Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation

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In eukaryotes, endoplasmic reticulum-associated degradation (ERAD) functions in cellular quality control and regulation of normal ER-resident proteins. ERAD proceeds by the ubiquitin–proteasome pathway, in which the covalent attachment of ubiquitin to proteins targets them for proteasomal degradation. Ubiquitin–protein ligases (E3s) play a crucial role in this process by recognizing target proteins and initiating their ubiquitination. Here we show that Hrd1p, which is identical to Der3p, is an E3 for ERAD. Hrd1p is required for the degradation and ubiquitination of several ERAD substrates and physically associates with relevant ubiquitin-conjugating enzymes (E2s). A soluble Hrd1 fusion protein shows E3 activity *in vitro* — catalysing the ubiquitination of itself and test proteins. In this capacity, Hrd1p has an apparent preference for misfolded proteins. We also show that Hrd1p functions as an E3 *in vivo*, using only Ubc7p or Ubc1p to specifically program the ubiquitination of ERAD substrates.

The substrates for ERAD include a diverse array of both misfolded proteins and regulated ER proteins[1]. Genetic analyses have shown that many ER proteins are degraded by the ubiquitin–proteasome system, whereby an E2 transfers multiple copies of the small protein ubiquitin to proteins in order to target them for degradation by the proteasome[2]. E3s specify E2-mediated ubiquitination by designating both the protein(s) targeted for degradation and the E2(s) that transfer(s) ubiquitin to the target protein. Despite their important function in specifying protein degradation, no E3 has been implicated in ERAD. Hrd1p is an ER-resident membrane protein that is required for ER degradation of many substrates including normal HMG-CoA reductase (HMGR)[3], a rate-limiting enzyme in sterol biosynthesis, and numerous misfolded proteins[4]. In fact, Hrd1p has a crucial function in removing aberrant proteins that are normally produced in cells[5,6]. The carboxy-terminal region of Hrd1p contains a RING-H2 motif that is required for Hrd1p function. Similar RING-H2 motifs are critical for the activity of several soluble E3s, including c-Cbl, the anaphase-promoting complex (APC), and the SCF complex[7–9]. Here, using a combination of *in vitro* and *in vivo* approaches, we show that Hrd1p functions as an ER-associated E3 that specifically requires Ubc7p or Ubc1p for its action. Hrd1p is thus the first membrane-anchored E3 characterized *in vivo*. As such, the study of Hrd1p promises to provide insight into the unknown mechanisms of ER-substrate selection, the nature of membrane-associated ubiquitin ligases, and the large family of related RING-finger proteins.

Results

Hrd1p is required for ubiquitination of the ERAD substrate Hmg2p. We directly examined the *in vivo* function of Hrd1p in ubiquitination of ER substrates. HMGR is an essential sterol synthetic enzyme that undergoes feedback-regulated ER degradation in eukaryotes[10,11]. ER degradation of yeast HMGR isozyme Hmg2p proceeds by ubiquitination[12] and requires Hrd1p (ref. 3). We assayed ubiquitination of Hmg2p by immunoprecipitating Hmg2p from cellular lysates and then immunoblotting for covalently attached ubiquitin (Fig. 1a). In a strain expressing the *hrd1Δ* allele, no ubiquitination of Hmg2p was observed, even when maximally stimulated by incubation of cells with zaragozic acid, a drug that increases sterol-pathway signals for degradation[13]. Hrd1p was also required for ubiquitination of an Hmg2p–green fluorescent protein (GFP) fusion[13,14], and of the unregulated, misfolded protein Myc_6–Hmg2p (ref. 3 and data not shown). Consistent with our earlier studies[12], Hmg2p ubiquitination was strongly dependent on ER-associated Ubc7p but was unaffected by loss of Ubc6p, the other ER-associated E2.

The C-terminal region of Hrd1p contains a RING-H2 motif that is required for Hrd1p-dependent degradation[15]. Similar RING-H2 motifs are critical in the function of several soluble ubiquitin E3s, including c-Cbl, APC and the SCF complex[8,16,17]. We therefore tested whether the Hrd1p RING-H2 motif is necessary for ubiquitination of Hmg2p. Cysteine 399 of Hrd1p is a conserved RING-H2 residue in c-Cbl and related E3 proteins[8,16]. Expression of the Hrd1p(C399S) mutant as the sole source of Hrd1p severely impaired *in vivo* Hmg2p ubiquitination (Fig. 1a) and degradation (data not shown).

