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Regulation of the Rsp5 HECT Domain Ubiquitin Ligase

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Michael E. French

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ABSTRACT

Regulation of the Rsp5 HECT Domain Ubiquitin Ligase

Michael E. French

Rsp5 is a ubiquitin ligase that controls a broad range of cellular processes in budding yeast and is part of a large family of proteins that controls analogous processes in mammalian cells. Although Rsp5 targets a number of different substrates for ubiquitination, the mechanisms that regulate Rsp5 catalytic activity are poorly characterized. This dissertation examines several previously unknown molecular factors that are likely to play a role in the regulation of Rsp5 activity. The first is the presence of a noncovalent ubiquitin-binding site located in the catalytic HECT domain of Rsp5. Protein interaction studies and mutagenesis were used to demonstrate that the N-terminal lobe of the HECT domain mediates binding to ubiquitin, and the results of *in vivo* growth assays and *in vitro* ubiquitination assays indicated that the Rsp5 ubiquitin-binding site regulates the ability of the Rsp5 HECT domain to assemble polyubiquitin chains. The second factor likely to regulate Rsp5 activity is an intramolecular interaction between the WW and catalytic HECT domains of the ligase. Finally, phosphorylation of Rsp5 is examined as a potential mechanism of regulation, and a kinase responsible for the phosphorylation known as Cbk1 is identified.

The results of these studies help contribute to our understanding of the mechanisms that regulate the activity of Rsp5 and related ubiquitin ligases. Mammalian homologues of Rsp5 such as Nedd4 and Itch are critical regulators of many important biological processes, and the identification of regulatory factors that control the catalytic activity of these ubiquitin ligases is critical to understanding how these enzymes carry out their functions. The results described in this thesis suggest that the activity of Rsp5 is regulated by at least three different factors, and

recent work from other labs indicates that additional regulatory factors exist. Finally, recent data indicates that mammalian homologues of Rsp5 are regulated by mechanisms that are similar to those described in this dissertation, suggesting that the results of these studies will be generally applicable to understanding how this family of ubiquitin ligases functions in higher eukaryotes.

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LIST OF ABBREVIATIONS

0K Ub	Lysine-less ubiquitin
A20 ZnF	A20 zinc finger
C-lobe	C-terminal lobe
CTD	Carboxy-terminal domain
CUE	Coupling of ubiquitin conjugation to ER degradation
DUIM	Double-sided ubiquitin ubiquitin-interacting motif
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
ENaC	Epithelial sodium ion channel
ER	Endoplasmic reticulum
GAT	GGA and target of Myb
GLUE	Gram-like ubiquitin binding in EAP45
GST	Glutathione S-transferase
HA	Hemagglutinin
HECT	Homologous to E6AP C-terminus
His_6	Hexahistidine
HIV-1	Human immuno-deficiency virus type I
HTLV-1	Human T cell leukemia virus type I
IsoT	Isopeptidase-T
K63, K48, K29 ubiquitin	Single-lysine ubiquitin mutants carrying K63, K48, or K29
MIU	Motif interacting with ubiquitin

N-lobe	N-terminal lobe
NZF	Npl4 zinc finger
PBS	Phosphate-buffered saline
PRD	Proline rich region
PRU	Pleckstrin-like receptor for ubiquitin
RING	Really interesting new gene
RIP	Receptor-interacting proteins
RSV	Rous sarcoma virus
SH3-3	Third Src homology 3 domain of Sla1
U-box	UFD2 homology box
Ub_4-Ub_n	Polyubiquitin chains longer than four subunits in length
UBA	Ubiquitin-associated
UBC	Ubiquitin-conjugating
UBD	Ubiquitin-binding domain
UBL	Ubiquitin-like protein
UBZ	Ubiquitin-binding zinc finger
UEV	Ubiquitin-conjugating E2 variant
UIM	Ubiquitin-interacting motif
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose
ZnF UBP	Zinc finger ubiquitin-specific processing protease

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CHAPTER I: INTRODUCTION

Overview

Ubiquitin is a important regulatory signal that plays a role in controlling and modulating a number of different cellular processes. Ubiquitin is the primary signal used to target proteins for degradation by the 26S proteasome (1) and it is an important nonproteolytic signal in many other biological pathways, including DNA repair, NF-κB signaling, pre-mRNA splicing, transcriptional regulation, and endocytosis (2-6). The ability of ubiquitin to act as a signal in a variety of different processes can be explained by at least three different factors. First, there are a large number of ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s) that function to select specific substrates for ubiquitination. Second, ubiquitin modifications can adopt distinct structural conformations that are specialized for different cellular functions. Third, ubiquitin signals are recognized by a diverse set of ubiquitin-binding domains (UBDs) found within proteins that participate in numerous cellular functions.

Although the general principles of ubiquitin conjugation and recognition have been established, there are many key questions that remain unanswered. What are the molecular principles that govern substrate specificity? How are distinct types of mono- and polyubiquitin signals generated? What are the factors that regulate catalytic activity? What are the *in vivo* targets and functions of UBDs? How are UBD-ubiquitin interactions regulated? This chapter begins by reviewing the known biochemical properties of ubiquitin and the enzymes that catalyze ubiquitination, then focuses on a specific family of E3s that includes the Rsp5 ubiquitin ligase, and concludes with a discussion of UBD characteristics and cellular functions. In each section, at attempt has been made to highlight recent advances in the field and draw attention to specific areas of investigation that either remain controversial or have not been adequately addressed in the literature.

Ubiquitin: Structure, Function, and Mechanisms of Conjugation

Ubiquitin: structure and function

The three-dimensional structure of ubiquitin is extremely compact (7-9) and contains several well-characterized features on its surface (Figure 1). Perhaps the most prominent feature of ubiquitin is its C-terminus, which adopts an extended conformation (Figure 1A) and consists of two glycine residues, G75 and G76, also present in most ubiquitin-like proteins capable of covalent conjugation (for an exception see ref 10). The surface of ubiquitin also features seven different lysine residues, and all of these appear to be capable of serving as sites of ubiquitin-ubiquitin conjugation (11). Three of the four most commonly used sites of conjugation are located on the front surface of ubiquitin, whereas the remaining four sites are located on the back surface of the molecule (Figure 1B). Finally, ubiquitin contains three exposed hydrophobic residues on its surface, L8, I44, and V70. These residues form a functional patch important for the recognition of ubiquitin by a number of other interacting cellular proteins (12,13).

Ubiquitin is typically conjugated to other proteins through an isopeptide bond, in which the C-terminal G76 of ubiquitin is attached to a free amino group within the protein. The amino group is most often contributed by an internal lysine residue, although in some cases the N-terminus of a protein can contribute the amino group (14). Ubiquitin can also be conjugated to cysteine, serine or threonine residues in rare instances (15-17), although the general relevance of ubiquitination on these residues remains to be determined. Unlike conjugation of the closely related ubiquitin-like protein SUMO, which generally occurs on lysine residues within the consensus sequence ΦKXD/E (Φ is a hydrophobic residue), ubiquitination is not site-specific and frequently occurs on several different lysines within a protein (18,19).

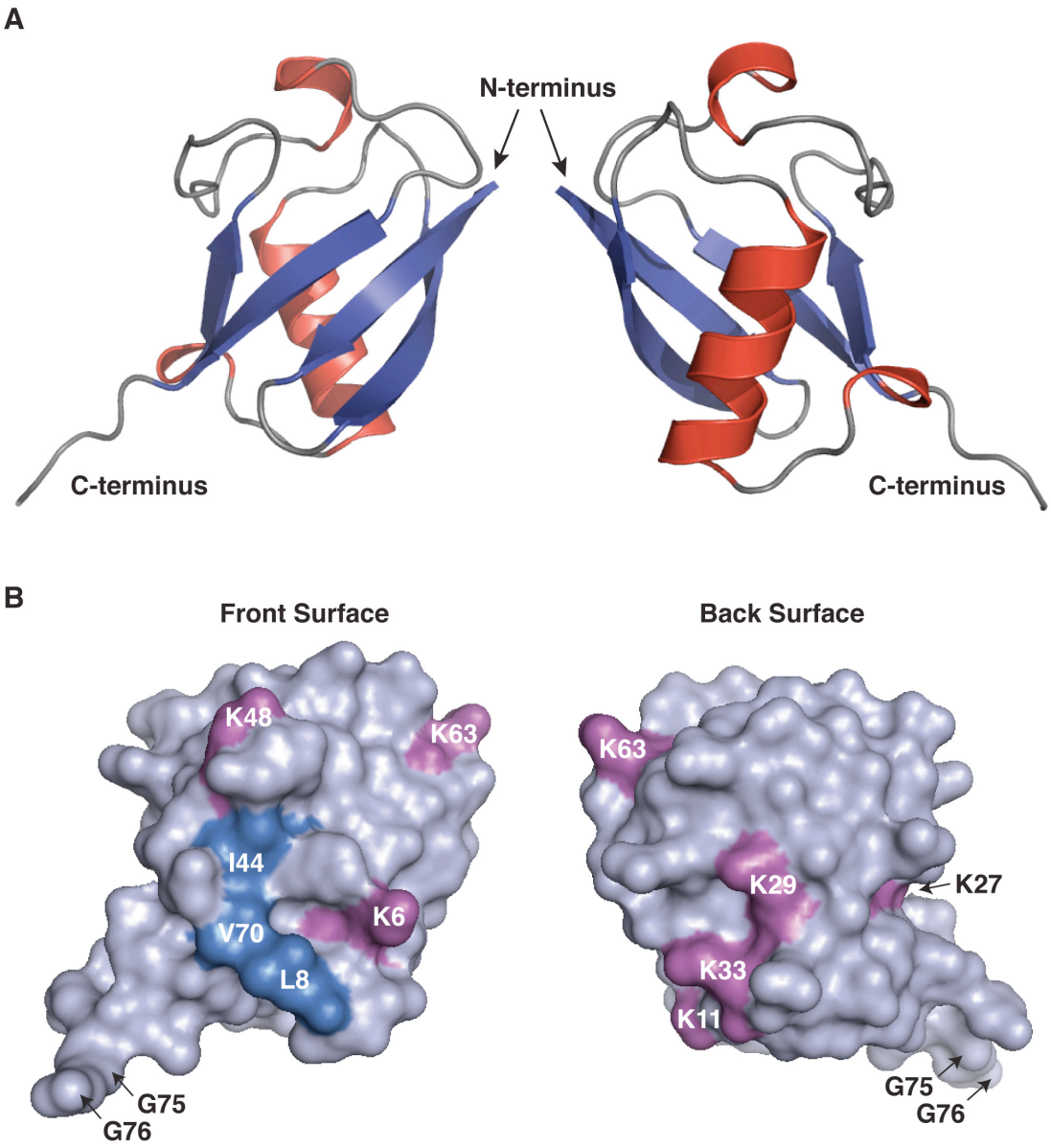

Figure 1. Three-dimensional structure and key surface features of ubiquitin. (A) Cartoon representation of ubiquitin based on its three-dimensional structure (PDB accession code 1UBQ). Helices are colored in red, β-strands are colored in blue, and the position of the N- and C-terminus is indicated. The structure on the right was generated by rotating the structure on the left by 180°. (B) Surface representation of ubiquitin (PDB accession code 1UBQ) highlighting the key surface features of the molecule. Lysines are colored in magenta, and residues comprising the I44 hydrophobic patch are colored in light blue. The position of the C-terminal G75 and G76 residues is indicated. Images were contructed in MacPyMOL.

Proteins targeted for ubiquitination can be modified with either monoubiquitin or polymeric ubiquitin chains. The conjugation of monoubiquitin to one or multiple lysine residues (Figure 2A) is an important regulatory signal that acts to alter the activity or location of many different proteins. Monoubiquitination does not generally function to target proteins for proteasomal degradation (for an exception see ref 20), but instead acts as a nonproteolytic signal to control a number of basic cellular processes, including gene expression, viral budding, DNA repair, and endocytosis (5,21,22). In contrast, polyubiquitin chains, which can adopt numerous structural conformations (Figure 2B), act as signals specialized for other cellular functions. For example, chains linked through K48 of ubiquitin play a well-characterized role in targeting proteins for degradation by the proteasome (23,24), whereas chains linked through K63 of ubiquitin act as nonproteolytic signals in translation (25), DNA repair (26-28), NF-κB signaling (29,30), mitochondrial inheritance (31), and endocytosis (32-34) (Figure 2C) .

The potential for diversity in polyubiquitin chain synthesis and signaling has become increasingly more apparent over the past several years. Chains linked through lysines other than K48 and K63 are still poorly characterized (Figure 2C), despite evidence that K6 and K11-linked chains are just as abundant as K48 and K63-linked chains in *S. cerevisiae* (35). K29-linked chains function to target proteins involved in Notch signaling for degradation in the lysosome (36,37) and have also been implicated in proteasomal degradation (38,39), whereas K6-linked chains play a poorly defined role in DNA repair (40). Cellular functions have not yet been ascribed to K11, K27, or K33-linked chains, although K11-linked chains are competent signals for degradation by proteasomes *in vitro* (41). K63-linked chains are also competent degradation signals *in vitro* (42-44), although genetic studies in yeast indicate that K63-linked chains are not likely to be a predominant targeting signal *in vivo* (28).

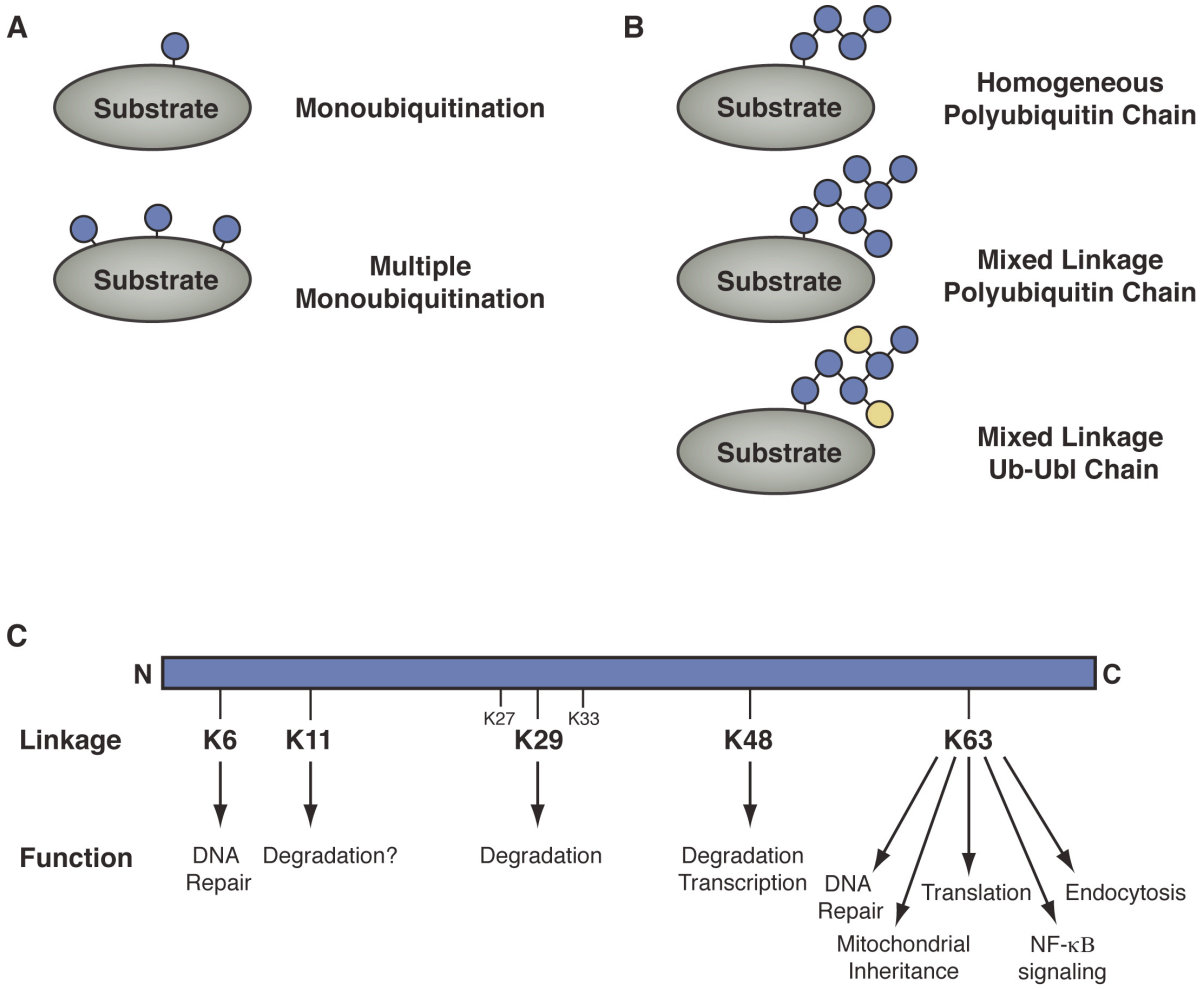

Figure 2. Schematic representations of the different types of ubiquitin modifications. (A) Monoubiquitination is the attachment of a single ubiquitin molecule (purple ball) to a single lysine residue within a protein. Multiple monoubiquitination refers to the modification of several substrate lysine residues with one ubiquitin. (B) Polyubiquitin chains can be homogeneously linked through the same lysine residue of ubiquitin or contain mixed linkages, in which two different lysines of the same ubiquitin are conjugated to different ubiquitin molecules (mixed linkage polyubiquitin chain) or ubiquitin-like proteins (yellow ball; mixed linkage Ub-Ubl chain). (C) Schematic indicating the known functional roles of the different types of ubiquitin chain linkages. Cellular functions have not yet been ascribed to K27 or K33-linked chains. All references are in the text, except for the role of K48-linked chains in transcriptional activation (see Flick et al. (2004) Nat. Cell Biol. *6*, 634-641).

Polyubiquitin chains can also adopt complex structures consisting of heterogeneous or mixed linkages, thus further expanding the potential signaling properties of a chain (Figure 2B). Branched chains containing mixed linkages, in which a single ubiquitin is linked to two distinct ubiquitin molecules through different lysines, have been synthesized *in vitro* (43) and exist in yeast cells (11). Interestingly, these 'forked' chains are more resistant to degradation by purified 26S proteasomes than chains uniformly linked through K48 or K63, suggesting that cells have evolved a mechanism to prevent the synthesis of these branched chains on substrates that need to be degraded (43). There is also at least one documented case in which a heterologous chain containing a SUMO-ubiquitin linkage has been reported (45). In this case, the mixed linkage chain appears to be functionally relevant because it targets a sumoylated protein for proteasomal degradation (45,46).

Diversity in signaling has also been achieved through the use of ubiquitin-like proteins and ubiquitin-like domains found within other proteins. Ubiquitin-like proteins are structurally related to ubiquitin, carry the signature diglycine motif, and can be covalently attached to other proteins to regulate their activities (47). Despite the fact that ubiquitin-like proteins share a common structural fold, these proteins are distantly related to ubiquitin in sequence and function in distinct cellular pathways. For example, SUMO-1 shares only 18% sequence identity with ubiquitin and acts as a signal in nuclear localization and transcriptional regulation (48), whereas FUB1 is only 37% identical to ubiquitin and functions in T-cell activation (49). In addition, there are a number of ubiquitin-like domains located within other proteins that are structurally related to ubiquitin, but cannot be conjugated because they lack a free C-terminus. One family of proteins, which includes the Rad23, Dsk2, and Ddi1 adaptors, contains a ubiquitin-like domain involved in the delivery of ubiquitinated proteins to the proteasome (50-53). These

built-in regulatory elements are likely to be more abundant than originally thought, since they typically share little sequence similarity with ubiquitin or each other and are therefore difficult to identify by bioinformatic approaches.

Mechanisms of conjugation: activating enzymes, conjugating enzymes, and ubiquitin ligases

The conjugation of ubiquitin to substrates requires the sequential actions of three different types of enzymes: a ubiquitin-activating enzyme (E1) that forms a thioester with the carboxyl group of G76 to activate the C-terminus of ubiquitin, a ubiquitin-conjugating enzyme (E2) that acts as an intermediate carrier of activated ubiquitin, and a ubiquitin ligase (E3) that facilitates the final transfer of activated ubiquitin to a substrate amino group (Figure 3; ref 54). In most organisms, there are only one or two E1s for ubiquitin, but the existence of a large set of E2s and E3s ensures that the substrate selection process occurs with exquisite specificity. The number of E2s and E3s that function in the conjugation of ubiquitin-like proteins is much smaller, although each ubiquitin-like protein appears to have its own dedicated E1 (55). The following discussion focuses mostly on enzymes that participate in the ubiquitin conjugation pathway, highlighting recent advances in understanding the catalytic mechanisms of ubiquitin transfer and substrate conjugation employed by these enzymes.

The chemistry of E1 catalysis is well defined and involves the formation of a ubiquitin adenylate intermediate that serves as a donor for the transfer of activated ubiquitin to a cysteine residue in the E1 active site (19). Structural studies of the E1s for ubiquitin, SUMO family members, and Nedd8 have shown that E1s contain at least three distinct domains that facilitate the major biochemical activities of the enzyme: an adenylation domain that binds to both ATP and either ubiquitin or the relevant ubiquitin-like protein, a catalytic cysteine domain that contains the E1 active site, and a C-terminal ubiquitin-fold domain that recruits specific E2s

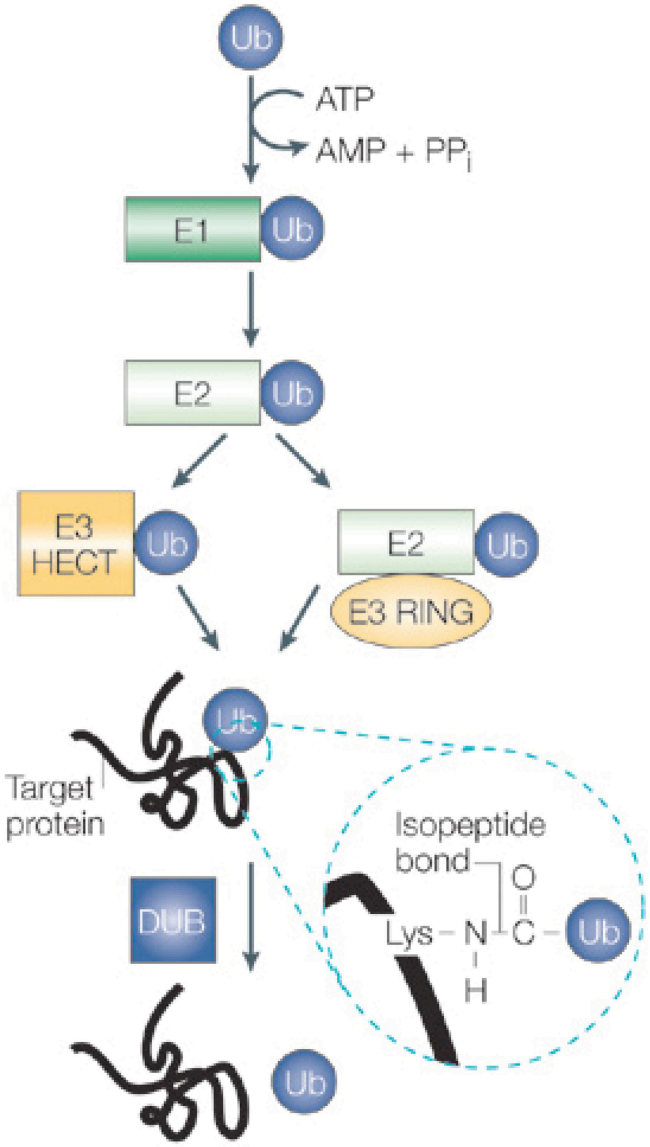

Figure 3. Enzymes that function in the ubiquitin conjugation pathway. The conjugation of ubiquitin to substrates involves three different types of enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). In most organisms, a single E1 is responsible for activating ubiquitin by forming a thioester bond with the C-terminus of ubiquitin in a reaction that requires ATP. Ubiquitin is then passed on to one of several E2s, which also form a thioester bond with C-terminus of ubiquitin. The final transfer of ubiquitin to a substrate almost always requires the presence of an E3, which binds directly to an E2 and a substrate to facilitate the transfer of ubiquitin to the substrate. HECT E3s form a thioester with ubiquitin and then directly transfer ubiquitin to the substrate, whereas RING E3s act as bridging factors to bring together an E2 charged with ubiquitin and a substrate. Finally, deubiquiting enzymes (DUBs) reverse the actions of these enzymes by cleaving the isopeptide bond formed between a substrate lysine amino group and the C-terminus of ubiquitin. This figure was adapted from Hicke et al., 2005 (ref 12).

