The EMBO Journal (2005) 24, 599–610 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
www.embojournal.org

THE EMBO JOURNAL

SCF^{Pof1}-ubiquitin and its target Zip1 transcription factor mediate cadmium response in fission yeast

Clare Harrison[1], Satoshi Katayama[1,4], Susheela Dhut[1], Dongrong Chen[2,3], Nic Jones[3], Jürg Bähler[2] and Takashi Toda[1,*]

[1]Laboratory of Cell Regulation, Lincoln's Inn Fields Laboratories, Cancer Research UK, London Research Institute, London, UK, [2]The Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK and [3]The Paterson Institute of Cancer Research, Manchester, UK

Ubiquitin-dependent proteolysis regulates gene expression in many eukaryotic systems. Pof1 is an essential fission yeast F-box protein that is homologous to budding yeast Met30. Temperature-sensitive *pof1* mutants display acute growth arrest with small cell size. Extragenic suppressor analysis identified Zip1, a bZIP (basic leucine zipper) transcription factor, as a target for Pof1. We show Zip1 is stabilized in *pof1* mutants, Pof1 binds only phosphorylated forms of Zip1, and Zip1 is ubiquitylated *in vivo*, indicating that Zip1 is a substrate of SCF^{Pof1}. Genome-wide DNA microarray assay shows that many cadmium-induced genes are under the control of Zip1, suggesting Zip1 plays a role in cadmium response. Consistently, *zip1* mutants are hypersensitive to cadmium and unlike wild type, lose cell viability under this stress. Intriguingly, cadmium exposure results in upregulation of Zip1 levels and leads wild-type cells to growth arrest with reduced cell size, reminiscent of *pof1* phenotypes. Our results indicate that Zip1 mediates growth arrest in cadmium response, which is essential to maintain viability. Normally growing cells prevent this response through constitutive ubiquitylation and degradation of Zip1 via SCF^{Pof1}.

The EMBO Journal (2005) **24,** 599–610. doi:10.1038/sj.emboj.7600536; Published online 20 January 2005
Subject Categories: proteins; cell cycle
Keywords: cadmium; DNA microarray; proteolysis; SCF; ubiquitin

Introduction

All cells must respond to environmental changes, such as UV-irradiation or genotoxic compounds, in order to survive and proliferate. Unicellular organisms in particular have evolved to utilize rapid adaptive responses at a single-cell level against various types of adverse stresses. This adaptation commonly occurs at the level of gene expression. Recent gene expression profiling experiments have shown both the global and selective transcriptional changes that occur in response to various cellular stresses (Gasch *et al*, 2000; Causton *et al*, 2001; Chen *et al*, 2003). These responses involve alterations in the activity of specific transcription factors. New transcription factors must be synthesized or existing ones modified and activated in order to respond to external cues. Cellular responses against these stresses are often accompanied with checkpoint-mediated cell cycle or division arrest (Hartwell and Weinert, 1989). Distinct classes of checkpoint pathways are activated in response to different types of damages, thereby preventing progression of the cell cycle to maintain cell viability. Then, when inappropriate stresses are overcome or removed, checkpoint signaling is turned off, leading to inactivation of these transcription factors and resulting recovery of cell proliferation.

Proteolysis often plays a crucial role in the feedback control underlying transcription-dependent stress responses (Pahl and Baeuerle, 1996). The ubiquitin–proteasome pathway is one of the major pathways for targeted proteolysis in the eukaryotic cell (Hochstrasser, 1996; Hershko and Ciechanover, 1998). It involves the covalent attachment of a polyubiquitin chain to a substrate, marking it for recognition and degradation by the 26S proteasome, a large multisubunit protease. The attachment of ubiquitin to a substrate occurs in a series of reactions, with the final step of substrate recognition being dependent upon a ubiquitin ligase, or E3. Different classes of ubiquitin ligase are now known and one of the largest and most versatile classes identified is that of the SCF (Skp1-Cul1/Cdc53-F-bpx) ubiquitin ligases (Feldman *et al*, 1997; Skowyra *et al*, 1997). The SCF ubiquitin ligases are multiprotein complexes. Each one contains at least three common components, Skp1, cullin-1/Cdc53 and Rbx1/Roc1/Hrt1. In addition to this core complex, they contain a variable receptor subunit, known as an F-box protein. Multiple F-box proteins can be identified in all eukaryotic organisms (Bai *et al*, 1996; Patton *et al*, 1998). They normally consist of an N-terminally proximal F-box motif, which is necessary for the interaction with Skp1, and a C-terminal protein–protein interaction motif. Each F-box protein recognizes and recruits specific substrates to the SCF complex, thus the F-box proteins are ultimately responsible for the timing and substrate specificity of proteolysis.

The fission yeast *Schizosaccharomyces pombe* genome contains at least 16 F-box proteins identified by genetic analysis and sequence homology. Of these, only two, Pof1 and Pof6, are essential for cell viability (Kominami *et al*, 1998; Ikebe *et al*, 2002; Hermand *et al*, 2003; Lehmann *et al*, 2004). Identification of the substrates recognized by these F-box proteins will help in our understanding of the essential role of SCF-mediated proteolysis within the cell. Pof1 is a WD40 repeat containing F-box protein with homology to the budding yeast *Saccharomyces cerevisiae* F-box protein Met30. The SCF^{Met30} complex is known to downregulate the activity of the transcription factor Met4 through both proteolytic and

*Corresponding author. Laboratory of Cell Regulation, Lincoln's Inn Fields Laboratories, Cancer Research UK, London Research Institute, PO Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.
Tel.: +44 20 7269 3535; Fax: +44 20 7269 3258;
E-mails: takashi.toda@cancer.org.uk or toda@cancer.org.uk
[4]Present address: Analytical Research Centre for Experimental Sciences, Saga University, Saga 840-8502 Japan

Received: 17 August 2004; accepted: 6 December 2004; published online: 20 January 2005

proteolysis-independent pathways (Kaiser *et al*, 2000; Patton *et al*, 2000; Rouillon *et al*, 2000; Kuras *et al*, 2002; Flick *et al*, 2004). Met4 is a bZIP (basic leucine zipper)-type transcription factor that is required for transcription of genes in the methionine biosynthesis pathway; thus, cells deficient in Met4 activity are methionine auxotrophs. In addition, upregulation of Met4, as seen in a *met30* mutant, results in a cell cycle arrest, the mechanism of which remains unknown (Patton *et al*, 2000).

In this study, we identify a bZIP transcription factor, Zip1, as the crucial substrate of SCF^{Pof1}. Inactivation of Zip1 rescues the lethality derived from Pof1 deficiency and overexpression of the *zip1*$^+$ gene results in a growth arrest phenotype, similar to *pof1* mutant cells. Unlike Met4, cells deleted for Zip1 are not auxotrophic for methionine or sulfur amino acids, instead showing acute sensitivity to cadmium stress. DNA microarray analysis suggests that Zip1 plays a critical role in transcription of genes specifically required for cadmium stress, thereby preventing cells from losing viability during exposure to this heavy metal. Thus, Pof1-Zip1-mediated responses comprise an essential network for the cadmium-responsive checkpoint pathway in fission yeast. Evolutional significance, in particular, in comparison with budding yeast Met30-Met4 and human systems, is discussed in the light of stress response mechanisms.

Results

Pof1 is a component of the SCF ubiquitin ligase

We previously showed that Pof1 binds Skp1 (Lehmann *et al*, 2004). In order to clarify whether Pof1 functions via an SCF complex, binding between Pof1 and cullin-1 (Pcu1) (Kominami *et al*, 1998) was examined. For this purpose, a strain was constructed in which the chromosomal *pof1*$^+$ and *pcu1*$^+$ genes were individually tagged with GST and 13Myc epitopes, respectively, at the C-terminus under the native promoter. Tagging did not interfere with protein function, as growth rate of these strains was indistinguishable compared to untagged wild-type cells. Immunoprecipitation was performed using anti-GST antibody. Immunoblotting showed that Pof1-GST co-precipitated with Pcu1-13Myc (Figure 1A). Thus, Pof1 is a component of the fission yeast SCF ubiquitin ligase.

Isolation of pof1 temperature-sensitive (ts) mutants

pof1$^+$ is essential for cell viability (Lehmann *et al*, 2004), and spores containing deleted *pof1* failed to germinate, which hampered the characterization of Pof1 function (C Harrison and T Toda, unpublished results). In order to examine the role of Pof1 further, we created ts *pof1* alleles using mutagenic PCR. Briefly, genomic DNA was prepared from a strain in which the *GFP* gene (linked with the G418-resistance marker gene *kan*^{r}) was inserted into the C-terminus of Pof1 under its endogenous promoter (*pof1*$^+$*-GFP-kan*^{r}). This 4.5 kb *pof1*$^+$*-GFP-kan*^{r} cassette was amplified with error-prone PCR, followed by transformation to a wild-type strain. G418-resistant colonies were selected at 27°C and ts transformants then isolated by replica plating at 36°C. This procedure resulted in the isolation of two ts *pof1* alleles, *pof1-6* and *pof1-12*. Nucleotide sequencing of the *pof1* gene in these ts mutants showed that both contained two mutations (Figure 1B). The *pof1-6* allele contained two point mutations