Hrd1p physically associates with E2s *in vivo*. The principal E2 involved in Hrd1p-dependent degradation is Ubc7p[4,12,18,19]. We directly tested for physical association of Hrd1p and Ubc7p using an *in vivo* crosslinking assay[20]. We treated intact cells expressing functional, haemagglutinin (HA)-epitope-tagged Ubc7p with the crosslinking agent dithiobissuccinimidyl propionate (DSP), and then lysed them and immunoprecipitated with anti-Hrd1p antisera. We then immunoblotted the precipitated proteins with an anti-HA antibody. HA–Ubc7p co-precipitated with Hrd1p in a crosslinker-dependent manner (Fig. 1b). The identical experiment carried out with strains lacking Hrd1p (*hrd1Δ*) or strains expressing only the C399S point mutant of Hrd1p ($HRD1^{C399S}$) showed no co-precipitation of Ubc7p. Because we determined that Ubc1p could also participate in the E3 activity of Hrd1p (see below), we carried out similar experiments using tagged Ubc1p, and found that Hrd1p also interacted with Ubc1p in a RING-H2-dependent manner (Fig. 1c). Thus, Hrd1p could directly associate with its two partner E2s *in vivo*, and each Hrd1p–Ubc interaction was completely dependent on the Hrd1p RING-H2 motif. In both cases, it was necessary to overexpress Hrd1p (roughly fourfold), probably because the interaction of Hrd1p with an E2 occurs with a fairly

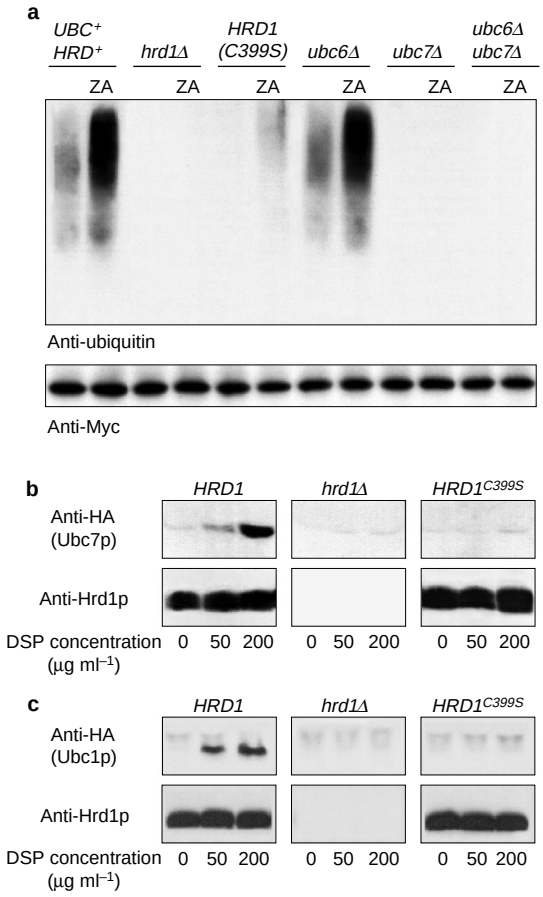

Figure 1 **Functions of Hrd1p *in vivo*. a**, *HRD1* and *UBC7* are required for ubiquitination of Hmg2p. Ubiquitination in the indicated strains was assayed by immunoprecipitation and subsequent immunoblotting for ubiquitin (upper panel) or Myc-tagged Hmg2p (lower panel). The RING-H2 mutant *HRD1(C399S)* was the only *HRD1* allele expressed in the indicated strain. ZA indicates cells treated with zaragozic acid for 10 min before the assay to stimulate ubiquitination[13]. **b**, Hrd1p and Ubc7p interact in a RING-dependent manner. Intact cells expressing both HA–Ubc7p and HA–Hrd1p were treated with crosslinker DSP, lysed, immunoprecipitated with anti-Hrd1p antiserum, and immunoblotted for crosslinked Ubc7p (upper panels) or Hrd1p (lower panels). DSP was added at 0, 50 and 200 μg ml^{-1} as indicated. **c**, Physical interaction of Hrd1p and Ubc1p *in vivo*. Analysis as in **b**.

low affinity to allow effective catalytic cycling. However, the interaction was absolutely dependent on an intact RING-H2 domain, as is the case for binding of E2s to other RING-H2 E3s such as c-Cbl[8].

Soluble Hrd1p fusion proteins catalyse multi-ubiquitination *in vitro*. Purified or recombinant ubiquitin–protein ligases can catalyze *in vitro* transfer of ubiquitin from E2s to target proteins or to themselves[7,8]. We directly assessed the E3 activity of Hrd1p using a soluble fusion of maltose-binding protein (MBP) and the C-terminal 203 residues of Hrd1p, which include the RING-H2 motif (residues 349–399). E3-catalysed ubiquitination requires ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (Ubc; E2), ubiquitin and ATP. Incubation of recombinant, purified MBP–Hrd1p, E1 and E2 with ubiquitin and ATP caused the production of ubiquitin immunoreactivity at many relative molecular mass (M_r) values (Fig. 2a). Exclusion of any reaction component completely inhibited the *in vitro* reaction (Fig. 2a, '–E1', '–E2', etc.), as did denaturation of the complete reaction mixture before incubation (Fig. 2a, '+SDS'). When the *in vitro* reaction was carried out with a fusion of glutathione-S-transferase (GST) and ubiquitin (M_r 32,000 (32K)) in place of native ubiquitin (M_r 8K), the resulting products had greatly separated M_r values, forming a ladder of discrete ubiquitin- or GST-immunoreactive bands (Fig. 2d and data not shown). Consistent with the *in vivo* data above, the Hrd1p RING-H2 domain was required for *in vitro* ubiquitination. MBP–Hrd1p(C399S) (numbered according to full-length Hrd1p) had no detectable ubiquitination activity at any concentration tested (Fig. 2b). Furthermore, the ubiquitination activity of MBP–Hrd1p was zinc-dependent, a feature of RING-H2-mediated action[7]. The *in vitro* activity of MBP–Hrd1p was completely inhibited by pretreatment with the zinc chelator TPEN, and was completely restored by reintroduction of free zinc ions (Fig. 2c).

