After the agar solidified, sterile filter disks soaked with 5ul of various concentrations of α-factor (Pepceuticals) were gently overlaid onto the cell lawn, and the plates incubated at 30°C for one day prior to scoring.
3.3 Results

3.3.1 *Ubp2 physically interacts with Rsp5 and Rup1*

As described in Chapter 2, purification of tagged Ubp2 by the tandem affinity purification (TAP) method resulted in the identification of physical interactions between the deubiquitinating enzyme Ubp2, the E3 ligase Rsp5, and a protein of previously unknown function, Rup1. I proceeded to further characterize the physical relationship between these proteins. The immunoprecipitation of either Ubp2-TAP or Rup1-TAP resulted in a similar yield of Ubp2 (Figure 3-1A); therefore, the interaction between Ubp2 and Rup1 is relatively stable and appears equimolar. In contrast, a substantial fraction of Rsp5 remained in solution and was not recovered with either Ubp2-TAP or Rup1-TAP after depletion of TAP-tagged proteins with IgG beads from soluble cell extracts (Figure 3-1B), indicating that a large fraction of Rsp5 exists in alternate protein complexes, or that Rsp5 binding is transient or labile. The relative fraction of the cellular Rsp5 pool present in a complex with Ubp2 and Rup1 is estimated to be ~10% of total. Since I have shown that only a modest fraction of the total cellular pool of Rsp5 is stably bound to Ubp2/Rup1, Ubp2 and Rup1 may be involved in only a subset of Rsp5-dependent pathways.

To further investigate the structure-function dependencies of the interaction of Ubp2/Rup1 with Rsp5, I constructed TAP-tagged strains bearing a targeted genomic deletion in either Ubp2 or Rup1. The composition of the resulting affinity-isolated protein complexes was then examined by Western blotting. Whereas Rsp5 remained bound to Rup1-TAP in the absence of Ubp2 (Figure 3-1C), the amount of Rsp5 precipitated by Ubp2-TAP was notably reduced in the absence of Rup1. These results support a previous report (Kee 2005) indicating that Rup1 likely mediates, at least in part, the interaction of Ubp2 with Rsp5. Consistent with this, I identified a putative WW domain consensus binding motif, PPPSY, encoded by amino acids 131-135 of Rup1. I generated a site-specific point mutant in which the invariant core tyrosine residue 135 was substituted with a phenylalanine (Y135F), and expressed this from a plasmid in a strain lacking endogenous *RUP1*. This type of mutation (Y->F) has previously been shown to markedly reduce or even abolish binding of other WW domain proteins to their interacting partners bearing analogous PY motifs (Chen 1997; Strano 2001). Indeed, as predicted, the interaction of this mutant variant with Rsp5 was impaired, although not abolished, relative to the
Figure 3-1: Ubp2, Rsp5, and Rup1 interact physically. (A) Ubp2 recovery from Ubp2-TAP, Ubp2-TAP rup1Δ, and Rup1-TAP strains. Arrow indicates gel band identified as Ubp2 by MALDI-ToF mass spectrometry. (B) Co-immunoprecipitation of a fraction of cellular Rsp5 with Ubp2 and Rup1. Cell extracts were depleted of endogenously TAP-tagged Ubp2 or Rup1 with IgG (IP lanes), and the remaining soluble Rsp5 (post-IP lanes) and bait proteins probed by Western blot with anti-Rsp5 antibodies which also recognized the protein A portion of the TAP tag. The ‘input’ lane shows the Rsp5 level in the extract prior to IP. Immunoprecipitations were performed with equal amounts of protein extracts in each lane (C) Rup1 tethers Ubp2 to Rsp5. IgG-based immunoprecipitation of Rup1-TAP and Ubp2-TAP from WT, ubp2Δ or rup1Δ strains. The co-purification of Rsp5 with the baits was determined by Western blotting using anti-Rsp5 antibodies. (D) Plasmids expressing FLAG (F/H)-tagged wildtype Rup1 or the Y135F point mutant were transformed into cells lacking endogenous Rup1. After immunoprecipitation with anti-FLAG antibodies, Rup1 and Rsp5 were detected by Western blotting. The bottom panel shows the expression levels of Rsp5 in whole cell extracts by Western blotting against Rsp5.
wild-type control (Figure 3-1D), implying that the motif mediates in part the recruitment of Rsp5. Similar results were obtained after expression of the Rup1 Y135F mutant in a \textit{ubp2}\textsuperscript{Δ} deletion mutant background (data not shown), indicating the residual binding was not mediated by Ubp2.

3.3.2 \textit{UBP2} and \textit{RUP1} interact genetically with \textit{RSP5}

To further explore the relationship of Ubp2 and Rup1 to Rsp5-related functions, I examined the effects of inducible ectopic overexpression of either Ubp2 or Rup1 in a strain harbouring a temperature-sensitive hypomorphic allele of \textit{RSP5} (\textit{rsp5-1}) (Wang 1999), as well as in a wild-type strain, looking for enhancement or suppression of the conditional slow-growth phenotype. As seen in the limiting dilution series shown in Figure 3-2A, Ubp2 overexpression was moderately toxic to the control strain as compared to an empty control vector, while the \textit{rsp5-1} mutant was extremely hypersensitive. This synthetic dosage lethality was specific to \textit{UBP2}, as the \textit{rsp5-1} strain did not shown any hypersensitivity to overexpression of two other unrelated deubiquitinating enzymes, \textit{UBP6} and \textit{UBP10} (Figure 3-2A). Indeed, although overproduction of \textit{UBP10} caused a noticeable growth defect in wildtype cells, this defect did not worsen when combined with the \textit{rsp5-1} mutation, in striking contrast to \textit{UBP2}. While wildtype yeast was not detectably sensitive to the overexpression of \textit{RUP1}, \textit{rsp5-1} and \textit{ubp2}\textsuperscript{Δ} mutants displayed modest hypersensitivity. On the other hand, a deletion of \textit{RUP1} resulted in a reduction in the growth impairment caused by the overexpression of \textit{UBP2}. These data support the notion that Rup1 functions coordinately with, but not equivalently to Ubp2. To confirm that these genetic interactions were not specific to the \textit{rsp5-1} allele, another allele, \textit{rsp5-326} (Katzmann 2004) was tested (Figure 3-2B). All of the above interactions were similar in this mutant background, with any small variances in growth due likely to slight differences in the hypomorphic effect of each \textit{RSP5} allele. When Rsp5 was expressed from a low copy number plasmid, pHA-\textit{RSP5} (Kaminska 2002), in \textit{rsp5-326} strains, the growth phenotypes seen were rescued to wildtype levels, indicating that these phenotypes were specific to \textit{RSP5}.
Figure 3-2: *UBP2* and *RUP1* interact genetically with *RSP5*. (A, B, D) The indicated over-expression plasmids, driven by a galactose inducible promoter, were transformed into either wildtype or mutant strains. Cell cultures were then serially diluted 10-fold (starting OD$_{600}$ of 0.5) and spotted onto SC-URA media containing glucose (non-inducing) or galactose (inducing). (B) Growth of *rsp5-326* mutant cells bearing pHA-*RSP5* (a low copy, but not galactose responsive plasmid), and/or a second overexpression plasmid as indicated. (C) Growth of cells deleted for either *UBP2* or *RUP1* alone, or in combination with an *RSP5* temperature sensitive allele (*rsp5-1*), on rich media (YPD) at either 30°C (permissive for *rsp5-1*) or 34°C (semi-permissive for *rsp5-1*).
Consistent with this apparent functional antagonism (Figure 3-2A; (Kee 2005)), deletion of the \textit{UBP2} open reading frame partially rescued the temperature-sensitivity of an \textit{rsp5-1} mutant strain (Figure 3-2C), although deletion of \textit{RUP1} had no observable effect. Taken together, these results were consistent with a model (Kee 2005) wherein Ubp2 directly antagonizes Rsp5 activity, and implied that Rup1 likely serves as a less critical ancillary role such as facilitating the interaction of Ubp2 with Rsp5 or possible substrates.

3.3.3 \textit{Ubp2 catalytic activity is important, but not essential for synthetic dosage lethality}

A key characteristic of Ubps is the presence of an evolutionarily conserved catalytic domain (Baker 1992). To test whether the catalytic activity of Ubp2 was necessary for the antagonistic interaction observed with Rsp5-deficient strains, I mutated cysteine 745, a critical residue of the core Cys box motif conserved across all members of the Ubp family, to serine, which is predicted to result in a severe or complete loss of deubiquitinating capacity (Cohen 2003a; Li 2002a). As expected, I observed that overexpression of this putatively inactive mutant, \textit{ubp2 C745S}, led to a marked reduction in the slow-growth phenotype caused by overexpression of \textit{UBP2} in either \textit{rsp5} strain background (Figure 3-2B and D), suggesting that inappropriate or excessive deubiquitination activity is largely, but not exclusively, responsible for the functional antagonism observed.

3.3.4 \textit{ubp2Δ cells are sensitive to 5-fluorouracil (5-FU)}

Given that Rsp5 has a well-established role in the internalization, trafficking, and subsequent vacuolar degradation of membrane-bound transporters (Horak 2003), I explored the possibility that Ubp2 might also modulate the efficiency or dynamics of these ubiquitination-dependent events using Fur4 as a model. \textit{rsp5} mutants have increased plasma membrane steady state levels of Fur4 (Galan 1996). The same is true for mutants in class E genes that accumulate an abnormal compartment, the class E compartment, from which Fur4 recycles to the plasma membrane (Bugnicourt 2004). All these mutants display increased sensitivity to the drug 5-Fluorouracil (5-FU) (Bugnicourt 2004), which is imported by Fur4. 5-FU is a toxic uracil
analogue which inhibits the nucleotide synthetic enzyme thymidylate synthase and incorporates aberrantly into cellular RNA and DNA (Longley 2003), leading to cell death. Therefore, in collaboration with Rosine Haguenauer-Tsapis’ lab we tested whether ubp2Δ is sensitive to 5-FU, using a class E mutant, vps37Δ, as a control (Figure 3-3). As shown in previous studies, cells in which the transporter is absent (fur4Δ) were completely resistant to the drug (Figure 3-3; (Jund 1970)). Compared to wildtype cells, ubp2Δ mutants were partially sensitive to the drug. However, they were not as sensitive as cells with a deletion in vps37Δ. The sensitivity of vps37Δ cells was not affected when combined with a mutation in UBP2, suggesting that VPS37 and UBP2 may act in the same pathway. These data show that Fur4 may be stabilized at the plasma membrane of ubp2Δ cells, leading to an increased sensitivity to 5-FU. The stabilization may not be complete as the sensitivity of ubp2Δ was less than that of strains defective in various sorting steps along the endocytic pathway.

3.3.5 ubp2Δ cells have increased steady state amounts of plasma membrane localized Fur4

In order to define whether the 5-FU sensitivity of ubp2Δ cells indeed comes from defects in trafficking, or merely from impaired RNA metabolism (Giaever 2004), I followed the fate of a GFP-tagged version of Fur4 in ubp2Δ and rup1Δ cells, using as control rsp5 mutant cells (Figure 3-4). A galactose-inducible version of Fur4-GFP was similarly targeted to the plasma membrane after galactose induction in all the strains. Glucose was then added to stop Fur4-GFP synthesis and chase to the plasma membrane any Fur4-GFP still in the secretory pathway. Uracil was then added to trigger Fur4-GFP endocytosis. Cells were harvested at various time points after the addition of uracil, and subjected to whole-cell imaging (Figure 3-4). Uracil triggered a progressive loss of plasma membrane GFP fluorescence in wildtype cells, a transient apparition of intracellular fluorescent dots (likely endosomes), followed by apparition of luminal vacuolar fluorescence (corresponding to free GFP, not immediately degraded by vacuolar proteases). Fur4-GFP displayed almost the same fate in rup1Δ cells. In contrast, in rsp5-1 cells, even at a permissive temperature, plasma membrane fluorescence was still detectable after two hours of uracil treatment, together with very faint vacuolar fluorescence. In ubp2Δ cells, the situation was intermediate between that observed in wildtype and rsp5 cells. Plasma membrane staining was still observed after 60 min of uracil treatment, and after two hours, vacuolar fluorescence was
Figure 3-3: Loss of *UBP2* results in hypersensitivity to 5-fluorouracil (5-FU). Wildtype, *ubp2Δ*, *vps37Δ*, *fur4Δ* single mutants, and *vps37Δubp2Δ* double mutant cells were transformed with a *URA3* plasmid and subsequently tested for sensitivity to 5-FU. Exponentially growing cells were serially diluted 10-fold (starting OD$_{600}$ ~0.7) and spotted onto SD plates with 5µM 5-FU. Plates were imaged either after 4 days (*left panel*) or 7 days (*right panel*).
Figure 3-4: Uracil-induced Fur4 sorting is perturbed in ubp2Δ mutant cells. A FUR4-GFP fusion reporter, along with a plasmid overexpressing ubiquitin (+) or an empty plasmid (-) were transformed into cells. Strains were grown in sucrose overnight, diluted to OD₆₀₀ = 0.5 in media containing galactose to induce synthesis of FUR4-GFP and 0.1mM copper sulphate to overexpress ubiquitin, and grown for 4 hours. Fur4 transcription was stopped by adding glucose for 1 hour to chase Fur4 to the plasma membrane. Uracil (40 μg/ml) was then added to induce Fur4 internalization. The GFP signal was viewed by fluorescence confocal microscopy at the indicated time points (min) after uracil addition. 0 mins indicates cells imaged immediately before uracil addition. Note that the left panel contains the same images as Figure 3-7, as the experiments were performed in parallel.
evidenced. Therefore, Ubp2 seems to play an important role in the efficient clearance of Fur4 from the plasma membrane.

