The EMBO Journal (2006) 25, 662–672 | © 2006 European Molecular Biology Organization | All Rights Reserved 0261-4189/06
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JOURNAL

Transferrin receptor-like proteins control the degradation of a yeast metal transporter

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Plasma membrane transporters are often downregulated by their substrates. The yeast manganese transporter Smf1 is subject to two levels of regulation: heavy metals induce its sequestration within the cell, and also its ubiquitination and degradation in the vacuole. Degradation requires Bsd2, a membrane protein with a PPxY motif that recruits the ubiquitin ligase Rsp5, and which has a role in the quality control of membrane proteins, that expose hydrophilic residues to the lipid bilayer. We show that degradation of Smf1 requires in addition one of a pair of related yeast proteins, Tre1 and Tre2, that also contain PPxY motifs. Tre1 can partially inhibit manganese uptake without Bsd2, but requires Bsd2 to induce Smf1 degradation. It has a relatively hydrophilic transmembrane domain and binds to Bsd2. We propose that the Tre proteins specifically link Smf1 to the Bsd2-dependent quality control system. Their luminal domains are related to the transferrin receptor, but these are dispensable for Smf1 regulation. Tre proteins and the transferrin receptors appear to have evolved independently from the same family of membrane-associated proteases.

The EMBO Journal (2006) **25,** 662–672. doi:10.1038/sj.emboj.7600984; Published online 2 February 2006
Subject Categories: membranes & transport; proteins
Keywords: Bsd2; metal transport; Rsp5; Smf1; ubiquitin

Introduction

Degradation of membrane proteins is an important aspect of cellular physiology. It is used both as a quality control mechanism, to eliminate damaged or misfolded proteins (reviewed by Trombetta and Parodi, 2003), and as a regulatory process to remove proteins that are no longer required (Hicke and Dunn, 2003). A common phenomenon is the downregulation of transporters in the presence of their substrates. Such substrate-induced destruction protects cells from self-poisoning, a particular problem with components such as heavy metals, which are essential for cell physiology but toxic in excess.

Transporter degradation occurs primarily in the vacuole (in yeast) or lysosomes (in higher cells). This is accomplished by sorting of the proteins into the internal vesicles of multivesicular bodies (MVBs), which fuse with the vacuole or lysosome and thus deliver the vesicles to the interior of the degradative compartment, where they are destroyed by hydrolytic enzymes (Futter *et al*, 1996; Odorizzi *et al*, 1998).

Targeting of proteins to MVBs requires, in most cases, the addition of single ubiquitin moieties to the protein (Hicke and Dunn, 2003). Ubiquitin serves as a sorting signal for transport from the Golgi to endosomes (Reggiori and Pelham, 2002), or for endocytosis from the cell surface (Hicke and Riezman, 1996), and is then used again as a signal for sorting into MVBs (Katzmann *et al*, 2001; Reggiori and Pelham, 2001). Thus, the initial decision to degrade a protein that has left the endoplasmic reticulum is reflected in its specific monoubiquitination, and the enzymes responsible for this are key to the correct selection of substrates.

Ubiquitination frequently involves a family of HECT domain ligases represented by Rsp5 in yeast, and by the Nedd4 proteins in animal cells. This is the case, for example, with sodium channels in kidney cells (Rougier *et al*, 2005). These channels contain PPxY motifs that can bind to WW domains, which are present in Rsp5 and Nedd4, and this accounts at least in part for their recognition. However, most transporters lack PPxY sequences. In yeast, Rsp5 is required for ubiquitination of several membrane proteins that undergo regulated degradation: the alpha factor receptor in the presence of its ligand (Dunn and Hicke, 2001), the general amino-acid permease Gap1 in amino-acid-rich growth media (Springael and Andre, 1998; De Craene *et al*, 2001), the uracil permease Fur4 when uracil is abundant (Galan *et al*, 1996; Blondel *et al*, 2004) and the maltose permease in substrate excess (Medintz *et al*, 1998). None of these proteins contain PPxY motifs and in no case is it known exactly how the presence of ligand or substrate triggers recruitment of Rsp5. Thus, a major unsolved problem is how the ubiquitination of such membrane proteins is controlled.

Work in yeast has shown that exposure to the lipid bilayer of polar residues within transmembrane domains (TMDs) is sufficient to induce ubiquitination and transport to the vacuole (Reggiori *et al*, 2000; Reggiori and Pelham, 2002). This appears to represent a general form of quality control, as well-folded membrane proteins do not normally expose polar residues to lipid. Two systems mediate this ubiquitination, which occurs after exit of the proteins from the endoplasmic reticulum. One consists of the RING-domain ubiquitin ligase Tul1, which has seven TMDs and apparently directly recognises the TMDs of its substrates (Reggiori and Pelham, 2002). The other involves the three-TMD protein Bsd2, which can also recognise polar substrates but is not itself a ubiquitin ligase. Instead, it recruits Rsp5 via a PPxY motif in its N-terminal cytoplasmic domain. Bsd2 thus serves as an adaptor that links Rsp5 to certain substrates (Hettema *et al*, 2004). Bsd2 and Tul1 have different but overlapping specificities, and the fungi are unusual in having both: plants lack Bsd2, whereas metazoa lack Tul1.

Bsd2 has also been implicated in downregulation of the manganese transporter Smf1 (Liu and Culotta, 1999b),

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Received: 24 May 2005; accepted: 11 January 2006; published online: 2 February 2006

a yeast homologue of the Nramp transporters of macrophages (Forbes and Gros, 2001). Removal of Smf1 is crucial for the survival of yeast in the presence of heavy metals. At least two distinct regulatory mechanisms have been described: removal of the transporter from the plasma membrane to internal organelles and degradation in the vacuole. Bsd2 is not required for the first step, but is required for degradation, as is Rsp5 (Liu and Culotta, 1999a, b; Eguez *et al*, 2004; Hettema *et al*, 2004). We have previously speculated that the metal-bound form of Smf1 adopts a conformation that exposes polar residues, and that these are recognised directly by Bsd2 (Hettema *et al*, 2004).

In the present study, we characterise two related proteins that bear a striking resemblance to the mammalian transferrin receptors (TFRs), which we named Tre1 and Tre2 (Transferrin REceptor-like). Deletion of both proteins prevents degradation of Smf1, increases manganese uptake and renders cells sensitive to cadmium. We show that Tre1 binds to Bsd2, and that the Tre proteins cooperate with Bsd2 to target Smf1 to the vacuole. Overexpression of Tre1 can partially inhibit manganese uptake even in the absence of Bsd2. We suggest that the Tre proteins serve as adaptors that link the unwanted metal transporters to the quality control machinery, thus ensuring their removal.

Results

The Tre proteins are relatives of the transferrin receptor that contain functional PPxY motifs

The Tre proteins were identified in a database search for yeast membrane proteins with PPxY motifs. They show a striking resemblance to mammalian TFRs. However, as TFRs do not have PPxY motifs and yeast does not use transferrin, the yeast proteins are likely to have a different function from their mammalian relatives.

Figure 1A shows a comparison of the domain structures of Tre1 (Ypl176c) and Tre2 (Yor256c) with that of the human TFR. Tre1 and Tre2 are 43% identical to each other, and show about 25% identity with the TFR. All three proteins share an N-terminal Y-x-x-hydrophobic motif, a single TMD, a 'protease-associated' (PA) domain, an M28 metalloprotease domain and a 'TFR-dimer' (TFRD) domain. Structural analysis of two proteases containing the M28 domain has identified five residues crucial for binding two zinc ions, and two catalytic residues, and the functional importance of these residues has been confirmed by mutagenesis of another member of the family (Chevrier *et al*, 1994; Greenblatt *et al*, 1997; Speno *et al*, 1999). Like the TFR, Tre1 and Tre2 lack almost all of these crucial residues and thus cannot be active proteases.

As we show below, Tre1 and Tre2 share similar properties and functions, and we analysed them in parallel. In almost all cases, the results were very similar for each, and so for clarity we will present mainly the Tre1 data. Where slight differences were observed, these are noted.