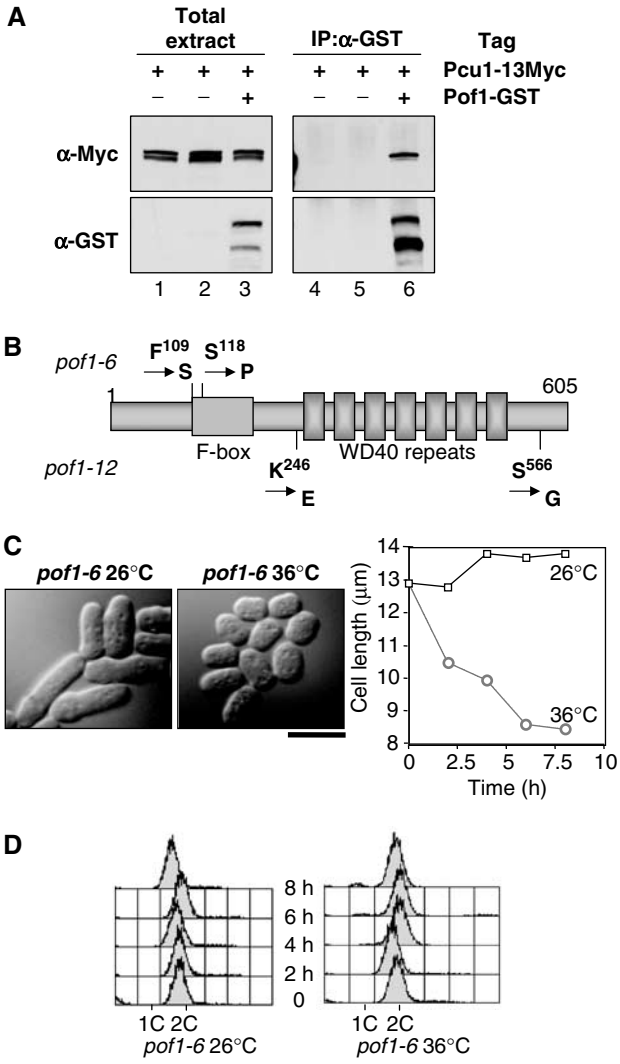

Figure 1 Complex formation between Pof1 and cullin-1 and creation of ts *pof1* mutants. (**A**) Interaction between Pof1 and cullin-1. Protein extracts were prepared from a single tagged (*pcu1*$^+$*-13myc*, lanes 1, 2, 4 and 5), or double-tagged strain (*pof1*$^+$*-GST pcu1*$^+$*-13myc*, lanes 3 and 6), and immunoprecipitation was performed with anti-GST antibody (lanes 4–6). After running on SDS–PAGE, immunoblotting was performed with anti-Myc (upper) or anti-GST antibody (lower). (**B**) Structure of Pof1 and mutation sites in *pof1-6* and *pof1-12*. Pof1-6 contains two amino-acid replacements at P109S and S118P, while Pof1-12 carries K246E and S566G mutations. F-box motif and WD40 repeats are shown with boxes. (**C**) Cell morphology. A *pof1-6* strain was grown at 26°C (left), shifted to 36°C and incubated for 6 h (middle). Phase-contrast microscopy images are shown. In addition, average cell length ($n = 200$) was plotted at each time point. The bar indicates 10 μm. (**D**) DNA content. A *pof1-6* strain was grown at 26°C (left) or 36°C (right) and samples were collected for flow cytometry at 2 h intervals. Note that a small G1 (1C) peak appeared at 6 and 8 h at 36°C.

in the F-box motif region, F109S and S118P, respectively. These would be predicted to interfere with Pof1 interactions with Skp1. The *pof1-12* allele contained two mutations in the C-terminal region following the F-box motif, K246E and S566G. These mutations, situated on either side of the WD40 repeat domain, would thus be predicted to interfere with Pof1-substrate binding.

Initial characterization of these mutants showed that both alleles arrested at 36°C as small cells consisting of a majority of G2 cells and a small G1 population (Figure 1C and D).

Table I Cell size comparison between wild type and *pof1-6* mutant

Strains	Cell length (μm)	Cell width (μm)
Wild type	12.3±2.1	4.3±0.4
pof1-6	8.5±1.2	4.4±0.4
wee1-50	6.6±0.7	4.3±0.3

Exponentially growing strains at 26°C were shifted to 36°C and incubated for 6 h. At least 300 cells were measured for each sample.

Average cell length was 8.5 μm after 6 h incubation at 36°C compared to 12.3 μm for wild-type cells under the same conditions (Table I, note that the cell width is indistinguishable between the two strains). This short cell phenotype appeared to be similar to *wee* mutants, in which mitosis occurs earlier than wild type due to premature activation of the Cdc2 kinase (Nurse, 1990). Unlike authentic *wee* mutants, however, the *pof1* mutants did not show any genetic compensation with the G2 arrest *cdc25-22* mutant, nor could any change in the phosphorylation state of Cdc2 at Y15 be observed (C Harrison and T Toda, unpublished results). This suggests that the *pof1* phenotype is not due to a '*wee*-like' cell cycle defect, but instead is most likely a direct deficiency in macromolecular synthesis *per se*, which leads to small cell size and growth arrest.

Identification of zip1 as extragenic suppressors of pof1-6

Pof1 is required for cell viability and ts *pof1* mutants displayed growth arrest at the restrictive temperature (Figure 1C). It is likely that uncoordinated accumulation or activation of some substrate(s) in the absence of SCF^{Pof1} function could result in this arrest. If this scenario was the case, the extragenic mutation in a gene encoding this substrate might rescue the ts *pof1* mutant. Given this prediction, we next sought to isolate Ts^+ suppressors from *pof1-6*. Lawns of cells ($\sim 10^8$ cells) carrying the *pof1-6* mutation were plated onto rich media and these plates left at the restrictive temperature, 36°C. After approximately 10 days, we could observe 23 colonies growing at this temperature. Backcrossing these revertants with a wild-type strain indicated that nine of these colonies contained some intragenic mutation, that is, the *pof1*-6 allele had reverted to wild-type function, whereas the remaining 14 isolates all contained an extragenic mutation, which suppressed the *pof1*-6 allele. Further genetic analysis showed that all 14 revertants contain the same, single locus, designated *sup9*.

Data from DNA microarray experiments (Chen *et al*, 2003) had identified $pof1^+$ as a gene specifically upregulated in response to cadmium stress. This suggested that SCF^{Pof1} activity is required in response to cadmium. This prompted us to test the sensitivity of cells containing the *sup9* mutation to cadmium stress. These cells were indeed hypersensitive to cadmium stress (0.5 mM cadmium sulfate, Figure 2A). To

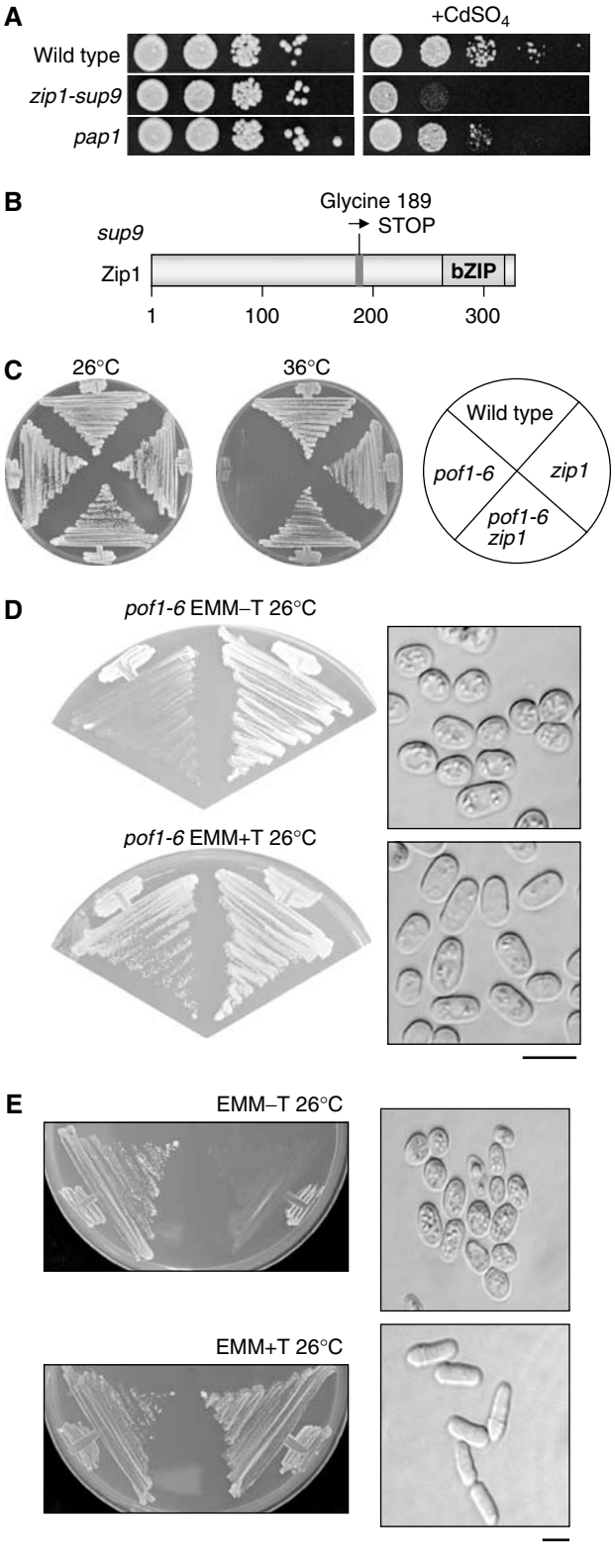

Figure 2 Identification of *zip1* as an extragenic suppressor locus of *pof1* mutants. (**A**) Cadmium hypersensitivity of *sup9* (*zip1*). Wild-type (top row), *sup9* (=*zip1*, middle) or *pap1*-deleted cells (bottom), which are defective in oxidative stress response (Toone and Jones, 1998), were spotted onto rich plates in the absence (left) or presence (right) of 0.5 mM cadmium sulfate (10^5 cells in the far-left spots for each plate and then diluted 10-fold in each subsequent spot rightwards) at 26°C and incubated for 4 days. (**B**) Schematic structure of Zip1. The position of the nonsense mutation in *sup9* (from glycine to TGA) and the C-terminal ZIP region are shown. (**C**) Suppression of *pof1-6* by *zip1* deletion. Four strains (wild type, *pof1-6*, *zip1* deletion and *pof1-6 zip1*) were streaked on rich medium and incubated at 26 or 36°C. (**D, E**) Toxicity of overexpressed $zip1^+$. Plasmids containing $zip1^+$ under the thiamine repressible mild *nmt41* promoter (D) or strongest *nmt1* promoter (E) were transformed into *pof1-6* (D) or wild-type cells (E), respectively, streaked on minimal plates in the absence (upper) or presence (lower) of thiamine and incubated at 26°C for 3 days. Cell morphology of *pof1-6* or wild-type cells containing $zip1^+$ plasmids under each condition is shown in the right panels together with morphology of cells containing empty plasmids. The bar indicates 10 μm.