In *in vitro* reactions, ubiquitin immunoreactivity was only observed at M_r values greater than that of MBP–Hrd1p (Fig. 2a–d, arrow), indicating that MBP–Hrd1p may catalyse self-ubiquitination, a feature of RING-H2 E3s[7,8]. We confirmed this by purification of MBP–Hrd1p from a reaction mix with amylose resin (which binds to MBP) and subsequent immunoblotting for ubiquitin (data not shown). Thus, at least a portion of the MBP–Hrd1p fusion itself was ubiquitinated, which is consistent with the ability of Hrd1p to autocatalyse its own degradation *in vivo*[20]. Identical behaviour was observed with a GST fusion of Hrd1p (see below).

***In vitro* Hrd1p-mediated transfer of ubiquitin to target proteins.** A variety of RING-H2 motifs, when incorporated into fusion proteins, will bind to E2s and catalyse self-addition of ubiquitin[7,8]. However, the principal *in vivo* function of an E3 is to catalyse processive transfer of ubiquitin from a charged E2 to a target protein. We demonstrated such a transfer activity for Hrd1p *in vitro* using two different test substrates: a protein with its binding site engineered into a Hrd1p fusion protein and a model misfolded quality-control substrate.

The M_r 10K S protein binds to the 15-amino-acid S peptide with high affinity[21,22] (Fig. 3a). We exploited this interaction by cloning the Hrd1p RING-H2 domain used above (the C-terminal 203 amino acids of Hrd1p) into a GST-fusion vector containing the S-peptide sequence, thereby producing a Hrd1p fusion with a high-affinity binding site for the S protein. The resulting GST–S-peptide–Hrd1p protein catalysed extremely efficient transfer of ubiquitin to S protein *in vitro*. This is shown by the appearance of bands of ubiquitin immunoreactivity at M_r values corresponding to mono- and di-ubiquitinated S protein when the S-protein substrate was included in the reaction (Fig. 3b, lane 2). Transfer of ubiquitin to S protein was completely inhibited when S-protein binding was blocked with free S peptide (lane 3). Similarly, neither of two Hrd1p fusions lacking the S-peptide sequence had any ability to transfer ubiquitin to S protein (Fig. 3b, lanes 5, 6), nor did a GST–S-peptide–Hrd1p(C399S) fusion (Fig. 3b, lane 4). The transfer-competent Hrd1p fusions also self-ubiquitinated in all cases in which they were active. This is reminiscent of Mdm2, a RING E3 that both self-ubiquitinates and transfers ubiquitin to p53 (ref. 23).

Hrd1p prefers a misfolded protein as a ubiquitination substrate *in vitro*. In some cases, E3 RING-H2 domains seem to participate in recognition of substrates[8,23,24]. *In vivo*, Hrd1p is involved in degradation of numerous misfolded proteins, and was independently isolated as Der3p in a screen for genes required for ERAD of misfolded proteins[4,15]. We therefore tested Hrd1p for any preferential E3 activity towards misfolded proteins *in vitro*. We used biotinylated BSA as a test substrate, comparing GST–Hrd1p-mediated ubiquitination of native or briefly boiled protein. We first carried out the reaction with methylated ubiquitin, which can be charged and transferred to target proteins, but cannot participate in formation of multi-ubiquitin chains[25]. Methylated ubiquitin facilitated detection of new ubiquitin immunoreactivity in the presence of strong Hrd1p auto-ubiquitination. Addition of boiled BSA to the *in vitro* reaction mix resulted in the appearance of a new ubiquitin-reactive band corresponding exactly to the M_r of BSA–ubiquitin (Fig. 3c, lane 2). In contrast, addition of normally folded BSA showed no new product (Fig. 3c, lane 1). Boiled BSA was also preferentially