The Tre proteins are involved in downregulation of the Smf1 manganese transporter

To investigate the function of the Tre proteins, we prepared mutant cells that lack them. Previous analysis had shown that deletion of *TRE1* does not generate an obvious phenotype, but deletion of *TRE2* is lethal (Giaever *et al*, 2002). *TRE2* is adjacent to an essential gene, *CDC31*, and we suspected that lethality might occur because the *TRE2* gene contains regulatory sequences that affect *CDC31* expression. Indeed, sporulation of a heterozygous *tre2* diploid containing a plasmid expressing *CDC31* allowed recovery of *tre2* null cells, which were dependent on the plasmid for growth. Deletion of *TRE1* from these cells produced a *tre1 tre2* double mutant.

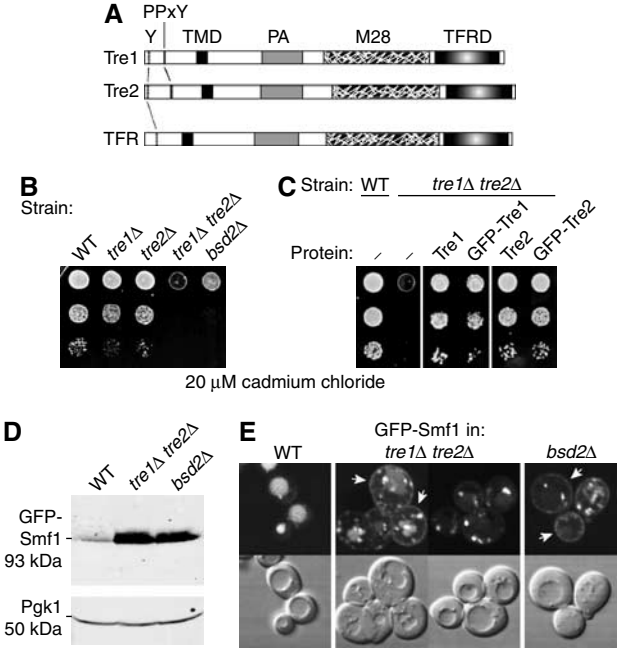

Figure 1 Downregulation of Smf1 requires Bsd2 and a Tre protein. (**A**) Diagram of Tre1 and Tre2 compared to human TFR. The Y-x-x-hydrophobic motif (Y), PPxY motif, PA domain, M28 protease domain and TFRD are indicated. (**B**) Sensitivity to cadmium. Cells of the indicated strains were grown on plates containing 20 μM cadmium chloride. Vertical columns represent serial dilutions of cells. All strains were plated at equivalent densities. (**C**) Complementation by Tre proteins and their GFP-tagged derivatives; assay as in panel B. (**D**) Immunoblot of GFP-Smf1 expressed in the indicated strains. Phosphoglycerate kinase (Pgk1) was detected on the same blot as a loading control. Cells were grown in YEPD medium, which is metal-replete. (**E**) GFP-Smf1 imaged in the indicated strains. Arrows indicate plasma membrane fluorescence, which was variably seen in both *tre1 tre2* and *bsd2* cells.

We found that the double mutant, unlike the single *tre* mutants, was unable to grow in the presence of modest levels of cadmium (Figure 1B). This defect could be complemented by expression of either Tre1 or Tre2, and also by GFP-tagged proteins (Figure 1C). Thus Tre1 and Tre2 have redundant functions in the protection of cells from cadmium. Cells lacking Bsd2 are also sensitive to cadmium, but in addition are sensitive to the amino-acid analogue canavanine, and are defective in the sorting of the vacuolar enzyme carboxypeptidase S (Hettema *et al*, 2004). The *tre1 tre2* cells did not show such defects, and targeting of Bsd2 to the vacuole was also unaffected (data not shown). Thus, the functions of the Tre proteins are distinct from those of Bsd2, but like Bsd2 they are required for cadmium resistance.

Cadmium uptake is mediated by the manganese transporter Smf1, and the cadmium sensitivity of *bsd2* cells is due to the lack of normal downregulation of Smf1 in the presence of metals—high levels of Smf1 result in excessive uptake

of cadmium, which is toxic (Liu *et al*, 1997). We therefore expressed GFP-tagged Smf1 from the constitutive *TPI1* promoter and examined levels of the protein in various mutants, grown under metal-replete conditions where downregulation normally occurs. Figure 1D shows that in *tre1 tre2* null cells, as in *bsd2* cells, the level of Smf1 was elevated. Fluorescence microscopy showed accumulation of Smf1 in punctate structures, instead of the normal exclusively vacuolar pattern (Figure 1E). Smf1 was frequently also visible on the plasma membrane, although the amount was very variable (Figure 1E). Similar results have previously been described for *bsd2* cells—Bsd2 is required only for vacuolar degradation, not for the retargeting of Smf1 from the plasma membrane to endosomes in metal-replete conditions (Liu and Culotta, 1999b). The clear conclusion from these results is that a Tre protein, together with Bsd2, is specifically required for the efficient vacuolar degradation of Smf1.

Tre1 can inhibit cadmium and manganese uptake in the absence of Bsd2

Although both Bsd2 and the Tre proteins are involved in Smf1 regulation, their effects could be distinguished. Bsd2 had no effect in the absence of Tre1 and Tre2—even when Bsd2 was overexpressed from the *TPI1* promoter, the cells remained cadmium sensitive (Figure 2A). In contrast, overexpression of Tre1, or to a lesser extent Tre2, restored growth of *bsd2* null cells in the presence of cadmium, suggesting that Tre1 can act directly on Smf1 even in the absence of Bsd2 (Figure 2B).

Surprisingly, this restoration of growth was not accompanied by any detectable reduction in Smf1 levels. Figure 2C shows an immunoblot of HA-tagged Smf1. As reported by others (Liu and Culotta, 1999b; Eguez *et al*, 2004), Smf1 stabilised in a *bsd2* mutant comprises a ladder of closely spaced bands, which likely reflect a progressive post-translational modification, such as phosphorylation. In wild-type cells, the upper bands are noticeably absent, consistent with the rapid ubiquitination and degradation of Smf1. Overexpression of Tre1 (or of active derivatives of it that are discussed below) did not significantly alter either the abundance or the qualitative pattern of Smf1 bands in the *bsd2* mutant, even though the cells became cadmium resistant. In contrast, expression of Bsd2 from a plasmid restored the downregulation of Smf1.

This surprising result raised the question of whether overexpressed Tre1 was acting by altering Smf1 activity and hence metal uptake, or whether it was inducing cadmium resistance in some indirect way. To test this, we measured the rate of ^{54}Mn uptake directly. Unlike cadmium sensitivity, which has been shown to depend on Smf1 specifically (Liu *et al*, 1997), Mn uptake measured in this way can be mediated both by Smf1 and by the related transporter Smf2 (Cohen *et al*, 2000), which is also regulated by Bsd2 (Portnoy *et al*, 2000). Figure 2D shows that deletion of either *bsd2* or both *tre1* and *tre2* increases the rate of Mn uptake to the same extent relative to wild-type cells, when the strains are grown in metal-replete medium. Significantly, expression of Tre1 in the *bsd2* cells consistently reduced Mn uptake, although it remained higher than in wild-type cells. We thus conclude that Tre1 can somehow inhibit the action of Smf1 and/or Smf2, even in the absence of Bsd2.

The rate of Mn uptake measured in this way corresponds to approximately 1000 Mn^{2+} ions per cell per second.

