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The Etp1 Ubiquitin Ligase Regulates Ubiquitin Homeostasis in Yeast

By

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M.S., D. Mendeleev University of Chemical Technology of Russia, 2002

Advisor: Keith D. Wilkinson, Ph.D.

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Abstract

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By Janetta A. Bryksin

Ubiquitination is a reversible post-translational modification of cellular proteins that regulates a wide array of cell processes and is implicated in many diseases. Although ubiquitin is a highly abundant protein, it is not produced in excess. Rather, there is a dynamic equilibrium between three forms of cellular ubiquitin: monomeric ubiquitin, a substrate-conjugated mono- and polyubiquitin, and unanchored ubiquitin chains. Maintenance of ubiquitin homeostasis is a tightly regulated process and involves several regulatory mechanisms.

Here, we characterize the role of Ethanol Tolerance Protein 1 or ETP1 in the regulation of ubiquitin homeostasis. *S. cerevisiae* Etp1 is a putative homologue of the human BRCA1 Associated Protein 2 or BRAP2. BRAP2 is a cytoplasmic E3 ubiquitin ligase that modulates the sensitivity of MAP kinase cascade. Also, BRAP2 binds to the NLS motif of various proteins and sequesters them in the cytoplasm. Our studies indicate that Etp1 has the same functional domains as BRAP2, which allows using a yeast model system to study the function of BRAP2.

ETP1 was previously described to play role in yeast adaptation to ethanol provided either as a sole carbon source or as a stressor. We hypothesize that, in addition to this function, ETP1 is implicated in the regulation of the ubiquitin homeostasis by mediating the formation of free ubiquitin chains. Upon loss of ETP1, yeast cells exhibit a decreased amount of polyubiquitin without changes in total ubiquitin levels. This function is dependent on the E3 ligase activity and the ubiquitin binding capability of Etp1. Loss of ETP1 leads to resistance of yeast to various stresses such as oxidative stress, translational inhibition, and an amino acid analog. Analysis of topology of ubiquitin chains catalyzed by Etp1 and its corresponding E2, Ubc4, has revealed the formation of K6, K11, K33, K48, and K63 ubiquitin chains. Doa4 is a deubiquitinating enzyme required for ubiquitin homeostasis. Deletion of ETP1 from *doa4Δ* yeast strain partially rescues the phenotypes of *doa4Δ*, indicating that ETP1 and DOA4 may act upon the same biological pathway. We therefore predict that Etp1 may be a ubiquitin sensor in a cell, controlling the pool of free ubiquitin chains.

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This work is dedicated to my husband, Anton V. Bryksin, without whose patience, support and encouragement it would not have been possible, and to my parents, Tatyana and Vladimir Kuzin, who raised me with a love of reading and respect for education.

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Chapter I

Background and Introduction

Overview of the ubiquitin system.

Ubiquitin, a small, 76-amino acid globular protein, was discovered by Goldstein and colleagues in 1975 (1). This protein is ubiquitously present in all eukaryotic cells, hence its name, differing in only 3 out of 76 residues from yeast to human (2). In a short period of time from 1978 to 1985, Awram Hershko, Aaron Ciechanover, and Irwin Rose, together with their collaborators, defined the essence of the ubiquitination process via biochemical assays and identified the major players in this pathway (3-7). Thus, in 2004 the Nobel Prize in chemistry was awarded jointly to Hershko, Ciechanover, and Rose for their collaborative effort of the discovery of ubiquitin-mediated protein degradation (8). Ubiquitin, they found, is covalently conjugated to target proteins, which leads to the degradation of the modified protein via the 26S proteasome (9). Even though this role of ubiquitination is the best characterized, post-translational modification by ubiquitin has also been linked to altered sub-cellular localization, protein-protein interaction, activity, and, function of the modified protein (10-12). We now know that ubiquitination plays important roles in the regulation of a wide array of cellular processes such as protein quality control, protein trafficking, cell-cycle progression, DNA repair, apoptosis, transcriptional regulation, endocytosis, receptor down-regulation, and signal transduction (9, 13).

The ubiquitin pathway

In general, there are three steps to the ubiquitination of a substrate catalyzed by the sequential action of three classes of enzymes, ubiquitin activating enzyme or E1, ubiquitin conjugating enzyme or E2, and ubiquitin ligase E3 (Figure 1). In the first step of ubiquitination, the E1 enzyme activates the ubiquitin molecule through an ATP-

dependent adenylation of the C-terminus, followed by thiol-ester bond formation between an active site cysteine of E1 and the C-terminus of ubiquitin. The E1 then binds to one of a few dozen E2 enzymes and the ubiquitin moiety is transferred to a thiol group on the E2. The thiol-ester between the active site cysteine of E2 and the C-terminus of ubiquitin creates the second intermediate in the pathway and is often referred to as the "charged E2". In the last step, E2 charged with ubiquitin associates with one of the E3 ubiquitin ligases, which also binds to a substrate targeted for ubiquitination. An isopeptide bond is formed between the C-terminal glycine residue of ubiquitin and ε-amino group on a lysine residue of the target protein, leading to a monoubiquitin of the target protein (12, 14, 15).

This process can be repeated with a lysine on the proximal ubiquitin (the one closest to the substrate) attacking another charged E2 resulting in a formation of a polyubiquitin chain on the target protein. Ubiquitination of a protein is often processive, i.e., the substrate is not released from the E3 until several ubiquitin molecules have been attached. However, in some cases, polyubiquitination requires the additional activity of E4 elongation factors (16) or a different E3.

Like most post-translational modifications, ubiquitination is a reversible process (17). Deubiquitinating enzymes or DUBs remove monoubiquitin or polyubiquitin from proteins and also disassemble polyubiquitin chains (18).

In yeast, there is only one E1 activating enzyme, several E2 conjugating enzymes, and several hundred of E3 ubiquitin ligases (Figure 2). This last step of ubiquitination is highly regulated and indeed offers a specific substrate-recognition element to the hierarchical process of the ubiquitination system.

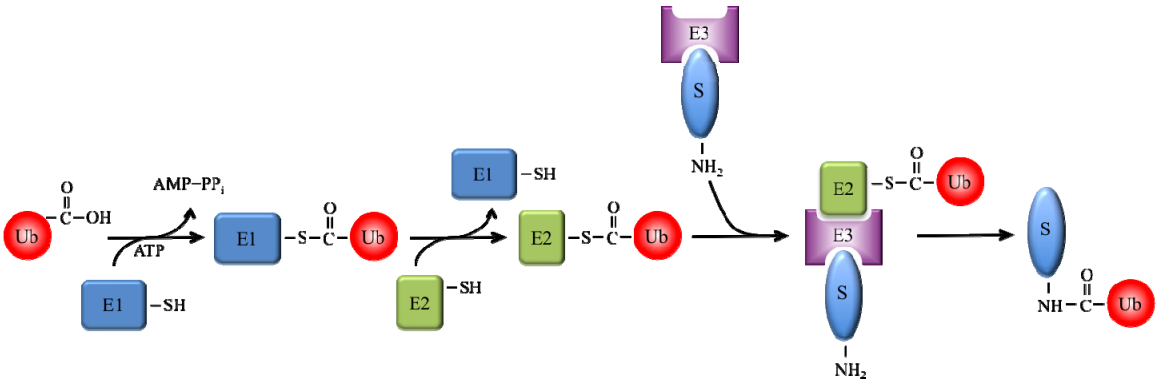

Figure 1. The ubiquitination pathway.

There are three steps to ubiquitination of a substrate. Ubiquitin is first activated through a formation of a thiol ester bond between the C-terminal glycine residue (G76) of ubiquitin and the active site cysteine of an E1 activating enzyme in an ATP-dependent manner. A reactive ubiquitin molecule is then transferred to an E2 conjugating enzyme through binding of an E1 to an E2. In the last step, E3 ubiquitin ligase binds both ubiquitin-charged E2 and a substrate for a transfer of ubiquitin onto a substrate.

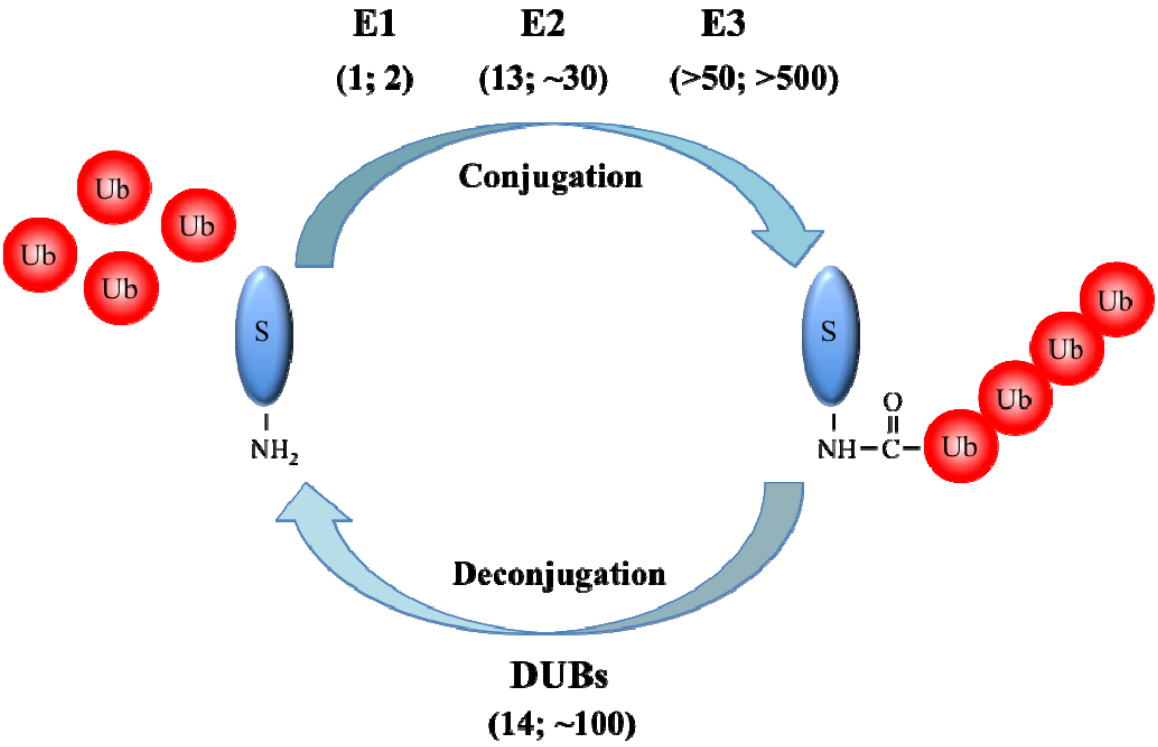

Figure 2. The ubiquitin conjugation cascade.

Ubiquitination is a hierarchical process. Conjugation of ubiquitin to a target protein requires a consequential action of activating enzyme or E1, conjugating enzymes or E2s, and ubiquitin ligases or E3s. Deubiquitinating enzymes or DUBs deconjugate ubiquitin from a substrate. The number of enzymes in the yeast *Saccharomyces cerevisiae* and in humans, respectively, are shown in parentheses for each step of ubiquitination.

Enzymes of the ubiquitin pathway

E1

In most organisms, a single essential E1 catalyzes the first step in the ubiquitination reaction. Studies utilizing a temperature-sensitive E1 led to the discovery that this enzyme is important for cell cycle progression via proteolysis of short-lived proteins, indicating the importance of E1 for cellular functions (19, 20).

To activate ubiquitin, the E1 enzyme binds MgATP and subsequently to ubiquitin causing the ubiquitin C-terminal acyl adenylation (7, 21). The ubiquitin adenylate serves as the donor of ubiquitin to the active cysteine in E1. Thus, a fully loaded E1 is conjugated to two molecules of ubiquitin: a thiol ester and an adenylate. Ubiquitin is then transferred from the E1-ubiquitin complex to an active site cysteine in E2 enzyme.

The C-terminal glycine of ubiquitin (G76) is essential for activation of ubiquitin by the E1 and is evolutionary conserved in most ubiquitin-like (UBL) protein modifiers (22, 23).

E2

E2 enzymes are present in all eukaryotes, underlining the importance of this element of ubiquitination system for cell function. There are ~30 E2 enzymes in humans and 13 E2-like proteins in *S. cerevisiae* (also called Ubiquitin-conjugating enzymes or Ubc1-Ubc13). Ubc9 and Ubc12 are E2 enzymes for SUMO and Nedd8, respectively, rather then ubiquitin (22).

General Properties of E2

The hallmark of E2 enzymes is a conserved ~ 150 amino acid catalytic core domain, UBC, which includes the active cysteine residue that accepts ubiquitin from E1. The UBC domain binds E1, E3s, and ubiquitin, therefore providing a platform for a transfer of ubiquitin to a target protein (24). Although all E2s have UBCs with a conserved architecture, some E2s have additional extensions to the catalytic core either at N- or C-terminus or both termini (25). These sequences may facilitate or impede the binding to specific E3s, may stabilize the interaction with the E1, or modulate the subcellular localization of E2. Also, depending on the extending sequences, E2s have either overlapping functions or more specific roles. For example, in *S. cerevisiae,* Ubc4 and Ubc5 have an interrelating function and are required for the degradation of many abnormal and short-lived normal proteins (26, 27), whereas Ubc3 is specifically required for the G1 to S-phase transition in the cell cycle by catalyzing Skp1-Cullin-F-box (SCF) ubiquitin protein ligase-mediated substrate ubiquitination (28-30). In some cases, E2 enzymes work in concert; for instance, the yeast anaphase-promoting complex (APC) uses Ubc4 to initiate ubiquitination, and a different E2, Ubc1, to elongate ubiquitin chains (Figure 3) (31).

Since there are hundreds of predicted E3s, it is not surprising that one E2 can bind multiple E3s. Even though there are examples of restricted E2-E3 pairs, in general, the function of a particular E2 is determined by its association with an individual E3, which in turn binds specific protein substrates. Similarly, a single E3 may bind different E2s for a specific type of ubiquitin modification that will occur on the substrate as it is, generally, the E2 that determines the type of assembled ubiquitin chains (9).

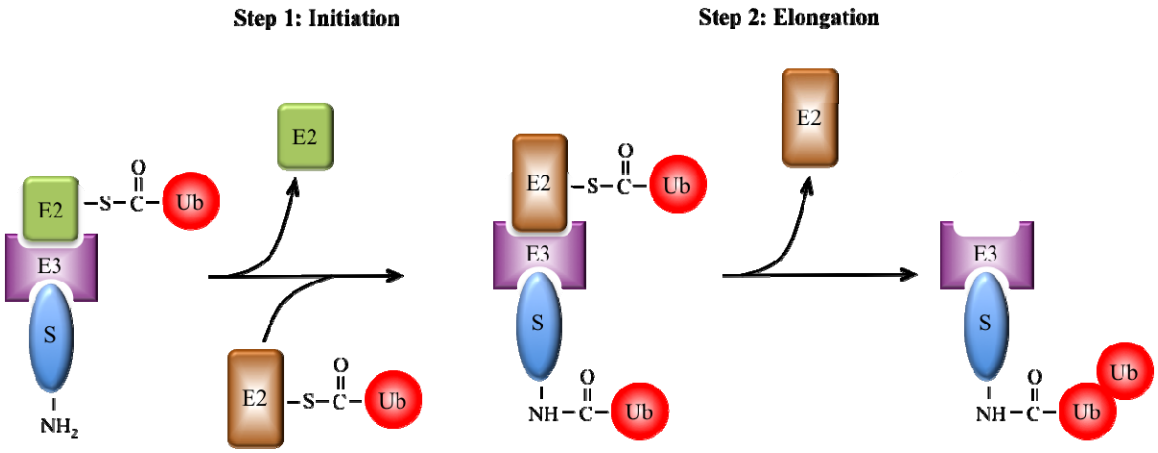

Figure 3. Different E2s may be required for substrate ubiquitination.

The yeast anaphase-promoting complex or cyclosome (APC/C) uses the ubiquitin-conjugating enzyme (E2) Ubc4 to initiate ubiquitination, and a different E2, Ubc1, to elongate Lys48-linked ubiquitin chains.

Ubc4 and Ubc5 are E2s relevant to these studies

S. cerevisiae UBC4 and UBC5 genes encode closely related 16 kDa proteins (92% identical residues), which irrefutably contribute to their overlapping and complementing functions. These E2 conjugating enzymes mediate turnover of bulk proteins by generating high molecular weight ubiquitin conjugates on the substrates targeting them for proteasomal degradation (32). Importantly, Ubc4/5 enzymes comprise a large part of the total ubiquitin-conjugation activity in cells subjected to various stresses. Loss of Ubc4/5 accounts for impaired cell growth and inviability at high temperatures or in the presence of an amino acid analog, and induction of the stress response (32). Expression of UBC4 and UBC5 genes is heat inducible and *ubc4ubc5* mutants are inviable at elevated temperatures signifying the role of these enzymes in the stress response. In addition, Ubc4 is important for yeast growth and polyubiquitination of overall cellular proteins in the presence of ethanol (33).

Although the majority of *S. cerevisiae* E2s contain N- and C-terminal extensions that facilitate interactions with the substrates or regulatory molecules, this is not the case for Ubc4 and Ubc5, which consist primarily of the catalytic domain (32). This suggests that Ubc4/5 may require additional factors (e.g. E3 ligases, the proteasome, or other substrate-recognition factors) to recruit substrate proteins for ubiquitination.

E3

After the E1 activating enzyme and the E2 ubiquitin conjugating enzyme(s), the E3 ubiquitin ligases are the last step in the E1-E2-E3 enzymatic cascade of a substrate ubiquitination. There are hundreds of E3 ubiquitin ligases encoded in the human genome, which is consistent with the role of E3s in conferring substrate specificity and regulation

to ubiquitination (34). Not surprisingly, E3s play an essential role in the control of innumerable cellular processes and are linked to multiple diseases (35). The abnormal regulation of some E3s, such as changes in catalytic activity or expression, may lead to such devastating effects as deregulated cell-cycle control and cancer. For example, loss or mutations of BRCA1 E3 ligase in humans, which eliminate its ubiquitin ligase activity, can lead to breast or ovarian cancer (36). The SCF and APC/C E3 ligases play an integral part in the highly ordered progression of the cell cycle, and their deregulation also contributes to tumorigenesis (37).

E3 ubiquitin ligases are broadly categorized into four major classes: RING-finger E3s, HECT-domain E3s, U-box-domain E3s, and PHD-domain E3s. RING-finger (Really Interesting New Gene) E3s and their variant U-box and PHD domain ligases, are the largest classes of E3s. They serve as an adapter between E2 and the substrate (38) juxtaposing E2, charged with ubiquitin and a substrate. The ubiquitin molecule is transferred directly from the E2 onto a substrate (Figure 4) by nucleophilic attack of the lysine □-amino group on the thiol ester of E2.

RING domain ubiquitin ligases

With 300 RING-finger genes in humans and 47 genes in *S. cerevisiae*, RING domain E3s make up the largest class of ubiquitin ligases so far (39). Up to date, nearly

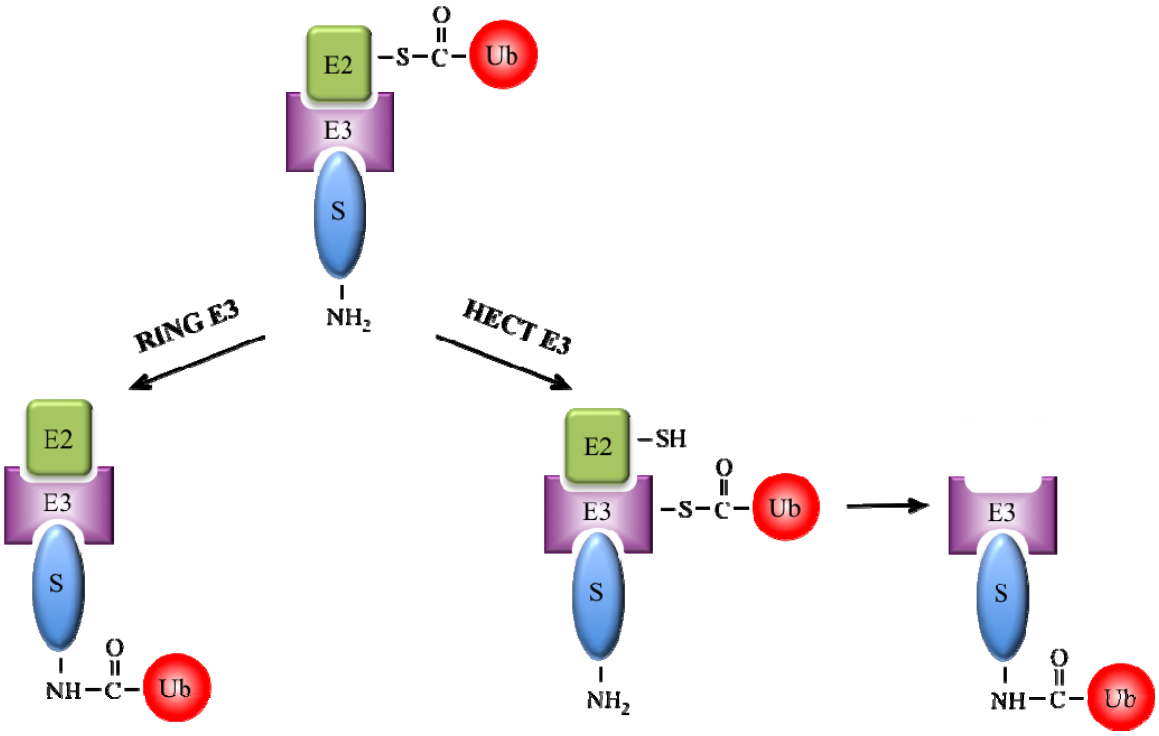

Figure 4. HECT and RING E3 ubiquitin ligases.

Two major classes of ligases are RING domain and HECT domain families of E3, which differ in the mechanism of ubiquitin transfer from the E2 to a target protein. In general, RING domain E3 ligases bind both the substrate and the E2, which has been charged with ubiquitin, bringing the enzymes into a close proximity for the ubiquitin transfer. For HECT domain E3s, ubiquitin is first transferred to the active site cysteine of the HECT domain followed by the transfer to a substrate.

half of 300 of human RING proteins have been described as ubiquitin ligases (reviewed in (38), while the rest have not been studied yet.

The characteristic feature of all RING ligases is the presence of $C_3H_2C_3$ or C_3HC_4 domains with a linear sequence of Cys-X_2-Cys-X_{9-39}-Cys-X_{1-3}-His-X_{2-3}-Cys/His-X_2-Cys-X_{4-48}-Cys-X_2-Cys, where X is any amino acid (Figure 5a) (40). The RING domain adopts a unique 'cross-brace' arrangement comprising a small central β sheet and, in some cases, an α helix, yielding a rigid platform for protein-protein interactions (41). Two coordinated Zn^{2+} ions form an integral part the RING finger domain: the first and the third pairs of cysteine/histidine bind the first Zn^{2+} ion, while the second and fourth pairs of cysteine/histidine bind the second Zn^{2+} ion with an inter-zinc distance of ~14 Å (Figure 5b). Mutations in these residues disrupt the binding to Zn^{2+} ions, and therefore lead to a formation of a 'catalytically dead' E3 ligase. Apart from the absolutely conserved cysteine and histidine residues, there is little sequence conservation among a variety of additional domains of RING proteins, such as PDZ, SH2, SH3, FHA, ubiquitin-like domains, and others. Likewise, the substrate-binding site may reside either in the RING domain itself or in the additional extension domains, or even require an additional protein subunit which adds great diversity to the E3 RING family (reviewed in (38). To add another layer of complexity, RING E3s can be either monomeric or be a part of a protein complex. For instance, the RING domain of Bard1, which does not possess intrinsic E3 activity, interacts with a RING domain of Brca1 stimulating the E3 activity of the latter (42).

RING domains underlie ubiquitin ligase activity by recruiting and directly binding E2 ubiquitin conjugating enzymes. Ironically, because E2s use overlapping residues to

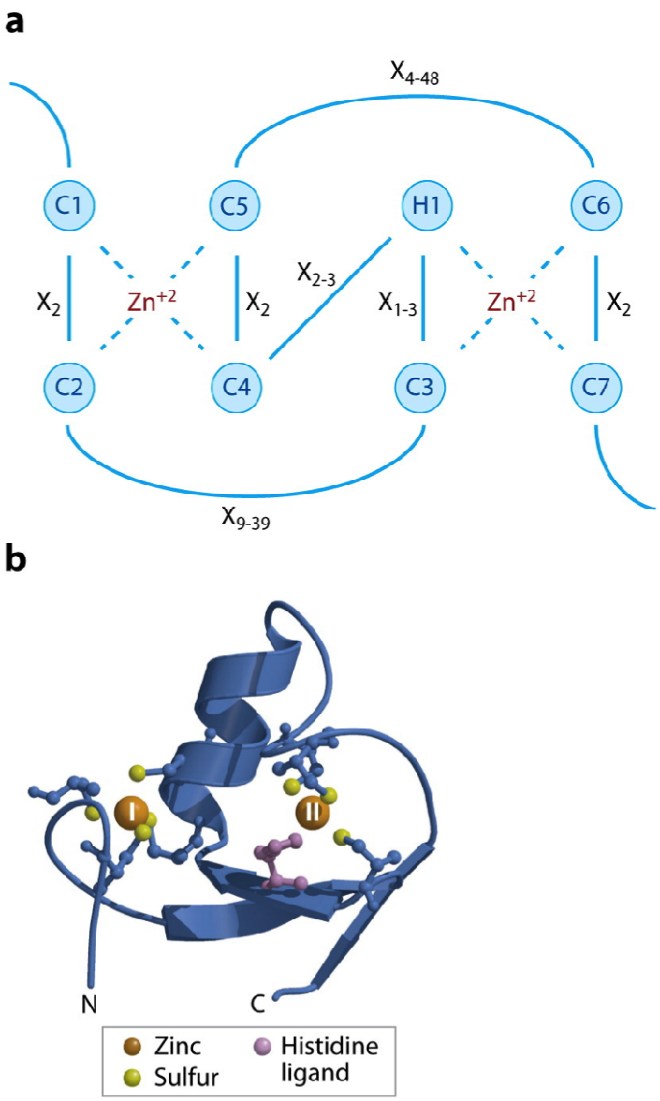

Figure 5. The RING finger domain (adapted from (38).

(a) Primary sequence organization of the RING-HC domain. The first cysteine that coordinates zinc is labeled as C1, and so on. H1 denotes the histidine ligand. Xn refers to the number of amino acid residues in the spacer regions between the zinc ligands. (b) Ribbon diagram of the three-dimensional crystal structure of the RING domain from c-Cbl. The zinc atoms in sites I and II are numbered. The termini are as marked.

bind both E1 and E3s (43), the affinity of RING domains for their partner E2s is usually low, and some highly active E2-E3 pairs do not display stable association. Hence, despite the impressive effort of structural, computational, and biochemical studies (38, 44, 45), identification of physiological E2-E3 partners still remains extremely challenging.

After binding to an E2 thioesterified with ubiquitin (E2□Ub), RING domain E3 ligases transfer ubiquitin from E2□Ub to substrate. It is commonly assumed that ubiquitin chains are built on a substrate one by one by the sequential mechanism (Figure 6a). However, polyubiquitination of a substrate can potentially occur via several different mechanisms (46). In case of Ube2g2 ubiquitin conjugating enzyme, the Lys48 of ubiquitin thioesterified to Ube2g2 can attack a second Ube2g2□Ub to form a diubiquitin chain on Ube2g2 (47). This mechanism of ubiquitin chain assembly requires proximity of two molecules of Ube2g2, which is achieved by binding of the both Ube2g2 to gp78 RING E3 ubiquitin ligase. Polyubiquitination of a substrate can then be achieved by transferring the preassembled ubiquitin chain from Ube2g2 to a lysine residue in a substrate *en bloc* (Figure 6b).

