The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme

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Saccharomyces cerevisiae Rsp5 is an essential HECT ubiquitin ligase involved in several biological processes. To gain further insight into regulation of this enzyme, we identified proteins that copurified with epitope-tagged Rsp5. Ubp2, a deubiquitinating enzyme, was a prominent copurifying protein. Rup1, a previously uncharacterized UBA domain protein, was required for binding of Rsp5 to Ubp2 both *in vitro* and *in vivo*. Overexpression of Ubp2 or Rup1 in the *rsp5-1* mutant elicited a strong growth defect, while overexpression of a catalytically inactive Ubp2 mutant or Rup1 deleted of the UBA domain did not, suggesting an antagonistic relationship between Rsp5 and the Ubp2/Rup1 complex. Consistent with this model, *rsp5-1* temperature sensitivity was suppressed by either *ubp2Δ* or *rup1Δ* mutations. Ubp2 reversed Rsp5-catalyzed substrate ubiquitination *in vitro*, and Rsp5 and Ubp2 preferentially assembled and disassembled, respectively, K63-linked polyubiquitin chains. Together, these results indicate that Rsp5 activity is modulated by being physically coupled to the Rup1/Ubp2 deubiquitinating enzyme complex, representing a novel mode of regulation for an HECT ubiquitin ligase.

The EMBO Journal (2005) 24, 2414–2424. doi:10.1038/sj.emboj.7600710; Published online 2 June 2005

Subject Categories: proteins

Keywords: deubiquitinating enzymes; HECT ubiquitin ligases; Rsp5; Rup1; Ubp2

Introduction

Ubiquitin is conjugated to substrates by a cooperating set of enzymes known as the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases, with the E3 enzymes conferring recognition of substrates (Pickart, 2001). The two major classes of E3 enzymes are the RING E3s and the HECT E3s. HECT E3s are mechanistically distinct from RING E3s in that they participate directly in the chemistry of protein ubiquitination by forming a reactive ubiquitin-thioester intermediate in the course of catalysis (Scheffner *et al*, 1995). RSP5 is one of five

Saccharomyces cerevisiae genes encoding HECT E3s and is the only one that is essential under normal growth conditions (Wang *et al*, 1999). Lethality of the *rsp5Δ* mutations can be suppressed by the addition of oleic acid to the growth media (Hoppe *et al*, 2000). The basis of oleic acid rescue is Rsp5-mediated ubiquitination of the Spt23 transcription factor, which activates Spt23 by stimulating a proteasome-catalyzed processing event (Rape and Jentsch, 2004). Processed Spt23 activates transcription of the *OLE1* gene, encoding a fatty acid desaturase. Rsp5 has been implicated in other cellular processes, most notably in trafficking of plasma membrane proteins. Rsp5 ubiquitinates several integral plasma membrane proteins, including Fur4 (Galan *et al*, 1996), Gap1 (Hein *et al*, 1995), and Ste2 (Dunn and Hicke, 2001), targeting them for ubiquitin-mediated endocytosis, and can also direct ubiquitin-mediated trafficking of proteins from the *trans*-Golgi network (TGN) to the vacuole (Helliwell *et al*, 2001). The latter requires the function of Bul1 and Bul2, two closely related proteins that interact with Rsp5 and influence some functions of Rsp5 (Kaida *et al*, 2003; Crespo *et al*, 2004).

A large family of deubiquitinating enzymes (DUBs) have the capacity to influence the fate of ubiquitinated proteins (Amerik and Hochstrasser, 2004). There are at least three broad functions for deubiquitinating enzymes: (1) the processing of ubiquitin precursor proteins to generate mature ubiquitin, (2) reversing the polyubiquitination of substrate proteins (Chen *et al*, 2002; Li *et al*, 2002), and (3) facilitating ubiquitin removal at the proteasome, allowing target proteins to be translocated into the proteasome (Amerik and Hochstrasser, 2004). DUBs are therefore capable of either promoting or antagonizing ubiquitin- and proteasome-dependent processes.

While several direct targets and functions of Rsp5 have been identified, the basis of regulation of Rsp5 activities is largely unknown. In order to gain insight into possible control or regulation of Rsp5 activity, we affinity purified Rsp5 along with associated proteins using the tandem affinity purification (TAP) method under conditions where interacting or regulatory proteins can be copurified (Puig *et al*, 2001). A prominent Rsp5-associated protein was the Ubp2 DUB. Rup1, a UBA (Ubiquitin-Associated) domain-containing protein, was found to mediate the Rsp5–Ubp2 interaction, and we present evidence for genetic interactions between Rsp5, Ubp2, and Rup1. Together, our *in vitro* and *in vivo* results indicate that at least a fraction of Rsp5 exists in a complex with Ubp2, and that the Ubp2/Rup1 complex serves to antagonize, and potentially regulate, Rsp5 *in vivo*.

Results

Purification of NTAP-Rsp5 and associated proteins

To identify proteins associated with Rsp5, a yeast strain was generated in which the NTAP (N-terminal TAP) epitope was integrated at the 5' end of the chromosomal RSP5 ORF, so that expression of NTAP-RSP5 was directed by the natural RSP5

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Received: 21 March 2005; accepted: 18 May 2005; published online: 2 June 2005

promoter. The *NTAP-RSP5* strain was fully viable at both 30 and 37°C, and the expression level of NTAP-Rsp5 was similar to that of endogenous Rsp5 in the parental strain (not shown). NTAP-Rsp5 protein was purified from extract of an 8l culture grown to mid-log phase. The first affinity purification step was performed on IgG Sepharose, with release of NTAP-Rsp5 by cleavage with TEV protease. The second affinity step was performed on calmodulin agarose with EGTA elution. The final eluate was concentrated and an aliquot was analyzed by SDS-PAGE and silver staining (Figure 1A). Several prominent bands in addition to Rsp5 were evident, and several were gel-isolated and identified by liquid chromatographic mass spectrometric analysis (LC/MS). The major bands less than 45 kDa were all breakdown products of NTAP-Rsp5, and the band at approximately 95 kDa was full-length Rsp5 (containing the residual CBP epitope). The three prominent bands that migrated with apparent molecular weights greater than Rsp5 were identified as Bul1, Ubp2, and Chc1. Bul1 is a previously identified Rsp5-interacting protein (Yashiroda *et al*, 1996, 1998). Chc1 is clathrin heavy chain and it has been previously suggested that Chc1 might interact with Rsp5 in a Pan1-dependent manner (Wendland and Emr, 1998). We were intrigued by the fact that Ubp2 copurified with Rsp5, since these enzymes have opposing biochemical activities.

