Zinc-regulated ubiquitin conjugation signals endocytosis of the yeast ZRT1 zinc transporter

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The yeast ZRT1 zinc transporter is regulated by zinc at both transcriptional and post-translational levels. At the post-translational level, zinc inactivates ZRT1 by inducing the removal of the protein from the plasma membrane by endocytosis. The zinc transporter is subsequently degraded in the vacuole. This regulatory system allows for the rapid shut off of zinc uptake activity in cells exposed to high zinc concentrations, thereby preventing overaccumulation of this potentially toxic metal. In this report, we examine the role of ubiquitin conjugation in this process. First, we show that ZRT1 is ubiquitinated shortly after zinc treatment and before endocytosis. Secondly, mutations in various components of the ubiquitin conjugation pathway, specifically the RSP5 ubiquitin–protein ligase and the UBC4 and UBC5 ubiquitin conjugating enzymes, inhibit both ubiquitination and endocytosis. Finally, mutation of a specific lysine residue in ZRT1 blocks both ubiquitination and endocytosis. This critical lysine, Lys-195, is located in a cytoplasmic loop region of the protein and may be the residue to which ubiquitin is attached. These results demonstrate that ubiquitin conjugation is a critical step in the signal transduction pathway that controls the rate of ZRT1 endocytosis in response to zinc.

Key words: internalization, protein trafficking, zinc uptake.

INTRODUCTION

Zinc is an essential nutrient that can also be toxic if accumulated in excessive amounts. Therefore, as extracellular zinc levels change, cells must maintain a balanced supply of intracellular zinc. In *Saccharomyces cerevisiae*, regulation of zinc uptake transporters located in the plasma membrane plays a major role in zinc homeostasis. Zinc uptake in *S. cerevisiae* is mediated by two different systems. A high-affinity system is active in zinc-limited cells; the *ZRT1* gene encodes the transporter for this system [1]. A low-affinity system, encoded by the *ZRT2* gene, is active in zinc-replete cells [2]. ZRT1 and ZRT2 are homologous proteins and are members of the ZIP family of metal ion transporters that are found in a diverse array of eukaryotic organisms [3]. Regulation of ZRT1 and ZRT2 activity in response to zinc availability occurs at both transcriptional and post-translational levels. At the transcriptional level, expression of *ZRT1* and *ZRT2* is induced in zinc-limited cells by the ZAP1 transcription factor [4,5]. Zinc inhibits ZAP1 function and down-regulates expression of these genes in zinc-replete cells.

Post-translational regulation of ZRT1 activity is mediated by controlling the rate at which this protein is internalized from the plasma membrane by endocytosis [6]. In zinc-limited cells, ZRT1 is stable in the plasma membrane. Subsequent exposure of these cells to high levels of extracellular zinc (i.e. $[Zn^{2+}]$ ranging from 1 μM–2 mM) triggers an accelerated rate of ZRT1 endocytosis, resulting in a rapid loss of ZRT1-mediated uptake activity. Once internalized, the protein is delivered to the vacuole where it is degraded by vacuolar proteases. Zinc-induced endocytosis is specific to ZRT1; zinc did not affect the endocytosis rates of several other plasma membrane proteins tested. Furthermore, the response is relatively specific for zinc; among the d-block transition metals, only cadmium and cobalt trigger the response, albeit less effectively than zinc. Therefore zinc-induced ZRT1 endocytosis is a specific regulatory system that shuts off zinc uptake activity in cells exposed to high zinc levels and thereby prevents zinc overload.

Regulated endocytosis of plasma membrane transport proteins in response to changing nutrient availability is an emerging theme in *S. cerevisiae*. For example, glucose, the preferred carbon source of yeast, signals the endocytosis of maltose permease [7,8] and GAL2 galactose permease [9]. Similarly, NH_4^+, a preferred nitrogen source, signals endocytosis of GAP1 general amino-acid permease [10], whereas the FUR4 uracil permease is internalized in response to nutrient starvation [11]. Post-translational regulation of ZRT1 in response to zinc and these further examples serve to illustrate the important role that regulated endocytosis of plasma-membrane proteins plays in controlling nutrient uptake.

The signal transduction pathways responsible for regulating the endocytosis of specific plasma membranes in response to certain signals are still poorly understood. In many cases, the signal that stimulates endocytosis does so by causing the attachment of ubiquitin to the plasma-membrane protein. This unusual role of ubiquitin was first shown in the case of the yeast STE2 α-factor mating-pheromone receptor, which is ubiquitinated and then endocytosed in response to ligand binding [12]. Mutations in components of the ubiquitin conjugation system that block ligand-induced ubiquitination of STE2 also prevented its efficient endocytosis. A sequence in the C-terminal cytoplasmic tail of STE2, SINNDAKSS, is sufficient for ligand-stimulated endocytosis. Mutation of the lysine residue, a potential site for ubiquitin conjugation, in this sequence blocks ligand-dependent ubiquitination and endocytosis. It was also found that fusion of ubiquitin in-frame to the cytoplasmic tail of STE2 caused constitutive endocytosis [13]. These data demonstrated

Abbreviations used: LZM-EDTA, low-zinc medium prepared without EDTA; HA, haemagglutinin; UBC, ubiquitin conjugating enzyme.

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that ubiquitination of STE2 is both necessary and sufficient to mark this protein for endocytosis.

Ubiquitination has also been implicated in signalling endocytosis of the STE3 a-factor receptor, the STE6 mating-factor transporter and the FUR4, MAL61 and GAP1 nutrient transporters [14–18]. In mammalian cells, endocytosis of the growth-hormone receptor occurs following ligand binding and ubiquitination has also been implicated as a signal in this process [19]. Thus ubiquitin serves as a signal for endocytosis of many different transporter proteins and receptors. In this report, we describe experiments demonstrating that ubiquitin conjugation in response to zinc treatment marks ZRT1 for endocytosis.

