A Cytosolic Domain of the Yeast Zrt1 Zinc Transporter Is Required for Its Post-translational Inactivation in Response to Zinc and Cadmium*

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Nutrient metals such as zinc are both essential to life and potentially toxic if overaccumulated by cells. Non-essential toxic metals like cadmium can enter cells through the uptake transporters responsible for nutrient metal acquisition. Therefore, in the face of ever changing extracellular metal levels, organisms tightly control their intracellular levels of nutrient metals and prevent accumulation of toxic metals. We show here that post-translational inactivation of the yeast Zrt1 zinc uptake transporter is important for zinc homeostasis. During the transition from zinc-limiting to zinc-replete growth conditions (i.e. zinc shock), the Zrt1 transporter is ubiquitinated, endocytosed, and subsequently degraded in the vacuole. To further understand this process at a molecular level, we mapped a region of Zrt1 required for ubiquitination and endocytosis in response to zinc to a domain located on the intracellular surface of the plasma membrane. This domain is a critical cis-acting component of the metal signaling pathway that controls Zrt1 protein trafficking. Using mutant alleles defective for metal-responsive inactivation, we also show that Zrt1 inactivation may be an important mechanism for preventing cadmium uptake and toxicity in zinc-limited cells.

Outside of the laboratory, microorganisms generally live in environments that are nutrient limiting due to low initial nutrient availability or depletion caused by their own growth and/or by the growth of competing microbes (1). Nutrient availability can change abruptly as new food sources enter a cell’s microenvironment. Thus, microbes in the wild are thought to experience a largely “feast or famine” existence of nutrient deficiency punctuated by pulses of increased nutrient levels. To deal with nutrient deficiency, many microbes have evolved with efficient nutrient uptake mechanisms that are induced by starvation conditions. Because these systems can be so highly expressed, cells must also have the ability to rapidly down-regulate uptake systems to prevent overaccumulation of potentially toxic nutrients (e.g. metal ions) during transitions in nutrient availability.

The transition from limiting to replete conditions is very stressful to microbial cells because of the insult this transition imposes on cellular homeostasis. In fact, this may be one reason that vast numbers of microbes in environmental samples are present in a “viable but nonculturable” state, i.e. nutrient-limited cells isolated from the environment are unable to survive the stress of transfer to the nutrient-rich media routinely used in microbial research (1, 2). These issues are the subject of our ongoing studies of zinc homeostasis in Saccharomyces cerevisiae. In S. cerevisiae, zinc uptake is mediated primarily by the Zrt1 and Zrt2 zinc uptake transporters (3, 4). These proteins are members of the ZIP family of transporters that are involved in metal uptake in bacteria, fungi, plants, and mammals (5). Transcription of the ZRT1 and ZRT2 genes is highly induced under low zinc conditions so zinc-limited cells have high levels of latent zinc uptake activity (3, 4). Therefore, when zinc-limited cells are resupplied with even low concentrations of zinc (e.g. 1 μM), large quantities of the metal are taken up by the cell, a condition that we refer to as “zinc shock” (6). This laboratory condition likely mimics the rapid fluctuations in nutrient levels experienced by yeast cells when growing in the wild.

The Zrc1 vacuolar zinc transporter plays an important role in tolerating zinc shock by sequestering excess zinc in the vacuole (6). A second possible mechanism for maintaining zinc homeostasis under zinc shock conditions is the post-translational inactivation of Zrt1 transport activity. Previous studies showed that following zinc treatment, Zrt1 is ubiquitinated on a lysine residue exposed on the cytosolic face of the plasma membrane, recruited into the endocytic pathway, and removed from the cell surface (7, 8). This removal leads directly to a loss of Zrt1 uptake activity thereby preventing additional zinc uptake. Without this inactivation, we calculate that total cellular zinc levels could rise from less than 100 μM to nearly 100 mM within 1 h of treating a zinc-limited cell with zinc.

