EMBO reports

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scientific report

Arrestin-like proteins mediate ubiquitination and endocytosis of the yeast metal transporter Smf1

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Many plasma membrane proteins in yeast are ubiquitinated and endocytosed, but how they are recognized for modification has remained unknown. Here, we show that the manganese transporter Smf1 is endocytosed when cells are exposed to cadmium ions, that this endocytosis depends on Rsp5-dependent ubiquitination of specific lysines and that it also requires phosphorylation at nearby sites. This phosphorylation is, however, constitutive rather than stress-induced. Efficient ubiquitination requires Ecm21 or Csr2, two members of a family of arrestin-like yeast proteins that contain several PY motifs and bind to Rsp5. Ecm21 also binds to phosphorylated Smf1, providing a link between Rsp5 and its substrate. PY motif-containing arrestin-like proteins are found in many species, including humans, and might have a general role as ubiquitin ligase adaptors.

Keywords: arrestin; endocytosis; Smf1; ubiquitin; yeast
EMBO reports (2008) **9**, 1216–1221. doi:10.1038/embor.2008.199

INTRODUCTION

Many plasma membrane proteins are subject to tight regulation, being actively removed from the cell surface under certain conditions. In yeast, such downregulation is achieved by ubiquitination of the protein, which acts as a signal for both endocytosis and entry into multivesicular bodies and hence the vacuole (Katzmann *et al*, 2002; Hicke & Dunn, 2003). In several cases, prior phosphorylation of the proteins has been shown to be essential for their ubiquitination (Hicke *et al*, 1998; Marchal *et al*, 1998). Ubiquitination of these proteins is carried out by Rsp5, a HECT domain ligase containing three WW domains (Shearwin-Whyatt *et al*, 2006). The WW domains recognize PPXY or related proline-containing sequences (PY elements); however, these are lacking in most plasma membrane proteins. A likely explanation is that specific adaptor molecules recognize the substrate and also bind, through PY motifs, to the Rsp5 ligase (Shearwin-Whyatt *et al*, 2006). Such a function has been suggested for the soluble Bul1 and Bul2 proteins (Helliwell *et al*, 2001), but it is at present unclear how specific ubiquitination of any protein on the yeast plasma membrane is achieved.

This endocytic regulation is distinct from the intracellular control observed for some proteins, including the manganese transporter Smf1, uracil permease Fur4 and general amino-acid transporter Gap1. When their substrates, or in the case of Gap1, a preferential source of nitrogen such as glutamate, are present in the medium, these proteins undergo Rsp5-dependent ubiquitination in the Golgi shortly after synthesis and are transported to the vacuole without ever reaching the cell surface (Liu & Culotta, 1999; Soetens *et al*, 2001; Blondel *et al*, 2004; Stimpson *et al*, 2006). In the case of Smf1, ubiquitination requires membrane protein adaptors with PY elements, namely Bsd2 and Tre1/2 (Hettema *et al*, 2004); however, these adaptors are not required for the endocytosis of Smf1 from the cell surface.

In animal cells, downregulation of ligand-activated G-protein-coupled receptors is mediated by β-arrestins (reviewed by Lefkowitz *et al*, 2006; Marchese *et al*, 2008). These are soluble proteins that recognize conformational features of the receptors and phosphorylated residues. Arrestins can directly recruit clathrin and other components of the endocytic machinery. In some cases, they can also recruit ubiquitin ligases, which modify both the arrestin and the receptor (Shenoy *et al*, 2008; Marchese *et al*, 2008).

Homology searches have shown that many species, including mammals, also contain a distinct family of arrestin-like proteins, which, unlike the β-arrestins, typically contain a pair of PY elements (Alvarez, 2008). This feature suggests that they could act as adaptors for HECT domain ubiquitin ligases. The Saccharomyces Genome Database (http://www.yeastgenome.org) identifies eight candidates in yeast with the essential sequence features of arrestins: Ecm21, Csr2, Aly1, Aly2, Rod1, Rog3, Ygr068c and Rim8. In addition, there are other proteins, such as Vps26, that are more distantly related to arrestins (Alvarez, 2008). At least seven of these proteins contain canonical PPXY sequences, and several have been shown to be ubiquitinated

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Received 15 September 2008; revised 25 September 2008; accepted 25 September 2008; published online 24 October 2008

in vivo and to be substrates for Rsp5 *in vitro* (Peng *et al*, 2003; Kee *et al*, 2006; Gupta *et al*, 2007).

