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Inducible dissociation of SCF^{Met30} ubiquitin ligase mediates a rapid transcriptional response to cadmium

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Activity of the Met4 transcription factor is antagonized by the SCF^{Met30} ubiquitin ligase by degradation-dependent and degradation-independent mechanisms, in minimal and rich nutrient conditions, respectively. In this study, we show that the heavy metal Cd^{2+} over-rides both mechanisms to enable rapid Met4-dependent induction of metabolic networks needed for production of the antioxidant and Cd^{2+}-chelating agent glutathione. Cd^{2+} inhibits SCF^{Met30} activity through rapid dissociation of the F-box protein Met30 from the holocomplex. In minimal medium, dissociation of SCF^{Met30} complex is sufficient to impair the methionine-induced degradation of Met4. In rich medium, dissociation of the SCF^{Met30} complex is accompanied by a deubiquitylation mechanism that rapidly removes inhibitory ubiquitin moieties from Met4. Post-translational control of SCF^{Met30} assembly by a physiological stress to allow rapid induction of a protective gene expression program represents a novel mode of regulation in the ubiquitin system.

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Introduction

The covalent modification of proteins by the small protein ubiquitin regulates myriad cellular processes (Hershko and Ciechanover, 1998). The best understood function of ubiquitylation is to target proteins for destruction by the 26S proteasome, a large compartmentalized protease particle that recognizes the polyubiquitin tag (Hochstrasser, 1996). More recently, nonproteolytic functions have been ascribed to ubiquitin conjugation, including targeting to different subcellular compartments and allosteric control of enzymatic events (Pickart, 2001). How ubiquitylation leads to different outcomes in different contexts is not fully understood.

Conjugation of ubiquitin to substrate proteins is achieved through a cascade of E1, E2 and E3 enzymes, which activate and then serially transfer ubiquitin to substrates (Hershko and Ciechanover, 1998). E3 enzymes, also called ubiquitin ligases, select specific substrates for ubiquitylation. The SCF (Skp1–Cdc53/cullin–F-box protein) and SCF-like complexes form a diverse family of ubiquitin ligases that regulate the cell cycle, the immune response, signaling cascades and developmental programs (Willems *et al*, 2004). SCF complexes recruit substrates through a repertoire of specific adapter subunits, termed F-box proteins, which are linked to a core ubiquitylation apparatus composed of the scaffold protein Cdc53/cullin, the RING finger protein Rbx1/Hrt1/Roc1, the F-box-binding protein Skp1 and an E2 enzyme (Skowyra *et al*, 1999; Willems *et al*, 2004). In budding yeast, over 20 different F-box proteins can be identified, but only three of these have been studied in detail, namely Cdc4, Grr1 and Met30 (Patton *et al*, 1998). SCF^{Cdc4} and SCF^{Grr1} each target multiple substrates in a manner that depends on substrate phosphorylation (Willems *et al*, 2004). To date, the only known substrate of the SCF^{Met30} complex is the Met4 transcriptional activator, which governs the *MET* gene network responsible for the biosynthesis of the sulfur-containing amino acids methionine and cysteine (Patton *et al*, 1998; Rouillon *et al*, 2000; McMillan *et al*, 2002). While SCF^{Met30} may target substrates other than Met4, for instance in the regulation of interorganelle phosphatidylserine trafficking (Schumacher *et al*, 2002), restraint of the Met4-dependent transcriptional activity is the only essential function of Met30 (Patton *et al*, 2000).

Ubiquitylation controls the *MET* gene network by both degradation-dependent and degradation-independent mechanisms. When yeast cells are grown in minimal medium and exposed to a high concentration of methionine, SCF^{Met30}-dependent ubiquitylation targets Met4 for degradation by the 26S proteasome (Rouillon *et al*, 2000). In contrast, when the cells are grown in rich medium, SCF^{Met30} oligoubiquitylates Met4 but without subsequent degradation (Kaiser *et al*, 2000; Kuras *et al*, 2002; Flick *et al*, 2004). In this circumstance, Met4 remains localized within the nucleus but is selectively excluded from most but not all *MET* gene promoters (Kuras *et al*, 2002). In particular, oligoubiquitylated Met4 retains activity at the *SAM* genes needed for the biosynthesis of *S*-adenosylmethionine (Kuras *et al*, 2002), a sulfur-containing compound widely used in intermediary metabolism as a methyl donor (Cantoni, 1977). The Met4–Met30 system thus enables selective activation of different promoters under different growth conditions. How nutrient conditions dictate the fate of ubiquitylated Met4 is not understood.

In addition to its regulation by methionine and by rich medium, control of Met4 activity was recently hypothesized to be modulated by other environmental changes (Fauchon

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et al, 2002; Aranda and Del Olmo, 2004). More especially, Met4 appears to mediate the adaptive response to cadmium (Cd^{2+}), a nonessential heavy metal that is highly toxic to eukaryotic cells (Fauchon *et al*, 2002). The stress response to Cd^{2+}, like that of many agents that induce oxidative stress, involves activation of sulfur metabolism pathways that generate compensating reducing equivalents (Jamieson, 2002; Pocsi *et al*, 2004). In yeast, Cd^{2+} activates the *MET* gene network in a Met4-dependent fashion, presumably to help build glutathione reserves necessary to complex and detoxify Cd^{2+} (Fauchon *et al*, 2002). Given this link, we thus examined if and how Cd^{2+} exposure might modify SCF^{Met30}-dependent regulation of Met4. We find that Cd^{2+} impairs both degradation-dependent and degradation-independent inhibition of Met4 by dissociation of Met30 from the core SCF complex. Further, in rich medium, Cd^{2+} elicits deubiquitylation of pre-existing pools of inactive oligoubiquitylated Met4. The Cd^{2+} adaptive response thus entails novel modes of transcriptional regulation by the ubiquitin system.

Results

Cd^{2+} inhibits degradation-dependent regulation of Met4 activity

To address how Cd^{2+} might affect the *MET* gene regulatory network, we first analyzed *MET* gene transcription in cells grown in minimal B medium and simultaneously exposed to methionine (1 mM) and Cd^{2+} (100 μM). The presence of Cd^{2+} compromised *MET* gene repression normally triggered by high methionine, as shown by the failure to repress *MET3*, *MET16* and *MET25* expression (Figure 1A). Cd^{2+} also blocked repression of *GSH1*, a Met4-dependent gene that encodes γ-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione biosynthesis from cysteine (Dormer *et al*, 2000; Wheeler *et al*, 2003). In all cases, the Cd^{2+} chelating agent EGTA prevented derepression of Met4-dependent genes by Cd^{2+}.