The regulated endocytosis of plasma membrane proteins is a critical component of cellular responses to changing environmental conditions in eukaryotic microbes. In S. cerevisiae, for example, the Ste2 and Ste3 mating pheromone receptors are endocytosed in response to binding of their ligand (9, 10). Nutrient transporters such as the Fur4 uracil permease (11), the Mal61 maltose permease (12), and the Gap1 amino acid permease (13) undergo endocytosis in response to changing growth conditions, nutrient content, or substrate availability. As with Zrt1, endocytosis of many of these proteins is triggered by the covalent attachment of the 76-residue ubiquitin protein to one or more lysine residues in their cytosolic domains (14). Ubiquitin contains a signal for endocytic internalization (15) and thus mediates the recruitment of the modified target pro-
teins into the endocytosis pathway.

Ubiquitination of plasma membrane proteins occurs through a series of events starting with the conjugation of ubiquitin to the E1 ubiquitin-activating enzyme. Ubiquitin is then transferred to active site cysteines of E2 ubiquitin-conjugating enzymes and then to a cysteine residue of an E3 ubiquitin protein ligase. In the last step of the process, ubiquitin is conjugated to a lysine residue of the target protein by the E3 ligase directly or via an E2 enzyme assisted by an E3 protein.

Analysis of these processes in yeast have implicated Ubc4 and Ubc5, two plasma membrane-localized E2 proteins, as playing important roles in regulated endocytosis of Zrt1, Ste2, Mal61, and others (8, 9, 16, 17). Similarly, the Rsp5 E3 ubiquitin protein ligase has been found to be involved in the ubiquitination of these and many other plasma membrane proteins (17). Given that many different target proteins are modified by apparently the same ubiquitination machinery, a key question is how specific proteins are targeted for modification while other potential targets are unaffected. Furthermore, very little is known about the signal transduction pathways by which environmental signals that trigger ubiquitination are sensed and how this signaling leads to altered ubiquitination. In this report, we build on our previous studies characterizing the mechanism of metal-induced Zrt1 inactivation. We have mapped a region of Zrt1 that is required for ubiquitination and endocytosis to occur. Furthermore, we address the physiological role of Zrt1 inactivation in zinc shock tolerance and cadmium resistance.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Strains used were DY1457 (a ade6 can1 his3 leu2 trp1 ura3), ZHY3 (DY1457 zrt1::LEU2 zrt2::HIS3), DEY1531 (DY1457 endd::LEU2), MSYS1 (a ade6 can1 his3 leu2 trp1 ura3), MSYS2 (MSYS1 ZRT1K205H), MSYS3 (MSYS1 zrc1A), MSYS4 (MSYS1 ZRT1K205H zrc1A), and MKY1 (MSYS1 ZRT1::HIS3). Yeast were grown in SD medium (0.67% yeast nitrogen base without amino acids) (18) supplemented with auxotrophic requirements and 2% galactose to induce expression of ZRT1 from the GAL1 promoter or 2% glucose to repress this expression. This medium was made zinc limiting by adding 1 mM EDTA. Low zinc medium (LZM) (7) was also used in some experiments. LZM is similar in composition to SD medium and contains 1 mM EDTA and 20 mM citrate as metal chelate buffers. CSD medium (19), SD medium made zinc limiting by removal of the metal cheleators, was also used. CSD does not contain strong chelators so the availability of added zinc in this medium is strong chelators so the availability of added zinc in this medium is strong chelators so the availability of added zinc in this medium is fully available. Unlike CSD, LZM contains 1 mM EDTA and 20 mM citrate as metal chelate buffers. CSD medium (19), SD medium made zinc limiting by removal of the metal cheleators, was also used. CSD does not contain strong chelators so the availability of added zinc in this medium is fully available. Unlike CSD, LZM contains 1 mM EDTA and 20 mM citrate as metal chelate buffers. CSD medium (19), SD medium made zinc limiting by removal of the metal cheleators, was also used. CSD does not contain strong chelators so the availability of added zinc in this medium is fully available. Unlike CSD, LZM contains 1 mM EDTA and 20 mM citrate as metal chelate buffers.