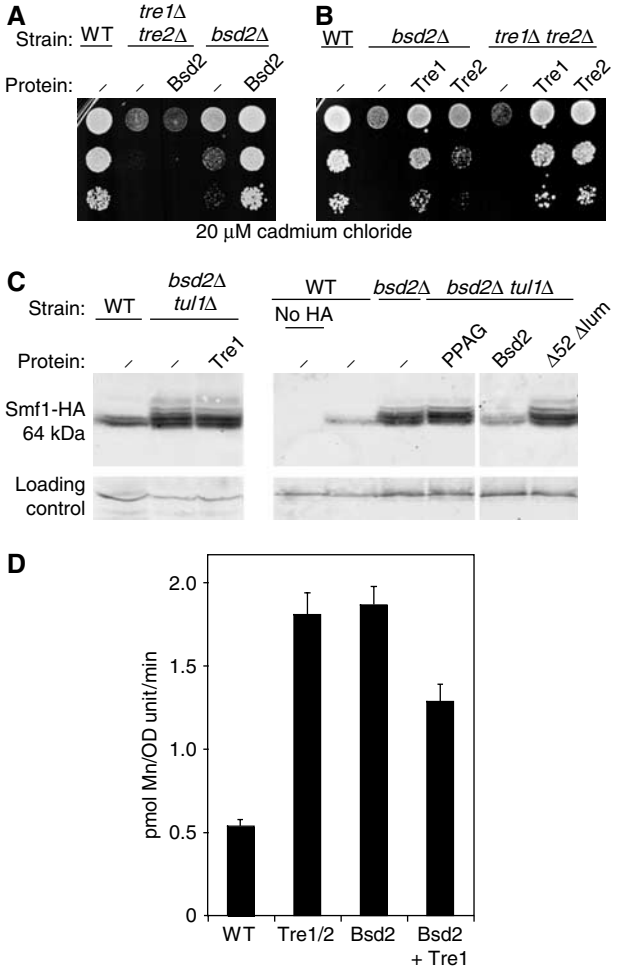

Figure 2 Tre proteins can affect Smf activity in the absence of Bsd2. (**A**) Overexpression of Bsd2 cannot restore cadmium resistance of a *tre1 tre2* strain. Bsd2 was expressed from the *TPI1* promoter, which results in at least 10-fold overexpression (Hettema *et al*, 2004), in the indicated strains. (**B**) Tre proteins, expressed from the *TPI1* promoter, can suppress cadmium sensitivity of a *bsd2* mutant. (**C**) Tre1 overexpression does not reduce Smf1 levels in a *bsd2* mutant. Tre1 (left) and mutant versions of it (right, PPAG and Δ52 Δlum) were expressed from the *TPI1* promoter in cells of the indicated strains that were also expressing Smf1-HA. Bsd2 was similarly expressed. The Smf1 was detected by alkaline lysis of the cells and immunoblotting with anti-HA. The same blot was probed with a rabbit antibody that recognises an unknown minor yeast protein, as loading control. No HA refers to a control strain that lacked the tagged Smf1. The right-hand panels and the left-hand panel are from two different gels. (**D**) Overexpression of Tre1 reduces uptake of Mn by Bsd2 mutant cells. Cells of the indicated strains were grown in metal-replete medium and ^{54}Mn uptake measured. The results are the mean of two separate experiments, each in duplicate. Bars indicate standard error of the mean.

Although the rate of transport by individual Smf molecules has not been measured, similar proton-linked transporters such as Na^+/H^+ exchangers in mammalian cells or an amino-acid transporter of *Arabidopsis* have been shown to have turnover numbers of several hundreds per second (Boorer *et al*, 1996; Cavet *et al*, 1999). Thus, Mn transport may require only a small number of transporters on the plasma membrane, consistent with the observation that GFP-Smf1 is mostly inside the cell. Uptake of ^{54}Mn is too rapid to be accounted for by the alternative route of fluid-phase endocytosis followed by extraction of the metal from

endosomes. Inhibition of activity by Tre1 could therefore be explained either by direct blocking of transporter function or by a reduction in the number of surface molecules. Both would be consistent with specific recognition of Smf1 by Tre1.

The Tre1 PPxY motif can bind the WW domains of Rsp5

We chose to study the Tre proteins initially because they have PPxY domains, which could potentially interact with Rsp5, and this would be consistent with a role in the ubiquitination and degradation of Smf1. However, the inability of Tre1 to cause Smf1 degradation without the help of Bsd2 raises the question of whether Tre1 is capable of binding Rsp5. To test this, we expressed the Tre1 cytoplasmic domain as a protein A fusion in *Escherichia coli*, and mixed extracts of these cells with glutathione-derivatised beads loaded with GST fusions containing each of the three Rsp5 WW domains. As shown in Figure 3A, the Tre1 fragment bound to each of the WW domains but not to GST alone. Mutation of the PPxY motif to PPAG greatly reduced binding. Thus, this qualitative assay suggests that Tre1 is at least capable of interacting with the Rsp5 ubiquitin ligase.

Tre1 and Smf1 ubiquitination depends on Bsd2

We next asked whether Tre1 was a substrate for ubiquitination. To improve resolution of the proteins, a GFP-tagged construct lacking the large luminal domain was analysed. This was expressed from the strong *TPI1* promoter and detected by immunoblotting with GFP antibodies; in parallel, it was immunoprecipitated with anti-GFP and then probed with anti-ubiquitin antibodies. Figure 3B shows that a series of bands larger than the main fusion protein were detected with both antibodies. This indicates that Tre1 does indeed undergo ubiquitination.

However, when we examined the modification of full-length Tre1 and of a mutant in which the PPxY motif was mutated to PPAG, we found that the PPxY motif was not required for ubiquitination. Instead, this was almost completely dependent on Bsd2. Only a very small amount of modification could be detected in a *bsd2* mutant (arrow in Figure 3B), which was completely abolished when the Tre1 PPxY was also mutated.

Prompted by this finding, we looked for interaction between Tre1 and Bsd2 coexpressed *in vivo*. Figure 3C shows that precipitation of a protein A-tagged Tre1 construct lacking the luminal domain resulted in the efficient co-purification of HA-tagged Bsd2. Identical results were obtained with full-length Tre1 (not shown). Together, these results indicate that Tre1 binds to Bsd2 and is ubiquitinated in a Bsd2-dependent manner, but that its own PPxY motif is not sufficient to mediate efficient ubiquitination *in vivo*. This suggests that Tre1 and Bsd2 may act together as a complex that mediates ubiquitination and degradation of Smf1.

Direct demonstration of Smf1 ubiquitination was complicated by its heterogeneity, but immunoprecipitation of HA-tagged Smf1 revealed a smear of material larger than the normal protein, which was absent in a *bsd2* strain (Figure 3D; note that the closely spaced bands observed in Figure 2C appear here as a single broad band). These experiments used protease-deficient *pep4* strains, in which we found the ubiquitinated forms to be much more prominent than in wild-type cells. To confirm that the larger material contained