The interaction of Rsp5-Ubp2 was confirmed by integrating an HA epitope at the 3' end of the chromosomal Ubp2 ORF in both the *NTAP-RSP5* and parental *RSP5* strains. Extracts from these strains were subject to IgG Sepharose affinity chromatography, and bound proteins were analyzed by SDS-PAGE and immunoblotting with anti-HA antibody. Figure 1B shows that HA-Ubp2 copurified with NTAP-Rsp5. In addition, a strain expressing CTAP-Ubp2 (C-terminal TAP epitope) was used to demonstrate that endogenous Rsp5 protein copurified with CTAP-Ubp2 (Figure 1C). These results strongly suggest that at least a fraction of the cellular pools of Rsp5 and Ubp2 exist in a stable complex *in vivo*.

The Rsp5-Ubp2 interaction is mediated by Rsp1

To determine whether Rsp5 and Ubp2 bound to each other directly *in vitro*, we performed binding assays with bacterially expressed GST-Rsp5 and *in vitro*-translated Ubp2. GST-Rsp5 is enzymatically active and has been shown to bind to several Rsp5-interacting proteins *in vitro* (Huibregtse *et al*, 1997; Salvat *et al*, 2004; Shcherbik *et al*, 2004), yet stable interaction was not detected with Ubp2 (Figure 2A, lane 1). The addition of total yeast cell extract or a high-salt DEAE fraction from cell extract stimulated the binding of Ubp2 to Rsp5 (Figure 2A, lanes 2 and 5), suggesting that an additional factor(s) might mediate the interaction. GST-E6AP, a human HECT E3, did not bind to Ubp2 in the absence or presence of yeast cell extract.

To identify the potential mediator of Rsp5-Ubp2 binding, we performed a large-scale purification of CTAP-tagged Ubp2 and identified copurifying proteins. As expected, Rsp5 was identified in the LC/MS analysis of CTAP-Ubp2-associated proteins (Figure 2B). A prominent Ubp2-associated protein with an apparent molecular weight of approximately 85 kDa was identified by mass spectrometry as Rup1 (YOR138C; calculated molecular weight 75 kDa). Rup1 has an N-terminal UBA domain (amino acids 1–41) but no other discernible functional domains. UBA domains of some proteins have

Figure 1 (A) Purification of NTAP-Rsp5 and associated proteins from extract of an 8l culture (YK001). The final eluate was concentrated and separated by SDS-PAGE and silver stained. The positions and identities of bands identified by LC/MS are indicated; Rsp5* indicates breakdown products of NTAP-Rsp5. Molecular weight markers (kDa) are indicated. (B) Confirmation of the Rsp5-Ubp2 interaction. Extracts were prepared from YK002 (*NTAP-RSP5*, *HA-UBP2*), YK008 (*RSP5*, *HA-UBP2*), and BY4741 (*RSP5*, *UBP2*). Proteins were affinity selected on IgG Sepharose and bound proteins were released with SDS-PAGE loading buffer. Total extracts (lanes 1–3) and eluates (lanes 4–6) were analyzed by SDS-PAGE and immunoblotted with anti-HA antibody. (C) Extracts were prepared from YK005 (*TAP-UBP2*) and BY4741 (*RSP5*, *UBP2*) and proteins were affinity selected on IgG Sepharose. Total extracts (lanes 1 and 2) and eluates (lanes 3 and 4) were analyzed by SDS-PAGE and immunoblotted with anti-Rsp5 antibody.

been shown to bind to polyubiquitin chains (Wilkinson *et al*, 2001; Chen and Madura, 2002; Rao and Sastry, 2002). *RUP1* has been reported to be a nonessential gene

(*Saccharomyces* Genome Database, www.yeastgenome.org), a result that we confirmed (below).

To confirm the Ubp2–Rup1 interaction, HA-Rup1 was expressed in the CTAP-Ubp2 strain and was shown to copurify with CTAP-Ubp2 (Figure 2C). HA-Rup1 also copurified with Rsp5 when expressed in the NTAP-Rsp5 strain (Figure 2C). Rup1 was not identified in the NTAP-Rsp5 purification

Figure 2 A cellular factor mediates the association of Rsp5 and Ubp2. (A) *In vitro*-translated ³⁵S-labelled Ubp2 was incubated with GST-Rsp5 or GST-E6AP immobilized on glutathione Sepharose, in the absence (–lanes) or presence of cell extracts (Ex.; lanes 2 and 7) or fractionated extracts (lanes 3–5) from the *ubp2Δ* strain. Bound Ubp2 was detected by SDS–PAGE and autoradiography. DEAE high-salt fractions were either 150 or 500 mM NaCl eluates; FT represents the flow-through fraction. Input (lane 8) shows 50% of translation mixture used in the binding assays. (B) TAP purification of Ubp2-associating proteins. Proteins from YK005 (*CTAP-UBP2*) were affinity selected on IgG Sepharose followed by elution and cleavage with TEV protease. The eluate (lane 2) was separated on 4–15% gradient gel and stained with Coomassie blue. A parallel purification was performed using the control BY4741 strain (lane 1). Arrows indicate the position of Ubp2, Rsp5, and Rup1 proteins, as identified by LC/MS. Molecular weight markers (kDa) are indicated. (C) HA-Rup1 binds to both NTAP-Rsp5 and CTAP-Ubp2. HA-Rup1 was expressed from a galactose-inducible promoter plasmid in strains YK001 (*NTAP-RSP5*), YK005 (*CTAP-UBP2*), and BY4741 (control, lane 1, not expressing any TAP-tagged protein). Extracts were prepared and TAP proteins purified on IgG Sepharose. Eluates (lanes 1–3) were analyzed by SDS–PAGE and immunoblotting with anti-HA antibody. Lane 4 shows HA-Rup1 in total lysate from BY4741. Detection of NTAP-Rsp5 and CTAP-Ubp2 (lanes 2 and 3, respectively) was a result of anti-mouse IgG secondary antibody recognizing the TAP epitope.

(Figure 1A), due to the fact that the region of the gel where Rup1 migrated was not analyzed in detail because of the large amount of breakdown products of NTAP-Rsp5 protein that migrated in the 80–90 kDa range.