Deubiquitinating enzymes such as Ubp4 (Doa4) have previously been linked to the overall recycling of ubiquitin prior to the proteasomal or vacuolar degradation of substrates (Dupre 2001; Swaminathan 1999). Depletion of the free ubiquitin pools in a yeast cell has the potential to lead to defects in protein sorting from the plasma membrane (Galan 1997), presumably by indirectly reducing the efficiency of Rsp5-mediated ubiquitination at various steps of the sorting pathway. To exclude the possibility that the Fur4-GFP sorting defect observed in \textit{ubp2}\textsuperscript{Δ} mutants was an artifact due to a general reduction in free ubiquitin levels, I assessed whether the phenotype could be suppressed by the forced overexpression of ubiquitin from a plasmid using a strongly inducible copper promoter. Impaired sorting in the \textit{ubp2} deletion mutant was not restored (Figure 3-4). This data, along with other previously reported data showing that \textit{ubp2} mutants exhibit wildtype levels of free ubiquitin (Amerik 2000), indicated that the impaired kinetics of Fur4-GFP sorting in \textit{ubp2}\textsuperscript{Δ} mutants are indeed likely a direct result of improper regulation of the sorting pathway, and not due to a deficit in cellular ubiquitin levels.

3.3.6 \textit{ubp2}\textsuperscript{Δ} cells display normal plasma membrane Fur4 ubiquitination and internalization

Plasma membrane stabilization of Fur4 subsequent to conditions normally triggering its endocytosis can be observed in two types of situations: in mutants impaired in the internalization step of endocytosis (Blondel 2004; Volland 1994), and in class E mutants with impaired MVB sorting. In the latter case, Fur4 recycles from the endosomal class E compartment back to the plasma membrane. In order to decipher the origin of plasma membrane stabilization of Fur4-GFP in \textit{ubp2}\textsuperscript{Δ} cells, we first searched, using \textit{vps} class E mutants, for conditions preventing any recycling.

Fur4-GFP was expressed in wildtype and \textit{vps37}\textsuperscript{Δ} cells, and cells were subjected to carbon starvation (CS) to induce internalization of Fur4-GFP from the plasma membrane. Glucose (carbon, CA), was then added, to induce recycling of Fur4 back up to the plasma membrane, in the presence or absence of an inhibitor of protein synthesis, cycloheximide (CHX).
Fur4-GFP localization was followed by both fluorescence microscopy (Figure 3-5A) and by monitoring uracil uptake (Figure 3-5B). In both WT and \(vps37\Delta\) cells, Fur4-GFP was internalized completely after carbon starvation as expected (Figure 3-5A, (Bugnicourt 2004)). Fur4-GFP was rapidly targeted back to the plasma membrane in \(vps37\Delta\) cells after the addition of glucose but not upon addition of glucose + CHX. Recycling only occurred in \(vps37\Delta\) cells, as the mutation blocks the entry of Fur4-GFP into the vacuolar lumen; in wildtype cells all Fur4-GFP was targeted to vacuolar lumen, where it was degraded (Bugnicourt 2004). As expected, uracil uptake was increased over 60 minutes post-glucose addition in \(vps37\Delta\) cells in the absence of CHX (Figure 3-5B). Taken together, these data show that recycling of Fur4-GFP in the presence of CHX was chosen as a good condition in which to define the level of action of Ubp2.

To determine whether Ubp2 was involved specifically at the internalization step, I examined the transport of Fur4 in the presence of CHX. After two hours of galactose induction of Fur4-GFP followed by glucose, CHX was added to wildtype and \(ubp2\Delta\) cells. The fate of Fur4-GFP was then followed by fluorescence imaging and uracil uptake measurement (Figure 3-6A and B). Plasma membrane fluorescence disappeared in a similar way in wildtype and \(ubp2\Delta\) cells (Figure 3-6A), and the loss of uracil uptake occurred with identical rate in both type of cells (Figure 3-6B). Endocytic internalization of Fur4 is thus normal in \(ubp2\Delta\) cells. In agreement with the observation of a normal rate of internalization, the pattern of ubiquitination (regularly spaced Fur4 species evidenced above the main Fur4-GFP signal) on Western blots of plasma membrane enriched fractions was identical in wildtype and \(ubp2\Delta\) cells (Figure 3-6C). This is in comparison to \(rsp5-1\) cells, which showed a distinct lack of Fur4-ubiquitin conjugates as expected. These data imply that Ubp2 is likely not involved in the deubiquitination of Fur4 at the plasma membrane.

3.3.7 Ubp2 is involved in efficient Fur4-GFP ubiquitination in the late steps of endocytosis

If Ubp2 is not required for ubiquitination at the plasma membrane or internalization, it is then likely required for subsequent steps of trafficking. In order to more closely decipher the
Figure 3-5: Recycling of Fur4-GFP cannot be detected in the presence of cycloheximide. WT and vps37Δ cells transformed with pFur4-GFP were cultured at 30°C and Fur4-GFP synthesis was induced for 90 min by adding galactose. Glucose was added to block Fur4-GFP synthesis (CC). 20 min later, cells were subjected to carbon starvation (CS) to trigger endocytosis of the permease for 60 minutes. Cultures were divided in two equal fractions. Glucose (CA) or glucose + cycloheximide (CA+CHX 0.1mg/ml) were then added. (A) Cells were visualized by fluorescence microscopy at t=0 (0’CC), after 60 minutes of carbon starvation (60’CS) and 60 minutes after the addition of carbon (60’CA) or carbon + cycloheximide (60’CA+CHX). Note that Fur4-GFP was efficiently internalized in all the strains upon CS. (B) Uracil uptake was measured 3, 10, 30 and 60 min after the addition of glucose. Results are expressed as a percentage of the initial uracil uptake measured immediately before carbon starvation, and plotted on a linear scale.
Figure 3-6: **UBP2 is not necessary for cycloheximide (CHX) triggered internalization of Fur4.** (A) pFUR4-GFP bearing cells were grown in raffinose overnight. Galactose was added for 2 hours to induce synthesis, and glucose was then added for 10 min to chase Fur4-GFP to the plasma membrane. CHX (0.1mg/ml) was added and GFP signal examined by fluorescence microscopy and Nomarski optics. Time refers to the time after addition of CHX, with 0 min as pre-induction. (B) Uracil uptake was measured at different times after the addition of CHX in WT and *ubp2Δ* strains. Results are expressed as a percentage of the initial uracil uptake, and plotted on a log scale. (C) Fur4 ubiquitin profile at the plasma membrane is unchanged in *ubp2Δ* mutants. pFUR4 was transformed into WT, *ubp2Δ*, *rup1Δ*, and *rsp5-1*, and cells, which were grown in raffinose overnight. Expression from pFUR4 was induced for 90 min with galactose before adding glucose for 15min to chase Fur4 to the plasma membrane. Total protein extracts (lysate) and enriched membrane fractions were collected and analyzed by Western blotting to visualize Fur4 and Fur4-ubiquitin conjugates. 3-phosphoglycerate kinase (PGK) and porin, a mitochondrial membrane protein, were used as loading controls.
origin of the Fur4 trafficking defect in *ubp2Δ* cells, I investigated the role of ubiquitination in uracil-induced endocytosis. Defects in Rsp5- and ubiquitin-dependent trafficking of a number of membrane-bound proteins, either at the plasma membrane, or at the MVB, can be bypassed using an in-frame fusion of ubiquitin to these various cargoes. This is notably the case for Fur4 (Blondel 2004). Hence, I examined whether expression of the Fur4-GFP reporter construct bearing ubiquitin as an N-terminal fusion (pUb-Fur4-GFP) could likewise suppress the sorting defect following the loss of Ubp2 activity. Control experiments (Figure 3-7) confirmed that, as predicted, the fusion construct rescued, at least partially, the internalization defect seen in *rsp5-1* cells at 60 minutes post-uracil addition. Likewise, the Ub-Fur4-GFP fusion protein was also efficiently cleared from the plasma membrane in the *ubp2* deletion mutants, as the fluorescence signal detected at the plasma membrane was virtually absent one hour after uracil induction (Figure 3-7). The concomitant increase in the background signal intensity detected in the vacuoles of *ubp2Δ* cells was expected due to missorting of nascent Ub-Fur4-GFP to the vacuole directly from the Golgi (Blondel 2004). Taken together, these data suggested that paradoxically, the loss of Ubp2 activity impaired proper Rsp5-mediated ubiquitination of Fur4-GFP, likely at the level of MVB sorting. This sorting step was restored with Ub-Fur4-GFP, and the protein, correctly sorted to MVB, did not recycle to the plasma membrane. Possible reasons for these observations are discussed in sections below.

3.3.8   *Ubp2 may modulate the sorting of other substrates such as the mating receptor protein Ste2*

To test the generality of Ubp2’s role in transport, I examined the effects of Ubp2 on the turnover of another well-characterized substrate of Rsp5, the *α*-factor receptor, Ste2 (Dunn 2001). Similar to Fur4, Ste2 is ubiquitinated, internalized, and subsequently degraded in the vacuole upon substrate-binding (Dunn 2001; Reneke 1988; Schandel 1994). Defects in the sorting and degradation of Ste2 result in hypersensitivity to *α*-factor (Raths 1993), as the presence of *α*-factor triggers cell cycle arrest in Mat a recipient cells (Bucking-Throm 1973).
Figure 3-7: Ub-Fur4-GFP is correctly sorted at the MVB in \textit{ubp2}\Delta mutant cells. \textit{pFUR4-GFP} or pUb-\textit{FUR4}-GFP expression plasmids were transformed into WT and \textit{ubp2}\Delta cells. Strains were grown in sucrose, diluted (OD\textsubscript{600} = 0.5) in media containing galactose and Fur4 synthesis induced for 4 hours. Transcription was stopped by adding glucose for 1 hour to chase Fur4 fusion reporters to the plasma membrane. Uracil (40 μg/ml) was then added to trigger internalization. GFP signal was viewed by fluorescence confocal microscopy both before (T0) and 60 min (T60) after uracil addition. (Note: cells in the left panel were also transformed with a control vector (YEp46\Delta) and incubated with 0.1mM copper sulphate, as the experiment was done in parallel to that shown in Figure 3-4.)
When a paper disk soaked with α-factor is placed on a lawn of MAT a cells, a gradient zone of growth inhibition forms, termed a halo. ubp2 deletion mutants exhibit hypersensitivity to α-factor by halo assay as compared to a WT control strain (Figure 3-8), possibly through a defect in clearing Ste2 from the plasma membrane of these cells. Surprisingly, the sensitivity of ubp2Δ cells to α-factor was slightly greater than of end3 deletion mutants, and rup1Δ cells are as sensitive as ubp2Δ cells. end3 cells lack an endocytic machinery component, and were previously found to be sensitive to α-factor (Raths 1993). These data suggest that Ubp2 modulates the ubiquitination and sorting of other plasma membrane associated proteins other than Fur4, but this effect may happen with slightly different kinetics depending on the cargo. The possible involvement of Rup1 in Ste2 transport is currently unclear and should be subject to further study.

3.4 Discussion

Despite the rapidly expanding interest in DUBs as likely critical regulators of core cellular processes such as protein trafficking (Amerik 2004; Nijman 2005), the physiological roles and targets of most of the known or predicted deubiquitinating enzymes in yeast remain unknown. In this report, I sought to confirm and extend the observations of previous studies regarding the molecular associations and putative functions of Ubp2 (Kee 2005; Kee 2006; Ren 2007). In principle, most of the genetic and physical interaction data reported in my study (Lam 2009) and previously by another group (Kee 2005) pointed to an antagonistic, and presumably regulatory relationship with Ubp2/Rup1 counteracting Rsp5. In light of this perspective, my finding that ubp2Δ mutants have a defect in Rsp5-mediated transporter and receptor sorting reported here was especially surprising.

The transport defect shown here could have been due to defects in endosome morphology or kinetics. Work by my collaborators in the same study (Lam 2009) showed that ubp2Δ cells displayed a normal rate of sorting of the fluorescent lipid-binding dye FM4-64 to endosomes and eventually to the vacuolar membrane, and that these cells also had normal endosome and vacuolar morphologies. Therefore, the defect in sorting seen in ubp2 is not due to indirect defects caused by abnormal endosome sorting and morphology. The accumulation of Fur4-GFP seen at
Figure 3-8: *ubp2Δ* cells are sensitive to alpha-factor consistent with a defect in Ste2 receptor sorting. alpha-factor (5µl of 100µM, 40µM or water alone) was spotted onto sterile filter disks, and placed onto solid media containing WT, *ubp2Δ*, *rup1Δ*, or *end3Δ MAT a* cells. Plates were then incubated at 30°C for one day and imaged. Halo diameters (mm) were measured and averaged from five replicates of 100µM alpha factor spots.
the plasma membrane under uracil induced conditions could be due to a defect in internalization or an increase in recycling of Fur4 to the plasma membrane. We saw that the internalization step itself did not seem to be impaired. Since the defect in Fur4-GFP trafficking appears only in recycling permissive conditions in \textit{ubp2}Δ cells, our data imply that the accumulation of Fur4-GFP at the plasma membrane is most likely due to a more efficient recycling of the permease. An aberrant accumulation of membrane proteins at the plasma membrane can result from a defect in MVB formation, as has been reported with ESCRT mutants (Bugnicourt 2004). Therefore, it is likely that \textit{ubp2}Δ mutants have a defect in internalization into MVBs. This defect is not complete, however, as Fur4-GFP eventually reaches the vacuolar lumen following a lag (i.e. 120 minutes post uracil addition versus 30 minutes in WT cells). In addition, Ubp2, Rup1, and Rsp5 have been shown in other studies to be present at the MVB. Rsp5 was found to be located at various sites within the endocytic pathway, including plasma membrane invaginations, late endosomes, and MVBs by colocalization with Pep12, an endosomal marker, and Vps32, a component of an ESCRT complex located at the MVB (Wang 2001). Rsp5, Rup1 and Ubp2 have also been shown to interact with Hse1 (Ren 2007), a component of the ESCRT-0 complex located at the MVB. In addition to Fur4, I obtained evidence for a role for Ubp2 in the proper clearance of another membrane-bound protein, Ste2, from the plasma membrane, suggesting Ubp2 is generally required for efficient sorting.