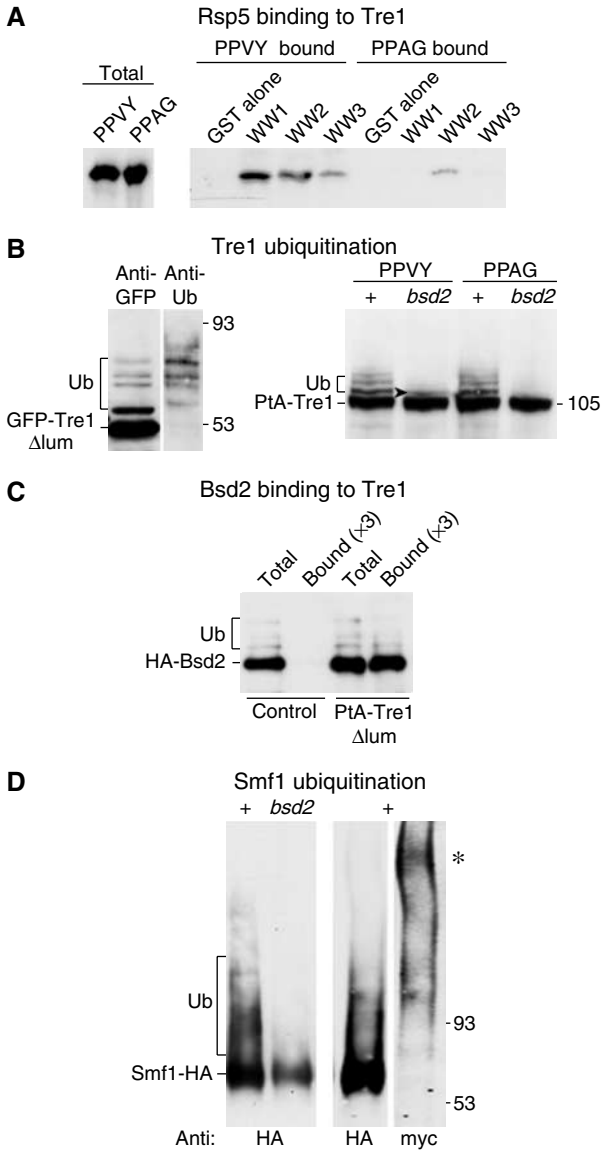

Figure 3 Binding and ubiquitination. (**A**) The PPxY motif binds the Rsp5 WW domains. Protein A fusions of the entire cytoplasmic domain of Tre1, with the wild-type PPVY sequence or a mutated PPAG motif, were incubated with GST fusions of each of the three WW domains of Rsp5 and bound protein detected by immunoblotting. (**B**) Tre1 is ubiquitinated. Left panel: a GFP-tagged version of Tre1 that lacked the luminal domain was detected either by immunoblotting of cell lysates with anti-GFP or by immunoblotting with anti-ubiquitin after immunoprecipitation with anti-GFP. The positions of size markers of 53 and 93 kDa are indicated. Right panel: full-length wild-type Tre1 (PPVY) and a PPxY mutant (PPAG), tagged with protein A, were expressed in the indicated strains and detected by immunoblotting. Ubiquitinated protein is indicated by Ub. The anti-ubiquitin antibodies do not efficiently recognise protein with a single ubiquitin moiety. (**C**) Tre1 binds Bsd2 *in vivo*. Cells expressing HA-tagged Bsd2 and either endogenous Tre1 only (Control) or a protein A-tagged Tre1 construct lacking the luminal domain were lysed in detergent (total), protein A-tagged Tre1 purified on IgG Sepharose beads and bound HA-Bsd2 detected by immunoblotting. (**D**) Smf1-HA was expressed, immunoprecipitated with anti-HA and immunoblotted. The left panel shows protein from BSD^+ (+) and *bsd2* cells. The right panel shows material from cells expressing myc-ubiquitin, probed simultaneously with anti-HA and anti-myc; the two fluorescent channels are shown side by side. Ub indicates ubiquitinated forms and the asterisk indicates polyubiquitinated protein. All yeast strains used for this figure were *pep4*.

ubiquitin, we repeated the experiment in cells expressing myc-tagged ubiquitin and probed the immunoblot with anti-HA and anti-myc. This revealed faint diffuse bands whose size suggests the addition of 1–4 ubiquitins. In addition, a small amount of much more slowly migrating polyubiquitinated Smf1 was observed, prominent owing to the presence of multiple myc epitopes (asterisk in Figure 3D). This was largely unaffected by a *bsd2* mutation (unpublished observations) and may represent material that is undergoing degradation in the endoplasmic reticulum. Nevertheless, our data are consistent with the idea that a major pathway for Smf1 downregulation involves the addition of single ubiquitins and consequent vacuolar targeting. As with the ubiquitination of Tre1, this requires the presence of Bsd2.

Intracellular targeting of Tre1

The ubiquitination of Tre1 suggests that it is itself targeted to the vacuole. To test this, we examined the location of Tre1 tagged at its cytoplasmic amino terminus with GFP. The Tre proteins have been estimated to be present at only 300–400 molecules per cell (Ghaemmaghami *et al*, 2003), so we again used the strong *TPI1* promoter for expression. Figure 4A shows that GFP-Tre1 was found mainly within vacuoles, with some additional punctate fluorescence typical of endosomal or Golgi structures. That the initially cytoplasmic GFP was inside the vacuole implied entry into the MVB pathway. A similar pattern was apparent in *end4* cells, in which endocytosis is blocked, indicating that Tre1 passes directly from the Golgi to endosomes rather than travelling via the plasma membrane (Figure 4A).

In agreement with the ubiquitination data, the Tre1 PPAG mutation did not affect targeting (Figure 4A), but in *bsd2* mutant cells even wild-type Tre1 was no longer in the vacuole, but instead was in punctate structures (Figure 4B). These corresponded to endosomes, as they could be labelled within 10 min by the endocytic tracer dye FM4-64 (Figure 4C). In contrast, removal of Tul1, a RING domain ubiquitin ligase with similar specificity to the Bsd2 system, had no effect on Tre1 targeting (Figure 4B).

These results were confirmed by immunoblotting. Cells expressing GFP-Tre1 contained both the full-length fusion protein and a 33 kDa band corresponding to free GFP (Figure 4D). The latter arises by vacuolar proteolysis, as shown by its absence in a strain lacking vacuolar proteases. In *bsd2* cells, the levels of free GFP were greatly reduced, indicating reduced vacuolar targeting (Figure 4D). We also examined cells expressing GFP-Tre1 from the *TRE1* promoter, but were unable to detect the protein either by fluorescence or by immunoblotting. However, we could detect a low level of GFP-Tre2 expressed from its own promoter, and confirm the effect of the *bsd2* mutation (Figure 4D). Bsd2-dependent vacuolar targeting is therefore not simply an artefact of overexpression.

From these results, we conclude that Tre1 itself has sorting determinants that target it to endosomes, but that its interaction with Bsd2 results in its ubiquitination and subsequent entry into the MVB pathway to the vacuole.

Bsd2 recognition and endosomal targeting involves the transmembrane and cytoplasmic domains of Tre1

As Bsd2 is thought primarily to recognise polar TMDs, we tested the role of the Tre1 TMD. Removal of the luminal domain did not affect targeting, but additional substitution of the TMD for that of the endosomal SNARE Pep12, or the