Regulation of RING domain E3 ubiquitin ligases

Ligase activity of RING domain ubiquitin ligases can be governed by a diverse set of regulatory mechanisms described below:

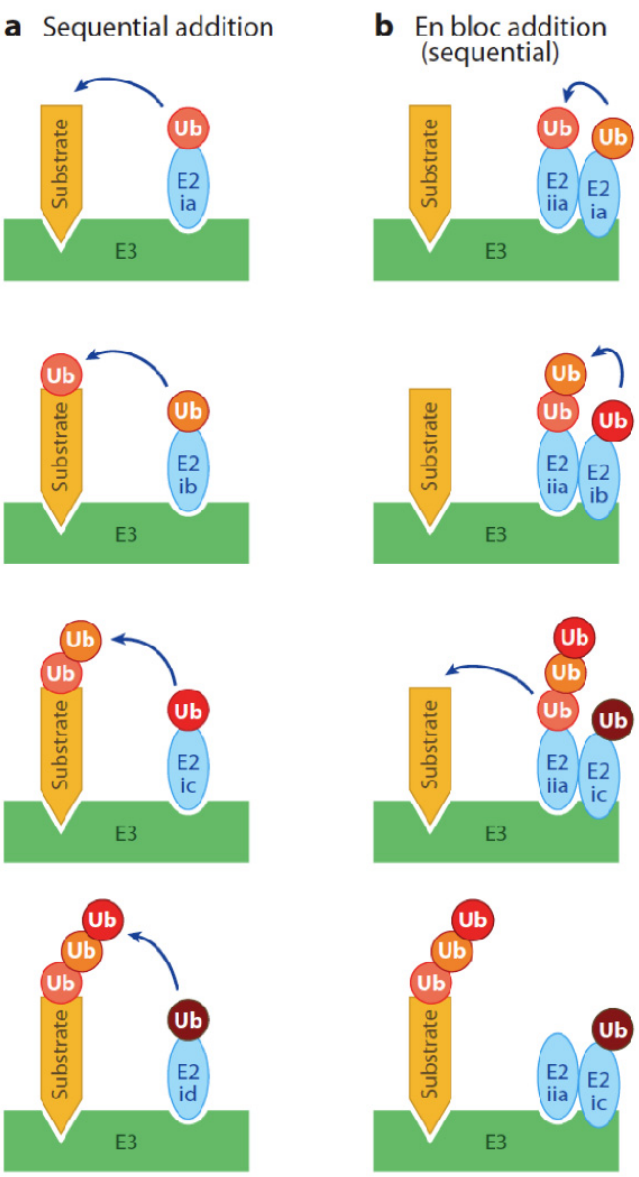

Figure 6. RING E3s may use different mechanisms to catalyze polyubiquitination of substrate (47).

(a) The sequential model of chain synthesis postulates that polyubiquitination is achieved by successive addition of ubiquitin molecules to a substrate. In between each round of transfer, the spent E2 dissociates to make way for a fresh molecule of E2□Ub. (b) An alternative possibility is that ubiquitin chains are preassembled on E2 and then transferred *en bloc* to substrate.

I. Binding Partners. One example of this form of regulation is binding of Brca1 to its partner Bard1 to stimulate the E3 activity of the former (42). Another example is the Cand1 protein, which binds to cullins and sequesters them in an inactive state (48). Also, in some cases, binding of pseudosubstrates to E3s can inhibit the ubiquitination of the authentic substrates (49).

II. Small Molecules. The plant signaling hormone auxin induces substrate ubiquitination by filling a cavity in the substrate-binding pocket of SCFTir1 ubiquitin E3 ligase (50). Auxin enhances the Tir1-substrate interactions by providing binding energy that stabilizes the substrate-E3 interaction.

III. Substrate Competition. APC/C degrades its substrates in a specific sequential manner, which depends on the processivity of the substrates. While highly processive substrates are ubiquitinated and degraded rapidly in early anaphase, substrates with poor processivity frequently dissociate from APC, which renders their polyubiquitination. Consequently, substrates with poor processivity acquire ubiquitin chains sufficient for targeting the substrate for degradation only in G1 phase (51).

IV. Post-translational Modification of E2, E3, or Substrate. Phosphorylation can directly affect the enzyme activity of either E2s or RING E3s. The Cdc34 ubiquitin-conjugating enzyme is phosphorylated in both human and yeast cells, which affects activity and subcellular localization of this E2 (52). Phosphorylation of yeast APC/C subunits enhances ubiquitin ligase activity of this E3 (53). Also, the protein substrate can require phosphorylation of one or several sites in order to be ubiquitinated. Yeast Sic1 binds the Cdc4 subunit

of SCFCdc4 ubiquitin ligase complex only when the former is phosphorylated (29). In addition to phosphorylation, other post-translational modifications can have an impact on the E3 ligase activity. For example, acetylation of RING domain of Mdm2 inhibits the ligase activity of this E3 toward p53 (54).

V. Covalent Conjugation of Ubiquitin Family Proteins. Many RING ligases can autoubiquitinate leading to either no functional outcome or to up- or down-regulation of ligase activity. In the case of Bard1-Brca1, autoubiquitination enhances the ligase activity of this complex (55), while enzymatic activity of Mdm2 is abrogated by autoubiquitination via targeting its own proteasomal degradation (56). Furthermore, autoubiquitination can also be nonproteolytic and, in the case of Traf6, activate signaling to its downstream target in the NF-κB pathway (57). In addition to regulation by autoubiquitination, ligase activity of RING E3s can be controlled via covalent conjugation of ubiquitin-like protein. For example, conjugation of such ubiquitin-like protein as Nedd8 activates the ligase activity of Cul1 E3 ligase enhancing the κ_{cat} for ubiquitin transfer to substrate (58).

HECT domain ligases

HECT E3 ligases (homologous to E6-associated protein [E6-AP] COOH-terminus), function in mechanistically different manners (34). The founding family member, E6-AP (59), contains an ~350 amino acid C-terminal region homologous to that of yeast RSP5 and utilizes a conserved active site cysteine residue near the C-terminus.

This cysteine accepts ubiquitin from bound E2 forming a covalent E3~ubiquitin thioester intermediate followed by the transfer of ubiquitin to the target protein (Figure 4) (60). In contrast, RING, U-box and PHD domain E3s do not form thioester intermediates with ubiquitin, but rather serve as an adapter between E2 and the substrate (38). When an E2 charged with ubiquitin is brought into close proximity with a substrate, the ubiquitin molecule is transferred directly from the E2 onto the substrate (Figure 4).

Ubiquitin chain topology

Substrate proteins can be ubiquitinated in several different ways (Figure 7). Monoubiquitinated substrates contain a single ubiquitin molecule conjugated to one or several lysine residues. Monoubiquitination is a reversible modification that usually has a nonproteolytic effect on a substrate. Depending on a substrate, monoubiquitination can signal to endocytosis, endosomal sorting, virus budding, histone regulation, DNA repair, receptor internalization, and nuclear export (13, 61-63).

Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), all of which could be used as acceptors for other ubiquitin moieties leading to the synthesis of the ubiquitin chain, or polyubiquitination (64, 65). Ubiquitin monomers are linked via an isopeptide bond between the C-terminus of one ubiquitin and any of the seven lysine residues of the preceding monomer thereby building ubiquitin chains of different topologies. In yeast, the relative abundance of polyubiquitin linkages corresponds with the following order: K48 (29%), K11 (28%), K63 (16%), K6 (11%), K27 (9%), K33 (3.5%), and K29 (3%) (66). In addition, linear ubiquitin chains consisting

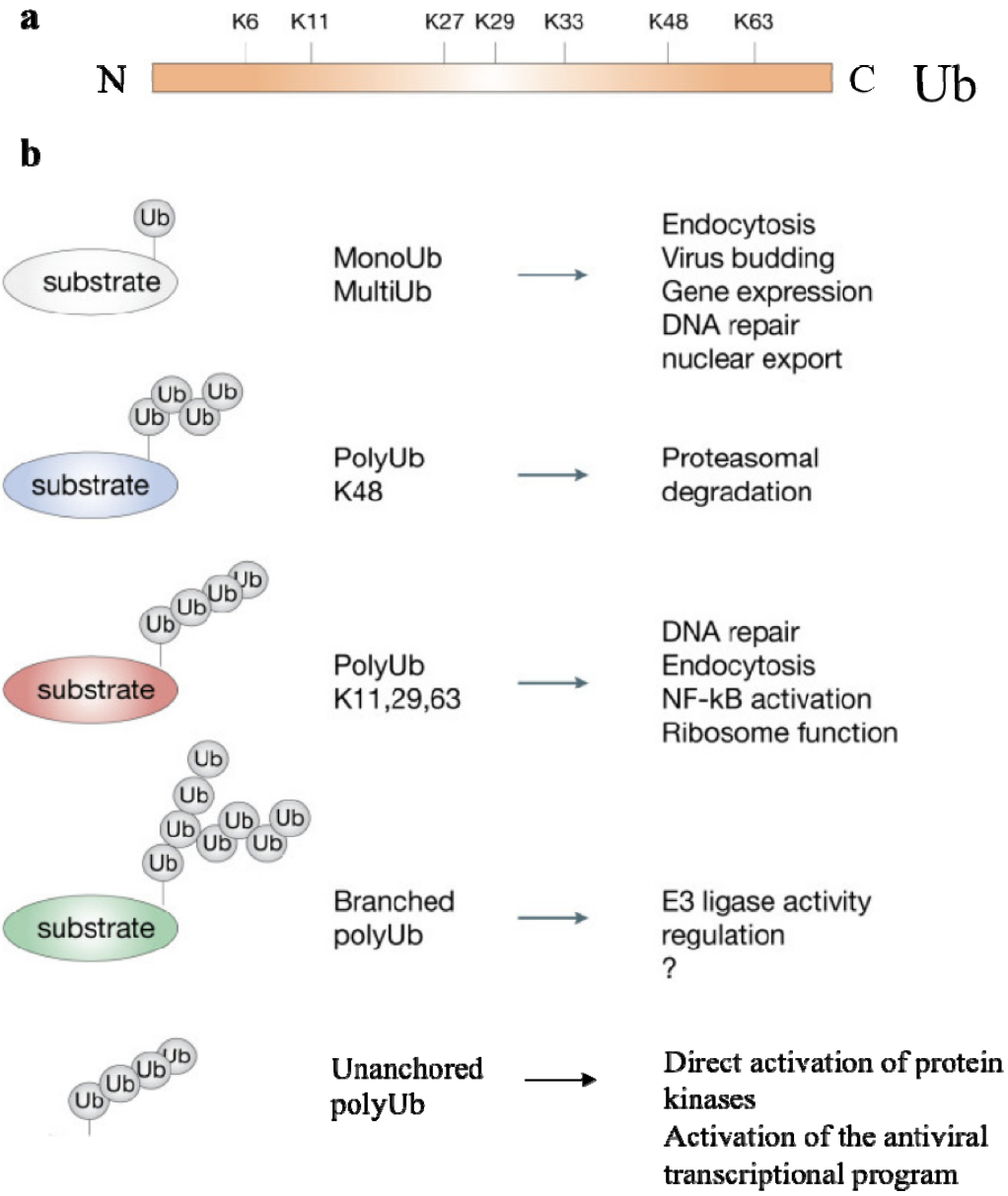

Figure 7. Ubiquitin chain topology (adapted from (67).

(a) Ubiquitin contains seven lysine residues that can be potentially used as acceptors for the attachment of other Ubiquitin molecules. (b) Schematic representation of the different Ub modifications with their functional roles. The question mark indicates that the functions of branched chains are largely unknown.

of a head-to-tail fusion of ubiquitin monomers can also be conjugated to substrate proteins (68). It is well known that the fate of the substrate is determined by the topology of the conjugated ubiquitin linkages (Figure 7).

The best-studies examples are K48- and K63-linked ubiquitin chains. When four or more ubiquitin monomers are linked via K48, the substrate is usually, but not always, targeted for degradation through the 26S proteasome (69, 70). Hydrophobic residues of adjacent ubiquitin molecules in a K48-linked chain are exposed at the interface and contact each other, which is required for the binding to the proteasome with high affinity (70, 71). A non-proteolytic function of K48-linked ubiquitin chains was described for the *S. cerevisiae* transcription factor Met4. Under certain conditions, Met4 becomes polyubiquitinatied with K48-linked chains, which abolishes its transcriptional activity without targeting the Met4 protein for degradation (72). Met4 contains an ubiquitin-binding domain that interacts with its own K48 ubiquitin chains restricting the chain length to below four, therefore hampering binding of the chain to the proteasome (72). Ubiquitin chains formed through K63, similarly to monoubiquitin, have been linked primarily to non-proteolytic signals involved in transcriptional regulation, endocytosis, DNA repair, and activation of protein kinases (73-75).

There are very few studies describing the function of other polyubiquitin isoforms. BRCA1 E3 complex can be autoubiquitinated with K6- or K29-linked chains, which may regulate the stability of BRCA1-Bard1 complex and consequently DNA repair (76). K11 chains have been suggested to target substrate proteins for proteasomal degradation (77-80). U-box-type E3 ligases assemble both K27 and K33 ubiquitin chains during stress response (81). K29-linked chains assembled on the Notch signaling

modulator DTX target DTX for lysosomal degradation (82). Also, both K29- and K33-linked ubiquitin chains were shown to regulate the enzymatic activity of AMPK-related kinases when these kinases are modified with such chains (83). One study revealed the surprisingly abundant levels of unconventional polyubiquitin chains on a broad range of substrates in yeast cells, underlining the significance of these chains for a cellular function (66).

Linear ubiquitin chain-assembly E3 ligase complex (LUBAC) catalyzes the formation of linear ubiquitin chains, which have been shown to regulate the NF-κB pathway (68). Branched ubiquitin chains containing different types of linkages have also been shown to be resistant to proteasomal degradation (79). For example NANOG, a homeobox transcription factor that plays a critical role in regulating embryonic stem cell pluripotency, can be ubiquitinated with forked K48- or K63-linked ubiquitin chains leading to NANOG stabilization (84).

Remarkably, ubiquitin chains that are not conjugated to a substrate (free ubiquitin chains) have been shown to have a function of their own (Figure 7) (85). Unconjugated K63 polyubiquitin chains synthesized by TRAF6 RING E3 ligase and UbcH5C E2 conjugating enzyme can directly activate the TAK1 kinase complex by binding to the ubiquitin receptor TAB2 (86). In addition, unanchored K63 polyubiquitin chains can directly activate RIG-I protein, a signaling protein involved in the immune response to viral infection (87).

Regulatory mechanism of ubiquitin homeostasis in yeast

Even though ubiquitin is a highly abundant protein, it is not produced in excess.

Rather, there is a dynamic equilibrium between three forms of cellular ubiquitin: monomeric ubiquitin, a substrate-conjugated mono- and polyubiquitin, and unanchored ubiquitin chains (Figure 8).

Ubiquitin is expressed from several ubiquitin encoding genes, or *UBI*. In *S. cerevisiae* there are four *UBI* genes (*UBI1-4*). *UBI1-3* encode fusion precursor proteins between ubiquitin and Ub_{L40} and Ub_{S27} ribosomal peptides, while *UBI4* encodes head-to-tail linear fusion ubiquitins, which are further cleaved by DUBs to monomeric ubiquitin (88, 89). Mutations in *UBI* genes as well as in several DUBs cause reduction of ubiquitin levels and various cell defects (reviewed in (90). Overexpression of ubiquitin also impairs cell growth as well as leads to cell sensitivity to several compounds (91). To prevent these undesirable effects, ubiquitin expression is tightly regulated by several control mechanisms:

I. Transcriptional regulation of ubiquitin-encoding genes. When yeast cells are exposed to such stress conditions as heat shock, starvation, or amino acid analog, misfolded proteins are accumulated, which need to be ubiquitinated and degraded by the 26S proteasome. Thus, more ubiquitin is required to dispose of misfolded proteins. In this case, it is beneficial to increase the transcription of *UBI4* gene which incodes a polyubiquitin gene to make more ubiquitin available. Indeed, transcription of *UBI4* is induced by heat or starvation (92), which also indicates that ubiquitin is an essential component of the stress response system.

II. Regulation by a change in proteasomal composition. Deubiquitinating enzyme Ubp6 binds reversibly to the proteasome and disassembles polyubiquitinatied

substrate proteins (93). Ubiquitin is therefore rescued from degradation by the proteasome and is recycled for other rounds of ubiquitination. The catalytic activity of Ubp6 is enhanced by its association with the proteasome (94), while the transcription of *UBP6* is increased in response to ubiquitin deficiency (95).

III. Regulation by deubiquitinating enzyme Doa4. The authors of one study proposed that unconjugated ubiquitin chains serve as a reservoir of ubiquitin pool in a cell under normal conditions (96). When the cell is subjected to heat shock, more monomeric ubiquitin is needed for rapid ubiquitination of numerous substrates. Rfu1 regulates the balance between monomeric ubiquitins and unanchored ubiquitin chains by inhibiting the Doa4 deubiquitinating enzyme (96). Doa4 and Rfu1 mediate rapid loss of free ubiquitin chains to cope with heat shock (Figure8).

IV. Other factors involved in ubiquitin homeostasis. In addition to Ubp6 and Doa4, described above, several other DUBs regulate the ubiquitin homeostasis in yeast. Cells lacking Ubp3, Ubp8, Ubp10, Ubp14, and Doa1 accumulate free ubiquitin chains of various lengths and topology depending on the DUB (97-99).

As described above, ubiquitin homeostasis is generally maintained by the expression of the ubiquitin genes, or by the expression of DUBs, which recycle polymeric ubiquitin to monoubiquitin. Intriguingly, loss of HECT E3 ubiquitin ligase Rsp5 leads to reduction of overall ubiquitin pool, and the reduced level of ubiquitin synthesis is sustained in *rsp1* mutant upon heat shock (100). Interestingly, Rsp5 contains

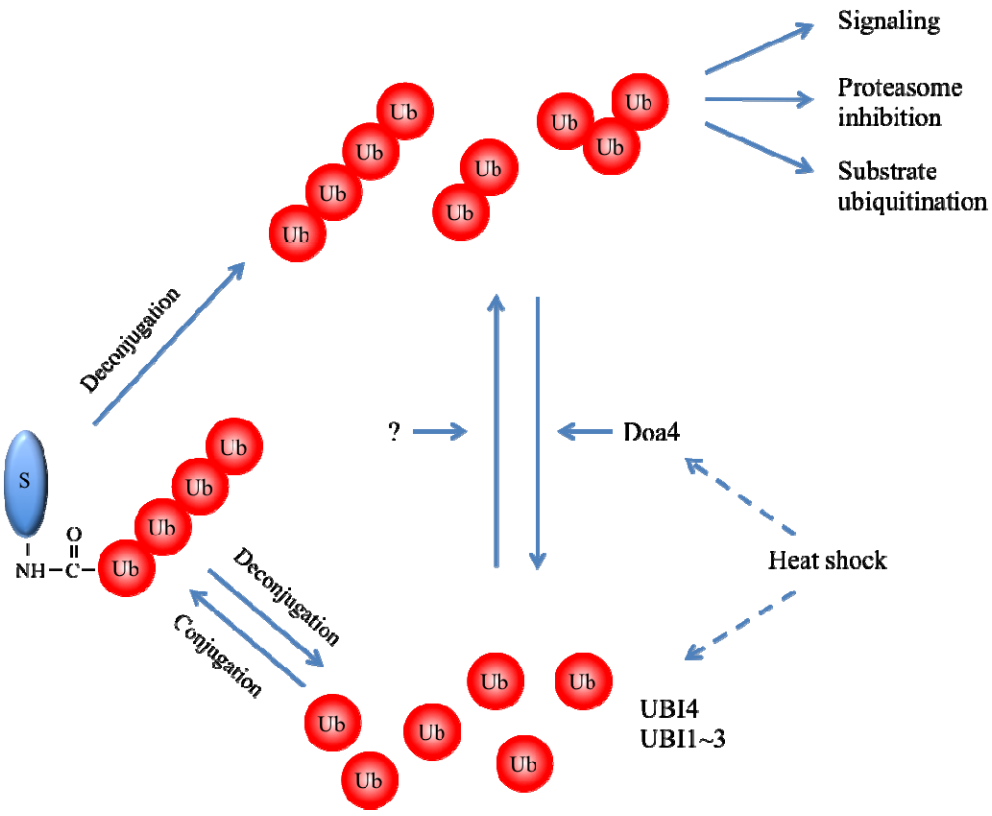

Figure 8. Ubiquitin homeostasis (adapted from (96).

The monomeric ubiquitin (Ub) pool is maintained through synthesis from Ub-encoding genes, *UBI1-4*, by release from protein-conjugated Ub chains, and by release from free Ub chains. DUB(s), such as Doa4, supply monomeric Ub by cleaving free Ub chains. Upon heat shock, transcription of *UBI4*-encoding polyubiquitin is increased and also more Doa4 is produced to increase monomeric Ub pool.

a noncovalent ubiquitin-binding site in its catalytic HECT domain, which is important for the ligase activity of Rsp5 (101). Rsp5 is the only E3 ubiquitin ligase known to play role in regulation of the yeast ubiquitin homeostasis up to date.

Clinical relevance of the ubiquitin system

Because the ubiquitin proteasomal system (UPS) is a major system controlling many cellular processes, it is not surprising that defects in components of the UPS system (both the loss and the overexpression) are implicated in numerous human diseases and disorders (9, 102). For example, the CYLD deubiquitinating enzyme is a negative regulator of the canonical NF-κB pathway, which controls processes such as inflammation, immunity and cell survival. Loss of CYLD is linked to a benign human tumor syndrome, or cylindromatosis (103). Autosomal recessive Parkinson's disease have been linked to dysfunctional interaction of another E3, Parkin, with its substrate (104). p53 is a classic tumor suppressor, and approximately 50% of all human tumors contain mutations in the p53 gene (105). Overexpression of the oncogenic E3 Mdm2 is implicated in tumor formation by promoting the degradation of the p53 protein (106).

There are many more examples of how mutations, loss or overexpression of components of the ubiquitin pathway may be linked to such diseases as various malignancies, neurodegenerative disorders (Alzheimer's disease, Parkinson's disease, Huntington's disease), genetic diseases (spinocerebellar ataxias), immune and inflammatory responses (asthma, arthritis), hypoxia, and muscle wasting (102). Therefore, targeting specific components of the UPS system for inhibition is therapeutically attractive.

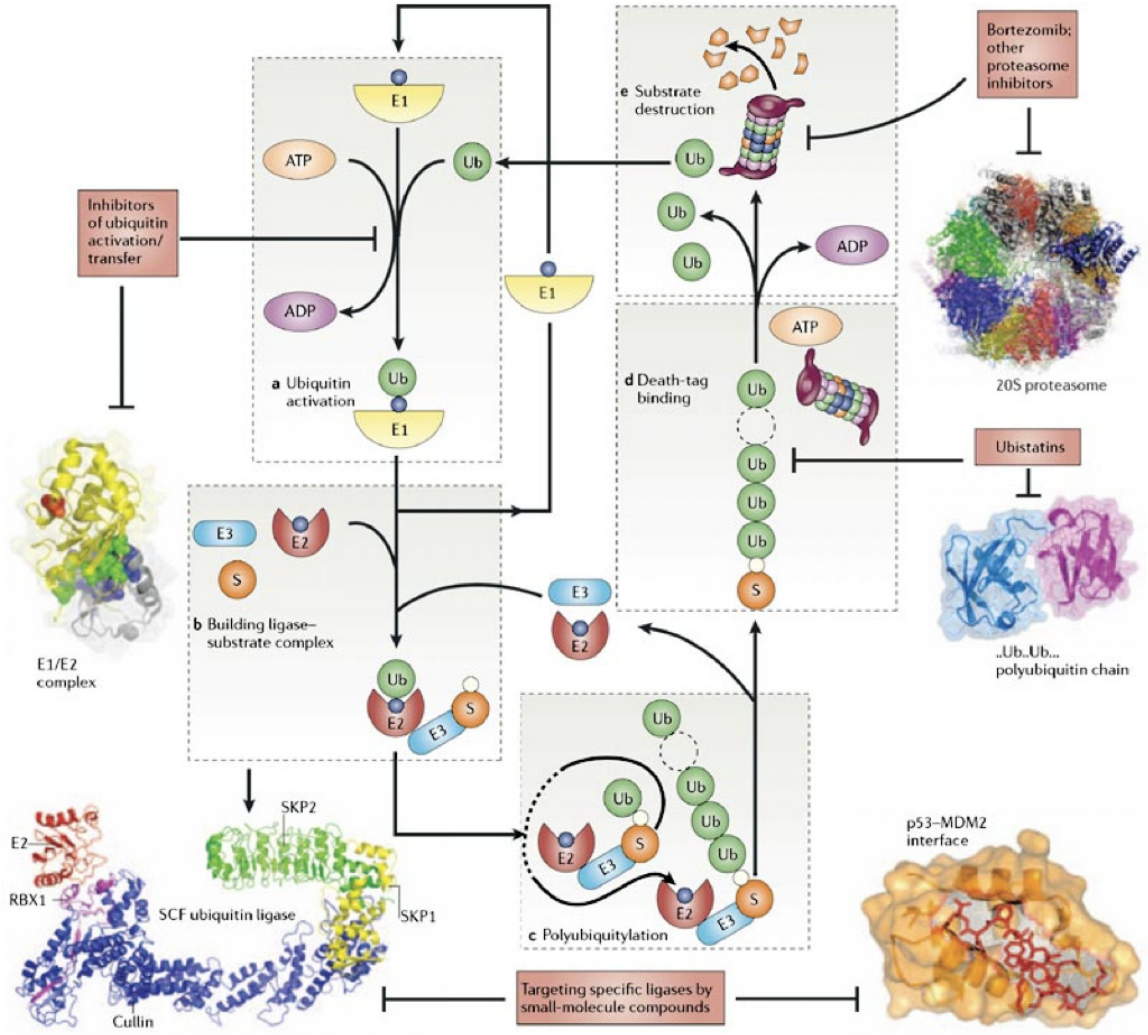

Figure 9. Potential sites for drug development in the ubiquitin-proteasomal system (UPS) (adapted from (102).

(a) Inhibition of activation of ubiquitin by E1 and transfer to E2; (b) Inhibition of the formation of the ligase-substrate complex; (c) Inhibiting specific ligases by small-molecules compounds; (d) Inhibition of polyubiquitin chain formation by ubistatins; (e) Inhibition of the proteasome.

The first potential site for drug development is targeting the most upstream components of the UPS, the E1 and the E2s (Figure 9). There are two possible approaches to inhibiting the E1 protein. First, activation of ubiquitin by the E1 may potentially be inhibited by blocking the access of the ubiquitin to the adenylate site or by blocking access of ATP. The later approach is quite feasible as drugs inhibiting ATP-binding sites already exist for several kinases (107).

Second, the E1-E2 protein-protein interaction can potentially be inhibited by small molecule inhibitors, which could be challenging because of difficulties with identification of small molecule binding pockets (107).

Next site for drug development is the proteasome, an important component of the UPS. Bortezomib (Velcade, PS-441) is the first drug approved by the Federal Drug Administration for treatment of multiple myeloma, and targets the proteasome (108, 109). Bortezomib selectively inhibits chymotrypsin-like activity of the 20S proteasome by reacting with a threonine residue of the active site of the proteasome. Interestingly, the activity of this drug seems to be directed only to a subset of hematopoietic malignancies.

Finally, because the greatest amount of specificity is present in the last step of ubiquitination process facilitated by E3s, it is very appealing to develop drugs targeting individual E3s, implicated in particular diseases. This increase in the specificity of therapeutic treatment could potentially improve the effectiveness of the treatment and, importantly, eliminate some nonspecific side effects at the same time.