MATERIALS AND METHODS

Yeast strains and growth conditions

The strains used are listed in Table 1. Yeast were grown in SD medium (0.67 % yeast nitrogen base without amino acids) [20] supplemented with auxotrophic requirements and 2 % (w/v) galactose to induce expression of *ZRT1* from the *GAL1* promoter or 2 % (w/v) glucose to repress this expression. Where indicated, 100 μM $CuSO_4$ was added to induce expression from the *CUP1* promoter. This medium was made zinc-limiting by adding 1 mM EDTA. ZRT1 endocytosis was induced by the addition of 2 mM $ZnCl_2$; although much lower concentrations (e.g. 1 μM) can trigger endocytosis, the higher concentration causes a greater response without loss of cell viability [6]. Unless stated otherwise, ZRT1 was expressed from the *GAL1* promoter to avoid the added complexity of zinc-responsive transcriptional control. Cell numbers in liquid cultures were determined by measuring the turbidity of the cell suspension at 600 nm (D_{600}) and estimating cell numbers from a standard curve.

Plasmid and DNA manipulations

Escherichia coli and yeast transformations were performed using standard methods [21,22]. Plasmid pOE1 is an episomal vector that expresses wild-type *ZRT1* from the *GAL1* promoter [1]. Epitope-tagged *ZRT1* alleles were used to allow detection of the ZRT1 protein. Plasmids pOE1-3ET and YIp306-3ET are episomal and integrating plasmids respectively, that express a *ZRT1* allele containing three haemagglutinin (HA) antigen epitopes from the *GAL1* promoter [6]. Plasmid pMC5-HSET is an episomal plasmid that expresses a *ZRT1* allele with a single HA tag from its own promoter [6]. Plasmid YEp96 contains the *UBI4* ubiquitin gene expressed from the copper-inducible *CUP1* promoter and YEp105 contains a ubiquitin allele epitope tagged with the myc antigen expressed from the *CUP1* promoter [23]. Site-directed mutagenesis of *ZRT1* was performed by either the overlap extension polymerase chain reaction (Lys-167 → Arg, Lys-195 → Arg) [24] or the Transformer system (Lys-154 → Arg) (Clontech). Mutations were verified by DNA sequencing.

Zinc-uptake assays

Zinc-uptake assays were performed as described previously for iron uptake [25], except that $^{65}ZnCl_2$ (Amersham) and LZM-EDTA (low-zinc medium prepared without EDTA) were substituted for $^{59}FeCl_3$ and LIM-EDTA (low-iron medium prepared without EDTA) respectively. Briefly, cells were harvested at 0–120 min, washed three times in LZM-EDTA and resuspended in LZM-EDTA. Cells were then incubated for 5 min in LZM-EDTA containing 1 μM ^{65}Zn at 30 °C, collected on glass fibre filters (Schleicher and Schuell) and washed with 10 ml ice-cold SSW (1 mM EDTA/20 mM sodium citrate, pH 4.2). Cell-associated radioactivity was measured with a γ-counter (Packard Auto-Gamma 5650).

Immunoblot and immunoprecipitation methods

Cells were grown to exponential phase (50 ml cultures, D_{600} 0.5–1), harvested by centrifugation at 1000 ***g*** for 5 min and washed with an equal volume of distilled water. The cells were resuspended in 0.5 ml of MIB (0.6 M mannitol, 20 mM Hepes/KOH, pH 7.4) plus protease inhibitors (1 mM EDTA, 1 mM PMSF, 1 mM pepstatin A). Glass beads were added to a volume of 0.5 ml and the cells were disrupted by vortexing for 4 × 20 s with incubation on ice for 1 min between vortexing. The homogenates were then centrifuged at 4000 ***g*** for 5 min at 4 °C and the pellet of unbroken cells was discarded. ZRT1 immunoblots were performed as described [26], using a monoclonal antibody specific to the HA epitope tag (12CA5; Boehringer Mannheim). Horseradish peroxidase (HRP)-conjugated goat anti-mouse (Pierce) was used as the secondary antibody and complexes were detected by enhanced chemiluminescence (ECL®, Amersham). Immunoblots of immunoprecipitates were performed using HRP-conjugated 12CA5 or an HRP-conjugated anti-myc monoclonal antibody (9E10; Boehringer Mannheim).

Immunoprecipitation was performed essentially as described by Springer [27]. Cells from exponential phase cultures (50 ml, D_{600} 0.5–10) were resuspended in 200 μl IP lysis buffer [25 mM Tris/HCl, pH 8.0, 300 mM NaCl, 2 % (v/v) Triton X-100, 2 % (w/v) BSA, 2 mM PMSF]. Cells were lysed by vortex mixing with glass beads for 2 min. The suspension was centrifuged at 3000 ***g*** for 10 min and the pellet of unbroken cells was discarded. 12CA5 antibody (1 μg in 1 μl) was added to the supernatant and incubated overnight at 4 °C with gentle mixing. Protein A–Sepharose (Pharmacia) (40 μl of a 50 % slurry) was added and mixed gently for 3 h at room temperature. The Sepharose beads were collected by centrifuging the suspension for 1 min at 200 ***g*** and the supernatant was removed and discarded. The beads were washed twice with 1 ml of IP dilution buffer [10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1 % (v/v) Triton X-100, 0.1 % (w/v) BSA, 1 % (v/v) Tween 20], once with 1 ml of wash buffer A (10 mM Tris/HCl, pH 8.0, 150 mM NaCl), once with 1 ml of wash buffer B (50 mM Tris/HCl, pH 6.8) and a final wash with 1 ml of distilled water. A 50 μl aliquot of SDS/PAGE sample buffer was added to the washed beads, the samples were boiled for 5 min, centrifuged at 12000 ***g*** for 1 min, and 25 μl of the supernatant was loaded on to a polyacrylamide gel, separated by SDS/PAGE and immunoblotted.

Endocytosis assay

Yeast cells were grown to exponential phase (D_{600} 0.5–1) in SD glucose medium, harvested and resuspended in 90 μl of fresh medium plus 10 μl of a 40 mg/ml Lucifer Yellow carbohydrazide (Sigma) stock solution. The cells were incubated at either 4 °C or 30 °C for 60 min. The cells were then centrifuged at 200 ***g*** for 1 min, washed three times in 1 ml of ice-cold washing buffer (50 mM succinate/NaOH, pH 5.0, 20 mM NaN_3) and then resuspended in 10 μl washing buffer. Cells were examined by epifluorescence microscopy using FITC optics. Although Lucifer Yellow carbohydrazide staining is a qualitative assay, its usefulness in the detection of defects in bulk endocytosis was confirmed in that little vacuolar Lucifer Yellow staining was observed in samples where endocytosis was blocked, i.e. in *end3* and *end4* mutants or in cells incubated at 4 °C.