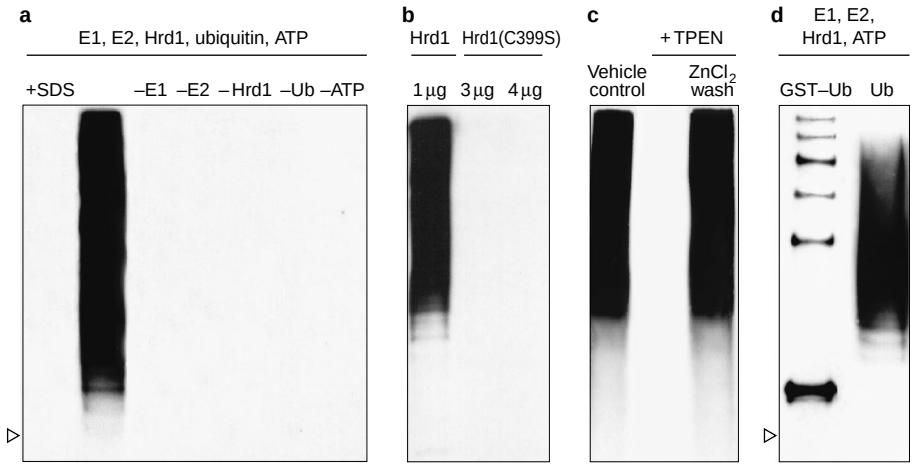

Figure 2 ***In vitro* auto-ubiquitination of MBP–Hrd1p. a**, Purified recombinant E1, E2 and MBP–Hrd1 were incubated with ATP and ubiquitin (Ub) at 25 °C for 90 min. The reaction mixture was then subjected to anti-ubiquitin immunoblotting. Reactions carried out with individual components removed (–E1, –E2, etc.) or after denaturation (+SDS) of complete mixture are also shown. **b**, An intact RING motif is required for Hrd1p E3 activity. In identical complete reactions, MBP–Hrd1p(C399S) did not catalyse ubiquitination *in vitro* at any concentration tested. **c**, The E3 activity of MBP–Hrd1p requires zinc. Treatment of MBP–Hrd1p with the zinc chelator TPEN destroyed Hrd1p E3 activity; subsequent treatment with $ZnCl_2$ restored activity. **d**, Effect of replacing ubiquitin (M_r 8K) with GST–ubiquitin (M_r 32K) on *in vitro* reaction product. Arrowheads indicate MBP–Hrd1p mobility.

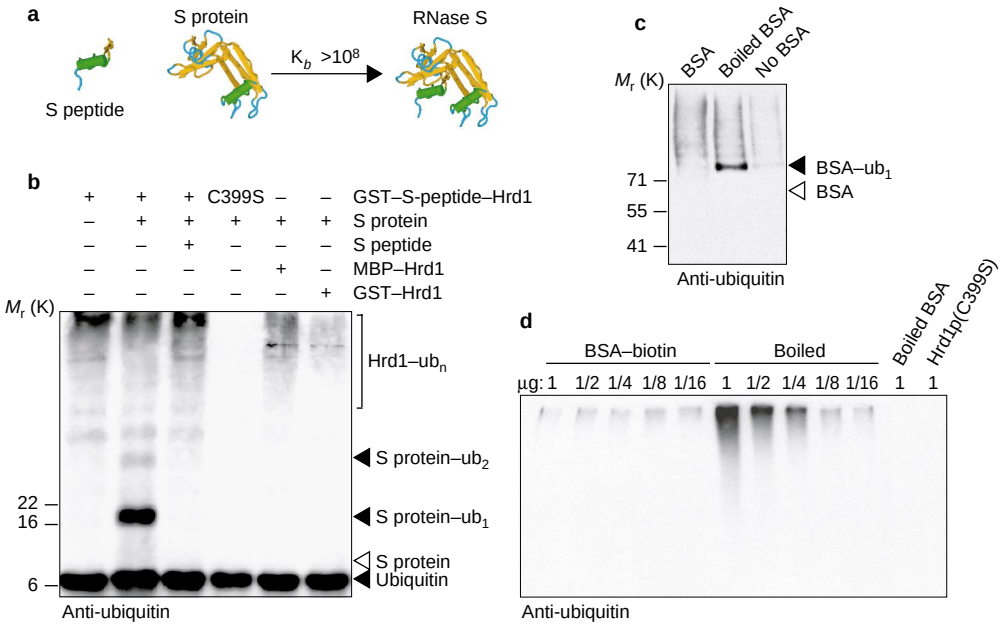

Figure 3 **Hrd1 promotes transfer of ubiquitin to specific proteins *in vitro*. a**, S protein binds to S peptide (and proteins containing the S-peptide sequence[38]) with high affinity. **b**, Binding of S protein to Hrd1 results in ubiquitination of S protein. Ubiquitination reactions were carried out with purified proteins and were subjected to immunoblotting for ubiquitin as in Fig. 2. **c**, Hrd1 promotes ubiquitination of a misfolded protein. Ubiquitination reactions were carried out with methylated ubiquitin to simplify the pattern of ubiquitin immunoreactivity. Equal amounts of boiled and unboiled BSA were added to the indicated reactions. **d**, The E3 activity of Hrd1p exhibits a preference for a misfolded protein. Indicated amounts of boiled or unboiled BSA–biotin were added to ubiquitination reactions. Streptavidin–agarose was used to selectively retrieve BSA–biotin from the reaction for subsequent analysis by SDS–PAGE and immunoblotting for ubiquitin. 'Boiled BSA' denotes addition of 1 µg of boiled, nonbiotinylated BSA; 'Hrd1p(C399S)' denotes addition of 1 µg of boiled biotinylated BSA to a reaction containing GST–Hrd1p(C399S). Open arrowheads indicate the M_r values of proteins not detected by immunoblotting (nonubiquitinated proteins).