Discovery of BRCA1 Associated protein 2

There has been widespread interest in proteins that regulate or associate with *BRCA1*, one of the key tumor suppressor genes affected by genetic alterations in breast cancer (110). At least 5% of breast cancer cases involve mutations in *BRCA1* that are inherited through the germ line (111-114), and such mutations can be detected in up to 90% of families with susceptibility to breast and ovarian cancer (112, 114). On the other hand, very few *BRCA1* mutations have been detected in sporadic breast cancer cases (115). In these cancers the gene product of *BRCA1* may be nonfunctional because of mislocalization. Normally a nuclear protein (116), BRCA1 accumulates in the cytoplasm in 80% sporadic breast cancers (117-119). This abrogates the nuclear functions of BRCA1 such as inhibition of growth (120), induction of apoptosis (121), and regulation of the cell cycle (122), as well as serving as a transcriptional co-activator (123), an E3 ubiquitin ligase (124) and a caretaker in maintaining genomic integrity (125, 126). Cytoplasmic sequestration of BRCA1 in malignant cells suggests an indirect suppression of protein function.

BRCA1 has two functional nuclear localization signals (NLS) (119, 127). Nuclear-cytoplasmic shuttling of BRCA1 is mediated by both NLS- independent and NLS-dependent mechanisms (128). In the NLS-independent mechanism, a binding partner, BARD1 (129), stimulates BRCA1 nuclear translocation by binding and escorting BRCA1 from cytoplasm to the nucleus via a piggyback mechanism (130).

Utilizing a fragment of BRCA1 containing two functional NLSs as bait for a yeast-two-hybrid system, several BRCA1 interacting proteins have been identified, including importin-α and a novel cytoplasmic protein BRCA1-associated protein 2, BRAP2 (131). While the function of importin-α in translocating NLS-containing proteins

through nuclear pores into the nucleus is well characterized (132), role of BRAP2 in the regulation of BRCA1 protein shuttling is poorly understood.

BRAP2 E3 ligase

BRAP2 is a 600-amino acid cytoplasmic RING E3 ubiquitin ligase (131). The emerging evidence points to a significant role of BRAP2 in human cells.

BRAP2 was discovered in a yeast-two-hybrid screen utilizing BRCA1 NLS as bait, hence the name – BRCA1 Associated Protein 2 (131). In addition to binding to BRCA1, BRAP2 binds to the NLS motifs SV40 large T antigen, and the bipartile NLS motif of mitosin (131). Also, BRAP2 functions as a cytoplasmic retention protein for the cell cycle regulating protein $p21^{Cip1}$ during monocyte differentiation, in a manner requiring the NLS of p21 (133).

Recent study from David Jans lab revealed the ability of BRAP2 to inhibit the nuclear import of specific viral proteins (134). Ectopic expression of BRAP2 in transfected African green monkey kidney COS-7 cells and HL-60 human promyelocytic leukemia cells lead to a significant reduction of NLS-dependent nuclear accumulation of either simian virus SV40 large-tumor antigen (T-ag) or human cytomegalovirus DNA polymerase processivity factor ppUL44. Both of these viral proteins have NLSs flanked by phosphorylation sites. Pulldown assays indicated direct, high-affinity binding of C-terminal region of BRAP2 to T-ag, which was strictly dependent on negative charge at T124 and the NLS. No effect of BRAP2 was observed on nuclear targeting of other viral proteins that lack a phosphorylation site near their NLS. These results are consistent with a model of BRAP2 function in which BRAP2 negatively regulates nuclear import of both endogenous and viral proteins, possibly by ubiquitination of these proteins.

Surprisingly, BPAP2 also negatively regulates the sensitivity of the MAP kinase cascade by limiting the formation of Raf/MEK complexes by preventing the dimerization and activation of the KSR1 scaffold protein (135). This function of BRAP2 is dependent its E3 ligase activity (136). BRAP2 acts as a Ras responsive E3 ubiquitin ligase that, on activation of Ras, is modified by autoubiquitination resulting in the release of inhibition of KSR. Therefore, BRAP2, through the MAP kinase cascade, may regulate various cellular activities such as gene expression, mitosis, differentiation, and cell survival/apoptosis (137).

Misregulation of BRAP2 is associated with several human diseases. A case-control study conducted with a total of 1480 cases and 2115 controls from Japanese and Korean populations, found a strong association of single nucleotide polymorphisms (SNPs) in BRAP2 gene locus with a coronary artery disease (138). BRAP2 is also overexpressed in primary breast cancer tissues pointing to a role of BRAP2 in tumorigenesis (139). In addition, BRAP2 has been linked to the myocardial hypertrophy in rats (140). Yet, the mechanism by which BRAP2 regulates cell processes in these diseases is not understood.

BRAP2 domain structure

BRAP2 domain structure is depicted in Figure 10. BRAP2 consists of a classical RING domain, the ubiquitin binding ZnF UBP domain, and the coiled-coil domain at the C-terminus. The RING domain of BRAP2 (residues 264-303) is responsible for its E3 ubiquitin ligase activity (135, 141). The ZnF UBP domain (residues 316-365) is

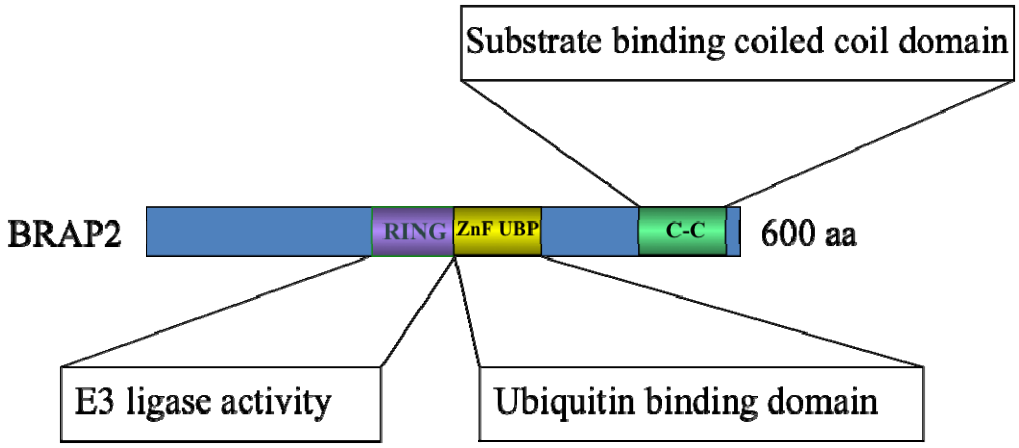

Figure 10. Domain structure of BRAP2.

Human BRAP2 is 600 amino-acid protein that contains the RING domain, responsible for its E3 ubiquitin ligase activity, the ZnF UBP ubiquitin binding domain, and the Coiled-Coil domain, which aid with substrate binding.

responsible for the binding of BRAP2 to ubiquitin (this study). The coiled-coiled domain of BRAP2 (residues 429-537) was shown to be important for protein-protein interaction in human cells with NLSs of various proteins (134) and for homo-oligomerization of BRAP2 homologues in plants (141).

The structure of BRAP2 is quite exceptional in that BRAP2 is the only E3 ligase that contains a ZnF UBP domain, otherwise found only in several DUBs and in hHDAC6 (136, 142, 143). The crystal and solution structure of the ZnF UBP domain from the DUB IsoT in complex with ubiquitin revealed the unique deep binding pocket within the domain, into which the C-terminal diglycine motif of free ubiquitin is inserted (Figure 11) (136, 143). Unlike other ubiquitin binding domains, which generally bind isoleucine 44 and valine 70 of the hydrophobic patch ubiquitin and leave the C-terminus of ubiquitin free for a further substrate conjugation (144), the ZnF UBP domain occupies the C-terminal tail of unconjugated ubiquitin. In the case of IsoT DUB, the ZnF UBP domain ensures that IsoT disassembles only unanchored polyubiquitin chains (98). Nevertheless, it is perplexing to observe such a domain in the structure of an E3 ligase.

BRAP2 orthologues

BRAP2 is highly conserved across eukaryotes, with a single orthologue present in each species (Figure 12). Very few studies have investigated the function of BRAP2 or its orthologues. One report described the role of *Caenorhabditis elegans* homolog of BRCA1-associated protein 2 (BRAP-2) in larvae development (145). A mutant containing a deletion of *brap-2* was highly sensitive to the oxidative stress-inducing drug

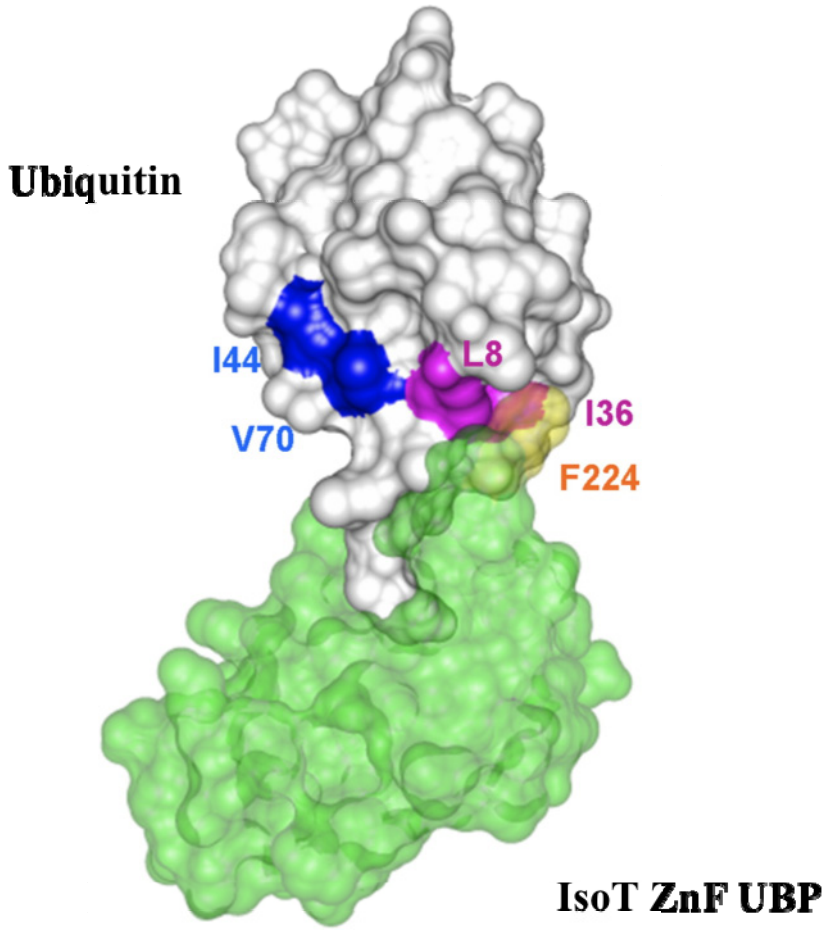

Figure 11. ZnF UBP domain (adapted from (136).

Stereo representation of the ZnF UBP domain/ubiquitin complex. The ZnF UBP domain is colored in green, and ubiquitin is colored in gray. I44 and V70 of the hydrophobic patch of ubiquitin are shown in blue. L8 and I36 of ubiquitin (shown in magenta) interact with F224 of the ZnF UBP domain (shown in yellow).

paraquat and demonstrated early larval arrest and lethality at low concentrations of paraquat compared with the wild-type. Developmental arrest subsequently leads to an increase in gene expression of the cyclin-dependent kinase inhibitor *cki-1*. This function of brap-2 is dependent specifically on the function of the *C. elegans* orthologue of BRCA-1 tumor suppressor *brc-1*, although no direct interaction between *brap-2* and *brc-1* or *cki-1* has been detected. Overall, BRAP-2 is necessary to prevent the aberrant induction of BRC-1 and CKI-1 triggered by excessive reactive oxygen species (ROS), the major contributor of oxidative damage and, consequently, cancer and aging processes.

Another study focused on the role of BRAP2 homologues in *Arabidopsis thaliana*. There are two homologues of human BRAP2 in *A. thaliana* named BRIZ1 and BRIZ2 (BRAP2 RING ZnF UBP domain-containing protein 1 and 2), which were shown to have the same functional domains as human BRAP2 (Figure 12) (141). Loss of either BRIZ1 or BRIZ2 results in a severe phenotype – heterozygous parents produce progeny that segregate 3:1 (3 wild-type : 1 growth-arrested seedlings). Both proteins exhibit E3 ligase activity in vitro, and the formation of BRIZ1-BRIZ2 heterodimer is required for the ligase activity of the complex in vivo and, consequently, for germination and post-germination growth.

Ethanol Tolerance Protein 1, or ETP1 is a 585-amino acid *S. cerevisiae* homologue of BRAP2 that shares 40.8% overall identity with BRAP2 with about ~75% homology in both RING and the ZnF UBP domain (135). Investigation of ETP1 function is the primary focus of this study.

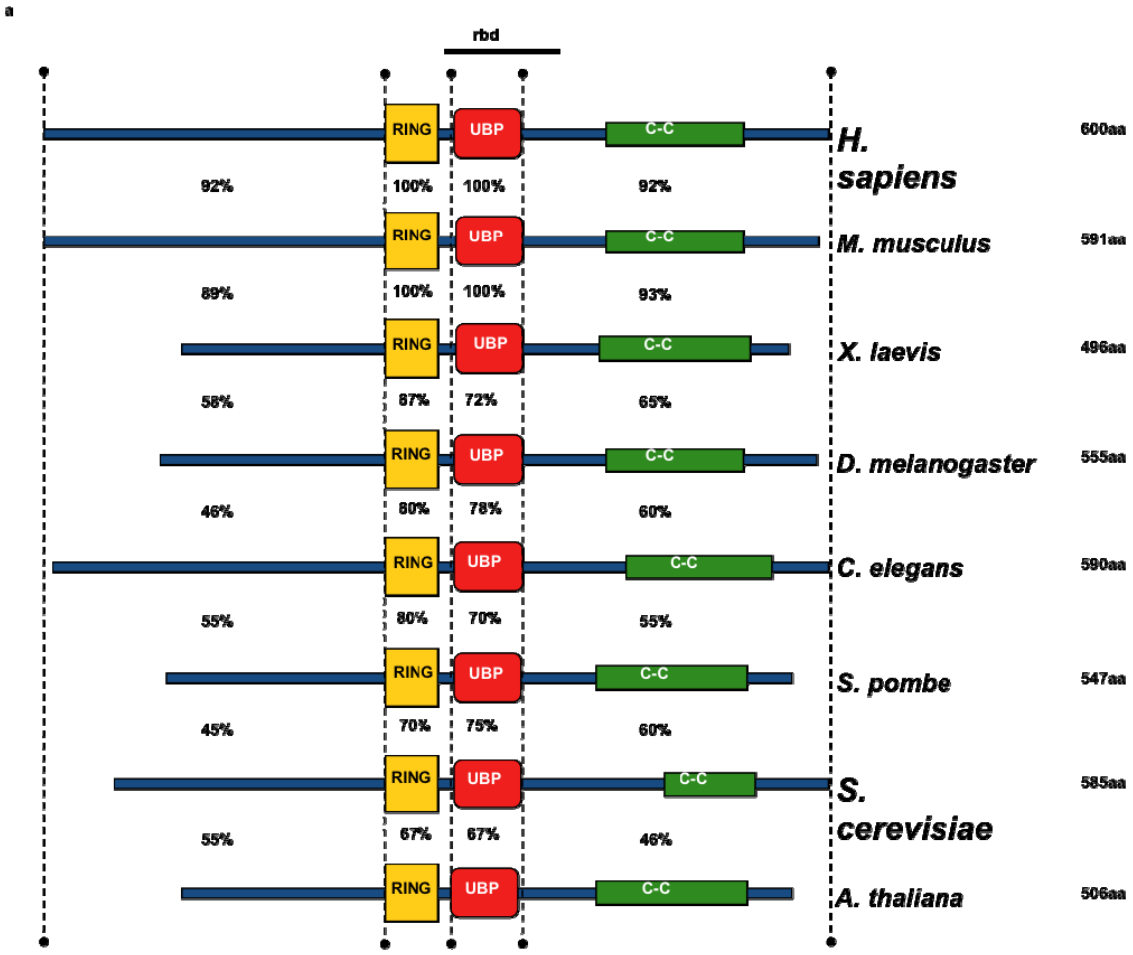

Figure 12. Orthologues of BRAP2 (adapted from (135).

BRAP2 is highly conserved among eukaryotic species with the most homology in the RING, the ZnF UBP, and the Coiled-Coil functional domains. Percent of homology of each domain is shown to those of human BRAP2.

ETP1 is yeast BRAP2

To date ETP1 was characterized only by George van der Merwe's laboratory in a study investigating *Saccharomyces cerevisiae* response to growth in ethanol-containing media (146). Yeast has the ability to use a variety of different carbon sources to support its growth (147). Abundant fermentable sugars such as glucose and fructose are utilized first and fermented to ethanol. After glucose is depleted, yeast cells adjust their gene expression patterns to be able to consume nonfermentable carbon sources, such as ethanol and glycerol. Nevertheless, even though yeast can tolerate up to 15% v/v ethanol, the growth rate of yeast in the media containing just 4–6% v/v of ethanol is reduced by 50% (148). ETP1 is required for yeast to adapt to the toxic environment of ethanol, whether as a sole carbon source or as a stressor (146). Loss of ETP1 leads to a growth defects in the presence of ethanol and to decreased ability to activate the transcription of ENA1 promoter and heat shock protein genes (HSP12 and HSP26). Also, upon a shift from glucose to ethanol, the turnover of some proteins, specifically Hxt3p, is delayed in an *etp1Δ* strain. Lastly, the hypersensitivity of *etp1Δ* to ethanol stress is linked to the control of the level of the cation/H^+ antiporter Nha1p.

Scope of this dissertation

Previous studies have implicated BRAP2 and its orthologues in such wide array of cell processes as nuclear-cytoplasmic protein shuffling to signaling through MAPK to cell response to various stresses. However, the biological function of BRAP2 still remains unknown. The goal if this dissertation is to understand the physiological role of

BRAP2 through the use of biochemical assays and yeast model system.

Studying functional homologues of human proteins in yeast offers several advantages. Yeast is easily transformed, has stable haploid and diploid states, and displays high levels of homologous recombination, all of which make studies in yeast more facile. Importantly for this proposal, yeast model system also offers an ease of testing protein-protein interactions, such as Yeast-2-Hybrid analysis.

Here, we characterize the yeast ETP1 protein, its functional domains, and its surprising role in the regulation of ubiquitin homeostasis. We also provide evidence that ETP1 is required for normal response of yeast to various stress conditions (Chapter 2).

Identification of substrates of specific E3s is a major goal in the ubiquitination field, but this has proven to be a difficult task. In this work, we have identified the binding partners of human BRAP2 via yeast-two-hybrid analysis, some of which could potentially be the substrates of BRAP2 E3 ligase (Chapter 3).

The findings presented in this dissertation are important for future studies that will aim to elucidate the role of human BRAP2 in regulation of ubiquitin homeostasis and to identify the substrates of BRAP3 E3 ligase (Chapter 4).

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Chapter II

The Ethanol Tolerance Protein 1 Ubiquitin Ligase Regulates Ubiquitin Homeostasis in Yeast

Abstract

Post-translational modification of cellular proteins by ubiquitination regulates a wide array of cell processes and is implicated in many diseases. Maintenance of ubiquitin homeostasis is tightly regulated by several mechanisms. Here, we characterize the role of Ethanol Tolerance Protein 1 or ETP1 in the regulation of ubiquitin homeostasis in *Saccharomyces cerevisiae*. We show that Etp1 is a short-lived homologue of mammalian BRCA1 Associated Protein 2, a RING ubiquitin ligase that is so named because it was first found to bind to the nuclear localization signal motif of BRCA1. ETP1 and BRAP2 have the same domain structure consisting of a functional RING, ZnF UBP, and Coiled-Coil domains. Upon loss of ETP1, yeast cells exhibit a significantly decreased amount of polyubiquitin chains without changes in total ubiquitin levels. The E3 ligase activity of Etp1 and its ubiquitin binding site are required for the function of Etp1. Deletion of ETP1 renders yeast resistant to oxidative stress, translational inhibition, and growth on an amino acid analog. Deletion of Doa4, a deubiquitinating enzyme required for ubiquitin homeostasis, results in a large increase in di- and tri-ubiquitin that is suppressed by deletion of ETP1. Deletion of ETP1 in a *doa4Δ* yeast strain also partially rescues the phenotypes of *doa4Δ*, indicating that ETP1 and DOA4 act upon the same biological pathway. We hypothesize that ETP1 is implicated in the regulation of the ubiquitin homeostasis by regulating the balance between free ubiquitin and short ubiquitin chains.

Introduction

Ubiquitination is a reversible post-translational modification of cellular proteins that plays important roles in the regulation of a wide array of cellular processes such as protein quality control, protein trafficking, cell-cycle progression, DNA repair, apoptosis, transcriptional regulation, endocytosis, receptor down-regulation, and signal transduction (1, 2). Ubiquitin is highly conserved, with orthologues present in every eukaryotic species and differing in only 3 out of 76 residues from yeast to human (3, 4). In 2004 the Nobel Prize in chemistry was awarded jointly to Awram Hershko, Aaron Ciechanover, and Irwin Rose for their pioneering discovery of ubiquitin-mediated protein degradation by the 26S proteasome (1). Even though this role of ubiquitination is the best characterized, post-translational modification by ubiquitin has also been linked to altered sub-cellular localization, protein-protein interaction, activity and function of the modified protein (5-7).

In general, ubiquitination of a target protein is catalyzed by the sequential action of three classes of enzymes; a ubiquitin activating enzyme E1, a ubiquitin conjugating enzyme E2, and a ubiquitin ligase E3. First, a thiol-ester is formed between the E1 and ubiquitin via an acyl adenylate intermediate and nucleophilic attack by an E1 thiol group. The E1 thiol ester then binds to one of a few dozen E2 enzymes and the ubiquitin moiety is transferred to a thiol group on the E2. Finally, the E2~ubiquitin thiol-ester (also referred to as the charged E2) associates with one of the E3 ubiquitin ligases, which also binds to a substrate targeted for ubiquitination. An isopeptide bond is then formed

between the C-terminal glycine residue of ubiquitin and ε-amino group on a lysine residue of the target protein, leading to a monoubiquitination of the target protein (7-9).

In humans, there are only two E1 activating enzymes, a few dozen E2 conjugating enzymes, and several hundred E3 ubiquitin ligases (10). This last step of ubiquitination is highly regulated and provides the specific substrate-recognition element to the hierarchical process of ubiquitination. E3 ubiquitin ligases are broadly categorized into two major classes based on structure and mechanism. RING-finger (Really Interesting New Gene) E3s and their variant U-box and PHD domain ligases, are the largest class of E3s with over 600 members (11). They act as adapters bringing the charged E2 and substrate together to achieve ubiquitination. HECT-domain (Homologous to E6AP C-terminus) E3s are named after the prototypical ligase, E6AP, which is activated by binding to the human papilloma virus E6 protein (12). HECT ligases first react with a charged E2 to form an E3~ubiquitin thiol ester and subsequently transfer the ubiquitin to the substrate protein (13).

The addition of the first ubiquitin to a target protein can nucleate the formation of a polyubiquitin chain on the target protein. There are seven lysine residues in ubiquitin: K6, K11, K27, K29, K33, K48, and K63, and all of them can be used in the polyubiquitination of a substrate. Polyubiquitin chains of various lengths and topology determine the fate of the substrate protein. Generally, K48 chains target proteins for proteasomal degradation, while K63 chains, similarly to monoubiquitin, have been linked primarily to generation of non-proteolytic signals involved in transcriptional regulation, endocytosis, DNA repair, and activation of protein kinases (14-16). The function of other ubiquitin chains in not well understood. However, several studies have revealed highly

abundant non-canonical ubiquitin chains in yeast (17) and man (18). Remarkably, ubiquitin chains that are not conjugated to a substrate (unanchored or free ubiquitin chains) have been shown to have a function in activating kinases and signaling in response to a viral infection (19-21).

The machinery that catalyzes ubiquitination, recognition of polyubiquitin, and disassembly of polyubiquitin chains must specifically bind to ubiquitin. More then twenty different families of ubiquitin-binding domains have been described (22). Most domains recognize a hydrophobic Ile44-containing surface on the ubiquitin molecule. In contrast, the ZnF UBP domain binds the free C-terminal RGG of ubiquitin and its binding is ablated by any changes to the free C-terminus (23). In USP5, a DUB that disassembles free ubiquitin chains, this domain imparts specificity for binding and catalysis of chains that bear a free C-terminus (24). This domain is present in several DUBs, as well as HDAC6, a tubulin deacetylase that binds polyubiquitinated proteins, and BRAP2, a ubiquitin ligase that participates in RAS signaling (23). To extend our understanding of the function of the ZnF UBP domain, we have begun to characterize the role of this domain in other proteins.

BRAP2 (BRCA1 Associated Protein 2) is a highly conserved RING E3 ubiquitin ligase that regulates several cellular processes (25). BPAP2 negatively controls the sensitivity of the MAP kinase cascade by limiting the formation of Raf/MEK complexes by preventing the dimerization and activation of the KSR1 scaffold protein (26). This function of BRAP2 is dependent on its E3 ligase activity. BRAP2 acts as a Ras responsive E3 ubiquitin ligase that, on activation of Ras, is modified by autoubiquitination resulting in the release of inhibition of KSR (26, 27). BRAP2 is

present in every eukaryotic species and has a conserved domain structure. Each orthologue contains three highly conserved domains, the RING domain, the ZnF UBP domain and the Coiled-Coil domain (28). However, most homologous are the RING domains and the ZnF UBP domain, suggesting the conserved function of these domains among the BRAP2 orthologues.

The RING domain is responsible for the E3 ubiquitin ligase activity of BRAP2 (26). However, the physiological substrates of this E3 ubiquitin ligase have not been identified. The function of the ZnF UBP domain of BRAP2 has not been studied. Finally, the coiled-coil domain of BRAP2 binds nuclear localization signal motifs (NLS) of BRCA1, SV40 large T antigen, and the bipartite NLS motif of mitosin (25). BRAP2 functions as a cytoplasmic retention protein for the cell cycle regulating protein $p21^{Cip1}$ during monocyte differentiation, in a manner requiring the NLS of p21 (29). In addition, BRAP2 inhibits the phosphorylated nuclear import of specific viral proteins (30).

Yeast is a powerful model system for interrogating structure-function relationships and we have exploited this to characterize the role of the ZnF UBP domain of BRAP2. The closest BRAP2 orthologue in budding yeast is ETP1 (Ethanol Tolerance Protein 1). ETP1 was previously described to play role in yeast adaptation to ethanol provided either as a sole carbon source or as a stressor (31). We report here that, in addition to this function, ETP1 is implicated in the regulation of the ubiquitin homeostasis by regulating the equilibrium between ubiquitin and free ubiquitin chains. Upon loss of ETP1, yeast cells display a significantly decreased amount of polyubiquitin without changes in total ubiquitin levels. This function is dependent on the E3 ligase

activity and the ZnF UBP ubiquitin binding site of Etp1. Loss of ETP1 leads to resistance of yeast to various stresses such as oxidative stress, translational inhibition, and an amino acid analog. Deletion of ETP1 from *doa4Δ* yeast strain partially rescues the phenotypes of *doa4Δ*, indicating that ETP1 and DOA4 may act upon the same biological pathway.