In minimal medium, Met4 is actively degraded upon exposure to high methionine (Kuras *et al*, 2002). To test whether the Cd^{2+}-mediated abrogation of *MET* gene repression might be due to Met4 stabilization, the stability of Met4 was analyzed in the presence of both methionine and Cd^{2+}. The rapid SCF^{Met30}-dependent elimination of Met4 can be measured in live cells by using a GFP-Met4 fusion protein expressed from the endogenous Met4 promoter (Kuras *et al*, 2002). Concurrent addition of Cd^{2+} and methionine to minimal medium prevented the disappearance of the GFP-Met4 fluorescent signal observed when methionine alone was added (Figure 1B). To corroborate this result, we determined the stability of an HA epitope-tagged version of Met4 expressed from the chromosomal locus. Consistently, the ^{HA3}Met4 protein abundance was maintained when Cd^{2+} was added together with methionine to cells grown in minimal medium (Figure 1C), whereas in the absence of Cd^{2+}, the ^{HA3}Met4 protein was rapidly degraded upon methionine exposure.

Chromatin immunoprecipitation (ChIP) was used to assess the abundance of Met4 at individual promoters in cells exposed to Cd^{2+}. Association of the general transcription factor TFIIB was analyzed in parallel to determine the correlation between the presence of Met4 and transcriptional activity. For this purpose, we used a strain in which the *MET4* and *SUA7* genes (encoding TFIIB) were replaced at the chromosomal locus with epitope-tagged derivatives under control of the endogenous promoters (*met4*::^{HA3}*MET4* and *sua7*::*SUA7*^{MYC9}, respectively). Crosslinked chromatin was prepared from cells grown in minimal B medium before and 40 min after the addition of 1 mM methionine, in the presence and absence of 100 μM Cd^{2+}. Addition of Cd^{2+} prevented the four- to 25-fold decrease in both Met4 and TFIIB occupancy at *MET* gene promoters caused by addition of repressive amounts of methionine in the absence of Cd^{2+} (Figure 1D). Taken together, these results demonstrate that Cd^{2+} impairs SCF^{Met30}-mediated degradation of Met4 that is normally induced by methionine in minimal medium.

Cd^{2+}-induced Met4 DNA recruitment in complete medium

In rich medium, Met4 is oligoubiquitylated by SCF^{Met30} but not degraded (Kaiser *et al*, 2000). Under these conditions, *MET* gene expression is repressed as ubiquitylated forms of Met4 are selectively excluded from most *MET* promoters (Kuras *et al*, 2002). To determine if Cd^{2+} affected this alternate mode of Met4 regulation, cells were grown in rich medium, exposed to Cd^{2+} and assessed for *MET* gene transcription. We note that in rich medium, a higher concentration of Cd^{2+} (0.5 mM) was needed to elicit a biological effect, perhaps because of sequestration by unknown components in this medium. We found that transcription of the *MET3, 10, 14, 16* and *25* genes was rapidly activated upon addition of 0.5 mM Cd^{2+} (Figure 2A). As expected, this activation was dependent upon a functional Met4 protein, as it was not observed in *met4*Δ cells (Figure 2B). Consistent with the observed transcriptional re-activation, Met4 occupancy at *MET* gene promoters was increased at least five-fold in cells grown in rich medium in the presence of Cd^{2+}, as compared to cells grown in the absence of Cd^{2+} (Figure 2C). The degree of TFIIB occupancy at these promoters was similarly increased by the presence of Cd^{2+}, in agreement with the high-level expression of *MET* genes under these

Figure 1 Cd^{2+} inhibits the degradation-dependent regulation of Met4. (**A**) Cd^{2+} prevents *MET* gene repression by methionine in minimal medium. Total RNA was extracted at the indicated times after the addition of 1 mM L-methionine, in the presence (+Cd^{2+}) or absence of 100 μM Cd^{2+}, or in the presence of 100 μM of both Cd^{2+} and EGTA (+Cd^{2+}, +EGTA), and *MET* gene expression was assessed by Northern analysis. (**B**) Cd^{2+} blocks elimination of endogenous GFP-Met4 by methionine. *met4*::*GFP-MET4* cells (strain CD240) were imaged at the indicated times after the addition (+Met) or not (−Met) of 1 mM L-methionine, in the presence (+Cd^{2+}) or absence of 100 μM Cd^{2+}, or in the presence of 100 μM of both Cd^{2+} and EGTA (+Cd^{2+}, +EGTA). (**C**) Cd^{2+} stabilizes endogenous Met4 protein. *met4*::^{HA3}*MET4* cells (strain CD233) were treated with (+Met) 1 mM L-methionine, in the presence (+Cd^{2+}) or absence of 100 μM Cd^{2+}, and total protein from TCA extracts was immunoblotted for the HA epitope or, as a control, lysyl-tRNA synthetase. (**D**) Met4p and TFIIB occupancy at *MET* promoters is elevated in response to Cd^{2+} exposure. A strain expressing both ^{HA3}Met4 and TFIIB^{9MYC} (CD269) or an untagged isogenic control strain (W303-1A) were grown in B minimal medium and exposed to 1 mM L-methionine, in the presence or absence of 100 μM Cd^{2+}. After crosslinking and immunoprecipitation with anti-HA or anti-Myc antibody, total DNA was analyzed by quantitative PCR with primer-pairs specific to the indicated Pol II promoters. ChIPs were performed at least twice and yielded similar results.