Zrt1 Inactivation Assays—Mutant zrt1 zrt2 cells have essentially no detectable zinc uptake in the assay conditions used. Unless noted otherwise, ZRT1 was expressed from the GAL1 promoter. Cells grown to exponential phase (OD600 = 0.5–1) in SD galactose medium were harvested by centrifugation (5 min at 1000 × g) and resuspended in SD glucose medium to shut off expression of the GAL1 promoter. Cycloheximide (100 μg/ml) was also added to block new protein synthesis. Previous studies demonstrated that these treatments do not interfere with Zrt1 inactivation and eliminate the added complexity of zinc-responsive transcriptional control (7). Zrt1 inactivation was routinely induced by the addition of 2 mM ZnCl2. Although much lower concentrations (e.g. 0.1 mM ZnCl2) can also trigger detection of Zrt1 inactivation, the higher concentration causes a greater response without loss of cell viability over the course of the assay (7). At various times, cells were harvested and assayed for Zn2+ uptake rates to assess plasma membrane Zrt1 activity. These assays were performed as described previously for iron uptake (21) except that ZnCl2 (Amersham Biosciences) and LZM prepared without EDTA (LZM-EDTA) were substituted for FeCl3 and LIM-EDTA, respectively. Briefly, cells were harvested, washed three times in LZM-EDTA and resuspended in LZM-EDTA. Cells were then incubated for 5 min in LZM-EDTA plus 1 μM ZnCl2 at 30 °C, collected on glass fiber filters (Schleicher and Schuell), and washed with 10 ml of ice-cold SSW (1 mM EDTA, 20 mM sodium citrate, pH 4.2). Cell-associated radioactivity was measured with a Packard Auto-Gamma 5650 γ-counter. For most alleles, uptake rates of cells at Tt differed by ~2-fold from experiment to experiment, perhaps due to stochastic differences in plasmid retention in each culture. Therefore, all data are normalized as % of Tt.

Immunoblot and Immunofluorescence Methods—Cultures were grown to exponential phase (50-ml cultures, OD600 = 0.5–1), harvested by centrifuging 5 min at 1000 × g, and washed with an equal volume of distilled water. The cells were resuspended in 0.5 ml MIB (0.6 M mannitol, 20 mM HEPES-KOH, pH 7.4) plus protease inhibitors (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg pepstatin A). Glass beads were added to a volume of 0.5 ml and the cells were disrupted by vortexing 5 × 1 min with incubation on ice for 1 min between vortexing. The homogenates were then centrifuged at 4,000 × g for 5 min at 4 °C and the pellet of unbroken cells was discarded. Zrt1 immunoblots were performed as described (22) using a monoclonal antibody specific to the HA epitope tag (12CA5, Roche Applied Science). Horseradish peroxidase-conjugated goat anti-mouse (Pierce) was used as the secondary antibody and complexes were detected by enhanced chemiluminescence (ECL, Amersham Biosciences). Indirect immunofluorescence microscopy was performed as described previously (7).
accumulated to similar levels as wild type but showed reduced plasma membrane localization and increased accumulation within intracellular organelles (data not shown).

To test the effects of these mutations on Zrt1 inactivation by zinc, zrt1 zrt2 cells expressing wild type Zrt1 or the Zrt13HQ mutant from the GAL1 promoter were grown to exponential phase in SD galactose medium. Cells were then harvested and resuspended in fresh medium with 1 mM EDTA (-Zn, □) and 2 mM ZnCl₂. At the indicated times, cells were harvested, washed, and assayed for initial ⁶⁵Zn uptake rates to assess the level of Zrt1 protein remaining at the cell surface. When normalized for uptake activity at T₀, both wild type and Zrt13HQ proteins showed identical profiles of inactivation by zinc at either 10 μM or 2 mM zinc (Fig. 1D). These data demonstrate that the histidine-rich motif of Zrt1 is important for Zrt1 function but does not play a major role in zinc sensing for inactivation.

Mapping a Domain in Zrt1 Required for Zinc-responsive Inactivation—Both Δ183–197 (Δ3) and Δ198–218 (Δ4) retained full uptake activity but were not inactivated in response to zinc treatment (Fig. 2). The slight loss of uptake activity observed after zinc treatment in these mutants is similar to that seen in mutants that block endocytosis (e.g. end4) and therefore may reflect direct inhibition of uptake activity by the high intracellular zinc levels that accumulate in these cells (23). The Δ183–197 mutation deleted lysine-195. When a lysine residue (in the tripeptide KNG) was reintroduced into Δ183–197 (Δ3 + K), full zinc-responsive inactivation was restored suggesting that the ubiquitinated lysine is the only important residue in the deleted interval. The importance of amino acids 196 and 197 (.NG.) was not directly examined by additional deletion mutations. However, an N196D mutation isolated serendipitously during these studies had no effect on inactivation (data not shown).