Experimental procedures

General – In all SDS-PAGE assays, proteins were separated on a 4-20% precast polyacrylamide gel (Bio-Rad). Proteins were transferred for 7 min onto a nitrocellulose membrane via iBlot (Invitrogen). The membrane was then probed with the appropriate antibody. When ubiquitin was being detected, the membrane was boiled for 10 min in dH_2O prior to blocking. Proteins were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Antibodies to the following epitopes have been used: ubiquitin (1:1000 diluted P4D1 antibodies from Santa Cruz); hexahistidine (1:1000 diluted antibodies from Sigma); poly-histidine affinity tag - HAT tag - KDHLIHNVHKEFHAHAHNK (1:1000 diluted antibodies from GenScript); hemagglutinin (6xHA tag; 1:4000 diluted antibodies from Maine Biotechnology Services); 3-phosphoglycerate kinase (PGK) (1:4000 diluted antibodies from Molecular Probes, Inc.). All plasmids used in this study are listed in Table 1. All primers were synthesized by Operon Biotechnologies, Inc. (Table 2). Standard yeast techniques were used in all phenotypic screens (32).

Protein Expression and Purification – To purify ETP1 protein, full length ETP1 was fused with a poly-histidine affinity tag, HAT tag, in pET28a(+) (Table 1). *E. coli* BL21 Gold (DE3) pLysS cells (Agilent Technologies) were transformed with pET28a(+)-HAT-ETP1 in the presence of 100 μg/ml ampicillin. To induce expression of ETP1, cells were grown to an A_{600} of 0.8 in Luria Broth containing 100 g/ml ampicillin at 30^{o}C, chilled to 15°C and incubated overnight at 15 ^{o}C in the presence of 50 μM IPTG. All

later steps were carried out at 4°C or on ice. Cells were harvested by centrifugation and subjected to osmotic shock to remove periplasmic proteins (33). The pellet was resuspended in 1.5 ml/g Lysis Buffer [50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 10 mM βME, 5 mM imidazole, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete Protease Inhibitors (EDTA-free, Roche Diagnostics). Cells were disrupted by sonication a total of 4 min at 60W in 30 sec pulses with 30 sec rests all on ice. The lysate was then spun at 12000 g for 20 min at 4°C. The supernatant was recovered and applied to a 5ml His Trap FastFlow (GE Healthcare) column at 1ml/min. The column was washed with 18 column volumes of modified Lysis Buffer (lacking Triton X-100 and Complete PIs). A wash of 10 column volumes of the modified Lysis Buffer at 20 mM final imidazole followed. HAT-Etp1 was eluted from the column with the modified Lysis Buffer at 100 mM final imidazole, collecting 1ml fractions for a total of 50 ml. Fractions were selected by Western using rabbit anti-HAT antibody. The pooled fractions were then dialyzed against 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM βME, 1 mM PMSF and stored at -20°C.

HAT-ETP1 C240A/C243A, HAT-ETP1 W311A/R323A, HAT-ETP1 C240A/C243A/W311A/R323A, HAT-BRAP2, His-Ubc1, His-Ubc3, His-Ubc4, His-Ubc7, His-Ubc8, His-Ubc10, His-Ubc11, and His-Ubc13 proteins (Table 1) were expressed and purified using a protocol described for the purification of a full-length ETP1 protein above.

In Vitro Ubiquitination Assay – E1 (100 nM) (Boston Biochem), E2 (350 nM), E3 (500 nM), ubiquitin (10 μM) (Boston Biochem), and Energy Regeneration System

(Boston Biochem) were combined in a total volume of 30 µl in ubiquitin assay buffer (50 mM Tris, pH7.4, 150 mM NaCl, 1 mM DTT). After incubation at 30°C for 2 hr, the reaction was quenched with 15 µl 3x SDS-loading buffer. The sample was boiled for 5 min and analyzed by a Western blot analysis with anti-Ub antibodies. Mass spectrometry analysis of ubiquitin chains present in the reactions with Etp1 or BRAP2 was performed as previously described (17).

Ubiquitin Binding Assay – 5 µg of HAT-tagged E3 ligase was diluted in a binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM DTT, 0.1% Triton X-100) and incubated with 50 µl of ubiquitin (12 mg/ml), or ubiquitin$^{1-75}$ (12 mg/ml) conjugated Sepharose resins (23) for 3 hr at 4°C on a rocker. Beads were collected by centrifugation, and unbound fraction was collected in the supernatant. Beads were washed three times with 1 ml of binding buffer and were boiled to release the ubiquitin bound fraction. Equal ratios of bound and unbound fractions were analyzed by an immunoblot analysis with anti-HAT antibodies.

Strains Used in This Study – S. cerevisiae strains are listed in Table 3. To create the ZAY1 strain in which ETP1 is C-terminally fused with 6xHA tags, BY4742 yeast cells were transformed by a LiAc method (32) with oligonucleotide made using the pYM17 plasmid (34) and ZAP108, ZAP109 primers (Table 2).

For complementation assays, ZAY2 strain was created, in which the *kanMX4* cassette of YSC1021-547582 (*etp1Δ*) strain was replaced with 6xHA-tagged ETP1. The

oligonucleotide used for a transformation was amplified from the genomic DNA of the ZAY1 strain using ZAP147 and ZAP148 primers (Table 2).

The *delitto perfetto* approach was utilized for site-directed mutagenesis of *ETP1* gene in ZAY2 strain background (35). First, a COunterselectable REporter (CORE) cassette was amplified from a pGSHU plasmid (35) using ZAP151 and ZAP152 primers. The CORE cassette was then integrated into the *ETP1* genomic locus of ZAY2 strain creating the ZAY3 strain. To create the ZAY4 strain, in which RING domain of ETP1 is mutated, or ZAY5 strain with the mutant ZnF UBP domain, or ZAY6 strain with both RING and ZnF UBP domains mutated, a CORE cassette in the ZAY2 strain was replaced with the appropriate targeting oligonucleotide containing the mutations. The ZD57, ZD59, and ZD61 plasmids were used as templates for an amplification of the oligonucleotides of ETP1 with the mutant RING domain, or the mutant ZnF UBP domain, or both mutant RING and ZnF UBP domains, respectively (Table 1). ZAP155 and ZAP156 primers were used in all three PCRs (Table 2).

To create, ZAY7 (*doa4Δ*) and ZAY8 (*doa4Δetp1Δ*) strains, the DOA4 gene of wild type BY4747 and *etp1Δ* was replaced with nourseothricin-resistance marker *natMX* that confers resistance to the antibiotic nourseothricin. The *natMX* cassette was amplified from the ZD-9 plasmid (36) using the ZAP118 and ZAP119 primers (Table 2).

Cycloheximide Chase – To measure a half-life of ETP1, ZAY1 cells, in which ETP1 is C-terminally fused with 6xHA tag, were grown to a mid-log phase in 50 ml YPD media (32). The cycloheximide chase assay was then performed as previously described (37). Equal amounts of cells were loaded onto a polyacrylamide gel (Bio-Rad) for an

immunoblot analysis. ETP1-6xHA protein presence was analyzed by anti-hemagglutinin antibodies. Loading was analyzed by immunoblot analysis with anti-PGK antibodies.

Yeast-Two-Hybrid – Two-hybrid analysis was performed in PJ69-4A strain according to the method of James et al (38). Full-length human BRAP2 was cloned between the EcoRI and BamHI sites (RHCP11 and RHCP13 primers, Table 2) of the "bait" pGBDUC1 plasmid encoding the *GAL4* DNA binding domain (38), generating the pGBDUC1-BRAP2 plasmid (Table 1). The pGBDUC1-BRAP2 plasmid was cotransformed with "prey" Human Testis Matchmaker cDNA library fused with *GAL4 activation domain* in pACT2 plasmid (Clontech) generously provided by Tamara Caspary. His^+ colonies were patched and replica plated onto medium containing 1 mM 3-aminotriazole (3-AT) and separately onto SD-Ade-His-Leu-Ura to identify strong interactors. Plasmids rescued from Ade^+ plates were sent for sequencing (Macrogen) to identify the insert. Subsequently, full-length yeast ETP1 was cloned into the pGBDUC1 generating pGBDUC1-ETP1 (Table 1). Binding of ETP1 to ubiquitin was confirmed by cotransforming pGBDUC1-ETP1 and pACT2-Ub (one of the interactors from the two-hybrid analysis of BRAP2 binding partners described above) in PJ69-4A strain. His^+ colonies were patched and replica plated onto SD-Ade-His-Leu-Ura to confirm a strong interaction.

Immunoblot Analysis of Ubiquitin Levels in Yeast Lysates – Cell cultures were grown to a stationary growth phase in liquid YPD media (32) at 30°C for 48 hours. Cultures were then diluted in YPD to $3x10^6$ cells/ml and were grown at 30°C for 4 hours

to mid-log. $3x10^7$ cells were spun down (4000 g, 10 min) and resuspended in SDS loading buffer, boiled for 10 min. Samples were subjected to SDS-PAGE and a Western blot analysis with anti-ubiquitin antibodies. Membrane was then stripped with OneMinute® Western Blot Stripping Buffer (GM Biosciences) and re-probed with anti-HA antibodies for the presence of ETP1-6xHA, and, subsequently, with an anti-PGK1 antibodies for a loading control.

Measuring Total Ubiquitin Levels by HPLC – YSC1049 and YSC1021-547582 yeast cell cultures were grown to mid-log phase in 50 ml YPD (32). $3x10^7$ of cells were collected for an immunoblot analysis. After centrifugation (4000 g, 10 min), $1x10^9$ cells were washed with dH_2O and then resuspended in 400 µl of a lysis buffer without protease and DUB inhibitors (50 mM Tris, pH 7.5, 150 mM NaCl). After lysing the cells using glass beads and a bead beater (five periods of disruption, 1 min each, with cooling on ice for 1 min in between of treatments), the whole cell lysate was spun down (12000 g, 10 min) and 300 µl of a supernatant was collected. To disassemble all ubiquitin chains in a lysate to monoubiquitin, the supernatant was incubated with 1 µM $Usp2_{CD}$ (Catalytic domain of Usp2, Boston Biochem) at 37°C for 90 min. The resulting monoubiquitin was further purified from a lysate as described previously (39). Briefly, lysate was incubated on ice with 5% by volume perchloric acid (PCA) for 10 min and was then spun down. The supernatant contained the purified monoubiquitin, which was analyzed by HPLC as described previously (40). Samples were collected for an immunoblot analysis with anti-ubiquitin antibody. Loading was analyzed by immunoblot analysis with anti-PGK antibodies. Experiment was done in triplicates.

Analysis of Free Ubiquitin Chains in Yeast Lysates - YSC1049 and YSC1021-547582 yeast cell cultures were grown to a mid-log phase for 4 hr at 30°C. $3x10^7$ cells were spun down (4000 g, 10 min), resuspended in 30 µl of SDS loading buffer, and boiled for 10 min for an immunoblot analysis. $7x10^8$ cell were spun down (4000 g, 10 min), washed in dH_2O, and resuspended in 400 µl of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 10 mM the deubiquitinating enzymes inhibitor *N*-Ethylmaleimide (NEM) (Sigma). After lysing the cells using glass beads and a bead beater (five periods of disruption, 1 min each, with cooling on ice for 1 min in between of treatments), the whole cell lysate was spun down (12000 g, 10min) and 300 µl of a supernatant was collected. To inactivate, NEM, the supernatant was incubated with *β*-mercaptoethanol (25 mM final concentration) for 30 min on ice. To cleave unconjugated ubiquitin chains exclusively, supernatant was incubated with purified deubiquitinating enzyme IsoT (20 µg/ml final concentration) at 30°C for 90 min (23). Proteins were separated on SDS-PAGE followed by a Western blot analysis with anti-ubiquitin and anti-PGK antibodies.

Yeast Media and Growth Conditions for Spot Assays – Cell cultures were grown to a stationary growth phase in liquid YPD media (32) at 30°C for 48 hours. Cultures were then diluted in YPD to $3x10^6$ cells/ml and were grown to a mid-log at 30°C for 4 hours. Cells were washed with dH_2O and were normalized to $1x10^7$ cells/ml. For paraquat, canavanine, and anisomycin phenotypic assays, 2 µl of 5-fold serial dilutions of cell cultures were spotted on YPD or SDM plates (32) (with necessary amino acid supplements) containing one of the following reagents: 1 mM, 1.5 mM, or 2 mM

paraquat (Sigma); 0.8 μg/ml, 1 μg/ml, or 1.2 μg/ml canavanine sulfate (Sigma); 15 μg/ml, 20 μg/ml, or 30 μg/ml anisomycin (Sigma). For hydrogen peroxide phenotypic assay, hydrogen peroxide (Sigma) was added to $1x10^7$ cells/ml cells to a final concentration of 15 mM, 20 mM, and 25 mM. Cells were then incubated for 30 min at 30°C on a shaker. 2 μl of 5-fold serial dilutions of cell cultures were spotted on YPD (32). All plates were incubated at 30°C for two days.

Table 1

Plasmids used in this study

Name	Description	Insertion Site	Source
ZD-9	TA::MX4-natR		(36)
ZD-54[a)]	pET28a(+)-HAT, Kan^+	NcoI/SacI	This study
ZD-52 [a)]	pET28a(+)-HAT-ETP1, Kan^+	SacI/NotI	This study
ZD-57 [a)]	pET28a(+)-HAT-ETP1 C240A/C243A, Kan^+	SacI/NotI	This study
ZD-59 [a)]	pET28a(+)-HAT-ETP1 W311A/R323A, Kan	SacI/NotI	This study
ZD-61 [a)]	pET28a(+)-HAT-ETP1 C240A/C243A/W311A/R323A, Kan^+	SacI/NotI	This study
ZD-53 [a)]	pET28a(+)-HAT-BRAP2, Kan^+	HindIII/NotI	This study
ZD-65	pET15b-Ubc1, Amp^+	BamHI/HindIII	(41)
ZD-66	pET15b-Ubc3, Amp^+	BamHI/HindIII	(41)
ZD-67	pET15b-Ubc4, Amp^+	BamHI/HindIII	(41)
ZD-68	pET15b-Ubc7, Amp^+	BamHI/HindIII	(41)
ZD-69	pET15b-Ubc8, Amp^+	BamHI/HindIII	(41)
ZD-70	pET15b-Ubc10, Amp^+	BamHI/HindIII	(41)
ZD-71	pET15b-Ubc11, Amp^+	BamHI/HindIII	(41)
ZD-72	pET15b-Ubc13, Amp^+	BamHI/HindIII	(41)
ZD-73	pGSHU, Ura^+, Amp^+		(35)

ZD-21	pYM17, NAT^+, Amp^+		(34)
ZD-74	pGBDUC1-BRAP2, Ura^+, Amp^+	EcoRI/BamHI	This study
ZD-75 [a)]	pGBDUC1-ETP1	PstI/BglII	This study

[a)] These plasmids were made by Custom Cloning Core Facility. Details are available upon request.

Table 2

Oligonucleotides used in this study

Name	Sequence
ZAP108	5′-GCACAGGCATCCAAAAGCAAGAAGAAGCGCAACAAAAATAAAAAAGCAGGGAAACGTACGCTGCAGGTCGAC-3′
ZAP109	5′-GAGATATAATAAATTTAGAATGCAAGAATGGTATGGCGGTGATGAAGATAATCAATCGATGAATTCGAGCTCG-3′
ZAP118	5′-GACTGAGTGTGCACGCTTCCAAAGTTTTTTTTACTATTTGATACATGCTTAAGTTACATGGAGGCCCAGAATACCC-3′
ZAP119	5′-TTGAACGGGAAAAAAAGTGTATAGACAACGGTTTTCAGTTATTTATTCAAATGAACAGTATAGCGACCAGCATTCAC-3′
ZAP147	5′-ATGTTGTCATACCCATGGACCTGC-3′
ZAP148	5′-AAAGGAAACCTCACCGGTTGGA-3′
ZAP151	5′-GGATTCAGAAACTACTGGATTAGTAACGATCCCGTGCCAGCACACTTTTCTTCGTACGCTGCAGGTCGAC-3′

ZAP152	5′- TCGACACACTGGACACCTTGAATTCTTCCACTTATTTAAGCAT TGGCAATTAGGGATAACAGGGTAATCCGCGCGTTGGCCGATT CAT-3′
ZAP155	5′- CCAAAATCAGGTTGAATCCGC-3′
ZAP156	5′- TTGTTGCGCTTCTTCTTGCT-3′
RHCP11	5′- GACGAATTCGACATGAGTGTGTCACTGGTTGTTATC-3′
RHCP13	5′- GACGGATCCGACTCACTTGCCCCTCTTGCTGCGGC-3′

Table 3

***S. cerevisiae* strains used in this study**

Strain	Relevant genotype	Source
YSC1049[a)]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Open Biosystems
YSC1021-547582[b)]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 etp1Δ::kanMX4*	Open Biosystems
ZAY1[b)]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 ETP1-6HA-NAT*	This study
ZAY2[b)]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 etp1Δ::ETP1-6HA-NAT*	This study
ZAY3[b)]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 etp1::*CORE-I-*Sce*I-*hyg*	This study
ZAY4[b)]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 etp1::C240A/C243A-6HA-NAT*	This study
ZAY5[b)]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 etp1::W311A/R323A -6HA-NAT*	This study
ZAY6[b)]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 etp1::C240A/C243A/W311A/R323A -6HA-NAT*	This study
ZAY7 [b)]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 doa4Δ::natR*	This study

ZAY8 [b)]	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 doa4Δ::natR etp1Δ::kanMX4*	This study
PJ69-4A	*MAT*a *trp1-901 leu2-3*, *112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2*::*GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ*	(38)

[a)] A wild type BY4742 strain.

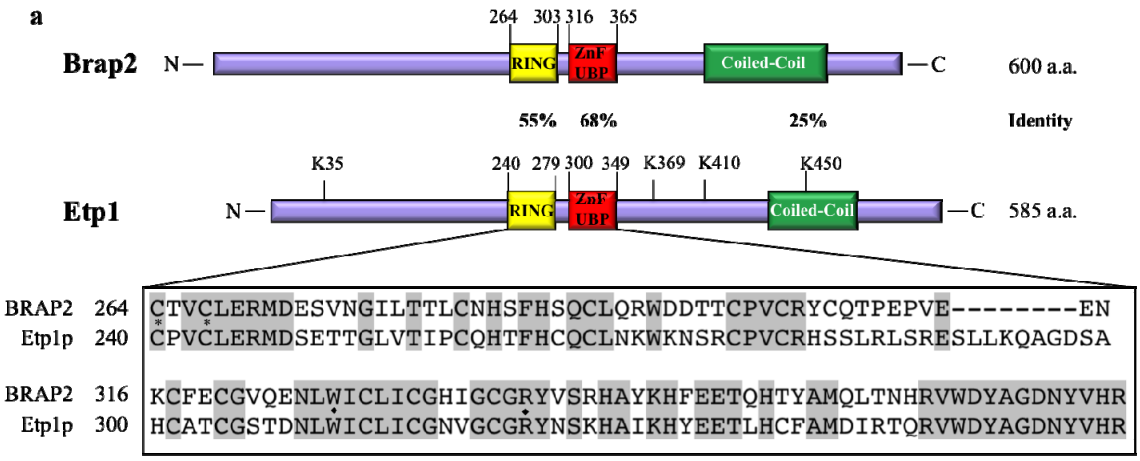
[b)] These strains are isogenic derivatives of YSC1049.

Results

Mutations to prove Etp1 domain functions

Based on the alignment of the primary amino acid sequences, ETP1 and BRAP2 are 40% identical with 55% identity (70% homology) between the RING domains and with 68% identity (75% homology) between the ZnF UBP domains (Figure 1a). To assess the function of these domains, we created the following mutants of Etp1: the RING domain mutant (*RING**), the ZnF UBP domain mutant (*ZnF**), and the double mutant (*RING*/ZnF **).

Cysteines 240 and 243 to alanines point mutation of Etp1 were made to disrupt the activity of the RING domain in analogy to the BRAP2 RING domain mutant (26). The ZnF UBP domain binds the C-terminal diglycine motif of ubiquitin (23). We mutated two residues in the ZnF UBP domain that come into a close contact with ubiquitin, tryptophan (W311A) and arginine (R323A), to abrogate the binding of Etp1 to ubiquitin. Also, the double mutant was made (C240A/C243A/W311A/R323A) to abrogate both the activity of the RING and the ZnF UBP domains.


a
Brap2
N—
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RING
ZnF UBP
Coiled-Coil
—C
600 a.a.
55% 68% 25%
Identity
Etp1
K35
240 279 300 349 K369 K410 K450
RING
ZnF UBP
Coiled-Coil
—C
585 a.a.
BRAP2 264 CTVCLERMDESVNGILTTLCNHSFHSQCLQRWDDTTCPVCRYCQTPEPVE--------EN
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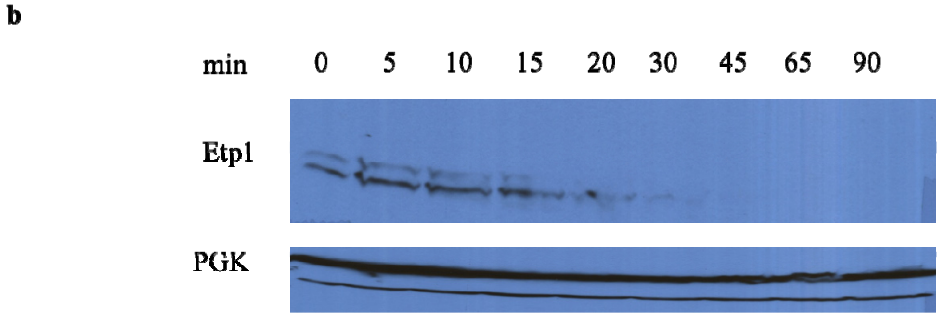

b
min 0 5 10 15 20 30 45 65 90
Etp1
PGK

Figure 1. Etp1 is a short-lived homologue of BRAP2.

(a) *S. cereviciae* Etp1 is an orthologue of human BRAP2 that shares the same domain structure with the most homology in the RING and ZnF UPB domains. Sequence alignment of RING and ZnF UBP domains of BRAP2 and Etp1 is shown in a zoom in box. Conserved residues are shaded gray. Alignments were done using the CLUSTALW algorithm. The residues that were mutated in Etp1 to disrupt the activity of the RING domain are indicated by asterisks. The residues that were mutated to disrupt binding of Etp1 with ubiquitin are shown with rhombi. Lysine residues 35, 369, and 450 of Etp1 were shown to be modified by ubiquitination *in vitro* (this study), while K369 and 410 of Etp1 are ubiquitinated *in vivo* (42). (b) Half-live of Etp1 was measured in a cycloheximide chase experiment. After the inhibition of translation by cycloheximide, the lysate was analyzed for the presence of Etp1-HA_6 at the indicated time points with anti-HA antibodies. PGK levels in these lysates were used as a loading control. Density of the bands were analyzed using LabWorks Software.

Etp1 is a short-lived protein

The fundamental way by which cell regulates its growth and developments is by controlling the amount of cellular proteins. Proteins are constantly synthesized and degraded, a process called turnover. Turnover rate of a particular protein often correlates with a role it plays in cellular processes. Depending on the function of the protein, the half-life of a protein can range form less then a minute to many days. In *S. cerevisiae*, the median half-life of cellular proteins is ~ 43 minutes (43). Accessing the half-life of a given protein is often a first step in understanding the role of this protein in a cell. For example, proteins that are required to maintain the cell structure usually have a long half-life of several days, while enzymes regulating the rate of metabolic pathways have half-lives of a few minutes (44).

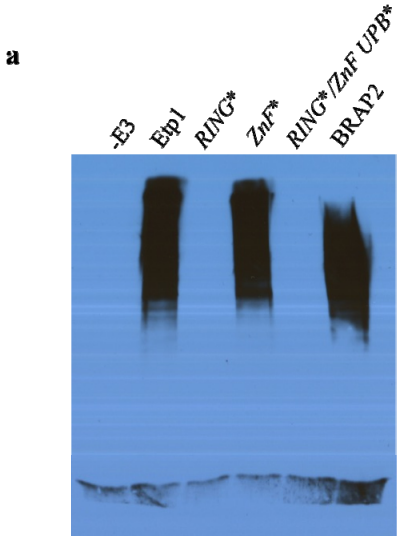
To examine half-life of Etp1, we created a yeast strain in which Etp1 is C-terminally tagged with six HA tags, Etp1-HA_6 and expressed from an endogenous promoter. As shown on Figure 1b, cells showed a progressing decline of levels of Etp1-HA_6 after 15 minutes of blocking protein synthesis with cycloheximide. After measuring the density of the bands of three western blots, the half-life of the Etp1 found to be ~ 17 minutes. This high turnover rate suggests tight regulation of the Etp1 protein levels in yeast. Interestingly, the Etp1 protein runs as a double band (Figure 1b). This is consistent with our earlier observation of TAP-tagged Etp1 (data not shown). The upper band of Etp1 could potentially indicate post-translational modification, for example, monoubiquitination.

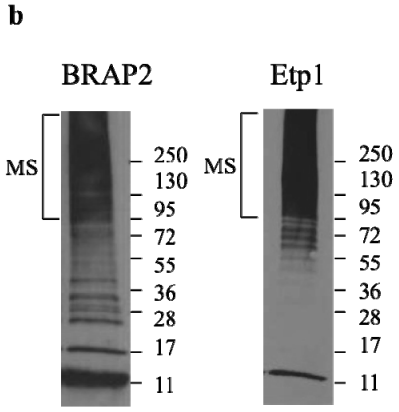
Etp1 is an E3 ubiquitin ligase

BRAP2 has been shown to autoubiquitinate *in vivo*, and this process is dependent on the RING domain E3 ubiquitin ligase activity of BRAP2 (26, 42). Most ligases ubiquitinate themselves (autoubiquitination) when the substrate is not present in the reaction (45-48). We investigated whether the RING domain of Etp1 exhibits ubiquitin ligase activity *in vitro* (Figure 2a). Human UbcH5b was used as the E2 because it was a preferred E2 for BRAP2 (A. Weissman, unpublished). When wild type Etp1 was used an E3, ubiquitin chains of high molecular weight were catalyzed in the reaction, demonstrating the E3 ubiquitin ligase activity of Etp1. Reaction with BRAP2 protein was used as a positive control (Figure 2a, lane 6), and reaction without an E3 as a negative control (Figure 2a, lane 1). In analogy to BRAP2, C240A/C243A mutation of the RING domain (*RING** and *RING*/ZnF*)* disrupted this activity of Etp1, indicating that RING domain of Etp1 is required for the E3 ubiquitin ligase activity. W311A/R323A mutation of ZnF UBP domain (*ZnF**) did not disrupt the ligase activity of Etp1 meaning that ubiquitin-binding site is not needed for ligase activity.