(Figure 4A; refs 56-61). Although the structural features of these enzymes are generally similar, there is at least one significant difference in the structure of the E1 for ubiquitin (Uba1) that may help to explain how this enzyme cooperates with multiple E2s, whereas the E1s for SUMO and Nedd8 each function with only a single E2– Uba1 features a much wider cleft between its catalytic domain and its ubiquitin-fold domain (Figure 4A) than the E1s for SUMO and Nedd8. This feature probably allows Uba1 to accommodate a variety of more bulky E2 partners carrying N- and C-terminal extensions that cannot be accommodated by the E1s for SUMO and Nedd8 (58).

Although the majority of ubiquitin activation in most organisms is carried out by a single E1 (Uba1/Ube1), higher eukaryotic organisms possess an additional E1 for ubiquitin known as E1-L2/Uba6 (62-64). Uba6 is only ~ 40% identical to Uba1/Ube1, is widely expressed in many different human tissues and cell types, and displays distinct biochemical properties from that of Uba1/Ube1. One key distinguishing factor is the presence of a unique ubiquitin-fold domain, which allows Uba6 to recruit and charge a distinct set of E2 enzymes, including the previously uncharacterized Uba6-specific E2 Use1 (63). Surprisingly, Uba6 also appears to be the major activating enzyme for the ubiquitin-like protein FAT10 in mammalian cells, suggesting that the enzyme has evolved to activate a related conjugation pathway in higher eukaryotes (62). The identification of specific E3s and substrates that participate in the Uba6-mediated activation pathway will be critical to understand the functions of this activation pathway in both ubiquitin and FAT10-regulated processes.

The next step in the ubiquitin conjugation pathway involves the transfer of activated ubiquitin to an E2. There are eleven E2s for ubiquitin in *S. cerevisiae* and many more in higher eukaryotes (54). E2s generally have two basic functions– to accept ubiquitin from an E1 and to

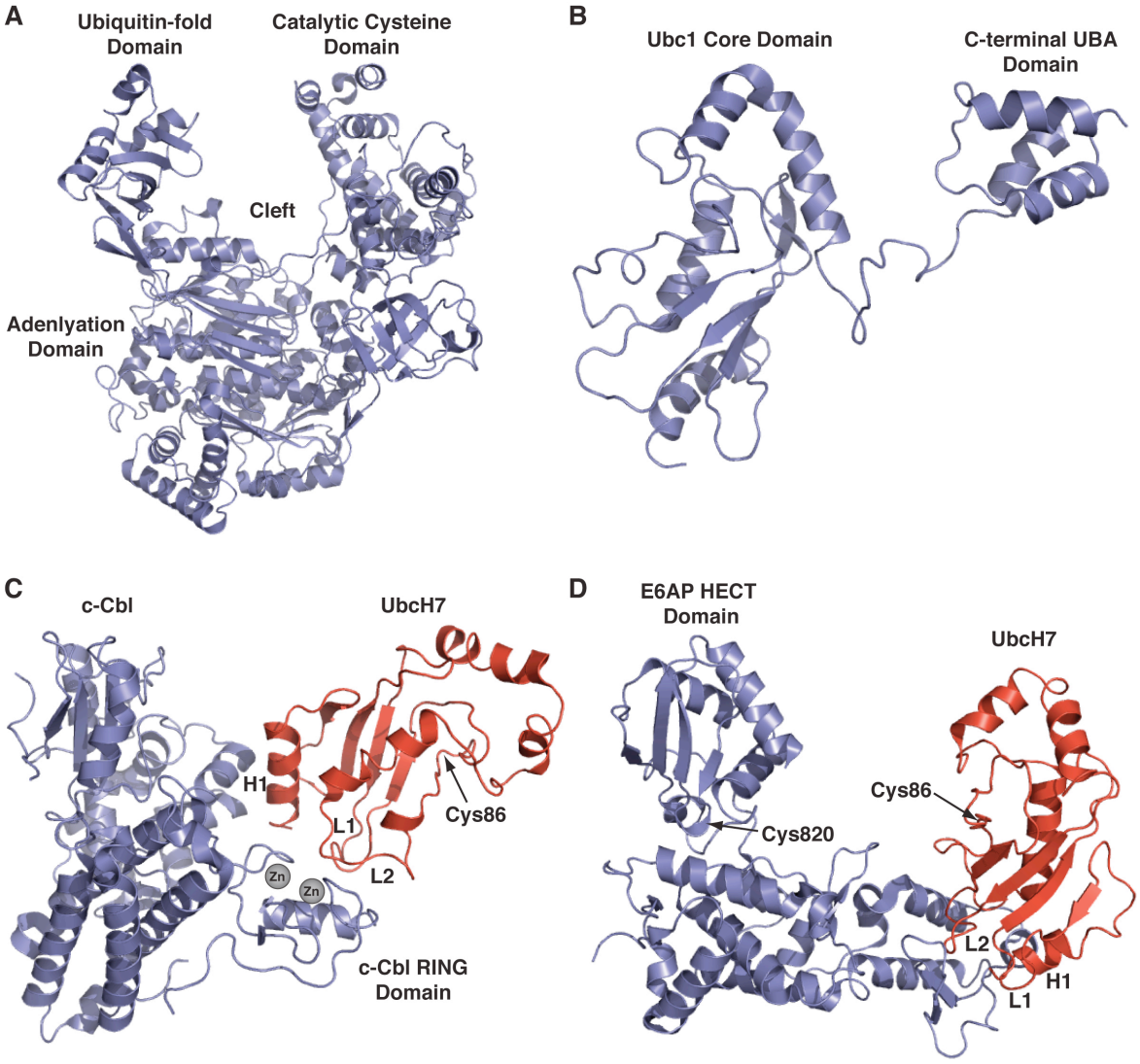

Figure 4. Three-dimensional structures of enzymes that function in the ubiquitin conjugation pathway. Cartoon representations of the E1 for ubiquitin (A) (Uba1; PDB accession code 3CCM), an E2 carring a C-terminal extension (B) (Ubc1; PDB accession code 1TTE), a RING E3-E2 complex (C) (c-Cbl7-UbcH7; PDB accession code 1FBV), and a HECT domain-E2 complex (D) (E6AP HECT-UbcH7; PDB accession code 1C47). Functional domains are indicated on the structures. In (C) and (D), the E2 is highlighted in red, and the position of cysteine residues that form a thioester bond with ubiquitin are indicated. Images were constructed in MacPyMOL.

transfer ubiquitin to either an E3 or a substrate. All E2s share a ~ 150 amino acid catalytic core domain that is required for formation of the ubiquitin thioester, and several E2s have additional N- or C-terminal extensions involved in substrate recognition (65), dimerization (66), or the regulation of polyubiquitin chain synthesis (Figure 4B; refs 67-69). The specificity of E2-E3 interactions has been engineered with a moderate degree of flexibility, such that a given E2 can interact with multiple E3s. For example, UbcH7 forms a complex with both the E6-AP HECT (Homologous to E6AP C-terminus) E3 and the c-Cbl RING (Really Interesting New Gene) E3 using similar surfaces of the E2, despite the fact that the HECT and RING catalytic modules are unrelated in sequence and structure (Figure 4C, 4D; refs 70,71). Conversely, a single E3 can function with multiple E2s. For instance, the BRCA1-BARD1 heterodimeric RING E3 complex cooperates with up to six different E2s to catalyze the transfer of monoubiquitin, K63-linked chains, or K48-linked chains (72).

Despite recent advances in understanding how an E2 cooperates with a given E3 to catalyze the ubiquitination of substrates, the specific catalytic roles of most E2s are still poorly understood. The best available model for catalysis by an E2 comes from structural studies of a specialized heterodimeric complex consisting of an E2, Ubc13, and a catalytically inactive E2-like protein Mms2 (73-75). This complex, in cooperation with one of two different RING E3s, assembles K63-linked polyubiquitin chains on substrates involved in DNA repair or NF-κB signaling (26,30,76). The assembly of a K63-linked chain by the Mms2-Ubc13 complex requires the formation of a covalent ubiquitin thioester bond at the Ubc13 active site and the noncovalent binding of a second acceptor ubiquitin to Mms2. Mms2 interacts with the acceptor ubiquitin in an orientation that promotes the selective insertion of K63 into the Ubc13 active site, thereby facilitating formation of the K63 linkage (73). Thus, the E2 specifies the linkage of a

chain that will ultimately be conjugated to a substrate in a RING E3-dependent reaction (see below for RING E3 catalysis).

The final step in the conjugation pathway is the transfer of ubiquitin to a substrate. This typically requires the presence of an E3 (for an exception see ref 77). The number of E3s in higher eukaryotes is predicted to range from several hundred to well over a thousand (54), thus explaining the extremely diverse nature of substrates targeted for ubiquitin conjugation. The two major types of E3s are defined by the presence of either a HECT domain or a RING domain (U-box domain E3s are structurally and mechanistically very similar to RING E3s and will not be considered as a separate family of E3s for the purposes of this discussion). Both types of E3s share the ability to bind to an E2 and to catalyze the transfer of ubiquitin to a substrate, but differ in the fundamental mechanism that they use to catalyze the ubiquitin transfer reaction. HECT E3s form a covalent thioester bond with ubiquitin before directly transferring ubiquitin to the substrate, whereas RING E3s act as bridging factors that function to increase the probability of reaction by bringing together a substrate lysine and an E2-ubiquitin thioester (54).

RING E3s are single-subunit or multi-subunit enzymes that recognize their substrates through diverse mechanisms and contain a RING domain, a short motif rich in cysteine and histidine residues that coordinates two zinc ions and binds to an E2 (54). The crystal structure of the RING E3 c-Cbl in complex with its E2, UbcH7, showed that the closest distance between the UbcH7 active site cysteine and any RING domain residue is about 15 Å, indicating that the RING domain does not provide reactive groups to the E2 active site (Figure 4C; ref 71). In addition, modeling of UbcH7 into the structure of the SCF^{Skp2} multi-subunit RING E3 complex revealed a large separation of ~ 50 Å between the substrate-binding domain of the E3 and the E2 active site cysteine (78). This latter structure, together with the finding that disassociation of the

Cdc34-ubiquitin thioester from the RING domain of SCF^{Cdc4} is required for ubiquitination of the SCF^{Cdc4}-bound substrate Sic1 (79), led to a proposed "hit-and-run" mechanism of ubiquitination. In this controversial model, the E2-ubiquitin thioester diffuses across the gap between the E2-binding and substrate-binding platforms until it collides with a substrate lysine. It is unclear how specificity for substrate targeting or subsequent rounds of ubiquitin transfer would be achieved in this model (80,81).

Despite a limited understanding of how RING E3s promote the transfer of the initial ubiquitin from an E2 to a substrate, several recent studies have shed light on the mechanisms of polyubiquitin chain synthesis employed by these E3s. The SCF-Cdc34 complex assembles K48-linked ubiquitin chains on Sic1 using a mode of chain assembly that requires an acidic loop conserved in Cdc34 E2 orthologs (82). Mutations that disrupt the Cdc34 acidic loop have no effect on the transfer of the first ubiquitin to Sic1, but impair the subsequent elongation of K48-linked chains on this substrate. These observations suggest that the role of the acidic loop is to position K48 of a substrate-linked ubiquitin in an orientation that promotes an attack of the SCF-bound Cdc34-ubiquitin thioester (82). A key feature of this model is that the growing K48-linked ubiquitin chain is assembled directly on the SCF-bound substrate. Yet, in at least one case, it has been demonstrated that an E2 can preassemble a K48-linked chain on its active site before transferring the chain to a RING E3-bound substrate (83). In this case, dimerization of the E2 is required for chain assembly. Thus, different RING E3s can utilize distinct mechanisms of chain synthesis, and properties of the E2 appear to play a critical role in determining the mechanism by which chains are assembled.

In contrast to RING E3s, HECT E3s participate directly in the chemistry of catalysis because they form an intermediate thioester bond with ubiquitin before transferring ubiquitin to

the substrate. HECT E3s consist of divergent N-terminal domains involved in either substrate recognition or localization and a large ~ 350 amino acid C-terminal HECT domain responsible accepting and transferring ubiquitin. The crystal structure of the E6AP HECT domain in complex with its E2, UbcH7, showed that the HECT domain is L-shaped in structure, with an elongated N-terminal lobe that binds to the E2 and a smaller C-terminal lobe that contains the active site cysteine (Figure 4D; ref 70). A remarkable feature of this structure is that the E2 and E3 active site cysteines are separated by a distance of 41 Å, indicating that large conformational changes must be required to bring the two active sites close together. The importance of large conformational changes in HECT domain catalysis is also supported by the structures of the HECT domains from WWP1 and Smurf2. Modeling of UbcH7 into these structures showed that the E2 active site cysteine is separated from the WWP1 HECT active site cysteine by a distance of 16 Å (84) and from the Smurf2 HECT cysteine by a distance of 50 Å (85). Importantly, rotation of the WWP1 HECT domain C-lobe around a flexible hinge loop connecting the N- and C-lobes of the domain demonstrated that the HECT and E2 active sites could be juxtaposed within ~ 5 Å of each other, a distance that might reasonably be bridged by local regorganization of both active sites (84).

The distinct conformations achieved in the E6AP, WWP1, and Smurf2 HECT domain structures suggest a model for how the HECT domain might catalyze polyubiquitination (84). The model proposes that a growing polyubiquitin chain is assembled on the HECT domain active site cysteine through a mechanism that requires the distal ubiquitin on the end of the growing chain to attack the E2-ubiquitin thioester intermediate. The proximal ubiquitin remains tethered to the active site through a thioester linkage, and the entire chain moves further away from the E2 active site with each successive round of ubiquitin transfer. At some point, steric limitations

would impede chain elongation, and the chain would be transferred to a bound substrate. This model was confirmed experimentally with E6AP, which assembles a K48-linked chain on its active site cysteine in a manner that depends on two thioester-linked ubiquitin molecules and E2 concentration (86). However, another HECT E3 known as KIAA10 uses a completely different mechanism of chain synthesis (86). In this case, the chain is built up directly on the substrate (either free ubiquitin or ubiquitin previously conjugated to a substrate) and involves a reaction in which the distal ubiquitin on the end of the growing chain attacks the HECT-ubiquitin thioester. Thus, like RING E3s, different HECT E3s employ diverse mechanisms of polyubiquitin chain synthesis– it is unclear if properties of the E2 play a role in determining the mechanism of chain formation or linkage selection.

Rsp5 and the Nedd4 Family of Ubiquitin Ligases

Rsp5 is the prototypical member of a family of ubiquitin ligases that share a common modular domain architecture and function in diverse biological processes in eukaryotes (87). These ubiquitin ligases, known as the Nedd4 family of E3s, are comprised of an N-terminal C2 domain involved in membrane localization, one to four central WW domains responsible for substrate recognition, and a C-terminal HECT domain that functions in catalyzing ubiquitination of substrates (Figure 5A). Whereas higher eukaryotes contain several Nedd4-like proteins that are specialized for different cellular functions, Rsp5 is the sole member of this family in budding yeast and therefore carries out many of the functions ascribed to individual Nedd4 family members in higher organisms. The following section summarizes the known properties of the individual domains that define these E3s and provides several examples of key physiological processes regulated by Rsp5 and Nedd4 family members.

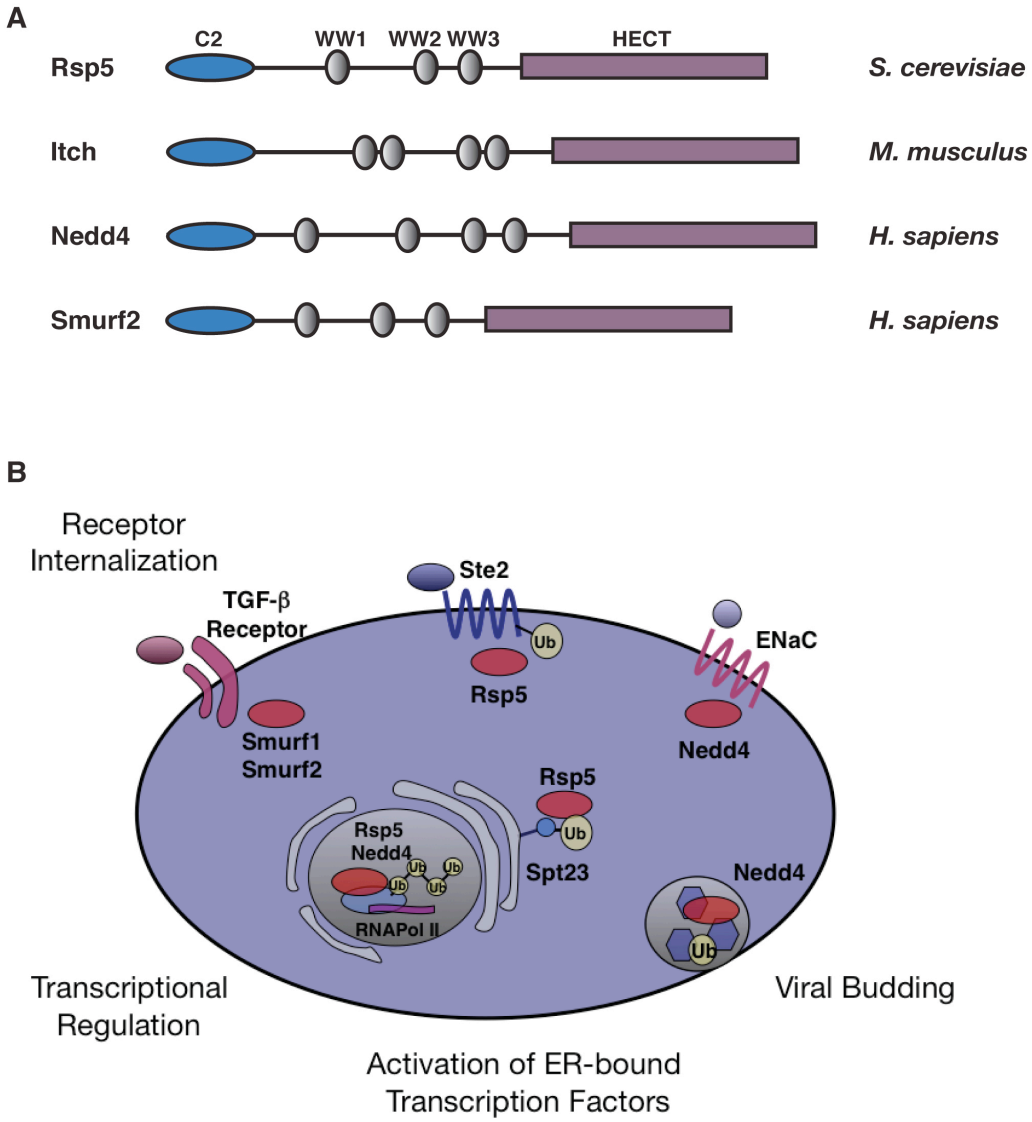

Figure 5. Rsp5 and the Nedd4 family of ubiquitin ligases. (A) Schematic representation of Rsp5 and several Nedd4 family members. The position of each functional domain and the organism that the E3 is found in is indicated. (B) Cellular processed regulated by Rsp5 and Nedd4 family members. A generic eukaryotic cell is depicted. E3s are shown as red ovals, and ubiquitin (Ub) is represented by a yellow ball. All references are in the text, except for Rsp5-mediated ubiquitination and partial processing of the membrane-bound transcription factor Spt23 (see Hoppe et al. (2000) Cell *102*, 577-586).

Domain architecture of Rsp5 and Nedd4 family members

The C2 domain is a 120-130 amino acid sequence that was originally identified in Protein Kinase C isoforms as a calcium-dependent phospolipid-binding domain (88). It interacts with a variety of phospholipids and proteins (89) and is thought to function as a membrane recruitment domain. The C2 domains of Rsp5 and Itch, for instance, associate with endosomal membranes to promote the ubiquitination of proteins involved in the biosynthetic and endocytic pathways (90,91). In yeast, the C2 domain of Rsp5 also directly interacts with proteins that appear to facilitate its association with membranes (92). The C2 domain has additional functions that are distinct from membrane recognition and association. For example, the C2 domain of Smurf2 participates in an intramolecular interaction with the Smurf2 HECT domain that acts to inhibit formation of the HECT-ubiquitin thioester (93). This autoinhibitory interaction also occurs in the Nedd4-1 and WWP2 family members, suggesting that the catalytic activity of these E3s is regulated in a similar manner.

WW domains are small modules (~ 35 amino acids) that obtained their name from the presence of two conserved tryptophan residues separated by 20-22 amino acids. They are found in a variety of different proteins and generally bind to proline-rich sequences. Nedd4 family members typically contain three or four WW domains (Figure 5A), and their primary function within these E3s is to interact with substrates targeted for ubiquitination. Nedd4 family WW domains primarily recognize PPxY motifs (where x is any amino acid), but have also been shown to bind to pS/pT (phosphoserine/phosphothreonine) residues (87). Like the C2 domain, the WW domains of Nedd4 family E3s can also participate in intramolecular interactions that inhibit the activity of the HECT domain. For example, the WW domains of Itch interact with and inhibit the activity of the Itch HECT domain. Interestingly, this autoinhibitory interaction is

relieved by a phosphorylation-induced conformational change in the E3, suggesting a regulatory mechanism for the activation of ubiquitin ligases within the Nedd4 family (94).

The HECT domain is a ~ 350 amino acid sequence found at the C-terminus of all Nedd4 family E3s that functions in catalysis (see previous section). Despite some recent progress in understanding the mechanisms by which other HECT domains catalyze ubiquitination, the biochemical activities of Nedd4 family HECT domains are still poorly characterized. The full-length Rsp5 and Nedd4 HECT E3s cooperate with the Ubc1/Ubc4/Ubc5 family of E2s (95-99) to transfer monoubiqutin or polyubiquitin chains to a variety of different substrates (see refs 33, 100-104 for examples). Both of these E3s preferentially assemble chains linked through K63 of ubiquitin *in vitro* (43,105), suggesting that K63-linked chain synthesis is an inherent property of the HECT domains in these E3s. Yet, several reports indicate that Rsp5 (both the full-length enzyme and the isolated HECT domain) can assemble chains linked through K48 of ubiquitin *in vitro* (see Chapter II; ref 105), and the enzyme targets several substrates for degradation in a proteasome-dependent manner *in vivo* (106,107). It is presently unclear if K48-linked chain synthesis represents a major activity of Rsp5 *in vivo*, although a dual mode of chain synthesis would help to explain the ability of this E3 to act as a multifunctional ligase in *S. cerevisiae*.

The HECT domains of other Nedd4 family members are not as well-characterized as those of Rsp5 and Nedd4. The Itch HECT domain is closely related in sequence to the HECT domain of Rsp5, yet Itch appears to catalyze the formation of K48 and K29-linked chains (36, 37,108). Thus, K63-linked chain synthesis is not a feature common to all HECT domains within the Nedd4 family of E3s. Although the types of chains assembled by other Nedd4 family members are unknown, functional studies suggest the existence of biochemical diversity within the family. For example, Smurf1 and Smurf2 target a number of different substrates involved in

TGF-β signaling for degradation (109), whereas WWP1 targets the p53 tumor suppressor for ubiquitination in a manner that stabilizes the protein and modulates its transcriptional activities (110).