Table 1 Yeast strains

Strain	Relevant genotype	Full genotype	Reference/source
DY1457	Wild type	*MATα ade6 can1 his3 leu2 trp1 ura3*	[1]
DEY1531	*end4*	*MATa ade6 can1 his3 leu2 trp1 ura3 end4::LEU2*	[6]
ZHY3	*zrt1 zrt2*	*MATα ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3*	[2]
23344c	Wild type	*MATa ura3*	B. Andre*
27038a	*rsp5*	*MATa ura3 rsp5*	B. Andre
RH144-3D	Wild type	*MATa bar1 leu2 his4 ura3*	H. Riezman†
RH3136	*ubc1*	*MATa bar1 leu2 his3 ura3 lys2 trp1 ubc1::HIS3*	[12]
RH3130	*ubc2*	*MATa bar1 leu2 his3 ura3 ubc2::HIS3*	[12]
RH3132	*ubc4*	*MATa bar1 leu2 his3 ura3 lys2 ade2 ubc4::TRP1*	[12]
RH3142	*ubc5*	*MATa bar1 leu2 his3 his4 ura3 lys2 trp1 ubc5::LEU2*	[12]
RH3097	*ubc4 ubc5*	*MATa bar1 leu2 his3 his4 ura3 lys2 trp1 ade2 ubc4::TRP1 ubc5::LEU2*	[12]
RH3143	*ubc7*	*MATa bar1 leu2 his3 ura3 lys2 trp1 ubc7::LEU2*	[12]

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RESULTS

Zinc induces ZRT1 ubiquitination

When zinc-limited cells expressing a high level of the ZRT1 zinc transporter are exposed to zinc concentrations ranging anywhere from 1 μM to 2 mM, a rapid decrease in ZRT1-mediated uptake activity is observed that is not apparent when cells are maintained on a zinc-limited medium [6]. This zinc-induced loss of ZRT1 activity also occurs in the presence or absence of cycloheximide and with cells expressing *ZRT1* from either its own or the non-zinc-responsive *GAL1* promoter ([6] and Figure 1A). These results demonstrated that zinc-induced loss of ZRT1 activity occurs at a post-translational level. Further analysis demonstrated that the observed decrease in zinc-uptake activity was due to endocytosis of the protein from the plasma membrane. For example, several genes in yeast, including the *END4* gene, are required for the initial internalization step of endocytosis to occur. Upon zinc treatment, inactivation of zinc-uptake activity occurred much more slowly in *end4* mutants than in wild-type cells (Figure 1A) and the protein is retained on the cell surface in the mutant [6]. Therefore, in the experiments described in the present work, we have used the loss of ZRT1-mediated uptake activity as an indirect assay of endocytosis of the transporter.

To assess the role of ubiquitin conjugation in zinc-induced ZRT1 endocytosis, we first performed immunoblots on protein extracts isolated from wild-type and *end4* mutant cells, with and without zinc treatment. To allow detection of the ZRT1 protein, an epitope-tagged allele of the *ZRT1* gene was used in which three tandem copies of the HA antigen epitope were fused to the N-terminus of ZRT1. Our previous studies demonstrated that the anti-HA antibody is specific for HA-tagged ZRT1 and does not detect any other yeast proteins [6]. In zinc-limited wild-type or *end4* cells, no change in ZRT1 protein level or mobility was observed (Figure 1B). In zinc-treated wild-type cells, however, two higher-molecular-mass forms of ZRT1 were detected after 30 min of zinc treatment (Figure 1B). The levels of unmodified and apparently modified forms of ZRT1 decreased over time, which was consistent with endocytosis and subsequent vacuolar degradation of the protein. In the *end4* mutant, similar zinc-dependent modified forms of ZRT1 were
observed but, in this case, no decrease in abundance was detected as the cells were incubated for longer times. These results show that ZRT1 is modified following zinc treatment. The persistence of the modified forms of the protein in the *end4* mutant also indicates that this modification occurs prior to endocytosis.

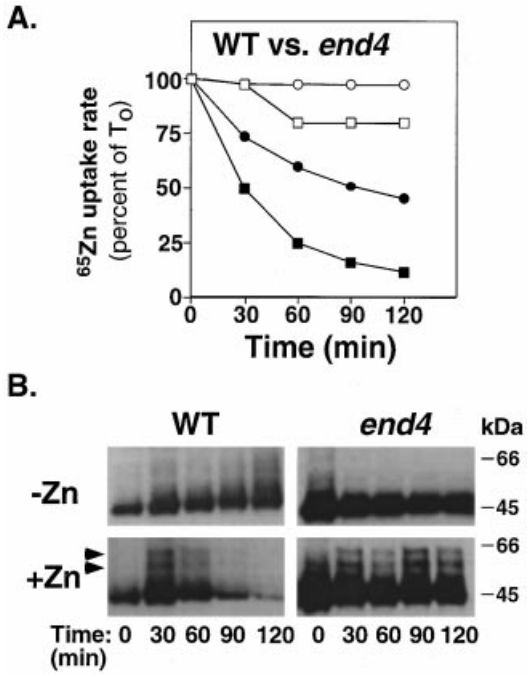

Figure 1 ZRT1 is modified in response to zinc and prior to endocytosis

(**A**) Wild-type (WT, DY1457; squares) and *end4* mutant (DEY1531; circles) cells expressing HA-tagged ZRT1 from the *GAL1* promoter (YIp306–3ET) were grown to exponential phase in SD medium plus 2% galactose. The cells were harvested and resuspended in SD glucose medium supplemented with 100 μg/ml cycloheximide and either 2 mM $ZnCl_2$ (+Zn; closed symbols) or 1 mM EDTA (−Zn; open symbols). At the indicated times, cells were harvested and assayed for ^{65}Zn-uptake rate. The standard deviations were less than 10% of the corresponding mean. (**B**) Total cell homogenates were prepared from cells grown as described for (**A**) and analysed by immunoblotting (2.5 μg protein/lane). Modified forms of ZRT1 are indicated by arrowheads and the position of molecular-mass markers are shown on the right.