Polyubiquitin synthesis

Depending on the E2-E3 pair, ubiquitin chains of various topologies can be catalyzed in the ubiquitination reaction. We investigated the abundance and the topology of ubiquitin chains catalyzed by both BRAP2 and Etp1 *in vitro* utilizing the human UbcH5b as an E2 conjugating enzyme (Figure 2b). Mass spectrometry analysis





Spectral counts

Ub chains	BRAP2	Etp1
K6	3	26
K11	4	24
K27	-	-
K29	-	-
K33	-	72
K48	19	65
K63	-	35

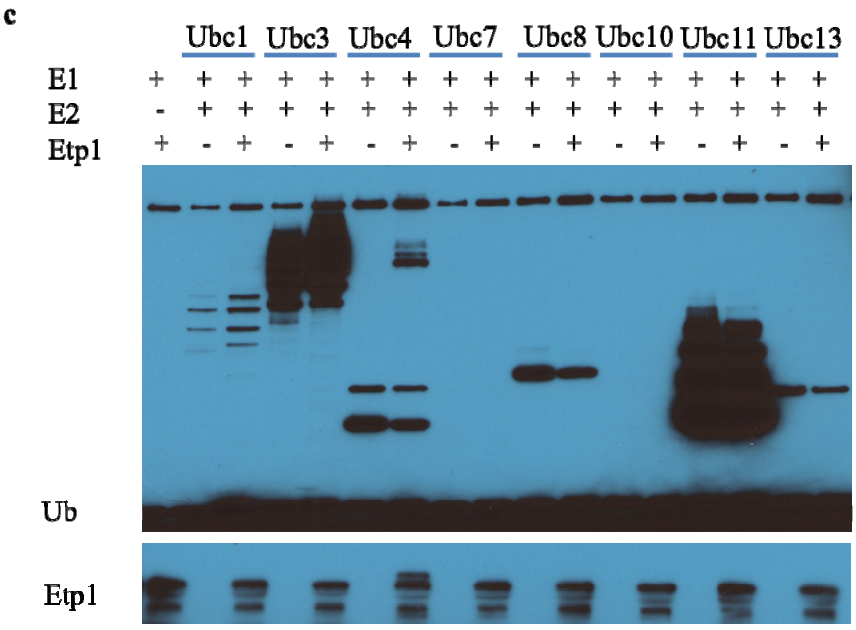


Figure 2. Etp1 is an E3 ubiquitin ligase.

(a) Etp1 autoubiquitinates *in vitro*. Human E1, human E2 (UbcH5b) and bacterial lysates expressing the depicted E3s (lanes 2-5) were mixed with human ubiquitin in the presence of ATP. The ubiquitin chain formation was detected with anti-Ub antibody. For a control, no E3 was included in the reaction (lane 1). (b) Mass spectrometry (MS) analysis of ubiquitin chains catalyzed either by human BRAP2 or yeast ETP1 *in vitro*. Human E1, human E2 (UbcH5b) and purified BRAP2 or Etp1 were incubated with human ubiquitin in the presence of ATP. Reactions were separated by SDS-PAGE, and the regions of the gels depicted by brackets were cut out and analyzed by MS to determine the topology of the Ub chains in the reactions. Specific Ub linkages found in each reaction are shown in a table; numbers represent the spectral counts for each chain type. (c) Ubc4 is the E2 relevant for Etp1. Eight purified yeast E2s were screened in the *in vitro* ubiquitination assay for Etp1. Yeast E1, yeast E2s, and yeast ubiquitin were incubated with or without Etp1 (controls) in the presence of ATP. A reaction with no E2 was also used as a control. Ubiquitin chain formation was analyzed by anti-Ub Ab. Membrane was stripped and probed with anti-HAT Ab to detect mono-ubiquitinated Etp1.

revealed that both BRAP2 and Etp1 enzymes catalyzed K6, K11, and K48 ubiquitin chains. In addition to that, Etp1 catalyzed K33 and K63 linkages. Each ubiquitin chain abundance was reflected by its spectral count, which is a tandem mass spectra of peptides eluted into the mass spectrometer (49-51). K33 and K48 chains were the most abundant in the reaction with Etp1 and represented 32% and 29% of total chains in the reaction, respectively (Figure 2b). Interestingly, we also detected free ubiquitin chains in the reaction with BRAP2, but not with Etp1, where all chains detected were conjugated to Etp1.

Etp1 is ubiquitinated *in vivo* at K369 and K410 sites (42). From the mass spectrometry analysis of in vitro ubiquitination reaction, we have identified the K35, K369, and K450 as residues of Etp1 modified by ubiquitin. Figure 1a depicts the position of each of these lysines with respect to the domain structure of Etp1.

E2 conjugating enzymes

There are thirteen known yeast E2s, Ubc1-13. However, Ubc9 and Ubc12 are E2 enzymes for SUMO and Nedd8, respectively, rather then ubiquitin (52). To determine the corresponding yeast E2 conjugating enzyme for Etp1, we have purified eight yeast E2s: Ubc1, Ubc3, Ubc4, Ubc7, Ubc8, Ubc10, Ubc11, and Ubc13. Each E2 was added to the ubiquitination reaction containing yeast E1, Etp1, and yeast ubiquitin (Figure 2c). Insofar as Ubc1, 3, 4, 8, 11 and 13 show E3 independent autoubiquitination, reactions without Etp1 were included as a control. Only addition of Ubc4 led to autoubiquitination of Etp1, indicating that, of those tested, Ubc4 is the most specific E2 for Etp1. Even though we

could not obtain Ubc5, the yeast homologue of UbcH5b, Ubc4 is 92% identical to Ubc5, and both proteins have a complementary function (53). However, we can not exclude the possibility of Ubc5 being a *bona fide* E2 for Etp1.

Etp1 binds ubiquitin

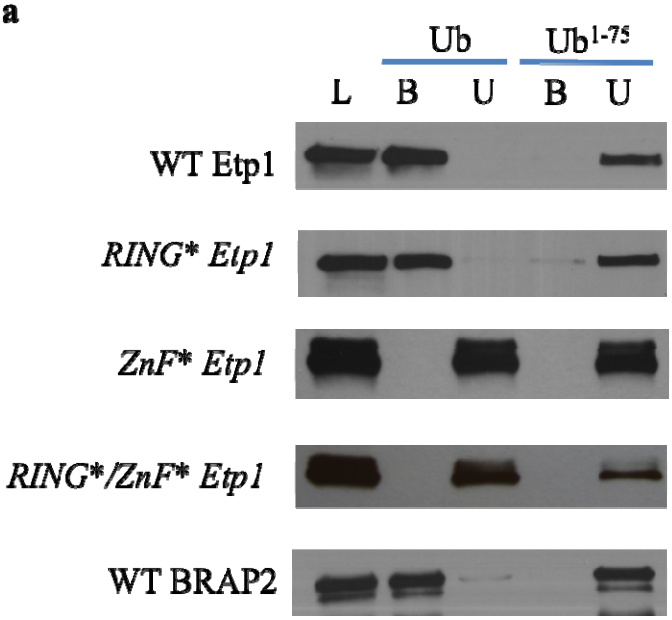
The ZnF UBP domain of Etp1 binds ubiquitin *in vitro* in a manner requiring the C-terminal glycine of ubiquitin (23). To test whether full length Etp1 binds ubiquitin, we utilized a binding assay using either wild type ubiquitin resin or ubiquitin 1-75 (Ub^{1-75}) resin as a control. As shown in Figure 3a, Etp1 was bound to the wild-type ubiquitin resin but not to the truncated ubiquitin resin. The RING domain mutant of Etp1 (RING*) also binds ubiquitin, which indicates the correct folding of this mutant. Etp1 proteins with a mutant ZnF UBP domain (UBP* and RING*/UBP*) failed to bind ubiquitin resin, demonstrating that the residues chosen for a mutation, indeed, abrogate the binding of Etp1 to ubiquitin.

Ubiquitin was also one of the strongest hits in the yeast-two-hybrid analysis using BRAP2 as bait (see Chapter 3). We have confirmed that Etp1 also binds ubiquitin in the yeast-two-hybrid assay (Figure 3b).

Etp1 regulates ubiquitin homeostasis

Because of the presence of the unique ZnF UBP domain in its structure, the hypothesized function of Etp1 is to be a sensor of the levels of unconjugated ubiquitin

a


Ub
Ub1-75
L
B
U
B
U
WT Etp1
RING* Etp1
ZnF* Etp1
RING*/ZnF* Etp1
WT BRAP2

b

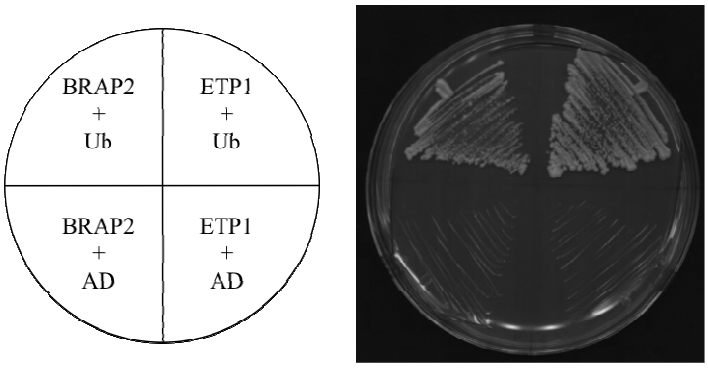

BRAP2
+
Ub
ETP1
+
Ub
BRAP2
+
AD
ETP1
+
AD

Figure 3. Etp1 binds ubiquitin.

(a) Both human BRAP2 and yeast ETP1 bind ubiquitin *in vitro* and require the C-terminal glycine of ubiquitin for binding. Mutations in the ZnF UBP domain (*ZnF** and *RING*/ZnF**), but not the RING domain (*RING**) abrogate the binding to ubiquitin (rows 3 and 4). Purified E3s were incubated with Ub-Sepharose resin or Ub^{1-75}-Sepharose resin as a control. Load (L), unbound (U), and bound (B) fractions were analyzed for the presence of E3s by anti-HAT Ab. (b) Both human BRAP2 and yeast Etp1 associate with ubiquitin, but not with the Activation Domain alone (AD) as shown by the yeast-two-hybrid assay.

(23, 54, 55). Therefore, we analyzed the levels of total ubiquitin in the wild type and the *etp1Δ* strains. Loss of ETP1 (*etp1Δ::kanMX4*) causes a decrease of polyubiquitin in whole cell lysates (Figure 4a, *etp1Δ* lane). Replacing the kanamycin cassette in the *etp1Δ* strain with ETP1-HA_6 complemented this phenotype (Figure 4a, *etp1Δ::ETP1* lane). Interestingly, this phenotype was not complement by either overexpression or underexpression of the plasmid-born Etp1 (data not shown). This suggests, that the cell needs a precise level of Etp1 for execution of its function.

To examine the role of the RING and the ZnF UBP domains in the function of Etp1, we have created a series of yeast strains in which either the RING domain, or the ZnF UBP domain, or both domains were mutated (*RING**, *UBP**, and *RING*/UBP**, respectively) replacing the endogenous chromosomal copy. All mutant proteins showed the same levels of expression as wild type Etp1 (Figure 4a). We then examined the ubiquitin levels in these strains. Mutation of either the RING domain, the ZnF UBP domain, or both caused a decrease of free ubiquitin chains (Figure 4a). The levels of di-, tri-, and tetraubiquitin decreased by 40%, 60%, and 40%, respectively, in these strains as measured by the intensity of ubiquitin immunostaining. To specifically identify the bands that correspond to the free ubiquitin chains, lysate from wild type and *etp1Δ* strains were treated with IsoT (56), a deubiquitinating enzyme that exclusively cleaves unconjugated ubiquitin chains. Prominent bands at 10, 17, and 24 kDa were abolished by IsoT treatment (Figure 4b, bands shown by asterisks).

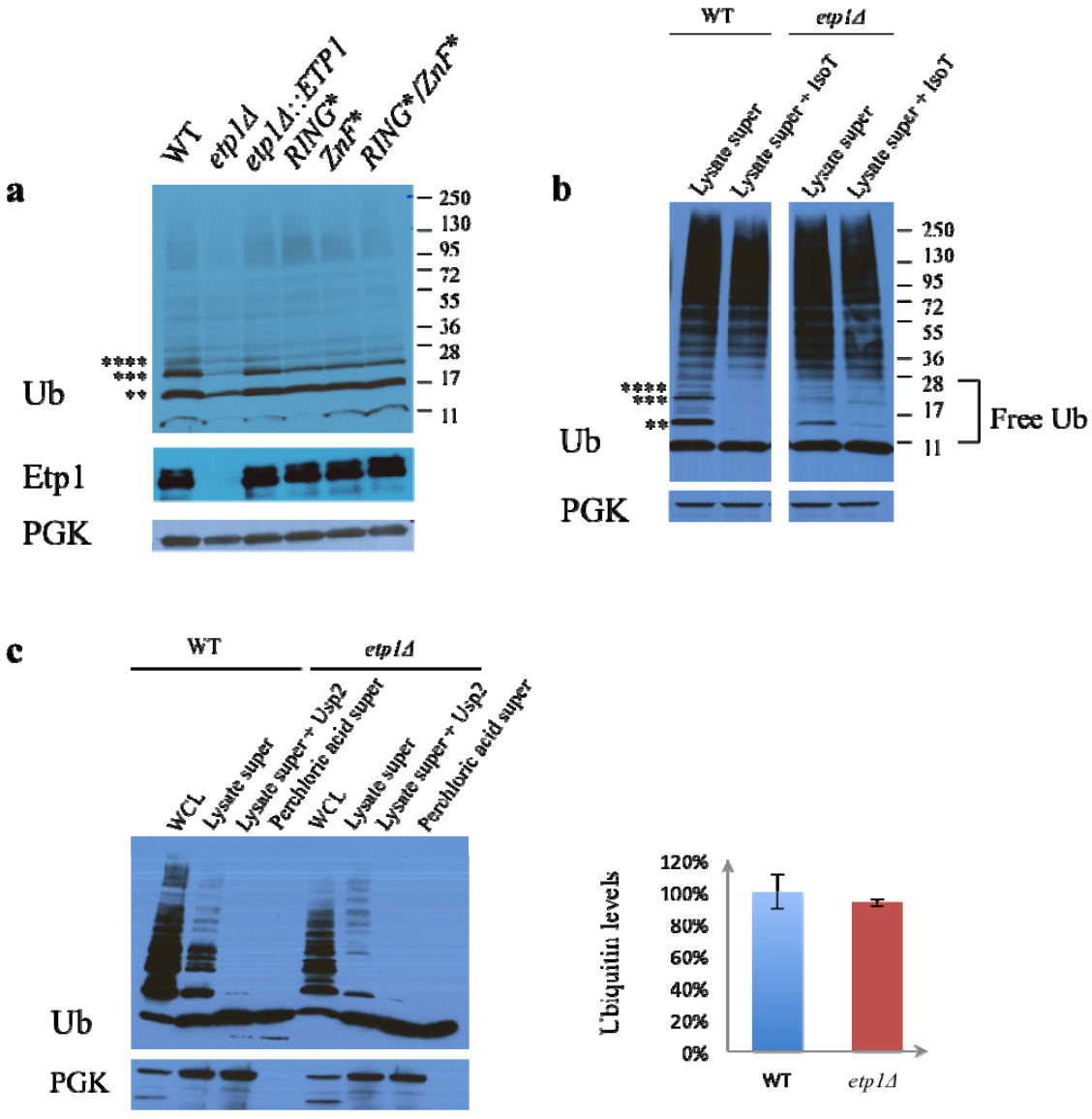

a
WT
etp1Δ
etp1Δ::ETP1
RING*
ZnF*
RING*/ZnF*
250
130
95
72
55
36
28
17
11
****
***
**
Ub
Etp1
PGK
b
WT
etp1Δ
Lysate super
Lysate super + IsoT
Lysate super
Lysate super + IsoT
250
130
95
72
55
36
28
17
11
Free Ub
****
***
**
Ub
PGK
c
WT
etp1Δ
WCL
Lysate super
Lysate super + Usp2
Perchloric acid super
WCL
Lysate super
Lysate super + Usp2
Perchloric acid super
Ub
PGK
Ubiquitin levels
120%
100%
80%
60%
40%
20%
0%
WT
etp1Δ

Figure 4. Levels of free ubiquitin chains are decreased in *etp1Δ* strain.

(a) Loss of ETP1 leads to a decrease of unconjugated ubiquitin chains. This phenotype is rescued by introduction of ETP1 into the *etp1Δ* genome. Both the RING and the ZnF UBP domains are required for Etp1 to control the levels of free Ub chains, as the levels of di-, tri-, and tetraubiquitin linkages (shown by asterisks) are decreased in the strains expressing mutant Etp1 proteins. Yeast whole cell lysates (WCL) expressing the indicated ETP1 variants from the endogenous promoter were analyzed for the distribution of the ubiquitin with anti-Ub Ab. The membrane was then stripped and probed with anti-HA Ab to compare the expression of Etp1 mutants to WT Etp1. PGK levels were used as a loading control. (b) To define the region of the gel that corresponds to free Ub chains, cell lysates were treated with IsoT, a DUB that specifically cleaves unconjugated Ub chains. Lysate proteins were separated by SDS-PAGE, and Ub was detected by anti-Ub Ab. Region of a gel corresponding to unanchored Ub chains is shown by brackets. PGK levels were used as a loading control. (c) Total Ub levels are the same in both WT and *etp1Δ* strains as measured by HPLC. Lysate supernatants were first treated with $Usp2_{CD}$ to cleave all ubiquitin chains to monoubiquitin, and then with perchloric acid to purify the monoubiquitin. Samples were analyzed by the HPLC for the amount of the monoubiquitin. Results are represented graphically as a percentage of the WT ubiquitin mean (mean $\pm$ SD, n = 6).

The anti-Ub antibody used does not detect free ubiquitin as effectively as polyubiquitin. To investigate whether the total levels of ubiquitin differ in the wild type and the *etp1Δ* strains, we have compared the amounts of ubiquitin purified from lysates of both yeast strains (Figure 4c). Importantly, all polyubiquitin was first disassembled to monoubiquitin by the catalytic domain of Usp2 ($Usp2_{CD}$) deubiquitinating enzyme, which nonspecifically cleaves all polyubiquitin to monoubiquitin (57). The levels of total monoubiquitin were then measured by HPLC. Remarkably, the levels of ubiquitin in wild type and *etp1Δ* were virtually the same (Figure 4c, graph), suggesting ETP1 deletion causes redistribution of the ubiquitin pool in these strains rather than changes in total ubiquitin levels.

Loss of ETP1 renders yeast resistant to various stresses

Mutation of the *C. elegance* homologue of BRAP2, *brap-2*, results in the high sensitivity of worms to the oxidative stress-inducing drug paraquat and to hydrogen peroxide (H_2O_2) (58). Surprisingly, loss of ETP1 leads to resistance to both paraquat and H_2O_2 (Figures 5a and 5b). When treated with 5 mM H_2O_2, the percent survival of wild type and *etp1Δ* strains were 20% and 70%, respectively. At 25 mM H_2O_2, survival was are 0% and 8%, respectively (Figure 5b, graph). Interestingly, yeast expressing mutant Etp1 either with impaired RING, or ZnF UBP, or both domains are more sensitive to oxidative damage then the wild type strain, indicating the negative gain of function of Etp1 mutations (Figure 5b).

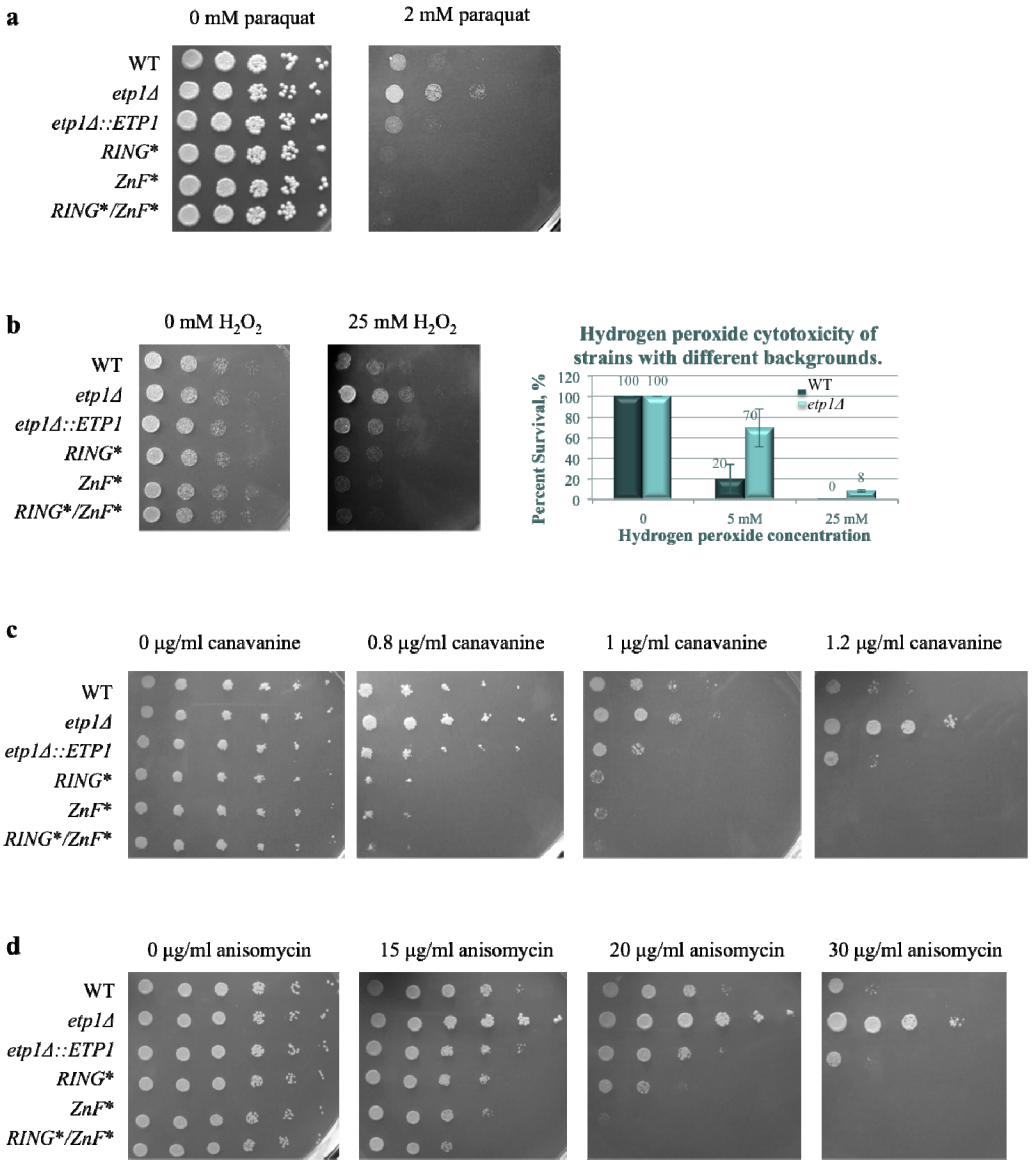

a
0 mM paraquat
2 mM paraquat
WT
etp1Δ
etp1Δ::ETP1
RING*
ZnF*
RING*/ZnF*
b
0 mM H2O2
25 mM H2O2
WT
etp1Δ
etp1Δ::ETP1
RING*
ZnF*
RING*/ZnF*
Hydrogen peroxide cytotoxicity of strains with different backgrounds.
Percent Survival, %
120
100
80
60
40
20
0
100 100
20
70
0
8
WT
etp1Δ
0
5 mM
25 mM
Hydrogen peroxide concentration
c
0 µg/ml canavanine
0.8 µg/ml canavanine
1 µg/ml canavanine
1.2 µg/ml canavanine
WT
etp1Δ
etp1Δ::ETP1
RING*
ZnF*
RING*/ZnF*
d
0 µg/ml anisomycin
15 µg/ml anisomycin
20 µg/ml anisomycin
30 µg/ml anisomycin
WT
etp1Δ
etp1Δ::ETP1
RING*
ZnF*
RING*/ZnF*

Figure 5. Phenotypes of *etp1Δ*.

Serial dilutions of the depicted strains were spotted one the media containing the following reagents: paraquat (a), canavanine (c), and anisomycin (d). In the hydrogen peroxide assay (b), cells were incubated with hydrogen peroxide for 30 min and serial dilutions of cell cultures were spotted on YPD. Graph represents the hydrogen peroxide cytotoxicity of WT and *etp1Δ* strains (mean $\pm$ SD, n = 6). Growth on YPD (a), (b) or on SDM (c), (d) is shown for a loading control.

Often defects of the ubiquitin-proteasomal system can be revealed by tolerance to such stresses as misfolding of proteins synthesized in the presence of canavanine and inhibition of translation by anisomycin. Deletion of the ETP1 gene leads to high tolerance of yeast cells to treatment with canavanine and anisomycin (Figures 5c and 5d, respectively). Inability of Etp1 to catalyze the ubiquitin chains (*RING** mutant) or bind ubiquitin (*ZnF** mutant) renders cells more sensitive to canavanine and anisomycin with an additive affect of both mutations (*RING*/ZnF**). A ZnF UBP mutant of Etp1 (*ZnF**) is more sensitive to treatment with anisomycin then the RING domain mutant (*RING**), indicating the significance of binding to ubiquitin to Etp1 function.

DOA4 **has an epistatic genetic relationship with** ***ETP1***

If Etp1 is regulating polyubiquitin levels it should show a genetic interaction with other genes regulating polyubiquitin. The Doa4 deubiquitinating enzyme has been shown to regulate ubiquitin homeostasis by mediating the cleavage of free ubiquitin chains in response to stress (59). To assess the genetic linkage between the *DOA4* and *ETP1*, we compared the phenotypes of *doa4Δ* and *etp1Δ* single and double deletion mutant strains. Loss of DOA4 causes an accumulation of free ubiquitin chains in yeast ((56) and Figure 6a, *doa4Δ* lane), deletion of ETP1 results in a reduction of free ubiquitin chains (Figure 6a, *etp1Δ* lane), while the double knock out shows an additive effect of *doa4Δ* and *etp1Δ* on di- and triubiquitin chains (Figure 6a, *doa4Δept1Δ* lane). By comparing the ubiquitin chains from the *doa4Δetp1Δ* with a ladder of $K48^{2-7}$ and $K63^{2-7}$, we have identified the triubiquitin species affected by these mutations as K63 linkages (Figure 6b).

The *doa4Δ* yeast is sensitive to elevated temperatures and oxidative stress with paraquat; and the temperature sensitivity phenotype is complemented by overexpression of ubiquitin (60, 61). We have confirmed the *doa4Δ* sensitivity to heat shock and also discovered *doa4Δ* sensitivity to hydrogen peroxide (Figure 6c, *doa4Δ* row). The *etp1Δ* yeast is not sensitive to elevated temperatures but is resistant to hydrogen peroxide (Figure 6c, *etp1Δ* row). We found that *doa4Δetp1Δ* is more resistant to elevated temperatures then *doa4Δ*, and is resistant to hydrogen peroxide similar to *etp1Δ* (Figure 6c, *doa4Δetp1Δ* row). That is, phenotypes of *doa4Δ* are suppressed by the loss of ETP1, suggesting that Doa4 and Etp1 have physiological roles in the same cellular pathway(s).