multi-ubiquitinated by GST–Hrd1p, as shown by the *in vitro* reaction with native ubiquitin (Fig. 3d). To detect multi-ubiquitinated BSA–biotin, we affinity-precipitated the post-reaction mix with streptavidin beads, and subjected the resulting precipitate to immunoblotting to detect ubiquitinated BSA (Fig. 3d). The resulting multi-ubiquitin bands were completely absent from a reaction

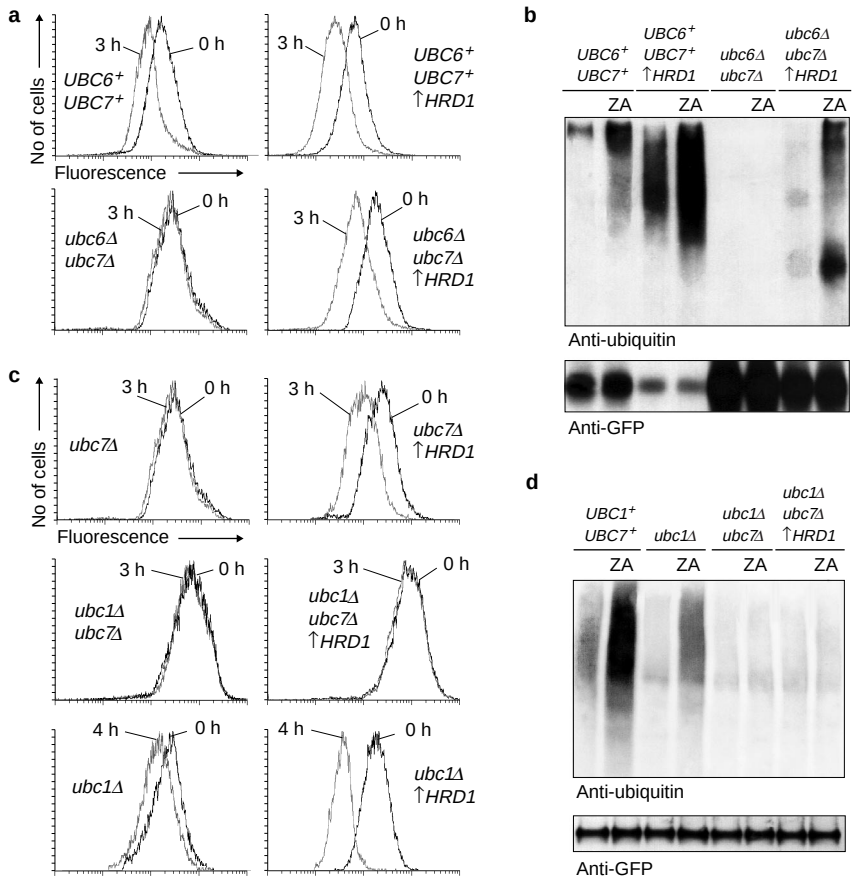

Figure 4 ***HRD1* promotes ubiquitination by specific E2s. a**, Increased expression of *HRD1* (↑*HRD1*) suppresses a *ubc6Δ ubc7Δ* degradation defect. Cycloheximide-chase analysis of Hmg2p–GFP; each pair of histograms shows Hmg2p–GFP degradation as loss of fluorescence. **b**, *HRD1* controls ubiquitination rate *in vivo*. The indicated strains were assayed for Hmg2p–GFP ubiquitination as in Fig. 1a, but Hmg2p–GFP was detected with an anti-GFP monoclonal antibody. **c**, Ubc1p or Ubc7p can participate in Hrd1p-stimulated degradation. Degradation of Hmg2p–GFP was evaluated in the indicated strains by cycloheximide treatment and flow cytometry as in **a**. **d**, Ubc1p is required for Hrd1p-stimulated ubiquitination in a *ubc7Δ* strain. Analysis as in **b**. In all cases, ↑*HRD1* denotes strains with fourfold overexpression of Hrd1p.

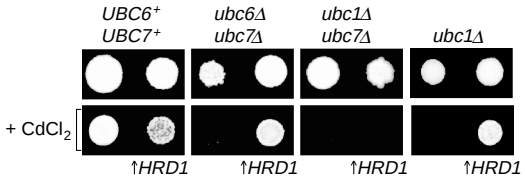

Figure 5 **HRD1 suppression of Cd^{2+} sensitivity.** Identical numbers of cells from the indicated strains were incubated on solid medium with 200 μM $CdCl_2$ (lower panels) or no $CdCl_2$ (upper panels). ↑*HRD1* denotes strains with increased expression of *HRD1*.

using boiled non-biotinylated BSA (Fig. 3d, 'boiled BSA') or GST–Hrd1p inactivated by the C399S mutation (Fig. 3d, 'Hrd1p(C399S)'). Blotting with streptavidin conjugated with horseradish peroxidase confirmed that equal amounts of BSA were precipitated from both sets of reactions (data not shown). Although equal amounts of both folded and boiled BSA were present in the indicated lanes, only misfolded BSA showed any detectable ubiquitin immunoreactivity. Together, these results indicate that the RING-H2 domain of Hrd1p can catalyse processive transfer of ubiquitin between an E2 and a variety of proteins, and so functions *in vitro* as a *bone fide* E3.