Here, we show that stress-induced endocytosis of the manganese transporter Smf1 is triggered by Rsp5-mediated ubiquitination of lysines in the amino-terminal region of the protein, and that this requires both phosphorylation of sites close to these lysines and the presence of at least one of the arrestin pair Ecm21/Csr2. We also show that Ecm21 binds specifically to phosphorylated Smf1 and, through its PY elements, is recognized by Rsp5. Thus, at least in this case, arrestins provide the missing link between Rsp5 and its plasma membrane substrates. By analogy, we suggest that ubiquitination of other plasma membrane proteins might also be mediated by members of the arrestin family.

RESULTS

Cadmium induces the endocytosis of Smf1

In metal-deficient medium, Smf1 is expressed on the cell surface; this also occurs in normal medium in a *bsd2* mutant. We have shown previously that Smf1 can be subsequently endocytosed in an apparently stress-induced manner (Sullivan *et al*, 2007). In exploring this phenomenon, we found that the endocytosis of Smf1 can be rapidly and efficiently induced by 0.1 mM cadmium chloride (Fig 1A). Cadmium is toxic and a substrate for Smf1; therefore, downregulation of the transporter might act to protect cells. Activity *per se* does not seem to be the trigger, however, as 5 mM manganese, a less toxic substrate, did not promote Smf1 internalization (data not shown). In our subsequent investigations of Smf1 endocytosis, we used cadmium as the inducer. We also added cycloheximide 10 min before the cadmium to ensure that we followed the fate of the pre-existing transporter rather than that of newly synthesized material. To study endocytic sorting specifically, experiments were conducted in *bsd2*Δ cells.

Endocytosis requires ubiquitination at lysines 33 and 34

We have shown previously that removal of 68 residues from the N-terminal cytoplasmic tail of Smf1 prevented its stress-induced endocytosis (Sullivan *et al*, 2007); this was also true when cadmium was used as the inducer (data not shown). Reasoning that this tail was probably the site of ubiquitination, we mutated the lysines at positions 20, 33, 34 and 65 to arginines, and found that this prevented the endocytosis of Smf1 as efficiently as did the *end3*Δ mutation, which blocks the internalization step of the endocytic pathway (Fig 1A). Mutation of just the two central lysines (K33,34) had almost as strong an effect, whereas mutation of the outer lysines (K20,65) did not greatly affect endocytosis. Interestingly, in the K33,34 mutant and to some extent in the K20,65 mutant, Smf1 tended to be internalized slowly and to accumulate in endosomal structures adjacent to the vacuole (Fig 1A). This suggests that limited ubiquitination can allow endocytosis, but is insufficient for efficient internalization into multivesicular bodies and delivery to the vacuole.

To detect ubiquitination, we immunoblotted green fluorescent protein (GFP)-Smf1, using *end3*Δ *bsd2*Δ cells to ensure that we studied cell surface-located transporters. With the wild-type protein, more slowly migrating bands, which became more intense after the addition of cadmium, were detected above the GFP-Smf1 band (Fig 1B). These correspond to ubiquitinated forms of Smf1, as shown by their labelling with Myc-tagged ubiquitin expressed in the same cells (Fig 1C). The bands were absent when GFP-Smf1 was expressed in *rsp5* mutant cells that have low levels of Rsp5 protein, suggesting that the modification was mediated by this enzyme. They were also missing from the GFP-Smf1-4KR samples and much reduced in the K33,34 mutant, confirming qthat these N-terminal lysines are the sites of stress-induced ubiquitination (Fig 1B).