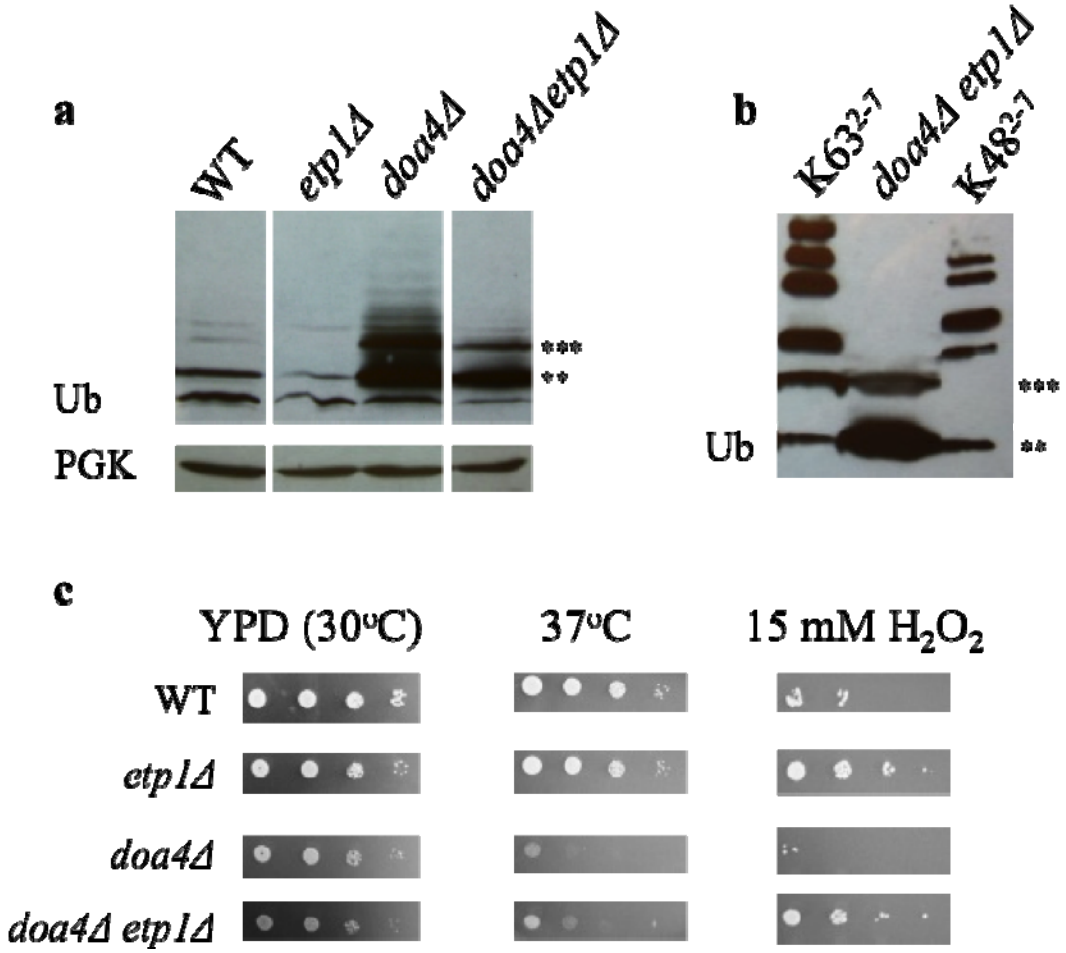

a
WT
etp1Δ
doa4Δ
doa4Δetp1Δ
***
**
Ub
PGK
b
K63^2-7
doa4Δ etp1Δ
K48^2-7
***
**
Ub
c
YPD (30°C)
37°C
15 mM H2O2
WT
etp1Δ
doa4Δ
doa4Δ etp1Δ

Figure 6. ETP1 and DOA4 act upon the same pathway.

(a) Whole cell lysates of WT, *etp1Δ*, *doa4Δ*, and *etp1Δdoa4Δ* were analyzed for the presence of ubiquitin on 10-20% SDS-PAGE gel with anti-Ub Ab. Di- and triubiquitin chains are shown by asterisks. Levels of PGK was measured with anti-PGK Ab for a loading control. (b) Do determine the topology of the free ubiquitin chains in the *doa4Δetp1Δ*, yeast cell lysates along with the $K63^{2-7}$ and $K48^{2-7}$ linkages were separated on 15% SDS-PAGE and analyzed with anti-Ub Ab. Di- and triubiquitin chains are shown by asterisks. (c) Serial dilution of of WT, *etp1Δ*, *doa4Δ*, and *etp1Δdoa4Δ* were spotted on YPD and incubated at $30^{o}C$ or $37^{o}C$. In the hydrogen peroxide phenotypic assay, cells were incubated with 15 mM hydrogen peroxide for 30 min and serial dilutions of cell cultures were spotted on YPD.

Discussion

In this work, we have characterized the yeast Etp1 protein, its functional domains, and demonstrated the postulated role in the regulation of ubiquitin homeostasis. First, we have shown that RING and ZnF UBP domains of Etp1 are functionally important. That is, the activity of the RING domain is required for Etp1 autoubiquitination *in vitro* (Figure 2a) and the ZnF UBP domain is required for binding of Etp1 to ubiquitin (Figures 3a). Etp1 catalyzes the formation of K6, K11, K33, K48, and K63 in the reaction with human E2 UbcH5b (Figure 2b), with K33 and K48 chains being the most abundant. While classical K48-linked polyubiquitination targets the substrate for the proteasomal degradation (62), the role of K33 linkages is still not well understood. K33 linkages are relatively resistant to proteasomal degradation (17) and have been implicated in nonproteolytic regulation of cellular processes and response to stress (63-65).

We found that the human BRAP2 catalyzes the formation of free ubiquitin chains *in vitro* (Figure 2b). Emerging evidence suggest that unconjugated polyubiquitin has a signaling function (19). Unconjugated K63 polyubiquitin chains synthesized by TRAF6 RING E3 ligase and UbcH5C E2 conjugating enzyme can directly activate the TAK1 kinase complex by binding to the ubiquitin receptor TAB2 (20). In addition, unanchored K63 polyubiquitin chains can directly activate RIG-I protein, a signaling protein involved in the immune response to viral infection (21). Moreover, it is currently speculated that free polyubiquitin can potentially inhibit the proteasome or could be used for substrate ubiquitination en bloc as opposed to sequential addition of ubiquitin. Although we have not observed free polyubiquitin synthesis by Etp1, we can not exclude the possibility that

Etp1 can catalyze the synthesis of free ubiquitin chains. We have identified yeast E2 conjugating enzyme, Ubc4, which works as a matching E2 for Etp1 E3 ubiquitin ligase (Figure 2c). In our screen we used 8 out of 13 known yeast E2s: Ubc1, Ubc3, Ubc4, Ubc7, Ubc8, Ubc10, Ubc11, and Ubc13. While Ubc9 and Ubc12 are E2 enzymes for SUMO and Nedd8, respectively, rather then ubiquitin (52), Ubc2, Ubc5, and Ubc6 could potentially act as the matching E2s for Etp1. Also, some E3s, e.g. Cdc34-SCF, use separate E2s for initiation and elongation of ubiquitination (66, 67) or require another partner for the ligase activity *in vivo*, e.g. BRCA1/BARD1 complex (11). However, in our ubiquitination assays we only used one E2 in each reaction and did not add any Etp1 substrates, so the results should be interpreted with caution. Analysis of the topology of ubiquitin linkages catalyzed by Ubc4-Etp1 pair is underway.

We provide evidence that Etp1 is required for the formation of free ubiquitin chains *in vivo*. Loss of ETP1 causes the reduction of di-, tri-, and tetraubiquitin (Figure 4a). Not only the ligase activity of Etp1 but also binding to ubiquitin is important for the formation of free ubiquitin chains, as mutation of the RING and ZnF UBP domain show a similar decrease of the amount of the chains compared to wild-type (Figure 4a). Interestingly, the total ubiquitin levels do not change upon deletion of ETP1 (Figure 4c), indicating that there is a redistribution of cellular ubiquitin rather then perturbation of ubiquitin synthesis or degradation. We did not detect more unanchored ubiquitin chains upon Etp1 overexpression (data not shown), suggesting that its effect is not rate-limiting for polyubiquitin synthesis and other binding partners may be necessary to precisely regulate the balance of cellular mono- and poly-ubiquitin.

Even though ubiquitin is a highly abundant protein, it is not produced in excess. Rather, there is a dynamic equilibrium between three forms of cellular ubiquitin: monomeric ubiquitin, a substrate-conjugated mono- and polyubiquitin, and unanchored ubiquitin chains (Figure 7) (59). Mutations in *UBI* genes as well as in several DUBs cause reduction of ubiquitin levels and contribute to various cellular defects (reviewed in (68). Overexpression of ubiquitin also impairs cell growth and leads to sensitivity to several compounds (69). To prevent these undesirable effects, ubiquitin expression is tightly regulated by several control mechanisms: transcriptional regulation of ubiquitin-encoding genes, regulation by the change of proteasomal composition, and regulation by deubiquitinating enzymes (68).

One example of this dynamic homeostatic response is exposure to such stresses as elevated temperatures, starvation, and the amino acid analog canavanine (69). All are thought to result in accumulation of misfolded cellular proteins, which are quickly ubiquitinated and targeted for the proteasomal degradation. Free ubiquitin levels must be increased to accomplish this degradation. Deletion of ETP1 renders yeast resistant to oxidative stress and treatment with canavanine and anisomycin (Figure 5). When a cell is exposed to such stress condition, misfolded proteins are formed which need to be quickly ubiquitinated and degraded. In *etp1Δ* there is less polyubiquitin available for DUBs to produce monoubiquitin for further substrate conjugation or, alternitavely, for en bloc substrate ubiquitination. Therefore, there is a slower response to various stresses and, hence, resistance of *etp1Δ* to paraquat, hydrogen peroxide, canavanine, and anisomycin (Figure 5).

The exact molecular mechanism of action of Etp1p is currently not known and should be investigated further. Curiously, Etp1 is the only E3 ubiquitin ligase that contains the ZnF UBP domain. We provide evidence that both RING and ZnF UBP domain are essential for the Etp1 function. Abrogation of ligase activity and the ability to bind ubiquitin shows a gain of function (Figure 5). In the case of translational inhibition with anisomycin, the Etp1 binding to ubiquitin is even more critical for cell survival then having a ligase activity.

A recent study proposed that unanchored ubiquitin chains function as a ubiquitin reservoir (59). When cells are exposed to stress conditions, free ubiquitin chains are rapidly disassembled by DUBs to monoubiquitin, which can be then used for substrate conjugation (68). The deubiquitinating enzyme Doa4 and its inhibitor Rfu1 were associated with the rapid disassembly of free ubiquitin chains upon heat shock (59). We propose a model for the function of Etp1 where short-lived Etp1 (Figure 1b) mediates a formation of free ubiquitin chains (Figure 7). Under normal conditions, these chains may be used for signaling or simply remain as a reserve pool of ubiquitin, while under stress the free polyubiquitin linkages are cleaved by Doa4 to provide ubiquitin for a further substrate conjugation. Indeed, our findings showed DOA4 and ETP1 genetically interact, as there is an additive effect of deletion of DOA4 and ETP1 on the amount of di- and triubiquitin, and tolerance to elevated temperature and hydrogen peroxide (Figure 6).

Interestingly, loss of Rsp5, a HECT E3 ubiquitin ligase, also leads to reduction of overall ubiquitin pool, and the reduced level of ubiquitin synthesis is sustained in *rsp1* mutant upon heat shock (70). Rsp5 contains a noncovalent ubiquitin-binding site in its catalytic HECT domain, which is important for the ligase activity of Rsp5 (71). Rsp5 was

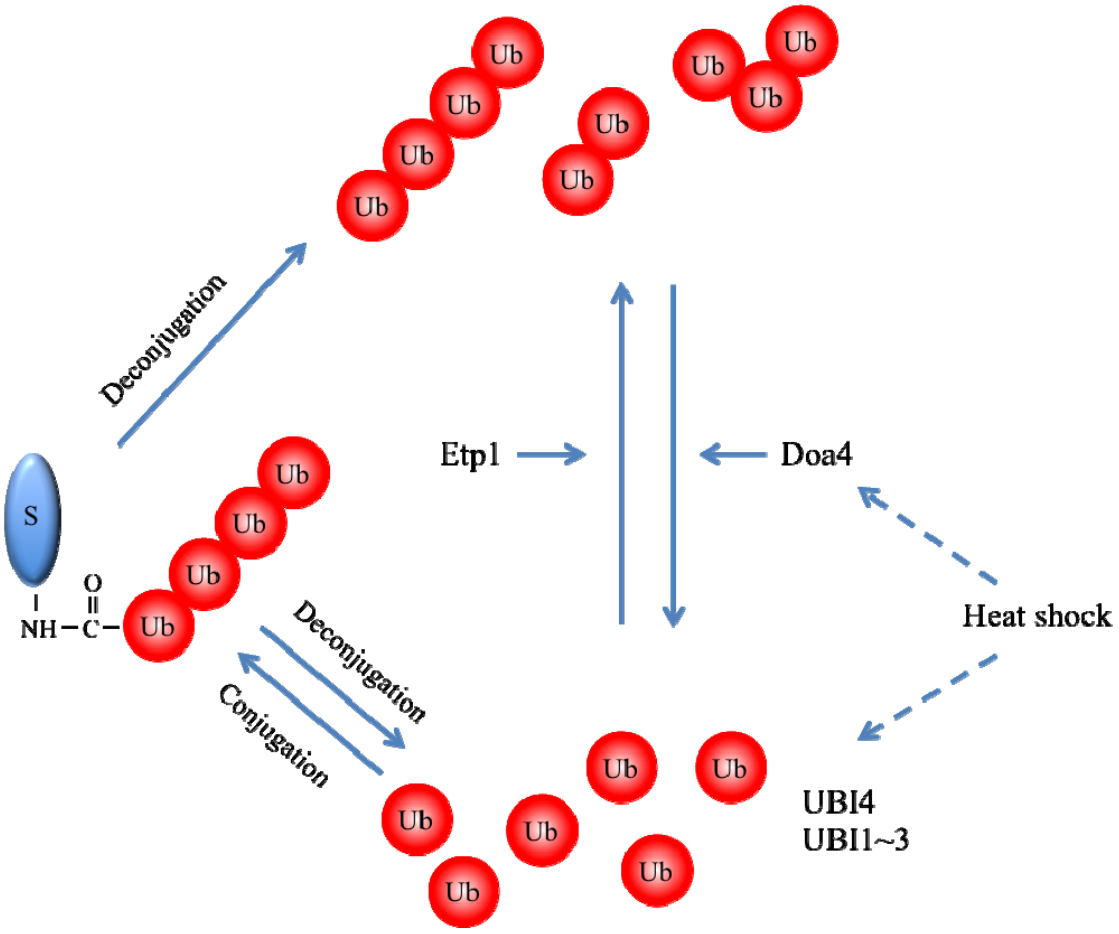


Figure 7. A model of regulation of Ub homeostasis by Etp1 and Doa4.

Cellular ubiquitin pool consists of monoubiquitin, free polyubiquitin chains and substrate-conjugated ubiquitin. Etp1 and Doa4 work in the opposite direction in balancing the amounts of free ubiquitin chains and monomeric ubiquitin.

the only E3 ubiquitin ligase known to play role in regulation of the yeast ubiquitin homeostasis up to date. In this work, we introduce an E3 of another class, the Etp1 RING E3 ubiquitin ligase that also plays role in the ubiquitin homeostasis in yeast.

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Chapter III

Yeast-Two-Hybrid Analysis of BRAP2 Binding Partners

An undergraduate student, Rachel Commander, conducted most of the experiments reported in this chapter. Janetta Bryksin wrote the chapter, designed the experiments, supervised Rachel, assisted in troubleshooting and analyzed the data.

Abstract

The post-translational modification of cellular proteins by ubiquitination is a key mechanism in regulating many cellular processes. E3 ligases confer specificity to ubiquitination by recognizing the target substrate protein and mediating the transfer of ubiquitin from a specific E2 conjugating enzyme to substrate. Identifying the substrates and the physiological binding partners of an E3 is fundamental in elucidating the cellular function of an E3.

BRAP2 is a highly conserved cytoplasmic E3 ubiquitin ligase that modulates the sensitivity of the MAP kinase cascade. In addition, BRAP2 binds to the NLS motif of BRCA1, SV40 large T antigen, mitosin, p21, and various viral proteins. Importantly, BRAP2 functions as a cytoplasmic retention protein for many cellular and viral proteins. However, the physiological substrate(s) of this E3 ubiquitin ligase has not been identified.

We have identified 24 binding partners of human BRAP2 E3 ubiquitin ligase via two yeast-two-hybrid (Y2H) assays. The most predominant hit in both Y2H assays was the mitochondrial protein CHCHD3. We have also identified ubiquitin as a binding partner of BRAP2. Remarkably, 46% of all hits terminate in either …KGG or …RGG sequences, including ubiquitin and the CHCHD3 proteins. We hypothesize that Etp1 binds the C-termini of proteins ending in R/KGG, such as CHCHD3 and ubiquitin, via the ZnF UBP domain.

Introduction

Ubiquitination, a post-translational modification of cellular proteins with the ubiquitin, targets the substrate proteins for the degradation by the 26S proteasome (1). In addition, ubiquitination has non-proteolytic functions such as regulation of cell cycle, apoptosis, DNA repair, signal transduction and endocytosis (1, 2). Consequently, the disruption of components of the ubiquitin-proteasomal system may lead to various malignancies, neurodegenerative disorders, genetic diseases, immune and inflammatory responses, hypoxia, and muscle wasting (1, 3, 4).

In general, the ubiquitination of a substrate requires a sequential action of three classes of enzymes: E1, E2, and E3 (5). Ubiquitin is first activated by the E1 ubiquitin activating enzyme in the ATP dependent manner. Activated ubiquitin is then transferred to one of the E2 ubiquitin conjugating enzymes. In the last step of ubiquitination, the E3 ubiquitin ligase mediates the transfer of ubiquitin from the E2 to the protein substrate. The ubiquitin cascade is hierarchical in nature. There are only two E1 enzymes, a few dozens of E2s, and several hundreds of E3s. This last step of ubiquitination offers a substrate specificity element to the ubiquitination process. Because of this, the E3s implicated in a particular disease are often considered as drug targets for inhibition (ref). Inhibition of E3 substrate binding or catalysis may achieve a maximal therapeutic potential with minimum abrogation of other cellular pathways and, hence, toxicity (3, 6).

Despite of the critical importance of the E3 ubiquitin ligases, our knowledge of the biological functions, mechanism of action, the physiological partners, and the substrates remains at a rudimentary stage for most E3s.

BRCA1 Associated Protein 2 or BRAP2 is a highly conserved E3 ubiquitin ligase, which has a unique domain structure containing a RING domain, a ZnF UBP domain and a Coiled-Coil domain (7). The RING domain is responsible for the E3 ubiquitin ligase activity of BRAP2 (8). The Coiled-Coil domain of BRAP2 binds the nuclear localization signal motifs (NLS) of several viral and cellular proteins, often with flanking phosphorylation sites (9), while the function of the ZnF UBP (ubiquitin binding domain) of BRAP2 has not been studied. BRAP2 is broadly expressed in the cytoplasm of multiple human and mouse tissues and is implicated in regulation of several cellular processes (8, 10). BPAP2 controls the sensitivity of the MAP kinase cascade, limiting the formation of Raf/MEK complexes by preventing the dimerization and activation of the KSR1 scaffold protein (7, 8, 11). BRAP2 acts as a Ras responsive E3 ubiquitin ligase that, on activation of Ras, is modified by autoubiquitination resulting in the release of inhibition of KSR (8, 11). In addition to this function, BRAP2 was shown to bind the NLS motifs of BRCA1, SV40 large T antigen, $p21^{Cip1}$, the bipartite NLS motif of mitosin, and NLSs of several viral proteins (9, 10, 12). However, the physiological substrate(s) of this E3 ubiquitin ligase has not been identified.

Here, we identify binding partners of the human BRAP2 utilizing two Yeast-Two-Hybrid (Y2H) screens. Y2H is a genetic method, which is based on activation of specific reporters upon binary interaction of ‘bait’ and ‘prey’ proteins (Figure 1) (13). In the first, small-scale Y2H screen, ubiquitin and CHCHD3 proteins from a Human Testes cDNA library were identified as binding partners of BRAP2. In the second screen, CHCHD3 was the predominant binding partner of BRAP2 from the normalized Universal Human cDNA library.

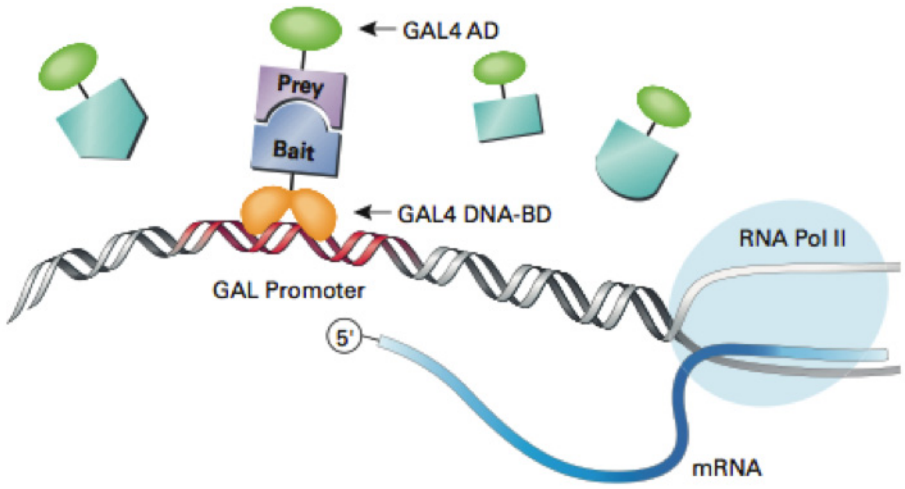


Figure 1. The two-hybrid principle (adapted from the Matchmaker protocol PT4084-1)

Two proteins are expressed separately, one (a bait protein) fused to the Gal4 DNA-binding domain (BD) and the other (a prey protein) fused to the Gal4 transcriptional activation domain (AD). Activation of the *HIS3* and *ADE2* reporter genes (used in both Y2H screens in this study) only occurs in a cell that contains proteins that interact and bind to the Gal4-responsive promoter.

Experimental procedures

Screen 1

Bait construction - Full-length human BRAP2 was cloned between the EcoRI and BamHI sites of the "bait" pGBDUC1 plasmid encoding the *GAL4* DNA binding domain (14), generating the pGBDUC1-BRAP2 plasmid. Insert was amplified from the pGEX-BRAP2 plasmid (full length, #1869) (15) using RCHP11 forward primer with EcoRI overhang (5'- GACGAATTCGACATGAGTGTGTCACTGGTTGTTATC) and RHCP13 reverse primer with BamHI overhang (5'-GACGGATCCGACTCACTTGCCCCTCTTGCTGCGGC). Sequence of the pGBDUC1-BRAP2 construct was confirmed by sequencing (Genewiz) by using forward sequencing primer 5'- GGCTTCAGTGGAGACTGATATGCC (RHCP6) and reverse sequencing primer 5'- GTATCTACGATTCATAGATCTCTG (ZAP44).

cDNA library - Human Testis Matchmaker cDNA library ("prey") fused with *GAL4* activation domain in pACT2 plasmid (Clontech) was generously provided by Tamara Caspary. The library was tittered according to Matchmaker protocol PT3247-1 (Clontech) and amplified as followed. The library was plated directly on Luria Broth (LB) plates containing 100 μg/ml ampicillin at a high density to reach nearly confluent colonies (~20,000–40,000 cfu per 150-mm plate) using glass beads for even spreading. Plates were incubated at 37°C for 18 hr. 5 ml of LB containing 20% glycerol was added to each plate and colonies were scraped into liquid medium. All the resuspended colonies

were pulled in one flask and mixed thoroughly. One third of the library culture (roughly equivalent to 3 L of overnight culture) was used to purify plasmids by QIAfilter Plasmid Giga Kit (QIAGEN). The rest of the library was aliquoted and stored at -80°C.

Transformation and Screen - - Two-hybrid analysis was performed in PJ69-4A strain (*MAT*a *trp1-901 leu2-3*, *112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2*::*GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ*) according to the method of James et al (14). PJ69-4A yeast strain was first transformed with pGBDUC1-BRAP2 plasmid by the high efficiency lithium acetate protocol (16). Cells were plated on SD-Ura to retain the plasmid. Next, cDNA library was cotransformed into the PJ69-4A yeast strain. 1 ml of cells was plated on 10 SD-His-Leu-Ura plates. His^+ colonies were patched and replica plated onto medium containing 3 mM 3-aminotriazole (3-AT) and separately onto SD-Ade-His-Leu-Ura to identify strong interactors. Each Ade^+ patch was restreaked on SD-Leu containing 1 mg/ml of 5-Fluoroorotic acid hydrate (5FOA from Sigma) to select against the bait pGBDUC1-BRAP2 plasmid, and then on SD-Leu. After the pGBDUC1-BRAP2 plasmid was lost, the cDNA library plasmids were recovered from yeast by using Zymoprep™ Yeast Plasmid Miniprep kit (Zymo Research). Plasmids were amplified in DH5α bacterial cells (Invitrogen). PCR was utilized to assess relative size of pACT2 insert using RHCP5 5'- AATACCACTACAATGGATG forward primer and ZAP28 5'-AGATGGTGCACGATGCACAG reverse primer. To confirm the interaction, plasmids containing inserts were retransformed into the PJ69-4A yeast cells already containing pGBDUC1-BRAP2 by the high-efficiency LiOAc method (16). Yeast cells were plated directly on SD- His-Leu-Ura. Three independent colonies were selected from SD- His-

Leu-Ura for each "hit" and streaked onto SD-Ade-His-Leu-Ura to confirm a strong interaction. Plasmids were isolated from Ade^+ yeast cells and were sent for sequencing (Macrogen) to identify the insert. RHCP5 forward primer (5'-AATACCACTACAATGGATG) was used for sequencing.

Screen 2

Bait construction - Full-length human BRAP2 was cloned between the EcoRI and BamHI sites of the "bait" pGBKT7 plasmid encoding the *GAL4* DNA binding domain (14), generating the pGBKT7-BRAP2 plasmid. Insert was amplified from the pGEX-BRAP2 plasmid (full length, #1869) (15) using RCHP11 forward primer with EcoRI overhang (5'- GACGAATTCGACATGAGTGTGTCACTGGTTGTTATC) and RHCP13 reverse primer with BamHI overhang (5'-GACGGATCCGACTCACTTGCCCCTCTTGCTGCGGC). Sequence of the pGBKT7-BRAP2 construct was confirmed by sequencing (Genewiz) by using the T7 forward sequencing primer 5'- TAATACGACTCACTATAGGG, ZAP29 forward sequencing primer 5'- CTGAAGATGGCGCCAGCCTC, and the ZAP26 reverse sequencing primer 5'- GGAATTAGCTTGGCTGCAAGCGCG.

cDNA library – The Y187 library strain (*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met–, gal80Δ, MEL1, URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*) pretransformed with the Universal Human cDNA library was purchased from Clontech

(catalogue number #630481). The library was tittered according to Matchmaker protocol PT3247-1.

Mating – Yeast-two-hybrid screen was carried out by 'mate and plate' method. PJ69-4A bait strain was transformed with pGBKT7-BRAP2 by the high-efficiency LiAc method (16). Yeast cells were plated on SD-Trp to retain the bait plasmid. A concentrated culture of the bait strain was prepared by growth of a single colony in 50 ml SD-Trp liquid medium for 20 hr. Cells were pelleted (1000g for 5 min) and resuspended to a cell density of $1x10^8$ cells per ml in SD-Trp (5 ml total). 1ml of the Y187 library strain pretransformed with the cDNA library was combined with 5 ml of the PJ69-4A bait strain transformed with pGBKT7-BRAP2 in a sterile 2 L flask. 45 ml of 2xYPDA (17) containing 50 µg/ml kanamycin was added to the flask. Cells were incubated at 30^{o}C, slowly shaking (40 rpm). After 20 hr, a drop of culture was analyzed under a phase contrast microscope (40X) for the presence of zygotes. Cells were pelleted by centrifugation (1000g for 10 min), washed with 50 ml 0.5xYPDA (with 50 µg/ml kanamycin), and resuspended in 10 ml of 0.5xYPDA/Kan liquid medium to obtain total volume of 11 ml. From the mated culture, 100 µl of 1:100, 1:1000, and 1:10,000 dilutions were plated on SD-Trp, SD-Leu, and SD-Leu-Trp. The remainder of the culture was plated on 55 150 mm SD-His-Leu-Trp + 3-AT plates (200 µl of culture for each plate); plates were incubated for 3 days at 30^{o}C. Mating efficiency was calculated by counting the colonies from the 1:10000 SD-Leu-Trp.