identify this suppressor gene, the suppressor mutant was transformed with a plasmid-based gene library and screened for transformants, that could restore growth on plates containing 1.0 mM cadmium sulfate. Two independent transformants able to grow in the presence of cadmium were isolated and plasmid stability tests showed this phenotype was plasmid dependent. DNA sequencing revealed that both transformants contained identical plasmids, containing the entire ORF encoding a bZIP transcription factor, Zip1 (Ohmiya *et al*, 1999). Zip1 was originally isolated as a multicopy suppressor gene that rescued the calcium-sensitive phenotype of the mutation in *atf1* encoding another bZIP. However, its physiological role remains to be determined.

The *zip1* gene in the *sup9* suppressor strain was sequenced and found to contain a GGA to TGA nonsense mutation corresponding to amino-acid residue glycine 189. This resulted in the insertion of a STOP opal codon in the middle of the *zip1* ORF prior to the C-terminal bZIP region (Figure 2B, *sup9* is referred to as *zip1* hereafter). This nonsense *zip1* allele was also able to rescue growth of ts *pof1-12* at 36°C (data not shown). To directly confirm that inactivation of Zip1 suppresses ts *pof1-6*, a complete deletion of the *zip1*$^+$ ORF was constructed and crossed with a *pof1-6* strain. The *pof1-6 zip1* double mutant could grow at 36°C, verifying that loss of Zip1 function rescues ts *pof1-6* (Figure 2C).

If Zip1 accumulation or activation was a direct cause for the phenotypic appearance of a *pof1-6* strain at the restrictive temperature, further overexpression of the *zip1*$^+$ gene in this mutant might result in inhibition of cell division even at the permissive temperature. To address this, the *zip1*$^+$ gene was subcloned into a multicopy plasmid under the control of a thiamine-repressible mild promoter (*nmt41-zip1*$^+$) (Basi *et al*, 1993). This plasmid was toxic to only *pof1*-6 cells, but not wild type, at 26°C and arrested cells mimicked the phenotype normally only seen at 36°C, with small cell size (Figure 2D). Moreover, when the *zip1*$^+$ gene was expressed under the strongest promoter (*nmt1-zip1*$^+$), growth was inhibited even in wild-type cells with reduced cell size (Figure 2E). Taken together, these data suggest that the small-size phenotype and temperature sensitivity of *pof1* mutants can be explained by an excess or hyperactivation of the *zip1*$^+$ gene product.

Zip1 is ubiquitylated in vivo

We addressed ubiquitylation of Zip1. In many cases, ubiquitylation of a protein leads to rapid proteasome-dependent degradation, which makes detection of ubiquitylated intermediates difficult. To circumvent this situation, ts *mts3-1*, a proteasome mutant (Seeger *et al*, 1996), was used and Zip1 was C-terminally tagged with an HA epitope (Zip1-HA) in this mutant. In order to visualize conjugated ubiquitin, this strain was transformed with a multicopy plasmid containing ubiquitin tagged with an HIS epitope (His-Ubi). After incubation for 1 h at the restrictive temperature to inactivate *mts3*, Ni^{2+} chelate resin was used to pull down all ubiquitylated conjugates in the cell and the resulting extract blotted with anti-HA antibody to detect the presence of Zip1-HA. A characteristic smear pattern was seen in the His-Ubi lane (Figure 3A, lanes 1 and 3), which was missing in the extract from cells transformed with empty vector (lanes 2 and 4). This indicates that Zip1 is ubiquitylated *in vivo*.

The next obvious question to ask was whether ubiquitylation of Zip1 was dependent upon Pof1 activity. In order to answer this question, we created *pof1-6mts3-1* double mutants carrying Zip1-HA. This strain was, however, extremely slow growing even at 26°C and practically impossible to propagate (Figure 3B, upper). Examination of these cells from plates showed that they displayed small-sized cell phenotype, identical to *pof1*-6 at the restrictive temperature (Figure 3B, lower). Although this meant dependency of Zip1 ubiquitylation upon Pof1 could not be directly addressed, these data in itself strongly imply that Pof1 and the proteasome are acting additively to inactivate the same substrate, Zip1.

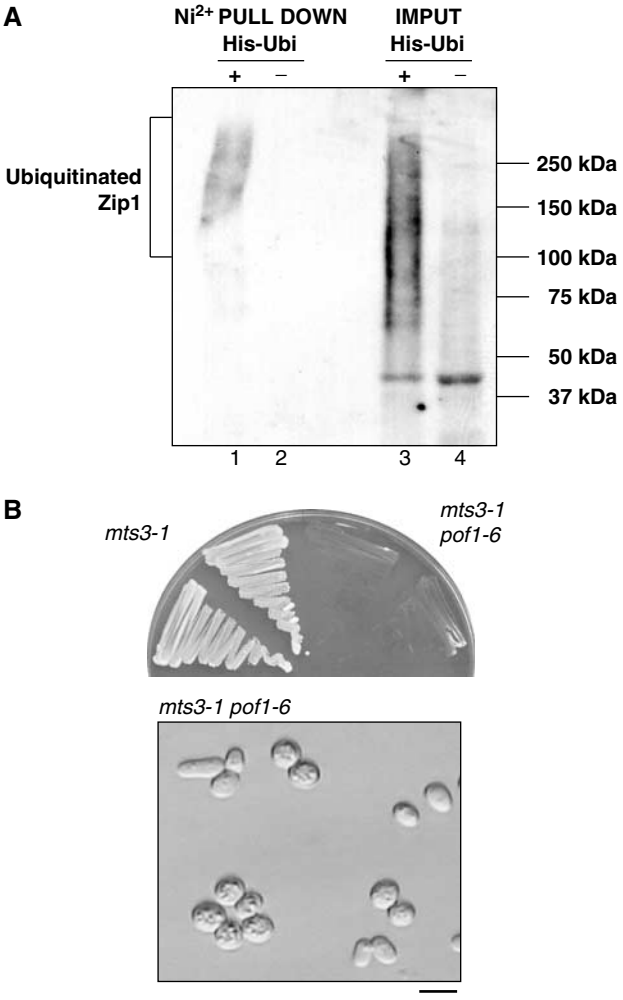

Figure 3 Ubiquitylation of Zip1. (**A**) Zip1 is polyubiquitylated *in vivo*. Plasmids containing 6His-ubiquitin (His-Ubi, lanes 1 and 3) or empty vector (lanes 2 and 4) were introduced into the *mts3-1* mutant containing Zip1-HA. After 1 h incubation at 36°C, ubiquitylated proteins were purified with Ni^{2+}-NTA beads (lanes 1 and 2). Total extracts (60 μg) are also run (lanes 3 and 4). Immunoblotting was performed with anti-HA antibody. (**B**) Synthetic phenotype between *pof1-6* and *mts3-1*. *mts3-1* single (left) or *mts3-1 pof1-6* double mutants (right) containing Zip1-HA were streaked on rich medium and incubated at 26°C for 3 days (upper). Cell morphology for *mts3-1 pof1-6* is also shown (lower). The bar indicates 10 μm.