Consistent with these results, Ubp2 was also shown in a parallel study by another group to be important for the Rsp5-mediated trafficking of a vacuolar protease, carboxypeptidase S (Cps), which is sorted to the vacuolar lumen from the Golgi via the MVB pathway (Ren 2007). In addition, a role for Ubp2 in properly sorting the membrane bound amino acid permease Gap1 directly from the Golgi to the MVB was also reported (Ren 2007). Interestingly, in a recent genome wide screen for genes involved in endocytosis, Ubp2 was not a high scoring hit (Burston 2009). In this screen, the localization of the membrane protein Snc1 was monitored by a colorimetric assay. This screen successfully detected genes involved in MVB trafficking and recycling, such as members of the ESCRT machinery (Burston 2009). It may be that the sorting defects seen in \textit{ubp2} mutants were too subtle for identification in this assay. Nevertheless, in my study (Lam 2009), I established for the first time, at least in the case of Fur4 (and possibly for Ste2), an additional involvement of Ubp2 in sorting of endocytic cargo from the plasma.
membrane to the MVB. It seemed, therefore, that Ubp2 plays an important role in modulating Rsp5-mediated trafficking of various types of membrane-associated cargo.

The role of Rup1 here is enigmatic. Residual Ubp2-Rsp5 binding was detected in the absence of Rup1, implying that Ubp2 and Rsp5 can interact, either directly, or possibly through another unknown adaptor protein. This could explain why Rup1 is not required for proper MVB sorting, although a minor sorting defect not apparent in the assay conditions tested cannot be ruled out. Experiments looking at the effect of mutating possible Ubp2-Rsp5 binding sites may be helpful in deciphering the biological significance of this direct Ubp2-Rsp5 interaction. Aspects of my genetic data imply that Rup1 has a positive role in Ubp2 activity. For example, deletion of RUP1 suppressed, albeit only partially, the temperature-sensitive phenotype of rsp5-1 mutants. The presence of a UBA domain in Rup1 is also suggestive. Ubiquitin-binding motifs, such as UBA or UIM (ubiquitin interacting motif) domains, are present in a sizeable fraction of proteins involved in intracellular protein trafficking (Hicke 2003), and have been suggested to facilitate the ‘passing-off’ of ubiquitinated endocytic cargo or components from one functional module in the processing machinery to another (Hicke 2003; Swanson 2003). Hence, Rup1 could play a complex role at the MVB, in both recruiting ubiquitinated proteins to the ternary complex and tethering Ubp2 to Rsp5.

What then, specifically, is the role of Ubp2? Although Ubp2 has been shown to deubiquitinate a non-essential ER-membrane bound transcription factor, Spt23, in vitro (Kee 2005), there is as yet no evidence for the physiological processing of Spt23 in vivo (M.L.; unpublished observations). Indeed, counterintuitive to expectation, our observation that an Ub-Fur4 fusion can rescue the sorting defect in ubp2Δ mutants suggests inefficient or improper ubiquitination of the internalized transporter.

One possibility is that Ubp2 is involved in ubiquitin chain length editing of internalized receptors and transporters at the MVB. Most substrates of the endocytic pathway are covalently attached with single ubiquitin moieties or short K63-linked ubiquitin chains (Galan 1997; Gitan 2000; Springael 1999b; Terrell 1998), although a Vps pathway substrate with a K63-linked poly-ubiquitin chain has been recently reported (Stawiecka-Mirota 2007). The finding that Ubp2 can efficiently and preferentially deubiquitinate K63-linked chains (Kee 2006) suggested a possible role for Ubp2 in editing Rsp5-catalyzed poly-ubiquitin chains down to a length suitable for
recognition by various proteins in the endocytic/ESCRT machinery. Although a difference in the ubiquitin profile of Fur4 in a WT versus ubp2Δ mutants was not detected, our Western blot assay represented the sum of all membrane compartments in the cell, and may not be sensitive enough to visualize a subtle kinetic defect at the MVB. It would be interesting and informative to look at the ubiquitination of Fur4 in conditions where more subtle differences in ubiquitination levels at the MVB could be detected.

A second possibility is a role of Ubp2 in the deubiquitination of the cargo (e.g. Fur4) at the MVB, which is then followed by a second, subsequent re-ubiquitination by Rsp5 for efficient sorting into the MVB. In support of this model, it was reported recently that Rsp5-dependent ubiquitination of cargo must occur at the MVB stage for proper sorting into MVBs (Leon 2008). Further support for this model was provided by my data showing a physical interaction between Rsp5 and Ubp2 through tethering by Rup1, allowing for a close physical proximity between the Ub ligase and the DUB at the MVB. Another DUB, Doa4, appears to play a somewhat related, but different, role at the MVB, as deubiquitination by Doa4 (present in the ubp2Δ strains used in my experiments) is not sufficient for the proper re-ubiquitination of Fur4. Although it has not yet been shown conclusively that deubiquitination must occur prior to cargo re-ubiquitination, this will be an interesting area to address in the future. In addition to cargo deubiquitination, I cannot currently rule out an (additional) indirect role of Ubp2 on the sorting machinery itself, which would add another possible level of complexity in the regulation of trafficking. I am also unable to exclude a role for Ubp2 at the plasma membrane, as there might have been subtle defects in ubp2Δ cells at the internalization step of endocytosis that remained undetected in my experimental conditions.

A third possibility is that Ubp2 may be involved in the regulation of members of the sorting machinery, specifically Rsp5-cargo adaptors. Ubp2 has been shown either in vitro or in vivo to deubiquitinate and/or stabilize three different members of the arrestin-related (ART) adaptor proteins (Kee 2006; Lu 2008). Although the ubiquitination of Fur4 appears to be regulated by multiple Rsp5 adaptors (Nikko 2009), a role for Ubp2 in the regulation of any of these ARTs, or other currently unknown adaptors may result in the sorting defect seen in ubp2Δ mutants cells. Some endocytic cargo, such as the lysine transporter Lyp1, have shown a preference for different ARTs when its internalization is triggered by different stimuli, such as lysine or cycloheximide (Lin 2008). Although Fur4 internalization has been shown to require the
same set of ART adaptors in the presence of substrate or cycloheximide (Nikko 2009), the
differential regulation of ARTs in these conditions, leading to the phenotype seen in ubp2Δ cells,
cannot currently be excluded. Further experiments, looking at both the stability of known ARTs
in ubp2Δ mutant cells, and at the involvement of any novel Rsp5 adaptors, may be useful in
elucidating the possible role of Ubp2 in the regulation of the trafficking machinery.

A fourth and especially attractive possibility to me is that Ubp2 is involved in modulating
the efficiency of Rsp5 ubiquitination, for example, by deubiquitinating auto-ubiquitinated Rsp5
or by sequestering Rsp5 to a specific cellular compartment or substrate. Deubiquitination of
Rsp5 would lead to the subsequent stabilization of the E3 Ub-ligase as is the case for many
previously identified E3-DUB physically interacting pairs as reviewed in Chapter 1 (Li 2004;
Mouchantaf 2006). Although an attractive hypothesis for Ubp2 function, the available evidence
actually pointed against this initially, as I detected no decrease in Rsp5 levels in ubp2Δ mutants
(data not shown), nor had Rsp5 been reported to be (auto)ubiquitinated in vivo. In Chapter 4, I
present evidence supporting this hypothesis, showing that Ubp2 may directly regulate the
activity of Rsp5.
Chapter 4

Ubp2 Regulates Rsp5 Ubiquitination Activity

*in vivo and in vitro*

This Chapter will form the basis of a manuscript which will be submitted for publication shortly:


In this Chapter, A. Emili supervised the experimentation and I performed all relevant experiments and analysis.
4. Ubp2 regulates Rsp5 ubiquitination activity \textit{in vivo} and \textit{in vitro}

4.1 Introduction

Rsp5, the sole member of the Nedd4 E3 ligase family in \textit{S. cerevisiae}, has been implicated in various steps in protein trafficking, including the endocytosis of proteins bound to the plasma membrane (reviewed in Horak 2003) such as the Fur4 uracil permease (Galan 1996) and the Mat \(\alpha\) receptor Ste2 (Dunn 2001). Apart from a key role in the ubiquitination of substrates immediately preceding internalization, Rsp5 has a known role in other steps in vesicle trafficking, such as sorting at the MVB (Morvan 2004) stage that precedes cargo delivery and degradation at the vacuole.

Similar to other Nedd4 ligases, Rsp5 contains specific domains which are important for function: an N-terminal C2 lipid binding domain, important for subcellular localization to endosomal membranes (Dunn 2004), three WW protein binding domains in the central region important for the recruitment of substrates and binding to accessory cofactors, and a HECT catalytic domain on the C-terminal end of the protein, responsible for ligase activity (reviewed in Ingham 2004). Various mutant strains of Rsp5 have been reported in the literature, including the temperature sensitive mutant, \textit{rsp5-1}, which has a leucine to serine substitution in the HECT domain (Wu 2001).

Regulation of Rsp5 activity occurs by various mechanisms, such as binding to adaptor proteins through specific protein-protein interactions. For example, the Rsp5 cofactors Bul1 and Bul2 have been implicated in dual roles in the Rsp5-mediated sorting of the amino acid permease Gap1 by facilitating ubiquitination at the plasma membrane (Soetens 2001), and by dictating the trafficking of newly synthesized Gap1 from the Golgi to the vacuole by specifying poly-ubiquitin chain length, presumably through modulation of Rsp5 activity (Helliwell 2001). In addition, binding of substrates by Rsp5 can occur either directly through one or more of its WW domains, or indirectly through additional adaptors such as Cvs7 (Art1) at the plasma membrane (Lin 2008) or Ear1 at the MVB (Leon 2008).
Association with a deubiquitination enzyme is another increasingly recognized mode of E3 regulation. As mentioned in Chapter 1, many E3s, for example the RING ligase Mdm2 (Li 2004), and the Itch ligase (Mouchantaf 2006), are complexed with DUBs. In general, deubiquitinating enzymes function to cleave auto-ubiquitinated ligases, resulting in their stabilization. In the case of yeast, my work (Chapter 3; Lam 2009) and that of others (Kee 2005) has shown that Rsp5 interacts both physically and functionally with Ubp2. This interaction is mediated in part through a specific co-factor, Rup1, that binds to both Ubp2 and Rsp5, but which has yet an unclear physiological role (Chapter 3; Kee 2005; Lam 2009). Ubp2 is important for proper trafficking at the MVB of Rsp5 membrane protein substrates, including the uracil permease Fur4 as described in Chapter 3 (Lam 2009). Although the data implied that Ubp2 may be involved in the proper ubiquitination of MVB substrates, the exact role and mechanism of regulation of Rsp5 by Ubp2 was unclear.

Deubiquitination by DUBs represent a common mode of ligase regulation (Li 2004; Mouchantaf 2006). Generally, E3 ubiquitination occurs through auto-ubiquitination, either through an intra-molecular (cis) or an inter-molecular (trans) mechanism. In almost all cases studied to date, E3 auto-ubiquitination results in the downregulation of the ligase itself through proteasome-associated degradation, such as in the case of the mammalian ligase Nedd4-2 (Bruce 2008), although cases of non-proteolytic ubiquitination, such as for Itch, have been reported (Scialpi 2008). Unlike many other E3 ubiquitin ligases, Rsp5 has not yet been reported to be ubiquitinated in vivo (prior to my own analyses elaborated below), and a catalytically inactive mutant is relatively stable compared to wildtype Rsp5 (Wang 1999). While Rsp5 has been reported to become auto-ubiquitinated under certain conditions in vitro (Huibregtse 1995), the physiological significance of this modification has not been demonstrated.

E3s can also be regulated through the intra-molecular folding of the enzyme. This has been reported in several cases, including the Itch ligase (Gallagher 2006) and the Nedd4-2 ligase (Bruce 2008). In the case of Nedd4-2, activity of the ligase is inhibited in the absence of substrate by physical associations between different domains on Nedd4-2 (Bruce 2008); the WW domains on Nedd4-2 can bind to a PY motif located within the HECT catalytic domain of the protein. Whether this occurs through a cis or trans (i.e. intra- vs inter-molecular) mechanism is not yet known. This binding results in an inactive enzyme, which then is thought to unfold/unbind only upon recognition and binding of a substrate, resulting in the activation of the
ligase (Bruce 2008). Activation of the ligase ‘on demand’, in turn, results in the ubiquitination and downregulation of both the substrate and the ligase itself by 26S proteasome degradation.

4.1.1  Project Rationale

My previous studies had revealed that Ubp2 has a complex regulatory relationship with the E3 Rsp5 and the cofactor Rup1. Although some initial studies had reported an antagonistic relationship between the two proteins (Kee 2005), my own work, and that of other laboratories have subsequently implicated Ubp2 as a positive effector of certain aspects of Rsp5-dependent ubiquitination and trans-membrane receptor internalization (Chapter 3, Lam 2009; Ren 2007).

Work by me and others has led to the hypothesis that this effect of Ubp2 may be due, in part, to editing of Rsp5 generated poly-ubiquitin chains to a suitable length for the substrate to participate in efficient trafficking processes. Ubp2 may also deubiquitinate the substrate upon completion of one event, such as the initial receptor internalization step, allowing Rsp5 to re-ubiquitinate the same substrate in later steps, such as during MVB formation. Although these models are promising, I could not exclude the possibility that Ubp2 exerts its primary effect on Rsp5 enzymatic function directly, perhaps by deubiquitination of a previously unreported ubiquitin modification on Rsp5 itself.