These results demonstrate that amino acids 198–218 are required for inactivation to occur. To more precisely map the required residues within this region, a series of smaller deletions were generated (Fig. 3A). This region was divided into three 7-amino acid subregions. Adding back single subregions into the Δ198–212 deletion interval indicated that no single subregion was capable of conferring Zrt1 inactivation (see Δ205–218, Δ198–204/212–218, Δ198–211, Fig. 3B). Adding back combinations of two of the three subdomains indicated that amino acids 205–218 were necessary for inactivation to occur (Fig. 3C), i.e. Δ212–218 and 205–211 were not zinc-responsive. The Δ198–204 mutant was inactivated normally indicating the deleted region is not involved.

To assess the importance of specific amino acids in the 205–
218 region in inactivation, polar or charged residues in the interval were substituted with alanine. Polar/charged residues were chosen because these are most likely to be surface exposed where they may participate in zinc sensing. Alanine substitutions were used because they are least likely to disrupt the overall structure of the domain (24). Alanine substitutions in the 212–218 region (i.e., Q213A, S214A, Q216A, and Q218A) had no effect on zinc responsiveness (data not shown). Similarly, T207A also had no effect on inactivation (Fig. 4A). However, mutations D205A, S208A, M209A, and D210A caused defective inactivation. S208A was particularly severe in its effects being indistinguishable from the end4 mutant (Fig. 2) and the K195R mutation (8).

Mutations that block inactivation of Zrt1 may potentially block zinc-induced ubiquitination or prevent endocytosis of the transporter after ubiquitination has occurred. Immunoblot analysis of wild type and mutant Zrt1 proteins indicated that these mutations block ubiquitination of the protein in response to zinc (Fig. 4B). Ubiquitination of wild-type Zrt1 in cells treated with zinc for 40 min was observed while no ubiquitination was detected for the control K195R mutant as well as Zrt1 Δ205–211, and S208A. Moreover, the mutations predicted to block endocytosis were shown by indirect immunofluorescence to have this effect. Wild-type Zrt1 was retained on the plasma membrane in –Zn conditions, as indicated by the bright staining at the cell periphery, but was lost in zinc-treated cells (Fig. 5). Our previous studies indicated that this loss was due to zinc-induced endocytosis (7). In contrast, K195R, Δ205–211, and S208A alleles were retained on the cell surface even in zinc-treated cells. Taken together, the experiments in Figs. 2−5 map a domain required for Zrt1 metal-responsive ubiquitination and subsequent endocytosis.

Additional experiments designed to assess if this region was sufficient as well as necessary in conferring zinc-induced endocytosis gave negative results. First, the entire loop between TM3 and TM4 (amino acids 153–218) was fused to the carboxyl-terminus of Pma1, the plasma membrane H^+-ATPase. Pma1 is normally a stable plasma membrane protein and this fusion approach was successful in showing the sufficiency of domains from Ste6 and Ste3 in mediating the ubiquitination and endocytosis of these proteins (25, 26). In the case of the Pma1-Zrt1 fusion, however, zinc-induced ubiquitination and endocytosis was not observed (data not shown). It was conceivable that the Zrt1 domain needs to attain a certain conformation to be functional and this conformation does not form in the Pma1-Zrt1 fusion. To address this possibility, Zrt1 alanine amino acids 195–218, which includes the ubiquitinated lysine, were inserted into two different sites in the TM3–4 loop of Zrt1, an iron transporter from *Arabidopsis* that can also transport zinc when expressed in yeast (27). These fusion proteins retained zinc transport activity, suggesting correct protein folding, but neither was inactivated in response to zinc (data not shown). Therefore, it remains possible that other regions of Zrt1 are required for inactivation to occur.
Assessing the Physiological Role of Zrt1 Inactivation in Metal Resistance—Zinc-limited cells have a high capacity for zinc uptake due to the transcriptional induction of ZRT1. For this reason, zinc-limited cells can undergo “zinc shock” when resupplied with the metal. We have found that the Zrc1 vacuolar zinc transporter is a critical component of zinc homeostasis during this transition period (6). Zinc-induced inactivation of Zrt1 may also play a role in zinc shock tolerance. To test this role and assess the relative importance of vacuolar sequestration and Zrt1 inactivation, we constructed isogenic strains defective in either vacuolar sequestration (zrc1Δ), inactivation of Zrt1 by zinc (ZRT1K195R) or both processes (zrc1Δ ZRT1K195R). The ZRT1K195 allele was introduced into the chromosome replacing the wild type ZRT1 locus and was expressed under the control of its own promoter. This creates an otherwise wild type context where zinc-responsive transcriptional control is intact. These cells were grown to exponential phase under zinc-limiting or replete conditions and then reincubated into media containing a range of zinc concentrations. The ability of cells to resist growth inhibition caused by these transitions was assessed by measuring the growth yield following 18 h incubation. Zinc-replete cells of all genotypes showed no sensitivity to zinc over the range of concentrations used (data not shown) consistent with the low level transcription of ZRT1 in these cells. For zinc-limited cells, all four strains grew well when transferred to a medium supplemented with no additional zinc (Fig. 6). Zinc-limited wild-type cells also grew well following transfer to media containing up to 10 μM zinc. (Lower growth yield was observed in medium with no added zinc because this is a zinc-limiting growth condition.) As expected, growth of zinc-limited zrc1Δ mutants defective in vacuolar zinc sequestration was severely inhibited by zinc shock at concentrations of 0.3 μM or more. Similarly, we found that cells defective in Zrt1 inactivation were also sensitive to zinc shock at concentrations of 3 μM and higher. Thus, both zinc sequestration in the vacuole and Zrt1 inactivation contribute to zinc shock tolerance.