Pof1 is required for Zip1 instability

We next asked whether the stability of the Zip1 protein is dependent upon Pof1 by examining the half-life of Zip1-HA in wild-type and *pof1*-6 cells. Exponentially growing cultures of

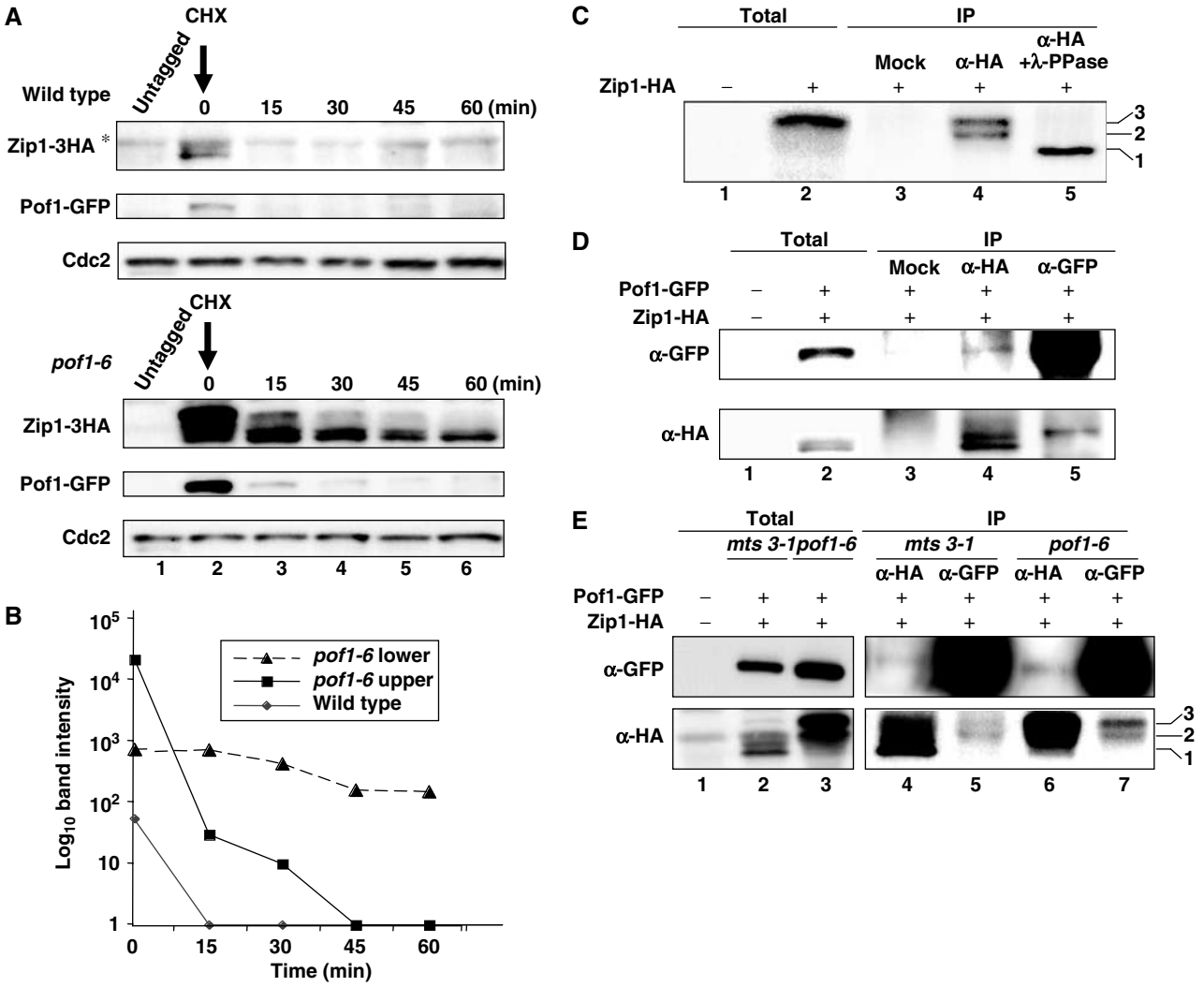

Figure 4 Stabilization of Zip1 in *pof1* mutants and binding between Pof1 and phosphorylated Zip1. (**A**) Accumulation and partial stabilization of Zip1 in *pof1-6*. Exponentially growing wild-type *pof1*$^+$-GFP (upper gel) or *pof1-6*-GFP (lower) cells containing Zip1-HA were shifted to 36°C for 2 h and CHX (100 μg/ml) was then added (time 0). Protein samples were prepared at indicated time points. Immunoblotting was performed with anti-HA (top), anti-GFP (middle) or anti-Cdc2 antibody (bottom). Samples from untagged strains were also run as a negative control (lane 1). Nonspecific band is marked with asterisk. (**B**) Quantification of Zip1 levels. Zip1-HA amount shown in (A) was quantified. Intensities of upper (squares), or lower bands (triangles) of Zip1-HA in *pof1-6* and a lower band (diamonds) in wild type were measured. (**C**) Phosphorylation of Zip1. *pof1-6* mutant cells containing Zip1-HA were shifted to 36°C for 4 h and immunoprecipitation was performed with mock (lane 3) or anti-HA antibody (lanes 4 and 5). Immunoprecipitates were treated with λ-protein phosphatase for 30 min at 30°C (lane 5). Total extract (30 μg) was also run (lane 2). As a negative control, protein samples prepared from untagged *pof1-6* were also run (lane 1). Immunoblotting was performed with anti-HA antibody. Three Zip1-HA bands are marked. (**D, E**) Binding between Zip1 and Pof1. Immunoprecipitation was performed with anti-HA (lanes 4 and 6), anti-GFP antibody (lanes 5 and 7) or mock (lane 3 in D), using protein extracts prepared from *mts3-1* (D and lanes 2, 4 and 5 in E) or *pof1-6* mutants containing Pof1-GFP Zip1-HA (lanes 3, 6 and 7 in E), which were incubated at 36°C for 1 h. Total extracts from a tagged (lane 2) or untagged strain (lane 1) were also run. Immunoblotting was performed with anti-GFP (upper) or anti-HA antibody (lower).

both strains were treated with protein synthesis inhibitor cycloheximide (CHX) to shut off *de novo* protein synthesis and Zip1 levels were examined at regular intervals afterwards. It was clear from the initial time point that *pof1*-6 cells have increased total Zip1 levels when compared to wild-type cells (Figure 4A, lane 2). In addition, Zip1 in this mutant accumulated in slower migrating forms, while only the fastest band was seen in wild-type cells. Zip1-HA disappeared quickly in wild-type cells upon addition of CHX; after 15 min, very little signal was detected (upper panel, lane 3). In clear contrast, in the *pof1-6* mutant, the faster migrating Zip1-HA band showed an increased half-life compared to wild type (lower panel and Figure 4B for quantification). Slower migrating bands, however, seemed to be still degraded relatively efficiently even in *pof1-6*. It is of note that not only Zip1 but also the Pof1 protein was upregulated in *pof1-6*, although in this case Pof1-GFP was as unstable as in wild-type cells (Figure 4A, lower panel). These increased levels of Pof1 in *pof1-6* are probably attributable to transcriptional activation of the *pof1*$^+$ gene via accumulation of Zip1, as *pof1*$^+$ gene expression is dependent upon Zip1 activity (see below).

Pof1 binds phosphorylated forms of Zip1

Phosphorylation is known to be a requirement of many F-box proteins for substrate recognition (Patton *et al*, 1998; Nash

et al, 2001). Slower migrating bands of Zip1-HA existing in *pof1-6* cells prompted us to examine whether these bands are phosphorylated forms. We tested this possibility by immunopurifying Zip1-HA from *pof1*-6 cells, then treating with λ-phosphatase. This confirmed that Zip1 accumulates in apparently two modified forms in *pof1*-6 cells, both of which are phosphorylated (Figure 4C, lanes 4 and 5, two phosphorylated bands are marked with 2 and 3, while the fastest migrating unphosphorylated band is shown as 1).

If Zip1 is a substrate for the SCF^{Pof1}, Pof1 is expected to bind Zip1. Immunoprecipitation was performed in a strain containing Pof1-GFP and Zip1-HA. To prevent degradation of Zip1, experiments were again carried out in an *mts3-1* background. Under this condition, Zip1 was detected as a doublet, the lower band of which corresponded to the band seen in wild-type cells (band 1 in Figure 4C) and the upper of which appeared to correlate to the faster migrating form (band 2 in Figure 4C) of the phosphorylated Zip1 bands (Figure 4D, lanes 2 and 4). Immunoprecipitation using anti-GFP antibody showed that Pof1 co-immunoprecipitated with Zip1, specifically the upper band of the doublet (Figure 4D, lane 5). Reciprocal immunoprecipitation with anti-HA antibody also co-precipitated Pof1-GFP, albeit very faintly (lane 4). This is probably due to the existence of an excess amount of free Zip1-HA dissociated from Pof1-GFP in the *mts3-1* mutant.

In order to examine which of Zip1 forms binds Pof1 in the *pof1-6* background, immunoprecipitation was performed using samples prepared from the *pof1-6* mutant used in Figure 4A (doubly tagged with Pof1-6-GFP and Zip1-HA). For comparison, parallel immunoprecipitation was also performed in extracts from *mts3-1* cells and precipitates were run side-by-side. As shown in Figure 4E, it is evident that two phosphorylated Zip1 forms interacted with Pof1-6 (lane 7, marked as bands 2 and 3). Taken together, these findings indicate that Zip1 is phosphorylated and this phosphorylation leads to an interaction with Pof1 and subsequent degradation.

Zip1 regulates expression of cadmium stress specific genes

Having identified Zip1 as a critical substrate of SCF^{Pof1}, we next explored the physiological role of Zip1. The hypersensitivity of *zip1* mutant cells to cadmium suggested that Zip1 plays some role in the cellular response against cadmium stress. In order to study this further, we utilized DNA microarrays to compare genome-wide transcriptional profiles of wild-type and *zip1* cells upon cadmium stress. Both strains were exposed to cadmium sulfate and at 0, 15 and 60 min time points, total RNA was prepared and microarray data were collected as performed previously (Chen *et al*, 2003). This analysis showed that a set of 27 genes appeared to be specific targets of Zip1, having less than 50% reduction in expression in a *zip1* strain compared to wild type at either 15 or 60 min after addition of cadmium (Table II). Noticeably, 20 out of these 27 genes were induced more than two-fold in wild-type cells in response to cadmium exposure (Figure 5A and see genes shown with bold letters in Table II).