Given data from concurrent studies showing auto-ubiquitination as an important mode of E3 regulation in mammalian systems, the latter mode of regulation appeared to be a particularly compelling scenario for the regulation of the yeast Rsp5. Therefore, I proceeded with experiments aimed at determining if Rsp5 is indeed ubiquitinated under certain physiological circumstances in yeast, and if so, whether this occurs via a cis or trans auto-enzymatic manner. In addition, I wanted to determine what role, if any, Ubp2 may have in this activity. In this Chapter, I show for the first time that a significant fraction of Rsp5 can indeed be extensively ubiquitinated in vivo, but that this event is normally transient and can only be stabilized and hence detected in the absence of Ubp2. This effect is direct since Ubp2 is able to efficiently deubiquitinate recombinant Rsp5 in vitro. Moreover, similar to its mammalian homologues such as Nedd4-2, the presence of a known substrate or the binding of a cofactor (Rup1) to Rsp5 markedly stimulates auto-ubiquitination activity in vitro, implicating protein binding as a method
in which normally inactive Rsp5 is activated. These data implicate Ubp2 in a novel regulatory feedback mechanism with Rsp5, and suggest a tightly coordinated pattern of auto-ubiquitination and deubiquitination as a mechanism in the regulation of Rsp5 activity. Given my previous work showing a role for Ubp2 in MVB sorting (Chapter 3), these data suggest that Ubp2 likely facilitates proper Fur4 trafficking by acting directly on Rsp5 at the MVB, specifically via the modulation of Rsp5 activity upon substrate recognition through rapid deubiquitination and restoration to an active state.

4.2 Materials and methods

4.2.1 Strains and plasmids

A yeast expression plasmid encoding wild-type Rsp5 under control of its natural promoter pHA-Rsp5 (p[HA-RSP5, CEN, LEU2]) (Kaminska 2002) and the mutant clone pHA-rsp5-1 (pPC33, unpublished) were obtained from Teresa Zoladek. Bacterial expression plasmids for generating recombinant substrates pET15b-UBC4, pET21a-GST-TEV-CTD, pGEX-6P2-RSP5 are described in (Gupta 2007). A strain carrying an UBP2 allele (ubp2 C745S) in the BY4741 background (Chapter 2), encoding catalytically inactive Ubp2 was constructed in two steps: First, I disrupted the endogenous wildtype UBP2 locus with a cassette encoding URA3 in a haploid strain, creating an ubp2::URA3 loss of function allele. Then, the sequence surrounding the ubp2C745S mutation in yeast expression plasmid pFH426-ubp2-C745S (described in Chapter 3) was PCR amplified and transformed into the ubp2::URA3 strain. Transformants missing the URA3 cassette were selected for on media containing 5-Fluoroorotic Acid (5-FOA). Since some transformants exhibited a slow-growth phenotype, which could be the result of secondary/unintended mutations in the genome, 5-FOA transformants were mated to haploid mat α ubp2Δ cells, diploids were selected, and haploid segregants selected and scored after tetrad dissection. Clones carrying the ubp2 C745S allele and showing a growth rate comparable to wildtype cells were identified by genomic amplification and sequencing of the UBP2 locus. The TAP strains Rup1-TAP ubp2Δ and Ubp2-TAP are as described in Chapters 2 and 3.
4.2.2  Cell lysis, immunoprecipitation, and Western blots

Yeast cells were cultured overnight in rich (YPD) media, harvested, and resuspended in Yeast Extraction Buffer (YEB) and lysed by bead beating as described in Chapter 2 but with the addition of 10mM of the potent DUB inhibitor N-ethylmaleimide (NEM; Sigma) and 0.1nM of the proteasome inhibitor MG132 (Sigma). For anti-HA immunoprecipitations, soluble cell lysates (approximately 6 mg) were first precleared for 2 hrs at 4°C with protein-G agarose (Millipore), followed by immunoprecipitation with 0.5μl mouse monoclonal anti-HA antibody (generated from a cultured 12CA5 cell line; a gift from Mike Tyers, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario) and 20μl of a 50% slurry of protein-G beads for 1hr. Beads were washed extensively with lysis buffer, and bound proteins eluted using sample loading buffer, electrophoresed and transferred onto a nitrocellulose membrane as described in Chapter 2. Western blots were probed with mouse monoclonal anti-GST antibody (B-14, Santa Cruz Biotechnology; 1:2000 dilution), a mouse monoclonal anti-ubiquitin antibody (clone 6C1; Sigma; 1:5000 dilution), or a rabbit anti-Rsp5 antibody (Stamenova 2004). Incubation with secondary antibody and detection of target proteins by ECL were performed essentially as described in Chapter 2.

4.2.3  Purification of recombinant Ubc4, Rsp5, and CTD

Inducible bacterial expression vectors bearing HIS-UBC4, GST-CTD, and GST-RSP5 fusion cassettes were transformed into E. coli strain BL21(DE3) and recombinant proteins expressed and affinity purified essentially as described in (Gupta 2007) with some modifications. Cells were grown in LB media at 37°C with vigorous shaking to an OD590 of 0.6, and 1mM isopropyl-β-1-thio-D-galactopyranoside (IPTG) added to induce protein expression. Cells were further incubated for 12 hrs at 16°C, then pelleted and lysed in sonication/binding buffer (20mM HEPES, pH 8.0, 500mM NaCl, 10% glycerol, and 0.5mM tris(2-chloroethyl) phosphate (TCEP)) containing a protease inhibitor cocktail (Complete, Roche). For HIS-Ubc4, 5mM imidazole was added to the buffer to minimize non-specific binding during purification. After sonication, whole cell lysates were clarified by centrifugation at 80,000 xg for 1 h at 4 °C, and soluble protein incubated with either nickel-nitrilotriacetic acid agarose (Ni-NTA, Qiagen) to isolate the HIS-Ubc4 fusion protein, or glutathione sepharose (Amersham) to recover the GST-tagged proteins.
Beads were washed repeatedly with sonication/binding buffer (with 30mM imidazole added to minimize non-specific background for the Ni-NTA purification). Bound HIS-Ubc4 was eluted with sonication/binding buffer containing 500mM imidazole, while the GST-tagged proteins were eluted with sonication/binding buffer containing 15mM glutathione. Protein eluates were concentrated by centrifugal filtration (MW cutoff 10kDa; Microcon, Amicon-Millipore) as needed, and the purity and yield assessed by electrophoresis on a 4-12% Bis-Tris polyacrylamide gel (Invitrogen) together with BSA control protein to estimate concentration.

4.2.4  *In vitro ubiquitination and deubiquitination assays*

Ubiquitination assays in microtube format were done essentially as described in (Gupta 2007) with some modifications. Each reaction (15μl total volume) consisted of 5x assay buffer (250mM HEPES, pH 7.4, 25mM MgOAc, 2.5mM TCEP, 500mM NaCl, and 50% glycerol), 1 μg of purified yeast recombinant ubiquitin (Boston Biochem), 0.16 μg of recombinant commercial yeast E1 (Ube1; Boston Biochem), 3.8 μg of purified E2 (HIS-Ubc4), 1.2 μg of purified E3 (GST-Rsp5), 8 pmol of purified substrate GST-CTD, and 3.3mM ATP (Sigma). In reactions supplemented with tandem affinity-purified yeast Rup1, 3.9 μl of a dilute Rup1-TAP preparation was added. Water was added to each reaction to bring the final volume of all reactions to 15 μl. ATP was added last to initiate enzyme activity, at time = 0, to all reactions. Samples were incubated for 4 hrs at room temperature and stopped at indicated times by boiling in SDS-PAGE sample buffer.

For the deubiquitination experiments, samples were first subject to the ubiquitination conditions described above, and approximately 0.3 or 0.6 μg of a concentrated preparation of tandem-affinity purified yeast Ubp2-TAP (together with 1μg of the DUB inhibitor Ub-aldehyde (Affinity Research) as a negative control, where indicated) was added. Calmodulin elution buffer (see Chapter 2) was added to bring the final reaction volume up to a total of 30 μl. Reactions were then incubated for a further 2 hrs at room temperature to allow for deubiquitination to proceed, then stopped by boiling in sample loading buffer. The reaction products were analyzed by separation on a 4-12% SDS-PAGE gel and visualized by Western blotting using anti-GST antibody.
4.2.5 TAP tag purifications and deubiquitinating activity assay

TAP tag purifications of Ubp2-TAP and Rup1-TAP were performed on 4L cultures essentially as described in Chapter 2, except the final elution buffer was DUB reaction buffer (10% glycerol, 50mM Tris-Cl pH 7.9, 50mM NaCl, 1mM EDTA, 2mM DTT, and 3mM EGTA). Yield and purity of the purifications were estimated by SDS-PAGE followed by staining with silver. To confirm the deubiquitination activity of the Ubp2-TAP preparation, 1-30μl of the TAP preparation (0.5μg/ml) was incubated with 1.5μl K63-linked ubiquitin chain (Boston Biochem) (2mg/ml). The reaction was incubated for 1 hour at room temperature to allow for deubiquitination, and stopped by the addition of 3.6μl of 100% trichloroacetic acid (TCA), followed by incubation on ice for 30 mins, and centrifugation to collect precipitated protein. Precipitates were resuspended in sample loading buffer, along with unbuffered Tris to neutralize the sample. The above was repeated but with 1-5μl of ubiquitin aldehyde (1mg/ml) to confirm that deubiquitination was Ubp dependent.

4.3 Results

4.3.1 Rsp5 is ubiquitinated and deubiquitinated by Ubp2 in vivo

As previous studies, by myself (Chapter 3; Lam 2009) and others (Kee 2005), have shown that Ubp2 physically interacts with Rsp5, I examined a possible role of Ubp2 in modulating the ubiquitination status of Rsp5. I hypothesized that Ub-Rsp5 species might accumulate in DUB deficient cells. To this end, I looked for evidence of ubiquitination of yeast Rsp5 in vivo by Western blotting of soluble cell extracts prepared from both wildtype and ubp2 null mutants. To enable sensitive detection of Rsp5, I transformed a plasmid expressing an ectopic N-terminal HA-tagged allele of Rsp5 under control of its natural endogenous promoter (Kaminska 2002) into both a haploid control strain and a mutant strain deleted of the genomic copy of UBP2. Cultures were grown overnight, cell free lysates prepared in the presence of DUB and protease inhibitors, and HA-Rsp5 immunoprecipitated with a monoclonal anti-HA antibody. Although only a single dominant MW species was apparent in WT cells, highly sensitive chemiluminescent Western blotting revealed the presence of shifted mobility forms,
corresponding in size to mono and poly/multi-ubiquitinated forms, for HA-Rsp5 purified from ubp2Δ cells (Figure 4-1A, right panel). To confirm the identity of these higher-weight bands, the experiment was repeated, but the blot was probed with a monoclonal anti-ubiquitin antibody. Indeed, the lower mobility bands correspond to ubiquitin modified species (Figure 4-1A, left panel), although ubiquitinated Rsp5 appears to constitute only a small fraction of all the Rsp5 detected. Nevertheless, these results suggest that Rsp5 is indeed ubiquitinated in vivo, albeit seemingly transiently, and that Ubp2 may have a role in deubiquitinating Rsp5.

The simplest interpretation is that Ubp2 directly removes Ub chains added by Rsp5 autocatalytically. To test whether the catalytic function of Ubp2 was required for this effect, I generated a strain bearing a catalytically inactivated point mutation mutant allele at cysteine 745, a critical residue of the core Cys box motif, in the sole endogenous genomic copy of UBP2 and then examined accumulation of Ub-Rsp5. Indeed, the level of mobility shifted Ub-Rsp5 species in the mutant ubp2 C745S strain was equivalent to that of an ubp2 deletion strain (Figure 4-1B), implicating the catalytic activity of Ubp2 in the reversal of Ub-Rsp5.

4.3.2 Rsp5 is ubiquitinated outside of its catalytic site

Since Rsp5, as an E3 ligase, is expected to transiently bind ubiquitin via the catalytic cysteine in its HECT domain, one possibility is that the shifted species seen may have resulted from the trapping of covalent reaction intermediates. Therefore, I looked at the effect of adding the reducing agent DTT to the sample just before electrophoresis on the stability of Ub-Rsp5 generated in vivo and purified from an ubp2 deletion strain. This method has previously been reported to break the thioester bond at the catalytic cysteine of Rsp5 (Huibregtse 1995). However, the addition of 100mM DTT, similar to the standard SDS-PAGE sample reduction using 10% β-mercaptoethanol, resulted in the retention of the shifted Ub-Rsp5 species (Figure 4-2B). This result implies that the Ub-Rsp5 species I detected are due to the presence of one or more isopeptide-linked ubiquitin chains attached to the polypeptide backbone of Rsp5 (i.e. ubiquitin modifications occurring outside of the catalytic pocket), and not simply a reaction intermediate. This indicates that, as with other E3 proteins, Rsp5 is ubiquitinated, and likely at a location outside the catalytic site of the protein.
Figure 4-1: Rsp5 is ubiquitinated in the absence of Ubp2. Western blot showing modified and unmodified forms of Rsp5 (A) A plasmid containing a tagged version of Rsp5 (pHA-Rsp5) was transformed into both wildtype and ubp2Δ cells. Cells were lysed, and tagged Rsp5 immunoprecipitated with anti-HA antibodies. After protein transfer to nitrocellulose, tagged (Ub-HA-Rsp5) and untagged (HA-Rsp5) species of Rsp5 were visualized using anti-ubiquitin (left panel) and anti-HA antibodies (right panel). Whole cell extract (WCE) and cells lacking plasmid (WT) were also run as positive and negative controls, respectively. (B) This experiment was performed essentially as in (A), except with an increased exposure time in order to visualize lower mobility Ub-HA-Rsp5 species. Accumulation of ubiquitinated HA-Rsp5 was also evident in a strain bearing a catalytically inactive mutant allele in UBP2 (ubp2 C745S).
Figure 4-2: **Rsp5 ubiquitination is not an intermediate of the active site cysteine reaction.** (A) Schematic of the domains (C2, WW, and HECT) of Rsp5. Numbers indicate the amino acid residue flanking each domain (UniProtKB protein knowledgebase; www.uniprot.org). Attachment of an ubiquitin chain to the active site ‘C’ in the HECT domain would be sensitive to reducing agents such as DTT, whereas covalent ubiquitination outside of the active site, through an isopeptide bond, would be insensitive to reduction. (B) Reduction of the catalytic cysteine residue does not reverse Rsp5 ubiquitination. HA-Rsp5 was visualized by Western blot analysis against ubiquitin (anti-Ub), or the HA tag (anti-HA). Modified and un-modified HA-Rsp5 were immunoprecipitated from cell lysates from an ubp2Δ strain with anti-HA antibodies. The agarose beads were then subjected to treatment with sample loading buffer containing β-mercaptoethanol (standard), 100mM DTT, or no reducing agent. The absence of reducing agent perturbs protein mobility by SDS-PAGE.
4.3.3 **Rsp5 is auto-ubiquitinated**