The apparent molecular mass of ZRT1 in zinc-limited cells is 45 kDa. The apparent molecular masses of the modified forms of ZRT1 are approx. 53 and 63 kDa, consistent with the addition of one and two 8 kDa ubiquitin proteins respectively. Modified ZRT1 was not observed in our previous studies because longer blot exposure times were required to detect these lower abundance forms. Modified forms of ZRT1, similar to those observed here,

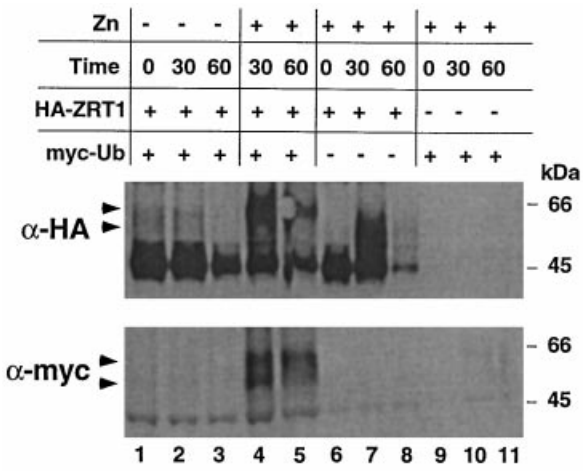

Figure 2 ZRT1 is ubiquitinated in response to zinc

Wild-type (DY1457) cells expressed HA-tagged ZRT1 (YIp306–3ET) or wild-type ZRT1 (pOE1) from the *GAL1* promoter and either myc-tagged ubiquitin (YEp105) or wild-type ubiquitin (YEp96) from the *CUP1* promoter. Cells were harvested at zero time and inoculated into SD glucose with 2 mM $ZnCl_2$ (+Zn) or 1 mM EDTA (−Zn) for the indicated times in min. Cell homogenates were prepared, immunoprecipitated using anti-HA (12CA5) antibodies and analysed by immunoblotting using anti-HA 12CA5 (α-HA) or anti-myc 9E10 (α-myc) antibodies. Modified forms of ZRT1 are indicated by arrowheads and the position of molecular-mass markers are shown on the right.

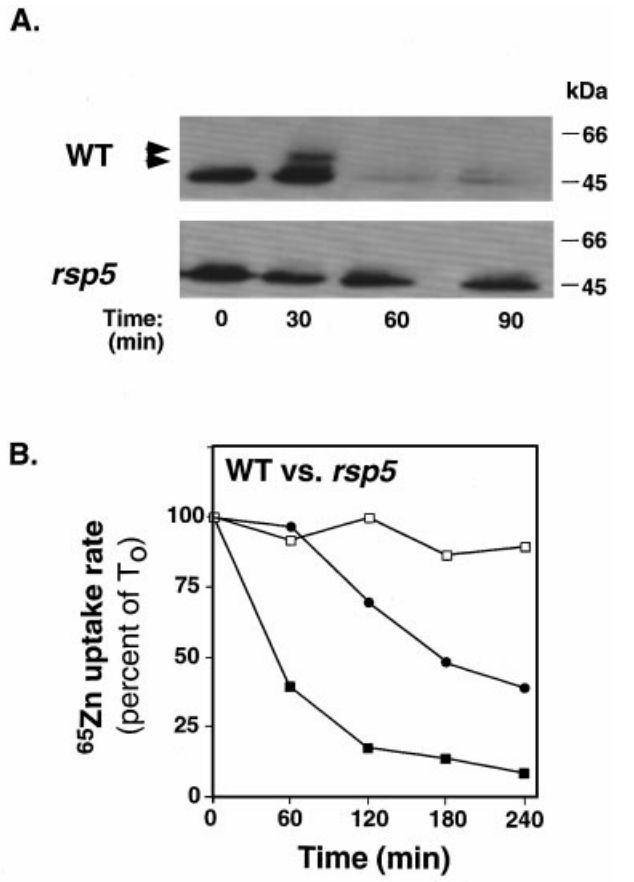

Figure 3 Ubiquitination and endocytosis of ZRT1 in response to zinc requires the RSP5 ubiquitin–protein ligase

(**A**) Wild-type (WT, 23344c) and *rsp5* (27038a) mutant cells expressing HA-tagged ZRT1 from *GAL1* promoter (YIp306–3ET) were grown to exponential phase in SD galactose medium. The cells were harvested and resuspended in SD glucose medium supplemented with 100 μg/ml cycloheximide and 2 mM $ZnCl_2$. At the indicated times, total cell homogenates were prepared and analysed by immunoblotting (2.5 μg protein/lane). Modified forms of ZRT1 are indicated by arrowheads and the position of molecular-mass markers are shown on the right. (**B**) Cells grown as described for (**A**) were harvested and assayed for ^{65}Zn-uptake rate. ■, Zinc-treated wild-type cells; ●, zinc-treated *rsp5* cells. Data obtained from wild-type cells incubated without Zn (open squares) are shown for comparison; uptake rates in zinc-limited *rsp5* mutants were similar. Standard deviations were less than 10% of the corresponding mean.

also accumulated in zinc-treated *pep4 prb1* mutants, which are unable to degrade endocytosed ZRT1 in the vacuole (results not shown) [6].

To test directly if the modified ZRT1 protein observed following zinc treatment was the result of ubiquitin conjugation, we used a combined immunoprecipitation/immunoblot method. In this experiment advantage was taken of a myc epitope-tagged variant of the *UBI4* ubiquitin gene [23]. Wild-type cells were transformed with two plasmids, one expressing either wild-type or myc-tagged ubiquitin from the *CUP1* promoter and another plasmid expressing either wild-type or HA-tagged ZRT1 from the *GAL1* promoter. The cells were grown to exponential phase in galactose-containing medium supplemented with 100 μM $CuSO_4$, to induce expression of the plasmid-borne genes. At zero time, the cultures were treated with either 2 mM $ZnCl_2$ (+Zn) or 1 mM EDTA (−Zn). Total protein extracts were harvested at 0–60 min, immunoprecipitated with anti-HA antibody, and then analysed by immunoblotting with either anti-HA or anti-myc antibodies (Figure 2). The anti-HA immunoblot reflects the immunoblot results shown in Figure 1. No ZRT1 was detected in extracts of cells expressing the untagged ZRT1 allele (Figure 2, lanes 9–11), indicating the specificity of the anti-HA antibody in immunoprecipitation. No modification was detected in zinc-limited cells (Figure 2, lanes 1–3) but modified forms did accumulate in zinc-treated cells expressing either the myc-tagged (Figure 2, lanes 4–5) or wild-type ubiquitin (Figure 2, lanes 6–8). The modified forms observed in cells expressing the wild-type ubiquitin gene were smaller in apparent molecular mass than those observed in cells expressing the myc-tagged ubiquitin. This result is consistent with the larger size of the myc-tagged ubiquitin compared with the untagged protein.