Table II Zip1 target genes

Gene name	Function	Budding yeast	Induction by cadmium	*zip1* versus wild type
SPAC869.05c	Sulfate transporter	Sul1	16.4	0.1
SPCC1739.06c	Uroporphyrin methyltransferase	Met1	7.8	0.1
SPCPB1C11.03	Membrane transporter		4.1	0.1
SPBPB10D8.02c	Arylsulfatase		7.3	0.1
SPBPB10D8.01	Membrane transporter		2.7	0.1
***pof1*$^+$**	F-box protein	Met30	5.7	0.2
SPBC106.17c	Homoserine *O*-acetyltransferase	Met6	3.5	0.1
SPBPB2B2.08	Unknown		25	0.1
SPBP16F5.08c	Flavin-dependent monooxygenase	Fmo1	4.4	0.1
SPAC10F6.01c	Sulfite reductase	Ecm17	1.3	0.3
***frp1*$^+$**	Ferric-chelate reductase	Fre1–4	3.8	0.4
SPBP8B7.05c	Carbonic anhydrase	Nce103	1.7	0.3
***cad1*$^+$/*hmt2*$^+$**	Sulfide-quinone oxidoreductase		2.2	0.4
SPBC725.04	Oxalyl-CoA decarboxylase		3.0	0.4
SPAC23A1.14c	Cystathionine γ-synthase		5.8	0.4
SPCC584.01c	Sulfite reductase	Met10	1.3	0.4
SPCC965.06	Potassium channel subunit		21.8	0.5
SPBPB10D8.04c	Malate permease	Ssu1	1.2	0.5
SPCC1827.03c	Long-chain fatty acid transporter	Fat2	1.2	0.3
SPBC27.04	Coiled-coil protein	Rad50	2.6	0.2
fip1$^+$	Iron permease	Fth1, Ftr1	1.3	0.2
SPBC1348.06c	Unknown Similarity to SPAC977.05c and SPBPB2B2.15	Fun19	21.0	0.3
SPAC977.05c	Unknown Similarity to SPBC1348.06c and SPBPB2B2.15	Fun19	20.0	0.3
cdc15$^+$	SH3 and FCH containing	Hof1	1.8	0.3
SPBPB2B2.05	GMP synthase		5.6	0.4
***cdc22*$^+$**	Ribonucleoside-diphosphate reductase (large subunit)	Rnr1, Rnr3	2.2	0.4
SPCC622.12c	NADP glutamate dehydrogenase	Gdh1, Gdh3	2.6	0.5

A total of 27 genes, in which the induction rate between *zip1* (*sup9*) and wild-type strains (*zip1* versus wild type) was less than 50%, are listed. Particularly, those (20 genes) that show more than two-fold induction at either 15 or 60 min upon cadmium stress in wild-type cells are emphasized by bold letters. Furthermore, among 20 of these genes, the induction of 15 genes is specific to cadmium stress (Chen *et al*, 2003) and these genes are shown with underlined bold letters (see Figure 5A).

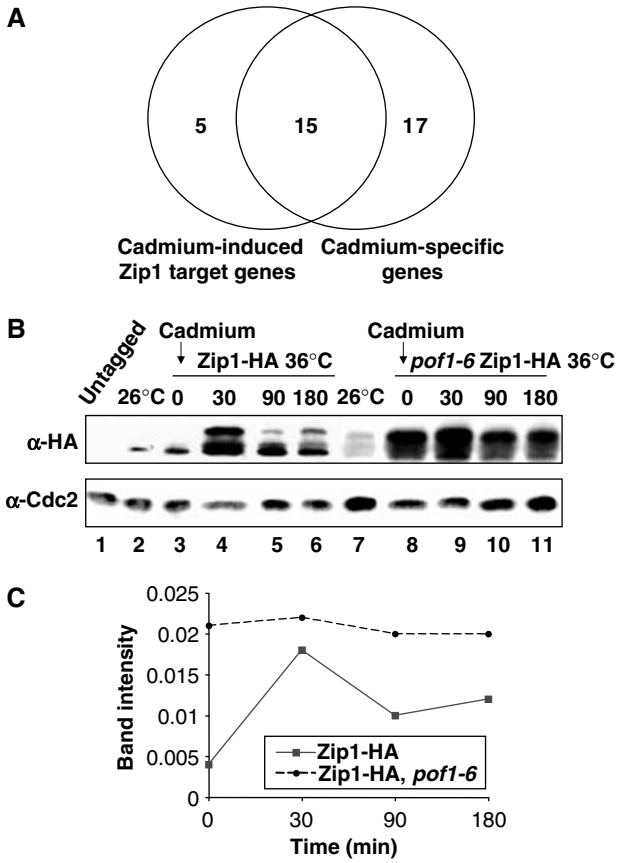

Figure 5 Comparison of Zip1-target and cadmium-specific genes and accumulation of Zip1 upon cadmium exposure. (**A**) Summary of DNA microarray analysis. Genes induced by cadmium stress in a Zip1-dependent manner and those induced in a cadmium stress-specific manner (Chen *et al*, 2003) are compared. (**B**) Increase of Zip1 upon cadmium exposure. Exponentially growing wild-type (lanes 2) or *pof1-6* cells containing Zip1-HA (lane 7) were shifted from 26 to 36°C and incubated for 1 h (lanes 3 and 8). Then, 0.5 mM cadmium sulfate was added, followed by protein extracts at 0, 30, 90 and 180 min time points (lanes 3–6 and 8–11). Immunoblotting was performed with anti-HA (upper) or anti-Cdc2 antibody (lower). Untagged wild type was used as a negative control (lane 1). (**C**) Quantification. Zip1-HA levels shown in (B) are quantified using Cdc2 as a control.

Previous global transcriptional analysis in response to various types of stresses has shown that responsive genes fall into two categories, core environmental stress response (CESR) that is common to most stresses and stress-specific responses (Chen *et al*, 2003). To explore which of these categories Zip1-dependent genes fell into, we compared our data with the previous stress data (Chen *et al*, 2003). Out of 20 Zip1-target genes, 15 belong to a subgroup consisting of the 32 cadmium-stress-specific genes (Figure 5A, these 15 genes are shown in underlined bold letters in Table II). It is worth noting that at least 10 of these 15 Zip1- and cadmium stress-specific target genes are independent of the Sty1-Atf1 stress-activated mitogen-activated protein kinase pathway, as shown previously (Chen *et al*, 2003). This substantiates the notion that Sty1-Atf1 primarily acts on CESR genes, while Pof1-Zip1 plays a specific role in cadmium stress response. Interestingly, *pof1*$^+$ itself is also seen to be induced in a cadmium-specific, Zip1-dependent manner, suggesting a negative feedback mechanism exists to ensure Zip1 degradation at some later time point following the cadmium response (Tables II and III). Overall, this microarray analysis shows that Zip1 is the major, if not sole, factor required for transcriptional induction upon cadmium stress.

Table III Cadmium induction of *pof1*$^+$ and *zip1*$^+$ genes

Strains	Wild type			*zip1* (=*sup9*)		
Cd^{2+} (min)	0	15	60	0	15	60
Gene expression						
pof1	1.0	5.7	2.1	1.0	1.0	0.8
zip1	ND	1.1	1.6	1.0	1.3	0.9

Comparative transcription levels of the *pof1* and *zip1* genes in response to cadmium stress (0, 15 and 60 min) are taken from microarray data in wild-type and *zip1-sup9* mutant cells. Note that, as *zip1-sup9* contains a nonsense mutation, *zip1* mRNA levels could be measured.
ND stands for not determined.

One of the most adverse consequences by the presence of cadmium in the cell is the absorption of sulfur molecules. Glutathione (GSH) functions to protect cells from cadmium stress by direct binding to this metal, leading to sequestration into the vacuole (Clemens and Simm, 2003). In budding yeast, GSH is synthesized, along with sulfur-containing amino acids, methionine and cysteine, through the sulfate assimilation pathway (Marzluf, 1997). Most of the enzymes in this pathway are induced under cadmium stress in this yeast (Fauchon *et al*, 2002). In contrast, in fission yeast only a subset of these sulfate assimilation genes appeared to be induced (Table II) (Chen *et al*, 2003), suggesting that the sulfate assimilation pathways are regulated differently between the two organisms. Nonetheless, the sulfate assimilation genes induced by cadmium in fission yeast were Zip1 dependent, suggesting that the primary role of Zip1 in response to cadmium is to upregulate uptake and assimilation of inorganic sulfur from the environment, presumably for the increased production of GSH.

Exogenous cadmium upregulates Zip1 protein levels

Given the critical role for Zip1 in cadmium response and tolerance, *zip1*$^+$ gene expression might have been expected to be upregulated upon cadmium stress. None of the wild-type microarray data, however, indicated the increase of *zip1*$^+$ gene expression upon exposure to cadmium (Table III). In order to examine Zip1 protein levels in response to cadmium stress, immunoblotting was performed in a Zip1-HA strain. As shown in Figure 5B, after 30 min in the presence of cadmium, cells had increased Zip1 levels (lane 4). Intriguingly, a slower migrating form of Zip1, apparently corresponding to the phosphorylated form observed in *pof1*-6 mutants, as well as unphosphorylated form, appeared after cadmium exposure. As *zip1*$^+$ gene expression does not change after cadmium stress (Table III), inhibition of Zip1 protein degradation is likely to be responsible for this increase in Zip1 levels upon cadmium addition. Prolonged exposure with cadmium appeared to result in a slight decrease of Zip1 levels (90 and 180 min, lanes 5 and 6 and Figure 5C for quantification). This may be ascribable to induction of the *pof1*$^+$ gene upon cadmium stress as a part

of negative feedback loop (Table III), although other possibilities can not be excluded.