Arrestins mediate ubiquitination of Smf1

To search for possible Rsp5 adaptors specific to Smf1, we screened single arrestin mutants for enhanced sensitivity to cadmium, reasoning that a defect in Smf1 endocytosis would lead to increased levels of the transporter at the cell surface and to increased uptake of the toxic metal. Some sensitivity was observed for *ecm21*Δ (data not shown); however, the stress-induced endocytosis of GFP-Smf1 in *ecm21*Δ *bsd2*Δ cells was mostly normal (Fig 2A).

The closest homologue of Ecm21 in yeast is Csr2. The internalization of GFP-Smf1 was normal in *csr2*Δ *bsd2*Δ cells, but when we combined mutations of both arrestins, there was a stronger effect on the internalization of Smf1, suggesting that Ecm21 and Csr2 have redundant functions (Fig 2A). In the double arrestin mutants, some Smf1 remained at the cell surface even after 90 min, but some accumulated in perivacuolar endosomes. As with the double lysine mutants, this is consistent with a low level of residual ubiquitination allowing endocytosis but not efficient delivery to the vacuole (Fig 2B).

In an attempt to eliminate this residual activity, we constructed a *bsd2*Δ strain in which the seven most closely related arrestins, all of which have canonical PPXY motifs, were deleted. In this strain, endocytosis of Smf1 was inhibited and the protein remaining mostly at the cell surface rather than reaching the endosomes (Fig 2A). Ubiquitination was also greatly reduced, more so than in the strain lacking only Ecm21 and Csr2 (Fig 2B). Thus, arrestins mediate Rsp5-dependent ubiquitination of Smf1, and there is considerable redundancy in their functions.

The arrestin Ecm21 interacts with Rsp5 and Smf1

Previous studies have shown that Ecm21 and Csr2 bind directly to and are ubiquitinated by Rsp5 (Peng *et al*, 2003; Kee *et al*, 2006); this is probably mediated by their PY elements. To confirm this, we mutated three potential elements in the carboxy terminus of Ecm21, LPTY, PPPP and PPRY (see Methods), and examined the ubiquitination of the mutants by Rsp5 *in vitro*. In the absence of Rsp5, wild-type Ecm21 migrated as a prominent single band on immunoblots, whereas, in the presence of the ligase, several slower migrating bands that correspond to ubiquitinated forms of Ecm21 were detected. Mutation of all three PY elements was sufficient to abolish these forms (Fig 3A).

PY elements were also required for the function of Ecm21 *in vivo*. Expression of wild-type Ecm21 in the seven arrestin mutant cells was sufficient to fully restore the endocytosis of Smf1, but the triple PY mutant version of Ecm21 did not significantly stimulate the endocytosis of Smf1 (Fig 3B).

If Ecm21 targets Rsp5 to Smf1, it should be able to bind not only to Rsp5 but also to Smf1. To test this, we co-expressed GFP-Smf1 with haemagglutinin (HA)-tagged Ecm21 and examined their interaction with or without treatment with a reversible cross-linker. HA-Ecm21 clearly co-immunoprecipitated with GFP-Smf1,