Although the mammalian homologues of yeast E3 ligases are generally auto-ubiquitinated (Fang 2000; Scialpi 2008), examples of cross-ubiquitination by other E3 ligases have also been documented (Itahana 2007). Hence, to determine if the ubiquitin modification on Rsp5 is the result of auto-ubiquitination, or of the activity of another E3 ligase, I examined the effects of inactivation of Rsp5 catalytic activity on ubiquitination levels in vivo. To this end, I transformed a plasmid bearing an HA-tagged version of the conditional hypomorphic mutant \( rsp5-1 \) allele (HA-\( rsp5-1 \)) into haploid yeast cells bearing a deletion of the genomic copy of \( UBP2 \). HA-tagged Rsp5-1 protein was then immunoprecipitated using anti-HA antibody, and the ubiquitination status of Rsp5 examined by Western blotting. In \( ubp2 \Delta \) cells, HA-Rsp5-1 showed a notable reduction in the level of shifted Ub-Rsp5-1 bands when compared with cells expressing wildtype tagged HA-Rsp5 (Figure 4-3). This result implies that Rsp5 is auto-ubiquitinated, possibly from an intra-molecular (\( cis \)) reaction.

The low level of residual ubiquitination still detectable in the \( ubp2 \Delta \) mutant background could have been due to residual \( cis \) \( rsp5-1 \) activity, a secondary route of \( trans \) ubiquitination from the endogenous Rsp5 still present in these cells, or from an alternate ubiquitin ligase other than Rsp5. To address this, I expressed and purified HA-Rsp5-1 from a yeast mutant strain wherein the native \( RSP5 \) locus had been converted to a conditional allele (\( ubp2 \Delta \) \( rsp5-1 \)). In this case, no ubiquitinated forms of Rsp5 were detectable (Figure 4-3). Therefore, the minor residual ubiquitination seen in \( ubp2 \Delta \) cells is most readily explained as an inter-molecular (i.e. \( trans \)) ubiquitination modification by wildtype Rsp5. Taken together, these data collectively indicate that the majority of Rsp5 ubiquitinated forms I have detected are likely not due to the activity of other E3s, and that the primary effect of deleting Ubp2 is to reveal an innate intra-molecular (\( cis \)) ubiquitination event occurring on Rsp5.

4.3.4 **Substrate recognition stimulates Rsp5 auto-ubiquitination**

In the case of the mammalian HECT E3 Nedd4-2, the ligase is thought to remain in a folded, catalytically inactive conformation in the absence of a substrate, likely through intra- or
Figure 4-3: Rsp5 is auto-ubiquitinated. Plasmids expressing either wildtype Rsp5 (pHA-Rsp5 WT) or a conditional catalytic mutant of Rsp5 (pHA-rsp5-1) were transformed into a haploid yeast strain lacking UBP2 alone or lacking both UBP2 and containing a hypomorphic copy of RSP5 (rsp5-1). Cells were grown overnight, diluted in fresh media in the morning, and further incubated at the non-permissive temperature (37°C) to inactivate Rsp5-1. Cell lysates were then immunoprecipitated with anti-HA antibodies, proteins transferred onto a nitrocellulose membrane, and Rsp5 probed for with an anti-HA antibody.
inter-molecular binding between the WW domain(s) and a PY motif in the HECT domain (Bruce 2008). However, once a substrate binds to Nedd4-2, this inhibitory interaction has been proposed to become disrupted, causing a change in the enzyme leading to an active conformation (Bruce 2008). To explore whether this mechanism is conserved in Rsp5, I performed an \textit{in vitro} ubiquitination reaction both in the absence and presence of substrate. For the reaction, I made a master mix by combining recombinant N-terminally GST-tagged Rsp5 affinity-purified from \textit{E. coli}, along with recombinant E1 and E2 enzymes, and ubiquitin. Then, a model substrate, the carboxy-terminal domain of RNA polymerase II (CTD) also purified from \textit{E. coli} as a GST-fusion protein (Gupta 2007), was either added (Figure 4-4; +CTD lanes) or omitted (-CTD lanes). RNAP is ubiquitinated by Rsp5 \textit{in vivo} in response to transcriptional arrest (Beaudenon 1999; Somesh 2005), and the unphosphorylated form has been reported to be an excellent substrate for Rsp5 \textit{in vitro} (Kus 2005). Ubiquitination was initiated by the addition of ATP and allowed to proceed at room temperature for various periods of time to monitor progression. The reactions were stopped by the addition of sample loading buffer, and the samples separated by SDS-PAGE. The ubiquitination of both the Rsp5 (GST-Rsp5) and the substrate (GST-CTD) were monitored by Western blotting using an antibody directed against the GST tag.

As seen in Figure 4-4 (upper panel), shifted (i.e. ubiquitinated) CTD substrate bands, as expected, appeared with the addition of ATP, and higher molecular weight species increased as reaction time increased indicating multi or poly-ubiquitination. Strikingly, the CTD containing reactions (Figure 4-4; upper panel) also resulted in a markedly enhanced amount of shifted GST-Rsp5, corresponding to both mono-ubiquitinated and more extensively auto-ubiquitinated forms, as compared to the lanes in which no CTD was added (right panel). This result was confirmed by blotting for Rsp5 only using anti-Rsp5 (lower panel). Auto-ubiquitination of Rsp5 in the presence of the substrate increased over time, with the non-modified form correspondingly decreasing in abundance. At 4 hours, there is a marked increase in the low-mobility forms of Ub-Rsp5 at the top of the gel, corresponding to Rsp5 conjugated to many ubiquitins (either multi-ubiquitination or long poly-ubiquitin chains). In contrast, in the absence of substrate, very little, if any, multi/poly-ubiquitinated Rsp5 species were detectable, although some species of Ub-Rsp5 can be seen peaking at 15 minutes of incubation, corresponding to short ubiquitin chains. Moreover, even though there is little ubiquitination in these substrate-free reactions, the non-Ub
**Figure 4-4: A substrate (CTD) increases Rsp5 auto-ubiquitination.** Western blot analysis of an *in vitro* ubiquitination reaction with CTD (+ CTD lanes) or lacking CTD (-CTD lanes). The ubiquitin reaction (E1, E2, Rsp5, ATP, buffer, Ub, +/- CTD) was incubated from 15 minutes to 4 hours as indicated, and stopped by the addition of sample loading buffer. Ubiquitinated and unmodified forms of Rsp5 were detected with anti-Rsp5 antibody. The mobility of reference molecular weight markers is shown at the left.
Rsp5 band can be seen as decreasing in intensity over time. This can probably be attributed to residual proteases contaminating any of the purified proteins which were used in the reaction. In spite of degradation in these samples, the important feature is the preferential multi/poly-ubiquitin profile of Rsp5 in the presence of substrate (CTD). It must also be noted that, since both the CTD and Rsp5 used in this assay are tagged with GST which is known to dimerize, that it is possible that the effects seen here may be an artifact of a GST-GST interaction. This could be ruled out by the cleavage of the GST tag of both these proteins prior to the ubiquitination assay.

4.3.5 The Ubp2 cofactor Rup1 stimulates Rsp5 auto-ubiquitination

Since I and others have shown that Rup1 also binds Rsp5 directly (Kee 2005) (Chapter 3), I next examined a possible influence of Rup1 on the auto-ubiquitination of Rsp5 in vitro. Rup1 was affinity-purified from a TAP-tagged yeast strain lacking a functional copy of UBP2 to remove the possible confounding effects of deubiquitination. Addition of a sub-stoichiometric amount of Rup1-TAP (since TAP purification results in a relatively small amount of purified protein) to the Rsp5 in vitro ubiquitination mixture described above, in the absence of substrate, resulted in a significant increase in band intensities corresponding to mono and multi/poly-Ub-Rsp5 species as compared to reactions in which Rup-TAP was omitted (Figure 4-5). Rsp5 auto-ubiquitination increased over time, with the greatest amount of poly/multi-Ub-Rsp5 accumulating after 4 hours of incubation. The effect of Rup1 on Rsp5 auto-ubiquitination appeared similar, but less pronounced to that of the CTD substrate.

Taken together, these data show that, similar to its mammalian homologue Nedd4-2 (Bruce 2008), Rsp5 auto-ubiquitination is stimulated by the presence of a substrate or a cofactor. In the presence of these proteins, Rsp5 may undergo an induced conformational change, which causes an increase in catalytic activity leading to both enhanced substrate and auto-ubiquitination. Moreover, these results suggest that Rup1 mediates a possible regulatory effect independent of simply facilitating the recruitment of Ubp2 to Rsp5.
Figure 4-5: Rup1 stimulates Rsp5 auto-ubiquitination. Western blot analysis of an in vitro ubiquitination reaction with CTD (+ CTD lanes) or lacking CTD (-CTD lanes) in the presence or absence of Rup1-TAP (+/- Rup1) purified from a strain lacking UBP2. The reaction was incubated from 5 minutes to 4 hours as indicated and stopped by the addition of sample loading buffer. Ubiquitinated and unmodified forms of GST-tagged Rsp5 were detected with anti-GST antibody.
4.3.6  

Ubp2 deubiquitinates auto-ubiquitinated Rsp5 in vitro

The in vivo data (Figure 4-1) indicate that auto-ubiquitinated Rsp5 is stabilized in the absence of Ubp2. This suggests that Ubp2 may have a role in deubiquitinating Rsp5 and hence minimizing the accumulation of auto-ubiquitinated species. To examine this directly, I tested the ability of purified TAP-tagged Ubp2 (Ubp2-TAP) isolated from otherwise wild-type yeast (containing endogenous Rup1) to deubiquitinate Ub-GST-Rsp5 generated in vitro. First, TAP purified Ubp2 was tested to confirm that it possessed deubiquitination activity in vitro. As Ubp2 has shown a preference for the deubiquitination of K63-linked Ub chains (Kee 2006), various amounts of the Ubp2 preparation were incubated along with K63-linked poly-ubiquitin chains. Ubp2 was able to deubiquitinate poly-Ub chains fully down to mono-ubiquitin, while the reactions containing lower amounts of Ubp2 correspondingly had less DUB activity, resulting in residual di- and tri-ubiquitin chains (Figure 4-6A). To confirm that proteolysis was due to deubiquitination by a DUB, the deubiquitination reactions were repeated, but with the addition of the DUB inhibitor Ub aldehyde (Figure 4-6B). As expected, increasing amounts of Ub-aldehyde resulted in the inhibition of Ubp2 deubiquitination (Figure 4-6B). Therefore, the Ubp2-TAP preparation was indeed positive for deubiquitination activity.

I next tested the ability of Ubp2 to deubiquitinate Rsp5. First, I performed an in vitro ubiquitination reaction in which CTD was added as a substrate to stimulate auto-ubiquitination. I allowed the reaction to proceed for 4 hrs to shift nearly all of the Rsp5 to the modified form (evident as an extensive smear on a Western blot). Various amounts of affinity-purified Ubp2-TAP, a DUB inhibitor (ubiquitin aldehyde) included as a negative control, or buffer alone, were then added to the reaction, and the mixture incubated at room temperature for a further 2 hrs. Finally, the ubiquitination status of GST-Rsp5 was monitored by a Western blot using antibodies directed against GST. As seen in Figure 4-7, the addition of Ubp2-TAP resulted in a dramatic reduction in the level of auto-ubiquitinated bands, and a corresponding increase in both the non-ubiquitinated and mono-ubiquitinated forms of Rsp5 compared to control (i.e. buffer only). It is interesting to note that certain shifted species (mono and short ubiquitin chains) directly above non-ubiquitinated Rsp5 remain visible, even after deubiquitination by Ubp2 for 2 hrs. This is in line with a previously reported preference of Ubp2 for processing longer ubiquitin chains,
Figure 4-6: The Ubp2-TAP preparation is catalytically active. (A) K63-linked ubiquitin chain was incubated in *vitro* with varying amounts of TAP purified Ubp2 to check for deubiquitination activity. The reaction was stopped and proteins precipitated by the addition of trichloroacetic acid (TCA), electrophoresed and visualized by staining with silver. The grey arrow bar indicates decreasing amounts of Ubp2 added, and the grey bar indicates an equivalent amount of Ub chain added in each reaction. Arrows point to the location of the various mono and poly-ubiquitin species. Mono ubiquitin (Ub) was located at the dye front (B) To test for DUB specificity, the experiment was repeated, with an equivalent amount of Ubp2 and K63 chain in each reaction. Increasing amounts of ubiquitin-aldehyde, a DUB inhibitor was added to each reaction (grey arrow bar). The mobility of reference molecular weight markers is shown at the left. Mono and di-ubiquitin (Ub, Ub-Ub) were located at the dye front.
Figure 4-7: Ubp2 deubiquitinates Rsp5 in vitro. Western blotting showing Ubp2 reversal of shifted Rsp5-Ub species after an ubiquitination reaction was performed in the presence of CTD to allow for Rsp5 auto-ubiquitination (Ub rxn). Ubp2-TAP (+ Ubp2), a two-fold amount of Ubp2-TAP (++ Ubp2), or buffer only (-Ubp2) was added, along with the DUB inhibitor ubiquitin aldehyde (+ Ub-ald) as a negative control, and allowed to incubate for a further 2 hours (+ incub). Modified and unmodified forms of GST-tagged Rsp5 were detected with anti-GST antibody. Highly ubiquitinated forms of Rsp5 are not visible, likely due to a difficulty in the transfer of large proteins.
wherein it is less effective at removing the short ubiquitin chains and the terminal ubiquitin (Kee 2006). In the case of the CTD substrate examined here, a substantial amount of unmodified and mono- Ub CTD is recovered. As with my in vivo results, I confirmed that the deubiquitination of Rsp5 was due to Ubp2 catalytic activity, and not degradation, as the effect was almost fully blocked by addition of the DUB inhibitor ubiquitin aldehyde to the reaction (Figure 4-7).