We showed previously that cadmium is also likely to signal endocytosis of Zrt1; Zrt1 activity is lost following cadmium treatment in an END4-dependent manner (7). To address if cadmium and zinc signal Zrt1 inactivation through the same pathway, we assayed the effects of cadmium on inactivation of wild type and various mutant Zrt1 proteins (Fig. 7A). While both zinc and cadmium inactivated wild-type Zrt1, this inactivation was impaired in cells expressing the ZRT1K195R, ZRT1Δ-K195R, and ZRT1Δ-S208A alleles. Moreover, immunofluorescence microscopy indicated that wild type Zrt1 was lost from the cell surface in response to cadmium and this trafficking did not occur for these mutant alleles (Fig. 5). These results suggest that zinc and cadmium share the same signal transduction pathway for inactivation of Zrt1.

Zinc uptake by Zrt1 is strongly inhibited by cadmium (data not shown) suggesting that this metal is also a potential substrate for the transporter. This hypothesis was supported by recent studies showing that zrt1 mutants absorb lower amounts of cadmium than wild type cells (28). Therefore, zinc-limited cells may have a high capacity for cadmium uptake due to ZRT1 up-regulation. These considerations suggested that Zrt1 inactivation might also protect zinc-limited cells from cadmium accumulation. To test this hypothesis, we performed...
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Fig. 7. Role of Zrt1 inactivation in cadmium resistance. A, ZHY3 (zrt1 zrt2) cells expressing wild-type or mutant Zrt1 from the GAL1 promoter were grown to exponential phase in SD galactose medium, harvested, and resuspended in SD glucose medium supplemented with 100 µM cycloheximide and either 1 mM EDTA, 2 mM ZnCl$_2$, or 100 µM CdCl$_2$. After 4 h of incubation, the cells were harvested and assayed for $^{65}$Zn uptake rate. Shown are the means of three replicates and the error bars represent ±1 S.D. B, wild-type (MSYS1), ZRT1K195R (MSYS2), zrc1Δ (MSYS3), and zrc1Δ ZRT1K195R (MSYS4) strains were grown to exponential phase under low zinc (LZM−) and replete (LZM+) conditions. Aliquots of CSD medium supplemented with the indicated cadmium concentration were then inoculated with these cells to an initial OD$_{600}$ of 0.01. After 18 h incubation, OD$_{600}$ of the cultures was determined. C, wild-type (MSYS1), ZRT1K195R (MSYS2), and ZRT1Δ205–211 (MKY1) were assayed as described in panel B. Data points are the means of three replicates, and error bars represent ±1 S.D.