To determine whether Rup1 was responsible for mediating the Rsp5–Ubp2 interaction, a *rup1Δ* mutation was created in the *CTAP-UBP2* strain. CTAP-Ubp2 was purified from this strain, and immunoblotting indicated that the degree of Ubp2–Rsp5 association was greatly decreased relative to the equivalent *CTAP-UBP2/RUP1* strain (Figure 3A, compare lanes 1 and 4). Figure 3A also shows that Rsp5 did not

Figure 3 Rup1 mediates the association of Rsp5 and Ubp2. (A) Cell extracts were prepared from strains expressing the indicated CTAP-tagged Ubp proteins in either an *RUP1* or *rup1Δ* background. Proteins were affinity selected on IgG Sepharose and eluates were analyzed by SDS–PAGE and immunoblotting with anti-Rsp5 antibody. (B) ³⁵S-labelled Ubp2 was assayed for binding to GST-Rsp5 (as in Figure 2A) in the presence of cell extract from either the *rup1Δ* strain (lane 1) or an *RUP1* wild-type strain (lane 2). Lane 3 shows 50% of the input amount of Ubp2 used in the binding reactions. (C) Purified Ubp2, Rup1, and Rup1ΔUBA proteins were used in GST-Rsp5 pull-down assays. A 100% of input amounts of each protein is shown in lanes 6–9. Bound proteins were analyzed by SDS–PAGE and Coomassie blue staining. (D) Rup1, Rsp5, and Ubp2 were ³⁵S-labeled by *in vitro* translation and assayed for binding to purified GST-Rsp5, E6AP, or Rup1. Inputs (lanes 7–9) show 50% of the translation mixture used in the binding assays.

significantly copurify with two other TAP-tagged Ubp proteins, Ubp3 or Ubp4, strongly suggesting that the interaction of Rsp5 with Ubp enzymes is specific for Ubp2. In addition, *RUP1*⁺ yeast cell extract stimulated the binding of *in vitro*-translated Ubp2 to GST-Rsp5, while cell extract from an *rup1Δ* strain did not (Figure 3B).

To determine if Rup1 was sufficient for mediating the association in a purified system, Rsp5, Ubp2, and Rup1 were individually expressed as GST fusion proteins in bacteria. The proteins were purified and the GST moieties were removed from Ubp2 and Rup1 by site-specific proteolysis. GST-Rsp5, on glutathione sepharose, did not stably bind to purified Ubp2 (Figure 3C, lane 1). The addition of purified Rup1 stimulated Ubp2 binding (lane 4), indicating that Rup1 is sufficient for mediating the Rsp5–Ubp2 interaction. Furthermore, GST-Rsp5 bound directly to Rup1 in the absence of Ubp2, indicating that the interaction between these two proteins is direct. These experiments were

also performed with a truncated form of Rup1, lacking the N-terminal UBA domain (Rup1-ΔUBA; lacking amino acids 2–40). Rup1-ΔUBA protein bound to Rsp5 similarly to full-length Rup1 and also mediated the interaction with Ubp2. Figure 3D confirms that GST-Rup1 binds to *in vitro*-translated Rsp5 in the absence of Ubp2, as well as to *in vitro*-translated Ubp2 in the absence of Rsp5. GST-E6AP did not interact with either Rup1, Ubp2, or Rsp5. Together, the *in vitro* binding results indicate that Rup1 can interact directly with both Rsp5 and Ubp2, as well as simultaneously with both proteins.

Domains that mediate formation of the ternary complex

To define the domains of Rsp5 necessary for binding to Rup1 and Ubp2, a series of N- and C-terminally truncated Rsp5 proteins were expressed either as GST fusion proteins or as *in vitro*-translated proteins (Rsp5 proteins A–I; Figure 4A). The smallest region of Rsp5 capable of interacting with GST-Rup1 included both the C2 domain and the first WW domain (Rsp5-H; Figure 4B). Figure 4C shows binding of *in vitro*-translated Ubp2 to GST-Rsp5 proteins A–E in the presence of purified Rup1 protein. Full-length GST-Rsp5 bound to Ubp2, but deletion of either the HECT domain or the region span-

Figure 4 Domains of Rsp5 required for binding to Rup1 and Ubp2. (A) Schematic of Rsp5 truncation mutants used in binding assays. (B) Binding of GST-Rup1 to Rsp5 proteins. ³⁵S-labeled *in vitro*-translated Rsp5 proteins were incubated with GST-Rup1 and bound proteins were resolved by SDS–PAGE and visualized by autoradiography. Input amounts (left panel) represent 50% of the translations used in the binding reaction (right panel). (C) Binding of GST-Rsp5 proteins to Ubp2. Purified GST fusion Rsp5 proteins, on glutathione Sepharose, were incubated with ³⁵S-labeled *in vitro*-translated Ubp2 in the presence (lanes 2–6) or absence (lane 1) of purified Rup1, and bound proteins were resolved by SDS–PAGE and visualized by autoradiography. Input (lane 7) indicates 50% of translation used in the binding reaction.

Figure 5 Domains of Ubp2 required for binding to Rsp5 and Rup1. (A) Schematic of Ubp2 truncation mutants used in binding assays. (B) Binding of GST-Rup1 to Ubp2 proteins. ³⁵S-labeled *in vitro*-translated Ubp2 proteins were incubated with GST-Rup1 and bound proteins were resolved by SDS–PAGE and visualized by autoradiography. Input amounts (left panel) represent 50% of the translations used in the binding reaction (right panel). * indicates predicted size of primary translation product for Ubp2-D. (C) Binding of GST-Rsp5 to Ubp2, in the presence of Rup1. ³⁵S-labeled *in vitro*-translated Ubp2 proteins were incubated with GST-Rsp5 in the presence of added Rup1 protein, and bound proteins were resolved by SDS–PAGE and visualized by autoradiography. Input amounts (left panel) represent 50% of the translations used in the binding reaction (right panel).

ning the C2 domain abrogated Ubp2 binding. Therefore, determinants for Ubp2 interaction span a large region of Rsp5 and extend beyond what is sufficient for binding to Rup1. In addition, Rsp5 deleted of the last six amino acids (Rsp5-E), which disrupts ubiquitination activity (Salvat *et al*, 2004), as well as full-length Rsp5 containing the active-site Cys to Ala mutation (not shown), bound to Ubp2 similarly to wild-type Rsp5, indicating that catalytic activity of Rsp5 is not necessary for Ubp2 association.