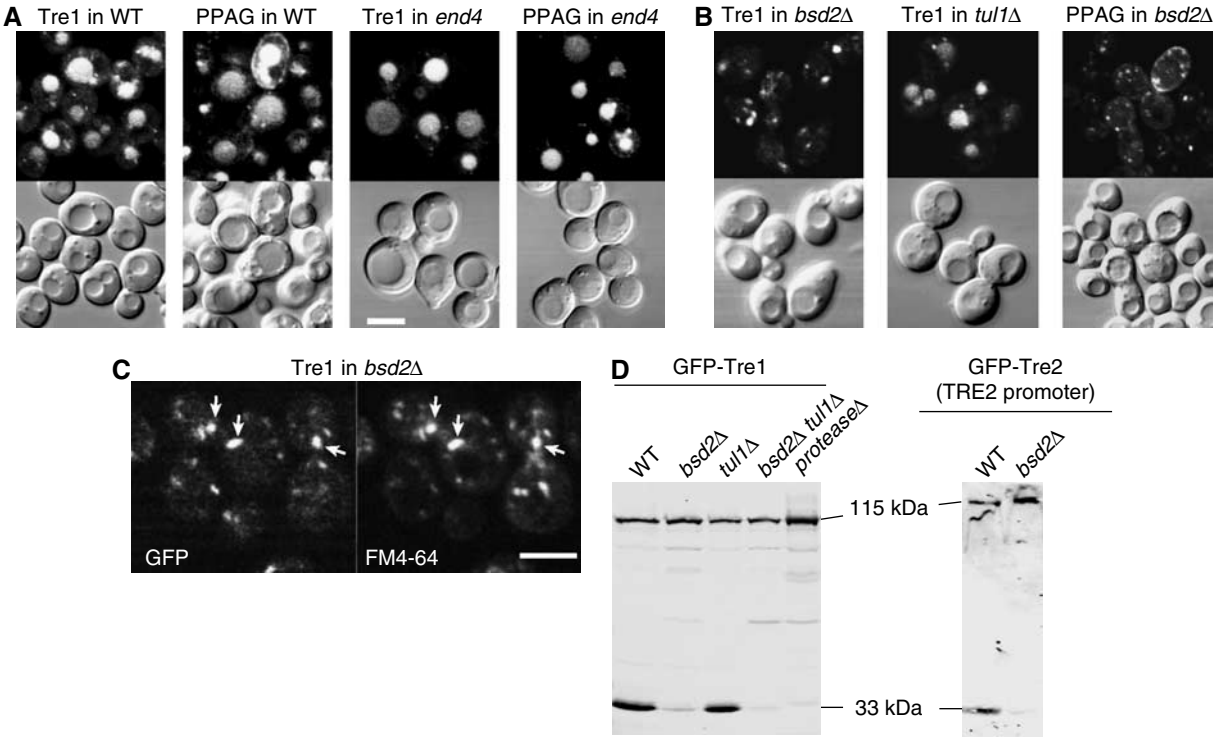

Figure 4 Tre1 is sorted to the vacuole by Bsd2. (**A, B**) GFP-Tre1, or the PPAG mutant, expressed from the *TPI1* promoter in the indicated strains. (**C**) Double label of GFP-Tre1 in *bsd2* cells that had been incubated for 10 min with FM4-64. (**D**) Immunoblot of GFP-Tre1, detected with anti-GFP, in the indicated strains. The left-hand panel shows proteins expressed from the *TPI1* promoter and the right-hand panel shows GFP-Tre2 expressed from its own promoter, exposed for much longer, as the signal was very weak. Bars in A and C, 3 μm.

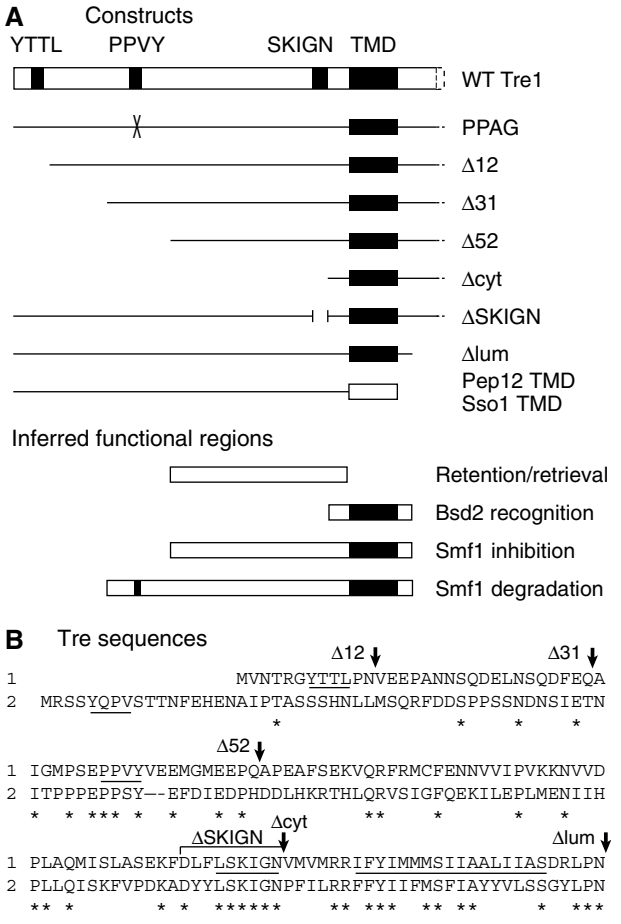

Figure 5 Tre1 mutants. (**A**) Diagrams of the various Tre1 mutants tested. The luminal domain is not shown. The regions implicated in various processes, as inferred from tests of these mutants, are summarised at the bottom. (**B**) Sequences of the cytoplasmic domains of Tre1 and Tre2 showing identities and deletion end points. Lines correspond to the features indicated by black boxes in panel A.

plasma membrane SNARE Sso1, abolished vacuolar delivery (Figures 5 and 6A). Instead, the protein accumulated on the plasma membrane, and in the case of the Pep12 TMD also in some endosome-like structures. Thus, the Tre1 TMD sequences are indeed required for Bsd2-dependent targeting.

Deletions of the Tre1 cytoplasmic domain did not prevent delivery to the vacuole in wild-type cells, as shown by immunoblotting and fluorescence microscopy (Figures 5 and 6B). However, mutations that disrupted the region near the membrane (Δ52, ΔSKIGN, Δcyt) had two striking effects. First, the protein became a substrate for Tul1 in addition to Bsd2—only in the absence of both was delivery into the vacuole abolished (Figure 6B). Thus, this region normally prevents interaction with Tul1. Second, when ubiquitination was prevented by removal of both Bsd2 and Tul1, the protein reached the vacuolar membrane instead of being retained in endosomes (Figure 6B), implying that this region also contains a signal that retains non-ubiquitinated protein in the Golgi/endosome system. Indeed, the mutants lacking it (Δ52, ΔSKIGN, Δcyt) showed less full-length protein even in wild-type cells, implying faster transit to the vacuole (Figure 6C). In contrast to the importance of the cytoplasmic domain, deletion of the luminal domain did not affect specific recognition of Tre1 by the Bsd2 system (Figure 3C and data not shown).

Smf1 regulation requires cytoplasmic sequences of Tre1

We next asked which features of Tre1 are responsible for regulating Smf1 activity. To distinguish this from the interaction with Bsd2, we first concentrated on regulation of Smf1 in the absence of Bsd2. To do this, we tested the various deletion mutants for their ability, when overexpressed, to restore growth of *bsd2* null cells on cadmium. Because some of the constructs are also substrates for Tul1, we used *bsd2 tul1* double mutant cells to eliminate any possible contribution of Tul1; however, the effects were indistinguishable from those obtained with the single *bsd2* mutant.

This activity of Tre1 did not require the luminal domain nor the PPxY motif. Indeed, a minimal fragment extending from position 53 to the luminal end of the TMD (Δ52 Δlum) was sufficient (Figure 7A). However, removal of the entire cytoplasmic domain, or disruption of the SKIGN region, abolished activity. As expected, the minimal active fragment and the PPAG mutant, although functional in the absence of Bsd2, were not able to reduce overall levels of Smf1 protein under these conditions (Figure 2C).

An essentially identical pattern of cadmium resistance was obtained when the same constructs were expressed at high level in *tre1 tre2* double mutant cells (Figure 7B). This demonstrated that endogenous wild-type Tre proteins, which are present in the *bsd2* suppression assay, are not required for the truncated Tre1 constructs to function. Testing of the TMD substitution mutants also showed that the TMD sequence was crucial for activity (Figure 7B), although this might be an indirect effect of the Tre protein being mislocalised. We conclude from these experiments that the TMD and immediately adjacent cytoplasmic sequences of Tre1 can modulate the location or activity of Smf1, in a way that is completely independent of Bsd2.

A different pattern emerged when we tested key deletion mutants by expressing them from the weak *TRE1* promoter in the *tre1 tre2* double mutant (Figure 7C). Under these conditions, Bsd2 is essential for cadmium resistance, and thus the assay measures the ability of Tre1 to cooperate with Bsd2 to downregulate Smf1. In addition to the TMD and adjacent sequences, this clearly also required the PPxY motif. Specifically, mutants lacking the N-terminal 52 residues, including the PPxY region (Δ52), or with a point mutation of the PPxY (PPAG) were inactive in this assay, even though they could inhibit Smf1 activity when expressed at high levels. Thus, degradation of Smf1 requires the PPxY motifs of both Bsd2 (Hettema *et al*, 2004) and Tre1.

In conclusion, three regions of Tre1 contribute to the regulation of Smf1. The TMD forms a recognition site for Bsd2, and the PPxY motif plays an important role, presumably in helping to recruit Rsp5. The intervening cytoplasmic region is able to reduce Smf1 activity in the absence of Bsd2, and hence is likely to be involved, directly or indirectly, in Smf1 recognition.