We were successful in mapping a region elsewhere in the TM3–4 loop that is critical for Zrt1 inactivation in response to metals. This region had the sequence DATSMRD (amino acids 205–211) with the underlined residues playing important roles. We have designated this domain as a potential “metal response domain” (MRD). Amino acids 212–218 were also important but their function was not affected by substitution mutations. It is our hypothesis that these seven residues are required as a spacer to place the more amino-terminal residues sufficiently distant from the membrane to allow their accessibility to the sensing and/or ubiquitination machinery.

A specific role for the putative MRD in Zrt1 inactivation is suggested by the observation that mutant proteins defective in inactivation have wild type uptake activity and are therefore not grossly misfolded. What role might the MRD play in the inactivation of Zrt1? Given that mutations in this domain block ubiquitination, the MRD is involved prior to or during that event. One possible role is as a site of phosphorylation that occurs in response to zinc and cadmium. Phosphorylation of experiments similar to those described in Fig. 6 to examine the ability of zinc-replete and zinc-limited wild-type and ZRT1K195R mutants to tolerate cadmium. Because Zrc1 had been previously implicated in cadmium as well as zinc tolerance (29), the contribution of this protein was also tested. As was observed for zinc sensitivity, zinc-replete cells of all genotypes showed little cadmium sensitivity; no growth inhibition was observed up to 3 µM cadmium (data not shown). In contrast, zinc-limited cells were very sensitive to cadmium treatment (Fig. 7B). The increased sensitivity of zinc-limited cells is consistent with an ability of Zrt1 to transport cadmium into the cell. Zinc-limited cells defective in Zrt1 inactivation (i.e., ZRT1K195R mutants) were sensitive to even lower levels of cadmium than cells expressing wild-type Zrt1. Moreover, cells expressing the Zrt1 Δ205–211 mutant also showed greater cadmium sensitivity (Fig. 7C). These results indicated that inactivation of Zrt1 by cadmium may be an important mechanism to protect zinc-limited cells from cadmium toxicity.
target proteins is an important signal for ubiquitination of cytosolic proteins such as IκB and cyclins (31). Moreover, phosphorylation of serine residues is required for ubiquitination and subsequent endocytosis of many yeast plasma membrane proteins including Ste2 and Fur4 (32, 33). These phosphorylated residues are located in regions of high acidic amino acid content. Ubiquitination of these proteins is dependent on the Rsp5 E3 ubiquitin ligase which led to the model that Rsp5 interacts directly or indirectly with regions whose net negative charge is further enhanced by phosphorylation (17). We showed previously that Zrt1 is also ubiquitinated in an Rsp5-dependent fashion (8) and the importance of acidic residues (Asp-205 and Asp-210) and phosphorylatable Ser-208 in Zrt1 inactivation is intriguing in this regard. However, several observations indicate that phosphorylation is not involved in Zrt1 inactivation. First, there was no apparent mobility shift in Zrt1 protein as assessed by immunoblots other than that which could be attributed to ubiquitination (8). Second, there was no effect of alkaline phosphatase treatment on the electrophoretic mobility of Zrt1 (data not shown). Third, immunoprecipitation of Zrt1 protein from cells grown with radiolabeled inorganic phosphate failed to recover any radiolabeled Zrt1 protein from either zinc-treated or untreated cells (data not shown). Fourth, the yeast casein kinase I homologues Yck1 and Yck2 have been shown to be directly or indirectly involved in phosphorylating Ste2, Fur4, and Pdr5 to promote their endocytosis (32, 34) (35).

A yck1 yck2 mutant showed no defect in Zrt1 inactivation in response to zinc at the nonpermissive temperature (not shown). First, substitution of Ser-208 with either aspartate or glutamate as potential phosphomimetic residues did not result in constitutive inactivation (data not shown). These data argue that phosphorylation is not involved in Zrt1 inactivation.

Two other models are currently under consideration. First, the MRD may be a zinc-binding site whose metal occupancy controls ubiquitination. The sequence we have identified as the MRD may be a zinc-binding site whose metal occupancy is further regulated by ubiquitination (8). Such a mechanism is unlikely to be directly relevant to Zrt1 because Rsp5 does not have a RING finger domain. However, it serves as a useful model of how zinc status might be communicated to the ubiquitination machinery through zinc-dependent protein-protein interactions.