N- and C-terminally truncated Ubp2 proteins (Ubp2 proteins A–E; Figure 5A) were expressed by *in vitro* translation and assayed for GST-Rup1 binding (Figure 5B) and GST-Rsp5 binding in the presence of Rup1 (Figure 5C). The C-terminal 322 residues of Ubp2 (Ubp2-E) were sufficient to mediate direct binding to Rup1, and the same region was sufficient for Rup1-mediated Rsp5 association. Together with the results shown in Figure 3C, we conclude that (1) the UBA domain of Rup1 is not necessary for formation of the ternary complex, (2) the C-terminal 322 residues of Ubp2 are sufficient for interaction with both Rup1 and the Rsp5/Rup1 complex, (3) the region spanning the C2 domain and first WW domain of Rsp5 are sufficient for interaction with Rup1, and (4) in addition to determinants required for Rup1 binding, determinants within the HECT domain are also necessary for stable association of Rsp5 with Ubp2.

Genetic interactions between Rsp5, Ubp2, and Rup1

Since Rsp5 and Ubp2 catalyze opposing reactions, we hypothesized that Ubp2 might exist in a complex with Rps5 in order to antagonize and potentially regulate Rsp5 activity. To test this hypothesis, we overexpressed Ubp2 under control of a galactose-inducible promoter in a wild-type *RSP5* strain (FY56) and in the *rsp5-1* mutant (FW1808). The premise was that overexpression of Ubp2 would cause a reduction in effective Rsp5 activity and therefore mimic *rsp5* loss-of-function mutations. The *rsp5-1* mutation is located within the HECT domain (L733S) and impairs enzymatic activity of the purified protein *in vitro* (Wang *et al*, 1999). The *rsp5-1* strain (FW1808) grows normally at 30°C, but undergoes a rapid non-cell-cycle-specific growth arrest at 37°C. A control vector (empty pYES2) and pYES2 expressing wild-type Ubp2 or the active-site mutant of Ubp2 (C745S) were introduced into the *rsp5-1* strain and a wild-type *RSP5* strain (FY56). A slight growth defect was seen in the *RSP5* background when expression of Ubp2 was induced by galactose, and this effect was not seen with the C745S mutant (Figure 6A). This effect was greatly enhanced in the *rsp5-1* background, suggesting that Ubp2 antagonizes Rsp5 activity. While overexpression of Ubp3 or Ubp4 resulted in a slight growth inhibition of the *rsp5-1* strain, a similar effect was seen in the *RSP5* strain (Figure 6B), suggesting that this is unlikely to be related to

Figure 6 Overexpression of Ubp2 or Rup1 inhibits growth of the *rsp5-1* mutant. (A) FY56 (*RSP5*) and FW1808 (*rsp5-1*) were transformed with pYES2 (vector), pYES-UBP2, or pYES-ubp2 C745S plasmids. The transformants were serially diluted (10-fold at each step) and plated onto either dextrose (Dex.) or galactose (Gal.) media and grown for 2 and 3 days, respectively, at 30°C. (B) FY56 (*RSP5*) and FW1808 (*rsp5-1*) were transformed with empty vector, or pYES-UBP2, UBP3, or UBP4 plasmids and serially diluted on galactose media and grown for 3 days at 30°C. (C) FW1808 (*rsp5-1*) was transformed with empty vector, pYES-RUP1, and pYES-rup1-ΔUBA plasmids and serial dilutions were plated onto either dextrose or galactose media and grown for 3 and 4 days, respectively, at 34°C.

effects on Rsp5 activity. Overexpression of HA-Rup1 did not inhibit growth of either the *RSP5* or *rsp5-1* strains at 30°C (not shown); however, a growth defect was seen upon overexpression of HA-Rup1 at 34°C in the *rsp5-1* strain (Figure 6C). Overexpression of HA-Rup1 deleted of the UBA domain did not elicit a growth defect, suggesting that, while the UBA domain is not necessary for stable ternary complex formation *in vitro*, this domain is important for mediating the antagonistic effect of Ubp2 on Rsp5 *in vivo*. Anti-HA immunoblotting showed that the full-length and Δ UBA proteins were expressed at the same levels (not shown).

We further predicted that if *UBP2* and *RUP1* cooperate to antagonize *RSP5* activity, then gene deletion of either *UBP2* or *RUP1* might rescue the temperature sensitivity of the *rsp5-1* mutant by effectively increasing Rsp5 activity. As shown in Figure 7A, both the *ubp2 Δ* and *rup1 Δ* mutations partially

rescued the temperature sensitivity of the *rsp5-1* mutation at 37°C. An essential function of Rsp5 at 30°C has been shown to be ubiquitin-mediated activation of the Spt23 and Mga2 transcription factors, which are necessary for *OLE1* gene expression and biosynthesis of oleic acid. We therefore tested whether the growth defect due to Ubp2 overexpression was related to a deficiency in oleic acid biosynthesis by attempting to suppress the *UBP2* overexpression phenotype with exogenous oleic acid. Growth was weakly rescued by oleic acid (Figure 7B), suggesting that Ubp2 functions, in part, to antagonize the ability of Rsp5 to activate the Spt23 and/or Mga2 transcription factors. However, the fact that rescue by oleic acid was weak indicates that the Rsp5–Ubp2 enzyme pair affects more than a single aspect of fitness at 30°C.