The anti-myc immunoblot demonstrated that the zinc-induced ZRT1 modification is attachment of ubiquitin. Modified ZRT1 protein was detected only in zinc-treated cells and only in cells expressing both the myc-tagged ubiquitin and the HA-tagged *ZRT1* alleles. Furthermore, the myc bands observed in the blot co-migrated with the modified ZRT1 bands observed in the anti-HA immunoblot.

Zinc-induced ZRT1 ubiquitination and endocytosis is impaired in mutants defective in ubiquitin conjugation

Taken together, the results shown in Figures 1 and 2 demonstrated that ubiquitin is conjugated to ZRT1 following zinc treatment and suggested that this modification signals ZRT1 endocytosis. If true, mutations that inactivate the relevant components of the ubiquitin conjugation system will inhibit both ubiquitination of ZRT1 and its zinc-induced endocytosis. Ubiquitination requires a multi-step pathway that begins with the ATP-dependent activation of the C-terminus of ubiquitin by E1-ubiquitin-activating enzymes [28]. Ubiquitin is then trans-

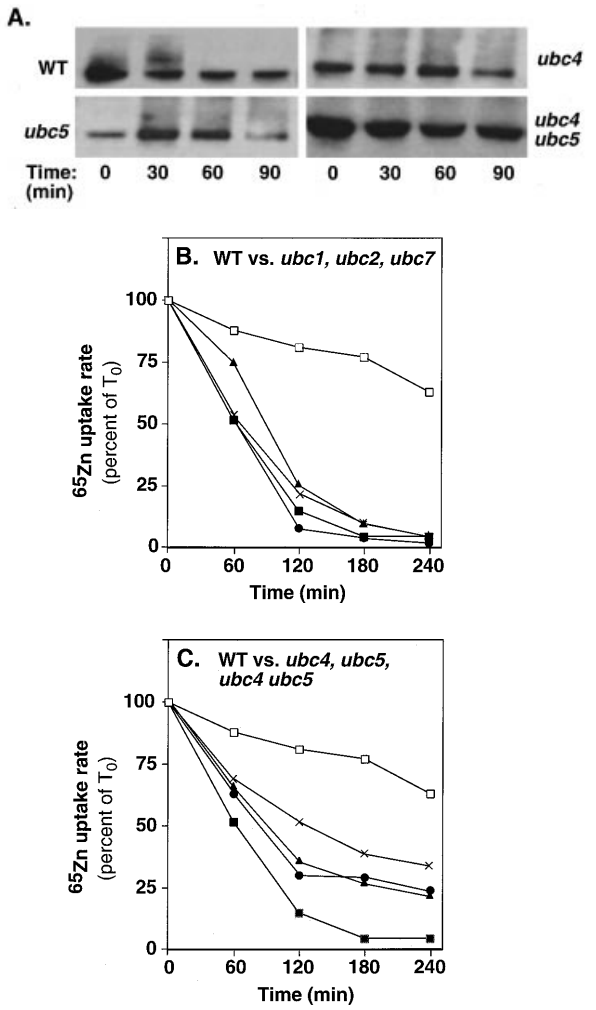

Figure 4 Effects of mutations in E2 ubiquitin conjugating enzymes on ZRT1 ubiquitination and endocytosis

(**A**) Wild-type (RH144–3D), *ubc4* (RH3132), *ubc5* (RH3142) and *ubc4*/*ubc5* (RH3097) mutant cells expressing epitope-tagged ZRT1 from its own promoter (pMC5-HSET) were grown to exponential phase in SD glucose medium supplemented with 1 mM EDTA. ZRT1 was expressed from its own promoter in this experiment because the *GAL1* promoter was expressed poorly in the genetic background of the mutant strains used (results not shown). The cells were harvested and resuspended in SD glucose medium supplemented with 100 μg/ml cycloheximide and 2 mM $ZnCl_2$. At the indicated times, total cell homogenates were prepared and analysed by immunoblotting (5 μg protein/lane). (**B**) Cells, grown as described for (**A**), were harvested and assayed for ^{65}Zn-uptake rate. Symbols are wild type (■), *ubc1* (●), *ubc2* (▲) and *ubc7* (×). Data obtained from wild-type cells incubated without Zn (□) are shown for comparison; analysis of uptake rates in zinc-limited *ubc* mutants gave similar results. Standard deviations were less than 10% of the corresponding mean. (**C**) The conditions were the same as described in the legend for (**B**). Wild type (■), *ubc4* (○), *ubc5* (▲), *ubc4*/*ubc5* (×) and wild type incubated without Zn (□).

ferred to ubiquitin conjugating (E2) enzymes that then attach the ubiquitin to the ϵ-amino group of lysine residues in substrate proteins, often with the participation of E3 ubiquitin–protein ligases. Several genes in yeast encode E3 ubiquitin–protein ligases or their subunits, including the *RSP5* gene [29]. Because *rsp5* null mutants are lethal, we used an *rsp5* promoter mutation that expresses less than 10% of the wild-type protein level [17]. As shown in Figure 3(A), the *rsp5* mutant was defective for zinc-induced ubiquitination of ZRT1, suggesting a specific role of this ubiquitin–protein ligase in the modification of ZRT1. Consistent with the hypothesized role of ubiquitination in signalling ZRT1 endocytosis, degradation of the ZRT1 protein was impaired in the *rsp5* mutant (Figure 3A), and inactivation of ZRT1 uptake activity in response to zinc was also defective (Figure 3B). The *rsp5* mutation did not completely eliminate zinc-dependent endocytosis of ZRT1 and this may be due to the 'leaky' nature of the *rsp5* allele used. Alternatively, an RSP5-independent mechanism may be present. The faster rate of ZRT1 turnover for the *RSP5* wild-type strain, shown in Figure 3(A), compared with the wild-type strain shown in Figure 1, was reproducible and may be due to the different genetic backgrounds of these strains.