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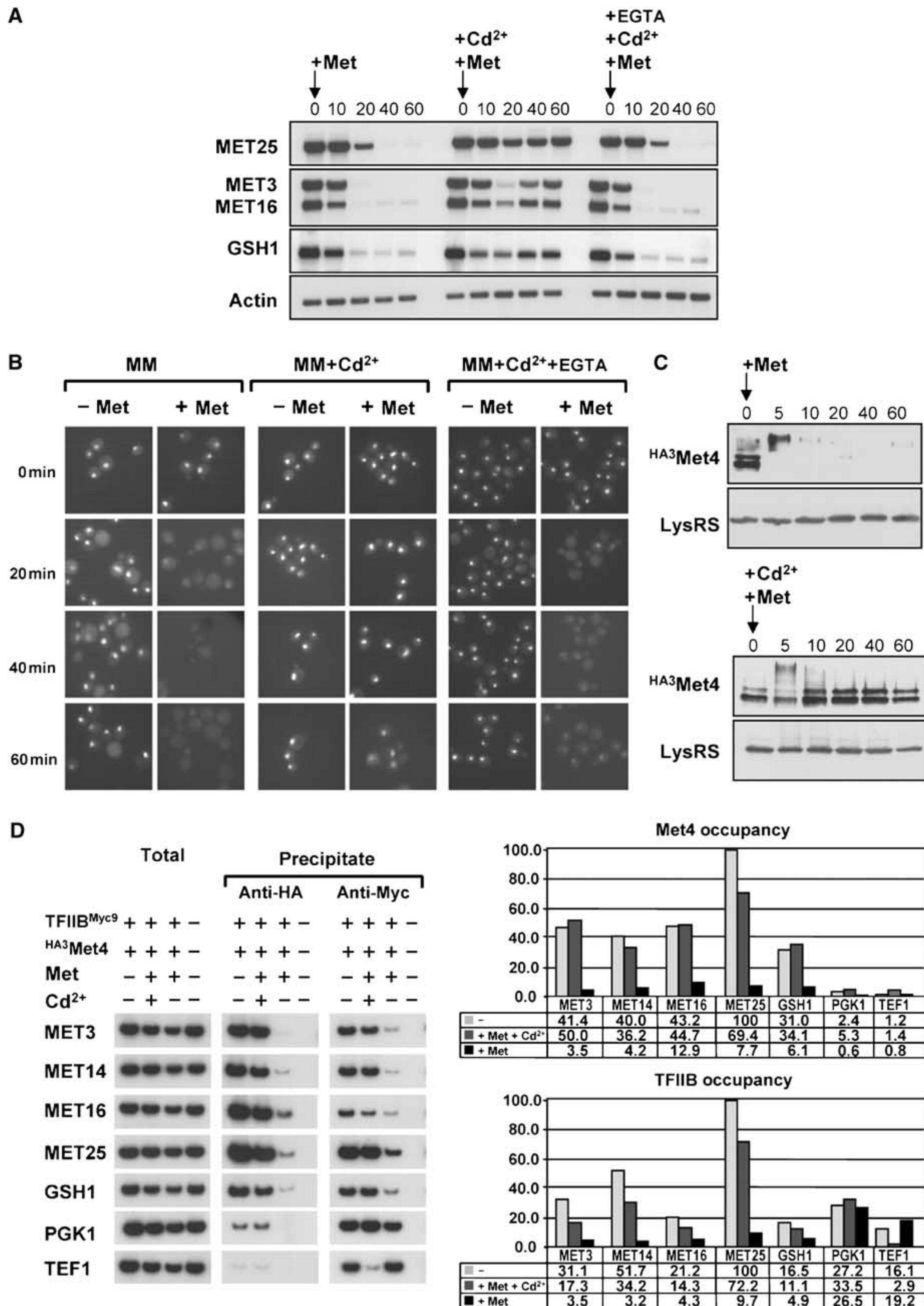
conditions. Finally, the specificity of the Cd^{2+} effect on Met4 activity was assessed by exposing cells grown in rich medium to other heavy metals including cobalt, copper, manganese, mercury, silver and zinc. Northern and quantitative real-time (RT)-PCR assays showed that in contrast to Cd^{2+}, these other heavy metals did not significantly activate *MET* gene expression in rich medium (Figure 2D). Cd^{2+} thus specifically reprieves Met4 from inactivation.



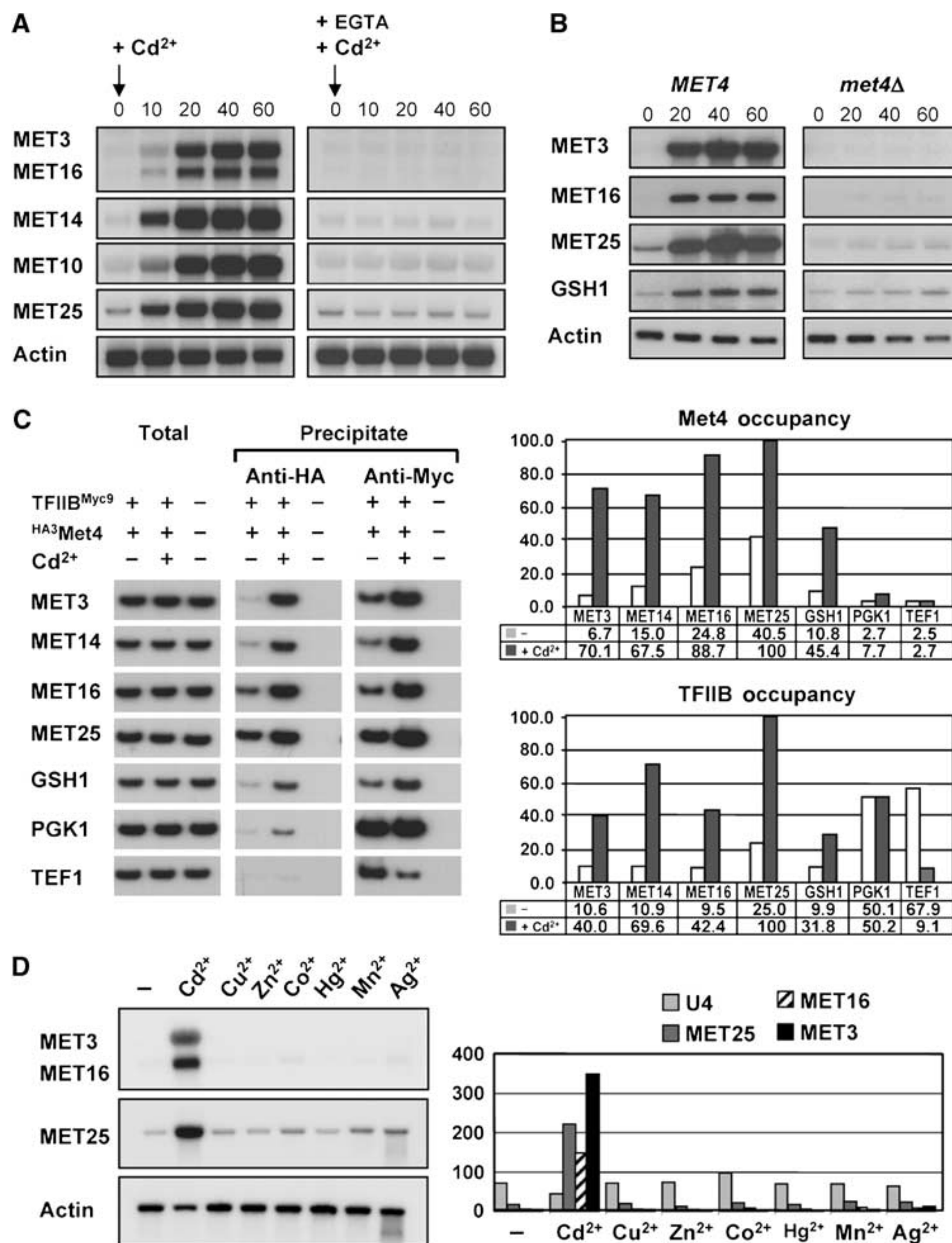


Figure 2 Cd^{2+} activates *MET* gene expression in rich medium. (**A**) Rapid induction of *MET* genes by Cd^{2+}. *MET* gene expression in wild-type cells was assessed at the indicated times after the addition of 0.5 mM Cd^{2+} in the absence or presence of 0.5 mM EGTA. (**B**) Cd^{2+}-mediated activation of *MET* genes depends on Met4. *MET* gene expression was assessed in wild-type and *met4*Δ (CC849-8A) cells at the indicated times after the addition of 0.5 mM Cd^{2+}. (**C**) Met4 and TFIIB recruitment to *MET* gene promoters in response to Cd^{2+}. ChIP experiments were carried out as in Figure 1 on wild-type cultures before and 40 min after the addition of 0.5 mM Cd^{2+}. (**D**) Cd^{2+} specifically induces *MET* gene expression. *MET* gene expression was assessed in wild-type cells before and 40 min after addition of the indicated heavy metals by Northern analysis (left) and quantitative real-time RT–PCR (right). Heavy metals were added at the following concentrations: Cu^{2+} 1 mM, Zn^{2+} 1 mM, Co^{2+} 2 mM, Hg^{2+} 0.3 μM, Mn^{2+} 0.5 mM and Ag^{2+} 30 μM.