Temperature sensitivity of the *rsp5-1* mutant was rescued by the addition of 1 M sorbitol to the growth media

Figure 7 Genetic interactions between *RSP5*, *RUP1*, and *UBP2*. (A) *ubp2 Δ* or *rup1 Δ* mutations partially rescue the temperature sensitivity phenotype of the *rsp5-1* mutant. *RSP5* (FY56), *rsp5-1* (FW1808), *rsp5-1, ubp2 Δ* (YK003), and *rsp5-1, rup1 Δ* (YK004) strains were serially diluted and grown on dextrose-containing media at 30 or 37°C for 2 and 4 days, respectively. (B) Exogenous oleic acid partially suppresses the growth inhibition due to Ubp2 overexpression. FW1808 (*rsp5-1*) transformed with pYES2 (vector), pYES-UBP2 (in duplicate), or pYES-ubp2 C745S was streaked onto galactose-containing media, in the absence or presence of added oleic acid, and grown at 30°C for 4 days. (C) The Ubp2 overexpression phenotype in the *rsp5-1* mutant is rescued by 1 M sorbitol. Left panel: *RSP5* (FY56) and *rsp5-1* (FW1808) strains were plated on dextrose-containing media at 37°C, with or without 1 M sorbitol. Right panel: The indicated FW1808 transformants were serially diluted and plated onto galactose-containing media at 30°C, with or without the addition of 1 M sorbitol to the media. The – sorbitol plate was grown for 3 days, and the + sorbitol plate was grown for 4 days. (D) Toxicity due to Spt23 overexpression is suppressed by *rsp5-1* and enhanced by *ubp2 Δ* . Upper panel: The pYES-SPT23 plasmid was transformed into FY56 (*RSP5*) or FW1808 (*rsp5-1*) and plated on galactose- or dextrose-containing media. Lower panel: The empty pYES vector or pYES-SPT23 plasmid was transformed into either *UBP2*, *ubp2 Δ* , or *ubp5 Δ* strains and plated onto galactose- or dextrose-containing media and grown for 4 days at 30°C.

(Figure 7C), as previously shown for the *rsp5-101* mutant (Yashiroda *et al*, 1996), suggesting that ubiquitination of one or more targets of Rsp5 is important for osmotic stability. We therefore predicted that the Ubp2 overexpression phenotype in the *rsp5-1* background at 30°C might be due, in part, to osmotic instability. This was confirmed, as shown in Figure 7C. The suppression of the *UBP2* overexpression phenotype by 1 M sorbitol was significantly more robust than rescue by oleic acid, suggesting that the predominant defect due to overexpression of Ubp2 is related to osmotic instability rather than oleic acid metabolism.

The genetic interactions described above indicate that elevated Ubp2 activity, relative to Rsp5 activity, mimics loss of function of Rsp5. We were interested in whether the opposite scenario—elevated Rsp5 activity relative to Ubp2—resulted in a discernible phenotype. While *S. cerevisiae* requires oleic acid for survival, an excess of oleic acid, through hyperactivation of *OLE1* gene transcription, for example, is also toxic (Stukey *et al*, 1989; Hoppe *et al*, 2000). Consistent with this, overexpression of Spt23 from a galactose-inducible promoter resulted in a strong growth defect in a wild-type *RSP5* background (Figure 7D). Because Rsp5 is required for ubiquitin-mediated activation of Spt23, we predicted that the growth defect due to overexpression of Spt23 would be diminished in the *rsp5-1* background, and this was indeed the case (Figure 7D). Furthermore, Spt23 overexpression in the *ubp2Δ* mutant enhanced the toxicity due to Spt23 overexpression in the *RSP5* background. These results indicate that elevated Rsp5 activity relative to Ubp2 activity results in hypersensitivity to the effects of Spt23 overexpression

and further substantiates an antagonistic relationship between Rsp5 and Ubp2.

Ubp2 reverses Rsp5-catalyzed ubiquitination *in vitro*

To test whether Ubp2 directly opposes Rsp5 activity *in vitro*, we performed *in vitro* ubiquitination assays with two substrates of Rsp5, Spt23 and WBP2. Spt23 is a biologically relevant Rsp5 substrate, while WBP2 is a human protein that is recognized by Rsp5 and Nedd4, a human homolog of Rsp5 (Salvat *et al*, 2004). These assays were performed with yeast Ubc1 as the E2 enzyme, which has been shown to function with Rsp5 *in vivo* (Dunn and Hicke, 2001). As shown in Figure 8A, Rsp5 efficiently catalyzed polyubiquitination of *in vitro*-translated Spt23 and WBP2. The addition of Rup1, 30 min after initiation of the ubiquitination reaction, did not affect ubiquitination of Spt23 or WBP2, while the addition of Ubp2 resulted in a significant disassembly of the ubiquitin conjugates. The addition of both Rup1 and Ubp2 resulted in enhanced deubiquitination. Therefore, Ubp2 can reverse Rsp5-catalyzed ubiquitination *in vitro*, and Rup1, while not absolutely required, stimulated this activity of Ubp2.

Ubp2 and Rup1 did not affect ubiquitination of two substrates of human E6AP (p53 and Scribble; Figure 8B). However, E6AP catalyzes almost exclusively K48-linked chains *in vitro* (Linares *et al*, 2003), while at least some of the natural substrates of Rsp5 are modified by K63-linked polyubiquitin chains *in vivo* (Galan and Haguenauer-Tsapis, 1997; Fisk and Yaffe, 1999; Dupre and Haguenauer-Tsapis, 2001; Soetens *et al*, 2001). We therefore investigated the relative preference of Rsp5 and Ubp2 for K63 chain assembly

Figure 8 Ubp2 antagonizes Rsp5-catalyzed ubiquitination *in vitro*. (A) Rsp5 ubiquitination assays utilized ³⁵S-labeled *in vitro*-translated Spt23 and WBP2. Each reaction contained added E1, E2 (Ubc1), Ub, and ATP in the absence or presence of Rsp5. At 30 min after initiating the ubiquitination reactions, Rup1 and/or Ubp2 (two different amounts) were added, followed by an additional 30 min incubation. Total reaction products were analyzed by SDS-PAGE and autoradiography. (B) E6AP ubiquitination of p53 and Scribble was performed in the presence of added HPV39 E6 protein, E1, and E2 (UbcH7), followed by the addition of Rup1 and/or Ubp2 as described above, and total reaction products were analyzed by SDS-PAGE and autoradiography.

and disassembly, respectively. We performed ubiquitination assays in the presence of ubiquitin in which all lysine residues were altered to arginine except for lysine 48 (K48-only ubiquitin) or lysine 63 (K63-only ubiquitin). The endogenous ubiquitin present in the *in vitro* translation reactions was first depleted by anion exchange chromatography, and as expected, there was no detectable ubiquitination in the absence of added ubiquitin (Figure 9A). The addition of wild-type ubiquitin, K48-only ubiquitin, or K63-only ubiquitin resulted in a similar degree of overall substrate modification (i.e., the amount of remaining unmodified substrate was similar; lanes 3–5). However, the average length of the polyubiquitin chains was shorter in the presence of K48-only ubiquitin relative to wild-type ubiquitin, while the average chain length was longer in the presence of K63-only ubiquitin. When Ubp2 was added, the extent of deubiquitination was similar in the reactions containing wild-type and K63-only ubiquitin, while there was significantly less deubiquitination seen in the K48-only reaction. Experiments with ubiquitin in which all lysines were mutated to arginines suggested that much of the multiubiquitination observed with K48-only ubiquitin was actually the result of monoubiquitination at multiple lysines of the substrate (not shown). Together, these results suggest that Rsp5 preferentially assembles K63-linked chains, while Ubp2 preferentially disassembles K63 chains. Similar experiments with E6AP confirmed that E6AP catalyzed almost exclusively K48-linked chains to both p53 and Scribble, and again, Ubp2 did not disassemble these conjugates (not shown).