Collectively, these results, similar to other studies on DUB-E3 complexes, provide novel mechanistic insight and establish Ubp2 as a direct regulator of Rsp5, capable of deubiquitinating auto-ubiquitinated Rsp5. As auto-ubiquitination of Rsp5 does not seemingly destabilize it in vivo (Figure 4-3), my data implicate Ubp2 in the possible regulation of Rsp5 through a non-degradative role, perhaps in the modification of Rsp5 activity, substrate turnover and/or subcellular localization.

4.4 Discussion

4.4.1 Detection of a novel ubiquitin modification on Rsp5

Many E3 ubiquitin ligases are auto-ubiquitinated and numerous examples exist in which E3s are associated with a deubiquitinating enzyme. These associated DUBs often appear to function to reverse auto-ubiquitination (Li 2004; Mouchantaf 2006) as well as possibly acting as deubiquitinating enzymes on E3 substrates (Li 2002a; Li 2005). In this Chapter, I present data showing that the yeast ligase Rsp5, like its mammalian homologues Itch and Nedd4-2, is auto-ubiquitinated. Although this modification has previously been detected in vitro, my work provides the first documentation of Rsp5 auto-ubiquitination in vivo, which suggests that the modification may be biologically significant. Rsp5 auto-ubiquitination may not have been apparent previously in a physiological context because it is transient and reversed by Ubp2 and possibly other deubiquitinating enzymes. This notion is supported by data showing that a catalytically inactive point mutant of Ubp2, ubp2C745S, accumulates Ub-Rsp5 comparable to a full ubp2 deletion, and my in vitro data showing that affinity purified Ubp2 is able to deubiquitinate extensively auto-ubiquitinated Rsp5.
I have established that the ubiquitin modification on Rsp5 is likely not simply a catalytic E3 intermediate present at the active cysteine on Rsp5, but rather one or more covalently bound chain(s) elsewhere on the enzyme that are insensitive to reducing agents. Mapping an ubiquitination site on Rsp5 would be helpful in determining what effect the modification could have on the ligase, and could guide follow-up experiments. For example, if the ubiquitination site was found to be in one of the WW protein binding domains, the modification may function to block interactions with substrates or co-factors. However, despite repeated efforts, shotgun tandem mass spectrometric analysis of proteolytic digests of affinity purified Ub-Rsp5 generated \textit{in vivo} in a \textit{ubp2} null mutant background did not reveal any compelling putative ubiquitination sites (ML and JH, unpublished observations), possibly because of the inefficient ionization or heterogeneity of the ubiquitinated Rsp5 species. Additional analysis of Ub-Rsp5 generated \textit{in vitro} could offer an alternative avenue of follow-up in the future.

It is currently unknown whether Rsp5 is subject to long chain (poly-ubiquitination), multi-site ubiquitination, or if the modification consists of covalent attachment of several short linked chains. Since most blots of ubiquitinated Rsp5 showed several discrete bands, this would suggest several levels of poly- or multi-ubiquitination. It should be possible to determine the type of ubiquitin chain present on Rsp5, either by using chain type specific antibodies (Newton 2008; Wang 2008), tandem mass spectrometry, or a panel of site–specific lysine mutant ubiquitin chains to prime the \textit{in vitro} ubiquitination reaction. Relevant to this, Rsp5 and Ubp2 have been reported to catalyze the ubiquitination and deubiquitination (Kee 2006), respectively, of K63-linked chains, which suggests the presence of auto-ubiquitinated K63-linked chains on Rsp5.

\subsection{Rsp5 is auto-ubiquitinated through an intra-molecular mechanism}

Another fascinating avenue to consider is the molecular mechanism by which the auto-ubiquitination reaction occurs. From previous studies on mammalian ubiquitin ligases (Fang 2000; Luhtala 2004; Mouchantaf 2006; Scialpi 2008), the attachment of a ubiquitination chain to the ligase can either result from an intra-molecular reaction, in that the activated enzyme itself transfers ubiquitin to a residue on the same molecule, or as an inter-molecular reaction, wherein the ligase will ubiquitinate another enzyme molecule, such as another subunit of a dimer. From my results, it seems that ubiquitination on Rsp5 happens preferentially as an intra-molecular
reaction *in vivo*, although some low level of *trans* ubiquitination does occur as the remaining (a small percentage of the total) Ub-Rsp5-1 smear is eliminated when the genomic wildtype copy of Rsp5 is deleted. *Trans* ubiquitination of Rsp5 is, however, consistent with reports showing that Rsp5 has the ability to form dimers (Dunn 2001). The disappearance of detectable Ub-Rsp5-1 species in the absence of functional endogenous Rsp5 also implies that no other ubiquitin ligases can stably ubiquitinate Rsp5 under standard yeast culture growth conditions, at least with a high enough efficiency to generate a band detectable by Western blot analysis.

4.4.3 *Binding to substrates or cofactors may unfold and activate Rsp5*

Given that intra-molecular ubiquitin transfer seems to be the most plausible route by which Rsp5 is auto-ubiquitinated *in vivo*, I wanted to determine if an Rsp5 substrate, such as a recombinant form of the carboxy-terminal domain (CTD) of RNA polymerase II, influences the rate or degree of auto-ubiquitination of Rsp5. As evidenced by my *in vitro* data, in the presence of CTD, ubiquitinated Rsp5 increases over time, with the Ub-Rsp5 species increasing in both in terms of chain length and/or the number of attached ubiquitins. This effect can be compared to reactions without ectopic substrate, for which there is a shifted ubiquitinated species corresponding only to mono-Ub or a short poly-Ub chain.

Since the addition of an Rsp5 substrate markedly increases Rsp5 auto-ubiquitination, it is tempting to speculate that, similar to the model proposed for its mammalian homologue Nedd4-2 (Bruce 2008), that Rsp5 is maintained in a ‘non-active’ conformation or a poised folded state in the absence of suitable substrates or co-factors such as Rup1. This ‘inhibited’ condition, as in the case with Nedd4-2, could be due to an intra- or inter-molecular interaction between one or more WW domains in the middle of Rsp5 with a sequence in the HECT domain. Upon inspection of the amino acid sequence of Rsp5, I noticed that, similar to mammalian Nedd4 family ligases, that Rsp5 does indeed possess a ‘LPQY’ motif in the HECT domain, which may serve as a candidate for binding to the WW domains. Upon recruitment of a binding partner or a substrate such as RNAPII CTD to the enzyme, Rsp5 may become unfolded as the WW domain shifts to bind the new factor, allowing the HECT-located PY motif on Rsp5 to become displaced such that the catalytic activity is engaged and the HECT domain free to ubiquitinate both the substrate and Rsp5 itself. This event may serve as a negative feedback mechanism, in which the presence of an
Rsp5 substrate leads to the inactivation of Rsp5 back to a resting ground state immediately after ubiquitination of the target. On the other hand, physical association of Rsp5 with Ubp2 would result in the deubiquitination and re-poising of Rsp5. Taken together, the data presented in this Chapter suggest that a deubiquitinating enzyme in yeast, similar to well characterized DUBs in mammalian systems, may be involved in a complex and important role in regulating ubiquitination through the direct modification of a ubiquitin ligase.
Chapter 5

Summary and Conclusion
5. Summary and Conclusion

5.1 Summary

Deubiquitination is an important mode of regulation in many cell processes. As shown by many examples of work in mammalian systems, DUBs affect cell processes by either regulating the ubiquitination of a substrate, or by modifying the ubiquitination machinery, in particular the E3 ubiquitin ligase itself. Deubiquitinating enzymes in the budding yeast *S. cerevisiae*, on the other hand, were not well characterized when I commenced my thesis research and there remain many open questions about their regulatory role(s). Studies published on a few yeast DUBs have described some interesting pathways and mechanisms in which these enzymes participate, implying that other DUBs may also participate in important processes in the cell.

In this thesis, I described my efforts to elucidate the functions of various Ubps via discovery of their interaction partners. In Chapter 2, I showed data stemming from my initial proteomics investigation into the protein binding partners of these enzymes, through protein tagging and affinity purification of stable protein complexes coupled with the identification of Ubp-interacting proteins by mass spectrometry. I described the protein partners of many of these Ubps, confirming some physical interactors which were reported by others around the start of my thesis studies, and others which both I and others have since validated and assessed for functional significance. From these protein-protein interaction networks, I selected two Ubp-containing complexes that I found particularly interesting for further focused investigation. In Chapter 2, I described my work on elaborating the biological significance of the Ubp6-Sem1-19S proteasome complex, which led to the discovery of a role for this complex in the repair of DNA damage.

In Chapters 3 and 4, I presented the results of my follow-up studies on a putative heterotrimeric Ubp2-Rsp5-Rup1 protein complex. Using a variety of assays, I showed that Ubp2 is involved in the regulation of protein trafficking, specifically at the step of MVB sorting of ubiquitinated cargo, a role in which Rsp5 had been previously been implicated. In addition, I found that Ubp2 may regulate sorting through the direct deubiquitination of Rsp5, which I have shown for the first time is auto-ubiquitinated *in vivo* under physiological conditions. Although my data show that Ubp2, Rsp5, and Rup1 have a complex regulatory relationship, many details
of their coordinated participation in intracellular protein trafficking currently remain unclear. As elaborated in this Chapter, I developed a testable working model that accommodates most of my major findings. While my discussions are centered on Ubp2, I also suggest a novel role for Rup1 as a cofactor in modulating Rsp5 activity.

5.2 The role of Ubp2 and Rup1 in regulating Rsp5-dependent processes

5.2.1 What happens to ubiquitinated Rsp5?

As presented in Chapter 4, I found that Rsp5 is auto-ubiquitinated in vivo, a modification which Ubp2 is able to reverse both in vivo and in vitro. Evidence, both from work reported in this thesis and from published literature (Wang 1999), has established that proteasomal degradation is an unlikely fate for Ub-Rsp5, as catalytic mutants of Rsp5 are as stable as the wildtype forms. Given that ubiquitinated Rsp5 is seemingly not rapidly degraded by the proteasome, if poly-ubiquitination occurs it likely involves K63 chains consistent with the known catalytic activity of Rsp5.

What then is the molecular fate of ubiquitinated Rsp5? Recent studies have pointed to several non-degradative roles of ubiquitination (reviewed in Chapter 1), particularly K63-linked chains, in regulating protein function. Indeed, the ubiquitination of the mammalian HECT ligase Itch results in an as yet unknown non-degradative fate for the enzyme (Scialpi 2008).

One possibility is that auto-ubiquitination could result in the re-localization of Rsp5 protein to another part of the cell. Rsp5 has been reported to be present in various parts of the cell, most notably in various sites along in the endocytic and vesicle trafficking pathways (Wang 2001). Recent studies have implicated ubiquitination as an important signal specifying the intracellular localization of proteins. For example, ubiquitination of the tumour suppressor protein, p53 can prevent its recognition by proteins mediating nuclear import, thus blocking its entry into the nucleus (Marchenko 2009). Therefore, it is possible that auto-ubiquitination of Rsp5 acts in a similar manner, and could represent one signal by which Rsp5 is re-directed to various compartments and new roles after ubiquitinating a substrate. ‘Activation’ of Rsp5 by
Ubp2 deubiquitination in this case then, may actually occur through a change in the localization of Rsp5, and therefore a change in the possible subset of Rsp5 substrates.

A second possibility is that auto-ubiquitination prevents binding of Rsp5 to one or more of its co-factors, interacting proteins, or even certain substrates. In this case, auto-ubiquitination would lead to differential substrate recognition or result in a change in Rsp5 activity. This could be tested easily by monitoring the binding of various known co-factors and substrates of Rsp5 to both ubiquitinated and non-ubiquitinated forms of Rsp5. In this case, the ubiquitination of Rsp5, and the reverse, deubiquitination by Ubp2, could therefore lead to shuttling between pathways or processes due to differential protein binding. Although this idea is speculative, the ubiquitination of Rsp5 may disturb one or more downstream events in the cell through this mechanism.