Physiological processes mediated by Rsp5 and Nedd4 family members

Rsp5 and Nedd4 family members function in the regulation of numerous biological processes by targeting a variety of different substrates for ubiquitination. In many cases, the ubiquitination event acts as a signal to target these substrates for degradation by the proteasome, but ubiquitin signals appended by these E3s can also alter the activity or location of substrates in a proteasome-independent manner. In mammalian cells, Nedd4 family members regulate the activity of a number of critical tumor suppressors, and consequently they are targets for a variety of anticancer drugs (111). The following discussion highlights three different physiological processes that are mediated by Rsp5 and Nedd4 family members: the internalization of receptors and ion channels from the cell surface, the regulation of RNA polymerase II activity, and the budding of enveloped viruses from the plasma membrane (Figure 5B).

In yeast, Rsp5 controls the ubiquitin-dependent internalization of a number of plasma membrane proteins, including signaling receptors, transporters, and ion channels (112-114). Most, if not all, of these cell surface proteins are modified by Rsp5 with monoubiquitin or short K63-linked polyubiquitin chains, and the internalization of these membrane proteins into endocytic vesicles is dependent on these ubiquitination events. For instance, the yeast α-factor receptor, Ste2, is monoubiquitinated by Rsp5 on multiple lysines, and the ubiquitination of just one of these lysines is a necessary and sufficient signal for receptor internalization (112,115). In contrast, the uracil permease, Fur4, is modified by Rsp5 with multiple K63-linked diubiquitin chains. Monoubiquitination of the permease is sufficient to trigger its internalization, although

the attachment of K63-linked chains enhances the rate of permease internalization by about two-fold (33,116). Both of these membrane proteins are ultimately degraded in the lysosome-like vacuole in response to ligand stimulation (117,118), and thus their regulated entry into the endocytic pathway by Rsp5 is a crucial step in their downregulation.

In mammalian cells, Nedd4-like E3s also function to regulate the internalization of membrane proteins from the cell surface. For instance, several Nedd4 family proteins control the activity of the epithelial sodium ion channel (ENaC), a critical regulator of sodium and fluid transport found in a variety of epithelia (119). Nedd4-2 appears to be the most important family member in the regulation of ENaC activity, and interactions with ENaC are mediated by PPXY sequences located in each of the three subunits that make up the ENaC complex (104,119,120). Likewise, the Smurf1 and Smurf2 family members control the activity of the TGF-β receptors at the cell surface by targeting the receptors for ubiquitination and degradation (121-123). The Smurf proteins are recruited to TGF-β receptor complexes by an adaptor protein that negatively regulates TGF-β signaling called Smad7. Intriguingly, the Smad7 adaptor protein does not simply function as a scaffolding protein, but rather it directly activates Smurf2 ubiquitin ligase activity by recruiting the E2, UbcH7, to the Smurf2 HECT domain (85).

Nedd4 family E3s also play a critical role in regulating the activity of proteins found in the nucleus, such as RNA polymerase II, a large twelve subunit complex that transcribes the majority of genes in eukaryotes. In yeast, the large subunit of RNA polymerase II, Rpb1, is polyubiquitinated by Rsp5 and degraded by the proteasome in response to a variety of different stimuli (106,124). As for most substrates of Rsp5, binding to Rpb1 is mediated by the Rsp5 WW domains and PPXY sequences in the substrate, which in this case are located in the carboxy-terminal domain (CTD) of Rpb1 (124,125). Intriguingly, the ubiquitination of Rpb1 occurs on

two different sites, and the mechanism of ubiquitination involves the formation of a complex that contains Rsp5 and an E2 dimer that functions to select each of the ubiquitination sites by directly interacting with the surrounding sequences (126). This represents one of the few cases in which the selection of a ubiquitination site by an E2/E3 pair is understood in some molecular detail. Importantly, the role of Nedd4 family members in regulating the activity of RNA polymerase II is also conserved and physiologically relevant in higher eukaryotes, where the ubiquitination and degradation of the polymerase is controlled by Nedd4-1 (127).

Nedd4-like proteins also play an important role in regulating the budding of enveloped viruses from the plasma membrane. For example, the budding activity of several retroviruses, including human T cell leukemia virus type I (HTLV-1), Rous sarcoma virus (RSV), and human immuno-deficiency virus type I (HIV-1), is dependent on multiple Nedd4 family member E3s (128-130). For HTLV-1 and RSV, these E3s regulate budding by interacting with a PPXY sequence in the viral Gag protein to promote Gag ubiquitination, a modification that probably facilitates interaction with several ubiquitin-binding class E vacuolar sorting proteins important for budding (129). For HIV-1, however, the mechanism by which the E3 (Nedd4-2) facilitates budding is unknown, since the HIV-1 Gag protein lacks PPXY sequences (128,130). Recent work has shown that Nedd4-2 targets several class E vacuolar sorting proteins for ubiquitination, including Tsg101, suggesting a role for the ligase in directly activating the budding machinery (128). The identification of additional cellular or viral cofactors targeted by Nedd4-2 will likely be required to define the precise role of this E3 in HIV-1 budding.

Ubiquitin-binding Domains

Ubiquitin-binding domains (UBDs) are modular sequences found within a variety of proteins that bind noncovalently to monoubiquitin or polyubiquitin chains. These small (~ 20-

150 amino acids), independently-folded domains function as receptors in proteins that bind to ubiquitin signals conjugated to substrates or as catalytic modules in enzymes that function in ubiquitination or deubiquitination. UBDs are known to play an important role in many different biological processes, and the structural features of many of these domains are well-characterized. However, the *in vivo* targets of most UBDs within the context of their full-length proteins are unknown, and their precise modes of action are poorly defined. This section examines the known biochemical and structural features of the UBDs that have been defined to date and provides a few examples of how these domains function in the cell.

Biochemical and structural characteristics of UBDs

The first ubiquitin-binding domain to be identified was in a regulatory subunit of the proteasome known as S5a/Rpn10 (131). The short sequence of amino acids responsible for binding to ubiquitin, which later came to be known as the ubiquitin-interacting motif (UIM), was subsequently identified in a number of diverse proteins through iterative database searches (132). Experimental confirmation that UIMs represents a general type of ubiquitin-binding domain soon followed (133-136), and another motif that had previously been implicated in ubiquitin conjugation and deconjugation (137), the ubiquitin-associated (UBA) domain, was also shown to bind ubiquitin (138,139). The discovery of these two UBDs led to a virtual explosion in the field– at least 17 additional unique ubiquitin-binding motifs have been identified since, bringing the total number of UBDs up to 19 (12,13,140-142). These domains share some fundamental biochemical properties, but they are structurally disparate and function in a wide variety of cellular processes.

UBDs typically bind to ubiquitin with weak-moderate affinity (K_d 10-500 μM; ref 13), although a wide range of binding affinities have been reported (see refs 140,143 for extreme

examples). Several different hypotheses have been proposed to explain why UBD-ubiquitin interactions are generally weak (12,13). One obvious reason for low affinity interactions could be the relatively high concentration of free ubiquitin present in cells (~ 10 μM in mammalian cells; ref 144). Exposed UBDs with high ubiquitin-binding affinities would be occupied with free ubiquitin, thus inhibiting their interaction with intended ubiquitinated targets. Another reason for low affinity interactions might be to promote the rapid assembly or disassembly of protein networks. For example, protein complexes that function in the endocytic pathway contain multiple UBDs and ubiquitinated substrates, and their stability is likely to be regulated by numerous low affinity, high specificity UBD-ubiquitin interactions (12). Finally, in some cases, the existence of low affinity UBD-ubiquitin interactions might be explained by a high local concentration of the ubiquitinated target. For example, some endocytic proteins that are monoubiquitinated contain UBDs that interact with the covalently attached monoubiquitin conjugate intramolecularly (145).

Another feature of UBDs that has gained considerable attention and remains poorly understood is their ability to selectively interact (or not) with distinct types of ubiquitin signals (see Figure 2). Some UBDs, for instance, bind preferentially to K63 or K48-linked chains (146-149), whereas others bind nondiscriminantly to monoubiquitin and different types of chains (148). In some cases, a preference for binding to a particular type of chain makes sense. For example, the hHR23A UBA2 domain selectively interacts with K48-linked chains, consistent with the known role of this protein in shuttling ubiquitinated proteins to the proteasome (148, 150,151). Yet, in other cases, the apparent selectivity is counter-intuitive. For example, the E2-25K UBA domain binds preferentially to K63-linked chains (148), even though the full-length enzyme synthesizes K48-linked chains *in vitro* (152). Another poorly understood observation is

that most UBDs that bind to monoubiquitin bind to polyubiquitin chains with higher affinity *in vitro*, even though the relevant target is likely to be monoubiquitin *in vivo* (12). Although an apparent preference for binding to chains *in vitro* might simply reflect the availability of muliple monomeric ubiquitin subunits, the basis for selective recognition of a monoubiquitinated target *in vivo* has yet to be defined.

A common theme in the regulation of UBDs is that their accessibility and ubiquitin-binding activity is often modulated by interactions with other proteins or domains. Many UBDs have a higher affinity for ubiquitin when they are in isolation than when they are within the context of their full-length proteins (12), indicating that the ubiquitin-binding efficiency of some UBDs is inhibited by intramolecular interactions. Some UBDs interact with other proteins besides ubiquitin (for examples see refs 141,153), suggesting that the ubiquitin-binding activity of these domains can also be regulated by intermolecular interactions. The ability of a ubiquitin-binding domain to interact with a ubiquitinated target can also be modulated by intramolecular interactions with a covalently attached ubiquitin molecule. For instance, monoubiquitination of the endocytic protein Sts2 prevents its UBA domain from interacting with other ubiquitinated partners (145). Finally, it is likely that UBD-ubiquitin interactions are also regulated by post-translational modifications. Although there are no known regulatory roles for post-translationally modified UBDs, several proteomic studies have shown that ubiquitin is phosphorylated (154, 155), and sumoylated forms of ubiquitin are also known to exist (45). The extent to which these and other post-translational modifications affect the ability of ubiquitin to interact with all or a subset of UBDs remains to be determined.

The structural features of UBDs are extremely diverse. The largest structural class of UBDs, which includes the UIM, DUIM, MIU, UBA, CUE, and GAT domains (see the list of

abbreviations), are α-helical and interact with the I44 hydrophobic patch of ubiquitin (13). These domains consist of either a single α-helix (Figure 6A) or a three-helix bundle (Figure 6B) and make use of conserved hydrophobic residues located on the surface of an α-helix to contact ubiquitin. Zinc finger domains are the second largest class of UBDs and include the NZF, A20 ZnF, ZnF UBP, and UBZ domains (13,156). Zinc finger UBDs interact with ubiquitin through vastly different structural surfaces and are unique in their ability to interact with one of three different regions on the surface of ubiquitin: the I44 hydrophobic patch, the C-terminal G75 and G76 residues (Figure 6C), or a polar patch centered around D58. The remaining UBDs whose structures are known share less in common with each other, but they can be grouped into one of two different classes– those that contain an E2-like fold (UEV and UBC domains) or those that contain a pleckstrin-homology domain fold (GLUE and PRU domains). These domains are not as well-characterized as the α-helical and zinc finger UBDs, but they all appear to share the ability to interact with canonical I44 hydrophobic patch of ubiquitin (13,140,157-159).

Physiological roles of UBDs

Although the biochemical properties and three-dimensional structures of many UBDs are well-characterized, our understanding of how these domains elicit various biological responses in the cell is less clear. The functions of most UBDs within the context of a full-length protein are unknown, and the relevant ubiquitinated targets of many UBD have not been identified. Yet, the sheer number of cellular proteins modified with ubiquitin (see ref 11 for a proteomic study that identified 1075 unique ubiquitinated proteins in yeast) and the existence of diverse types of ubiquitin modifications (see Figure 2) suggests that UBDs are likely to function in numerous cellular events. The following discussion highlights several examples of UBDs that have been functionally characterized in at least some detail. The role of UBDs are specifically examined in

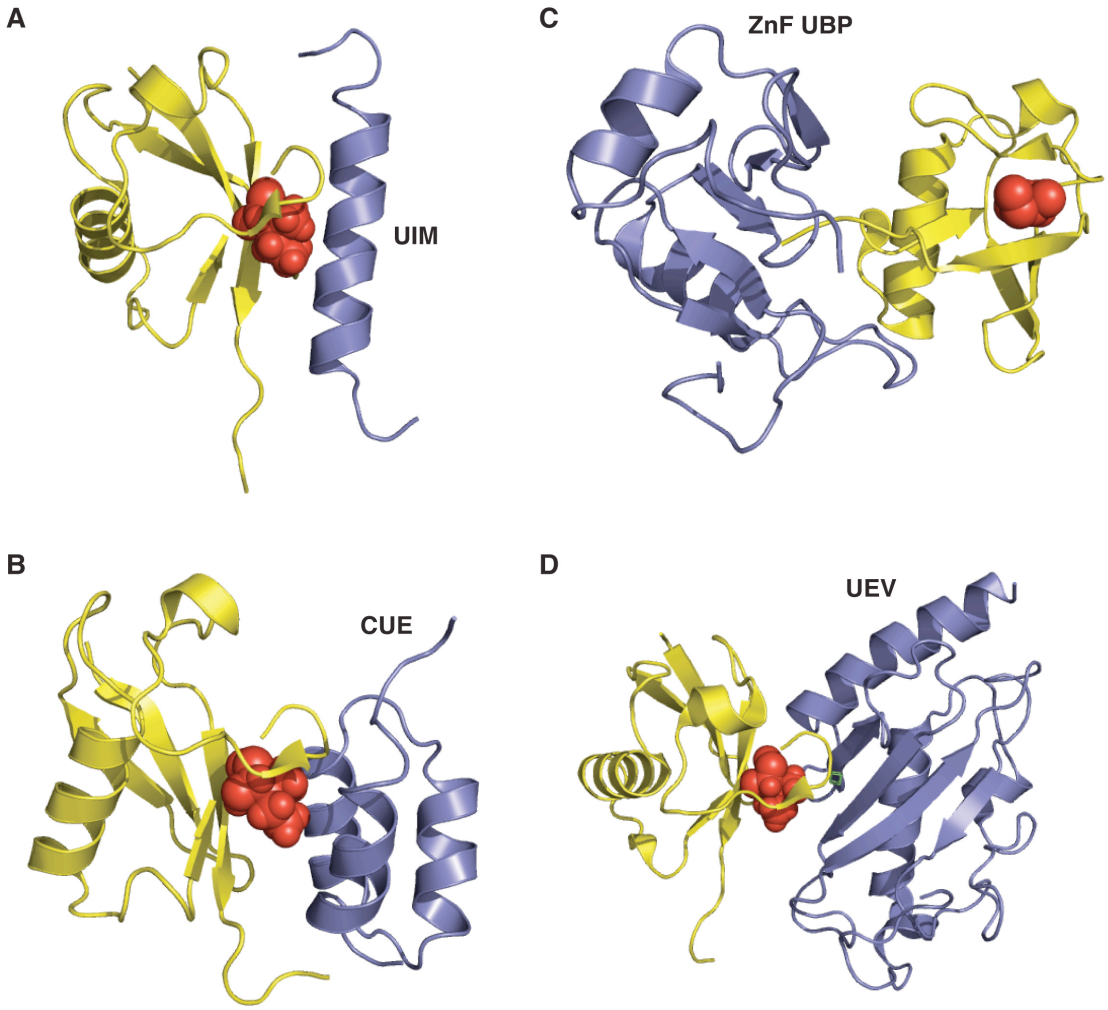

Figure 6. Three-dimensional structures of select ubiquitin-binding domains in complex with ubiquitin. Cartoon representations of several ubiquitin-binding domains (slate blue) in complex with ubiquitin (yellow) are illustrated as follows: (A) Vps27 UIM (PDB accession code 1QOW), (B) Cue2 CUE domain (PDB accession code 1OTR), (C) ZnF UBP domain (PDB accession code 2G45), and (D) Mms2 UEV domain (PDB accession code 1ZGU). The I44 residue is depicted as a red sphere for reference. All images were constructed in MacPyMOL. The full names of each individual ubiquitin-binding domain are indicated in the list of abbreviations.

three different contexts– as catalytic modules in ubiquitination and deubiquitination, as ubiquitin receptors in proteasomal degradation, and as regulatory modules in NF-κB signaling.

Perhaps the most clearly defined role of any UBD is in polyubiquitin chain formation and involves a UEV domain-containing protein called Mms2/Uev1a. As discussed previously, this UEV-containing protein forms a complex with an active E2, Ubc13, that together functions to specifically assemble K63-linked polyubiquitin chains (27,30). Structural and biochemical studies demonstrated that the UEV domain binds to an acceptor ubiquitin noncovalently to position K63 close to the Ubc13 active site, such that only K63 can attack the Ubc13 thioester-linked donor ubiquitin (73-75,160). Thus, the UEV domain functions as an orientation-specific ubiquitin-binding module that determines the chain linkage properties of an E2. This complex cooperates with at least two different RING E3s to conjugate K63-linked chains to substrates involved in DNA repair and NF-κB signaling (26,30,76), and, in both cases, the K63-linked chains are functionally important signals (3,161). Other E2s also carry UBDs that have been implicated in the regulation of polyubiquitin chain synthesis (67-69,162), indicating that coupled ubiquitin binding might be a general requirement for chain synthesis catalyzed by E2s.

Another example of a UBD with a clearly defined role in catalysis comes from structural and biochemical studies of isopeptidase-T (IsoT), a deubiquitinating enzyme responsible for disassembling the majority of unanchored polyubiquitin chains in cells (163-165). IsoT contains four ubiquitin-binding domains, which together function to coordinate the binding and hydrolysis of unanchored polyubiquitin chains (166,167). A structural analysis of one of these domains, the ZnF UBP domain, showed that it contains a deep binding pocket where the C-terminal diglycine motif of ubiquitin binds (167). The structure explains the specificity of isoT for an unmodified C-terminus on the proximal subunit of polyubiquitin (the subunit that can be conjugated to a

substrate through an isopeptide bond) and supports a model for the catalytic activation of isoT that involves the initial recognition of a free polyubiquitin chain by the ZnF UBP domain (167). Intriguingly, the ZnF UBP domain from an E3 also requires the C-terminus of ubiquitin for binding, suggesting that this mode of ubiquitin recognition is also functionally relevant in other catalytic reactions (167).

UBDs play an important role in controlling the delivery of polyubiquitinated proteins to the proteasome. The recognition of polyubiquitinated substrates by the proteaseome requires the actions of several different types of ubiquitin receptors. Some of these receptors, which include the Rpn10/S5a and Rpn13 subunits, are intrinsically associated with the proteasome and contain UBDs that directly recognize the polyubiquitin signal (131,140). Other types of receptors, which include the Rad23, Dsk2, and Ddi1 proteins, appear to act as adaptors that function to escort polyubiquitinated proteins to the proteasome. These adaptor proteins are defined by the presence of an N-terminal ubiquitin-like (UBL) domain, which binds to the proteasome, and one or more UBA domains, which bind to polyubiquitinated proteins (reviewed in ref 53). Surprisingly, there is a significant amount of functional redundancy between these different types of receptors– a recent study reported that abrogating the ubiquitin-binding function of all five receptors present in yeast is required for stabilization of endogenous ubiquitin conjugates (140). Although it is not completely clear why there is such substantial functional overlap, the requirement of distinct substrates for different types of receptors *in vivo* suggests that these ubiquitin receptors are specialized for specific targets (53).

A role for multiple UBDs in the recognition of diverse ubiquitin signals is also evident in the NF-κB signaling pathway, which plays a critical role in controlling key immune responses, cell survival, and proliferation (168,169). NF-κB proteins are activated by an upstream kinase

complex (145) that consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit essential for NF-κB activation called NEMO. NEMO binds specifically to K63-linked chains (via its coiled-coil leucine zipper UBD) attached to another class of regulatory proteins known as RIPs (receptor-interacting proteins), which link activated receptors at the cell surface to the IKK complex (170,171). Mutations in NEMO that abolish binding to these K63-linked chains block the activation of IKK and NF-κB proteins, thus providing direct evidence that the ubiquitin-binding activity of NEMO is required for its signaling properties. Interactions between UBDs and ubiquitinated proteins also play additional roles in the NF-κB signaling pathway. For instance, the ABIN proteins are negative regulators of NF-κB signaling that contain a UBD that interacts with ubiquitinated NEMO and is required for inhibition of NF-κB signaling (142). Several other proteins that function in NF-κB signaling are known to be ubiquitinated, indicating that UBD-ubiquitin interactions are likely to play an extensive role in the regulation of this signaling pathway (reviewed in 172).

Questions for Thesis Studies

Despite the availability of high resolution structures of several RING and HECT catalytic modules (70,71,78,84,85), the mechanisms of ubiquitination employed by most ubiquitin ligases are poorly understood. Large conformational changes are clearly necessary to achieve catalytic competence, and, for HECT E3s, these conformational changes appear to be a requirement for polyubiquitin chain synthesis (84,86). Although we are beginning to understand the mechanisms of chain formation empolyed by RING E3s in some detail (82,113,173), our understanding of how HECT E3s assemble chains is extremely limited. Most of our basic knowledge regarding the biochemical activities of HECT E3s comes from *in vitro* studies examining the assembly of unanchored polyubiquitin chains (86,174), whereas little is known about how these E3s catalyze

ubiquitination in the presence of a substrate. In addition, recent work has demonstrated that the activities of several HECT E3s are regulated by auxiliary factors (85,94,105), but the generality and precise roles of these regulatory factors in modulating HECT E3 activity are poorly defined.

This thesis examines the mechanisms of ubiquitination employed by the Rsp5 ubiquitin ligase, a HECT E3 that represents the sole member of a family of ubiquitin ligases conserved from yeast to humans. This E3 controls a variety of cellular events and is biochemically unique because it has the ability to catalyze several different types of ubiquitination– substrates of Rsp5 can be modified with either monoubiquitin, K63-linked ubiquitin chains, or K48-linked ubiquitin chains. Therefore, the study of this model enzyme provides an opportunity to investigate basic mechanisms of mono- and polyubiquitination and to examine regulatory switches that may determine the catalytic mode of an E3. Chapter II of this thesis describes the identification and characterization of a previously unknown noncovalent ubiquitin-binding site located in the Rsp5 HECT domain and provides evidence that the Rsp5 ubiquitin-binding site plays an important role in the regulation of polyubiquitin chain synthesis. In Appendix I, two additional factors that are likely to modulate Rsp5 activity are described, an intramolecular interaction and phosphorylation of the ligase. Finally, data describing a regulatory function for another type of ubiquitin-binding domain (a variant SH3 domain) unrelated to Rsp5 is presented. These studies contribute to our understanding of how Rsp5 and related ubiquitin ligases function and provide new insights into the mechanistic roles of UBDs.

CHAPTER II: REGULATION OF THE RSP5 UBIQUITIN LIGASE BY AN INTRINSIC UBIQUITIN-BINDING SITE

Abstract

Rsp5 is a Homologous to E6AP C-terminus (HECT) ubiquitin ligase (E3) that controls many different cellular processes in budding yeast. Although Rsp5 targets a number of different substrates for ubiquitination, the mechanisms that regulate Rsp5 activity remain poorly defined. Here we demonstrate that Rsp5 carries a noncovalent ubiquitin-binding site in its catalytic HECT domain. The N-terminal lobe of the HECT domain mediates binding to ubiquitin, and point mutations that disrupt interactions with ubiquitin alter the ability of the Rsp5 HECT domain to assemble polyubiquitin chains *in vitro*. Point mutations that disrupt ubiquitin binding result in temperature-sensitive growth defects in yeast, indicating that the Rsp5 ubiquitin-binding site is important for Rsp5 function *in vivo*. The Nedd4 HECT domain N-lobe also contains ubiquitin-binding activity, suggesting that interactions between the N-lobe and ubiquitin are conserved within the Nedd4 family of ubiquitin ligases. We propose that a subset of HECT E3s are regulated by a conserved noncovalent ubiquitin-binding site that functions to restrict the length of polyubiquitin chains synthesized by the HECT domain.