Discussion

We have shown that the TFR-like proteins Tre1 and Tre2 have a specific role in the degradation of the divalent metal ion

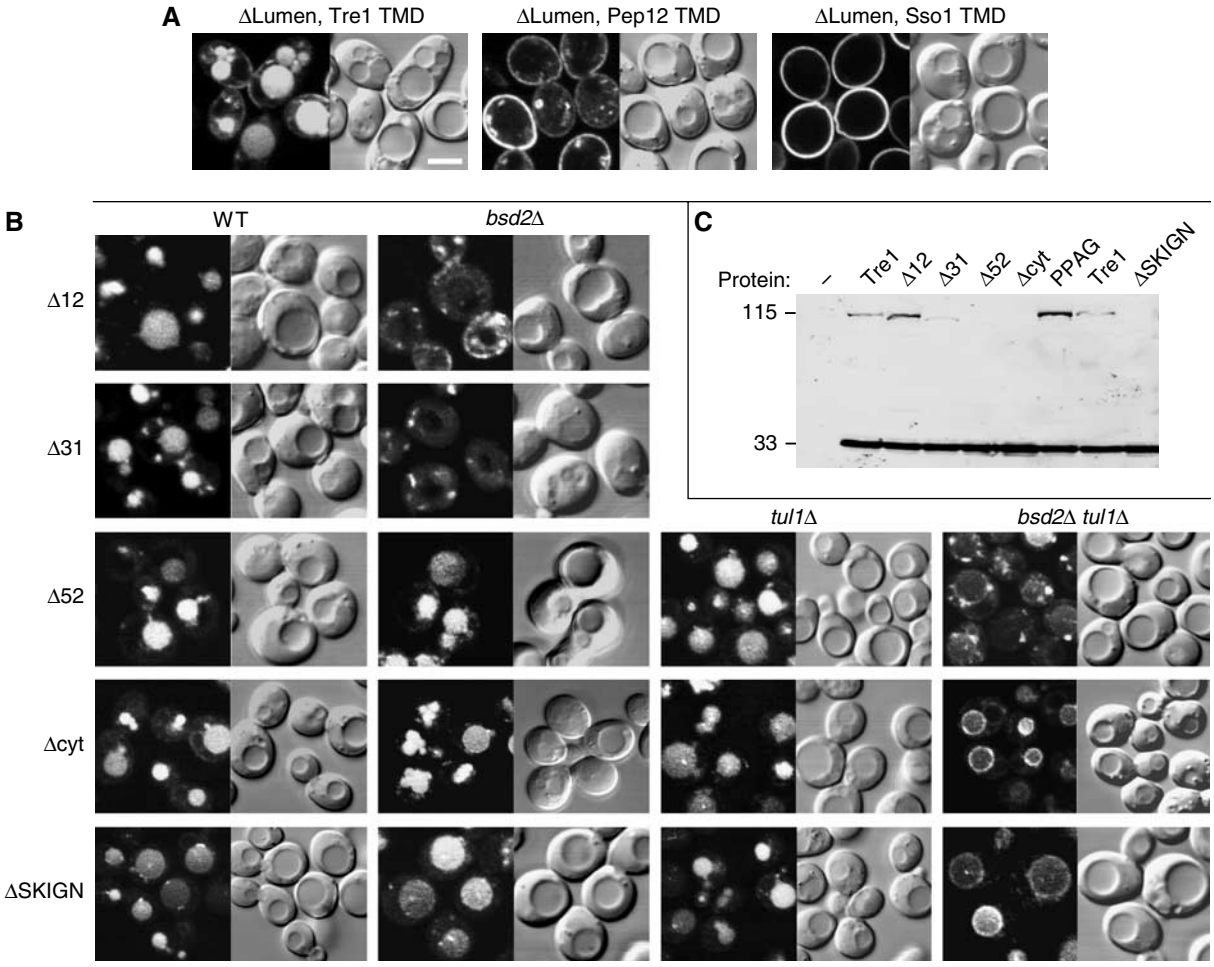

Figure 6 Sorting of Tre1 mutants. (**A**) Effect of the TMD on sorting of truncated Tre1 to the vacuole in wild-type cells. GFP-Tre1 constructs that lacked the luminal domain (Δlumen) or in addition had the TMD replaced by that of Pep12 or Sso1 are shown. Bar, 3 μm. (**B**) GFP-tagged cytoplasmic domain mutants of Tre1 expressed in wild-type (WT) *bsd2*, *tul1*, and *bsd2 tul1* double mutant cells. See Figure 5 for details of the mutations. (**C**) Immunoblot of the mutants expressed in *tre1 tre2* cells. The prominent band at 33 kDa is free GFP generated by vacuolar proteolysis.

transporter Smf1, a role that is important in the protection of cells from heavy metal poisoning. Although Bsd2 is also required for degradation, the ability of the Tre proteins to influence Smf1 activity even in the absence of Bsd2 makes them candidates for the elusive class of regulators that directly recognise transporters and control their abundance.

A model for Smf1 regulation

Our model for Tre1 action is diagrammed in Figure 8A. Transporters such as Smf1 typically consist of two rigid domains, which move relative to each other in the membrane, alternately exposing the substrate binding site to the extracytoplasmic side and the cytoplasm (Lemieux *et al*, 2004). In the case of Smf1, uptake is driven by the co-transport of protons, which, together with the metal ions, are released on the cytoplasmic side of the membrane. We propose that the Tre proteins recognise Smf1 while simultaneously binding Bsd2. The PPxY motifs on Bsd2 and Tre1/2 recruit Rsp5, which then ubiquitinates Smf1, as well as Tre1/2 and Bsd2. We cannot say precisely where this occurs, but as Bsd2 and Tre1 do not normally pass through the plasma membrane, and nor does Smf1 in metal-replete conditions (Liu and Culotta, 1999b), it is likely to be in the Golgi and/or endosomes. All three ubiquitinated proteins then enter the MVB pathway to the vacuole.

In support of this model, we can readily detect a Tre1–Bsd2 complex. However, under comparable conditions we have been unable to detect a Tre1–Smf1 complex, even when *in vivo* crosslinking is used. Nevertheless, the ability of Tre1 to modulate cadmium and manganese uptake in the absence of Bsd2 suggests that it is capable of interacting with Smf1, and can either inhibit its action or help to sequester it away from the plasma membrane. The failure to detect a complex may simply reflect the weak and transient nature of this essentially catalytic interaction, although it remains possible that recognition is more indirect, perhaps involving an additional protein.

Analysis of inactive Smf1 mutants has shown that they escape Bsd2-mediated degradation, and it has been suggested that binding of metals to Smf1 is required to induce a conformation that is preferentially degraded (Liu and Culotta, 1999a). An attractive possibility is thus that it is the metal-bound conformation of Smf1 that the Tre proteins recognise, allowing them to respond directly to the presence of metals.

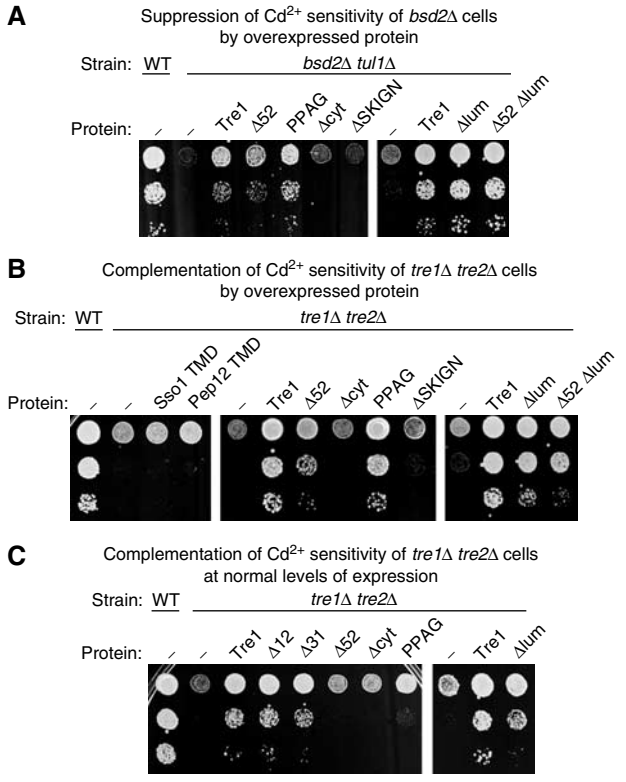

Figure 7 Regions of Tre1 required for Smf1 regulation. (**A**) Suppression of *bsd2* cadmium sensitivity by overexpressed protein. GFP-tagged Tre1 and derivatives of it (see Figure 5 for details) were expressed from the *TPI1* promoter in the indicated strains. Growth was on plates containing 20 μM cadmium. (**B**) Suppression of *tre1 tre2* cadmium sensitivity by Tre1 derivatives expressed from the *TPI1* promoter. (**C**) Suppression of *tre1 tre2* cadmium sensitivity by Tre1 mutants expressed from the *TRE1* promoter, at low levels.