Cd^{2+} inhibits ubiquitylation of Met4

In rich medium, Met4 is stable but rendered inactive because of its oligoubiquitylation by SCF^{Met30} (Kaiser *et al*, 2000; Kuras *et al*, 2002). The above results suggested that Cd^{2+} might abrogate this effect. Indeed, the high-molecular-weight ubiquitylated forms of an HA epitope-tagged version of Met4 observed in rich medium were rapidly replaced by unmodified Met4 upon exposure to Cd^{2+} (Figure 3A). As expected, this collapse was prevented by concomitant addition of EGTA. After the initial collapse of the Met4 isoforms caused by Cd^{2+}, new higher molecular weight forms gradually appeared. To investigate the nature of these subsequent isoforms, endogenous Met4 was immunoprecipitated with an anti-Met4 polyclonal antibody, followed by immunoblot with either anti-Met4 or anti-ubiquitin antibodies. This experiment confirmed that while Met4 was oligoubiquitylated in rich medium, the secondary modified forms induced after Cd^{2+} addition were not due to ubiquitylation (Figure 3B). Rather, the Cd^{2+}-dependent modifications were due to phosphorylation, as shown by collapse to the lowest mobility form upon

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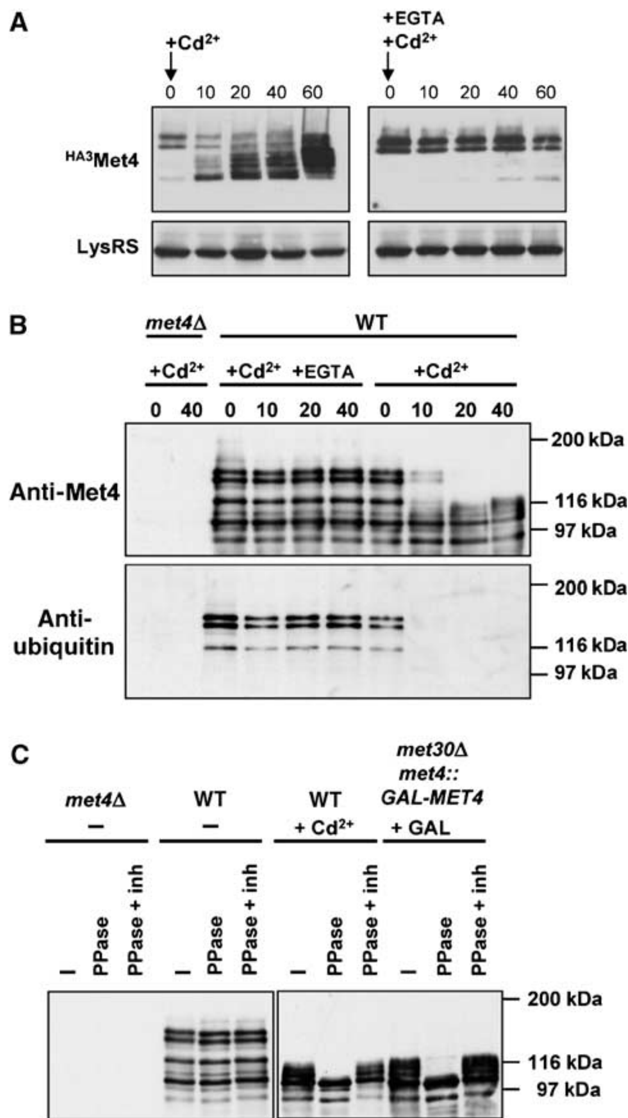

Figure 3 Cd^{2+} impairs SCF^{Met30}-dependent ubiquitylation of Met4 in rich medium. (**A**) Collapse of Met4 isoforms in response to Cd^{2+}. *met4*::^{HA3}*MET4* cells (strain CD233) were exposed to 0.5 mM Cd^{2+} in the absence or presence of 0.5 mM EGTA and extracted proteins analyzed for Met4 and lysyl-tRNA synthetase abundance by immunoblot. (**B**) Loss of ubiquitinated Met4 species upon Cd^{2+} treatment. Endogenous Met4 was immunoprecipitated with anti-Met4 antibody and then immunoblotted with anti-Met4 and anti-ubiquitin antibodies. (**C**) Accumulation of phosphorylated Met4 species in response to Cd^{2+} and in the absence of Met30. Wild-type, *met4*Δ and *met4*::*GAL1-MET4, met30*Δ strains were grown in rich medium containing 2% raffinose and harvested before and after 40 min of the indicated treatment with either 0.5 mM Cd^{2+} or galactose. Anti-Met4 immunoprecipitates were treated with lambda phosphatase in the presence or absence of phosphatase inhibitors and then immunoblotted with anti-Met4 antibody.

lambda phosphatase treatment (Figure 3C). Although the function of these phosphorylation events remains to be deciphered, similar phosphorylated Met4 species were observed in cells that lack Met30 when grown in rich medium in the absence of Cd^{2+} (Figure 3C), as reported recently (Flick *et al*, 2004). All told, these observations suggest that Cd^{2+} acts by inhibiting SCF^{Met30} activity.

Cd^{2+} induces dissociation of the SCF^{Met30} ubiquitin ligase

The above results suggest that SCF^{Met30} activity is a target of the Cd^{2+} signal, regardless of whether cells are grown in minimal medium and exposed to high methionine concentrations, or are grown in rich medium. One possible mechanism for Cd^{2+}-mediated inhibition of SCF^{Met30} might be the complete elimination of Met30 itself. However, a promoter shutoff experiment showed that while Met30 was a moderately unstable protein, as shown previously (Galan and Peter, 1999), the *in vivo* half-life of Met30 was unaffected by Cd^{2+} (Figure 4A). We next considered whether the interactions between Met4 and Met30 and/or the assembly of the SCF^{Met30} complex might be affected by Cd^{2+}. To test the former possibility, we overexpressed Met30 to facilitate detection of the Met4–Met30 interaction, which is constitutive but is normally limited by the low-level expression of *MET30* in minimal medium (Rouillon *et al*, 2000). In this context, Cd^{2+} treatment did not affect formation of the Met4–Met30 complex, in either minimal medium (Figure 4B) or in rich medium (data not shown). We therefore determined if the critical interaction between Met30 and the F-box-binding protein Skp1 was altered by Cd^{2+} treatment. As judged by co-immunoprecipitation from yeast extracts, the interaction between Skp1 and Met30 was abolished in cells exposed to Cd^{2+} (Figure 4C and D). This result was obtained with cells grown in either minimal or rich medium and by immunoprecipitating first either Met30 or Skp1 immune complexes. The loss of the interaction between Skp1 and Met30 was specific since another interaction within the SCF^{Met30} complex, that between Skp1 and Cdc53, was not altered by Cd^{2+} (Figure 4C and D). Thus, exposure of cells to Cd^{2+} cripples the ability of the SCF^{Met30} complex to ubiquitylate Met4 by disrupting the interaction between the substrate-binding subunit Met30 and the core catalytic apparatus.