Introduction

Modification of proteins with ubiquitin plays an important role in controlling and modulating a number of cellular processes. Ubiquitination is the primary signal used to target cellular proteins for degradation by 26S proteasomes (1), and it is an important nonproteolytic signal in many other biological pathways, including DNA repair, NF-κB signaling, transcription, and endocytosis (3-5). The ability of ubiquitin to function in a variety of cellular processes can be explained by the existence of structurally distinct ubiquitin modifications and the recognition of ubiquitination signals by a diverse set of ubiquitin-binding domains (UBDs) found within a host of cellular proteins (12,13).

Ubiquitin is conjugated to other proteins by a series of enzymes that act in a well-defined, sequential manner (54). The final transfer of ubiquitin to a cellular protein is usually carried out by an E3 ubiquitin ligase. E3s contain the primary determinants for substrate recognition and generally belong to one of two families. Really Interesting New Gene (92) E3s are thought to act primarily as molecular scaffolds to bring together a ubiquitin-conjugating enzyme (E2) charged with ubiquitin and a substrate targeted for ubiquitination (71,78). In contrast, HECT E3s form an obligatory covalent thioester intermediate with ubiquitin before transferring ubiquitin to the substrate. A conserved cysteine residue located within the active site of the HECT domain is required for formation of this intermediate (175,176).

Ubiquitin modifications adopt distinct structural conformations, and these structural differences are functionally important because they have the ability to target proteins for different fates in the cell. Protein monoubiquitination is an important regulatory signal in a variety of basic cellular processes, including endocytosis, histone remodeling and viral budding (22). In contrast, polyubiquitin chains act as signals specialized for other cellular functions. For example, chains linked through K48 of ubiquitin play a well-characterized role in targeting proteins for degradation by the proteasome, whereas chains linked through K63 of ubiquitin act as nonproteolytic signals in DNA repair, kinase activation and endocytosis (177). Despite the known importance of diverse ubiquitination signals, little is known about the mechanisms used by E3 enzymes to ensure that substrates targeted for ubiquitination are modified with the appropriate type of signal.

Rps5 is a HECT E3 that controls a broad range of cellular processes in *Saccharomyces cerevisiae* and is part of a large family of proteins that controls analogous processes in mammalian cells (87). For example, Rsp5 and its well-characterized mammalian homologue,

Nedd4, are both required for efficient internalization of cell surface receptors from the plasma membrane (4,178). Both E3s also play a role in regulating the stability of RNA polymerase II by targeting the polymerase for ubiquitination and degradation (106,124,127). In yeast, Rsp5 has been implicated in many other cellular processes, including the nuclear export of mRNA, mitochondrial inheritance, and the activation of ER-bound transcription factors (31,179-181).

The ability of Rsp5 to act as a multi-functional E3 in yeast is due, at least in part, to its capacity to modify different substrates with distinct mono- and polyubiquitin signals. Several proteins that function in endocytosis are monoubiquitinated in an Rsp5-dependent manner (100,101,114). In contrast, Rsp5 targets a number of cellular proteins for polyubiquitination, including the large subunit of RNA polymerase II, the vacuolar membrane protein Sna3, and the mRNA nuclear export factor Hpr1 (124,179,182,183). Although Rsp5 possesses an intrinsic preference for K63-linked chain synthesis *in vitro* (105) and modifies a number of its substrates with K63-linked chains *in vivo* (33,102,184), the enzyme can also assemble polyubiquitin chains linked through K48 of ubiquitin. In general, however, the mechanisms that control the linkage and length of polyubiquitin chains synthesized by Rsp5 remain poorly defined. Here we identify a previously unknown noncovalent ubiquitin-binding site located in the Rsp5 HECT domain that regulates the length of K63-linked and K48-linked polyubiquitin chains assembled by the Rsp5 HECT domain.

Materials and Methods

Plasmid construction and mutagenesis

Plasmids encoding fragments of Rps5 fused to glutathione S-transferase (GST) were constructed in pGEX vectors (GE Healthcare, Waukesha, Wisconsin). The GST (LHP497), GST-3xWW (aa 228-430, LHP703), and GST-HECT C-terminal lobe (C-lobe) (aa 691-809,

LHP2468) plasmids were constructed by PCR-amplifying the relevant DNA sequence and ligating into the pGEX-6P-2 vector. The GST-HECT domain plasmid (aa 425-809, LHP1434) was generated in a similar fashion but with ligation into the pGEX-4T-3 vector. A plasmid encoding the GST-HECT N-terminal lobe (N-lobe) (aa 425-691, LHP2325) was created by introducing a STOP codon after amino acid 691 in LHP1434 using QuikChange site-directed mutagenesis (Stratagene, La Jolla, California). The GST-C2 domain plasmid was obtained from Hilary Godwin (University of California, Los Angeles, Los Angeles, California). All point mutations in LHP2325 and LHP1434 were introduced by QuikChange mutagenesis and verified by automated sequencing.

Plasmids encoding hexahistidine (His_6)-tagged ubiquitin (LHP1404), Rvs167 SH3 domain (aa 428-482, LHP1496), and the HECT domain N-lobes from Rsp5 (aa 426-691, LHP 2381), Nedd4 (aa 501-767, LHP2443), and Tom1 (aa 2880-3148, LHP2442) were generated by ligation-independent cloning of the relevant PCR-amplified fragment into the pET-30 vector (EMD Chemicals, La Jolla, California). All point mutations in LHP1404 were introduced by QuikChange mutagenesis. A yeast expression vector carrying untagged wildtype *RSP5* under the control of its endogenous promoter was provided by Jon Huibregtse (University of Texas at Austin, Austin, Texas) and modified to remove a NotI site in the multiple cloning region, generating LHP472. QuikChange mutagenesis was used to introduce the Y516A (LHP2735) and F618A (LHP2737) mutations into this plasmid. Construction of the hemagglutinin (HA)-tagged Rsp5 plasmid (LHP478) has been described previously (112). Multicopy plasmids encoding wildtype (LHP308) or lysine-less (0K) (LHP306) ubiquitin were adapted from plasmids pES7 and pTER62 obtained from Mike Ellison (University of Alberta, Edmonton, Canada) by removing the c-myc epitope tag.

Yeast strains and growth media

All yeast strains were propagated in synthetic minimal medium (YNB; US Biological, Swampscott, Massachusetts) or rich YPD medium (2% bacto peptone, 1% yeast extract, 2% glucose supplemented with 10 mg/l adenine, uracil, and tryptophan). Yeast transformations were performed using standard techniques (185). Strains carrying *RSP5* (LHY5653), *rsp5*^{Y516A} (LHY 5655), or *rsp5*^{F618A} (LHY5657) as the sole source of Rsp5 were constructed by transforming the wildtype or mutant plasmids into LHY4507 (*rsp5Δ* p*GFP-Rsp5[URA3]*) and selecting for loss of p*GFP-RSP5*[URA3] as described previously (112).

Ubiquitin-binding assays

Binding assays carried out with ubiquitin-agarose beads (Boston Biochem, Cambridge, Massachusetts) and lysates from yeast cells expressing HA-tagged Rsp5 were performed as previously described (136), except that 7.5 mg of lysate protein was incubated with the beads for 1 hour at 4°C. Recombinant proteins for all other binding experiments were expressed in *E. coli* (BL21-CodonPlus cells, Stratagene) by inducing cultures with 1 mM isopropyl-β-D-thiogalacto-pyranoside for 3-5 hours at 18°C-37°C. Preparation of bacterial cell lysates has been described previously (136). Immobilization of His_6-tagged proteins on TALON metal affinity resin (Clontech) and GST-tagged proteins on glutathione resin (GE Healthcare) was performed according to the manufacturer's instructions.

Binding of bacterially expressed GST-tagged Rsp5 fragments to ubiquitin-agarose or agarose beads was performed by incubating the lysates and the beads for 1 hour at 4°C. The beads were then washed four times in phosphate-buffered saline (PBS) lysis buffer (115 mM NaCl, 16 mM Na_2HPO_4, 4 mM KH_2PO_4, 1% Triton X-100, 5% glycerol, pH 7.3), and bound proteins were eluted by boiling in 1x Laemmli sample buffer (125 mM Tris-HCl pH 6.8, 4%

SDS, 500 mM 2-mercaptoethanol, 20% glycerol, .02% bromophenol blue). Binding of bacterially expressed GST-tagged HECT fragments or purified N-lobes to immobilized His_6-ubiquitin or His_6-Rvs167SH3 was carried out in the same manner, except that the beads were washed twice in PBS lysis buffer containing 10 mM imidazole and twice in PBS lysis buffer containing 20 mM imidazole. Bound proteins were resolved by SDS-PAGE and analyzed by either immunoblotting with anti-GST antiserum (GE Healthcare) or staining with Coomassie Brilliant Blue G250 (Bio-Rad Laboratories, Hercules, California).

Binding to polyubiquitin chains was assayed by incubating 10 μg of purified K63-linked or K48-linked chains (Boston Biochem) with immobilized GST, GST-HECT or GST-N-lobe proteins for 1 hour at 4°C. The beads were washed four times in PBS lysis buffer, and bound chains were eluted by boiling in Tris-Tricine sample loading buffer (10 mM Tris-HCl pH 6.8, 3% SDS, 500 mM 2-mercaptoethanol, 2 mM EDTA, 25% glycerol, 0.02% bromophenol blue). Bound chains were resolved by Tris-Tricine gel electrophoresis and analyzed by anti-His immunoblotting (Bethyl Laboratories, Montgomery, Texas).

Analysis of protein expression levels in yeast lysate

Protein expression levels of Rsp5 and free ubiquitin were analyzed as follows: $2x10^8$ cells from each strain were harvested before and after shifting cultures to 37°C for 1 hour. Cells were lysed under denaturing conditions in 1 mL lysis buffer (10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.2 N NaOH, 0.5% 2-mercaptoethanol) and then precipitated with 10% trichlororoacetic acid. Protein precipitates were collected by centrifugation, resuspended in 1x Laemmli sample buffer, and analyzed by SDS-PAGE and immunoblotting with Rsp5 antiserum (101) or ubiquitin antiserum (Millipore, Temecula, California).

In vitro ubiquitination assays

Yeast E1, UbcH5a, ubiquitin, and all ubiquitin mutants were purchased from Boston Biochem. All GST-HECT proteins were expressed in *E. coli* (BL21-CodonPlus cells), purified on glutathione resin according to the manufacturer's instructions, and eluted from the resin in 10 mM Tris-HCl, pH 8.0 buffer containing 10 mM glutathione. Recovered proteins were assayed for purity by SDS-PAGE and Coomassie staining, and protein concentrations were determined using the Bradford assay. HECT domain autoubiquitination assays were carried out with 0.1 μM yeast E1, 0.2 μM UbcH5a, 0.3 μM GST-HECT, and 75 μM of the indicated ubiquitin. Reactions were initiated by adding buffer containing ATP (final concentrations: 25 mM Tris pH 7.5, 50 mM NaCl, 4 mM $MgCl_2$, 0.1 μM DTT, 4 mM ATP). After brief mixing, the zero time point was withdrawn on ice, and reactions were immediately transferred to a 30°C water bath. Reaction aliquots were removed at the indicated times, added to an equal volume of 2x Laemmli sample buffer, and analyzed by SDS-PAGE and anti-GST immunoblotting.

Results

The Rsp5 HECT domain binds to ubiquitin

To identify cellular proteins that bind to ubiquitin, a yeast genomic two-hybrid library was screened with a ubiquitin mutant defective in K48-linked chain formation (Ub^{K48R}) as bait. A fragment of Rsp5 encoding residues 195-809 was identified in this screen (M. Sutanto, S. Shih, and L. Hicke, unpublished data), and an interaction with Ub^{K48R} was confirmed in an independent two-hybrid assay (Figure 7A). To verify that the full-length Rsp5 protein binds to ubiquitin, we tested the ability of an HA-tagged version of Rsp5 expressed in yeast to bind to ubiquitin-agarose beads. HA-Rsp5 bound to ubiquitin-agarose beads but not to control agarose beads (Figure 7B), confirming that full-length Rsp5 is a ubiquitin-binding protein.

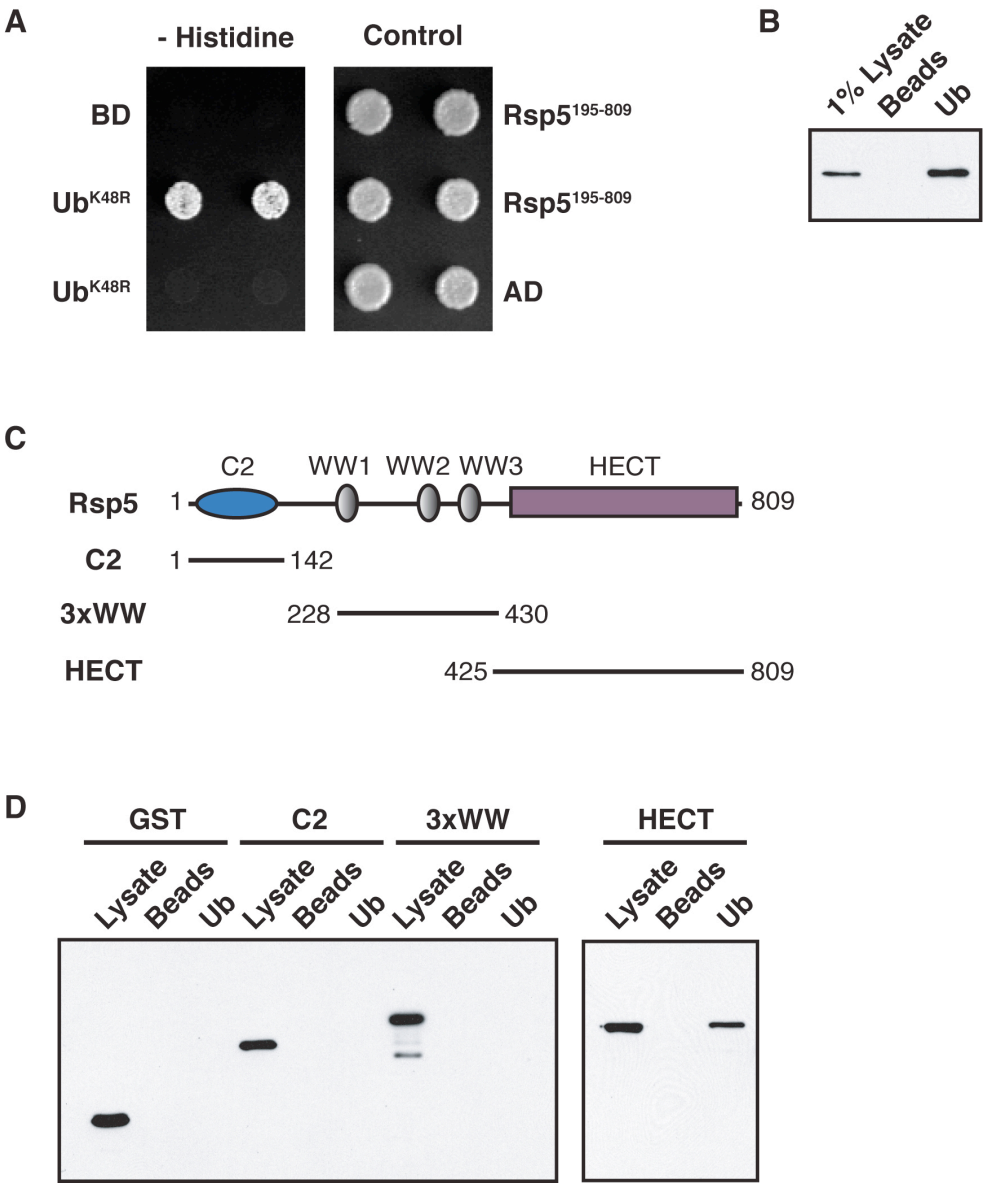

Figure 7. The Rsp5 HECT domain binds directly to ubiquitin. (A) A fragment of Rsp5 containing residues 195-809 fused to the Gal4 activation domain (AD) was assayed for interaction with Ub^{K48R} fused to the Gal4 DNA-binding domain (BD) by the yeast two-hybrid method. Two different transformants were assayed for growth on media lacking histidine to test for an interaction or control media lacking leucine and tryptophan. **(**B) A lysate prepared from yeast cells expressing HA-tagged Rps5 (LHY856) was incubated with ubiquitin-agarose beads (Ub) or agarose beads alone (Beads). Bound proteins were eluted, and Rsp5 was detected on an anti-HA immunoblot. (C) Schematic representation of Rsp5 indicating the position of its functional domains. Fragments tested for ubiquitin binding in C are shown. (D) Bacterial lysates from cells expressing the indicated GST-tagged Rsp5 domains were incubated with ubiquitin-agarose beads or agarose beads alone. Total lysates and bound proteins were analyzed by anti-GST immunoblotting.

Rsp5 is part of a large family of proteins found throughout eukaryotes that share a common modular domain architecture (87). All family members contain an N-terminal C2 domain, one to four central WW domains, and a large ~ 350 amino acid HECT domain at the C-terminus (Figure 7C). Rsp5 does not carry any of the numerous UBDs that have been described to date (12,13,140-142). To test which region of the Rsp5 protein is responsible for its ubiquitin-binding activity, we assayed different fragments of Rsp5 for binding to ubiquitin-agarose beads. A GST-HECT domain fusion protein expressed in *E. coli* bound specifically to the ubiquitin-agarose beads in this assay, whereas fragments of Rsp5 containing the C2 or three WW domains did not (Figure 7D). These observations indicate that the Rsp5 HECT domain binds directly to monoubiquitin.

The Rsp5 and Nedd4 HECT domain N-lobes bind to ubiquitin

The HECT domain is highly structured, with an elongated, α-helical N-terminal lobe (N-lobe) and a smaller, globular C-terminal lobe (C-lobe) (70,84,85). The C-lobe contains the conserved cysteine residue that forms a thioester with ubiquitin, whereas the N-lobe interacts with an E2 enzyme to enable the transfer of ubiquitin from the E2 to the HECT active site cysteine. To determine which lobe of the Rsp5 HECT domain is responsible for ubiquitin binding, we expressed the isolated N- and C-lobes (Figure 8A) in bacteria and tested them for interaction with immobilized His_6-tagged ubiquitin. A GST-N-lobe fusion protein bound to ubiquitin just as well a GST-HECT domain fusion, whereas the isolated C-lobe did not bind to ubiquitin at all (Figure 8B). The HECT domain N-lobe also bound to polyubiquitin chains linked through either K63 or K48 of ubiquitin, with a preference for binding to longer chains (Ub_4-Ub_n) over shorter di- and triubiquitin chains (Figure 8C). From these experiments, we

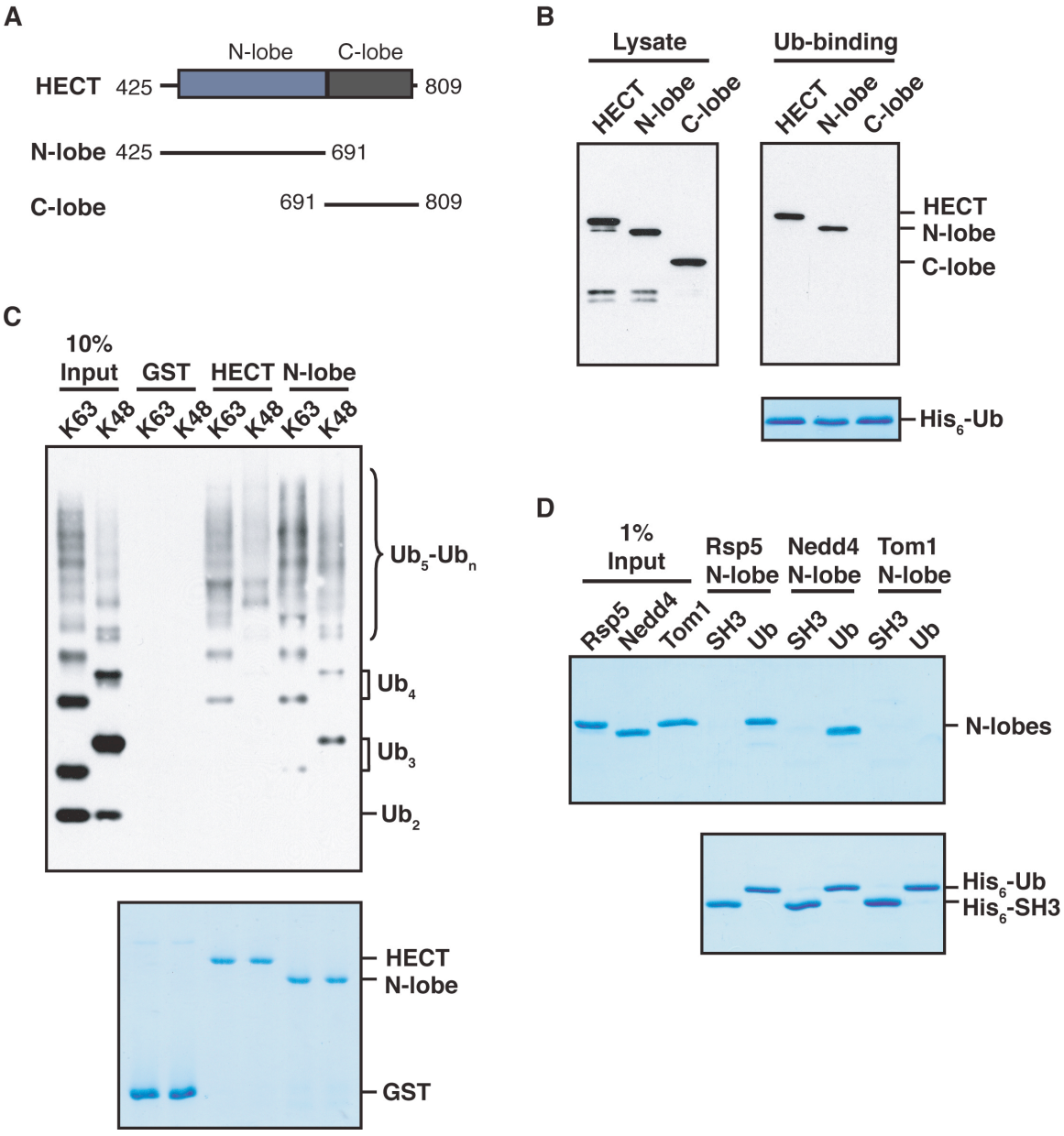

Figure 8. The Rsp5 and Nedd4 HECT domain N-lobes bind to ubiquitin. (A) Schematic representation of the Rsp5 HECT domain. Fragments tested for ubiquitin binding in B are shown. (B) Bacterial lysates from cells expressing the indicated GST-tagged HECT domain fragments were incubated with beads carrying immobilized His_6-tagged ubiquitin (His_6-Ub). Lysates and proteins eluted from the beads were analyzed by anti-GST immunoblotting (top panels) or Coomassie staining (bottom panel). (C) GST-HECT domain and GST-HECT N-lobe fusions were immobilized on beads, and the beads were incubated with purified His_6-tagged K63-linked or K48-linked polyubiquitin chains. Purified chains (10% Input) and proteins eluted from the beads were analyzed by anti-His immunoblotting (top panel) or Coomassie staining (bottom panel). (D) The indicated N-lobes were purified from an *E. coli* lysate and incubated with equivalent amounts of immobilized His_6-Ub or a control His_6-tagged SH3 domain from Rvs167 (His_6-SH3). Purified N-lobes (1% Input) and proteins eluted from Ub or SH3 beads were detected by Coomassie staining.

conclude that all of the Rsp5 ubiquitin-binding activity resides in the N-lobe of its HECT domain.

To test if the ability of the N-lobe to bind ubiquitin is a general property of HECT domains, we assayed the N-lobes from the mammalian Nedd4 and yeast Tom1 HECT domains for ubiquitin binding. Nedd4 is both structurally and functionally related to Rsp5, and its HECT domain N-lobe is 55% identical to the Rsp5 N-lobe in sequence. In contrast, Tom1 is unrelated to Rsp5, and its HECT domain N-lobe is only 40% identical to the Rsp5 N-lobe. In a ubiquitin-binding assay carried out with both of these N-lobes, the Nedd4 N-lobe bound specifically to immobilized His_6-tagged ubiquitin, whereas the Tom1 N-lobe did not detectably bind to ubiquitin or a negative control His_6-tagged SH3 domain (Figure 8D). These observations indicate that the N-lobe ubiquitin-binding site is conserved in the Nedd4 HECT domain, but is not a feature common to all HECT domains.