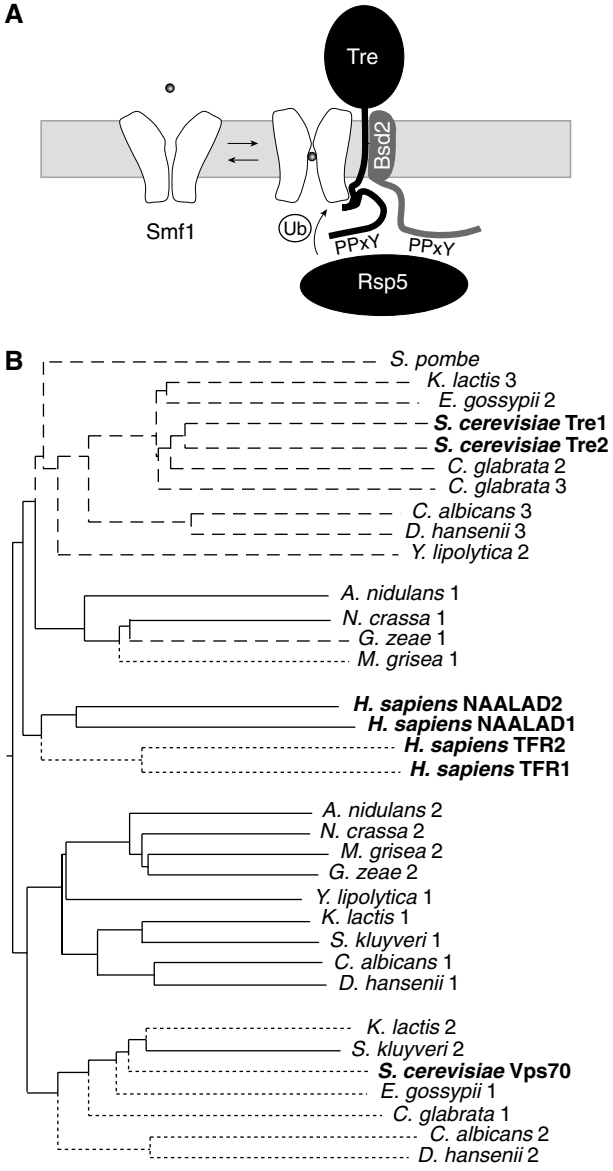

Figure 8 Model for Tre protein function and relationship to the TFR. (**A**) Model. Smf1, in a metal-bound conformation, is recognised by Tre1. Tre1 is in a complex with Bsd2, which, through the PPxY motifs of both proteins, recruits Rsp5. Rsp5 ubiquitinates all three membrane proteins, which are thereby directed into the MVB pathway to the vacuole. (**B**) Evolutionary relationships of the Tre proteins. A family tree of ascomycete proteins that share the domain structure of the Tre proteins, together with human relatives, was prepared with the program clustal W using related proteins from *Physarum* and *Gloeobacter* as outliers. Proteins were categorised according to the presence of six residues shown to be crucial for protease activity. Solid lines indicate proteins likely to be active proteases. Dotted lines indicate a non-functional protease domain, and dashed lines indicate both lack of protease activity and the presence of a PPxY motif in the cytoplasmic domain. A, *Aspergillus*; C, *Candida*; D, *Debaromyces*; E, *Eremothecium*; G, *Gibberella*; H, *Homo*; K, *Kluyveromyces*; M, *Magnaporthe*; N, *Neurospora*; S, *Schizosaccharomyces* (*pombe* only) or *Saccharomyces*; Y, *Yarrowia.*

The second form of regulation of Smf1, whereby plasma membrane levels are reduced under metal-replete conditions, appears independent of the Tre proteins—although the *tre1 tre2* double mutation stabilises Smf1, the stabilised protein mostly does not appear on the plasma membrane but rather has a Golgi/endosomal distribution. Hence, some other mechanism must be capable of removing Smf1 from the cell surface in response to metals. This unknown mechanism may aid the degradation of the transporter by ensuring that it is exposed to Tre1/2 and Bsd2 in endosomes. The nature of the Tre-independent step is clearly an area for future investigation.

One puzzling observation is that Smf1 degradation requires the PPxY motif of the Tre protein, in addition to that on Bsd2 (Hettema *et al*, 2004). Bsd2 itself is clearly capable of acting on other substrates, such as carboxypeptidase S, without the aid of Tre1. By analogy, one might expect Tre1 simply to recruit Rsp5 directly, without Bsd2. However, recruitment of Rsp5 has different requirements at different membranes (Dunn *et al*, 2004). Modification of carboxypeptidase S likely occurs in the Golgi and requires both the lipid-binding C2 domain and the WW2 domain of Rsp5 (Dunn *et al*, 2004; Katzmann *et al*, 2004; Morvan *et al*, 2004); possibly, Smf1 modification occurs elsewhere, such as in endosomes, and uses a different combinatorial strategy for Rsp5 recruitment that involves two WW domains and two PPxY motifs.

A further question is why both *TRE* genes should have been retained, given that they perform much the same function and that many duplicated genes have been lost from the yeast genome. Similarly, one can ask why the luminal domain, which seems to play at most a limited role

in Smf1 downregulation, should have been retained. The most obvious possibility is that Tre1 and Tre2 have other substrates that we have not so far identified. Their structure would suit them to a role in the recognition of damaged or misfolded luminal domains, or of other specific membrane protein substrates, coupling such recognition to ubiquitination and destruction. Such a role may have escaped detection because of the redundancy of quality control mechanisms, and the limited number of growth conditions that we have tested.

Evolution of the Tre family

What is the relationship of the Tre proteins to the mammalian TFRs? Both are part of a larger family of protease-like membrane proteins, all of which share the same domain structure. A family tree of the protein sequences from ascomycete fungi suggests several ancestral gene duplications, with some genes having been lost from modern species (Figure 8B). Inspection of the key functional residues in the protease domain indicates that some of the proteins have retained protease activity (solid lines in Figure 8B), and it seems likely that they are vacuolar proteases.

The Tre proteins are derived from a separate gene that appears to have lost protease activity early on (dashed lines in Figure 8B). All the descendants have PPxY motifs and most have recognisable similarity in the SKIGN region, suggesting a conserved function. They may have evolved from an ancestral protease that evolved an ability to interact with Bsd2 as an aid to vacuolar delivery, as is the case with other vacuolar enzymes such as carboxypeptidase S (Hettema *et al*, 2004). Subsequently, they may have lost protease activity but gained the ability to recognise Smf1 and/or other substrates, thus preserving their utility. Interestingly, filamentous fungi seem to have diverged before evolution of the protease-deficient Tre gene, but in two of them (*Gibberella zeae* and *Magnaporthe grisea*) there is evidence for independent loss of protease activity in a related gene, and in one case the gain of a PPxY motif.