To confirm the apparent preference of Ubp2 for K63-linked chains, deubiquitination assays were carried out using free purified K63 or K48 chains as substrates over a 100-fold range of Ubp2 concentration. As shown in Figure 9B, at equivalent enzyme concentrations, Ubp2 showed a strong preference for hydrolysis of K63-linked chains, although there was reactivity against K48-linked chains at higher concentrations of Ubp2. Figure 9C shows that, at the lower concentrations of Ubp2, Rup1 enhanced the activity of Ubp2 against free K63 chains. This effect was dependent on the UBA domain of Rup1, suggesting that the UBA domain plays a role in antagonizing Rsp5 function beyond simply bridging the Rsp5–Ubp2 interaction.

Discussion

The results presented here represent a unique demonstration of the physical coupling of an HECT ubiquitin ligase with a DUB for the purpose of modulating substrate modification. At least four examples of specific interactions between ubiquitin ligases and DUBs have been previously reported. These involve the human Nrdp1 ligase and USP8 (Wu *et al*, 2004), the herpes simplex virus ICP0 ligase and USP7 (Canning *et al*, 2004), the TRAF2/TRAF6 ligases and CYLD (Brummelkamp *et al*, 2003; Kovalenko *et al*, 2003), and the VHL ligase and the VDU1 and VDU2 DUBs. The ligases in the first three cases are RING domain E3s that undergo auto-ubiquitination, and in all cases, the DUB has been proposed to reverse the auto-ubiquitination. In contrast, our results strongly suggest that Ubp2 modulates ubiquitination of Rsp5 substrates, including Spt23, rather than self-ubiquitination of Rsp5. Rsp5 has not been shown to undergo auto-ubiquitination *in vivo*, consistent with the observations that

Figure 9 Rsp5 and Ubp2 preferentially assemble and disassemble K63-linked polyubiquitin chains. **(A)** Rsp5-catalyzed ubiquitination reactions were carried out with WBP2 as a substrate, as in Figure 8A, except that the endogenous ubiquitin present in the translation reaction was first removed by anion exchange chromatography. The reactions were then performed in the absence of added ubiquitin (left panel, lane 2) or the presence of wild-type ubiquitin, K48-only ubiquitin, or K63-only ubiquitin (left panel, lanes 3–5). Deubiquitination (lanes 6–8) was initiated after 30 min by the addition of Ubp2 and Rup1. Control reactions (lanes 9–11) show that the ubiquitination reaction was dependent on added E2 (Ubc1) and Rsp5. **(B)** Purified K63 and K48 polyubiquitin chains (Ub₃–Ub₇; Boston Biochem) were assayed as substrates of purified Ubp2 protein over a 100-fold range of Ubp2 concentration. The deubiquitination reactions were performed for 30 min at room temperature. Products were analyzed by 12% SDS–PAGE and Coomassie blue staining. **(C)** Rup1 stimulates Ubp2-catalyzed disassembly of K63 chains, dependent on the UBA domain. Disassembly of free K63 chains was assayed at the two lowest concentrations of Ubp2 shown in panel B, in the absence (–) or presence of purified Rup1 (WT) or Rup1ΔUBA (Δ) proteins. Products were analyzed by SDS–PAGE and Coomassie blue staining.

the half-life of wild-type Rsp5 is similar to that of the active-site cysteine mutant of Rsp5 (Wang *et al*, 1999), and overexpression or deletion of Ubp2 does not affect the steady-state level of Rsp5 (data not shown). More similar to the Rsp5–Ubp2 relationship, the VHL-associated DUB, VDU2, appears to rescue a VHL substrate (HIF-1α) from degradation (Li *et al*, 2005). In this case, however, VDU2 interacts directly with the substrate, whereas our results suggest that substrate specificity of Ubp2 is likely to be conferred through its Rup1-dependent association with Rsp5.

Rsp5 and Ubp2 only formed a stable complex in the presence of Rup1, and genetic relationships were consistent with the notion that Rup1 cooperates with Ubp2 to antagonize Rsp5 activity. While the UBA domain of Rup1 was not required to mediate formation of the ternary complex *in vitro*, the UBA domain was necessary for stimulation of Ubp2 activity against free K63 chains at limiting concentrations of Ubp2. It will be of interest to determine whether the Rup1 UBA domain binds polyubiquitin chains, as reported for other UBA domains (Wilkinson *et al*, 2001; Chen and Madura, 2002; Rao and Sastry, 2002), and whether it interacts preferentially with K63-linked chains. We speculate that the UBA domain might aid in presenting polyubiquitin chains to Ubp2 or, alternatively, might aid in stabilizing an active conformation of Ubp2. The chain specificity of Ubp2 was the same in the absence or presence of Rup1, indicating that this is an inherent characteristic of the enzyme, rather than an Rsp5- or Rup1-dependent effect. A few DUBs have been reported to disassemble both K48- and K63-linked chains *in vitro*, including Cezanne/A20 (Evans *et al*, 2004; Wertz *et al*, 2004), Ubp14/isopeptidase T (Falquet *et al*, 1995), and CYLD (Kovalenko *et al*, 2003; Trompouki *et al*, 2003), while AMSH, a JAMM motif isopeptidase, has been shown to have a strong preference for disassembly of K63 chains (McCullough *et al*, 2004).