Screen – His^+ colonies from 150 mm SD-His-Leu-Trp + 3-AT plates were streaked in patches onto SD-Ade-His-Leu-Trp + X-Gal (Sigma) (QDO/X-Gal) to confirm strong interactions by ADE2 and lacZ expression. Plates were incubated for 2 days at 30°C. Each patch showing the ADE2 and lacZ expression was used for the purification of both bait and library plasmids by Zymoprep™ Yeast Plasmid Miniprep kit (Zymo Research). 100 microliters of DH5α cells were transformed with 15 microliters of plasmid from each of the plasmid miniprep. Cells were plated onto LB/Amp in order to select for the library plasmid. In most cases, one DH5α colony per putative hit was selected to recover the library plasmid.

To confirm the interaction, PJ69-4A yeast cells already containing pGBKT7-BRAP2 were retransformed with recovered library plasmids by the high-efficiency LiOAc method and plated on SD-Ade-His-Leu-Trp. Library plasmids were recovered from Ade^+ colonies and were sent for sequencing (Macrogen) to identify the insert. Forward T7 sequencing primer (5'- TAATACGACTCACTATAGGG**)** was used for sequencing.

Results

BRAP2 binds Ubiquitin and CHCHD3 as shown in Y2H screen 1

First, a small scale Y2H screen was carried out using BRAP2 as bait and Human Testis Matchmaker cDNA library proteins as prey. A human testis cDNA library was selected because BRAP2 is predominantly expressed in testes (Figure 2). Out of ~ 1×10^5 colonies screened, 352 colonies showed HIS3 expression. To identify strong interactions, colonies were also screened for the expression of ADE2. Out of 352 His^+ patches, 79 showed ADE2 expression. We were able to recover plasmids from 47 Ade^+ patches. Interactions were confirmed by retransformation of recovered plasmids into the yeast strain containing the bait BRAP2 plasmid. We confirmed the interaction for 8 hits. This represents a "hit rate" of 0.01%. After sequencing the cDNA library inserts, we identified 6 plasmids containing the ubiquitin insert and 2 plasmids containing the coiled-coil-helix-coiled-coil-helix domain containing 3 mitochondrial protein (CHCHD3) insert. All 8 inserts terminated in the C-terminal sequences for both proteins, indicating that the C-terminus of ubiquitin and CHCHD3 is important for binding to BRAP2.

Importantly, the following negative controls were conducted in evaluating the Y2H screen:

1. The empty bait pGBDUC1 and prey pACT2 vectors did not show the interaction.
2. The bait pGBDUC1-BRAP2 did not interact with the empty pACT2.
3. The empty pGBDUC1 did not interact with pACT2-CHCHD3.
4. The empty pGBDUC1 did not interact with pACT2-Ub.

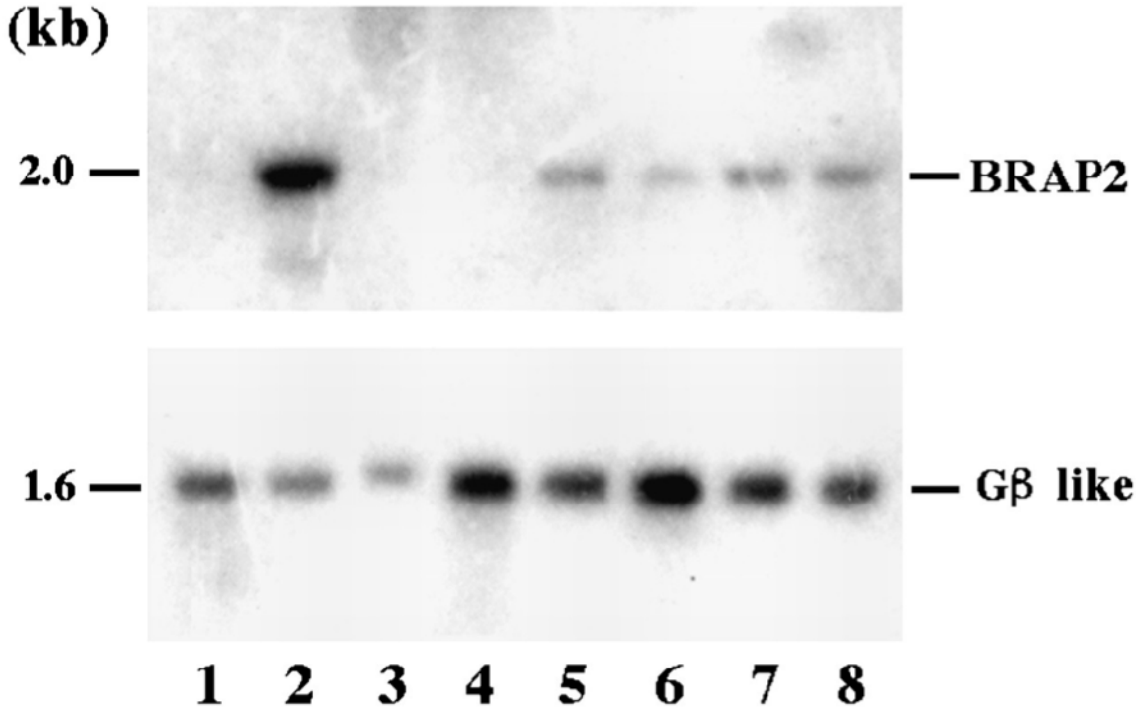


Figure 2. Northern hybridization of BRAP2 (adapted from (10)).

Expression of BRAP2 in adult mouse tissue. About 10 μg of total RNA from adult male mouse tissues were analysed (lane 1, small intestine; lane 2, testis; lane 3, thymus; lane 4, spleen; lane 5, kidney; lane 6, lung; lane 7, liver; lane 8, brain). In the upper panel, the blot was hybridized with the32P-labeled BRAP2 cDNA probe; only a single message of about 2.0-kb was detected in each lane. The lower panel shows the same blot hybridized with the32P-labeled Gβ-like cDNA probe. A single message of about 1.6-kb was detected in every tissue.

CHCHD3 is the main binding partner of BRAP2 identified in Y2H screen 2

A second, large scale Y2H screen was carried out using the 'mate and plate' method. In this method, a yeast strain containing the bait (BRAP2) was mated directly with the yeast library containing the cDNA library inserted into a prey vector. In this screen, we used the normalized Universal Human cDNA library. Out of ~ 3×10^7 colonies screened, ~ 5 x 10^3 colonies showed HIS3 expression. We randomly selected 500 colonies to patch onto SD-Ade-His-Leu-Trp + X-Gal to detect strong interactions. Out of 500, 320 colonies showed ADE2 and lacZ expression. Plasmids were recovered from all 320 colonies. All the hits were tested further on the individual basis by co-transforming each prey hit into the yeast stain containing BRAP2 to confirm the interaction. Out of 320 transformed yeast, 302 grew on SD-Ade-His-Leu-Trp (a "hit rate of 0.01% as observed in screen 1). The summary of the hits is presented in Appendix I.

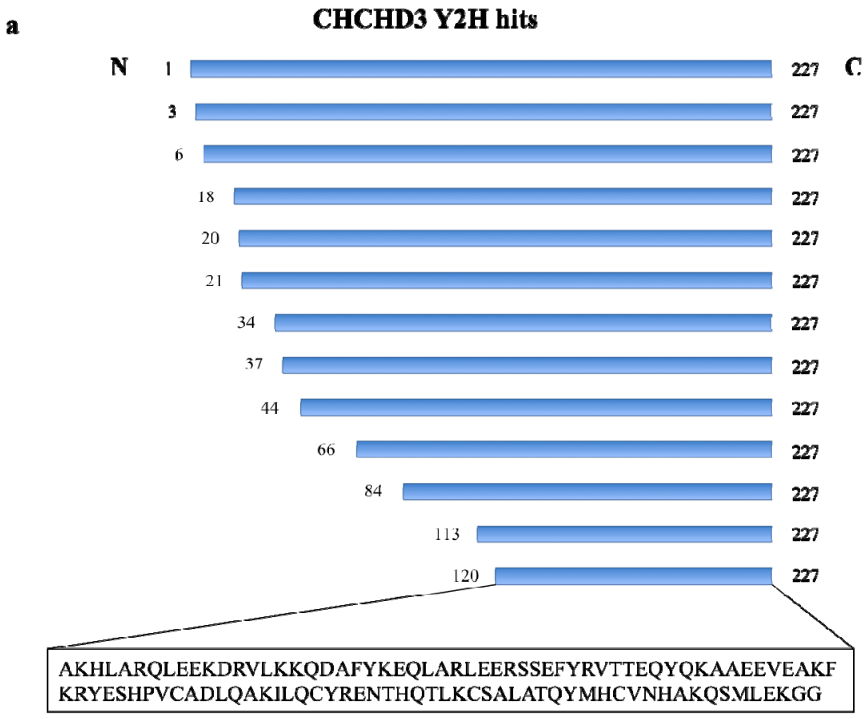
The following controls were used in Y2H:

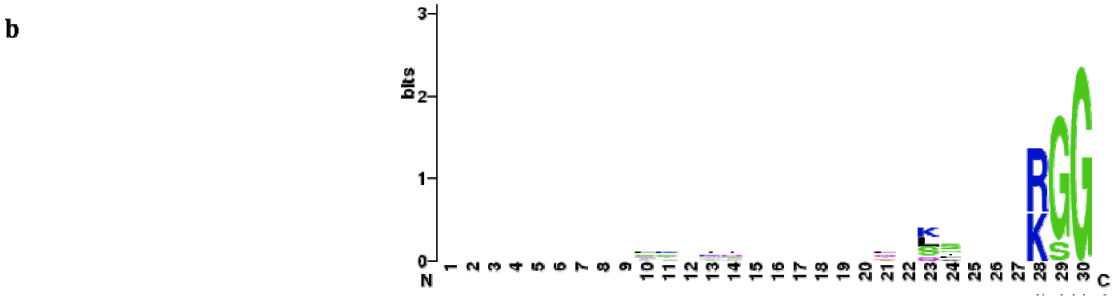
1. Nonspecific binding control: pGBKT7-BRAP2 and pGADT7-T did not interact.
2. Mating control: pGBKT7-53 (in PJ69-4A) with pGADT7-T (in Y187) did interact.

CHCHD3 was identified as the main hit (~39%). All CHCHD3 clones were truncated from the N-terminus and had an intact C-terminal KGG (Figure 3a), indicating that the binding of BRAP2 to CHCHD3 requires the C-terminus of the later protein. Another major hit observed 19 times was a collection of out of frame peptides derived from the TMX1 cDNA and terminating in RGG. Finally, five additional peptides from the untranslated regions of four different cDNAs fit this general pattern. The shortest of

all fragments was 30 amino acids long, making it unlikely that a specific secondary structure was being detected. Figure 3b shows the alignment of all these hits and a sequence logo derived from these sequences. It is apparent that the only thing in common in these sequences is the C-terminal R/KGG sequence characteristic of ubiquitin. While this analysis strongly implicates the ZnF UBP domain as the site of binding, approximately half of the hits do not show this C-terminal motif. We presume that these are binding to different regions on BRAP2, perhaps the coiled coil domain known to being nuclear localization signals (see discussion).

All protein hits were categorized into distinct groups based on their biological function in a cell (Table 1). The largest group (class I) consists of 10 proteins, all of which play role in the regulation of transcription. Class II is composed of proteins, which play role in the ubiquitin-proteasomal pathway. Class III proteins participate in the cell inflammatory response, while class IV proteins play role in the protein phosphorylation. The rest of proteins, each with a distinct biological function, were grouped into class V.





UB	GKQLEDGRTLSDYNIQKESTLHLVLRLRGG
CHCHD3	QTTAIPIPFKKIIIRICTTFEKSGGGTRGG
TMX1 (out of frame)	THQTLKCSALATQYMHCVNHAKQSMLEKGG
Untranslated #1	IEHAKNLEISKLSFLKNPKTYPLLPQDKGG
Untranslated #2	KYLCYHVLKNIPKIICQRFDLVKSEVERGG
Untranslated #3	IRDGIWPKLSLCKNHGEDINQSSSGTRKSG
Untranslated #4	VRPQSIGITFSAITAPVTNCQMKISYKRGG

Figure 3. Alignment of CHCHD3 hits.

(a) Full length CHCHD3 protein consists of 227 amino acids. All CHCHD3 hits are truncated from the N-terminus and have the intact C-terminus. The sequence of the shortest CHCHD3 clone is shown in a zoom out box. (b) Alignment and sequence logo from all hits conforming to the C-terminal motif of ubiquitin.

Table 1. Classification of Y2H hits for BRAP2.

Proteins were categorized in five classes based on their biological function.

Class	Biological functions	Proteins
I	Transcription regulation	ZNF251, SETDB1, ARID2, ZNF350, GTF3A, ZBTB38, ZNF177, ZNF302, SETDB1, KDM6A
II	Protein ubiquitination	CUL1, CACYBP, RNF2
III	Inflammatory response	MRVI1, KNG1
IV	Protein phosphorylation	ROCK2, YSK4
V	Various functions	CHCHD3, PPIE, OSBPL1A, AHNAK, FILIP1, LRRC6, XPA, ATP1B1

Discussion

The YH2 system is widely used to detect protein-protein interactions on a large scale (18). In the Y2H assay, a 'bait' protein is expressed as a fusion to the Gal4 DNA-binding domain (DNA-BD), while cDNA library of 'prey' proteins are expressed as fusions to the Gal4 activation domain (AD) (13). When bait and prey fusion proteins interact, the DNA-BD and AD are brought into close proximity to activate transcription of reporter genes (Figure 1).

In this study, we have identified binding partners of BRAP2 in two Y2H screens. In the first screen (small scale), a Human Testes cDNA library was used as prey, while in the second screen (large scale), the normalized Universal Human cDNA library was used as prey. Ubiquitin and CHCHD3 were identified as main hits in the first screen. CHCHD3 was also the main hit in the second screen, although we did not detect ubiquitin as an interacting protein in this screen. The lack of ubiquitin in a second screen can be explained by a nature of the used cDNA library. 'Normalization' reduces the proportion of highly abundant transcripts in a cDNA pool (Figure 4). This means that many of the most highly abundant housekeeping genes are significantly reduced in copy number so fewer clones could be screened to identify proteins with less abundant messages. Since ubiquitin is a highly abundant protein (19, 20), its transcripts were most likely depleted from the library almost entirely.

Remarkably, half of all hits terminate in either …KGG or …RGG sequences. This includes ubiquitin, which ends with …RGG, the CHCHD3 protein, which ends with

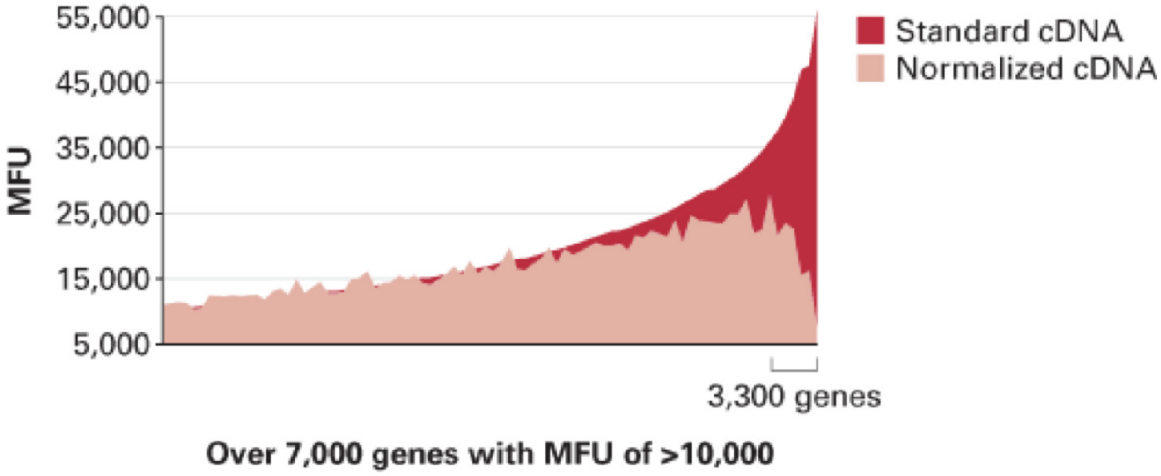


Figure 4. Reduction in abundance of highly expressed gene transcripts following cDNA normalization (adapted from the Matchmaker protocol PT4084-1).

cDNA normalization reduces the abundance of highly expressed gene transcripts. Data are shown for genes from mixed tissues, before and after normalization, which exhibit greater than 10,000 Mean Fluorescence Units (MFU), representing over 7,000 genes. Approximately 3,300 genes show a significant reduction in intensity, and thus abundance, following normalization. Due to the large volume of data obtained, the median MFU was plotted for groups of 100 genes before and after normalization.

…KGG, and several out of frame inserts ending with similar sequences (figure 3b). All of these inserts terminated in the GG sequences and no other motif were identifiable in the last 30 amino acids, the shortest insert detected. This strongly suggests that the C-terminus is required for binding of these proteins to BRAP2. We have shown that human BRAP2 binds ubiquitin via the ZnF UPB domain in a manner requiring the C-terminal glycine of ubiquitin (see Chapter 1). We hypothesize that ZnF UBP domain of BRAP2 not only binds ubiquitin, but also additional proteins, either substrates or physiological binding partners via interaction with a K/RGG motif at the C-terminus. This hypothesis is also supported by the recent finding that ZnF UBP domains of the tubulin deacetylase HDAC6 and the deubiquitinating enzyme Usp16 preferentially bind the C-terminus of various peptides ending with a Gly-Gly motif (21). ZnF UBP domain of HDAC6 bound ubiquitin and other peptides ending with …RGG, …KGG, …LGG, …FGG, …MGG, …IGG, and …AGG tightly with K_D values ranging from 0.23 μM to 8.8 μM (21). This suggests that ZnF UBP domain could play a role in the function of BRAP2 that doesn't involve binding of ubiquitin.

This preference for peptides (as opposed to ubiquitin) can be explained by comparing the structure of the USP5 ZnF USP and a homology model of the BRAP2 domain. The ZnF UBP domain of Etp1 does not contain the L2 loop, which is present in the ZnF UBP domain of USP5 and makes extensive contacts with ubiquitin (Figure 5). The lack of the L2 loop suggests that if ubiquitin binding to the ZnF UBP domain is important it is likely a much weaker interaction than that reported for USP5. In fact, preliminary evidence suggests that BRAP2 binds ubiquitin 1000 fold weaker than does USP5 (Zhanetta Astakhova, John Shanks and Keith D. Wilkinson, unpublished).

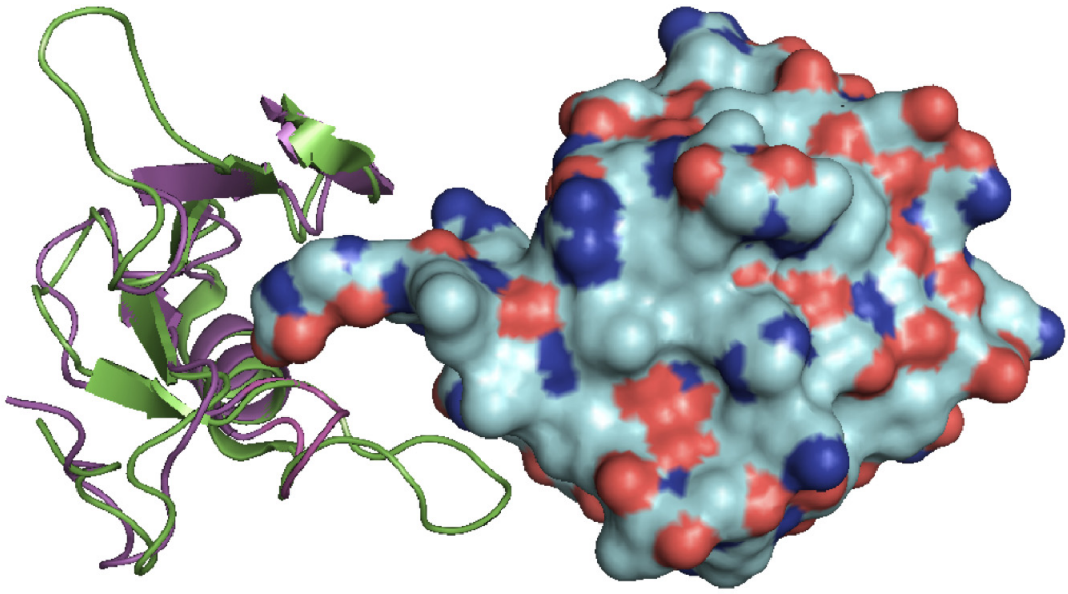


Figure 5. Homology model of ETP1 and USP5 ZnF UBP domains.

Etp1 ZnF UBP domain (magenta) superimposed on USP5 ZnF UBP domain (green) bound to ubiquitin (space filling). ETP1 lacks the L2 loop of USP5 that makes extensive contact with ubiquitin. ETP1 homology model is generated from accession number **P38748** (ETP1_YEAST, residues 298-358) at http://swissmodel.expasy.org/repository/ and Usp5/Ub structure is accession number 2G45 (residues 197-267) from http://PDB.org.

In addition to these peptides ending in K/RGG we also found another 20 proteins that interact in the Y2H assay but do not fit the consensus profile described above. It is possible that these are interacting with another region of BRAP2 such as the coiled coil domain involved in binding phosphorylated NLS signals. Additional analyses of these binding interactions are currently underway.

Except for ubiquitin, we have not detected any previously known binding partners of BRAP2, notably KSR1, UBCH5b, or nuclear localization signals. The latter exception might be explained by the fact that in order for BRAP2 bind the NLSs of some viral proteins these proteins have to be specifically phosphorylated at the sites flanking the NLS (9). If this were a requirement for BRAP2 to bind cellular proteins as well, the lack of specific kinases in yeast would prevent the phosphorylation and, consequently, the binding of protein substrates to BRAP2.

In considering the possible significance of the CHCHD3 interaction it became apparent that BRAP2 and CHCHD3 exist in separate cellular compartments. CHCHD3, an inner mitochondrial membrane protein, was first identified as a substrate of the cAMP-dependent protein kinase (PKA) (22). It is found in a complex with mitofilin, SAM50, metaxins 1 and 2, CHCHD6 and DnaJC11 in the inner mitochondrial membrane. Very recent studies indicate that CHCHD3 is essential for maintaining cristae integrity and mitochondrial structure in both human cells and worms (23, 24). Perhaps most intriguing is the fact that, upon apoptosis, the outer mitochondrial membrane is lysed and the CHCHD3 containing complex becomes exposed to the cytoplasmic contents. During that process mitofilin becomes ubiquitinated and participates in delivery of these damaged mitochondria to the autophagic machinery. Thus, it may be that exposure of the C-

terminus of CHCHD3 recruits BRAP2 to ubiquitinate mitofilin. Consistent with this hypothesis, all animal homologs of CHCHD3 end in KGG.

In summary, we have identified the ubiquitin and the CHCHD3 protein as main binding partners of BRAP2 in two Y2H screens. The physiological role of these associations should be investigated further, arguably, with a special emphasis on the role of ZnF UBP domain in the function of BRAP2.

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Appendix I

Compete list of YH2 hits for screen 2

Name	Description	Biological function	# of hits
CHCHD3	An inner mitochondrial membrane protein, essential for maintaining crista integrity and mitochondrial function.	Mitochondrial morphology and dynamics.	118
ZNF251	Nuclear protein, which may be involved in transcriptional regulation.	Transcription regulation	21
MRVI1	Part of cGMP kinase signaling complex; NO/PRKG1-dependent regulator of IP3-induced calcium release.	Platelet activation, inflammatory response	14
KNG1	Secreted to plasma; inhibitor of thiol proteases; plays an important role in blood coagulation.	Inflammatory response	10
SETDB1	Histone methyltransferase that specifically trimethylates 'Lys-9' of histone H3.	Chromatin regulator, transcription regulation	9
ARID2	Subunit of the PBAF chromatin-remodeling complex, which facilitates ligand-dependent transcriptional activation by nuclear receptors. Highly expressed in testis.	Transcription regulation	7

PPIE	Member of the peptidyl-prolyl cis-trans isomerase (PPIase) family, which possesses PPIase and protein folding activities, and it also exhibits RNA-binding activity.	mRNA processing	6
OSBPL1A	Member of the oxysterol-binding protein (OSBP) family, a group of intracellular lipid receptors.	Lipid transport	5
AHNAK	Nucleoprotein, which may be required for neuronal cell differentiation. Modulates L-type Ca(2+) channel inactivation.	Ca(2+) signalling	4
FILIP1	May control the start of neocortical cell migration from the ventricular zone (by similarity).	Unknown	4
ZNF350	Transcriptional repressor. Binds to a specific sequence, 5'-GGGxxxCAGxxxTTT-3', within GADD45 intron 3. Interacts with BRCA1 and RNF11.	Transcription regulation	4
CUL1	Core component of multiple SCF E3 ubiquitin-protein ligase complexes, which mediate the ubiquitination of proteins involved in cell cycle progression, signal transduction and transcription.	Negative regulation of cell proliferation, protein ubiquitination	3
GTF3A	Interacts with the internal control region	Transcription	3

	within the 5S RNA genes, is required for correct transcription of these genes by RNA polymerase III. Also binds the transcribed 5S RNA's. May initiate transcription of the 5S ribosomal RNA gene and maintain the stability of transcription of other genes.	regulation	
LRRC6	Testis-specific leucine-rich repeat protein, which may be involved in spermatocytogenesis or prophase of meiosis (by similarity).	Unknown function	4
ROCK2	Regulates the assembly of the actin cytoskeleton. Promotes formation of stress fibers and of focal adhesion complexes. Plays a role in smooth muscle contraction.	Protein phosphorylation	3
XPA	Involved in DNA excision repair. Required for UV-induced CHK1 phosphorylation and the recruitment of CEP164 to cyclobutane pyrimidine dimmers (CPD), sites of DNA damage after UV irradiation.	DNA repair	3
ZBTB38	A transcriptional activator. May be involved in the differentiation and/or survival of late postmitotic neurons.	Transcription regulation	3
ZNF177	Negative regulator of transcription from	Transcription	3

	RNA polymerase II promoter.	regulation	
KDM6A	Histone demethylase that specifically demethylates 'Lys-27' of histone H3, thereby playing a central role in histone code.	Chromatin regulator, transcription regulation	2
YSK4	Belongs to the STE Ser/Thr protein kinase superfamily.	Protein phosphorylation	2
ZNF302	Nuclear proteins, which may be involved in transcriptional regulation.	Transcription regulation	2
ATP1B1	Non-catalytic component of the active enzyme, which catalyzes the hydrolysis of ATP coupled with the exchange of Na^+ and K^+ ions across the plasma membrane.	Sodium and potassium transport	2
CACYBP	May be involved in calcium-dependent ubiquitination and subsequent proteasomal degradation of target proteins. Probably serves as a molecular bridge in ubiquitin E3 complexes.	Protein ubiquitination	1
RNF2	E3 ubiquitin-protein ligase that mediates monoubiquitination of 'Lys-119' of histone H2A, thereby playing a central role in histone code and gene regulation.	Protein ubiquitination, transcription regulation	1
(TMX1)[a)]	Peptide, which ends with …RGG	N/A	19

(LARP7)[a)]	Peptide, which ends with ...RKR	N/A	9
(YES1)[a)]	Peptide, which ends with …RFE	N/A	4
(DNAJB)[a)]	Peptide, which ends with …SPT	N/A	3
(OAZ3)[a)]	Peptide, which ends with…RRT	N/A	1
(USP48)[a)]	Peptide, which ends with …IKR	N/A	1

Chapter IV

Conclusions and Future Directions

Overall summary

Our interest in the BRCA1 Associated Protein 2 (BRAP2) enzyme rose after the ubiquitin-binding domain, ZnF UBP was crystallized in our lab for the deubiquitinating enzyme IsoT (1). The ZnF UBP (zinc finger ubiquitin-specific processing protease) domain, also known as the DAUP (deacetylase/ubiquitin-specific protease) domain, PAZ (polyubiquitin-associated zinc finger) domain, or BUZ (binder of ubiquitin zinc finger) domain, is found in the cytoplasmic tubulin deacetylase HDAC6, a group of ubiquitin-specific proteases (USPs), and in BRAP2 (1-6). Thus, BRAP2 is the only E3 ubiquitin ligase that contains the ZnF UBP domain. The puzzling part of this observation is that the ZnF UBP domain specifically binds the free C-terminal RGG of ubiquitin, and in the catalytic mechanism of E3 ligases ubiquitin is always present as a thiol ester with an E2 conjugating enzyme or an amide with another protein. Thus the question, what is the role of binding to free ubiquitin in a BRAP2 function?