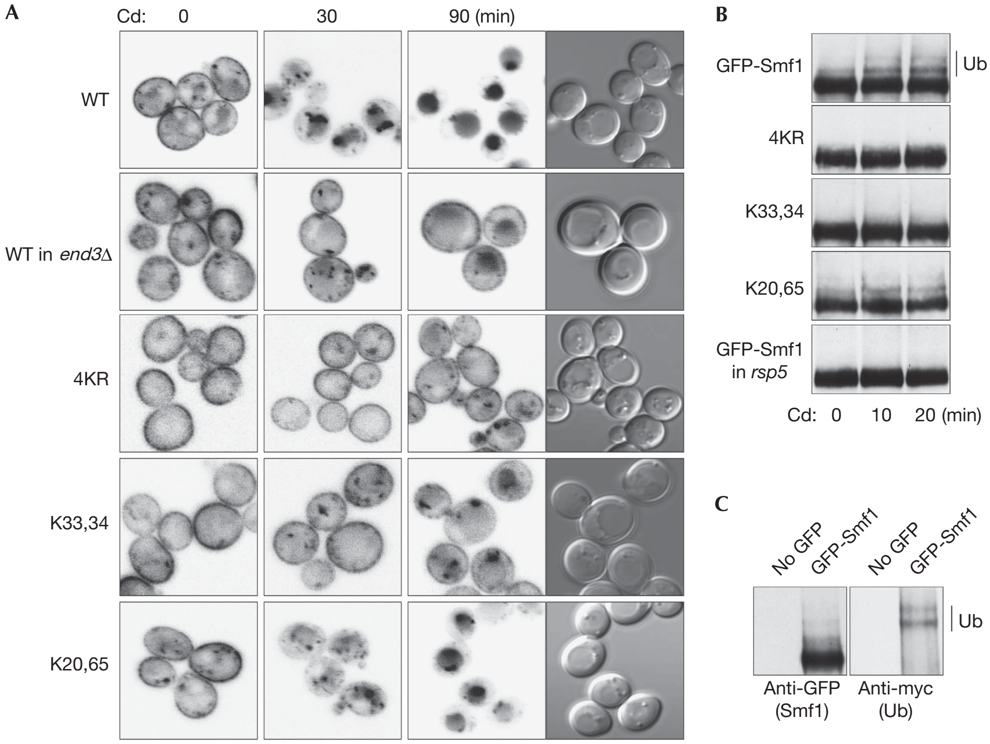

Fig 1 | Cadmium induces Smf1 internalization through the ubiquitination of lysines 33 and 34. (**A**) Fluorescent images showing the time course of internalization following the addition of cadmium (Cd), for wild-type GFP-Smf1 (WT) or mutants as indicated; all cells are *bsd2*Δ. Differential interference contrast images of the cells at the last time point are also shown. (**B**) Immunoblot of GFP-Smf1 and mutants exposed to cadmium for the indicated times. Cells were *end3*Δ *bsd2*Δ, or *rsp5* where indicated. Smf1 is found on the surface of both types of cell ((**A**) and data not shown). (**C**) GFP-Smf1 immunoprecipitated from cadmium-treated cells expressing Myc-tagged ubiquitin was blotted with anti-GFP and with anti-Myc, which shows ubiquitinated forms. Monoubiquitinated Smf1 is not efficiently detected under these conditions. GFP, green fluorescent protein; Ub, ubiquitin.

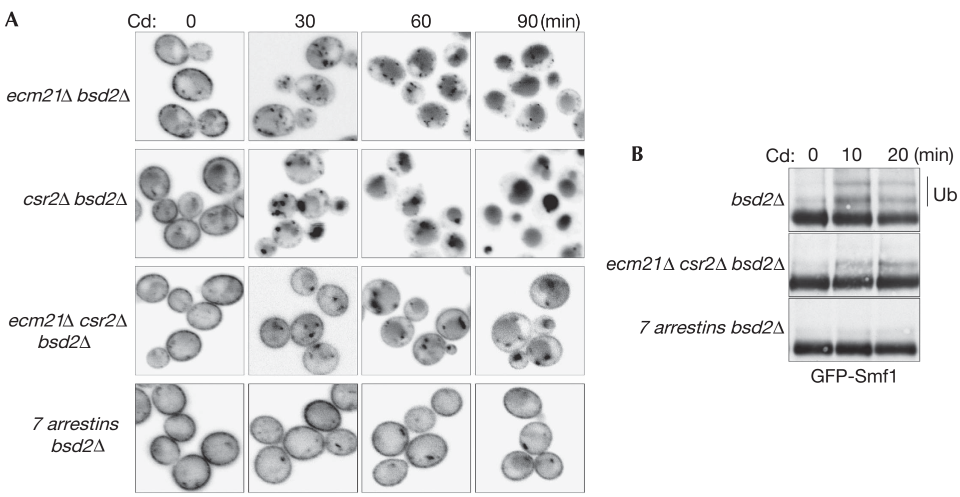

Fig 2 | Arrestins are required for the efficient ubiquitination and endocytosis of Smf1. (**A**) Fluorescent images are shown of GFP-Smf1 in the indicated strains, exposed to cadmium for the times indicated. The seven arrestins mutant lacked Ecm21, Csr2, Aly1, Aly2, Rod1, Rog3 and Ygr068c. (**B**) Immunoblots of GFP-Smf1 in the indicated strains. Cd, cadmium; GFP, green fluorescent protein; Ub, ubiquitin.

although only after crosslinking (Fig 4). Ecm21 appeared as several bands *in vivo*, which might reflect its known ubiquitination, but we have not investigated this in detail.