Determining the E2 specificity of Hrd1p *in vivo*. E3s orchestrate protein degradation by facilitating transfer of ubiquitin from selected E2s to specific target proteins. Our crosslinking and *in vitro* studies described above, as well as other work on RING-H2 E3s[7], have shown that the RING-H2 domain can physically and functionally interact with diverse E2s. Taken alone, these studies leave open the question of E2 selectivity in Hrd1p action. To address this question, we directly evaluated the E2(s) that participate in Hrd1p-mediated degradation *in vivo*. Like other E3s[26–28], Hrd1p is rate-limiting for ER degradation, such that increasing levels of Hrd1p hasten degradation of Hrd1p-specific substrates *in vivo*[15], including Hmg2p–GFP[20] (Fig. 4a, degradation; Fig. 4b, ubiquitination). We therefore determined the E2 specificity of Hrd1p by examining the effects of *ubc* null mutants on Hrd1p-stimulated degradation. We used Hmg2p–GFP to allow analysis by both flow cytometry and biochemistry[14]. Identical results were obtained using wild-type Hmg2p as the substrate (data not shown). We observed that Hrd1p could promote ubiquitination by ubiquitin-conjugating enzymes *in vivo* — thereby exhibiting E3 activity *in vivo* — and showed a marked specificity for Ubc7p and Ubc1p.

We first examined the role of the two ER-associated E2s Ubc7p and Ubc6p in Hrd1p function. These two E2s (although predominantly Ubc7p) have been implicated in Hrd1p-dependent

degradation of numerous substrates[4,18,19,29]. Hmg2p–GFP is strongly stabilized in both *ubc7Δ* and *ubc6Δ ubc7Δ* strains[12] (Fig. 4a, c). Nevertheless, modest (fourfold) overexpression of Hrd1p in the *ubc6Δ ubc7Δ* strain fully restored Hmg2p–GFP ubiquitination (Fig. 4b) and degradation, as measured by cycloheximide chase followed by flow cytometry (Fig. 4a). Furthermore, Hrd1p-restored ubiquitination and degradation was normally regulated by the sterol pathway (Fig. 4b, 'ZA', and data not shown). Thus, Hrd1p was still rate-limiting for ER ubiquitination and degradation in the absence of Ubc6p and Ubc7p — the two known ER-associated E2s. In fact, fourfold elevation of Hrd1p levels in the absence of Ubc7p and Ubc6p led to a restoration of degradation of Hmg2p that was identical in magnitude and regulation to that in wild-type strains (although not in ubiquitination pattern).

Hrd1p uses only Ubc7p or Ubc1p *in vivo*. Because Hrd1p could function in the absence of Ubc6p and Ubc7p, we tested which other E2s could participate in Hrd1p function. Hrd1p-stimulated degradation of Hmg2p–GFP in the *ubc7Δ* strain (Fig. 4c) or the *ubc6Δ ubc7Δ* strain (data not shown) was absolutely dependent on Ubc1p, an E2 that is not widely known to participate in ER degradation. Increasing Hrd1p dosage in a *ubc1Δ ubc7Δ* strain had no effect on Hmg2p–GFP degradation (Fig. 4c) or ubiquitination (Fig. 4d), despite the presence of all other *UBC* genes including *UBC6*. In contrast, removal of Ubc5p — an E2 that is quite similar to Ubc1p in sequence and function[30] — still allowed ERAD by Hrd1p overexpression in *ubc7Δ* or *ubc6Δ ubc7Δ* strains (data not shown). Thus, in the absence of Ubc1p and Ubc7p, Hrd1p failed to function in any detectable manner. However, Hrd1p could stimulate degradation of Hmg2p–GFP in either the *ubc1Δ* or *ubc7Δ* strains (Fig. 4c), indicating that Hrd1p can function as an E3 *in vivo* with either Ubc7p or Ubc1p, but only with these two E2s.