There are more than twenty known families of ubiquitin binding domains. However, all of them bind ubiquitin non-covalently in such a way that C-terminal tail of ubiquitin is free and available, as a thiol ester or amide, for example, for a further substrate conjugation (7, 8). In contrast, the ZnF UBP domain binds free C-terminus of unanchored ubiquitin. It has been proposed that the binding of free C-terminal ubiquitin to its ZnF UBP domain could regulate the activity of HDAC6 and several deubiquitinating enzymes (2, 8, 9).

We chose to approach this question using the yeast homologue of BRAP2, Ethanol Tolerance Protein 1 (ETP1). Through biochemical and genetic analysis of

BRAP2 and its yeast orthologue Etp1, we discovered a surprising role of Etp1 in regulation of ubiquitin homeostasis. In addition, we have identified binding partners of human BRAP2, which can potentially be the protein substrates for this E3 ubiquitin ligase.

BRAP2 binding partners; scaffolds or substrates

We have utilized two yeast two hybrid (Y2H) screens to identify the binding partners of BRAP2. Not surprisingly, one of the hits was ubiquitin. The CHCHD3 protein was the main binding partner of BRAP2 detected in both Y2H screens. Interestingly, both ubiquitin and CHCHD2 have a C-terminal diglycine motif. In fact, 46% of all hits, including the out of frame peptides, terminate in either …KGG or …RGG sequences. All identified CHCHD3 and ubiquitin truncated library proteins had an intact C-terminus, indicating that the C-terminus of these proteins is a likely binding site for BRAP2. We have shown that human BRAP2 binds ubiquitin via the ZnF UBP domain in a manner requiring the C-terminal glycine of ubiquitin (Chapter 1). We hypothesize that ZnF UBP domain of BRAP2 not only binds ubiquitin, but also additional proteins, either as substrates or physiological binding partners. Our hypothesis is also supported by the recent finding that the ZnF UBP domains of deacetylase HDAC6 and the deubiquitinating enzyme Usp16 preferentially bind the C-terminus of various peptides ending with the Gly-Gly motif (10). This suggests that the ZnF UBP domain could play a bigger role in the function of BRAP2 via binding to proteins other than ubiquitin.

We have also proposed a role for CHCHD3 binding in autophagy of damaged mitochondria, especially those that have lost outer membrane integrity. CHCHD3 is an inner mitochondrial protein that would not normally encounter BRAP2. However, upon lysis of the mitochondrial outer membrane, an access to CHCHD3 would be possible. The combined interaction between Coiled-Coil domains in each protein and the binding of the highly conserved KGG C-terminal sequence of CHCHD3 with the ZnF UBP domain of BRAP2 are likely to account for the apparent strength of the binding interactions detected in Y2H.

Interestingly, the vast majority of other Y2H hits are proteins that are implicated in the transcriptional regulation (ZNF251, SETDB1, ARID2, ZNF350, GTF3A, ZBTB38, ZNF177, ZNF302, SETDB1, and KDM6A). The physiological role of these associations should be investigated further, arguably, with a special emphasis on the role of the Coiled-Coil domain in the function of BRAP2.

Work not described in this dissertation

Our early efforts to elucidate the function of Etp1 focused on the determination of the binding partners by pull-down assays and the genetic interactions by the synthetic genetic array (SGA) analysis. Because purified BRAP2 and Etp1 are unstable during purification and in lysates, they are not suitable for pull-down assays. After trying several different fusion tags (hexahistidine, SUMO, GST, TAP) for purification of Etp1, we employed the Histidine Affinity Tag (HAT), which allowed a better stabilization of both proteins. Because of the enhanced stability, purified HAT-Etp1 and HAT-BRAP2 could

potentially be used for pull-down assays in the future. Mutants of HAT-tagged Etp1 (RING domain, ZnF UBP domains, and a double RING/ZnF UBP domain mutants) were also expressed in *E. coli* and purified. These proteins were characterized in biochemical assays and then used in vivo to elucidate the role RING and ZnF UBP domains in the function of Etp1.

Our collaborative effort with Munira Basrai's laboratory to identify the genetic interactions for ETP1 via an SGA screen produced 31 'sick' and 12 lethal synthetic interactions in the initial screen. A list of these genes is presented in Appendix I. However, when tested on the individual basis, none of these interactions were confirmed. Nevertheless, ETP1 was detected as one of the hits in SGA screen for GRE1 (11). Deletion of both ETP1 and GRE1 genes from the genome caused the yeast cell lethality, indicating that these genes are epistatic (11). GRE1 is a hydrophilin of unknown function, which is induced by various stresses (osmotic, ionic, oxidative, heat shock and heavy metals) (12-15). Interestingly, another SGA hit for GRE1 was SET2 (11). SET2 is a histone methyltransferase, which plays role in transcriptional elongation by methylating a lysine residue of histone H3 (16). We have also identified SETDB1, a homologue of SET2, as a binding partner of human BRAP2 in Y2H assay (Chapter 3). Altogether, these data suggest a putative role of BRAP2/ETP1 in regulation of transcription, potentially, by regulating the nuclear-cytoplasmic shuttling of SETDB1/SET2 (see below). The genetic and protein interactions of ETP1, GRE1, and SET2 should be investigated further.

Since the Coiled-Coil region of human BRAP2 binds the nuclear localization signal motifs (NLS) of numerous proteins (5, 17, 18), including the NLS of SV40 large T-antigen protein, we also tested binding of Etp1 to purified mono- and bipartite NLS of

SV40 large T-antigen and the NLS of the yeast Swi6 protein. Etp1 did not bind either one of the tested NLSs (data not shown). In the light of the recent discovery, we, however, do not exclude the possibility that Etp1 binds NLSs. In order for BRAP2 to bind the NLS of SV40 large T-antigen, the latter protein has to be phosphorylated at the sites flanking the NLS (18). However, in our binding assays for Etp1, we used short NLS peptides on a GFP protein carrier, which could not be phosphorylated. Given that the substrates of Etp1 are known and contain an NLS, the future studies should focus on the role of Etp1 in binding of Etp1 to NLS of a full-length substrate and retaining it in the cytoplasm in analogy with BRAP2.

Tools used in these studies

Etp1 was previously described to play role in yeast adaptation to ethanol provided either as a sole carbon source or as a stressor (19). We have confirmed the ethanol sensitivity phenotype of *etp1Δ*. Interestingly, all the observed phenotypes, including the ethanol sensitivity phenotype of *etp1Δ*, were complemented only by introducing ETP1 back into its endogenous chromosomal location, but not by the plasmid born Etp1. It is worth mentioning, that the constructs we made for the expression of Etp1 lead to either overexpression of Etp1 (CUP1 promoter) or underexpression of Etp1 (partial endogenous promoter). Therefore, a precisely regulated amount of Etp1 is needed for the complementation of *etp1Δ* sensitivity to ethanol. This may be also reflected in our finding that Etp1 is a short-lived protein.

We have also employed conservative point mutations in the RING domain, ZnF UBP domain, or both domains to probe biochemical and physiological functions of Etp1. These mutations are quite precise in their consequences; ablation of RING domain function has no effect on ubiquitin binding and destroying the ubiquitin binding affinity of the ZnF UBP domain has no effect on the catalytic activity of Etp1 in autoubiquitination assays.

Finally, we have obtained expression clones for eight of the thirteen yeast E2 ubiquitin conjugating enzymes from the SIDNET cDNA Archive (Daniela Rotin, University of Toronto, Canada). These proteins were expressed and purified from bacteria to see which would function with ETP1. The majority of those tested either functioned with Etp1 (Ubc4) or were efficiently autoubiquitinated in the presence of yeast E1 and ubiquitin (all except Ubc7 and 10).

Etp1 and BRAP2 have the same functional domains

When we first started our studies, Etp1 was a putative protein of unknown function. We set out to evaluate the functional homology of Etp1 and BRAP2 and focused our attention on the RING and the ZnF UBP domains, since these were the most homologous regions of the Etp1 and BRAP2 proteins. Indeed we showed that both the RING and the ZnF UBP domains are functional in both Etp1 and BRAP2. The RING domain is required for the E3 ubiquitin ligase activity of BRAP2 (6). We have shown that the ability of Etp1 to form ubiquitin chains *in vitro* is also dependent on its RING domain (Chapter 2). The purified ZnF UBP domain of Etp1 binds ubiquitin (1). We have shown

that full-length Etp1 can also bind ubiquitin. In addition, we provide evidence that full-length BRAP2 also binds ubiquitin as shown by our biochemical assays (Chapter 2). Therefore, BRAP2 and Etp1 share the same functional domains, allowing the use of yeast Etp1 to elucidate potential functions of human BRAP2. Nevertheless, we have not analyzed the function of the Coiled-Coil domains of BRAP2 and Etp1. The Coiled-Coil domain of BRAP2 binds the NLS of various viral and cellular proteins (18). Since the Coiled-Coil domains of both proteins are 60% homologous (20), the function of these domains could be overlapping. Future studies may focus on the analysis of phenotypes of yeast strains expressing the truncated Etp1 lacking the C-terminal Coiled-Coiled domain.

BRAP2 and Etp1 catalyze the formation of ubiquitin chains *in vitro*

Human BRAP2 efficiently utilizes the UbcH5b E2 conjugating enzyme (in collaboration with Allan Weissman and coworkers, data not shown) to produce ubiquitin chains of different topology. BRAP2 and UbcH5b form predominantly free polyubiquitin chains with K6, 11 and 48 linkages as shown by mass spectrometry analysis (Chapter 2). BRAP2 has not been reported to autoubiquitinate and we found no evidence of this in our analysis either. Analysis of the topology of ubiquitin chains catalyzed by Etp1 and a heterologous E2, UbcH5b, has revealed the formation of K6, K11, K33, K48, and K63 ubiquitin chains attached to Etp1, with K48 and K33 being the most abundant chains. While classical K48-linked polyubiquitination targets substrates for proteasomal degradation (21), the role of K33 linkages is still not well understood. K33 linkages are

relatively resistant to proteasomal degradation (22) and have been implicated in nonproteolytic regulation of cellular processes and response to stress (23-25).

We did not detect the formation of free ubiquitin chains in the reaction with Etp1; rather, Etp1 was autoubiquitinated with ubiquitin chains of different lengths at lysines 35, 369, and 450. A large-scale analysis of yeast ubiquitinated proteins indicated ubiquitination of Etp1 at lysines 369 and 410 (26). Interestingly, the Etp1 protein runs as a double band with a difference in bands' sizes corresponding to modification by monoubiquitin. However, the effect of Etp1 post-translational modification by ubiquitination has not been analyzed and should be investigated further. We also cannot exclude the possibility that Etp1 may form free ubiquitin chains *in vivo* with the physiologically relevant E2.

Ubc4 is a competent E2 conjugating enzyme for Etp1

We have identified Ubc4 as a relevant E2 conjugating enzyme for Etp1. Ubc4 efficiently supports addition of a small number of ubiquitin molecules attached to Etp1. Analysis of the topology of ubiquitin linkages catalyzed by Ubc4~Etp1 pair is underway. Our results are consistent with the findings for BRAP2~UbcH5b pairing. Human UbcH5b is a homologue of both the yeast Ubc4 and Ubc5 E2s. Even though Ubc4 and Ubc5 are 92% identical and consist mainly of the catalytic domain (27), it is possible that Ubc5 could play an additional role in the function of Etp1. The question whether Ubc5 is another physiologically relevant E2 for Etp1 should be studied further.

It should be noted that in our screen we only used 8 out of 13 known yeast E2s: Ubc1, Ubc3, Ubc4, Ubc7, Ubc8, Ubc10, Ubc11, and Ubc13. Ubc9 and Ubc12 are E2 enzymes for SUMO and Nedd8, respectively (28), while Ubc13 also forms a heterodimer with MMS2 to catalyze K63-linked chains (29). Thus, Ubc2, Ubc5, Ubc6 should be characterized in the future.

We also noted a slight increase in the efficiency of Ubc1 autoubiquitination in the presence of Etp1, suggesting that that they may interact. If further work confirms this binding, it is reminiscent of the properties of the BRCA1/BARD1 ubiquitin ligase that binds to many E2s but utilizes only some and catalyzes different types of linkages depending on the E2 used. A closer analogy is also noted with the Anaphase promoting Complex Ubiquitin ligase. This ligase uses Ubc4 to monoubiquitinated substrates and then Ubc1 to extend the chains (30). We must examine more closely the combinatorial action of different E2s to see if there are similar combinatorial interactions determining the final ubiquitination products of Etp1.

Etp1 regulates ubiquitin homeostasis in yeast

The work reported in this dissertation has shed insights into many aspects of Etp1 function. Arguably, the most significant finding was that the Etp1 ubiquitin ligase is implicated in the regulation of the ubiquitin homeostasis (Chapter 2). Upon loss of ETP1, yeast cells display the decreased amount of free di-, tri-, and tetraubiquitin without changes in total ubiquitin levels. This implies that Etp1 modulates the redistribution of ubiquitin polymers rather than affecting the balance between ubiquitin synthesis and

degradation. Both the ligase activity of Etp1 and ubiquitin binding are important for the formation of free ubiquitin chains *in vivo*, as mutation of the RING and ZnF UBP domain of Etp1 led to a decreased amount of free chains compared to wild-type. However, abrogation of both the RING and the ZnF UBP domains did not cause any more significant loss of free ubiquitin chains than did each domain alone. Thus, *etp1Δ* caused a much more significant loss of free ubiquitin chains than did mutation of either or both the domains studied. This indicates that additional domains of Etp1, perhaps, the Coiled-Coil domain, could play role in the function of Etp1 by binding to another protein that itself influences free ubiquitin chain levels. The future *in vivo* analysis of ubiquitin chains in cells expressing truncated Etp1 can elucidate the role of the Coiled-Coil domain in the function of Etp1.

Another interesting finding was that Etp1 is a short-lived protein with a half-life of ~17 minutes. This high turnover rate suggests tight regulation of the Etp1 protein levels in yeast, arguably for a quick response to changing environmental conditions.

etp1Δ is resistant to various stresses

In this work, we have discovered new phenotypes of *etp1Δ.* Loss of ETP1 renders yeast resistant to various stresses such as oxidative stress by paraquat or hydrogen peroxide, translational inhibition by anisomycin, and an amino acid analog canavanine.

Interestingly, loss of both the E3 ligase activity and ubiquitin binding ability of Etp1, led to a higher sensitivity to above mentioned drugs, higher even than the wild type strain. This implies a negative gain of function by these mutations and suggests that there

might be an additional binding interaction responsible for the Etp1 function. The effect of the expression of the truncated Etp1 lacking the Coiled-Coil domain on the ubiquitin levels and tolerance to paraquat, hydrogen peroxide, canavanine and anisomycin should be compared to strains expressing wild type Etp1, *etp1Δ* strain, and strains expressing the RING domain, ZnF UBP domain and RING/ZnF UBP domain mutant of Etp1.

ETP1 and DOA4 act upon the same pathway

Several regulatory mechanisms are involved in the maintenance of ubiquitin homeostasis. Doa4 deubiquitinating enzyme has been recently shown to regulate the amount of monoubiquitin by cleaving free ubiquitin chains in yeast under stress (31). We have studied the effect of deletion of ETP1 on the *doa4Δ* phenotypes. Indeed, our findings showed that DOA4 and ETP1 are epistatic, as there is an additive effect of deletion of DOA4 and ETP1 on the amount of di- and triubiquitin, and tolerance to elevated temperatures and hydrogen peroxide (Chapter 2).

Model for Etp1 function

In this work, we have characterized the yeast Etp1 protein, its functional domains, and demonstrated a role for Etp1 in the regulation of ubiquitin homeostasis. Even though ubiquitin is a highly abundant protein, it is not produced in excess. Rather, there is a dynamic equilibrium between three forms of cellular ubiquitin: monomeric ubiquitin, a substrate-conjugated mono- and polyubiquitin, and unanchored ubiquitin chains (Figure

1) (31). Mutations in *UBI* genes as well as in several DUBs cause reduction of ubiquitin levels and contribute to various cellular defects (32). Overexpression of ubiquitin also impairs cell growth and leads to sensitivity to several compounds (33). To prevent these undesirable effects, ubiquitin expression is tightly regulated by several control mechanisms: transcriptional regulation of ubiquitin-encoding genes, regulation by the change of proteasomal composition, and regulation by deubiquitinating enzymes (32).

One example of this dynamic homeostatic response is exposure to such stresses as elevated temperatures, starvation, and the amino acid analog canavanine (33). All are thought to result in accumulation of misfolded cellular proteins, which are quickly ubiquitinated and targeted for the proteasomal degradation. Free ubiquitin levels must be increased to accomplish this degradation. Deletion of ETP1 renders yeast resistant to oxidative stress and treatment with canavanine and anisomycin. In *etp1Δ* there is more free ubiquitin available to ubiquitinate and degrade these damaged proteins and thus, more resistance of *etp1Δ* to paraquat, hydrogen peroxide, canavanine, and anisomycin. Conversely, there is less need for DUBs to produce monoubiquitin from polyubiquitin and this may be the basis of the genetic interactions between Etp1 and Doa4 deubiquitianting enzyme.

We found that human BRAP2 catalyzes the formation of free ubiquitin chains *in vitro*. Emerging evidence suggests that unconjugated polyubiquitin has a signaling function (34). Unconjugated K63 polyubiquitin chains synthesized by TRAF6 RING E3 ligase and UbcH5C E2 conjugating enzyme can directly activate the TAK1 kinase complex by binding to the ubiquitin receptor TAB2 (35). In addition, unanchored K63 polyubiquitin chains can directly activate RIG-I protein, a signaling protein involved in

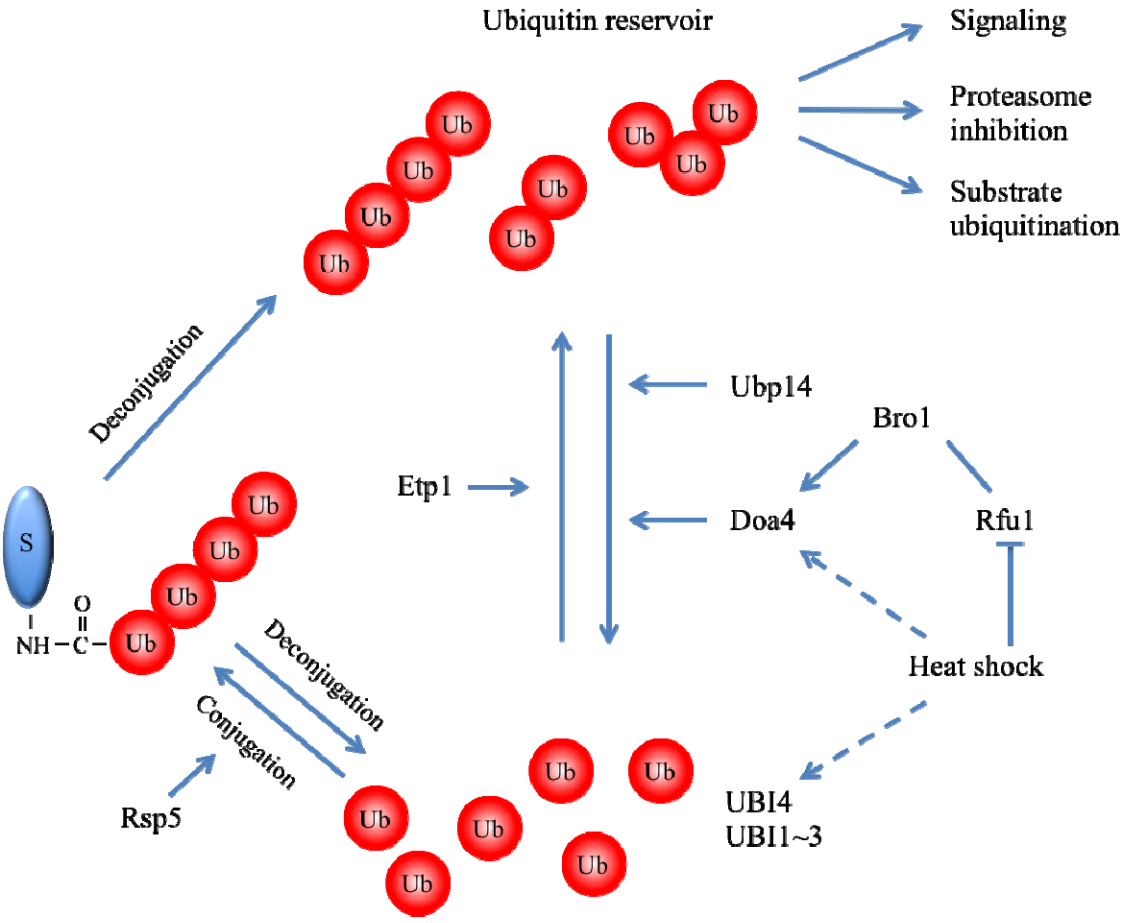
Ubiquitin reservoir
Signaling
Proteasome inhibition
Substrate ubiquitination
Deconjugation
Ubp14
Bro1
Etp1
Doa4
Rfu1
S
NH
C
O
Heat shock
Deconjugation
Conjugation
Rsp5
UBI4
UBI1~3
Ub

Figure 1. A model of regulation of Ub homeostasis by deubiquitinating enzymes and ubiquitin ligases.

The monomeric ubiquitin (Ub) pool is maintained through synthesis from Ub-encoding genes, UBI1–4, by release from protein-conjugated Ub chains, and by release from free Ub chains. DUB(s), such as Doa4 and Ubp14, supply monomeric Ub by cleaving free Ub chains. Under normal conditions, Rfu1 inhibits the activity of Doa4, resulting in the maintenance of the monomeric Ub pool, and consequently enhances the formation of free Ub chains. Upon heat shock, Rfu1 levels decrease and more Doa4 is produced therefore, monomeric Ub would be produced from free Ub chains by Doa4. In addition, transcription of UBI4-encoding polyubiquitin is increased. Since the activity of Doa4 is enhanced by Bro1, Doa4 would be controlled by a balance between its activator (Bro1) and inhibitor (Rfu1). Etp1 acts as a ubiquitin sensor, influencing formation of free ubiquitin chains that the cell may use as a reservoir of polyubiquitin. Under normal conditions, these chains may be used for signaling or simply remain as a reserve pool of ubiquitin, while under stress the free polyubiquitin linkages are cleaved by DUBs for a further substrate conjugation.

the immune response to viral infection (36). Moreover, free polyubiquitin inhibits the proteasome (37) or potentially could be used for substrate ubiquitination via *en bloc* transfer of a polyubiquitin chain as opposed to sequential addition of ubiquitin.

Although we have not observed free polyubiquitin synthesis by Etp1, we do not exclude the possibility that Etp1 can catalyze the synthesis of free ubiquitin chains under some conditions. We have identified a yeast E2 conjugating enzyme, Ubc4, which works as a matching E2 for Etp1 E3 ubiquitin ligase. Analysis of the topology of ubiquitin linkages catalyzed by Ubc4~Etp1 pair is underway.

A recent study proposed that unanchored ubiquitin chains function as an ubiquitin reservoir (31). When cells are exposed to stress conditions, free ubiquitin chains are rapidly disassembled by DUBs to monoubiquitin, which can be then used for substrate conjugation (32). The deubiquitinating enzyme Doa4 and its inhibitor Rfu1 were associated with the rapid disassembly of free ubiquitin chains upon heat shock (31). We propose a model for the function of Etp1 where short-lived Etp1 mediates a formation of free ubiquitin chains (Figure 1). Under normal conditions, these chains may be used for signaling or simply remain as a reserve pool of ubiquitin, while under stress the free polyubiquitin linkages are cleaved by Doa4 to provide ubiquitin for a further substrate conjugation. Indeed, our findings showed DOA4 and ETP1 genetically interact, as there is an additive effect of deletion of DOA4 and ETP1 on the amount of di- and triubiquitin, and tolerance to elevated temperature and hydrogen peroxide.

It is also important to investigate the relationship between the Etp1 and Ubp14. Ubp14 is a DUB that specifically disassembles unanchored ubiquitin chains in yeast (38). Loss of UBP14 has similar effect on yeast phenotypes as loss of DOA4, and deletion of

both genes has an additive effect on the yeast phenotypes (39). We attempted to conduct these analysis but we could not recover the double mutant, suggesting that there might be genetic interactions between ETP1 and UBP14 also. If it turns out that these strains can be constructed we expect that the of deletion of ETP1 from *doa4Δ, ubp14Δ,* and *doa4Δubp14Δ* yeast strains will at least partially rescue the phenotypes of these strains.

Notably, while loss of Etp1 induces drug resistance, mutations of both the RING and ZnF UBP domains confer a gain of function phenotype manifested by an increased sensitivity to paraquat, canavanine, anisomycin, and hydrogen peroxide compared to wild type. This, and the fact that enzymatically inactive Etp1 almost completely restores polyubiquitin levels, strongly suggests that Etp1 forms a complex with other partners that directly catalyze or control these processes.

Interestingly, loss of Rsp5, a HECT E3 ubiquitin ligase, also leads to reduction of overall ubiquitin pool, and the reduced level of ubiquitin synthesis is sustained in *rsp1* mutant upon heat shock (40). Rsp5 contains a noncovalent ubiquitin-binding site in its catalytic HECT domain, which is important for the ligase activity of Rsp5 (41). Rsp5 was the only E3 ubiquitin ligase known to play role in regulation of the yeast ubiquitin homeostasis up to date. In this work, we introduce an E3 of another class, the Etp1 RING E3 ubiquitin ligase that also plays role in the ubiquitin homeostasis in yeast.

In conclusion, we propose that Etp1 is one of the regulators of ubiquitin homeostasis in yeast. Etp1 acts as a ubiquitin sensor, influencing formation of free ubiquitin chains that the cell may use as a reservoir of polyubiquitin. Under normal conditions, these chains may be used for signaling or simply remain as a reserve pool of ubiquitin, while under stress the free polyubiquitin linkages are cleaved by DUBs for a

further substrate conjugation. The exact molecular mechanism of action of Etp1 action is currently not known and is a fertile ground for future studies.

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Appendix I

Preliminary Genetic interactions of ETP1[a)]

1.**MDM10**	13. SCO2	25. MET18	37. MDM34
2. **RPS8A**	14. ETR1	26. INO1	38. DBF2
3. **NPL4**	15. CCZ1	27. RCY1	39. BUB1
4. **BRE1**	16. BUD31	28. CTK1	40. GYP1
5. **INO2**	17. OST4	29. SPE1	41. NEW1
6. **KRE28**	18. HMO1	30. SIC1	42. YAR1
7. **SRB2**	19. UME6	31. RPL37A	43. RPL43A
8. **SWI6**	20. UBP6	32. TSA1	
9. **RSC2**	21. SOH1	33. RPS16A	
10. **SIN4**	22. PMR1	34. EOS1	
11. **LEA1**	23. HUR1	35. IES2	
12. **VPS4**	24. KEM1	36. COQ2	

[a)] This list of 43 genes represents all hits from the SGA analysis.

1-12: Synthetic lethal interaction of the double knock-out strains.

13-43: Growth of the double knock-out strains (size of the colony) is worsened.