Interaction surfaces on the Rsp5 HECT domain N-lobe and ubiquitin

To map the ubiquitin-binding surface on the Rsp5 HECT domain N-lobe, we used an alanine-scanning mutagenesis approach. Although the three-dimensional structure of the Rsp5 HECT domain has not been determined, modeling of the Rsp5 HECT domain onto the known structure of the closely related WWP1 HECT domain (84) allowed us to target surface-exposed residues on the Rsp5 N-lobe for mutagenesis. Individual residues and stretches of up to three contiguous residues were mutated to alanine, and the resulting GST-tagged N-lobe mutants were tested for binding to immobilized His_6-tagged ubiquitin (Figure 9A, data not shown). Most of the ~ 50 mutations that were made had no effect on binding to ubiquitin. Three mutations in the N-lobe (Y516A, F618A and $VV \rightarrow AA^{621\text{-}622}$) completely abolished ubiquitin binding. An additional three mutations (N513A, Y521A and R651A) caused a reduction in binding. Finally,

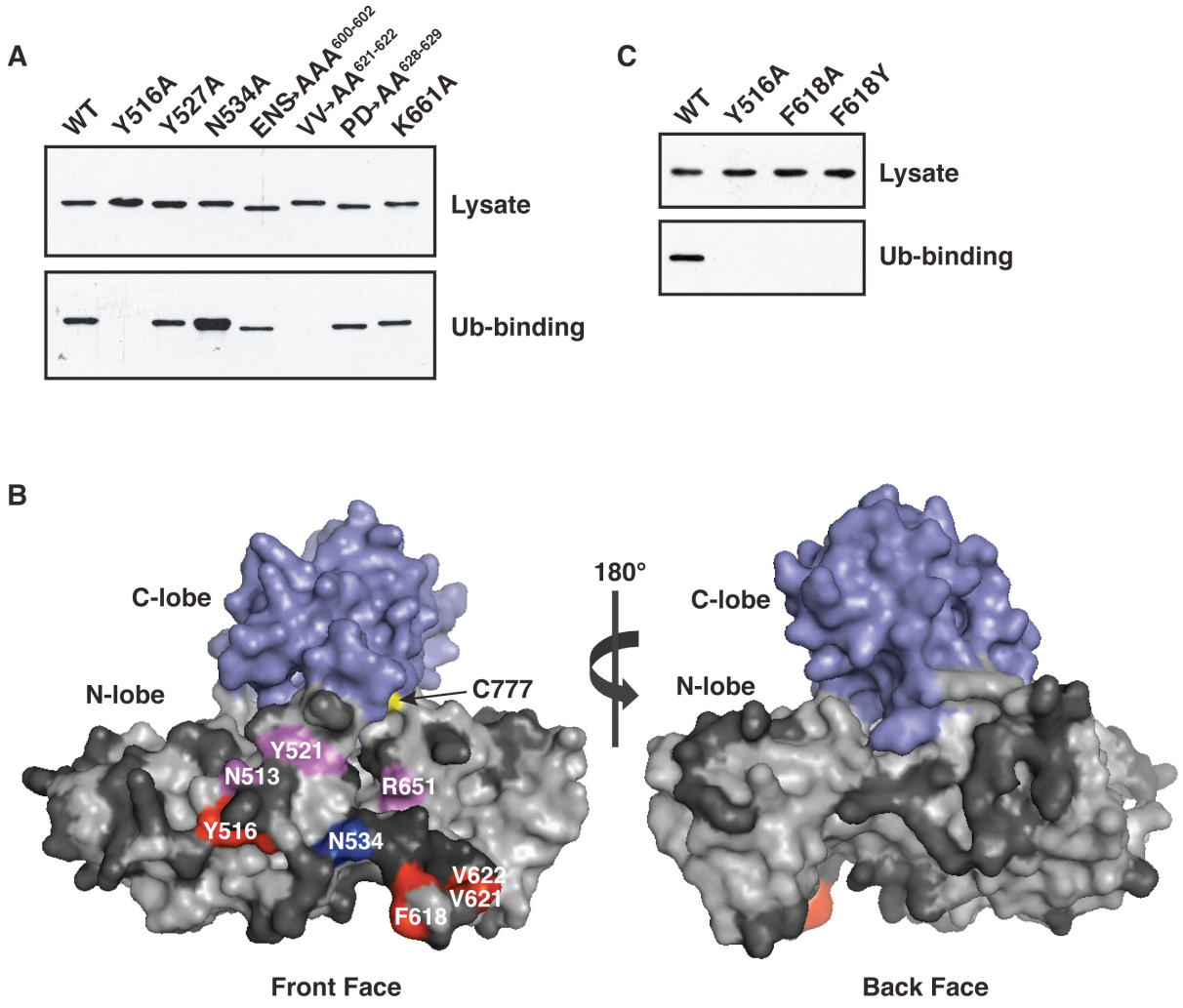

Figure 9. Ubiquitin binds to a region on the front surface of the Rsp5 HECT domain N-lobe. (A) A representative experiment from the alanine-scanning mutagenesis of residues in the Rsp5 HECT domain N-lobe. Bacterial lysates from cells expressing the indicated GST-tagged N-lobe mutants were incubated with beads carrying immobilized His_6-tagged ubiquitin. Lysates and proteins bound to ubiquitin were analyzed by anti-GST immunoblotting. Mutation of the acidic residues in the ENS→$AAA^{600-602}$ and PD→$AA^{628-629}$ mutants resulted in slightly altered electrophoretic mobility. (B) Surface representation of the Rsp5 HECT domain, created by modeling onto the WWP1 HECT domain crystal structure (PDB accession code 1ND7). Results of the alanine mutagenesis are summarized as follows: red = mutation abolished binding; magenta = mutation reduced binding; blue = mutation enhanced binding; dark grey = mutation had no effect. (C) Bacterial lysates from cells expressing the indicated GST-tagged HECT domain mutants were incubated with immobilized His_6-tagged ubiquitin. Lysates and proteins bound to ubiquitin were analyzed by anti-GST immunoblotting.

one mutation (N534A) reproducibly enhanced binding ~ 2-3 fold. Mapping of the results from the alanine mutagenesis onto the modeled three-dimensional structure of the Rsp5 HECT domain showed that ubiquitin binds to a region on the front surface of the N-lobe that lies ~ 15-20 Å from the HECT active site cysteine (Figure 9B). Importantly, none of the mutations made on the back face of the N-lobe had any effect on binding to ubiquitin, confirming the specificity of the alanine-scanning mutagenesis.

To test if amino acids required for ubiquitin binding in the Rsp5 N-lobe are also important in the context of the entire HECT domain, we assayed GST-HECT domain mutants carrying the Y516A or F618A mutations for binding to immobilized His_6-tagged ubiquitin. As in the N-lobe, these mutations abolished binding of the entire HECT domain to ubiquitin (Figure 9C). We also constructed an F618Y HECT domain mutant for analysis because this residue is an F in the ubiquitin-binding Rsp5 and Nedd4 N-lobes, but is a Y in the non-binding Tom1 N-lobe. The F618Y mutation eliminated binding of the Rsp5 HECT domain to ubiquitin (Figure 9C), indicating that this residue is a crucial component of the HECT domain ubiquitin-binding site.

Most of the previously characterized UBDs bind to a hydrophobic surface patch on ubiquitin surrounding a key isoleucine residue, I44. To test if the Rsp5 HECT domain N-lobe also uses this surface of ubiquitin for binding, we mutated a select number of ubiquitin surface residues to alanine and tested these mutants for their ability to bind to the N-lobe (Figure 10A). Mutation of I44 and its neighboring residues, G47, H68, R42 and R72, abolished binding to the N-lobe, and mutation of two additional residues, K48 and V70, caused a reduction in binding. In contrast, mutations in and around K63 of ubiquitin (Y59A, Q62A and K63A) and in residues encompassing another functionally important surface of ubiquitin (F4A, T14A) (186) had no

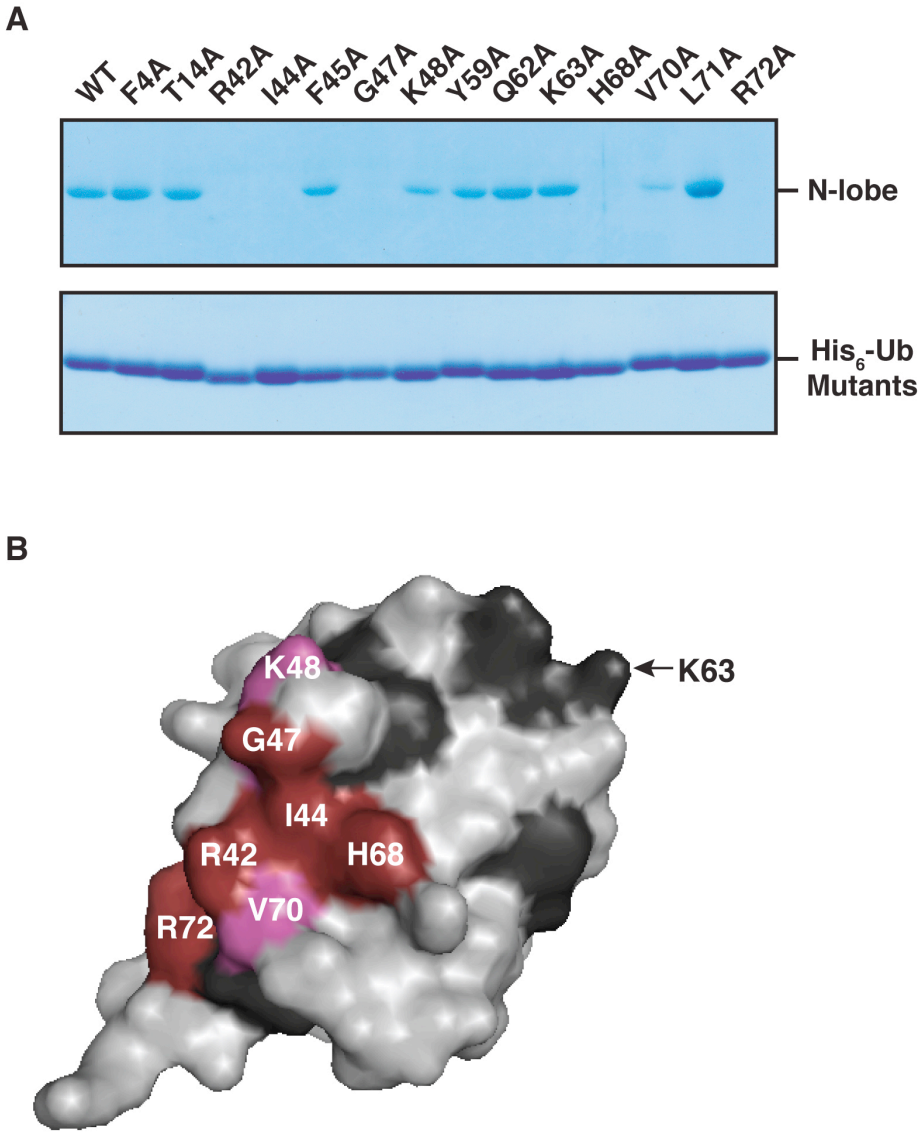

Figure 10. The Ile44 hydrophobic patch of ubiquitin is required for binding. (A) The indicated His_6-tagged ubiquitin mutants were immobilized on beads and incubated with a bacterial lysate from cells expressing a wildtype GST-tagged N-lobe. Bound proteins and ubiquitin mutants eluted from the beads were detected by Coomassie staining. (B) Surface representation of ubiquitin based on its three-dimensional structure (PDB accession code 1UBQ). Results from the alanine mutagenesis are summarized as follows: red = mutation abolished binding; magenta = mutation reduced binding; dark grey = mutation had no effect. The position of K63 is shown for reference. No mutations were made on the back face of ubiquitin (not shown).

effect on binding (Figure 10A and 10B). Thus, like most other UBDs characterized to date, the Rsp5 HECT domain N-lobe binds to the I44 hydrophobic patch of ubiquitin.

The Rsp5 ubiquitin-binding site is important for Rsp5 activity in vivo

Rsp5 controls a number of cellular processes in yeast, and most, if not all, of these are dependent on the catalytic activity of the ligase. To test if mutations that disrupt the Rsp5 ubiquitin-binding site affect Rsp5 activity in yeast, we constructed yeast strains expressing the $Rsp5^{Y516A}$ or $Rsp5^{F618A}$ mutants as the only source of Rsp5 in the cell. Both of these strains grew normally at 30°C, but exhibited modest to severe temperature-sensitive growth defects at 37°C (Figure 11A). The observed growth defects were not due to altered expression or stability of the Rsp5 proteins because both mutants were expressed and stable, even at the elevated temperature (Figure 11B). We conclude that the Rsp5 ubiquitin-binding site is important for Rsp5 activity in yeast cells.

To test if the growth defects of the $rsp5^{Y516A}$ and $rsp5^{F618A}$ mutants could be suppressed by overexpression of ubiquitin, we expressed ubiquitin from a multicopy plasmid in each of these mutant backgrounds and assayed growth at 37°C. Strikingly, overexpression of ubiquitin almost completely rescued the temperature-sensitive growth defect of the $rsp5^{F618A}$ mutant (Figure 11C). A similar result was obtained with the $rsp5^{Y516A}$ mutant (data not shown). To test whether these observations are simply due to a reduced ability of the $rsp5^{F618A}$ and $rsp5^{Y516A}$ mutants to synthesize free ubiquitin at the elevated temperature, as has been observed for the *rsp5-1* mutant (187), we analyzed free ubiquitin levels in the $rsp5^{F618A}$ and $rsp5^{Y516A}$ mutants. Both of these mutants expressed ubiquitin at levels comparable to wildtype cells, even after shifting the cells to 37°C (Figure 11D). Thus, the $rsp5^{F618A}$ and $rsp5^{Y516A}$ growth defects cannot simply be due to limiting free ubiquitin. Instead, these observations suggest that the $rsp5^{F618A}$

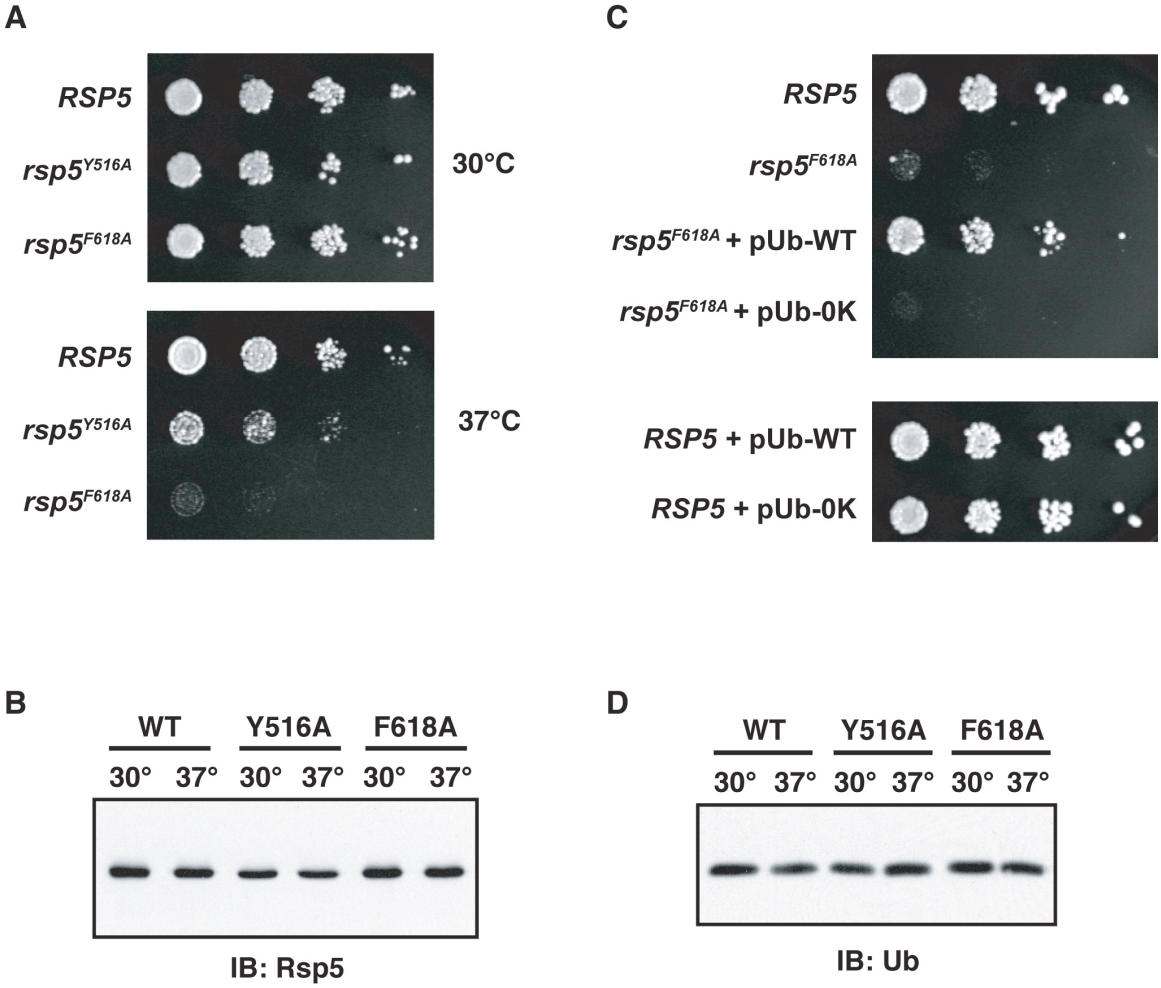

Figure 11. Phenotypic analysis of the *rsp5*^{Y516A} and *rsp5*^{F618A} mutants. (A) *RSP5* (LHY5653), *rsp5*^{Y516A} (LHY5655) and *rsp5*^{F618A} (LHY5657) cells were serially diluted, plated onto rich media and grown at 30°C or 37°C for 2 days. (B) Yeast strains described in A were grown to mid-log phase at 30°C and then shifted to 37°C for 1 hour. Cells were harvested before and after the temperature shift. Cell lysates were prepared and analyzed for Rsp5 expression by immunoblotting with Rsp5 antiserum. (C) *RSP5* and *rsp5*^{F618A} yeast strains used in A were transformed with multicopy plasmids encoding either wildtype ubiquitin (pUb-WT) or 0K ubiquitin (pUb-0K). Serial dilutions of each strain were plated onto rich media and grown at 37°C for 2 days. (D) Yeast strains tested in A were treated as described in B, except that cell lysates were analyzed for free ubiquitin levels by anti-ubiquitin immunoblotting.

and *rsp5*^{Y516A} phenotypes are due to an effect on Rsp5 catalytic activity that alters the ubiquitination of one or more substrates required for growth at 37°C.

To determine if the ability of ubiquitin to form chains is required for rescue of the *rsp5*^{F618A} temperature-sensitive growth defect, we overexpressed lysine-less (0K) ubiquitin, in which all seven lysines have been mutated to arginine, in *rsp5*^{F618A} cells. Overexpression of 0K ubiquitin did not rescue the growth defect of *rsp5*^{F618A} cells (Figure 11C), indicating that the ability of ubiquitin to form chains is required for this effect. Together, the results presented in Figure 11C and 11D suggest that the *rsp5*^{F618A} growth phenotype is due to a specific effect on Rsp5-catalyzed polyubiquitination of one or more substrates required for growth at 37°C.

The Rsp5 ubiquitin-binding site regulates the assembly of polyubiquitin chains

To more directly test the role of the Rsp5 ubiquitin-binding site in Rsp5-catalyzed ubiquitination, we used an *in vitro* autoubiquitination assay with the purified Rsp5 HECT domain. We first evaluated the types of polyubiquitin chains assembled by the Rsp5 HECT domain by performing ubiquitination assays with a series of ubiquitin mutants carrying a single lysine residue (K63, K48 or K29 ubiquitin). The Rsp5 HECT domain preferentially assembled chains linked through K63 of ubiquitin, consistent with previous observations for full-length Rsp5 (105). The synthesis of K48-linked chains was noticeably less efficient, and there was little to no chain synthesis activity through K29 because the conjugation pattern observed with K29 ubiquitin was similar to the conjugation pattern observed with 0K ubiquitin (Figure 12A).

We next analyzed the effect of mutations that disrupt the Rsp5 ubiquitin-binding site on HECT domain autoubiquitination. In an assay carried out with the F618Y HECT domain and wildtype ubiquitin, the pattern of conjugates observed with the F618Y mutant was markedly different from the pattern observed with the wildtype HECT domain. Specifically, there was a

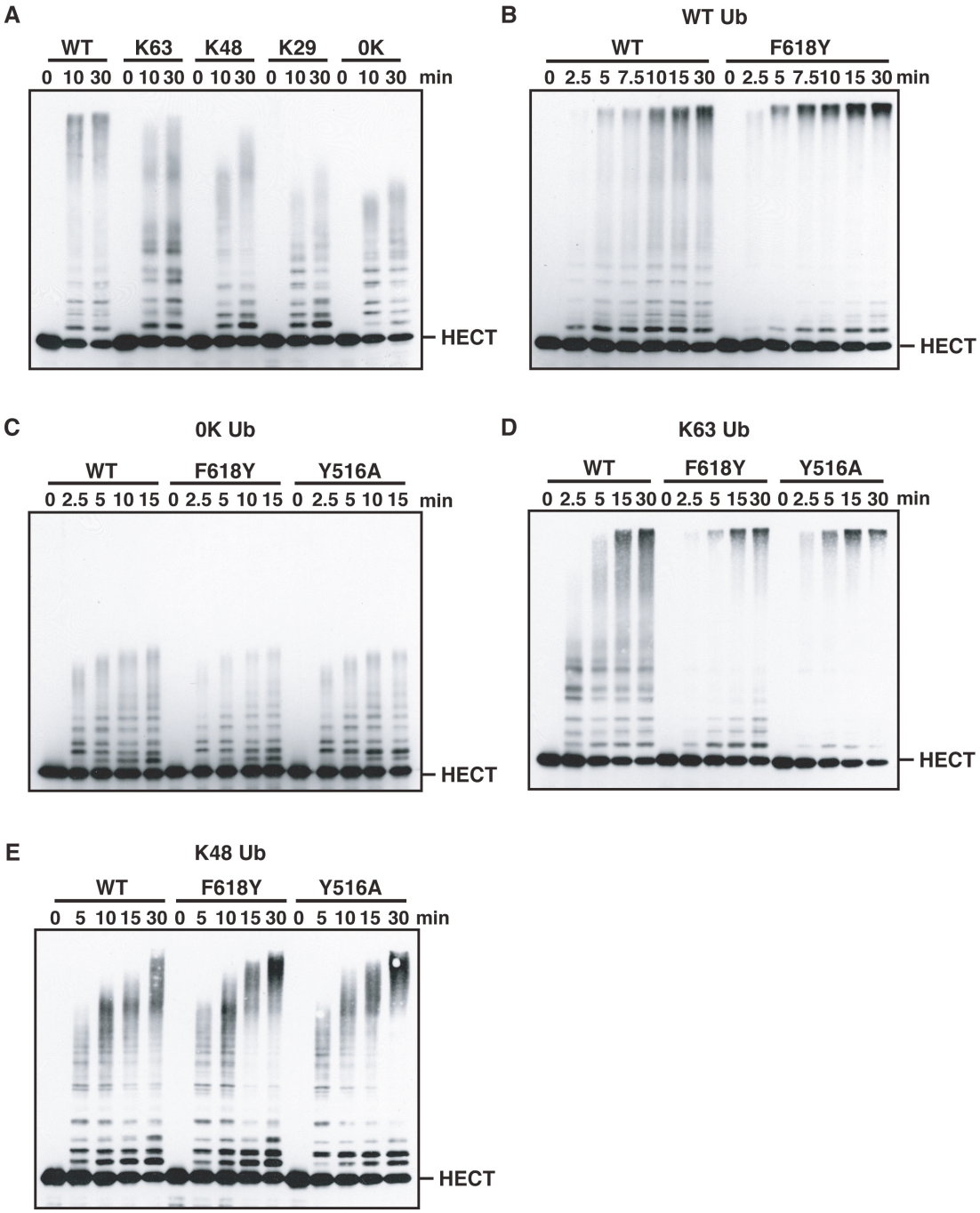

Figure 12. The Rsp5 ubiquitin-binding site regulates the length of polyubiquitin chains assembled by the HECT domain. (A) In vitro autoubiquitination assays were carried out with a wildtype GST-HECT fusion protein and either wildtype ubiquitin (WT), lysine-less ubiquitin (0K) or one of the indicated single- lysine ubiquitins (K63, K48 or K29). Reactions were quenched at the indicated times and ubiquitin conjugates were detected by anti-GST immunoblotting. The position of the unmodified HECT domain is indicated. (B), (C), (D) and (E) Reactions were carried out as described in A but in the presence of either a wildtype or mutant GST-HECT fusion protein (WT, F618Y or Y516A) and the indicated ubiquitin: WT ubiquitin for B, 0K ubiquitin for C, K63 ubiquitin for D and K48 ubiquitin for E.

strong accumulation of high molecular weight polyubiquitin conjugates at the top of the gel and a concomitant decrease in low molecular weight conjugates in the lower to middle region of the gel (Figure 12B). A similar effect was observed with the Y516A and F618A HECT domain mutants (data not shown). These observations indicate that HECT domains carrying mutations in the ubiquitin-binding site are enzymatically active and that these mutations alter the length and/or linkage of polyubiquitin chains assembled by the Rsp5 HECT domain.