As Skp1 recruits many other F-box-containing proteins to the SCF core apparatus, we tested the specificity of the Cd^{2+} effect by examining the assembly and activity of another yeast SCF complex, SCF^{Cdc4}, which mediates degradation of the CDK inhibitor Sic1 at the G1/S transition (Willems *et al*, 2004). Unlike the Skp1–Met30 complex, addition of Cd^{2+} to cells in rich medium did not disrupt the Skp1–Cdc4 complex (Figure 4E). If anything, the amount of Skp1 bound to Cdc4 was increased by Cd^{2+}, perhaps because more Skp1 was available upon its liberation from Met30. As a further control for specificity of the response, Cd^{2+} also did not affect abundance of the Cdc4 substrate Sic1 (Figure 4F). Moreover, quantitative flow cytometry of a GFP-Sic1 reporter construct showed that the kinetics of Sic1 degradation upon release from a G1 arrest imposed by mating pheromone was not impaired by Cd^{2+} (Figure 4G).

To begin to address the mechanism of Cd^{2+} action, we examined whether Cd^{2+} affected the activity of a recombinant SCF^{Met30} complex. For this purpose, an active SCF^{Met30} complex was affinity purified from insect cells that had been coinfected with Skp1, Cdc53, Rbx1 and Met30 baculovirus constructs. This SCF^{Met30} complex efficiently ubiquitylated recombinant Met4, which was also produced in insect cells (Figure 4H). Despite its potent effects *in vivo*, Cd^{2+} did not discernibly inhibit the ability of the recombinant complex to ubiquitylate Met4 *in vitro* (Figure 4H). Addition of crude cell extracts, from either untreated or Cd^{2+}-treated cultures, also

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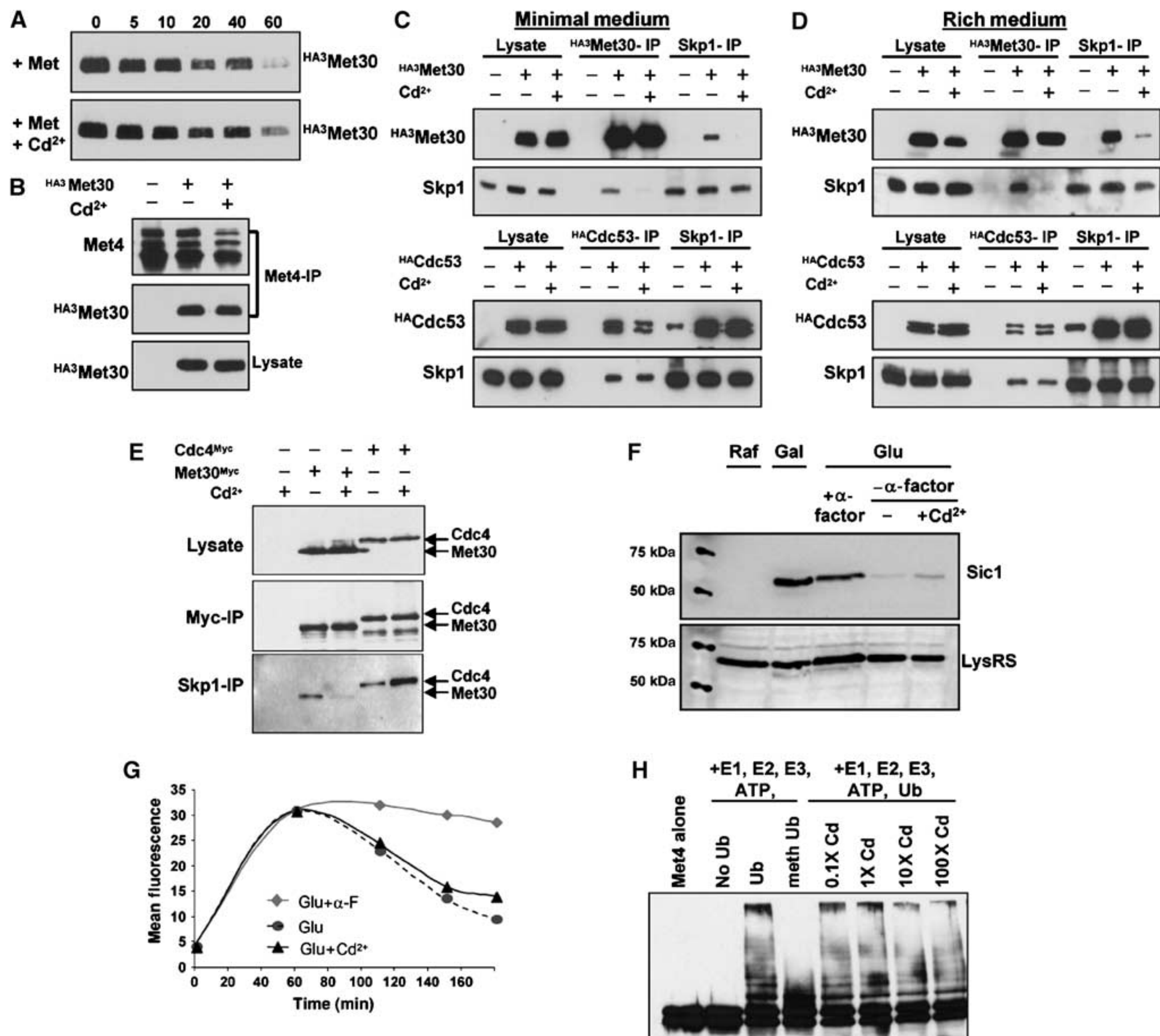