In similar experiments, we tested the role of several different E2 ubiquitin conjugating enzymes (UBCs) in ZRT1 ubiquitination and endocytosis. At least 13 yeast genes encode E2 enzymes and these proteins each confer a distinct substrate specificity on the conjugation process. Cells mutant in *UBC1*, *UBC2* or *UBC7* had no defect in zinc-induced ubiquitination of ZRT1 (results not shown) whereas, both *ubc4* and *ubc5* single mutants were affected (Figure 4A). UBC4 and UBC5 are known to perform overlapping functions in ubiquitin conjugation [30]. Therefore we also assayed the effects of a *ubc4*/*ubc5* double mutant on ZRT1 ubiquitination and also found it to be defective. In addition, these mutants were assayed for effects on zinc-induced loss of ZRT1 uptake activity. The *ubc1*, *ubc2* and *ubc7* mutants were not defective for zinc-induced ZRT1 endocytosis (Figure 4B). The *ubc4*, *ubc5* and *ubc4*/*ubc5* strains were defective for ZRT1 endocytosis (Figure 4C). Consistent with the overlapping functions of UBC4 and UBC5, the *ubc4*/*ubc5* double mutant was even more defective for ZRT1 internalization than either single mutant. This additivity was reproducible in several replicate experiments.

These results support the hypothesis that the UBC4 and UBC5 E2 enzymes and the RSP5 E3 enzyme are responsible for ubiquitination and subsequent endocytosis of ZRT1 in response to zinc. An alternative explanation is that *ubc4*, *ubc5* and *rsp5* mutants reduce the rate of bulk endocytosis and indirectly affect ZRT1 trafficking. To test this hypothesis, we assessed bulk endocytosis in these cells with the soluble endocytosis marker Lucifer Yellow. Whereas Lucifer Yellow accumulation was greatly inhibited in the *end4* mutant, no defect in accumulation was detected in *rsp5* or any of the *ubc* mutants used in this study (results not shown). Thus the defects observed in mutants defective for UBCs are due to specific defects in ZRT1 endocytosis.

Mutation of Lys-195 blocks both ubiquitination and endocytosis of ZRT1

To further assess the role of ubiquitination in signalling ZRT1 endocytosis, we used site-directed mutagenesis to identify the specific site(s) of ubiquitin attachment within ZRT1. The ubiquitin conjugation machinery covalently attaches ubiquitin to target protein lysines. Polyubiquitin chains can then form when additional ubiquitin moieties are attached to lysines in previously attached ubiquitins. Therefore the appearance of mono- and di-ubiquitinated forms of ZRT1 (see Figure 1) is consistent with attachment of ubiquitin to either one or two lysine residues in the zinc transporter. ZRT1 contains a total of 12 lysine residues. Based on the predicted locations of the ZRT1 transmembrane domains and the predicted topology of the protein [1], seven of these 12 lysine residues are exposed on the cytoplasmic surface of the plasma membrane. Of these seven, four residues are located in very short cytoplasmic loops of the protein, which we suspected may not be readily accessible for ubiquitin conjugation. Therefore we focused our analysis on Lys-154, Lys-167 and Lys-195, which

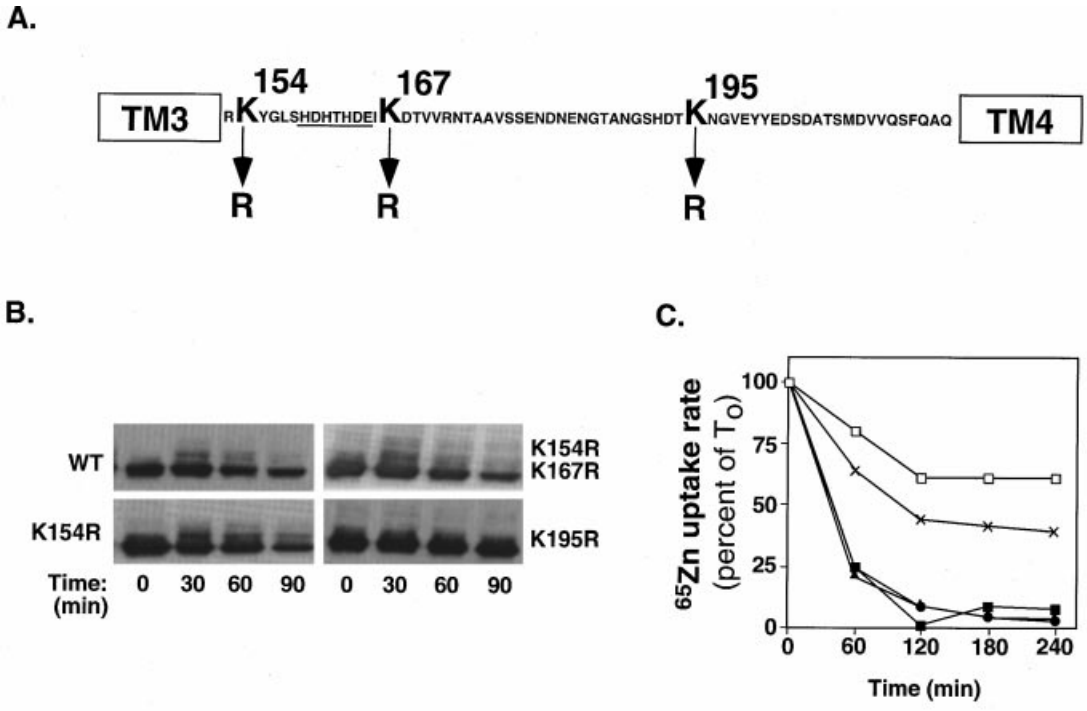

Figure 5 Lys-195 is required for zinc-dependent ubiquitination and endocytosis of ZRT1

(**A**) The amino acid sequence of wild-type and mutant ZRT1 proteins between transmembrane (TM) domains 3 and 4. The potential metal binding domain is underlined. (**B**) Wild-type (WT, DY1457) cells expressing wild-type or mutant *ZRT1* alleles from the *GAL1* promoter (pOE1-3ET and derivatives) were grown to exponential phase in SD galactose medium. The cells were harvested and resuspended in SD glucose medium supplemented with 100 μg/ml cycloheximide and 2 mM $ZnCl_2$. Total cell homogenates were prepared at the indicated times and analysed by immunoblotting (2.5 μg protein/lane). (**C**) Cells, grown as described in the legend of (**B**) were harvested and assayed for ^{65}Zn-uptake rate. Symbols are wild type (■), Lys-154 → Arg (▲), Lys-154 → Arg/Lys-167 → Arg (●) and Lys-195 → Arg (×). Data obtained from cells expressing the wild-type protein incubated without Zn (□) are also shown for comparison; analysis of uptake rates in zinc-limited cells expressing *ZRT1* mutant alleles gave similar results. Standard deviations were less than 10% of the corresponding mean.

are located in the only large cytoplasmic loop in the protein, a 66-amino-acid segment between transmembrane domains III and IV (Figure 5A).