To test if mutations that inactivate the Rsp5 ubiquitin-binding site have an effect on the ability of the HECT domain to catalyze monoubiquitination, we assayed the F618Y and Y516A HECT domain mutants for autoubiquitination in the presence of 0K ubiquitin. The pattern of 0K ubiquitin conjugates observed with both of these mutants was virtually indistinguishable from the pattern observed with the wildtype HECT domain (Figure 12C). We conclude that the F618Y and Y516A mutations have no effect on the ability of the HECT domain to accept monoubiquitin from an E2 or to transfer monoubiquitin to acceptor lysine residues targeted for ubiquitination. Together, the results presented in Figures 12B and 12C indicate that the Rsp5 ubiquitin-binding site plays a specific role in regulating the assembly of polyubiquitin chains.

To determine whether the Rsp5 ubiquitin-binding site plays a role in the assembly of a specific type of polyubiquitin chain, we next assayed the F618Y and Y516A HECT domain mutants for chain synthesis in the presence of either K63 or K48 ubiquitin. Both the F618Y and Y516A mutations significantly altered the distribution of K63-linked conjugates in a manner similar to that observed in reactions carried out with wildtype ubiquitin (Figure 12D). In contrast, there was a more modest effect on the distribution of K48-linked conjugates, with the most significant differences in conjugation patterns appearing at the 15 and 30 minute time points (Figure 12E). We conclude that the F618Y and Y516A mutations alter the distribution of

both K63-linked and, to a lesser extent, K48-linked polyubiquitin chains. The accumulation of high molecular weight polyubiquitinated species observed with the F618Y and Y516A HECT domains suggests a role for the Rsp5 ubiquitin-binding site in limiting the length of K63-linked and K48-linked polyubiquitin chains.

Discussion

UBDs are often found in proteins that recognize ubiquitinated targets, where they interpret the information carried by ubiquitin signals to regulate downstream events. UBDs are also found in enzymes that catalyze the attachment or removal of ubiquitin to other proteins, where they presumably function to aid in the catalytic steps required for ubiquitination or deubiquitination (12,13). Here we identify a previously unknown UBD in the HECT domain of Rsp5 and demonstrate that interactions with ubiquitin play a critical role in the regulation of Rsp5 activity *in vitro* and *in vivo*.

Ubiquitin binds to a region on the front surface of the Rsp5 HECT domain N-lobe that lies approximately 15-20 Å from the conserved active site cysteine residue in the modeled structure. The site of interaction is adjacent to, but non-overlapping with, the putative E2-binding site (70). The binding site on ubiquitin is centered around the I44 hydrophobic patch, the site of interaction for almost every UBD characterized to date. Mutations in and around K63 of ubiquitin had no effect on binding, whereas a subset of mutations in and around K48 disrupted binding. These observations are consistent with a model in which the N-lobe binds to ubiquitin in an orientation that favors polyubiquitin chain linkage through K63. This model is also supported by the finding that mutations that inactivate the ubiquitin-binding site have a more pronounced effect on the distribution of K63-linked chains than they do on the distribution of K48-linked chains.

Although the chain synthesis activities of several E2 enyzmes, including E2-25K, Ubc1 and the Mms2/Ubc13 complex, are known to be regulated by UBDs (67-69,75,160), it has been hypothesized that HECT E3s might also use a noncovalent ubiquitin-binding site to aid in polyubiquitin chain synthesis. The existence of a ubiquitin-binding site within the KIAA10 HECT domain has been inferred from mechanistic studies (86,174), but direct interactions with ubiquitin have not been confirmed experimentally. Furthermore, the Rsp5 and KIAA10 HECT domains probably carry distinct UBDs, since a 60 amino acid sequence upstream of the KIAA10 HECT domain is required for presumed interaction with ubiquitin (86,188). Here we show that the Rsp5 and Nedd4 HECT domain N-lobes carry a ubiquitin-binding site, but the N-lobe of the more distantly related Tom1 HECT domain does not. Thus, the N-lobe UBD is likely to be a specific feature of a subset of HECT E3s within the Nedd4/Rsp5 family of ubiquitin ligases.

Mechanistic role of the Rsp5 ubiquitin-binding site in polyubiquitin chain assembly

The results presented here demonstrate that the Rsp5 ubiquitin-binding site plays a specific role in regulating the assembly of a polyubiquitin chain. Mutations that disrupt the Rsp5 ubiquitin-binding site alter the ability of the HECT domain to assemble K63-linked and K48-linked polyubiquitin chains. These same mutations have no effect on the conjugation of lysine-less ubiquitin, indicating that the Rsp5 ubiquitin-binding site does not affect the ability of the HECT domain to transfer monoubiquitin to lysine residues targeted for ubiquitination. Consistent with the idea that the Rsp5 ubiquitin-binding site is specifically important for polyubiquitination, the temperature-sensitive growth defects of *rsp5*^{F618A} and *rsp5*^{Y516A} cells could be rescued by overexpression of wildtype but not lysine-less ubiquitin. Thus, the *rsp5*^{F618A} and *rsp5*^{Y516A} *in vivo* phenotypes are likely due to an effect on Rsp5 catalytic activity that alters

the polyubiquitination of one or more physiological substrates important for growth at the restrictive temperature.

HECT E3s can use at least two distinct mechanisms of polyubiquitin chain synthesis (86). The KIAA10 HECT domain builds up chains by catalyzing the sequential addition of ubiquitin monomers onto the end of a free or substrate-anchored polyubiquitin chain. The key feature of this model is the existence of a putative noncovalent ubiquitin-binding site in KIAA10, which nucleates the formation of chains by positioning a lysine residue within the bound 'acceptor' ubiquitin in an orientation that facilitates attack on the HECT-ubiquitin thioester. In contrast, the E6AP HECT domain builds up chains on its active site cysteine prior to transferring the chain to a substrate. This mode of chain assembly requires an E3-E2 heterodimer and involves an attack by the HECT thioester-linked ubiquitin on the E2-ubiquitin thioester. Although the mechanism of polyubiquitin chain synthesis employed by the Rsp5 HECT domain is currently unknown, both the Rsp5 HECT domain and full-length Rsp5 assemble free ubiquitin chains inefficiently *in vitro* (our unpublished results). This is diagnostic of an E6AP-like mode of chain synthesis, suggesting that Rsp5 assembles chains on its active site cysteine. However, the presence of a noncovalent ubiquitin-binding site within the Rsp5 HECT domain suggests that a KIAA10-like mode of chain synthesis might also be possible. Further work is needed to determine if Rsp5 uses one or both modes of chain assembly and to determine the predominant mechanism of chain synthesis used on Rsp5 substrates.

The results presented here are consistent with a model in which the Rsp5 ubiquitin-binding site restricts the length of polyubiquitin chains assembled by the Rsp5 HECT domain. The basis for this model comes from the finding that mutations that inactivate the Rsp5 ubiquitin-binding site result in the accumulation of high molecular weight K63-linked and K48-

linked polyubiquitinated species. We cannot formally exclude the possibility that these high molecular weight conjugates represent an accumulation of many short polyubiquitin chains attached to multiple sites of ubiquitination. However, the observation that HECT domain ubiquitin-binding mutants transfer lysine-less ubiquitin to the same number of ubiquitination sites as the wildtype HECT domain argues against this possibility. A role for the Rsp5 ubiquitin-binding site in limiting chain length is also supported by the finding that the Rsp5 HECT domain N-lobe binds preferentially to longer polyubiquitin chains (Ub_4-Ub_n) over shorter di- and triubiquitin chains. Consequently, we propose a model in which the Rsp5 N-lobe binds to the distal ubiquitin on the end of a growing chain to limit chain elongation. A similar model has been proposed to explain the role of the Ubc1 ubiquitin-associated domain in restricting polyubiquitin chain length (68,69) and to explain the role of the Met4 ubiquitin-interacting motif in preventing the extension of a polyubiquitin chain on Met4 (146).

Here we describe a previously unknown noncovalent ubiquitin-binding site located in the Rsp5 HECT domain that plays a role in the regulation of polyubiquitin chain length. Rsp5 is part of a large family of proteins that control diverse cellular processes in both yeast and mammalian cells (87). The existence of a ubiquitin-binding site within the Nedd4 HECT domain N-lobe suggests that the chain synthesis activities of other family members are likely to be regulated by an analogous ubiquitin-binding site. Thus, the results described here reveal a new mode of regulation for HECT E3s within the Nedd4 family of ubiquitin ligases and shed light on the diverse role of UBDs in the dynamic assembly of polyubiquitin chains.

CHAPTER III: SUMMARY AND DISCUSSION

Overview

Ubiquitin ligases (E3s) are the key regulatory enzymes in the ubiquitin conjugation pathway because they contain the primary determinants for substrate recognition. Despite the availability of high resolution structures of several E2-E3 complexes, the mechanisms that control the catalytic activity of most E3s are poorly characterized. The experiments described in this thesis address the mechanisms of ubiquitination employed by the Rsp5 ubiquitin ligase, an E3 that is part of a family of structurally and functionally related enzymes found throughout eukaryotes. Three different specific modes of Rsp5 regulation that were previously unknown are described: a noncovalent ubiquitin-binding site located in the catalytic HECT domain (Chapter II), an intramolecular interaction that occurs through the WW and HECT domains of Rsp5 (Appendix I), and direct phosphorylation of the ligase by a serine/threonine protein kinase known as Cbk1 (Appendix I). The data presented in Chapter II provides direct evidence that the Rsp5 ubiquitin-binding site plays a role in the regulation of polyubiquitin chain synthesis, whereas the experiments described in Appendix I are preliminary and require further work to determine the mechanism of Rsp5 regulation.

The results described in this thesis are important because they help contribute to our understanding of how E3s work and how the catalytic activity of these enzymes is modulated. Mammalian orthologues of Rsp5 such as Nedd4 and Itch/AIP4 are critical regulators of many fundamental biological processes, including the maintenance of blood pressure and the activation of key immune responses (119,189). In addition, HECT E3s have been implicated in the development of many different types of tumors and are the targets of a variety of anticancer therapeutic agents (111). Consequently, an investigation of the molecular mechanisms that control the catalytic activity of these E3s contributes to our understanding of how these

regulatory enzymes function in a variety of important biological events. In the following discussion, the results described in Chapter II are evaluated in conjunction with additional relevant data, and related questions regarding the regulation of Rsp5 catalytic activity are discussed. Results describing the regulation of Rsp5 by intramolecular interactions and phosphorylation are discussed in more detail in Appendix I.

Characteristics of the Rsp5 Ubiquitin-binding Site

The experiments described in Chapter II demonstrate that Rsp5 contains a previously unknown noncovalent ubiquitin-binding site located in the catalytic HECT domain. Although nineteen different types of ubiquitin-binding domains (UBDs) have been described to date (12,13,140-142), inspection of the Rsp5 protein coding sequence showed that the HECT domain does not contain any of these previously characterized domains. Protein interaction studies were used to map the ubiquitin-binding site to the N-terminal lobe (N-lobe) of the HECT domain, a large ~ 265 amino acid sequence whose only known function is to bind to ubiquitin-conjugating enzymes (E2s). Thus, the Rsp5 HECT domain N-lobe contains a new type of UBD responsible for a ubiquitin-binding activity that had not been previously recognized. The location of this ubiquitin-binding site immediately suggested that it is likely to play an essential or regulatory role in controlling the catalytic activity of Rsp5.

To identify the surface of the Rsp5 N-lobe responsible for ubiquitin binding, we used two different approaches. First, we attempted to further narrow down the region of the N-lobe responsible for ubiquitin binding by expressing smaller fragments of the N-lobe in *E. coli*. These experiments were largely unsuccessful because many of the truncated fragments that were constructed were poorly expressed or unstable in *E. coli* (data not shown). Second, we used a large-scale alanine-scanning mutagenesis approach, in which individual residues or stretches of

up to three contiguous residues were mutated to alanine in the context of the N-lobe. Modeling of the Rsp5 HECT domain onto the known structure of the closely related WWP1 HECT domain (84) (Swiss-PdbViewer, The Swiss Institute of Bioinformatics) allowed us to selectively target surface-exposed residues for the mutagenesis. The results of these experiments demonstrated that ubiquitin binds to a region on the front surface of the Rsp5 N-lobe that lies approximately 15-20 Å from the conserved cysteine residue (C777) that forms a thioester with ubiquitin. Importantly, none of the mutations made on the back surface of the N-lobe had any effect on ubiquitin binding, confirming the specificity of the alanine mutagenesis.

To define the binding site on ubiquitin, a select number of residues on the surface of ubiquitin were mutated to alanine and tested for their ability to bind to the Rsp5 N-lobe. The results of these experiments showed that the Rsp5 N-lobe interacts with the I44 hydrophobic surface patch of ubiquitin, the site of interaction for almost every other UBD characterized to date. The results of the ubiquitin mutagenesis also showed that mutations in and around K63 of ubiquitin had no effect on binding, indicating that K63 is likely to be free and accessible for chain synthesis when ubiquitin is bound to the N-lobe. These findings are consistent with a model in which the N-lobe binds to ubiquitin in an orientation that favors the assembly of chains linked through K63. Although we were unable to obtain direct evidence for this hypothesis (see below), the results presented in Chapter 2 are not inconsistent this model. It is therefore possible that the presence of a noncovalent ubiquitin-binding site in the Rsp5 HECT domain is the key feature that determines the ability of this E3 to synthesize K63-linked chains.

The results of binding experiments carried out with K63-linked and K48-linked chains demonstrated that the Rsp5 N-lobe binds to both types of chains with roughly equal efficiency. The lack of selectivity for binding to K63-linked chains was somewhat surprising, given the

known preference that Rsp5 has for K63-linked chain synthesis *in vitro* and *in vivo*. This observation, together with the finding that mutations in and around K63 of ubiquitin had no effect on binding, suggests that the mode of recognition employed by the Rsp5 N-lobe does not involve linkage-specific determinants within a chain. Instead, the N-lobe probably recognizes a ubiquitin monomer in the context of a chain, perhaps targeting the distal ubiquitin on the end of a growing chain for interaction (see below). Although there was no apparent preference for binding to chains linked through K63 or K48 of ubiquitin, the Rsp5 N-lobe bound selectively to longer chains (Ub_4-Ub_n) over shorter di- and triubiquitin chains. The basis for this selectivity is not yet understood, although the observed preference for binding to chains of increased length is not simply due to the availability of multiple monomeric ubiquitin subunits because the molar concentration of each chain tested in this assay was equivalent.

To determine the general relevance of this ubiquitin-binding site in other HECT E3s, we tested the N-lobes from two additional HECT domains for their ability to bind ubiquitin. For this analysis, we chose a mammalian HECT E3 that is both structurally and functionally related to Rsp5 called Nedd4 and another yeast HECT E3 that shares little in common with Rsp5 known as Tom1. The results of these experiments demonstrated that the N-lobe ubiquitin-binding site is conserved in the Nedd4 HECT domain, but not present in the Tom1 HECT domain. Thus, this ubiquitin-binding site is not likely to play an essential role in controlling the general catalytic activity of HECT E3s, but instead probably functions to modulate the activity of a subset of HECT E3s. A role for the HECT domain ubiquitin-binding site in specifically regulating the assembly of a K63-linked chain is supported by the recent finding that Nedd4, like its yeast counterpart Rsp5, preferentially assembles K63-linked chains *in vitro* (43). However, a more thorough analysis of HECT domains that assemble chains linked through other lysines of

ubiquitin would help to establish whether or not there is a correlation between HECT domain ubiquitin-binding activity and K63-linked chain synthesis.

Although a more quantitative determination of the ubiquitin-binding affinity of the Rsp5 N-lobe would have been informative, we have estimated (from pull-downs) that the Rsp5 N-lobe binds to monoubiquitin with a K_d of approximately 200 μM. This value is well within the range of typical ubiquitin-binding affinities for other UBDs (10-500 μM). Given the functional role of the Rsp5 ubiquitin-binding in polyubiquitin chain synthesis (see below) and the relatively high concentration of free ubiquitin in cells (~ 10 μM in mammalian cells; ref 144), a weak-modest ubiquitin-binding affinity is probably of functional significance. The high local concentration of a thioester-linked ubiqutin attached to the HECT domain or an isopeptide-linked ubiquitin conjugated to a bound substrate should saturate the Rsp5 ubiquitin-binding site despite its weak affinity. Thus, a low affinity ubiquitin-binding site would strongly bias the polymerization reaction in favor of E3-linked or substrate-bound ubiquitin chains and disfavor the synthesis of free ubiquitin chains, which have no known functional role in the cell.

Role of the Rsp5 Ubiquitin-binding Site in Chain Synthesis

The results presented in Chapter II demonstrate that the Rsp5 ubiquitin-binding site plays an important role in the regulation of Rsp5 activity *in vitro* and *in vivo*. Autoubiquitination assays carried out with the purified Rsp5 HECT domain were used to define the role of the Rsp5 ubiquitin-binding site in HECT domain ubiquitination. These assays showed that mutations that inactivate the Rsp5 ubiquitin-binding site alter the ability of the HECT domain to assemble both K63-linked and K48-linked polyubiquitin chains. Importantly, these same mutations had no effect on the ability of the Rsp5 HECT domain to accept monoubiquitin from an E2 or to transfer monoubiquitin to lysine residues targeted for ubiquitination. Thus, the Rsp5 ubiquitin-binding

site plays a specific role in regulating the assembly of a polyubiquitin chain. Consistent with the idea that the Rsp5 ubiquitin-binding site is specifically important for polyubiquitination, the temperature-sensitive growth defects of the *rsp5*^{Y516A} and *rsp5*^{F618A} mutants could be rescued by overexpression of wildtype but not lysine-less ubiquitin (the results of overexpression studies carried out with ubiquitin K→R mutants were uninformative because the K63R mutant caused a growth defect in wildtype *rsp5* cells; K48R and K29R rescued growth). Thus, the *in vivo* phenotypes of the *rsp5*^{Y516A} and *rsp5*^{F618A} mutants are likely due to an effect on Rsp5 catalytic activity that alters the polyubiquitination of one or more key physiological substrates.

The results of the autoubiquitination assays are consistent with a model in which the Rps5 ubiquitin-binding site functions to restrict the length of a polyubiquitin chain. This model is based on the finding that mutations that inactivate the Rsp5 ubiquitin-binding site result in the accumulation of high molecular weight K63-linked and K48-linked polyubiquitinated species. Control reactions carried out with lysine-less ubiquitin were used to demonstrate that these high molecular weight conjugates represent authentic polyubiquitinated species. We cannot formally exclude the possibility that these high molecular weight conjugates represent an accumulation of many short polyubiquitin chains attached to multiple sites of ubiquitination. However, the observation that Rsp5 HECT domain mutants carrying a defective ubiqutin-binding site transfer lysine-less ubiquitin to the same number of ubiquitination sites as the wildtype HECT domain (Figure 12C) argues against this possibility. A role for the Rsp5 ubiquitin-binding site in limiting chain length is also supported by the finding that the Rsp5 N-lobe interacts selectively with longer chains (Ub_4-Ub_n) over shorter di- and triubiquitin chains.

The mechanism by which the Rsp5 ubiquitin-binding site functions to restrict the length of a polyubiquitin chain is unknown (Figure 13). The most likely model is that the Rsp5 N-lobe

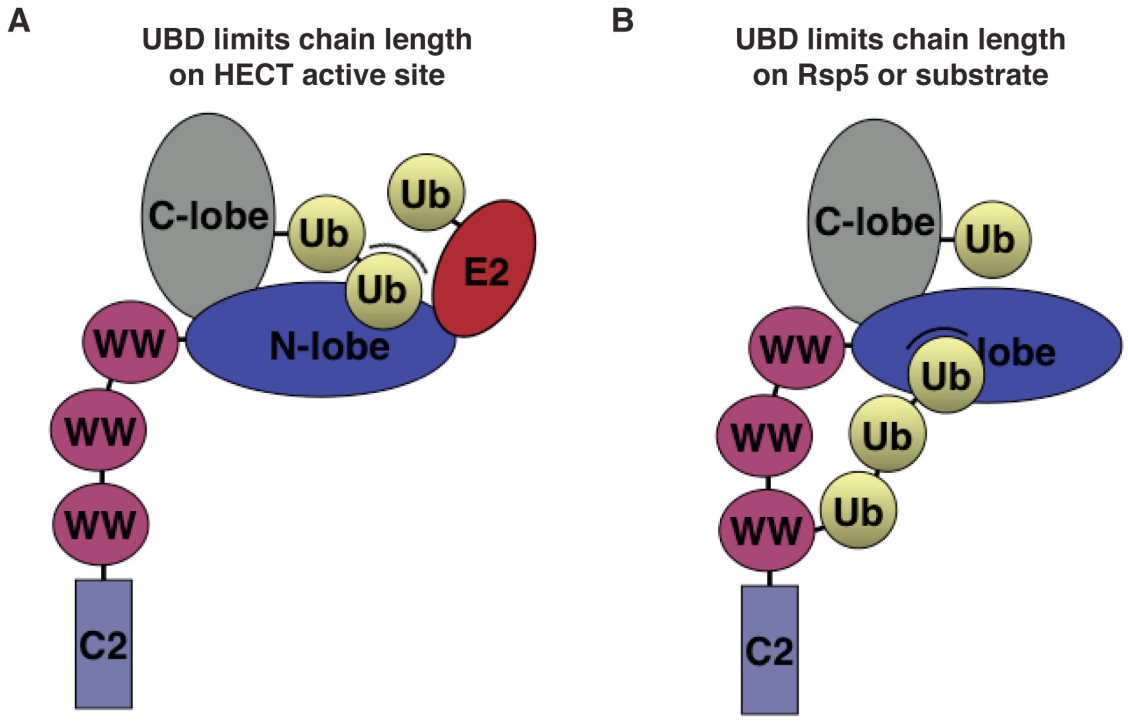

Figure 13. Models for the role of the Rsp5 ubiquitin-binding site in resticting chain length. In (A), the Rsp5 N-lobe interacts with the distal ubiquitin on the end of a growing chain that is attached to the HECT domain active site cysteine through a thioester bond. In (B), the Rsp5 N-lobe binds to the distal ubiquitin on the end of a growing chain that is conjuated to a lysine residue in Rsp5 or a substrate through an isopeptide bond. In both cases, the interaction between the N-lobe and the distal ubiquitin prevents the chain from accessing the relevant active site: the E2 active site for (A) or the HECT E3 active site for (B).

interacts with the distal ubiquitin on the end of a growing chain to prevent the chain from accessing the E2 or E3 active site. The growing chain could be tethered to the HECT domain active site cysteine through a thioester linkage (Figure 13A) or conjugated to a substrate (or Rsp5) through an isopeptide bond (Figure 13B), depending on the mechanism of chain synthesis used by the Rsp5 HECT domain. Two key preliminary observations suggest that the Rsp5 HECT domain can synthesize chains using both modes of assembly depicted in Figure 13: first, the Rsp5 HECT domain synthesizes free chains inefficiently (data not shown), suggesting an E6AP-like mode of chain synthesis that depends on two thioester-linked ubiquitins (Figure 13A), and second, full-length Rsp5 can utilize a Ub-GST fusion that cannot be activated by the E1 in free chain synthesis assays (data not shown), indicating that free ubiquitin (or ubiquitin conjugated to a substrate) can act as an acceptor in chain formation (Figure 13B). An alternative model similar to the one described above involves an interaction between the Rsp5 N-lobe and an E2 or E3 thioester-linked ubiquitin that prevents the growing chain from approaching the E2 or E3 active site. In this model, the ability of the Rsp5 N-lobe to bind to a thioester-linked ubiquitin would need to be regulated such that sufficient rounds of productive chain synthesis could occur before the inhibition of chain elongation.