Phosphorylation of Smf1 is required for arrestin binding

Treatment of GFP-Smf1 with alkaline phosphatase increased its electrophoretic mobility, suggesting that it is phosphorylated

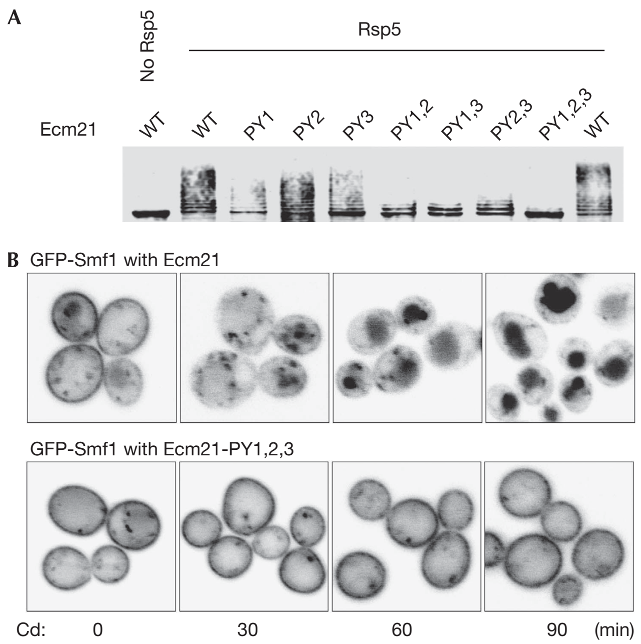

Fig 3 | Recruitment of Rsp5 by Ecm21. (**A**) Rsp5-mediated *in vitro* ubiquitination of Ecm21 and mutants lacking one, two or all three of the carboxy-terminal PY elements. (**B**) Fluorescent images of GFP-Smf1 co-expressed with Ecm21 (upper panels) or the triple PY mutant of Ecm21 (lower panels) in the seven arrestin mutant cells. Cd, cadmium; GFP, green fluorescent protein; WT, wild type.

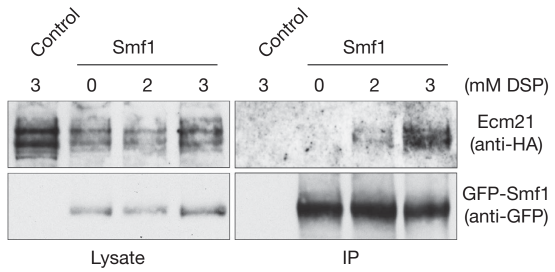

Fig 4 | Crosslinking of Ecm21 to GFP-Smf1. GFP-Smf1 (omitted in the control) and HA-tagged Ecm21 were co-expressed in *ecm21Δ bsd2Δ* cells. After spheroplasting and treatment with the indicated concentrations of the DSP crosslinker, the cells were lysed and immunoprecipitated with anti-GFP. Immunoblots of the lysate and immunoprecipitates (IP) with the indicated antibodies are shown. DSP, dithiobis(succinimidyl propionate); GFP, green fluorescent protein; HA, haemagglutinin.

(Fig 5A). A significantly smaller shift was observed when six serines in the N-terminal tail (at positions 24, 36, 51–54) were changed either to alanine (Smf1-SA) or, to mimic phosphorylation, to aspartic acid (Smf1-SD). This indicates that at least some of these serines are phosphorylated, although residual phosphatase sensitivity of the mutants indicates that phosphorylation also occurs at other sites. The phosphorylation state of Smf1, as judged by its electrophoretic mobility, was not altered by stress (for example, see Figs 1B,5C).