Hrd1p shows identical E2 specificity in a global phenotype. We also examined the E2 specificity of Hrd1p in a global phenotype associated with normal protein degradation: cellular resistance to Cd^{2+} toxicity[31]. Wild-type yeast can grow in medium with 200 μg ml^{-1} $CdCl_2$. Loss of *UBC7* or *UBC1* causes profound sensitivity to cadmium[31] (Fig. 5), presumably because of the increased burden of misfolded proteins that are normally degraded by ER-associated mechanisms. Increased levels of Hrd1p suppressed cadmium sensitivity in *ubc6Δ ubc7Δ* and *ubc1Δ* strains, restoring cell growth on medium containing $CdCl_2$ (Fig. 5). However, the cadmium sensitivity of the *ubc1Δ ubc7Δ* strain was not relieved by increased expression of Hrd1p. Thus, Hrd1p exhibits the same E2 selectivity in suppression of cadmium sensitivity as it does in Hmg2p degradation — either Ubc1p or Ubc7p allows Hrd1p suppression of cadmium sensitivity, but only these two E2s can participate in this activity of Hrd1p.

Ubc1p normally participates in Hmg2p degradation. These studies have shown that Ubc1p can function in Hrd1p-mediated ERAD. Direct examination revealed that Ubc1p has a demonstrable function in ER degradation even at normal levels of Hrd1p. A *ubc1Δ* strain was clearly deficient in both Hmg2p–GFP ubiquitination (Fig. 4d) and degradation (Fig. 4a, c; compare the 3-h chase for *UBC6*$^+$ *UBC7*$^+$ and the 4-h chase for *ubc1Δ*; and data not shown). Furthermore, Ubc1p has recently been implicated in the degradation of misfolded carboxy peptidase Y (CPY*), another well-studied, Hrd1p-dependent ERAD substrate[5]. At native levels of Hrd1p, *ubc7Δ* strains showed a greater defect than *ubc1Δ* strains in Hmg2p–GFP degradation[12] (Fig. 4c). Thus, Hrd1p can function as an E3 with two distinct E2s, Ubc1p and Ubc7p, but has a preference for Ubc7p. Whether Ubc1p, like Ubc7p[32], requires localization to the ER surface to function in ERAD remains an unanswered and clearly relevant question.

Discussion

We have shown that Hrd1p is the ER-associated ubiquitin–protein ligase for degradation of misfolded proteins and regulated HMGR. The isolated Hrd1p RING-H2 domain catalysed processive transfer of ubiquitin to test proteins *in vitro*, and seemed to have some intrinsic preference for misfolded proteins in this activity, although the structural basis for this intriguing selectivity remains to be determined. The RING-H2 domain of Hrd1p faces the cytosol, where it can engage Ubc7p or Ubc1p[19], whereas the amino-terminal transmembrane anchor forms a complex with the ER-membrane protein Hrd3p, which regulates the activity of the Hrd1p RING-H2 domain across the ER membrane[20]. Study of the *HRD* E3 complex will reveal the unknown manner in which highly diverse ER substrates are selected for degradation, and will provide insights into the biology of the seemingly abundant, but poorly understood, family of membrane-anchored ubiquitin–protein ligases — a family that may well include the Hrd1p homologues in humans, *Drosophila* and *Caenorhabditis elegans*.

It is clear that some ER substrates undergo ubiquitin-mediated degradation independently of Hrd1p/Hrd3p[4,33], indicating that other ubiquitin–protein ligases may work in conjunction with Hrd1p. Nevertheless, Hrd1p has a broad and central function in ERAD of both normal and misfolded proteins[3,5,6,15,29]. We have delineated the molecular details of this function and extended the action of RING-domain ubiquitin ligases to membrane surfaces and substrates. □

Methods

Immunoprecipitation, immunoblotting and protein crosslinking.

Immunoprecipitation was carried out as described[11] with additional protease inhibitors (*n*-ethylmaleimide, 4-(2-aminoethyl)benzene sulphonyl fluoride (AEBSF) , E-64, benzamidine and ε-amino-*n*-caproic acid). Immunoblotting was also carried out as described[11], except that Tris-buffered saline contained 0.45% Tween 20, and 20% heat-inactivated bovine calf serum was used as the blocking agent. Ubiquitin immunoblots were processed as described[34]. SDS–PAGE was carried out using 3–8% NuPage Tris–acetate gels (Novex/Invitrogen, San Diego, California; Figs 2, 3c and 4d) or 14% Tris–glycine gels (Fig. 3b). All other SDS–PAGE was carried out using 8% Tris–glycine gels. *In vivo* protein crosslinking was carried out as described[20]. Briefly, intact cells were placed in amine-free buffer, treated with the crosslinker DSP, and subsequently lysed, immunoprecipitated with anti-Hrd1p antiserum and immunoblotted for Ubc7p. For crosslinking experiments, *HRD1* and *UBC7* were expressed from the same (*TDH3*) promoter.

Antibodies.

GFP was detected using a polyclonal rabbit antibody for cycloheximide chases (provided by C. Zuker, UCSD) and a mouse monoclonal antibody following immunoprecipitations (Zymed). Ubiquitin was detected with an anti-bovine ubiquitin monoclonal antibody (Zymed). The Myc epitope was detected with the 9e10 monoclonal antibody (ATCC hybridoma); the HA epitope was detected with the 12CA5 monoclonal antibody (Babco); polyclonal antibodies against Hmg2p were generated previously[3].

Protein purification and *in vitro* ubiquitination.