Cadmium was also added to *pof1-6* mutants preincubated at 36°C. In this mutant, as shown earlier (Figure 4A), 1 h incubation at 36°C prior to cadmium exposure resulted in the increase of Zip1-HA levels together with the appearance of slower migrating bands (lane 8). In clear contrast to wild-type cells, *pof1-6* mutants did not show any further increase of Zip1-HA levels upon cadmium addition (lanes 9–11 and Figure 5C for quantification). These results indicate that one of the cellular responses to cadmium includes Zip1 stabilization and in *pof1-6* mutants, Zip1 levels are maximally upregulated. This also suggests that upregulation of Zip1 levels under cadmium stress is mediated by inhibition of Pof1-dependent ubiquitylation and degradation of Zip1.

Zip1 is not necessary for functioning of the sulfur amino-acid biosynthesis genes

Pof1 is most homologous to budding yeast Met30, the F-box protein required for inactivation of bZIP transcription factor Met4 (Patton *et al*, 2000). Met4 is known to be responsible for the transcription of many of the genes in the sulfate assimilation pathway, specifically for the production of methionine and cysteine when these are missing from the environment. Thus, *met4*-deleted cells cannot grow without an external source of methionine. As our microarray data suggested that Zip1 is able to activate transcription of only some, if not many, of the genes in this pathway, we tested if Zip1 is similarly essential for growth on media lacking sulfur amino acids. In contrast to cells with mutations in genes in the sulfur amino-acid pathway, such as *met5-1*, *zip1*-deleted mutant cells were able to grow on minimal media containing only the inorganic sulfur source, Na_2SO_4 (Figure 6). This suggests that while Zip1 is capable of upregulating some of the genes in the sulfur assimilation pathway in response to cadmium stress, it is not essential for the cells to synthesize sulfur amino acids under conditions where these amino acids are lacking.

Zip1 is required for maintenance of viability in cells exposed to cadmium

Previous results led us to the possibility that Zip1 is specifically required to initiate cadmium stress response and that phenotypic consequences of *pof1* mutants, in which Zip1 accumulates, are in fact mimicking those of cells exposed to cadmium. In line with this notion, as shown earlier, cadmium exposure also results in elevated Zip1 levels (see Figure 5B and C). If this was the case, wild-type cells exposed to cadmium should show some of the defective phenotypes seen in a *pof1* mutant, such as small cell size and growth arrest. We investigated this by exposing wild-type, *pof1*-6- and *zip1*-deleted cells growing in culture to cadmium stress and observing their response. Cadmium sulfate (0.5 mM) stopped growth of all cells at 26°C in liquid medium. However, if samples of these liquid cultures were plated onto cadmium-free plates, even after 6 h exposure to cadmium, wild-type and *pof1*-6 cells retained high viability (Figure 7A). This implies that growth arrest observed in these two strains is reversible. In sharp contrast, *zip1* cells lost viability, with only 15% of cells surviving after 6 h in the presence of cadmium. This indicates that apparent division arrest in wild-type and *pof1-6* cells upon cadmium stress is not due to cell death; instead, that cells arrest growth but remain viable and, importantly, that this response is Zip1 dependent.

We next examined whether cadmium exposure could induce small cells as seen in the *pof1* mutant at the restrictive temperature. In agreement with our prediction, even at the permissive temperature, *pof1*-6 cells became smaller in response to cadmium as did wild-type cells (Figure 7B and C). On the contrary, the cell size of *zip1* mutants did not show any reduction, rather it was increased (Figure 7B).

Finally, while observing the cadmium-exposed cells, we noticed that a larger than average number of them were septating. We tested whether this response was Zip1 dependent. Again, consistent with our notion, this was indeed the case. Wild-type and *pof1*-6 cells showed a peak of septation 2–4 h after cadmium exposure, while the percentage of septated cells in *zip1* mutants did not display a clear increase (Figure 7D). The biological significance of this remains unclear, but it is obviously a Zip1-dependent response. In summary, this analysis has unveiled the physiological role for Zip1 in cadmium stress, namely that Zip1 plays a key role in survival under adverse cadmium exposure.

Discussion

This report provides evidence that the F-box protein Pof1 is essential because of its ability to inactivate Zip1. This conclusion is drawn from the following results. Firstly, genetic data show that nonsense mutation or deletion of $zip1^+$ rescues the growth defects of *pof1* mutants. Secondly, $zip1^+$

Figure 6 The absence of Zip1 does not result in sulfur amino-acid auxotrophy. Wild-type, $zip1^+$-deleted and *met5-1* mutant cells were streaked on minimal medium whose sulfur sources are provided from only Na_2SO_4 (middle) or methionine (right) and incubated at 26°C for 5 days. Some growth of *met5-1* in the presence of Na_2SO_4 is most likely ascribable to the existence of residual methionine pool inside *met5-1* cells.

overexpression is toxic in wild-type cells, which mimic the phenotypes of *pof1* mutants at the restrictive temperature. Thirdly, Zip1 is stabilized in a *pof1* mutant and Zip1 itself is ubiquitylated *in vivo*. Finally, Pof1 binds to phosphorylated forms of Zip1 in the cell.

Pof1 is homologous to the essential budding yeast F-box protein Met30, known to regulate the Met4 transcription factor (Kaiser *et al*, 2000; Patton *et al*, 2000; Rouillon *et al*, 2000; Kuras *et al*, 2002; Flick *et al*, 2004). Met4 transcribes, among others, genes of the *MET* network required for the biosynthesis of methionine (Patton *et al*, 2000; Rouillon *et al*, 2000). It has been shown that Met30 downregulates Met4 activity in response to extracellular methionine or intracellular *S*-adenosylmethionine levels (Kuras and Thomas, 1995; Thomas *et al*, 1995; Kaiser *et al*, 2000; Rouillon *et al*, 2000) and this is achieved through both proteolytic and nonproteolytic mechanisms (Kaiser *et al*, 2000; Rouillon *et al*, 2000; Kuras *et al*, 2002). Our observations, however, suggest that only proteolytic mechanism may be shared in fission yeast. Firstly, *pof1-6* mutants clearly have increased levels of Zip1. Secondly, Zip1 is a protein of short half-life (<15 min) in wild-type cells and displays an increased stability in *pof1* mutants. Thirdly, unlike Met4 (Flick *et al*, 2004), no monoubiquitylated form of Zip1 has been found. Finally, *pof1* mutants are lethal when combined with a proteasome mutation. All of these data suggest that Pof1 is responsible for the degradation of Zip1.

The differences in regulation of Met4 and Zip1 may be attributable to their different functions. As described above, Met4 is crucial for the production of sulfur amino acids when none are available in the environment, thus the *met4* mutant cannot grow without the addition of methionine exogenously to the growth media. However, in addition to the *MET* genes, Met4 is responsible for the regulation of many other genes; most importantly, Met4 has been shown, like Zip1, to be involved in the upregulation of GSH in response to cadmium stress (Dormer *et al*, 2000) and this appears to be regulated independently of the sulfate assimilation pathway (Wheeler *et al*, 2002). Zip1, on the other hand, plays no role in the sulfate assimilation pathway in terms of normal growth, as the *zip1* mutant can grow on media containing only the inorganic sulfur source. This would suggest that although both transcription factors are able to induce transcription of genes in the sulfate assimilation pathway, *S. cerevisiae* has evolved to rely upon Met4 for all sulfur amino-acid biosynthesis, whereas *S. pombe* uses Zip1 only to upregulate this pathway to make more glutathione in response to cadmium stress. Thus, *S. pombe* could be envisaged to use a simple system as described in Figure 8, where Zip1 is constantly made and degraded via SCF^{Pof1} and only stabilized Zip1 is capable of transcribing downstream genes in the presence of cadmium stress. This system allows Zip1 to be readily available – presumably cadmium was a commonly encountered stress for fission yeast – yet protects cells from the dangerous effects of Zip1 transcription, noticeably complete cell growth arrest. *S. cerevisiae*, on the other hand, cannot just switch Met4 completely on and off as it relies on Met4 for sulfur