Strikingly, stress-induced endocytosis of the GFP-Smf1-SA mutant was markedly inhibited. By contrast, the phosphomimic SD variant was efficiently endocytosed and targeted to the vacuole (Fig 5B). Endocytosis of this variant remained stress inducible, indicating that phosphorylation, or its simulation, is necessary but not sufficient for internalization.

We assessed the ubiquitination of these Smf1 forms, using *end3Δ bsd2Δ* cells to ensure that they were at the cell surface (Fig 5C). The Smf1-SA mutant was less ubiquitinated than the wild-type Smf1, and seemed to be modified predominantly by a single ubiquitin. By contrast, the phosphomimic mutant was ubiquitinated at least as well as the wild-type transporter. As with its endocytosis, ubiquitination of the SD mutant remained cadmium inducible.

A simple explanation for these results is that phosphorylation of Smf1 is required for arrestin binding. To test this, we repeated the crosslinking of Ecm21 to Smf1 using the GFP-Smf1-SA and GFP-Smf1-SD mutants. Fig 5D shows that the SA mutant co-precipitated with Ecm21 much less efficiently than did the wild-type Smf1, whereas the SD variant bound well to Ecm21. We conclude that phosphorylation of the N terminus of Smf1 is necessary to allow binding of Ecm21. Ecm21 can, in turn, recruit Rsp5, triggering the ubiquitination of both Ecm21 and Smf1 and subsequent internalization.

DISCUSSION

Arrestins as ubiquitin ligase adaptors

We have shown that yeast arrestins mediate ubiquitination of the divalent metal transporter Smf1 and hence control its endocytosis. Ecm21 can interact with both Smf1 and Rsp5, suggesting that it acts by recruiting Rsp5 to Smf1. Subsequent ubiquitination of Smf1, and of the associated arrestin, provides an endocytic signal. Such an adaptor mechanism is conceptually similar to the mechanism of metal-dependent modification of Smf1 by Rsp5 at intracellular sites. However, it is distinct in that intracellular sorting does not require the N terminus, and thus must involve ubiquitination of different lysine residues and also requires different PY-containing adaptor molecules, namely Tre1/2 and Bsd2 (Stimpson *et al*, 2006; Sullivan *et al*, 2007).

Arrestins are best known for their ability to interact with rhodopsin and the related G-protein-coupled receptors in animal cells. The first arrestin to be identified in fungi, the palF of *Aspergillus*, also interacts with a seven transmembrane receptor and is involved in ambient pH signalling (Herranz *et al*, 2005). However, there are indications that even the classical arrestins have a wider role (Lefkowitz *et al*, 2006), and the functions of the arrestin-like proteins that are found in many species, and typically contain tandem PY motifs, are largely unknown (Alvarez, 2008). The Smf1 example suggests that they might act as ubiquitin ligase adaptors for diverse substrates, including perhaps the many plasma membrane proteins in yeast that are known to be substrates for Rsp5, but for which the recognition mechanism has remained unknown.

The role of phosphorylation

One feature that is clearly shared by the mammalian and yeast arrestins is that their binding depends, in part, on interactions with phosphorylated peptide sequences. In the case of Smf1, it seems that phosphorylation of the protein, although essential, is not the trigger for its endocytosis: we have observed no evidence that cadmium induces phosphorylation of Smf1, and the SD mutant