A third possibility and the most intriguing to me for the role of auto-ubiquitination is the direct regulation of Rsp5 activity. Ubiquitination has been reported to result in the downregulation of E3 ligase activity without resulting in proteolysis. For example, auto-ubiquitination of the E3 ligase Mdm2 results in the modification of Mdm2 activity by a non-proteolytic mechanism, in addition to resulting in degradation of Mdm2 itself, as a mutation in an auto-ubiquitination site of the E3 ligase Mdm2 results in an increase in ligase activity (Buschmann 2000). Similarly, in the case of Rsp5, ubiquitination may result in a decrease in enzymatic activity, possibly through a conformation change in the ligase. Auto-ubiquitination of Rsp5 may proceed until a suitable Ub chain length is reached for the inhibition of Rsp5 activity. Specifically, since the deletion of UBP2 somewhat paradoxically causes a phenotype of Fur4 internalization similar to a substrate level ubiquitination defect (Chapter 3), the accumulation of ubiquitinated Rsp5 in vivo (albeit to substoichiometric levels) may result in a down-regulation of Rsp5 ligase activity at the MVB, leading to the protein trafficking defect I observed in ubp2 mutant cells. The activity of both ubiquitinated and non-ubiquitinated forms of Rsp5 could be tested in vitro by examining the ubiquitination levels of well characterized in vitro substrates such as the RNAPII CTD used in Chapter 4. However, it must be noted that a loss of Ubp2 activity may only result in a mis-regulation of the specific subset of Rsp5 ligase actually associated with Ubp2/Rup1 in vivo, as the essential function of Rsp5 is seemingly unperturbed in ubp2Δ deletion mutants (i.e. ubp2Δ cells do not exhibit a growth defect when assayed on rich media as compared to conditional rsp5 alleles such as rsp5-1).
5.2.2 The role of Rup1 in Rsp5 regulation

Rup1 was identified and named by me, in work performed as part of this thesis (Chapter 3; Lam 2009), as well as in the published literature by a competing group (Kee 2005), as a protein which interacts physically with Rsp5. Although some data, such as the enhanced deubiquitination by Ubp2 of an *in vitro* substrate in the presence of Rup1 (Kee 2005), hint at a role in regulating Ubp2 functions, the exact role of Rup1 remains to be elucidated. It is telling then, that Rup1 was found, similarly to CTD, to increase auto-ubiquitination of Rsp5 *in vitro* (Chapter 4). Since Rup1 has a PY motif, which is predicted to bind the WW domains of Rsp5 (Chapter 3), Rup1 binding, therefore, may result in a conformational change in Rsp5 to an active state, poised for substrate modification. Examination of Rsp5 substrate ubiquitination efficiency in the presence of Rup1 may be helpful in elucidating the role of this protein.

A related possibility for Rup1 and Ubp2 function maybe in the cooperative stimulation of Rsp5 ligase activity (Figure 5-1). In this model, Rup1 binding would cause the unfolding and activation of Rsp5, while Ubp2 keeps Rsp5 un-ubiquitinated and therefore active and poised for substrates. The net effect would be a stabilization of Rsp5 activity along a particular pathway. This is similar to a model proposed for Itch (Azakir 2009), in which an extracellular signal results in the phosphorylation of, and increase of Itch activity through unfolding of the ligase. Simultaneously, an increase in the association of Itch with USP9X (a DUB) results in the stabilization of Itch from degradation by the proteasome due to the reversal of auto-ubiquitination.

In addition, the participation of the putative ubiquitin binding domain (UBA) of Rup1 in Rsp5 regulation remains to be elucidated, although it is tempting to speculate that it may function in binding to ubiquitinated Rsp5, perhaps resulting in the strengthening of the physical interaction between the two proteins once Rsp5 is activated. Another interesting but unanswered question is whether Ubp2 and Rup1 are involved in the regulation of all of the Rsp5 in the cell. While both Ubp2 and Rup1 form a 1:1 complex, they appear to interact substoichiometrically with Rsp5, at least in the buffer conditions tested. As silver staining is semi-quantitative, staining with Coomassie blue may be a better choice in determining the exact stoichiometries of this complex. It is likely that most Rsp5 in the cell is not bound by Ubp2/Rup1, implying that Ubp2/Rup1-dependent regulation of Rsp5 occurs on only in a small percentage of cellular Rsp5,
and by extension, in a small subset of Rsp5 functions, although it cannot be ruled out that Ubp2-Rup1 may interact with the majority of Rsp5 in the cell, but only transiently. Non-Ubp2/Rup1 bound Rsp5 then, would presumably be in a folded, inactive state until binding by a substrate or possibly another cofactor (Figure 5-1).

5.3 Conclusion

In this thesis, I show that a proteomic-based approach can be very useful in elucidating the functions of a specific group of deubiquitinating enzymes, the yeast Ubps. Future work, including refinements to the microarray deubiquitination assay I devised and elaboration of the functional relationships suggested by the genetic interaction screens could potentially fill in the gaps in our knowledge about this set of enzymes and their crosstalk with E3 ligases. Although the studies presented here were focused in yeast, the generally high conservation of basic DUB structure and function relationships, including their relevant pathways, from yeast to higher Eukaryotes permit the extrapolation of the insights gained from these assays to human systems.
Figure 5-1: Model of Ubp2 function. (A) Rsp5 is in a folded, non-active conformation. (B) In the absence of Ubp2-Rup1, Rsp5 binds a substrate, (S), and is unfolded and active, ubiquitinating both the substrate and itself. After the substrate is ubiquitinated, Rsp5 remains ubiquitinated, which may render it inactive or lead to re-localization. (C) In the presence of Ubp2 and Rup1, the tertiary complex forms. Rup1 binding results in the activation of Rsp5, leading to both substrate and Rsp5 ubiquitination. Ubp2 is able to deubiquitinate Rsp5, likely producing a mono-ubiquitin form, and Rsp5 therefore can remain active. Ubp2 may also deubiquitinate the substrate itself.
References


Chen L, Davis NG (2002a) Ubiquitin-independent entry into the yeast recycling pathway. Traffic 3: 110-123.


Ribar B, Prakash L, Prakash S (2007) ELA1 and CUL3 are required along with ELC1 for RNA polymerase II polyubiquitylation and degradation in DNA-damaged yeast cells. Mol Cell Biol 27: 3211-3216.


Rittberg DA, Wright JA (1989) Relationships between sensitivity to hydroxyurea and 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (MAIO) and ribonucleotide reductase
RNR2 mRNA levels in strains of Saccharomyces cerevisiae. Biochem Cell Biol 67: 352-357.


Appendix

Promising Preliminary Experimental Avenues and Future Directions

In this Chapter, A. Emili supervised the experimentation and I performed all relevant analysis. I also performed all of the described experimentation, with the exception of the proteome array screen, where I was provided with some purified protein reagents from the Rotin lab, the SGA screens, in which I was assisted by J. Rilstone, and some tetrad analysis of genetic interactions which was performed by MQ. Huangfu and M. Raghavan under my supervision.
Appendix: Promising Preliminary Experimental Avenues and Future Directions

An experimental approach to identify substrates of deubiquitinating enzymes

From the data presented in Chapters 3 and 4, my results suggested that Rsp5, and perhaps trafficking related protein cargo substrates, are targets for Ubp2-mediated deubiquitination. In addition to sorting at the MVB, Rsp5 has been implicated in many other functions in the cell, including protein sorting at other cellular organelles (Belgareh-Touze 2008), events in the nucleus such as the ubiquitination of RNAPII (Beaudenon 1999; Somesh 2005), and the partial cleavage and release of transcription factors at the ER (Hoppe 2000; Shcherbik 2003). As Rsp5 is involved in these events, it is also possible that Ubp2 may also modulate these and other Rsp5-related functions, perhaps by deubiquitinating Rsp5 substrates in addition to Rsp5 itself.

An affinity-tagging approach such as that described in Chapter 2 may not be the best method by which to search for additional substrates, as some substrate-Ubp2 interactions may be transient and not survive the TAP purification protocol in order to be identified. Therefore, I decided to take a complementary in vitro proteomic screening strategy to identify additional Ubp2 substrates by performing a direct deubiquitination assay using a yeast proteome microarray. The microarray chip, on a single glass slide, is spotted with thousands of purified yeast proteins. It has been used successfully for various types of assays, including a search for novel substrates of Rsp5 and Nedd4 ligases by the use of fluorescently tagged ubiquitin which can be detected on the microarray when substrates are ubiquitinated (Gupta 2007; Persaud 2009).

I adapted this assay to probe for Ubp2 mediated deubiquitination of substrates after Rsp5 catalyzed labeling. In this Appendix, I report my preliminary data showing successful ubiquitination and deubiquitination of select proteins on the chip. From my results, I have generated a list of putative Ubp2 substrates, and I describe my efforts to confirm one of these high scoring substrates, Fra1, as a physiologically relevant in vivo target of both Rsp5 and Ubp2. The method and data described in this section provide a novel means to identify substrates of DUBs in general, and can be extended to screen for substrates of other yeast Ubps or mammalian DUBs in the future.
Materials and methods

Purification of recombinant proteins

E1, E2, and Rsp5 were purified essentially as described in Chapter 4.

Purification of Ubp2-TAP

TAP purification of Ubp2-TAP was done essentially as described in Chapter 4, with DUB reaction buffer as the final elution buffer.

Ubiquitination and deubiquitination reactions

Ubiquitination using recombinant Rsp5 and associated factors was reconstituted in vitro using a proteome microarray chip (ProtoArray Yeast Proteome Microarray chip v1.0; Invitrogen) essentially as in (Gupta 2007). Briefly, the chip was first washed with 0.5% PBST, and nonspecific adsorption blocked with 5% milk in PBST for 1 hour. After washing the slide extensively with PBST, a 600µl ubiquitination mixture consisting of 50mM HEPES, 5mM MgCl₂, 0.5mM TCEP, 10% glycerol, 4μg FITC-ubiquitin (Boston Biochem), 0.64μg E1 (Boston Biochem), 15.2μg E2 (Ubc4), 4.8μg Rsp5, and 2μl of 100mM ATP was applied to the slide. The slide was then carefully covered with a coverslip to assure an even distribution of the mixture, and incubated for 3 hours at room temperature in the dark to let the reaction proceed. The slide was then washed extensively with PBST, dried by centrifugation, and scanned to visualize fluorescent spots indicating incorporation of the fluorescent (FITC)-ubiquitin with a ProScan Array HT scanner (Perkin Elmer) at 10µm resolution with a 488nm laser. For the subsequent deubiquitination reaction, the slide was washed extensively with PBST, followed by the addition of a highly purified preparation of Ubp2-TAP (0.3μg) in the presence of DUB reaction buffer (Chapter 4). The slide was again covered with a coverslip and incubated at room temperature for a further 2 hours, after which it was rinsed, dried, and re-scanned as described above.
**Data analysis**

Data were analyzed essentially as described in (Gupta 2007). Spot intensities were quantified with ProScan Array HT software (Perkin Elmer). Proper scanning and quantification were first confirmed by checking for the presence of Alexa fluor antibody control spots on all subarrays. To select positive hits, an ubiquitination cutoff criterion was implemented, in which the signal from at least 50% of pixels on a given spot must be greater than two standard deviations above the background. Hits were considered if both spots for a substrate (as each protein was spotted in duplicate on the slide) passed this threshold. For all proteins that matched this initial cutoff criteria, an ‘ub score’ (defined as (the mean intensity of the spot - background) divided by the protein concentration) was calculated. This method of scoring was repeated to quantify the deubiquitination reaction, resulting in an analogous ‘dub score’. To define Ubp2 substrates, comparison of the two scores, ‘ub/dub’, was calculated by dividing the ub score with the dub score. To correct for slight variances in spot intensity due to individual spot position (systematic errors), a normalized score ub/dub score was calculated by plotting spot position vs ub/dub score. A linear line of best fit was generated and used to normalize the scores.

**Fra1 Western blots**

Expression of GST-tagged Fra1 was performed using a previously described plasmid construct (pGST-Fra1; Sopko 2006). Expression was induced by growth of log-phase cells (OD$_{600}$ = 0.5) in YP media upon addition of 1% galactose at 30°C or 37°C for 4 hours. Cells were harvested and lysed by glass bead beating in YEB (as described in Chapter 2), but with the addition of 10mM N-ethylmaleimide (NEM, a general DUB inhibitor; Sigma) and 0.1nM MG132 (proteasome inhibitor; Sigma). GST-Fra1 was precipitated from the lysate with glutathione sepharose beads (Amersham), washed extensively with lysis buffer, and the proteins eluted, electrophoresed and transferred onto a Western Blot membrane as described in Chapter 2. Western blots were probed with mouse monoclonal anti-GST antibody (B-14, Santa Cruz Biotechnology; 1:2000 dilution) or with mouse monoclonal anti-ubiquitin antibody (clone 6C1; Sigma). Incubation with a horseradish peroxidase-conjugated sheep anti-mouse secondary antibody and detection of target proteins by enhanced chemiluminescence were performed as described in Chapter 2.
Monensin sensitivity assay

Strains were grown overnight to saturation in YPD media, and diluted with sterile water to OD_{600} = 0.5. 10-fold dilutions with sterile water were then made, and 3μl of the cell suspension spotted onto media (SC) plates containing monensin (50μg/ml; Sigma) dissolved in ethanol, or ethanol only. Plates were incubated for 2 days at 30°C and imaged.

Results

Ubiquitination and deubiquitination of the proteome chip

As evidenced in recent studies, protein-array based assays are useful for finding novel substrates for the ubiquitin system. In a screen for Rsp5 substrates (Gupta 2007), many previously known substrates were confirmed, and several novel substrates were discovered. Many of the hits were validated with both in vitro and in vivo methods, demonstrating that microarray screens of this type are useful for the examination of in vivo roles of proteins. Therefore, to discover possible Ubp2 substrates, I subjected the proteins on a protein-array chip to a step-wise sequence of Rsp5-mediated ubiquitination followed by deubiquitination using affinity purified Ubp2 (Appendix Figure 1). First, the slide was blocked with milk to prevent non-specific protein interactions. Rsp5, along with E1, E2, ATP, buffer, and fluorescently labelled ubiquitin (FITC-Ub) were incubated with the slide, and all possible Rsp5 substrates were ubiquitinated. The slide was then dried, scanned, and quantified to determine which proteins were successfully ubiquitinated by monitoring for fluorescent signals. After scanning, putative substrates of Ubp2 were deubiquitinated by incubation with Ubp2-TAP. The slide was again dried and quantified. To determine which proteins were deubiquitinated, data from both the ubiquitination and deubiquitination reactions were analyzed (see materials and methods) and an ‘ub score’, ‘dub score’, and ‘ub/dub score’ were generated. This entire procedure was repeated on two separate slides in order to monitor for the reproducibility of the results.