Figure 4 Cd^{2+} induces dissociation of the SCF^{Met30} ubiquitin ligase. (**A**) Met30 half-life is not altered by Cd^{2+} exposure. A strain bearing a *GAL1-^{HA3}MET30* construct was induced in galactose medium for 2 h, then transferred from galactose to 2% glucose- and 1 mM methionine-containing medium, in the presence or absence of 100 μM Cd^{2+}, and ^{HA3}Met30 abundance assessed by anti-HA immunoblot at the indicated times after glucose repression. (**B**) The Met4–Met30 interaction is unaffected by Cd^{2+}. An ^{HA3}Met30 fusion protein was expressed from a *GAL1* promoter in B medium containing 2% galactose in the presence or absence of 100 μM Cd^{2+}, Met4 immunoprecipitated from extracts with anti-Met4 antibody and immunoblotted with anti-Met4 and anti-HA antibodies. (**C**) The Skp1–Met30 interaction is specifically abolished by Cd^{2+} in minimal medium. ^{HA3}Met30 and ^{HA3}Cdc53 fusion proteins were expressed as in (B), extracts subjected to immunoprecipitation with the indicated antibodies and immunoblotted with anti-Skp1 and anti-HA antibodies. (**D**) The Skp1–Met30 interaction is specifically abolished upon Cd^{2+} exposure in rich medium. The experiments were performed as in (C) but cells were grown in rich medium and in the presence of 0.5 mM Cd^{2+}. (**E**) The Skp1–Cdc4 interaction is not abolished upon Cd^{2+} exposure in rich medium. Met30^{MYC3} and Cdc4^{MYC3} fusion proteins were expressed from the constitutive *CDC53* promoter (Ho *et al*, 2002) in rich medium in the presence or absence of 0.5 mM Cd^{2+} and protein interactions tested as in (C) with anti-MYC and anti-Skp1 antibodies. (**F**) Sic1 instability is not affected by Cd^{2+}. A strain (CYS37) expressing a GFP-Sic1 fusion protein from the *GAL1* promoter was grown in minimal medium, arrested in G1 phase by α-factor mating pheromone for 1.5 h, released into glucose medium in the presence and absence of 100 μM Cd^{2+}, with or without α-factor, and GFP-Sic1 abundance assessed 120 min after the promoter shutoff by immunoblot with anti-GFP or, as a control, anti-lysyl-tRNA synthetase (LysRS) antibodies. (**G**) Quantification by flow cytometry of GFP-Sic stability in either an α-factor block or after release from the block in the presence and absence of 100 μM Cd^{2+}. Cells were grown as described in (F) and the GFP-Sic1 fluorescent signal recorded and quantified at the indicated times using a Beckton Dickinson FacScalibur™ flow cytometer. (**H**) Met4 ubiquitylation by recombinant SCF^{Met30} is not affected by Cd^{2+}. Purified recombinant Met4 and SCF^{Met30} produced in insect cells were incubated with E1 enzyme, Cdc34, ubiquitin and ATP, either in the absence or presence of the indicated amounts of Cd^{2+}. Methyl-ubiquitin served as a control to demonstrate polyubiquitin chain formation on Met4.

did not impair the extent of Met4 ubiquitylation in the *in vitro* system (data not shown). SCF^{Met30} is thus specifically dissociated in response to Cd^{2+} in a manner that appears to require active cellular metabolism.

Cd^{2+} triggers Met4 deubiquitylation

In rich medium, highly ubiquitylated forms of Met4 are localized within the nucleus of the cells and are stable, being apparently immune to the proteasome (Kaiser *et al*,

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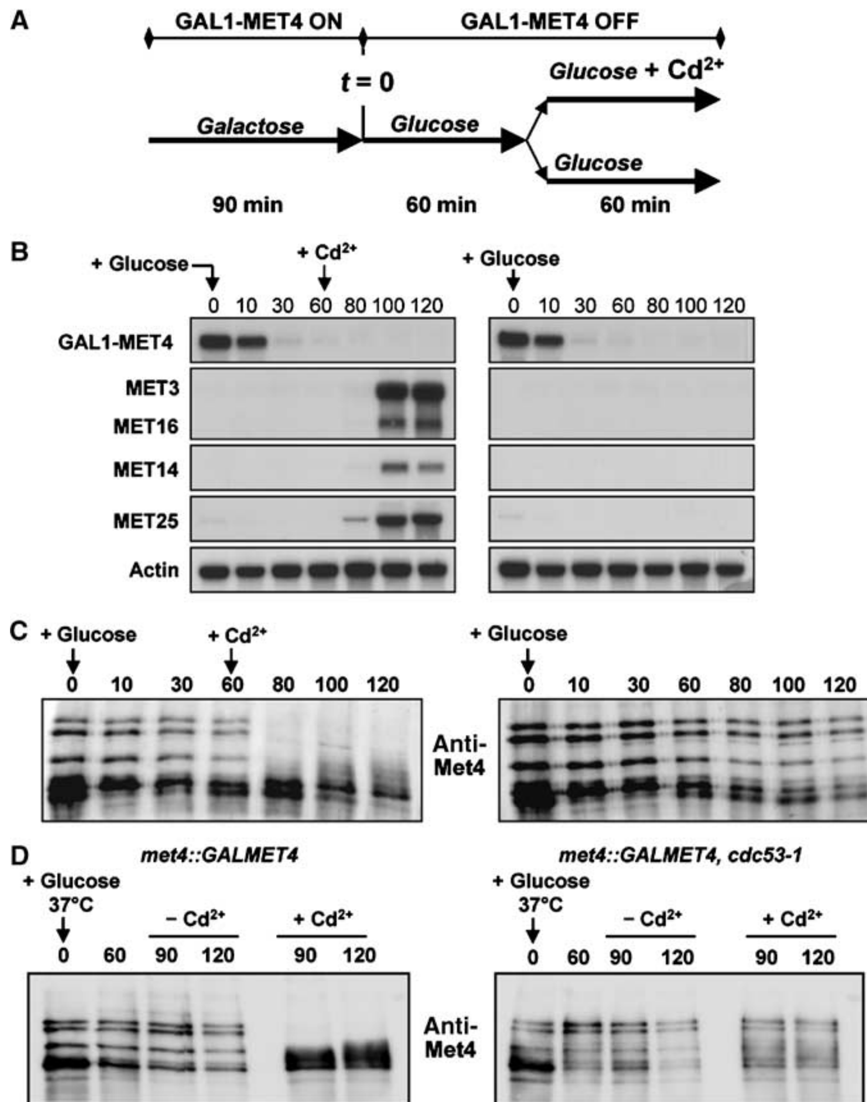

Figure 5 Cd^{2+} triggers deubiquitylation of Met4 in rich medium in the absence of *de novo* Met4 synthesis. (**A**) Schematic representation of the experiment. (**B**) Cd^{2+}-activated *MET* gene expression does not require *de novo* Met4 synthesis. A *met4*::*GAL1-MET4* strain (CC932-6D) was subjected to the regimen in (A) and assessed for *MET* gene expression at the indicated time points. (**C**) Cd^{2+} induces Met4 deubiquitylation. The same experiment as in (B) was performed except that Met4 was immunoprecipitated and immunoblotted with anti-Met4 antibody. (**D**) Inactivation of SCF^{Met30} does not induce Met4 deubiquitylation. Cultures of wild-type and *cdc53-1* strains were shifted to the nonpermissive temperature of 37°C at the same time as repression of *GAL1-MET4* by glucose. Cd^{2+} was either added or not at 60 min postshift.