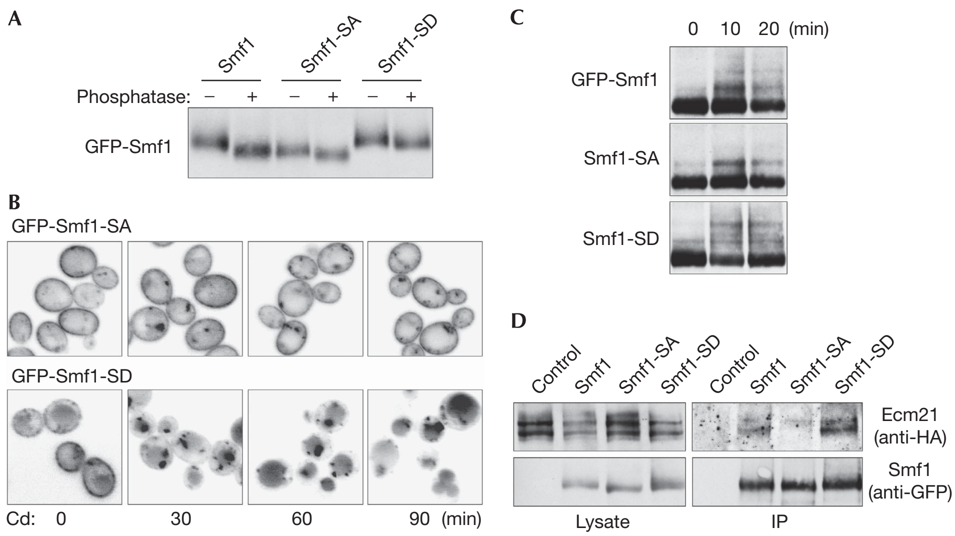

Fig 5 | Phosphorylation of Smf1 enhances arrestin binding, ubiquitination and endocytosis. (**A**) Immunoblot of GFP-Smf1 and the phosphorylation mutants before and after treatment with calf intestinal phosphatase. The SA mutant has the six serines at positions 24, 36 and 51–54 changed to alanines; the SD mutant has the same residues changed to aspartic acid. (**B**) Fluorescent images of the phosphorylation mutants of GFP-Smf1 in *bsd2Δ* cells exposed to cadmium (Cd) for the indicated times. (**C**) Immunoblots of GFP-Smf1 and the phosphorylation mutants, expressed in *end3Δ bsd2Δ* cells that were exposed to cadmium for the indicated times. Bands above the principal one correspond to ubiquitinated forms. (**D**) Crosslinking of Ecm21 to GFP-Smf1 and the phosphorylation mutants. The experiment was performed as in Fig 4, with 3 mM DSP crosslinker; control samples lacked GFP-Smf1. DSP, dithiobis(succinimidyl propionate); GFP, green fluorescent protein.

that mimics constitutive phosphorylation is still subject to control. Furthermore, we have not detected any change in the interaction between Ecm21 and Smf1 as a result of stress, although our assay requires spheroplasting of cells and it is fairly likely that this itself induces stress. It thus remains to be determined how cadmium and other stressors control the ubiquitination process.

If phosphorylation of Smf1 itself is not involved in the stress response, it might have another function. It seems most likely that Smf1 is modified by constitutive plasma membrane-associated kinases such as the YCK1/2 pair, which are known to be involved in the modification of Fur4 and Ste2 (Hicke *et al*, 1998; Marchal *et al*, 2000). If so, phosphorylation would specifically mark those molecules that had reached the cell surface. This would ensure that the arrestins are targeted to the molecules that require rapid removal, and not to the intracellular pool of Smf1.

METHODS

Strains, plasmids and media. All strains were derivatives of BY4741 and BY4742, which were obtained from the EUROSCARF (European Saccharomyces Cerevisiae Archive for Functional analysis) consortium. Deletions of the *BSD2* gene, as well as of the *ALY1*, *ALY2*, *ROD1* and *YGR068C* genes, were made by replacing the entire coding sequence of the gene with the *Schizosaccharomyces pombe HIS5* cassette. The *ROG3* gene was replaced by the *nat1* gene from *Streptomyces noursei* (natMX cassette). For the strain lacking seven arrestins, the *HIS5* gene was removed after each deletion using the Cre-Lox system. The *rsp5* mutant consisted of the insertion of the natMX cassette just upstream from the ATG codon; this recreates a lesion similar to that in the *npi1* allele, which expresses reduced levels of wild-type protein (Springael *et al*, 1999).