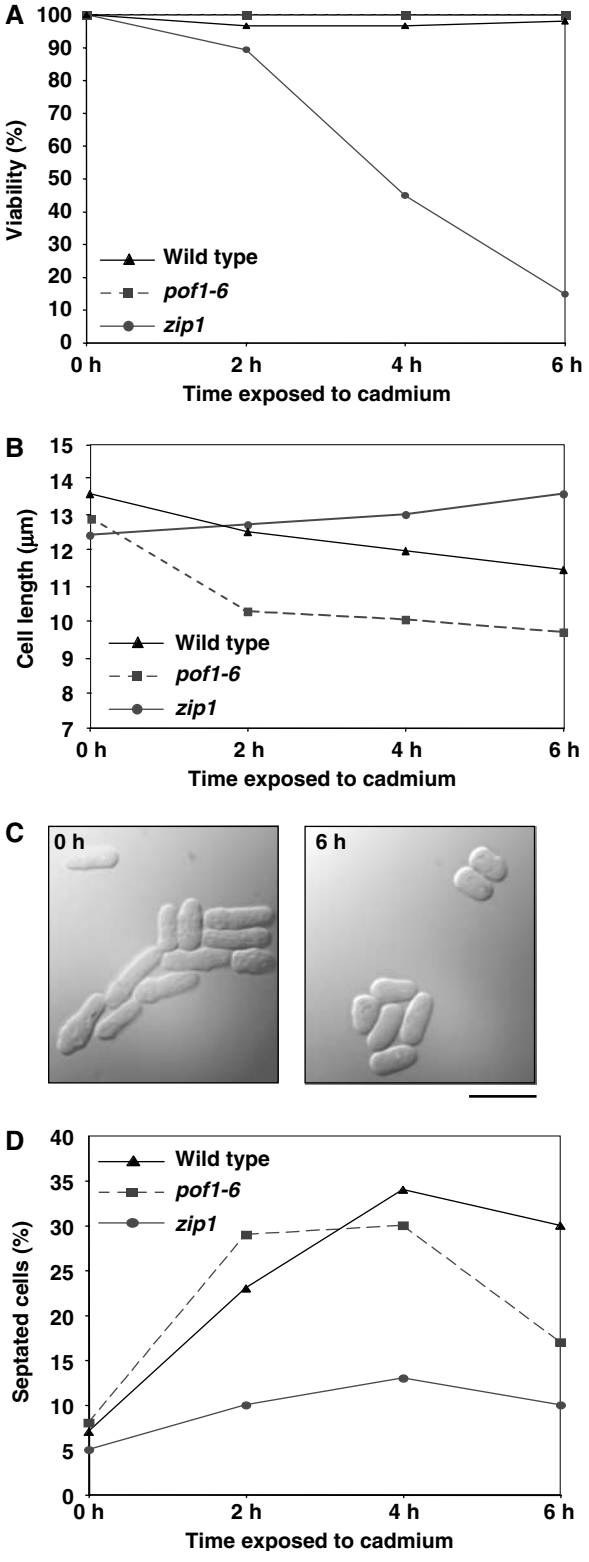

Figure 7 Zip1 is required for maintenance of viability during cadmium exposure. (**A**) Viability under cadmium stress. Wild-type (triangles), *pof1-6* (squares) or *zip1*-deleted cells (circles) were treated with 0.5 mM cadmium sulfate at 26°C and cell viability was examined at every 2 h interval. At each time point, 2 × 200 cells were plated onto rich medium (lacking cadmium) and incubated at 26°C for 5 days. The number of viable colonies was counted and viability was calculated by dividing this number by 200 and calculating an average. (**B**) Cell size change upon cadmium exposure. The cell size was measured and plotted ($n = 100$ per time point). (**C**) Cell morphology under cadmium stress. Phase-contrast micrographs of wild-type cells before (left) or after cadmium treatment (6 h, right) are shown. The bar indicates 10 μm. (**D**) Percentage of septated cells.

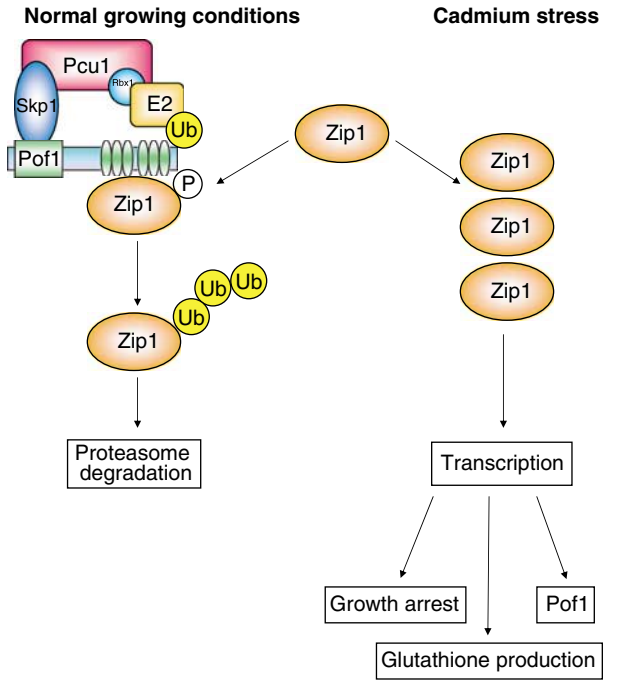

Figure 8 A model for the function and regulation of Zip1. Zip1 is constitutively produced, but its cellular levels are kept low under normal, noncadmium, growing conditions (left). This is achieved by constitutive SCF^{Pof1}-dependent ubiquitylation and proteasome-mediated degradation. Binding between Pof1 and Zip1 is dependent upon Zip1 phosphorylation. Under cadmium stress, some switch triggers an increase in Zip1 protein levels and its transcriptional activity. This results in an increased production of glutathione coupled with an arrest of cell growth, thereby ensuring high cell viability during cadmium exposure. The mechanism of this switch is unknown, but the accumulation of phosphorylated Zip1 species during cadmium exposure suggests that the phosphorylation of Zip1 could play some role in transcriptional activity.

amino-acid biosynthesis, and so has evolved a more complex level of regulation involving ubiquitylation without degradation.

How the cell regulates Zip1 activity in response to cadmium remains unknown. We have shown that Zip1 protein levels increase under cadmium stress without transcriptional stimulation and it is, therefore, tempting to speculate that Pof1-dependent degradation of Zip1 is switched off under this condition. Pof1 interacts with a phosphorylated form of Zip1, so the kinase responsible for this phosphorylation would be a candidate effector of this molecular switch. However, the data suggest the mechanism of Zip1 regulation is more complex, as a phosphorylated form of Zip1 appears to accumulate in cadmium-exposed cells. It may be that this form is capable of, or even required for, Zip1 transcriptional activity.

At the moment, it remains to be determined whether slower migrating phosphorylated forms of Zip1 in *pof1-6* are identical to those seen in *mts3-1* or wild-type cells exposed to cadmium. What is clear, on the other hand, is that the slowest form in *pof1-6* (band 3) is still unstable. This phosphorylated form might either be degraded by some pathway other than SCF^{Pof1} or be easily dephosphorylated. It is also possible that Zip1 phosphorylation under cadmium stress might be performed by a kinase different from that phosphorylating Zip1 under normally growing conditions or *pof1-6* mutant background. One such candidate kinase includes the Sty1/Spc1 stress-activated protein kinase (Toone and Jones, 1998). In any case, Pof1 is required for Zip1 ubiquitylation and degradation under normal growing conditions, but it should be noted that in the presence of cadmium stress, Zip1 levels might be regulated in a different manner.

The most interesting aspect of the Zip1 transcription factor is its ability to induce growth arrest. This is seen in *pof1* mutants, which arrest as small cells in a Zip1-dependent manner, and also when $zip1^+$ is overexpressed. This growth arrest does inhibit cell division, but is not lethal to the cells, as *pof1* mutants grown at the restrictive temperature still retain high viability even after 8 h (C Harrison and T Toda, unpublished observations). The growth arrest seen in both wild-type cells treated with cadmium stress and *pof1* mutants at the restrictive temperature is likely to be caused by the same mechanism, as in either case, Zip1 accumulates and the arrest is Zip1 dependent. We envisage two possibilities for the mechanisms underlying Zip1-dependent growth arrest. One is that genes, which encode growth inhibitor-type molecules, are induced in a Zip1-dependent manner. The other possibility is that growth arrest is derived from intracellular sulfur deficiency. In particular, the *pof1-6* small cell phenotype is remarkably similar to that of spermidine-deprived *S. pombe* cells (Chattopadhyay *et al*, 2002). Spermidine is synthesized via the sulfur assimilation pathway, and absolutely required for eukaryotic cell growth (Tabor and Tabor, 1984). These two possibilities are currently under investigation.

Cadmium has a biological half-life of 10–30 years and one of the most serious environmental pollutants (Hengstler *et al*, 2003). Given that many cells in the body will be exposed to cadmium, it is likely that higher organisms also have some conserved aspects of cadmium stress response seen in lower eukaryotes. Consistent with this notion, the mammalian bZIP Nrf2 protein has been shown to be an important transcription factor for cadmium response and its overexpression leads to upregulation of genes involved in glutathione production in certain cell types (Hayes *et al*, 2000). Interestingly, Nrf2 is an extremely labile protein and is degraded by the ubiquitin–proteasome system, while in the presence of cadmium it becomes stabilized (Stewart *et al*, 2003). This is an identical mechanism to that which seems to exist to control Zip1 activity in fission yeast. Recent reports indicate that Nrf2 is degraded via the SCF-related Cul3 pathway (Cullinan *et al*, 2004; Kobayashi *et al*, 2004), suggesting that Zip1 and Nrf2 systems are evolutionarily interconnected.

Oncoprotein transcription factors such as c-Jun and c-Myc have recently been shown to be degraded via SCF (Cardozo and Pagano, 2004). Here, we describe how a growth arrest mechanism can be coupled to cadmium stress response through a bZIP transcription factor. This mechanism should be conserved through evolution and its elucidation is of great significance for our understanding of eukaryotic growth control upon environmental stresses in general.