To determine if the role of the Rsp5 ubiquitin-binding site in restricting chain length is relevant in the context of substrate ubiquitination catalyzed by full-length Rsp5, we investigated the ubiquitination of a native substrate of Rsp5 called Sna3. Sna3 is a membrane protein of unknown function that is polyubiquitinated by Rsp5 on a single lysine residue (K125) *in vivo* (102) and *in vitro* (Figure 14A). The results of ubiquitination assays carried out with the Sna3 substrate were unexpected– the same mutations that resulted in accumulation of high molecular weight polyubiquitinated species in the HECT domain autoubiquitination assays (Figure 14) had

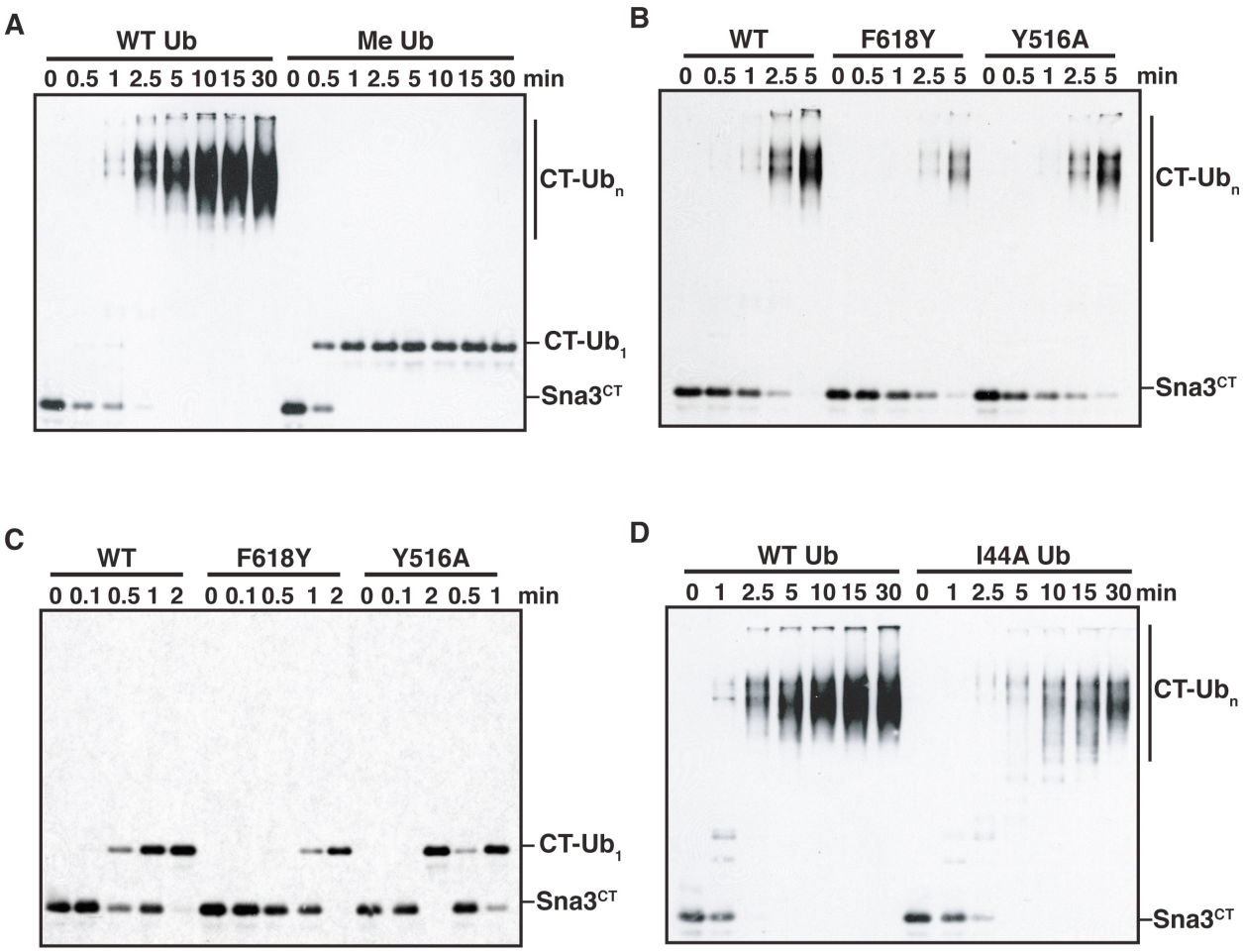

Figure 14. Effects of mutations in the Rsp5 ubiquitin-binding site on Sna3^{CT} ubiquitination. (A) In vitro ubiquitination assays were carried out with wildtype Rsp5 and either wildtype ubiquitin (WT Ub) or methylated ubiquitin (Me Ub). Reactions were quenched at the indicated times, and conjugates were detected by anti-His$_6$ immunoblotting. The positions of unmodified Sna3^{CT} (Sna3^{CT}), mono-ubiquitinated Sna3^{CT} (CT-Ub$_1$), and polyubiquitinated Sna3^{CT} (CT-Ub$_n$) are indicated. (B) and (C) Reactions were performed as described in (A) but in the presence of either wildtype Rsp5 (WT) or one of the indicated Rsp5 mutants (F618Y or Y516A) and either wildtype ubiquitin (B) or methylated ubiquitin (C). (D) Reactions were carried out as described in (A), except that wildtype Rsp5 was assayed in the presence of either wildtype or I44A ubiquitin (I44A Ub).

little to no effect on the polyubiquitination of Sna3 (Figure 14B). Although the F618Y mutation caused a modest defect in chain synthesis, this effect was probably due to a general decrease in the activity of the enzyme because the F618Y mutation also caused a defect in the conjugation of methylated ubiquitin in control reactions (Figure 14C). A decrease in the efficiency of Sna3 polyubiquitination was also observed in reactions carried out with I44A ubiquitin (Figure 14D), but this effect is likely due to a deficiency in a conjugation reaction that occurs upstream of Rsp5-catalyzed ubiquitination (174).

The reason for the discrepancy between the results of the substrate ubiquitination assays carried out with full-length Rsp5 and the HECT domain autoubiquitination assays is not clear. A simple explanation for these findings is that the Rsp5 ubiquitin-binding site does not play a role in regulating the assembly of chains conjugated to a substrate by full-length Rsp5. The Rsp5 ubiquitin-binding site could specifically function to restrict the length of chains assembled on Rsp5, perhaps as a way of preventing the elongation of chains on the E3 in the presence of a bound substrate. This explanation would make sense if the acceptor ubiquitin that interacts with the Rsp5 N-lobe during chain synthesis must be conjugated to the E3 in cis (Figure 13B). An alternative explanation is that the Rsp5 ubiquitin-binding site might function to restrict the length of a chain as it is assembled on the Rsp5 HECT domain (Figure 13A) in a manner that affects autoubiquitination specifically. This explanation would make sense only if the mechanism of chain synthesis depicted in Figure 13A occurs in the context of autoubiquitination but not in the context of substrate ubiquitination– this is a dinstinct possibility, since the mechanisms of chain formation in both autoubiquitination and substrate ubiquitination are unknown.

Another possible explanation for the discrepancy between the results of the substrate ubiquitination assays carried out with full-length Rsp5 and the autoubiquitination assays carried

out with the HECT domain is that the point mutations introduced have different effects on the ubiquitin-binding activity of full-length Rsp5 versus the isolated HECT domain. Both the F618Y and Y516A mutations clearly abolished binding of the isolated Rsp5 HECT domain to ubiquitin (Figure 9C), but the effects of these mutations on the ubiquitin-binding activity of full-length Rsp5 were not determined (due to technical difficulties). It is therefore possible that the F618Y and Y516A mutations have only a weak-modest effect on the ubiquitin-binding activity of full-length Rsp5 or that the interaction properties of the Rsp5 N-lobe are different within the full-length protein than they are in the HECT domain. Although attemps were made to construct more severe mutations in full-length Rsp5 for analysis of ubiquitination with the Sna3 single-lysine substrate, these experiments were not informative because all the mutations introduced resulted in defects in both mono- and polyubiquitination.

Remaining Questions and Future Directions

The presence of a noncovalent ubiquitin-binding site located in the Rsp5 N-lobe is one key feature that regulates the activity of Rsp5, but there are likely to be many other factors responsible for modulating the activity of this E3. Rsp5 has the ability to catalyze the synthesis of ubiquitin chains up to ten-twelve subunits in length in the presence of only an E1 and an E2 (Figure 14A). Yet, many substrates of Rps5 are modified with monoubiquitin or short K63-linked chains *in vivo*, indicating that additional cellular factors are responsible for regulating the catalytic activity of the ligase. One key regulatory factor is a deubiquitinating enzyme, Ubp2, that forms a complex with Rsp5 and disassembles K63-linked chains attached to Rsp5 substrates (105,190). Other important regulatory factors that are likely to affect ligase activity include an intramolecular interaction between the WW and HECT domains of Rsp5 and phosphorylation of the ligase by a serine/threonine kinase known as Cbk1 (Appendix I). Although the basis for the

selective mono- or polyubiquitination of a substrate catalyzed by Rsp5 is not known, direct regulation of the E3 by post-translational modification, E2 recruitment, or regulatory proteins is likely to play a role.

To investigate the regulatory mechanisms of ubiquitination carried out by an E3, a meaningful and reliable *in vitro* reconstitution system is essential. Although the information that we obtained from the single-lysine substrate ubiquitination assays was limited with respect to the role of the Rsp5 ubiquitin-binding site in chain synthesis (see above), this assay could be used in future studies to examine the effects of other potential regulatory factors on Rsp5-catalyzed ubiquitination. The significance of this assay is that it allows one to immediately distinguish between the synthesis of a ubiquitin chain versus the attachment of monoubiquitin (Figure 14A). Quantitative ubiquitination assays similar to the ones depicted in Figure 15 could be used to determine the kinetic properties of the enzyme or to determine how the presence or absence of specific factors affects the initial rate of mono- or polyubiquitination. One valuable piece of information that should be considered in the design of these assays is that the initial rate of polyubiquitination catalyzed by Rsp5 reaches a plateau when the concentration of ubiquitin is ~ 50 μM (Figure 15). Reactions carried out with 200 μM ubiquitin resulted in a decrease in the initial rate of polyubiquitination and the synthesis of longer chains, whereas reactions carried out at lower concentrations of ubiquitin (1-5 μM) resulted in the synthesis of shorter chains and a more distributive pattern of ubiquitination. The results of these preliminary experiments and the general scheme of the assay depicted and described in Figure 15 might be used to address many unanswered questions regarding the biochemical activities of Rsp5. The factors that determine the length and linkage of chains assembled by the E3 and the basis for the selective mono- or polyubiquitination of a substrate are two key topics that warrant investigation.

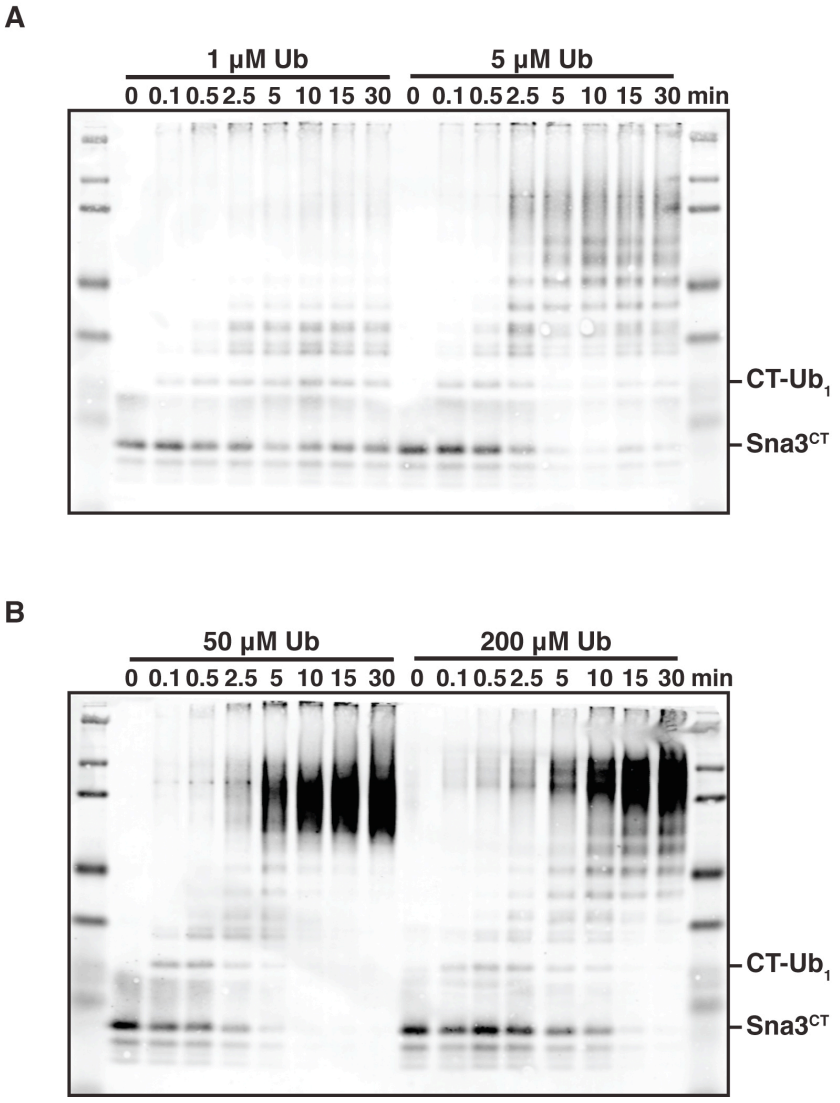

Figure 15. Quantitative Sna3^{CT} ubiquitination assay with full-length Rsp5. In vitro ubiquitination assays were carried out under the following conditions: 0.1 μM yeast E1, 0.2 μM UbcH5a, 0.3 μM full-length Rsp5, 0.5 μM Sna3^{CT}, and wildtype ubiquitin (1, 5 μM for A; 50, 200 μM for B). The Sna3^{CT} construct (LHP2785, aa 64-133) is His_6-tagged at the N- and C-terminus and was purified from the BL21DE3 strain as described previously (LHB738; ref 182). Reactions were carried out as described in the methods section of Chapter II, except that reactions were quenched at the indicated times in an equal volume of 2x Tris-Tricine loading buffer (6% SDS, 20 mM Tris pH 6.8, 1 M BME, 4 mM EDTA, 55% glycerol, .04% bromo-phenol blue). Reaction products were separated on big 10% Tris-Tricine gels, and conjugates were detected by immunoblotting with an anti-His_6 primary antibody (Bethyl laboratories) and a fluorescent anti-rabbit secondary antibody (Molecular Probes). Images were scanned and analyzed on the Odyssey (LI-COR Biosciences). The disappearance of unmodified substrate (Sna3^{CT}) and the appearance of mono- (CT-Ub_1) or polyubiquitinated species (all conjugates running above CT-Ub_1) can be quantified by measuring the relative fluorescent intensity.

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APPENDIX I: REGULATION OF RSP5 BY INTRAMOLECULAR INTERACTIONS AND PHOSPHORYLATION

Background

Although HECT E3s were originally thought to be constitutively active enzymes, it is now apparent that many different factors regulate the activity of these E3s. Regulation at the level of substrate recognition and subcellular localization plays an important role in determining the target specificities and cellular functions of these enzymes (191). The direct regulation of HECT E3 catalytic activity also plays a critical role in specifying the actions of these enzymes, and several recent reports have demonstrated that the direct regulation of ligase activity occurs through multiple mechanisms. Phosphorylation, recruitment of an E2, and association with a deubiquitinating enzyme are three factors that are known to modulate the catalytic activity of HECT E3s (192). Given that the basic mechanisms of mono- and polyubiquitination are poorly characterized for almost every HECT E3 studied to date, additional regulatory factors are likely to exist (see Chapter II of this thesis).

To identify additional factors that might regulate the activity of Rsp5, we took advantage of several key previous findings suggesting a role for the regulation of E3 activity. Previous work carried out by Rebecca Dunn, a former graduate student in the lab, demonstrated that Rsp5 interacts with itself *in vitro* and *in vivo* (112), indicating that the enzyme participates in either inter- or intramolecular interactions. Either type of interaction could function to regulate the catalytic activity of Rsp5, and, at the time these studies were initiated, a mechanism of HECT E3 regulation based on self-association had not been described. Therefore, we set out to investigate the ability of Rsp5 to associate with itself as a potential mode of E3 regulation. At around the time we began this line of investigation, another lab discovered that Itch, a closely related HECT E3 that controls several key immune responses, is engaged in an intramolecular interaction that directly inhibits its ubiquitin ligase activity (94).

Another key experiment suggesting a role for the regulation of Rsp5 activity was carried out by a former member of the lab, who demonstrated that Rsp5 is phosphorylated (J. Ptasienski and L. Hicke, unpublished data). Phosphorylation is a common post-translational modification that regulates the activity of many different proteins, and several previous reports had suggested a role for phosphorylation in the regulation of HECT E3 activity around the time that these studies were initiated. Phosphorylation of Nedd4-2 was found to negatively regulate E3 activity at the level of substrate recognition by interfering with E3-substrate interactions (193). In contrast, phosphorylation of the HECT E3 Itch was demonstrated to directly stimulate its E3 activity, although the mechanism of ligase activation had not been defined (194). We therefore set out to investigate the phosphorylation of Rsp5 as a potential mode of E3 regulation, with the expectation that we might either identify a new mode of regulation or determine the mechanism of phosphorylation-induced activation for a HECT E3.

Materials and Methods

Plasmid construction and mutagenesis

Plasmids encoding the full-length Rsp5 protein or fragments of the Rsp5 HECT domain fused to glutathione S-transferase (GST) were constructed in pGEX vectors (GE Healthcare, Waukesha, Wisconsin). The GST-Rsp5 encoding plasmid (aa 1-809, LHP562) was constructed by PCR-amplifying the relevant DNA sequence and ligating into the pGEX-6P-2 vector. Plasmids encoding fragments of the Rsp5 HECT domain fused to GST (LHP1434, 2325 and 2468) were subcloned into the pGEX-4T-3 or pGEX-6P-2 vectors and have been described previoulsy (see Chapter II). The hexahistidine (His_6)-tagged 3xWW domain plasmid (aa 228-426, LHP1645) was generated by ligation-independent cloning of the relevant PCR-amplified fragment into the pET-30 vector (EMD Chemicals, La Jolla, California). Point mutations in

LHP1434 (pGEX-4T-3 HECT) were introduced by QuikChange mutagenesis (Stratagene, La Jolla, California) and verified by automated sequencing. A plasmid encoding the carboxy-terminal domain (CTD) of Rpb1 fused to GST (aa 1525-1733, LHP2697) was provided by Jon Huibregtse (University of Texas at Austin, Austin, Texas).

Binding assays

Recombinant proteins for binding assays were expressed in *E. coli* (BL21-CodonPlus cells, Stratagene) as described previously (see Chapter II), except that the His_6-3xWW fusion protein was induced for 5 hours at 18°C. The His_6-3xWW fusion was immobilized on TALON metal affinity resin according to the manufacturer's instructions (Clontech, Mountain View, California). Binding experiments carried out with bacterially expressed GST-HECT fragments and immobilized His_6-3xWW domains were performed by incubating the bacterial lysates with the beads for 1 hour at 4°C. The beads were washed twice in PBS lysis buffer (115 mM NaCl, 16 mM Na_2HPO_4, 4 mM KH_2PO_4, 1% Triton X-100, 5% glycerol, pH 7.3) containing 10 mM imidazole and twice in PBS lysis buffer containing 20 mM imidazole. Bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-GST (GE Healthcare) or staining with Coomassie Brilliant Blue G250 (Bio-Rad Laboratories, Hercules, California).

In vitro kinase and ubiquitination assays

The Cbk1 kinase assays were performed in collaboration with Charlie Yoo and Jaclyn Jansen (Weiss lab, Northwestern University). Briefly, Cbk1 was immunoprecipitated from a yeast lysate, the beads were incubated with 0.3 μM of bacterially-expressed Rsp5 and P-32 labeled phosphate, and the phosphorylated species were resolved by SDS-PAGE and detected by autoradiography. Phosphorylated Rsp5 prepared in this manner was then added in an *in vitro*

ubiquitination assay containing 0.1 μM yeast E1, 0.2 μM UbcH5a, 0.3 μM GST-CTD and 75 μM of ubiquitin. Reactions were initiated by adding buffer containing 4 mM ATP (final concentrations: 25 mM Tris pH 7.5, 50 mM NaCl, 4 mM $MgCl_2$, 0.1 μM DTT). The zero time point was withdrawn on ice after brief mixing, and the reactions were immediately transferred to a 30°C water bath. Reaction aliquots were removed at the indicated times, added to an equal volume of 2x Laemmli sample buffer (see Chapter II), and analyzed by SDS-PAGE and anti-GST (GE Healthcare) immunoblotting.

Results and Discussion

Regulation of Rsp5 by intramolecular interactions

To determine if the ability of Rsp5 to interact with itself might play a role in regulating the catalytic activity of the E3, we tested different fragments of Rsp5 for binding to the catalytic HECT domain. A GST-C2 domain fusion protein (amino acids 1-142) expressed in bacteria did not bind to an immobilized His_6-tagged fragment containing the HECT domain (data not shown), consistent with a recent published report demonstrating that deletion of the Rsp5 C2 domain has no effect on the catalytic activity of the ligase (93). However, a bacterially expressed GST-HECT domain fusion protein bound specifically to an immobilized His_6-tagged fragment of Rsp5 (amino acids 228-426) containing all three of its WW domains (Figure 16A). The isolated GST-HECT domain N- and C-lobe fusion proteins did not bind to this fragment of Rsp5 (Figure 16A), suggesting that the key determinants of binding are located in both lobes of the HECT domain. These results indicate that one or several of the Rsp5 WW domains interact with the catalytic HECT domain and that inter- or intramolecular interactions might regulate the catalytic activity of Rsp5.