The ub scores for both slides were plotted to check for reproducibility for the ubiquitination reaction (Appendix Figure 2A). Although the correlation was not exact, especially
Appendix Figure 1: Workflow for the *in vitro* coupled ubiquitination-deubiquitination screen. Yeast proteins were spotted in duplicate on the microarray (Protoarray). The slide was blocked with milk, washed, and proteins ubiquitinated using purified Rsp5 and related cofactors. After washing and drying, the slide was scanned to record the fluorescent (ubiquitinated) spot intensities. The chip was then exposed to purified Ubp2-TAP isolated from yeast, and deubiquitination allowed to proceed for 2 hours. Finally, the slide was again scanned and the decrease in signal quantified to monitor deubiquitination efficiency.
Appendix Figure 2: Reproducibility of the ubiquitination reaction. (A) The ubiquitination score (ub score = (the mean intensity of the spot - background) divided by the protein concentration) for my biological replicates (slide 1 and slide 2) are plotted, and the correlation between the datasets determined. (B) Comparison of the ub scores obtained for a representative experiment (slide 1) with the mean ub score calculated for the results reported by Gupta et al. (2007).
for spots at the higher end of the score range, there was a general correlation of ub scores from the two slides, with a $R^2$ value of 0.78. It must be noted that the ranges of scores between the two slides were quite different, with one slide having a maximum ub score of around 60, while the other slide had a maximum score of around 40. The ub score for one of my slides was then compared to the data set from (Gupta 2007). Again, the two data sets correlated generally, but the correlation was not exact, with a $R^2$ value of 0.78 (Appendix Figure 2B).

The ubiquitination scores between the two replicate slides did not have a very high correlation, although spots that scored highly on one slide tended to score highly on the other slide also. In addition, my ubiquitination assay captured many ubiquitination hits from (Gupta 2007), although the range of scores was higher in the (Gupta 2007) data set. Next, I compared the scores from the ubiquitination and deubiquitination reactions by calculating the ub/dub ratio. In comparing the two slides on which I had repeated the entire experiment, I found that there was a small subset of 11 proteins that scored highly on either replicate (Appendix Figure 3; Appendix Table 1). Although scoring highly on only one replicate, one particularly noteworthy Ubp2 target was Fra1 (YLL029w). Fra1, at the time of the deubiquitination screen, was a protein of unknown function, although a recent study implicated it in the mediation of the transcription of iron induced genes (Kumanovics 2008). While it was not found as a putative Rsp5 candidate in a previous screen (Gupta 2007), Fra1 was detected in both my Ubp2-TAP and Rup1-TAP protein-protein interaction results (Krogan 2006), suggesting that it is physically associated with the Ubp2- Rup1-Rsp5 complex. As this protein was not pulled down consistently in each Ubp2/Rup1 TAP purification, Fra1 was not followed-up at the time as an Ubp2-Rup1-Rsp5 complex member. As the transient nature of the interaction may implicate Fra1 as an Ubp2 substrate, I proceeded to test if this was indeed the case.

In vitro confirmation of Fra1 as a Ubp2 substrate

To test whether Fra1 is a substrate of Rsp5 and/or deubiquitinated by Ubp2 in vivo, I transformed a plasmid expressing GST-tagged Fra1 into both wildtype and various mutant yeast strains in order to visualize the protein levels and ubiquitination status of Fra1. Soluble protein lysates were prepared, and GST-Fra1 was precipitated from the extract with glutathione
Appendix Figure 3: Putative Ubp2 deubiquitination substrates. The ub/dub ratio score was calculated for all protein spots passing the cutoff criteria (see materials and methods). The ub/dub score of the two replicate slides (slide 1 and slide 2) were then plotted. High scoring hits from either of the replicates are shown as red circles.
<table>
<thead>
<tr>
<th>ORF</th>
<th>Protein</th>
<th>Ub/dub score slide 1</th>
<th>Ub/dub score slide 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFL017W-A</td>
<td>SMX2</td>
<td>7.50</td>
<td>30.91</td>
</tr>
<tr>
<td>YHR185C</td>
<td>PFS1</td>
<td>3.23</td>
<td>14.59</td>
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<tr>
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<td>YDR391C</td>
<td>1.64</td>
<td>6.84</td>
</tr>
<tr>
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<td>BRE2</td>
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<td>YPL257W-A</td>
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</tr>
<tr>
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<td>YRF1-5</td>
<td>3.35</td>
<td>1.87</td>
</tr>
<tr>
<td>YLL029W</td>
<td>FRA1</td>
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</tr>
<tr>
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<td>BDF2</td>
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<tr>
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<tr>
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<td>YPR137C-A</td>
<td>1.23</td>
<td>18.12</td>
</tr>
</tbody>
</table>

**Appendix Table 1: Ub/dub scores for putative Ubp2 deubiquitination substrates.** The ub/dub ratio score for high scoring hits (red circles in Appendix Figure 3) are listed, along with the identity of the protein and open reading frame (ORF).
sepharose beads. Proteins were then electrophoresed, transferred onto a Western blot, and Fra1 visualized with an anti-GST antibody. In addition, any ubiquitinated species were visualized using anti-ubiquitin antibody.

Ubiquitinated species of Fra1 were detected in wildtype cells (Appendix Figure 4); however no decrease in Ub-Fra1 levels was observed in a strain bearing a hypomorphic \( rsp5-1 \) allele at either the permissive or restrictive temperature (37°C). Moreover, when comparing the GST-Fra1 pattern in wildtype and \( ubp2\Delta \) cells, there did not seem to be any increase in Ub-Fra1 signal in the absence of the DUB. Taken together, these data imply that, although Fra1 is ubiquitinated, Rsp5 either does not detectably target Fra1 under standard growth conditions or is not the primary E3 ligase for Fra1 \textit{in vivo}, and that likewise, Ubp2 also does not seem to influence the basal ubiquitination level of Fra1. Interestingly, when I perform hierarchical clustering on recent genetic interaction data (Costanzo 2010) using Cluster 3.0 (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/), \( FRA1 \) appears to cluster together with \( RUP1 \) and \( UBP2 \), implying that these three genes share many genetic interactors. Therefore, although Fra1 is likely not a target of Ubp2, the \textit{in vivo} function of Fra1 may be related to the functions of both Ubp2 and Rup1.

Discussion

I have devised a novel proteome-scale assay to systematically screen for substrates of Ubp2. Although the biological significance of my initial results has not been validated and there are currently still a few technical and analytical challenges that need to be addressed, I believe this type of assay shows promise for screening for substrates of deubiquitinating enzymes. Through the analysis of my deubiquitination screen, I generated a set of putative Ubp2 substrates, and focused specifically on one, Fra1, on which I attempted to validate as an \textit{in vivo} Ubp2 substrate. Although I found that Fra1 was ubiquitinated \textit{in vivo}, my results show that it is unlikely an Rsp5 or Ubp2 substrate. Despite the preliminary nature of my results, there were other promising Ubp2 targets that were found through this screen, which would require confirmation and follow-up work in the future.
Appendix Figure 4: Testing of Fra1 as a potential Ubp2 substrate \textit{in vivo}. GST-Fra1, expressed in either wildtype (WT), \textit{rsp5-1} (grown at the non-permissive temperature of 37°C), or \textit{ubp2Δ} cells was precipitated with glutathione sepharose beads, electrophoresed and transferred onto a Western blot. Non-modified and ubiquitinated Fra1 species were visualized using anti-GST and anti-ub antibodies, as indicated.
On the positive side, Ubp2 deubiquitination of the proteins was very specific, as only a very small subset of all Rsp5 ubiquitinated proteins were deubiquitinated. This suggests that Ubp2 deubiquitinates Rsp5 as well as only a select subset of Rsp5 targets. However, this limited coverage is suspect for a few reasons. Many potential substrates of Ubp2 may have been missed in this screen as the assay described is dependent first upon successful ubiquitination by Rsp5. In addition, certain substrates of Rsp5, such as Fur4, must also be phosphorylated and/or require adaptor proteins for recognition by Rsp5 (Leon 2008; Marchal 2000). Conversely, it is also possible that Ubp2 substrates may be ubiquitinated by a ligase other than Rsp5. Moreover, although the ubiquitination reaction was successful and specific in that only a subset of the proteome was ubiquitinated by Rsp5 (as expected), reproducibility between the two replicate chips was not very high with $R^2 = 0.78$ for my dataset versus $R^2 = 0.97$ for the dataset from Gupta et al. (calculated from raw data provided by the Rotin laboratory). Since this was my first attempt at using microarray technology, there could have been a few basic technical issues. Scans of my slides showed a large area of increased intensity, possibly due to an uneven application of the reaction mixture or uneven distribution of the mixture under the coverslip, which may have caused a systematic increase or decrease in signal in certain areas of the slide. Although I attempted to compensate for signal variability, a better normalization calculation should be used in future analyses. A similar problem occurred with the deubiquitination reaction, likely aggravating the results and resulting in a varied range of ub/dub scores between the two replicates.

This assay can be extended and modified in many ways. In particular, an improvement in the sensitivity of the assay, and a possible increase in the number of Ubp2 substrates, may have been achieved if recombinant Ubp2 was used instead of affinity purified Ubp2-TAP from yeast. While my preparation of Ubp2-TAP was active, it also contained Rsp5 and Rup1, which may have affected the results. Although Rsp5 could not have re-ubiquitinated any substrates in the deubiquitination reaction (as there was no additional ATP added in the deubiquitination reaction), it is possible that other contaminants may have impacted the screen. In addition, the effects of any putative interactors or cofactors of Ubp2 can be easily assayed using this system, simply by adding these proteins to the deubiquitination reaction. Moreover, the assay can be adapted to screen other deubiquitinating enzymes for which specific substrates are currently unknown. In addition to yeast Ubps, mammalian DUBs could be assayed with this type of screen.
as human proteome microarray chips are commercially available (Invitrogen), perhaps using a combination of recombinant E3 ligases for maximum coverage of proteins. In fact, a successful ubiquitination screen has recently been reported for human and rat Nedd4 ligases using this microarray chip (Persaud 2009), and a deubiquitination screen could easily be performed after ubiquitination reactions are completed and scored.

Genomic screens for *UBP2* functions

Large scale genetic screens performed using the synthetic genetic array (SGA) method (Costanzo 2010; Tong 2002; Tong 2001) provide an alternative approach by which to elucidate functional relationships. In this assay, haploid yeast bearing single gene deletions or hypomorphic alleles of essential genes of interest, are mated to a collection of marked deletion mutant strains of the opposite mating type lacking each of the non-essential genes in a high-throughput manner using robotics. Through replica pinning and different selection steps, the final result is a collection of colonies representing cells with mutations in two genes, one being the gene of interest (query). The double mutants are scored for growth defects, either by examining colony sizes, or potentially through other types of assays. If a deletion in two genes results in a loss of fitness (synthetic sick) or no growth (synthetic lethal), these phenotypes often imply that two genes are involved in similar functions possibly in parallel or converging pathways in the cell.

Both *UBP2* and *RUP1* have been screened using the SGA method both by me and by members of the Boone laboratory here at the University of Toronto, and mutations in each gene caused convincing synthetic growth defects (as defined using a stringent cutoff score) in conjunction with mutations in genes linked to specific cell processes, in particular protein transport pathways (Costanzo 2010). Although promising, these putative interactions would benefit from independent confirmation by tetrad analysis (to eliminate possible false positives), and elaboration by retesting other pathway components (to reduce false negatives). The data implicate both Ubp2 and Rup1 in other functions in the cell. For example, both *UBP2* and *RUP1* were found to genetically interact with *VPS9*, encoding a guanine nucleotide exchange factor for the endosomal GTPase Vps21 required for vacuolar sorting of cargo from both the Golgi and cell surface (Gerrard 2000; Hama 1999), while *RUP1* interacts with *CCZ1*, encoding a protein
implicated in vesicle fusion in various transport steps (Kucharczyk 2009). Consistent with these results, Vps9 has previously been reported to be mono-ubiquitinated by Rsp5 both in vivo and in vitro (Davies 2003), implicating Ubp2 and Rup1 in the deubiquitination of Vps9 or the regulation of Rsp5 at this sorting step. The interaction of RUP1 with CCZ1 suggests that Rup1, and possibly Ubp2, may also serve a role during vesicle fusion.

Another gene of interest that was revealed in my own SGA screens was MON2. I found that MON2 showed a strong aggravating genetic interaction with RUP1. MON2 was originally identified in a screen for mutants displaying hypersensitivity to monensin, a drug which blocks transport from the Golgi (Muren 2001). It has since been implicated in Golgi and ER functions, where it is thought to act in the recruitment of the protein machinery involved in vesicle formation and transport between the ER and Golgi (Gillingham 2006; Singer-Kruger 2008). Although the synthetic lethality between RUP1 and MON2 could not be confirmed by tetrad analysis (data not shown), I did find that the ubp2Δmon2Δ double mutant had a significantly increased sensitivity to monensin as compared to mon2Δ cells, while rup1Δmon2Δ cells appeared to have similar sensitivity as the mon2Δ single mutant (Appendix Figure 5). Therefore, the genetic data implicate Ubp2, and perhaps Rup1, in additional roles at both the ER and Golgi in addition to the MVB (Chapter 3).
Appendix Figure 5: *ubp2Δ mon2Δ* cells are sensitive to monensin. Various mutant yeast strains were serially diluted 10-fold (starting OD$_{600}$ of 0.5), and spotted onto SC + glucose media containing monensin (dissolved in ethanol), or media containing an equal concentration of ethanol (EtOH) only. Cells were grown for 2 days at 30°C and the plates imaged.