Rsp5 is the first HECT E3 to be shown to display a distinct preference for assembly of K63-linked polyubiquitin chains *in vitro*. Human E6AP preferentially catalyzes K48 linkages, consistent with its role in targeting p53 for proteasomal degradation (Scheffner and Whitaker, 2003), while the synthesis of K63 chains by Rsp5 is consistent with reports that have shown that some Rsp5-mediated functions are dependent on K63 of ubiquitin (Galan and Haguenaer-Tsapis, 1997; Fisk and Yaffe, 1999; Soetens *et al*, 2001). Rsp5 has also been linked to proteasomal degradation pathways (Beaudenon *et al*, 1999; Erdeniz and Rothstein, 2000), which are predicted to involve K48 or K29 linkages (Pickart and Fushman, 2004). The type of chain synthesized by Rsp5 might be regulated by additional factors or perhaps substrate dependent. It should be noted that the preferential assembly of K63 chains by Rsp5 was seen when the activating E2 enzyme was either yeast Ubc1, human UbcH7, or *Arabidopsis* Ubc8 (not shown), suggesting that K63 chain assembly is an

inherent characteristic of Rsp5 and not a function of the activating E2 enzyme.

While Ubp2 and Rsp5 catalyze opposing reactions, it was conceivable that coupling of Ubp2 to Rsp5 activities might promote, rather than antagonize, at least a subset of Rsp5 functions. For example, Spt23 has been reported to be monoubiquitinated *in vivo* (Rape *et al*, 2001). Ubp2 could conceivably promote Rsp5 function by limiting chain extension to favor monoubiquitination. In contrast, there are examples of DUBs that rescue substrates from ubiquitination (Chen *et al*, 2002; Li *et al*, 2002). The observed genetic interactions between *RSP5* and *UBP2* are most consistent with the latter examples, and suggest a model in which Ubp2 rescues Rsp5 substrates from ubiquitination. An antagonistic relationship between Rsp5 and Ubp2 was supported by the observations that (1) overexpression of Ubp2 in the *rsp5-1* background resulted in a strong growth suppression, (2) *rsp5-1* temperature sensitivity was rescued by either the *ubp2Δ* or *rup1Δ* mutations, and (3) the *ubp2Δ* mutation sensitized cells to the effects of Spt23 overexpression. It is not known whether all functions of Rsp5, or only a subset, are subject to modulation by Ubp2.

There are nine human homologs of Rsp5 (e.g., WWP1/2, Smurf1/2, Nedd4, Itch) involved in various functions, including disease states (Liddle's syndrome, Epstein-Barr virus latency), the life cycle of several budding viruses, and TGFβ signaling (reviewed in Ingham *et al*, 2004). The results presented here raise the question of whether the activities of these enzymes are also modulated by physically coupled DUBs. The fact that many of the functions of the mammalian Rsp5 homologs are also related to trafficking of membrane proteins suggests that similar regulatory mechanisms might be utilized.

Materials and methods

Yeast strains, media, and plasmids

A list of yeast strains is shown in Table I. Growth media containing oleic acid contained 0.5 mM oleic acid (Sigma) in the presence of 1% Tergitol. The *NTAP-RSP5* strain (YK001) was generated using FY56 as the parental strain. A C-terminally truncated *RSP5* mutant fragment was subcloned into pYES2 vector (Invitrogen) deleted of the 2μ origin, with 500 bp of the *RSP5* upstream sequence inserted in place of *P_{GALI}*. The construct was transformed into FY56 and colonies were selected on Ura⁻ plates. Clones that expressed

Table I Yeast strains used in this study

Strain	Genotype	Source
FY56	MATα <i>RSP5 his4-912δR5 lys2-128δ ura3-52</i>	Huibregtse <i>et al</i> (1997)
FW1808	MATα <i>rsp5-1 his4912δ lys2-128δ ura3-52</i>	Huibregtse <i>et al</i> , (1997)
GW047	MATa <i>rsp5Δ::LEU2 ade2-1 his3-11 can1-100</i> [pRS414- <i>rsp5prom-RSP5</i>]	Wang <i>et al</i> (2001)
GW048	MATa <i>rsp5Δ::LEU2 ade2-1 his3-11 can1-100</i> [pRS414- <i>rsp5prom-rsp5-1</i>]	Wang <i>et al</i> (2001)
YK001	MATα <i>NTAP-RSP5 his4-912δR5 lys2-128δ ura3-52</i>	This study
YK002	MATα <i>NTAP-RSP5 ubp2Δ::UBP2-3XHA::KanMX6 his4-912δR5 lys2-128δ ura3-52</i>	This study
YK003	MATα <i>rsp5-1 ubp2Δ::KanMX6 his4912δ lys2-128δ ura3-52</i>	This study
YK004	MATα <i>rsp5-1 rup1Δ::KanMX6 his4912δ lys2-128δ ura3-52</i>	This study
BY4741	MATa <i>his3 leu2 met15 ura3</i>	Open Biosystems
YK005	MATa <i>TAP-UBP2::HIS3 leu2 met15 ura3</i>	Open Biosystems
YK006	MATa <i>TAP-UBP2::HIS3 rup1Δ::KanMX6 leu2 met15 ura3</i>	This study
YK007	MATa <i>TAP-RUP1::HIS3 leu2 met15 ura3</i>	This study
YK008	MATa <i>ubp2Δ::UBP2-3XHA::KanMX6 his3 leu2 met15 ura3</i>	This study
YK009	MATa <i>ubp2Δ::KanMX6 his3 leu2 met15 ura3</i>	Open Biosystems
YK010	MATa <i>rup1Δ::KanMX6 his3 leu2 met15 ura3</i>	Open Biosystems
YK011	MATa <i>TAP-UBP3::HIS3 leu2 met15 ura3</i>	Open Biosystems
YK012	MATa <i>TAP-UBP4::HIS3 leu2 met15 ura3</i>	Open Biosystems

full-length NTAP-Rsp5 and truncated (untagged) Rsp5 protein were screened by anti-Rsp5 immunoblotting. Clones were then selected on uracil/5-FOA-containing plates, and clones that expressed NTAP-Rsp5 as the sole source of Rsp5 were isolated (strain YK001). Deletion of the complete *UBP2* or *RUP1* ORFs (strains YK003, YK004, and YK006) was carried out using a PCR-based method with KanMX6 selection (Longtine *et al*, 1998). C-terminal 3xHA tagging of genomic *UBP2* in YK001 and BY4741 strains (YK002 and YK008, respectively) was carried out similarly, using a *HIS3* marker. Full-length ORFs of HA-*UBP2*, HA-*RUP1*, HA-*UBP3*, HA-*UBP4*, and Flag-SPT23 were PCR-amplified from yeast genomic DNA. The active-site mutation of *UBP2* (*ubp2-C745S*) was generated by site-directed mutagenesis. Other *RSP5*, *UBP2*, and *RUP1* mutants were generated by PCR amplification from each full-length ORF. All ORFs were subcloned into pYES2 vector for *in vitro/in vivo* expression or pGEX-6p-1 vector (Amersham) for bacterial expression of GST fusion proteins.