2000; Kuras *et al*, 2002). However, when cells grown in rich medium are exposed to Cd^{2+}, ubiquitylated Met4 species disappear very quickly (Figure 3B). As Met4 forms are stable in rich medium, these rapid changes in Met4 modification pattern could not be explained simply by the inhibition of SCF^{Met30} activity. We therefore examined whether Cd^{2+} might also induce an active Met4 deubiquitylation response. That is, pre-existing transcriptionally inactive ubiquitylated isoforms of Met4 might be converted into transcriptionally active nonubiquitylated forms. To test this hypothesis, Met4 was conditionally expressed from the *GAL1* promoter, which was then repressed by glucose for 60 min prior to addition of Cd^{2+}. Northern analysis demonstrated that *MET* gene expression was strongly activated by Cd^{2+} in the complete absence of any *GAL1-MET4* transcript, consistent with the conversion of pre-existing inactive Met4 into transcriptionally competent Met4 (Figure 5B). Parallel immunoprecipitation analysis of Met4 revealed that pre-existing ubiquitylated Met4 species were indeed converted into nonubiquitylated forms upon Cd^{2+} exposure (Figure 5C).

In principle, the deubiquitylation of Met4 in rich medium might result from a basal level of deubiquitylation that dominates only when SCF^{Met30} is fully inhibited, or from an induction of ubiquitin hydrolase activity by Cd^{2+}. To discriminate between these possibilities, we inactivated SCF^{Met30} by shifting *cdc53-1* strain to the restrictive temperature and examined the status of preformed Met4–ubiquitin conjugates. Inactivation of Cdc53 did not trigger Met4 deubiquitylation (Figure 5D). From this result, we conclude that Cd^{2+} induces a Met4-specific ubiquitin hydrolase activity. Intriguingly, Cd^{2+} did not induce Met4 deubiquitylation when the *cdc53-1* strain was inactivated, perhaps suggesting that an intact SCF^{Met30} complex is needed for recognition of Met4 by the ubiquitin hydrolase. In summary, in rich

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medium, *MET* gene activation by Cd^{2+} appears to result from both the disassembly of the SCF^{Met30} complex and the rapid deubiquitylation of Met4.

Met4 is required for growth in the presence of Cd^{2+}

To corroborate the inferred role of Met4 in the detoxification of Cd^{2+} (Fauchon *et al*, 2002), we assessed the Cd^{2+} sensitivity of cells that do not express a functional Met4 protein or one of its associated cofactors, Cbf1, Met28, Met31 and Met32, which variously tether Met4 to promoter DNA in a gene-specific manner (Kuras and Thomas, 1995; Blaiseau and Thomas, 1998). Serial dilutions of *met4*Δ, *met28*Δ, *met31*Δ, *met32*Δ and the double *met31*Δ *met32*Δ mutant strains on minimal medium plates containing 20 μM Cd^{2+} demonstrated that the single *met4*Δ and the double *met31*Δ *met32*Δ deleted cells are exquisitely sensitive to Cd^{2+} (Figure 6). The Cd^{2+} sensitivity of the *met31*Δ *met32*Δ strain further suggests that Met31/Met32 factors furnish the predominant DNA-binding platform for Met4 (Blaiseau and Thomas, 1998), consistent with genome-wide expression profiles of *met31*Δ *met32*Δ and *cbf1*Δ strains (TA Lee and M Tyers, unpublished data). We note that Cd^{2+} sensitivity of these strains is not particularly manifest in rich medium, perhaps because ample reducing equivalents are supplied by the complex bacterial and yeast extracts used in this formulation (data not shown). Met4 thus plays an essential role in Cd^{2+} detoxification under limiting nutrient conditions.

Discussion

The diverse roles of the ubiquitin system derive from its ability to selectively modify many different substrates in a highly regulated manner, a feat achieved by the large number of E3 ubiquitin ligases in the cell (Hershko and Ciechanover, 1998; Pickart, 2001). Regulation of E3–substrate interactions may occur at the level of substrate modification, E3 expression or E3 modification and assembly. One of the most highly regulated E3 activities is that of the anaphase-promoting complex/cyclosome (APC/C), which targets many substrates in mitosis and G1 phase. The APC/C is controlled primarily by the availability of substrate recruitment factors and their phosphorylation-dependent association with the core APC/C particle (Willems *et al*, 2004). In contrast, substrate ubiquitylation by SCF complexes is usually dictated by substrate level modification, predominantly phosphorylation, and/or by F-box protein expression.

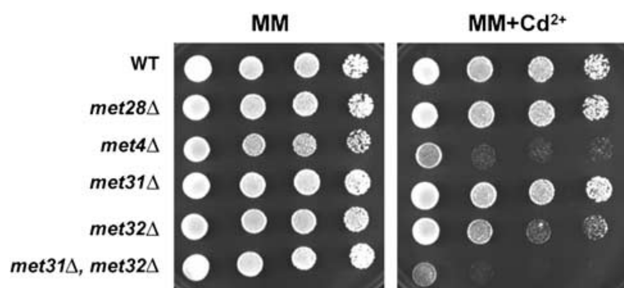

Figure 6 Cd^{2+} sensitivity of *met4*Δ and *met31*Δ *met32*Δ strains. Serial dilutions of wild-type (WT, W303-1A), *met28*Δ (CC769-7D), *met4*Δ (CC849-1B), *met31*Δ (CC867-1C), *met32*Δ (CC845-1C) and *met31*Δ *met32*Δ (CC845-1A) strains were plated onto YNB minimal medium in the presence and absence of 20 μM Cd^{2+} and grown for 2 days.

Met30 differs from most characterized F-box proteins in that it appears to recognize its substrate Met4 in a constitutive manner. Regulation is instead conferred at the level of *MET30* expression, which itself is controlled by Met4 in an autoregulatory loop (Rouillon *et al*, 2000), and by an as yet undiscovered mechanism that links the fate of Met4–ubiquitin conjugates to nutrient conditions (Kaiser *et al*, 2000; Rouillon *et al*, 2000; Kuras *et al*, 2002). Here we report that the activity of Met4 is also modulated by the heavy metal Cd^{2+} so as to allow constitutive activation of the sulfate assimilation pathway and build the glutathione reserves required to chelate and detoxify Cd^{2+}. The ubiquitylation status of Met4 and its attendant transcriptional activity are dictated by at least two Cd^{2+}-dependent pathways. First, in both minimal and rich medium, Cd^{2+} triggers the specific disengagement of Met30 from its binding partner Skp1, thereby preventing the ubiquitylation of Met4. Second, in rich medium, pre-existing inactive Met4–ubiquitin conjugates are rapidly deubiquitylated in the presence of Cd^{2+}. This deubiquitylation activity appears to be induced in response to Cd^{2+}. The redundancy of the 17 predicted deubiquitinylating enzymes in yeast has so far precluded genetic identification of the relevant activity. Regardless of the precise mechanism, the rapid deubiquitylation of Met4 poises cells to respond rapidly to Cd^{2+} in all nutrient conditions. These supernumerary regulatory mechanisms serve to link tightly the regulation of sulfur metabolism to the heavy metal stress response.