Substitution of either Lys-154 or both Lys-154 and Lys-167 with arginine had no effect on ubiquitination of ZRT1 in response to zinc (Figure 5B). In contrast, substitution of Lys-195 with arginine resulted in a complete loss of detectable ZRT1 ubiquitination in zinc-treated cells. These results suggest that Lys-195 is the single site of zinc-responsive ubiquitination within ZRT1 and that di-ubiquitination occurs through attachment of a second ubiquitin to the first moiety. Zinc-uptake assays were performed to examine the effect of these mutations on ZRT1-mediated uptake activity and endocytosis. All three mutant alleles of ZRT1 retained wild-type uptake activity. The Lys-154 → Arg and Lys-154 → Arg/Lys-167 → Arg alleles were inactivated normally in response to zinc, indicating wild-type rates of endocytosis (Figure 5C). In contrast, the Lys-195 → Arg mutant was severely defective for zinc-induced endocytosis. These data support the hypothesis that ubiquitination of Lys-195 in response to zinc serves as the signal for endocytosis of ZRT1.

DISCUSSION

In this report, we have described our initial characterization of the signal transduction pathway that controls ZRT1 endocytosis in response to zinc. Our results establish that ubiquitination of the zinc transporter is an essential part of this pathway. This conclusion is based on the following observations. First, we demonstrated that ZRT1 is ubiquitinated in response to zinc treatment. Zinc-dependent ubiquitin conjugation occurs prior to endocytosis; the ubiquitinated protein accumulated in a mutant strain that is blocked for the initial internalization step of endocytosis. Secondly, mutations that inactivate components of the ubiquitin conjugation system blocked both ubiquitination and endocytosis of ZRT1 in zinc-treated cells. Specifically, mutations in the functionally overlapping UBC4 and UBC5 E2 enzymes or the RSP5 E3 ubiquitin–protein ligase were defective in these processes. These components are known to provide substrate specificity to ubiquitin conjugation and, consistent with this fact, mutants defective for other E2 activities had no effect on zinc-induced ZRT1 ubiquitination. Thirdly, a mutation within ZRT1 that alters a potential ubiquitination site, Lys-195, strongly inhibited both ubiquitination and endocytosis. Given that all ZRT1 ubiquitination is blocked by the Lys-195 → Arg mutation, we suggest that Lys-195 is the only ubiquitinated residue in the protein. Finally, studies by Terrell et al. [13] demonstrated that ubiquitin is not only necessary but sufficient to signal endocytosis of the STE2 α-factor receptor. Together, these data argue strongly that ubiquitination of ZRT1 in response to zinc is an essential step in signalling the subsequent endocytosis of the protein.

How does ubiquitin signal the endocytosis of ZRT1? One model is that ubiquitination introduces a signal that marks the protein for endocytosis. Endocytosis of many plasma membrane proteins is known to be dependent on small sequences called 'internalization signals' that are located in the cytoplasmic

domains of these proteins (for a review see [31]). These include the so-called 'tyrosine-based' and 'dileucine-based' signals. Studies have shown that internalization signals such as these interact with components of the clathrin-coated pits to recruit proteins into these nascent sites of endocytosis. Ubiquitination of ZRT1 may result in a conformational change in the transporter that exposes an internalization signal found within ZRT1. A more compelling hypothesis comes from the work on STE2 by Terrell et al. [13]. The observation that ubiquitin can be fused in-frame to the STE2 receptor and still signal endocytosis suggests that ubiquitin itself is the signal for endocytosis rather than a precise alteration in target protein conformation. Thus an uncharacterized internalization signal may be present within ubiquitin that interacts with clathrin-coated pit constituents.

The ubiquitin conjugation machinery that is required for ZRT1 ubiquitination has also been implicated in the ubiquitination of other plasma membrane proteins in yeast. The RSP5 ubiquitin–protein ligase has been shown previously to be required for modification (and subsequent endocytosis) of FUR4, GAP1 and the maltose permease [16–18]. RSP5 is thought to be associated with the plasma membrane as part of a multi-protein complex consisting of PAN1, END3, yAP1801, yAP1802 and INP51, which co-ordinates the early stages of endocytosis, including ubiquitination, actin depolymerization and assembly of clathrin-containing structures [32]. UBC4 and UBC5 have also been implicated in mediating the ubiquitination and endocytosis of STE2, STE3 and STE6 [12,14,33]. Given that the same proteins seemingly mediate ubiquitination of several plasma membrane proteins in response to different signals, an important question of substrate specificity arises. A likely hypothesis is that for each of these target proteins, their respective signal introduces or exposes a similar recognition sequence in their structure that facilitates its interaction with the ubiquitin conjugation machinery. In the case of UBC4 and UBC5, that recognition signal has been identified as small clusters of hydrophobic residues in the target protein [34].

How then might zinc signal ubiquitination of ZRT1? One model is that zinc signals phosphorylation of ZRT1 and this modification signals its ubiquitination. The phosphorylation state of many proteins plays an important role in regulating their rate of ubiquitination. Cytosolic proteins such as cyclins and IκB (inhibitor of nuclear factor κB) are phosphorylated prior to ubiquitination [35]. Phosphorylation is also a critical determinant of STE2 ubiquitination. Ligand binding causes hyperphosphorylation of the cytoplasmic tail of the STE2 receptor [36]. Mutation of all three serine residues in the tail region SINNDAKSS motif, which is required for regulated endocytosis, prevented phosphorylation, ubiquitination and endocytosis. Furthermore, mutation of the *YCK1* and *YCK2* genes, which encode the two yeast casein kinase orthologues, also prevented phosphorylation, ubiquitination and endocytosis of the receptor, suggesting that these kinases are involved. Phosphorylation of the FUR4 uracil permease also regulates its ubiquitination and subsequent endocytosis [37]. This protein is phosphorylated before ubiquitin conjugation and this process requires a cytosolic 'PEST' domain, rich in aspartate/glutamate and serine and threonine residues. In contrast, dephosphorylation of GAP1 coincides with its ubiquitination, suggesting that phosphorylation masks a ubiquitination signal within this protein [10].