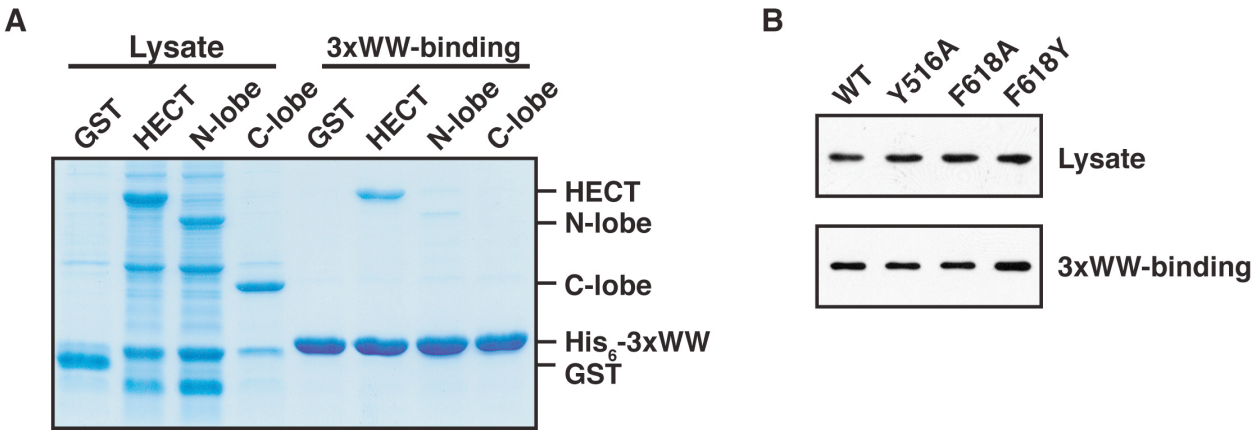

Figure 16. The central WW domains interact with the catalytic HECT domain of Rsp5. (A) Bacterial lysates from cells expressing the indicated GST-tagged HECT domain fragments were incubated with an immobilized His_6-tagged fragment of Rsp5 carrying the three WW domains (aa 228-426). Lysates and proteins eluted from the beads were analyzed by Coomassie staining. (B) Bacterial lysates containing the indicated GST-tagged HECT domain mutants were incubated with the immobilized His_6-tagged WW domain fragment. Lysates and bound proteins were analyzed by immunoblotting with anti-GST. The same lysates were used for assaying binding of these mutants to immobilized His_6-tagged ubiquitin (Figure 8C).

Around the time that these studies were initiated, another lab discovered that the closely related Nedd4 family member Itch is engaged in an intramolecular interaction between a region of the protein containing all four of its WW domains and the catalytic HECT domain (94). This interaction inhibits the activity of the Itch HECT domain and is relieved by a phosphorylation-induced conformational change in the protein. Although we have not yet determined if the interaction between the WW and HECT domains of Rsp5 inhibits the the activity of Rsp5 in a similar manner, given the strong structural and functional similarities between these E3s this is likely to be the case. Autoubiquitination assays carried out with a fragment of Rsp5 containing the three WW and HECT domains versus a fragment containing the isolated HECT domain could be used to determine if an analogous mode of autoinhibition regulates the catalytic activity of Rsp5. In addition, point mutations in the HECT domain that disrupt binding to the WW domain containing fragment might be used to examine the role of this interaction in regulating substrate ubiquitination catalyzed by Rsp5.

To determine if the Rsp5 ubiqutin-binding site is regulated by interactions between the WW and HECT domains of Rsp5, we tested the effect of point mutations in the HECT domain that abolish interaction with ubiquitin on binding to the WW domains. None of the mutations tested had any effect on binding to the immobilized WW domain containing fragment of Rsp5 (Figure 16B), suggesting that the HECT domain binding sites for ubiquitin and the WW domains are nonoverlapping. The existence of two independent binding sites is also supported by the observation that the isolated HECT domain N-lobe is both necessary and sufficient for binding to ubiquitin, but is not sufficient for binding to the WW domains. Although direct competition binding experiments would be useful in addressing the possibility that these binding sites are

partially overlapping, the results presented here indicate that the WW domains are not likely to regulate the ubiquitin-binding activity of the Rsp5 N-lobe.

Regulation of Rsp5 by phosphorylation

Previous work demonstrated that phosphorylated forms of the full-length Rsp5 protein could be detected in a yeast lysate prepared from P-32 labeled cells (J. Ptasienski and L. Hicke, unpublished data). The site of modification and kinase responsible for phosphorylation of Rsp5 were not determined. However, in a separate line of investigation, it was discovered that Rsp5 contains multiple phosphorylation consensus motifs for a kinase known as Cbk1 (E.L. Weiss and M. Yaffe, unpublished data). Cbk1 is a serine/threonine kinase involved in cell morphogenesis pathways, including cell wall biosynthesis, apical growth, and bud site selection (195,196). To determine if Rsp5 is a target of Cbk1-mediated phosphorylation, we immunopurified Cbk1 from a yeast lysate and performed an *in vitro* kinase assay with bacterialy-expressed full-length Rsp5. Cbk1 displayed robust kinase activity towards Rsp5 in this assay (Figure 17A), indicating that Rsp5 is an efficient substrate of the kinase *in vitro*.

To determine if Cbk1-mediated phosphorylation of Rsp5 regulates its ubiquitin ligase activity, we carried out an *in vitro* ubiquitination assay with phosphorylated Rsp5 (prepared as described in Figure 17A) and the carboxy-terminal domain (CTD) of Rpb1 (the large subunit of RNA polymerase II) as a substrate. There was little to no effect on the ubiquitination of a GST-CTD fusion protein (Figure 17B), suggesting that Cbk1-mediated phosphorylation of Rsp5 has no effect on Rsp5 catalytic activity. The ratio of phosphorylated to unmodified Rsp5 was not determined prior to the *in vitro* ubiquitination assay, thus it is possible that an effect on CTD ubiquitination was masked by the presence of excess unmodified Rsp5. Future studies should address this possibility and determine if mutations in Cbk1 consensus phosphorylation sites of

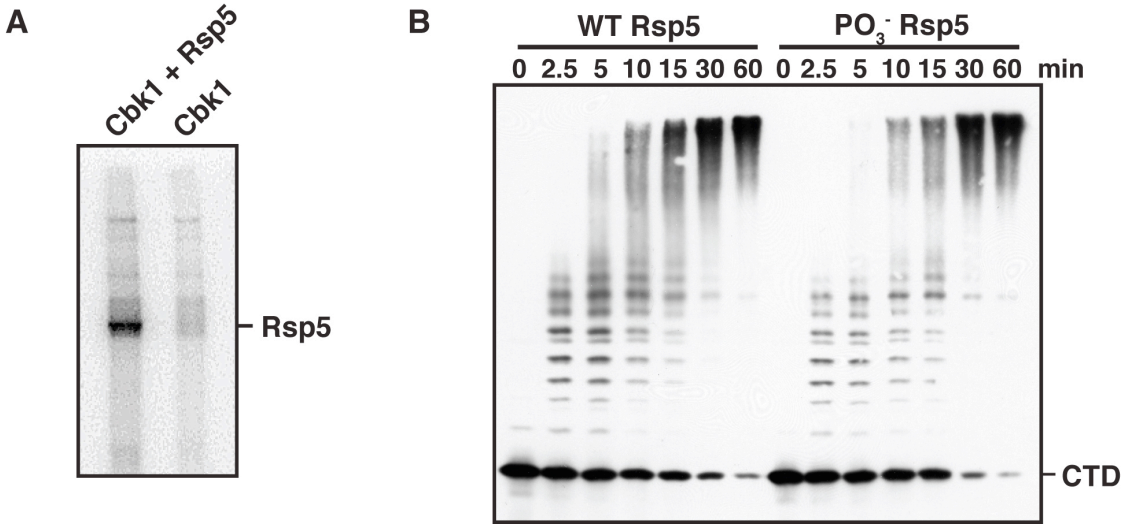

Figure 17. Rsp5 is phosphorylated by Cbk1 in vitro. (A) Immunopurified Cbk1 was incubated with bacterially-expressed Rsp5, and P-32 labeled phosphate. Reaction products were resolved by SDS-PAGE and detected by autoradiography. Ckb1 alone was included as a control because the kinase autophosphorylates in vitro. (B) In vitro ubiquitination assays were carried out with phosphorylated Rsp5 (PO3- Rsp5) prepared as described in (A), the carboxy-terminal domain (CTD) of Rpb1 as a substrate, and wildtype ubiquitin. Reactions were carried out for the indicated times, quenched with SDS-PAGE loading buffer, and analyzed by anti-GST immunoblotting. Mock-treated unphosphorylated Rsp5 (WT Rsp5) was prepared as described in (A) but in the absence of Cbk1.

Rsp5 abrogate the function or activity of the E3 *in vivo*. Given the role that phosphorylation plays in activating the closely related HECT E3 Itch, it is likely that phosphorylation also acts to regulate the catalytic activity of Rsp5 in some manner.

APPENDIX II: UBIQUITIN COMPETES WITH BINDING OF PXXP-CONTAINING LIGANDS TO THE SLA1 SH3-3 DOMAIN

Background

Monoubiquitin and polyubiquitin chains attached to proteins are recognized by an array of downstream effector proteins that generally contain small, modular ubiquitin-binding domains (UBDs). These domains interact directly with various types of ubiqutin modifications (Figure 2) and have been implicated in controlling a variety of different cellular events (12,13). The internalization of receptors from the plasma membrane is one such event that is thought to be controlled by multiple UBD-ubiquitin interactions (12), although the precise role of many UBDs in the endocytic machinery is poorly defined. In a screen for yeast endocytic proteins that bind to monoubiquitin, a previous member of our lab identified a protein required for receptor internalization known as Sla1 (141). Further analysis demonstrated that the Sla1 ubiquitin-binding site is located in the third SH3 domain of the protein (SH3-3), suggesting an unexpected role for ubiquitin in the regulation of SH3 domain function.

SH3 domains are modular sequences of 50-70 amino acids that promote protein-protein interactions during the assembly of large dynamic complexes. These globular domains contain a hydrophobic groove that typically binds to proline-rich peptide ligands containing a core PXXP motif (197). The finding that an SH3 domain binds ubiquitin was unexpected because ubiquitin does not carry a PXXP or closely related sequence. SH3 domains that bind to ubiquitin are found in several proteins that play an important role in the internalization step of endocytosis, including Sla1, CIN85, amphiphysin I, and amphiphysin II (141). At the time the experiments desribed in this appendix were carried out, the role of ubiquitin binding in the regulation of SH3 domain function was unknown, although the location of the ubiquitin-binding site on the SH3-3 domain indicated that ubiquitin might negatively regulate interactions with PXXP-containing ligands. To directly test this hypothesis, we performed competition binding experiments with the

SH3-3 domain in the presence of ubiquitin and a PXXP ligand and discovered that ubiquitin binding plays a role in negatively regulating the interaction properties of this SH3 domain.

Materials and Methods

Plasmid construction

Plasmids encoding the hexahistidine (His_6)-tagged SH3 domains of Sla1 (SH3-3, aa 350-420, LHP2167), amphiphysin I (aa 625-695, LHP2408), and Grb2 (aa 1-57, LHP2408) were constructed by ligation-independent cloning of the relevant PCR-amplified DNA fragment into the pET-30 vector (EMD Chemicals, La Jolla, California). Templates for the PCR amplification of DNA encoding the Grb2 and amphiphysin I SH3 domains were obtained from the American Type Culture Collection (Manassus, Virginia, Grb2) or Pietro De Camilli (Yale University, New Haven, Connecticut, amphiphysin I). Plasmids encoding glutathione S-transferase (GST) fused to the proline-rich domain of human dynamin (GST-PRD) or to the dynamin PRD carrying the PSRPNR→PSDANR mutation (GST-PRD^{mut}) were provided by Pietro De Camilli and have been previously described (198).

Binding assays

E. coli cultures (BL21-CodonPlus cells, Stratagene) were induced to express His_6-tagged SH3 domains or GST-PRD fusions as described previously (Chapter II). All His_6-tagged SH3 domains were immobilized on TALON metal affinity resin according to the manufacturer's instructions (Clontech, Mountain View, California). Binding assays carried out with GST-PRD proteins from bacterial lysates and the immobilized SH3-3 or amphiphysin I SH3 domains were performed by incubating the beads and lysates for 1 hour at 4°C. The beads were washed twice in PBS lysis buffer (115 mM NaCl, 16 mM Na_2HPO_4, 4 mM KH_2PO_4, 1% Triton X-100, 5%

glycerol, pH 7.3) containing 10 mM imidazole and twice in PBS lysis buffer containing 20 mM imidazole. Competition binding experiments carried out with the SH3-3 and Grb2 SH3 domains were performed in a similar manner, except that bacterial lysates containing a constant amount of GST-PRD (~ 5 μM) were titrated with increasing amounts of GST-Ub (0-40 μM) prior to the binding assay. Total lysates and bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-GST (GE Healthcare) or staining with Coomassie Brilliant Blue G250 (Bio-Rad Laboratories, Hercules, California).

Results and Discussion

The Sla1 SH3-3 domain has no known interacting partners besides ubiquitin, but several PXXP-containing sequences were predicted by phage display screens to be ligands for this SH3 domain (199). Surprisingly, we were unable to detect binding of the SH3-3 domain to any of these ligands that we tested (S. Stamenova and L. Hicke, unpublished data). However, we discovered that the SH3-3 domain interacted with a proline rich region (PRD, aa 751-838) of the dynamin GTPase, a native ligand of the amphiphysin SH3 domain (Figure 18A; ref 198). To determine if the SH3-3 domain, like the amphiphysin SH3 domain, binds to the PXXPXR sequence in the dynamin PRD, we assayed binding of the SH3-3 domain to a mutant version of the dynamin PRD carrying the PSRPNR→PSDANR mutation (PRD^{mut}). This mutation partially disrupted binding of the dynamin PRD to both the SH3-3 and the amphiphysin SH3 domain (Figure 18A), indicating that the SH3-3 domain, like most other SH3 domains, interacts with conventional PXXP-containing ligands.

Previous work carried out by a former member of the lab indicated that ubiquitin binds to a hydrophobic groove on the surface of the SH3-3 domain that is also the site of interaction for PXXP-containing ligands (141). The presence of overlapping binding sites suggested that

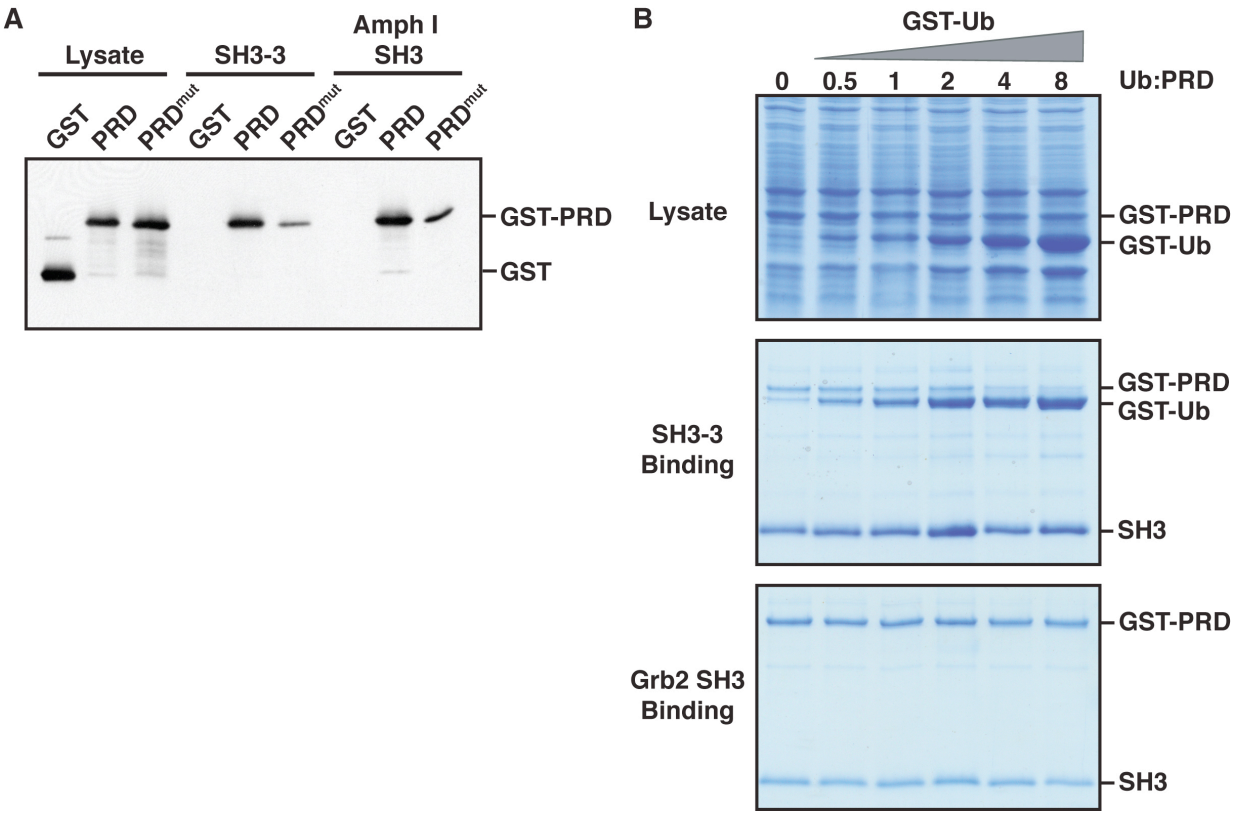

Figure 18. Ubiquitin and a PXXP-containing ligand compete for binding to the Sla1 SH3-3 domain. (A) Bacterial lysates containing GST, GST-PRD, or GST-PRD^{mut} fusion proteins were incubated with the indicated immobilized His$_6$-tagged SH3 domains. Lysates and bound proteins were analyzed on an anti-GST immunoblot. (B) A lysate containing GST-PRD was mixed with increasing concentrations of GST-Ub and then incubated with immobilized His$_6$-tagged Sla1 SH3-3 or N-terminal Grb2 SH3 domains. To enhance detection of proteins in the binding assay, SH3-3 levels were raised to a concentration in which GST-PRD did not saturate the SH3-3 domain binding sites; hence, competition by GST-Ub only became evident after saturation of the binding site by GST-Ub (at 2x Ub:PRD). Under conditions in which SH3-3 domain binding sites were saturated by GST-PRD, competition by GST-Ub was observed at equimolar Ub and PRD concentrations (not shown). Lysates and proteins bound to the SH3 domains were analyzed by tris-tricine gel electrophoresis and Coomassie staining.

ubiquitin and a PXXP-containing ligand might compete for binding to an SH3 domain. To test this hypothesis, we performed a competition binding experiment with the SH3-3 domain by incubating the immobilized domain with bacterial lysates containing a constant amount of GST-PRD and increasing amounts of GST-Ub. As a control, we also carried out the competition experiment with the N-terminal Grb2 SH3 domain, an SH3 domain that binds to the dynamin PRD but does not bind to ubiquitin (141). The binding of GST-PRD to the SH3-3 domain was inhibited by the presence of increasing concentrations of GST-Ub, whereas the binding of GST-PRD to the Grb2 SH3 domain was unaffected (Figure 18B). These results demonstrate that ubiquitin competes with binding of a PXXP-containing ligand to the SH3-3 domain, suggesting a role for ubiquitin in negatively regulating the interaction properties of an SH3 domain.

Although the interaction between the SH3-3 domain and the dynamin PRD is probably not physiologically relevant because there is no known dynamin-like protein in yeast, two key observations suggest that competition between ubiquitin and PXXP ligands for binding to an SH3 domain is feasible *in vivo*. First, the affinity of ubiquitin for the SH3-3 domain ($K_d \sim 40$ μM) is similar to that of low affinity SH3 domain-PXXP ligand interactions (141), indicating that competition could occur in the presence of equimolar concentrations of these ligands. Second, the SH3-3 domain binds to ubiquitinated proteins in the context of a yeast lysate (141), indicating that interactions with ubiquitinated targets might regulate the ability of the domain to interact with other ligands in the cell. While the identification of native SH3-3 domain ligands that compete with ubiquitin for binding to this SH3 domain *in vivo* will be required to establish physiological relevance, the results presented here suggest that the interaction properties of some SH3 domains are regulated by a novel ubiquitin-dependent mechanism.

MICHAEL E. FRENCH
Department of Biochemistry, Molecular Biology and Cell Biology
Interdepartmental Biological Sciences Program
Northwestern University
2205 Tech Drive, Hogan 2-100
Evanston, IL 60208
Lab: (847) 467-4485, Mobile: (847) 644-1943
m-french@northwestern.edu

EDUCATION

2002-2008 Ph.D. Candidate
Interdepartmental Biological Sciences Program
Department of Biochemistry, Molecular Biology, and Cell Biology
Northwestern University, Evanston, Illinois

2000-2002 M.S., Biology
Department of Biology
Bucknell University, Lewisburg, Pennsylvania

1996-2000 B.S., Biology
Cell Biology and Biochemistry Program
Bucknell University, Lewisburg, Pennsylvania

RESEARCH EXPERIENCE

2002-Present Graduate Student
Department of Biochemistry, Molecular Biology, and Cell Biology
Northwestern University, Evanston, Illinois
Advisor: Linda Hicke, Ph.D.
Thesis: Regulation of the Rsp5 HECT Domain Ubiquitin Ligase

2000-2002 Graduate Student
Department of Biology
Bucknell University, Lewisburg, Pennsylvania
Advisor: Kathleen Page, Ph.D.
Thesis: Prenatal programming of the hypothalamic-pituitary-adrenal axis by exposure to dexamethasone

2002 Summer Intern
Division of Drug Metabolism
Merck Research Laboratories, Rahway, New Jersey
Advisor: Eugene Tan, Ph.D.
Research Project: Developed a pharmacokinetic model for rat liver perfusion

2001 Summer Intern
Division of Analytical Chemistry
Merck Research Laboratories, Rahway, New Jersey
Advisor: Lili Zhou, Ph.D.
Research Project: Characterized the electrophoretic separation properties of a drug candidate compound

TEACHING EXPERIENCE

2004-2006 Mentor for two undergraduate students researching in Dr. Linda Hicke's lab, Northwestern University

2005 Teaching Assistant
Biological Sciences 309 Principles of Biochemistry
Northwestern University
Instructor: Erik Sontheimer, Ph.D.
Led a literature-based weekly discussion section, graded exams, and advised undergraduates during office hours

2004 Teaching Assistant Fellows Program
Searle Center for Teaching Excellence
Northwestern University
Designed and instructed workshops for new graduate teaching assistants in the Chemistry and Interdepartmental Biological Sciences programs

2004 Teaching Assistant
Biological Sciences 210-2 Biochemistry and Molecular Biology
Northwestern University
Instructors: Neil Welker, Ph.D., Andreas Matouschek, Ph.D.
Instructed two undergraduate laboratory sections and graded quizzes, exams, and laboratory reports

2002 Course Instructor
Medical College Admission Test Preparatory Course
The Princeton Review
Instructed the Biology section of a ten-week preparatory course surveying basic concepts in biochemistry, molecular and cell biology, genetics, neuroscience, physiology, and immunology.

2001 Teaching Assistant
Biology 352 Cell Biology
Bucknell University
Instructor: Sally Nyquist, Ph.D.
Assisted course instructor by preparing reagents for laboratory sections and demonstrating laboratory techniques

HONORS AND AWARDS

2007	Interdepartmental Biological Sciences Program Travel Award
2005	Teaching Assistant Fellow, Searle Center for Teaching Excellence, Northwestern University
1999	Bucknell University Undergraduate Research Grant

PUBLICATIONS

French ME, Kretzmann BR, Hicke L. (2008) Regulation of the Rsp5 ubiquitin ligase by an intrinsic ubiquitin-binding site. Submitted.

Stamenova SD, French ME, He Y, Francis SA, Kramer ZB, Hicke L. (2007). Ubiquitin binds to and regulates a subset of SH3 domains. *Molecular Cell,* 25(2):273-84.

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CONFERENCE PRESENTATIONS

French ME, Hicke L. (2008). Regulation of the Rsp5 Ubiquitin ligase by an intrinsic ubiquitin-binding site. Poster presented at the Fifth International Conference on Ubiquitin, Ubiquitin-like Proteins and Cancer, Houston, Texas

French ME, Kretzmann BR, Hicke L. (2006). Regulation of the Rsp5 Ubiquitin ligase by an intrinsic ubiquitin-binding site. Poster presented at the Ubiquitin and Cellular Regulation FASEB Conference, Saxtons Rivers, Vermont.