MBP fusions were constructed and purified with amylose resin according to the manufacturer's instructions (New England Biolabs, Beverly, Massachusetts) as were His_6 fusions using TALON resin (Clontech) and GST fusions using glutathione–Sepharose (Pharmacia). Both MBP–Hrd1p and GST–S-peptide–Hrd1p were fusions with the C-terminal 203 amino acids of Hrd1p (containing the RING-H2 motif). The parent vector for GST–S-peptide constructs was pET42(b) (Novagen). Assays for *in vitro* ubiquitination were carried out as described[8]. Briefly, 0.1 μg HIS_6–E1 (human, ref. 35), 0.2 μg HIS_6–UBC4 (human, ref. 35), 2.5 μg of ubiquitin (bovine, Sigma) and the indicated amounts of Hrd1p fusions were incubated at 25 °C for 90 min in 2 mM ATP, 50 mM Tris–HCl pH 7.5, 2.5 mM $MgCl_2$ and 0.5 mM dithiothreitol. Reactions contained TPEN (Fluka, 100 mM stock in absolute ethanol), S protein (Sigma), or S peptide (Biozyme, San Diego, California) as indicated. Biotinylated BSA (Sigma) was resuspended to a stock concentration of 10 mg ml^{-1} in HDB (25 mM HEPES, 0.7 mM Na_2HPO_4, 137 mM NaCl and 5 mM KCl, pH 7.4) containing 10% glycerol. Where indicated, BSA was boiled in a microfuge tube for 5 min. To precipitate biotinylated BSA, 60 μl of a 50% Streptavidin–agarose (Fluka, HDB-equilibrated) suspension was added to each reaction along with 300 μl HDB. Reactions were incubated for 1 h at 25 °C with gentle shaking and then washed 3 times with 1 ml HDB, 3 times with 1 ml HDB containing 0.25% Triton X-100 and 0.5% deoxycholate, and then washed twice more with 1 ml HDB. The supernantant was then aspirated to dryness, 50 μl of 2 × USB was added, and the sample was boiled for 5 min before being subjected to SDS–PAGE. Boiling had no effect on precipitation efficiency, as determined by SDS–PAGE of precipitates and blotting using horseradish-peroxidase-conjugated strepavidin. All proteins were resuspended and/or stored in HDB buffer.

Assays for protein degradation.

Cycloheximide chase was used to measure protein stability as described[3]. Degradation was measured by immunoblotting or flow cytometry (Hmg2p–GFP strains). Flow cytometry was carried out using a FACScalibur machine (Becton Dickinson) as described[14]. Statistical analysis was carried out to quantify loss of fluorescence[36].

Plasmids.

pRH808 (P_{TDH3}-*HRD1*,*TRP1* YIP), which was used to increase expression of *HRD1*, was constructed by

placing the yeast *TDH3* promoter immediately before the *HRD1* coding region using polymerase chain reaction (PCR) and Vent polymerase. pRH808 increased steady-state levels of Hrd1p roughly fourfold in *ubc6Δ ubc7Δ*, *ubc7Δ* and *ubc1Δ ubc7Δ* strains. pRH1256 (*ubc1Δ::KanMX* disruption plasmid) was constructed by inserting the *KanMX* gene into *PstI*–*BstZ17I* sites of *UBC1*.

Strains and media.

The parent for all yeast strains was JRY527, an S288C derivative. The parent strain for experiments with Hmg2p–GFP was RHY853 *MATα ade2–101 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ lys2–801 met2 trp1::hisG URA3::*P_{TDH3}*–HMG2cd::*P_{TDH3}*–hmg2–GFP*. The parent strain for experiments with Hmg2p was RHY1167 *MATα ade2–100 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 leu2Δ lys2–801 met2 trp1::hisG ura3–52::*P_{TDH3}*–1MYC–HMG2*. RHY853 and RHY1167 alleles were described previously[3,11,13], as were the *hrd1Δ::TRP1*, *ubc6Δ::KanMX* (ref. 4) and *ubc7Δ::HIS3* (ref. 12) alleles. The *ubc1Δ::KanMX* allele was created by transforming RHY853 with the *ubc1Δ::KanMX* fragment from pRH1256. All strains were isogenic and were constructed using standard techniques in yeast genetics. Yeast strains were grown in yeast minimal media at 30 °C with shaking as described[11]. Yeast were transformed by the lithium-acetate method[37]. When strains expressed the *KanMX* gene, G-418 sulphate was added to a final concentration of 500 μg ml^{-1}.

RECEIVED 3 MAY 2000; REVISED 21 AUGUST 2000; ACCEPTED 2 OCTOBER 2000;
PUBLISHED 4 DECEMBER 2000.

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ACKNOWLEDGMENTS
This work was supported by grants from the American Heart Association, Western States Affiliate (to N.W.B.), ARCS Foundation, San Diego (to N.W.B.) and the National Institutes of Health (to R.Y.H.), and by a Searle Scholarship (to R.Y.H.).
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