Experiments designed to detect if ZRT1 is phosphorylated or dephosphorylated in response to zinc have indicated that this modification does not occur and mutations in YCK1 and YCK2 have no effect on zinc-induced endocytosis (results not shown). Therefore, ZRT1 ubiquitination must be regulated by a novel mechanism different from that regulating STE2, FUR4 and GAP1. An alternative model for ZRT1 is that zinc sensing involves a direct mechanism (i.e. zinc binding directly to the transporter) that exposes the hydrophobic recognition signal. It is interesting to note that a potential metal binding domain, HDHTHED (Figure 5A), that is conserved in several other ZIP transporters is located in the same inter-transmembrane domain loop as the ubiquitinated lysine residue [3]. Perhaps intracellular Zn^{2+} binds to this site and signals a conformational change that allows Lys-195 to be ubiquitinated. We are currently addressing this hypothesis.

In an interesting parallel, zinc-induced endocytosis has recently been observed in mammalian cells. The prion protein (PrP^C) is a cell surface glycoprotein expressed in brain, spinal cord and several other tissues. While the function of this protein has remained a mystery, a conformational alteration of PrP^C into a protease-resistant form is responsible for prion neurodegenerative diseases. It has recently been noted that endocytosis of PrP^C is stimulated by zinc as well as copper in cultured neuroblastoma cells [38]. The present studies could shed light on the mechanism of metal-regulated endocytosis of other plasma membrane proteins like PrP^C.

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REFERENCES

1 Zhao, H. and Eide, D. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 2454–2458
2 Zhao, H. and Eide, D. (1996) J. Biol. Chem. **271**, 23203–23210
3 Eng, B. H., Guerinot, M. L., Eide, D. and Saier, M. H. (1998) J. Membr. Biol. **166**, 1–7
4 Zhao, H. and Eide, D. J. (1997) Mol. Cell. Biol. **17**, 5044–5052
5 Zhao, H., Butler, E., Rodgers, J., Spizzo, T. and Duesterhoeft, S. (1998) J. Biol. Chem. **273**, 28713–28720
6 Gitan, R. S., Lou, H., Rodgers, J., Broderius, M. and Eide, D. (1998) J. Biol. Chem. **273**, 28617–28624
7 Medintz, I., Jiang, H., Han, E., Cui, W. and Michels, C. A. (1996) J. Bacteriol. **178**, 2245–2254
8 Riballo, E., Herweijer, M., Wolf, D. H. and Lagunas, R. (1995) J. Bacteriol. **177**, 5622–5627
9 Chiang, H., Schekman, R. and Hamamoto, S. (1996) J. Biol. Chem. **271**, 9934–9941
10 Stanbrough, M. and Magasanik, B. (1995) J. Bacteriol. **177**, 94–102
11 Volland, C., Urban-Grimal, D., Geraud, G. and Haguenauer-Tsapis, R. (1994) J. Biol. Chem. **269**, 9833–9841
12 Hicke, L. and Riezman, H. (1996) Cell **84**, 277–287
13 Terrell, J., Shih, S., Dunn, R. and Hicke, L. (1998) Mol. Cell **1**, 193–202
14 Roth, A. F. and Davis, N. G. (1996) J. Cell Biol. **134**, 661–674
15 Kölling, R. and Hollenberg, C. P. (1994) EMBO J. **13**, 3261–3271
16 Galan, J. M., Moreau, V., Andre, B., Volland, C. and Haguenauer-Tsapis, R. (1996) J. Biol. Chem. **271**, 10946–10952
17 Springael, J. and Andre, B. (1998) Mol. Biol. Cell **9**, 1253–1263
18 Medintz, I., Jiang, H. and Michels, C. A. (1998) J. Biol. Chem. **273**, 34454–34462
19 Govers, R., van Kerkhof, P., Schwartz, A. L. and Strous, G. J. (1997) EMBO J. **16**, 4851–4858
20 Sherman, F., Fink, G. R. and Hicks, J. B. (1986) Methods in Yeast Genetics, Cold Spring Harbor Press, Cold Spring Harbor, NY
21 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Press, Cold Spring Harbor, NY
22 Schiestl, R. H. and Gietz, R. D. (1989) Curr. Genet. **16**, 339–346
23 Hochstrasser, M., Ellison, M. J., Chau, V. and Varshavsky, A. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 4606–4610
24 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Gene **77**, 51–59
25 Eide, D., Davis-Kaplan, S., Jordan, I., Sipe, D. and Kaplan, J. (1992) J. Biol. Chem. **267**, 20774–20781
26 Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY
27 Springer, T. A. (1991) in Current Protocols in Molecular Biology (Ausubel, F. M., ed.), p. 10.16.1, John Wiley & Sons, New York

28 Ciechanover, A. (1994) Cell **79**, 13–21
29 Huibregtse, J. M., Scheffner, M., Beaudenon, S. and Howley, P. M. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 2563–2567
30 Seufert, W., McGrath, J. P. and Jentsch, S. (1990) EMBO J. **9**, 4535–4541
31 Kirchhausen, T., Bonifacino, J. S. and Riezman, H. (1997) Curr. Opin. Cell Biol. **9**, 488–495
32 Wendland, B., Emr, S.D. and Riezman, H. (1998) Curr. Opin. Cell Biol. **10**, 513–522
33 Loayza, D. and Michaelis, S. (1998) Mol. Cell. Biol. **18**, 779–789
34 Sadis, S., Atienza, C. and Finley, D. (1995) Mol. Cell. Biol. **15**, 4086–4094
35 Laney, J. D. and Hochstrasser, M. (1999) Cell **97**, 427–430
36 Hicke, L., Zanolari, B. and Riezman, H. (1998) J. Cell Biol. **141**, 349–358
37 Marchal, C., Haguenauer-Tsapis, R. and Urban-Grimal, D. (1998) Mol. Cell. Biol. **18**, 314–321
38 Pauly, P. C. and Harris, D. A. (1998) J. Biol. Chem. **273**, 33107–33110

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