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REFERENCES

1. Colwell, R. R. (2000) J. Infect. Chemother. 6, 121–125
2. Koch, A. L. (1997) Microbiol. Mol. Biol. Rev. 61, 305–318
3. Zhao, H., and Eide, D. (1996) J. Biol. Chem. 271, 23203–23210
4. Zhao, H., and Eide, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2454–2458
5. Gaither, L. A., and Eide, D. (2001) BioMetals 14, 251–270
6. MacDiarmid, C. W., Milanick, M. A., and Eide, D. (2003) J. Biol. Chem. 278, 15065–15072
7. Gitan, R. S., Luo, H., Rodgers, J., Broderius, M., and Eide, D. (1998) J. Biol. Chem. 273, 28617–28624
8. Gitan, R. S., and Eide, D. J. (2000) Biochem. J. 346, 329–336
9. Hicke, L., and Riezman, H. (1996) Cell 84, 277–287
10. Roth, A. F., and Davis, N. G. (1996) J. Cell Biol. 134, 661–674
11. Volland, C., Urban-Grimal, D., Gerard, G., and Haguener-Tsapis, R. (1994) J. Biol. Chem. 269, 9833–9841
12. Medintz, I., Jiang, H., Han, E., Cui, W., and Michels, C. A. (1996) J. Bacteriol. 178, 2245–2254
13. Stein, W. D. (1990) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
15. Shih, S. C., Sloper-Mould, K. E., and Hicke, L. (2000) EMBO J. 19, 187–198
16. Rotin, D., Staub, O., and Haguener-Tsapis, R. (2000) J. Membrane Biol. 176, 1–17
17. Rotin, D., Starb, O., and Haguener-Tsapis, R. (2000) J. Membrane Biol. 176, 1–17
18. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
19. Lyons, T. J., Gasch, A. P., Gaither, L. A., Rotin, D., Brown, P. O., and Eide, D. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7957–7962
20. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
21. Eide, D., Davis-Kaplan, S., Jordan, I., Sipe, D., and Kaplan, J. (1992) J. Biol. Chem. 267, 20774–20781
22. Harlow, E., and Lane, D. (1988) Antibodies: a Laboratory Manual, pp. 471–510, Cold Spring Harbor Press, Cold Spring Harbor, NY
23. Stein, W. D. (1990) Channels, Carriers, and Pumps. An Introduction to Membrane Transport, pp. 127–172, Academic Press, Inc., San Diego, CA
24. Wertman, R. P., Druhan, D. G., and Rotstein, D. (1992) Genetics 132, 337–350
25. Kolling, R., and Losko, S. (1997) EMBO J. 16, 2251–2261
26. Roth, A. F., Sullivan, D. M., and Davis, N. G. (1998) J. Cell Biol. 142, 949–961
27. Koshikawa, Y., Eide, D., Clarke, W. G., Guerinot, M. L., and Fukasawa, H. B. (1999) Plant Mol. Biol. 40, 37–44
28. Gomes, D. S., Fragoso, L. C., Ribeiro, C. J., Panek, A. D., and Eleutherio, E. C. (2002) Biochim. Biophys. Acta 1573, 21–25
29. Kamiono, A., Nishiuzawa, M., Teranishi, Y., Murata, K., and Kimura, A. (1989) Mol. Gen. Genet. 219, 161–167
30. Mason, B., and Moore, C. B. (1982) Principles of Geochemistry. 4th Ed., Wiley, New York
31. Laney, J. D., and Hochstrasser, M. (1999) Cell 97, 427–430
32. Hicke, L., Zanolari, B., and Riezman, H. (1998) J. Cell Biol. 141, 349–358
33. Marchal, C., Haguener-Tsapis, R., and Urban-Grimal, D. (1998) Mol. Cell. Biol. 18, 314–321
34. Marchal, C., Haguener-Tsapis, R., and Urban-Grimal, D. (2000) J. Biol. Chem. 275, 23608–23614
35. Decottignies, A., Owsianik, G., and Ghislain, M. (1999) J. Biol. Chem. 274, 37139–37146
36. Auld, D. S. (2001) BioMetals 14, 271–314
37. Thien, C. B., Walker, F., and Langdon, W. Y. (2001) Mol. Cell 7, 355–365