TAP protein purification

The TAP purification procedure was similar to that described previously (Puig *et al*, 2001). An 8 l culture of strain YK001 was grown in YPD to an OD₆₀₀ of 1.2, at 30°C. The cell pellet was resuspended in NP-40 lysis buffer (0.5% NP-40, 150 mM NaCl, 10 mM Tris 8.0, supplemented with protease inhibitors) and cells were disrupted with a bead beater. The extract was cleared by centrifugation at 31000g for 15 min at 4°C. IgG Sepharose (Amersham) was added and incubated with mixing for 4 h at 4°C. The beads were washed in NP-40 lysis buffer, followed by TEV cleavage buffer (0.1% NP-40, 150 mM NaCl, 10 mM Tris pH 8.0, 0.5 mM EDTA, 1.0 mM DTT), and TEV protease was added and incubated at room temperature for 2 h. The supernatant was diluted with calmodulin binding buffer (150 mM NaCl, 10 mM Tris pH 8.0, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl₂, 10 mM β-mercaptoethanol) and Calmodulin Sepharose (Amersham) was added and incubated for 1.5 h at 4°C. After washing, bound proteins were eluted in buffer containing 150 mM NaCl, 10 mM Tris pH 8.0, 0.02% NP-40, 1 mM Mg²⁺ acetate, 1 mM imidazole, 20 mM EGTA, 10 mM DTT). The final eluate was TCA-precipitated, resuspended in 1 × SDS-PAGE loading buffer, and loaded on a 4–15% gradient SDS-PAGE gel. Gels were stained with either silver or Coomassie blue. Bands were excised from a Coomassie blue-stained gel and subjected to in-gel tryptic digest. The fragmented peptides were analyzed by LC/MS. The peptide sequence information was used to search for protein identification using the Mascot (Matrix Sciences) search engine. For confirmation of protein interactions, small-scale TAP purifications were performed. Cell extracts from each TAP strain was subject to IgG Sepharose purification, with release of bound proteins by SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE and immunoblotting was performed using indicated antibodies.

In vitro protein interaction assays

GST fusion proteins were expressed from the pGEX-6p-1 vector in *Escherichia coli* DH5α and purified by standard methods on glutathione Sepharose. ³⁵S-labeled proteins were synthesized *in vitro* using a coupled transcription-translation rabbit reticulocyte system (Promega). Yeast cell extracts for binding assays were prepared by growing cells in YPD until mid-log phase, resuspending the pellets in NP-40 lysis buffer, and lysing the cells with a bead

beater. Cell extracts were cleared by centrifugation at 27000g for 10 min. Either 50 μg of whole-cell extracts was used for each binding assay or equal volumes of fractions from a DEAE ion exchange column. Complex formation using purified proteins (Rsp5, Ubp2, and Rup1 or Rup1ΔUBA) was performed using GST fusions. Ubp2, Rup1, and Rup1ΔUBA were cleaved from GST by PreScission protease (Amersham). Binding reactions were performed using GST-Rsp5 on glutathione beads and each of the other free proteins for 2 h at 4°C. Total protein was recovered from the washed beads by elution with SDS-PAGE loading buffer.

For mapping of domains that mediate the ternary complex, bacterially expressed and purified GST-Rsp5 proteins (Wang *et al*, 1999) and *in vitro*-translated wild-type Ubp2 were used, along with purified Rup1 protein from bacteria. To map regions of Rsp5 that are required for binding to Rup1, bacterially purified GST-Rup1 and *in vitro*-translated Rsp5 proteins were used. To map regions of Ubp2 that are required for binding to Rsp5 or Rup1, purified GST-Rsp5, Rup1, and *in vitro*-translated Ubp2 proteins were used. Binding reactions were performed as described above.

In vitro ubiquitination and deubiquitination assays

In vitro ubiquitination/deubiquitination assays were performed in the presence of 10 mM Tris pH 7.5, 50 mM NaCl, 5 mM ATP, 5 mM MgCl₂, 0.1 mM DTT, and 50 μg/ml ubiquitin (Sigma). Purification of baculovirus-expressed E6AP, HPV39 E6, human E1, and human E2 (UbcH7) was performed as described previously (Salvat *et al*, 2004). Bacterially expressed wild-type Rsp5, Rsp5-C777A, Ubp2, Rup1, Rup1ΔUBA, and yeast E2 (Ubc1) were purified on glutathione Sepharose and cut with PreScission protease (Amersham). WBP2, p53, hScribble, and Spt23 were *in vitro* translated in the presence of [³⁵S]methionine. The translation reaction (3–4 μl) was used for each ubiquitination/deubiquitination reaction. The ubiquitination reactions were carried out for 30 min at room temperature, followed by an additional 30 min incubation in the presence or absence of Rup1 and/or Ubp2. For the assays using K48-only and K63-only ubiquitin (Boston Biochem), the *in vitro*-translated substrates were first partially purified by DEAE anion exchange column to remove endogenous ubiquitin. The reactions were performed as described above and stopped by addition of SDS-PAGE loading buffer and products were analyzed by SDS-PAGE and autoradiography. The deubiquitination assays using free ubiquitin chains utilized 3 μg of either K48 or K63 polyubiquitin chains_{3–7} (Boston Biochem), and were incubated with 0.15–15 ng of Ubp2 with or without Rup1 or Rup1ΔUBA for 1 h at room temperature in buffer containing 10 mM Tris pH 7.5, 50 mM NaCl, 5 mM MgCl₂, and 0.1 mM DTT. The reactions were stopped by SDS-PAGE loading buffer and products were analyzed by 12% SDS-PAGE and staining with Coomassie blue.

Acknowledgements

We thank Klaus Linse (Institute for Cellular and Molecular Biology Core Facility) for performing LC/MS protein identification, Sylvie Beaudenon for helpful suggestions and discussions, and Melissa Kelley for critical reading of the manuscript. This work was supported by grants to JMH from the National Institutes of Health (CA072943) and the Advanced Research Program of the Texas Higher Education Coordinating Board.

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