Materials and methods

Strains used in this study are listed in Table IV. See Supplementary data for Materials and methods.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Table IV Strains used in this study

Strain name	Genotype	Derivation
22	*h*$^-$ *leu1*	Our stock
TP108-3C	*h*$^-$ *leu1 ura4 pap1*::*ura4*$^+$	Our stock
wee1	*h*$^+$ *wee1-50*	Paul Nurse
met5	*h*$^+$ *met5-1*	Paul Nurse
SKP407	*h*$^-$ *leu1 ura4 pof1*$^+$*-GST-kan*^{r}	This study
SKP414-17	*h*$^-$ *leu1 ura4 pcu1*$^+$*-13myc-kan*^{r}	Our stock
CLP30	*h*$^-$ *leu1 pof1*$^+$*-GFP-kan*^{r}	This study
CLP30-6	*h*$^-$ *leu1 pof1-6-GFP-kan*^{r}	This study
CLP30-12	*h*$^-$ *leu1 pof1-12-GFP-kan*^{r}	This study
CLP41	*h*$^-$ *pof1-6-GFP-kan*^{R} *sup9*	This study
CLP32	*h*$^-$ *pof1-6-GFP-kan*^{R}	This study
CLP52-1	*h*$^-$ *sup9*	This study
CLP064-1	*h*$^+$ *leu1 ura4 his2 zip1*::*kan*^{r}	This study
CLP066-1	*h*$^+$ *leu1 his2 ura4 pof1-6-GFP-kan*^{r} *zip1*::*kan*^{r}	This study
CLP057-2A	*h*$^-$ *leu1 mts3-1 zip1-3HA-kan*^{r} *pof1-6-GFP-kan*^{r}	This study
CLP053-5	*h*$^-$ *leu1 ura4 pof1-6-GFP-kan*^{r} *zip1*$^+$*-3HA- kan*^{r}	This study
CLP054-5	*h*$^+$ *leu1 ura4 his2 zip1*$^+$*-3HA- kan*^{r}	This study
CLP056-5C	*h*$^-$ *leu1 mts3-1 zip1*$^+$*-3HA-kan*^{r}	This study

Acknowledgements

We thank Drs Paul Nurse, Hiroaki Seino and Minoru Yoshida for providing strains and materials used in this study. We thank Dr Yasmine Mamnun for useful discussion and suggestions, and Dr Axel Behrens for critical reading of the manuscript. DC was supported by the EMF Biological Research Trust. This work was supported by the Cancer Research UK (to JB, NJ and TT) and the HFSP research grant (to TT).

References

Bai C, Sen P, Hofmann K, Ma L, Goebl M, Harper JW, Elledge SJ (1996) *SKP1* connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86:** 263–274

Basi G, Schmid E, Maundrell K (1993) TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcriptional efficiency but not the transcription start point or thiamine repressibility. *Gene* **123:** 131–136

Cardozo T, Pagano M (2004) The SCF ubiquitin ligase: insights into a molecular machine. *Nat Rev Mol Cell Biol* **5:** 739–751

Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA (2001) Remodeling of yeast genome expression in response to environmental changes. *Mol Biol Cell* **12:** 323–337

Chattopadhyay MK, Tabor CW, Tabor H (2002) Absolute requirement of spermidine for growth and cell cycle progression of fission yeast (*Schizosaccharomyces pombe*). *Proc Nat Acad Sci USA* **99:** 10330–10334

Chen D, Toone WM, Mata J, Lyne R, Burns G, Kivinen K, Brazma A, Jones N, Bahler J (2003) Global transcriptional responses of fission yeast to environmental stress. *Mol Cell Biol* **14:** 214–229

Clemens S, Simm C (2003) *Schizosaccharomyces pombe* as a model for metal homeostasis in plant cells: the phytochelatin-dependent pathway is the main cadmium detoxification mechanism. *New Phytol* **159:** 323–330

Cullinan SB, Gordan JD, Jin J, Harper W, Diehl JA (2004) The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. *Mol Cell Biol* **24:** 8477–8486

Dormer UH, Westwater J, McLaren NF, Kent NA, Mellor J, Jamieson DJ (2000) Cadmium-inducible expression of the yeast *GSH1* gene requires a functional sulfur-amino acid regulatory network. *J Biol Chem* **275:** 32611–32616

Fauchon M, Lagniel G, Aude J-C, Lombardia L, Soularue P, Petat C, Marguerie G, Sentenac A, Werner M, Labarre J (2002) Sulfur sparing in the yeast proteome in response to sulfur demand. *Mol Cell* **9:** 713–723

Feldman RMR, Correll CC, Kaplan KB, Deshaies RJ (1997) A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* **91:** 221–230

Flick K, Ouni I, Wohlschlegel JA, Capati C, McDonald WH, Yates III JR, Kaiser P (2004) Proteolysis-independent regulation of the transcription factor Met4 by a single Lys 48-linked ubiquitin chain. *Nat Cell Biol* **6:** 634–641

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol Cell Biol* **11:** 4241–4257

Hartwell LH, Weinert TA (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science* **246:** 629–634

Hayes JD, Chanas SA, Henderson CJ, McMahon M, Sun C, Moffat GJ, Wolf CR, Yamamoto M (2000) The Nrf2 transcription factor contributes both to the basal expression of glutathione *S*-transferases in mouse liver and to their induction by the chemopreventive synthetic antioxidants, butylated hydroxyanisole and ethoxyquin. *Biochem Soc Trans* **28:** 33–41

Hengstler JG, Bolm-Audorff U, Faldum A, Janssen K, Reifenrath M, Gotte W, Jung D, Mayer-Popken O, Fuchs J, Gebhard S, Bienfait HG, Schlink K, Dietrich C, Faust D, Epe B, Oesch F (2003) Occupational exposure to heavy metals: DNA damage induction and DNA repair inhibition prove co-exposures to cadmium, cobalt and lead as more dangerous than hitherto expected. *Carcinogenesis* **24:** 63–73

Hermand D, Bamps S, Tafforeau L, Vandenhaute J, Makela TP (2003) Skp1 and the F-box protein Pof6 are essential for cell separation in fission yeast. *J Biol Chem* **278:** 9671–9677

Hershko A, Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* **67:** 425–479

Hochstrasser M (1996) Ubiquitin-dependent protein degradation. *Annu Rev Genet* **30:** 405–439

Ikebe C, Kominami K, Toda T, Nakayama K (2002) Isolation and characterization of a novel F-box protein Pof10 in fission yeast. *Biochem Biophys Res Commun* **290:** 1399–1407

Kaiser P, Flick K, Wittenberg C, Reed SI (2000) Regulation of transcription by ubiquitination without proteolysis: Cdc34/SCF^{Met30}-mediated inactivation of the transcription factor Met4. *Cell* **102:** 303–314

Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, Yamamoto M (2004) Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol Cell Biol* **24:** 7130–7139

Kominami K, Ochotorena I, Toda T (1998) Two F-box/WD-repeat proteins Pop1 and Pop2 form hetero- and homo-complexes together with cullin-1 in the fission yeast SCF (Skp1-Cullin-1-F-box) ubiquitin ligase. *Genes Cells* **3:** 721–735

Kuras L, Rouillon A, Lee T, Barbey R, Tyers M, Thomas D (2002) Dual regulation of the Met4 transcription factor by ubiquitin-dependent degradation and inhibition of promoter recruitment. *Mol Cell* **10:** 69–80

Kuras L, Thomas D (1995) Functional analysis of Met4, a yeast transcriptional activator responsive to *S*-adenosylmethionione. *Mol Cell Biol* **15:** 208–216

Lehmann A, Katayama S, Harrison C, Dhut S, Kitamura K, McDonald N, Toda T (2004) Molecular interactions of fission yeast Skp1 and its role in the DNA damage checkpoint. *Genes Cells* **9:** 367–382

Marzluf GA (1997) Molecular genetics of sulfur assimilation in filamentous fungi and yeast. *Annu Rev Microbiol* **51:** 73–96

Nash P, Tang X, Orlicky S, Chen Q, Gertler FB, Mendenhall MD, Sicheri F, Pawson T, Tyers M (2001) Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. *Nature* **414:** 514–521

Nurse P (1990) Universal control mechanism regulating onset of M-phase. *Nature (Lond.)* **344:** 503–508

Ohmiya R, Kato C, Yamada H, Aiba H, Mizuno T (1999) Isolation of multicopy suppressors of the calcium sensitivity of a mutant lacking the bZIP transcription factor Atf1 in fission yeast. *Mol Gen Genet* **261:** 297–306

Pahl HL, Baeuerle PA (1996) Control of gene expression by proteolysis. *Curr Opin Cell Biol* **8:** 340–347

Patton EE, Peyraud C, Rouillon A, Surdin-Kerjan Y, Tyers M, Thomas D (2000) SCF^{Met30}-mediated control of the transcriptional activator Met4 is required for the G1-S transition. *EMBO J* **19:** 1613–1624

Patton EE, Willems AR, Tyers M (1998) Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. *Trends Genet* **14:** 236–243

Rouillon A, Barbey R, Patton EE, Tyers M, Thomas D (2000) Feedback-regulated degradation of the transcriptional activator Met4 is triggered by the SCF^{Met30} complex. *EMBO J* **19:** 282–294

Seeger M, Gordon C, Ferrell K, Dubiel W (1996) Characteristics of 26 S proteasomes from fission yeast mutants, which arrest in mitosis. *J Mol Biol* **263:** 423–431

Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW (1997) F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin–ligase complex. *Cell* **91:** 209–219

Stewart D, Killeen E, Naquin R, Alam S, Alam J (2003) Degradation of transcription factor Nrf2 via the ubiquitin–proteosome pathway and stabilization by cadmium. *J Biol Chem* **278:** 2396–2402

Tabor CW, Tabor H (1984) Polyamines. *Annu Rev Biochem* **53:** 749–790

Thomas D, Kuras L, Barbey R, Cherest H, Blaiseau P-L, Surdin-Kerjan Y (1995) Met30p, a yeast transcriptional inhibitor that responds to *S*-adenosylmethionine, is an essential protein with WD40 repeats. *Mol Cell Biol* **15:** 6526–6534

Toone WM, Jones N (1998) Stress-activated signalling pathways in yeast. *Genes Cells* **3:** 485–498

Wheeler GL, Quinn KA, Perrone G, Dawes IW, Grant CM (2002) Glutathione regulates the expression of γ-glutamycysteine synthetase via the Met4 transcription factor. *Mol Micobiol* **46:** 545–556