Plasmids expressing Smf1 and its derivates from the TPI promoter were based on the YCplac111 *CEN LEU2* vector as described previously (Stimpson *et al*, 2006; Sullivan *et al*, 2007). All the GFP-Smf1 variants (4KR; K33,34; K20,65; SA; SD) were generated by PCR. *ECM21* was cloned from yeast DNA by PCR, and mutations in the PY1- (T887A, Y888G), PY2- (P916A, P917G) and PY3- (Y1106A) elements were created using the QuikChange system (Stratagene, Amsterdam, The Netherlands). Plasmids for yeast expression of Ecm21 were based on the YCplac33 *CEN URA3* vector. Ecm21 and variants were N-terminally tagged with 3 × HA, and expressed from the Ecm21 promoter for complementation of seven arrestin mutant cells, or the TPI promoter for dithiobis(succinimidyl propionate) (DSP) crosslinking. Myc-epitope-tagged ubiquitin was expressed from the YEp105 (*CUP1*-MycUb) plasmid (Ellison & Hochstrasser, 1991). Cells transformed with the plasmid were cultured in the absence of additional $CuSO_4$ to avoid ubiquitin overproduction.

Immunoprecipitation, crosslinking and detection of proteins. To study the ubiquitination state of Smf1, membrane-enriched fractions were prepared as described by Springael & Andre (1998) from cells co-transformed with the YEp105 plasmid. For phosphatase treatment, membrane-enriched fractions of cells collected in the absence of cycloheximide were incubated for 90 min at 37 °C with or without 30 U of calf intestine alkaline phosphatase (Roche, Welwyn Garden City, UK) before tricarboxylic acid precipitation. For the detection of ubiquitinated GFP-Smf1 in immunoprecipitates, cells co-transformed with YEp105 plasmid were collected 10 min after the addition of 0.1 mM $CdCl_2$, and bead-bashed in lysis buffer (1% Triton X-100, 50 mM Tris pH 7.4 and 150 mM NaCl) added with protease inhibitors (Complete EDTA-free; Roche) and 10 mM *N*-ethylmaleimide

(Sigma, Gillingham, UK). GFP-Smf1 was immunoprecipitated from the lysate with anti-GFP microbeads (Miltenyi Biotec, Bisley, UK) according to the manufacturer's instructions, except that the proteins were eluted from the beads with low pH (100 mM glycine-HCl pH 2.7, 0.1% Triton X-100 and 150 mM NaCl). For *in vivo* crosslinking experiments, spheroplasts were prepared after 10 min treatment with 0.36 mM cycloheximide, resuspended in 0.6 ml of 25 mM potassium phosphate pH 7.4 and 200 mM sorbitol containing 0–3 mM DSP (Pierce, Rockford, IL, USA), and incubated for 30 min at 4 °C. The crosslinker was quenched by the addition of 100 mM Tris pH 7.4 and an equal volume of 2 × lysis buffer (2% Triton X-100, 100 mM Tris pH 7.4 and 300 mM NaCl) with protease inhibitors (Complete EDTA-free; Roche) and 10 mM *N*-ethylmaleimide (Sigma) was added to the cell suspensions. After 40 min at 4 °C, GFP-Smf1 was precipitated with anti-GFP microbeads (Miltenyi Biotec) as above. Proteins were detected on Western blots with rabbit GFP, Myc or HA antibodies (Sigma).

Fluorescence imaging. Cells expressing GFP-tagged Smf1 or its derivates were imaged in growth medium on a Zeiss LSM510 confocal microscope. Typically, cells were first imaged 10 min after the addition of 0.36 mM cycloheximide ($t = 0$ min), and then at the indicated times after the addition of 0.1 mM $CdCl_2$. The images were adjusted for contrast and brightness, and in some cases they were blurred to filter noise, by using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA). The images are shown inverted for clarity.

***In vitro* ubiquitination assay.** The *in vitro* ubiquitination assay and production of recombinant Rsp5 were carried out as described previously (Sullivan *et al*, 2007). Ecm21 and derivates were expressed from pET30, and the recombinant proteins were purified using the 6 × HIS tag as described, except that the desalting buffer contained 1 mM $MgCl_2$; they were detected with antibodies against the S-tag (Novagen, Darmstadt, Germany).

ACKNOWLEDGEMENTS
We thank Mike Lewis for advice and some preliminary studies, and Ben Nichols and Thomas Mund for helpful comments on the paper.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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