An additional related gene family is represented in *Saccharomyces cerevisiae* by Vps70, a protein whose absence causes mis-sorting of soluble vacuolar proteases (Bonangelino *et al*, 2002). It is possible that this was originally a protease with a role in the processing of vacuolar enzymes, which are often synthesised as inactive precursors, and then evolved a role in transporting its substrates to the vacuole. There have been multiple independent occasions during evolution when protease activity was lost from this protein, as a result of different mutations (dotted lines in Figure 8B), although in the case of *Saccharomyces kluyveri* activity seems to have been retained.

The Tre and Vps70 families are restricted to fungi, perhaps reflecting the particular needs of these organisms. Vertebrate TFRs appear to have evolved independently from a gene that also gave rise to the plasma membrane glutamate carboxypeptidases (also known as N-acetylated-alpha-linked acidic dipeptidase) (Figure 8B). Thus, there is no direct evolutionary relationship between the TFRs and the Tre proteins. Rather, both have evolved from the same family of proteases. It seems that the ability to recognise protein substrates, together with a transmembrane and cytoplasmic domain that allows specific sorting, has allowed this protease family to contribute on several evolutionarily distinct occasions to the sorting of other proteins.

The Tre proteins illustrate a strategy of coupling the destruction of a particular substrate to a more general quality control system. Although the Tre proteins themselves have no precise homologues in animal cells, there are proteins related to Bsd2 (Shearwin-Whyatt *et al*, 2004). An open question is whether these are involved, perhaps through quite different adaptors, in regulating the abundance of mammalian transporters or other membrane proteins.

Materials and methods

Strains and plasmids

All strains were derivatives of BY4742 (*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0*) obtained from the EUROSCARF consortium, except for the *end4* mutant, which was in the SEY6210 background (*MATα ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*), and the protease-deficient strain used in Figure 4D (*MATα pep4 prb1 prc1 cps1 ura3 leu2*) (Heinemeyer *et al*, 1991). Deletions were made by replacing the entire coding sequence of the mutated gene with a marker (*Schizosaccharomyces pombe HIS5*, or the KanMX4 cassette). Ubiquitination and Tre1-Bsd2 binding were analysed in BY4742 strains deleted for *pep4* or, where indicated, both *pep4* and *bsd2*. To construct a *tre1 tre2* double deletion mutant, a heterozygous *tre2* mutant (EUROSCARF) was first transformed with a pRS316 *CEN* plasmid that carried *URA3* and *CDC31* but lacked any of the coding sequences of *TRE2*. Sporulation of this allowed recovery of a haploid *MATα tre2Δ* strain harboring the plasmid, and the *TRE1* gene was then replaced by *S. pombe HIS5*. The *tre1 tre2* strain was grown without selection for the *CDC31* plasmid, which was nevertheless retained as it was essential for growth. A second strain containing a pRS415 plasmid with *LEU2* and *CDC31* was generated by plasmid shuffling. This was used for the assay of Tre derivatives that were expressed from *URA3* plasmids.

Plasmids expressing Tre proteins or Pep12 and their derivatives were based on either the Ycplac33 *CEN URA3* vector or the similar pRS416 vector with tags, deletion end points and promoters, as indicated in the text and figures. Deletions were generated by PCR using appropriate primers. Point mutation of the PPxY motif was achieved using PCR. Smf1 was expressed similarly using the *TPI1* promoter, and tagged at the C terminus with a triple HA tag or GFP. For Figure 1E, it was GFP tagged at the N terminus, which gave brighter fluorescence. Tre1 was tagged at the N terminus with either GFP or protein A and expressed from Ycplac33 using the *TPI1* promoter or, where indicated, the *TRE1* promoter. The sequences of all PCR-generated clone fragments were confirmed by DNA sequencing.

Binding of Tre cytoplasmic domain to Rsp5 WW domains

This was carried out essentially as described by Hettema *et al* (2004). Briefly, the Tre1 cytoplasmic domain was expressed between an N-terminal 6-His tag and a C-terminal protein A tag in pET30a+. The WW domains of RSP5 were cloned into pGEX6 (WW1, residues 221–273; WW2, residues 324–372; WW3 residues 382–428). *E. coli*-produced GST fusion proteins were bound to glutathione-Sepharose beads, washed in binding buffer (20 mM Tris–HCl, pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM PMSF, 0.05% Triton X-100) and incubated for 2 h with *E. coli* cytosol prepared from cells expressing the Tre1 cytoplasmic domain, lysed in binding buffer. Beads were subsequently spun down and washed extensively with binding buffer and once with binding buffer containing 1.5 M KCl. Proteins were eluted from beads with sample buffer and analysed by immunoblotting.

Immunoprecipitation and detection of proteins

For routine immunoblotting protein samples were prepared using an adaptation of the alkaline lysis method of Volland *et al* (1994), to reduce proteolysis. Up to 10 OD_{600} units of yeast in log phase were harvested by centrifugation at 4000 r.p.m. for 5 min, resuspended in 500 μl cold 0.2 M NaOH and 0.2% β-mercaptoethanol and incubated for 10 min on ice. Trichloroacetic acid was added to 5%, the mixture incubated on ice for 10 min, centrifuged at 13 000 r.p.m. for 5 min

and the pellet resuspended in 15 µl 1 M Tris base and 35 µl 2 × SDS sample buffer. For detection of ubiquitinated proteins and co-precipitation experiments, spheroblasts were lysed and precipitated with anti-GFP or anti-HA antibodies coupled to agarose beads, or IgG Sepharose for protein A-tagged proteins, as described previously (Reggiori and Pelham, 2002; Hettema *et al*, 2003, 2004). Proteins were detected with anti-GFP (Roche Diagnostics), anti-ubiquitin (Santa Cruz Biotech), anti-HA (12CA5) or anti-Pgk1 (Molecular Probes) monoclonal antibodies, or rabbit anti-Tlg1 or anti-myc (Santa Cruz Biotech). Protein A was detected with peroxidase-coupled anti-peroxidase (DakoCytomation). Secondary antibodies were Alexa Fluor 680-labelled goat anti-mouse (Molecular Probes) and IRDye 800-labelled donkey anti-rabbit (Rockland), and were visualised using a Li-Cor Biosciences Odyssey infrared scanner.

Microscopy

GFP-labelled cells in early log phase were imaged in water using a BioRad Radiance confocal microscope. To label endosomes, FM4-64 (Molecular Probes) was added to 1 ml of cells (at an OD_{600} of 1) to a final concentration of 1 µM. Cells were incubated on ice for 10 min, pelleted by centrifugation, resuspended in approximately 50 µl of the media and uptake followed by confocal microscopy.

Assay of cadmium sensitivity and manganese uptake

To test cadmium sensitivity, cells were grown to stationary phase, and adjusted to the same concentration. Serial 10-fold dilutions were transferred to plates containing the appropriate selective media together with 20 µM $CdCl_2$ using a device with flat-ended pins to create circular patches of cells. Plates were photographed after growth for 3 days at 30°C. ^{54}Mn uptake was measured as described by Cohen *et al* (2000). Briefly, cells were grown to an OD_{600} of 0.5, washed twice with ice-cold water and once with choline medium (100 mM choline chloride, 10 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 25 mM MES pH 4.5, 1 µM $MnCl_2$, 2% glucose) and then resuspended at an OD_{600} of 1.25 in the same medium. Aliquots (0.5 ml) in 1.5 ml conical tubes were pelleted in a microfuge and resuspended in 0.5 ml of choline medium containing about 100 000 c.p.m. of ^{54}Mn. After incubation for 15 min at 30°C, cells were chilled on ice, pelleted, washed twice with ice-cold choline medium containing 1 mM $MnCl_2$ and radioactivity measured in a gamma counter. No more than about 5% of the input radioactivity was taken up under these conditions. Background counts from samples maintained on ice, which were at least 10-fold lower than any of the experimental samples, were subtracted.

Acknowledgements

We thank John Kilmartin for the CDC31 plasmid, Ewald Hettema and Javier Valdez-Taubas for plasmids, advice and many helpful discussions, Elina Nikko for help with the GFP-Smf1 and Miguel Peñalva for comments on the manuscript.

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