Cd^{2+}-induced dissociation of the SCF^{Met30} ubiquitin ligase

The fact that Cd^{2+} compromised both the degradation-dependent and degradation-independent regulation of Met4, under minimal and rich nutrient conditions respectively, suggested that the divalent metal might target the common mediator of the two mechanisms, the SCF^{Met30} ubiquitin ligase. Of several possible targets in the SCF^{Met30} pathway, we found that Cd^{2+} impairs the assembly of the Skp1–Met30 subcomplex. The dissociation of the Skp1–Met30 interaction in response to Cd^{2+} is specific, as the Skp1–Cdc53 interaction is not compromised by Cd^{2+} nor is the assembly and *in vivo* activity of SCF^{Cdc4} affected. The interaction of Skp1 with F-box proteins occurs via a bipartite interface comprised of a conserved core F-box domain and an N-terminal variable region (Schulman *et al*, 2000). The variable region of Met30 is thus the logical potential target of the Cd^{2+} pathway. Based on the structures of the SCF^{Skp2}, SCF^{Cdc4} and SCF$^{\beta\text{-TrCP}}$ complexes, all of which indicate fixed juxtaposition of the substrate to the E2 by the F-box protein (Zheng *et al*, 2002; Orlicky *et al*, 2003; Wu *et al*, 2003), perturbation of the F-box protein–Skp1 interaction would afford a specific means to inactivate any given SCF complex. Indeed, under some circumstances, carbon source appears to control the interaction between Skp1 and the F-box protein Grr1, which helps dictate the response to glucose (Li and Johnston, 1997). The mechanism whereby the Skp1–Met30 interface is disrupted is unknown. Because Cd^{2+} appears not to affect purified recombinant SCF^{Met30} activity *in vitro*, direct binding of the metal ion to Met30 seems unlikely. Whether Cd^{2+} triggers post-translational modification of Met30 or the association of an inhibitory factor remains to be seen.

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The regulation of SCF^{Met30} by Cd^{2+} also bears on the somewhat controversial issue of degradation-dependent versus degradation-independent regulation of Met4. While our data strongly suggest that SCF^{Met30}-dependent ubiquitylation of Met4 leads to Met4 degradation specifically in minimal media (Rouillon *et al*, 2000; Kuras *et al*, 2002), whereas Met4 is stably ubiquitylated in rich medium (Kaiser *et al*, 2000; Kuras *et al*, 2002), the degradation of Met4 under minimal conditions has been disputed (Flick *et al*, 2004). Experiments in this study demonstrate that Cd^{2+} causes the accumulation of unmodified forms of Met4, association of Met4 with cognate promoters and transactivation of *MET* genes in the presence of methionine in minimal medium (see Figure 3). Because a single agent can switch Met4 from a highly unstable to stable state in otherwise identical experimental conditions, it is unlikely that the instability of Met4 derives from artifacts of epitope tags, media conditions or protein extraction (Kaiser *et al*, 2000). Rather, the central role of sulfur metabolism appears to have selected for multiple modes of Met4-dependent transcriptional regulation, each attuned to respond to particular environmental conditions.

Role of Met4 in Cd^{2+} detoxification

In yeast cells, the primary Cd^{2+} detoxification mechanism involves the formation of a Cd^{2+}-glutathione chelate, which is subsequently sequestered in the vacuole (Li *et al*, 1997). Efficient elimination of Cd^{2+} therefore requires high levels of glutathione, a sulfur-containing tripeptide whose biosynthesis is rate limited by the cysteine consuming step. Cd^{2+} detoxification is dependent upon a fully active sulfate assimilation pathway, which provides the reduced sulfur atom required for cysteine and hence glutathione biosynthesis. The transcriptional regulation of the sulfate assimilation pathway requires Met4 and its cofactors Cbf1, Met28, Met31 and Met32 (Thomas and Surdin-Kerjan, 1997). Of these factors, only Met4 is endowed with intrinsic transcription activation function, while Cbf1, Met28, Met31 and Met32 act by tethering Met4 to promoter DNA (Kuras and Thomas, 1995; Blaiseau and Thomas, 1998). Thus, the direct regulation of Met4 activity is an efficient means to meet the requirements for reduced sulfur in glutathione biosynthesis during Cd^{2+} detoxification. As the Met4–Met30 interaction is not perturbed by Cd^{2+}, the presence of Met30 apparently does not interfere with the activity of Met4 transcriptional complexes, a feature that may also enable dynamic regulation of Met4 activity without the need for *de novo* synthesis. The fact that the Cd^{2+} signaling pathway is epistatic to both degradation-dependent and degradation-independent regulation of Met4 allows the cell to cope with Cd^{2+} toxicity regardless of nutrient conditions (Figure 7). In addition to direct regulation of sulfur metabolism under Cd^{2+} stress, Met4 also helps control a proteome-wide response to Cd^{2+}, whereby the production of abundant sulfur-rich proteins is repressed, thus redirecting reduced sulfur equivalents toward glutathione biosynthesis (Fauchon *et al*, 2002; Jamieson, 2002). The major role of Met4 in Cd^{2+} detoxification is underscored by the sensitivity of *met4*Δ and *met31*Δ *met32*Δ strains to this heavy metal. The cellular response to Cd^{2+} in yeast thus occurs at a system-wide level that relies on metabolic, transcriptional and post-transcriptional effectors.

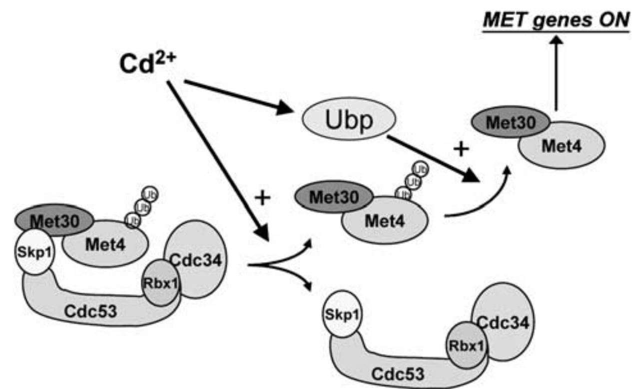

Figure 7 Model for Cd^{2+}-mediated activation of the *MET* gene network through the inhibition of the SCF^{Met30} ubiquitin ligase and activation of an uncharacterized deubiquitinylating enzyme (Ubp).

Conservation of the oxidative stress response

Cd^{2+} causes oxidative stress by depletion of intracellular glutathione pools (Ercal *et al*, 2001). The oxidative stress response in mammals and that in yeast bear striking similarities. In mammalian cells, upon oxidative stress, the bZIP transcription factor Nrf2, in association with a family of coactivators, directs the expression of phase II detoxification enzymes, which in part mediate glutathione biosynthesis (Nguyen *et al*, 2004). Nrf2 activity is controlled in part through its regulated localization (Itoh *et al*, 1999), and in part through its degradation by an SCF-related ubiquitin ligase complex composed of Cul3, Rbx1 and a BTB-Kelch domain adaptor protein called Keap1 (Cullinan *et al*, 2004; Kobayashi *et al*, 2004). Similar to the rescue of *met30* lethality by a *met4* deletion, the lethality of *Keap1*$^{-/-}$ mice is rescued by codeletion of *Nrf2* (Wakabayashi *et al*, 2003). In a manner also analogous to the Cd^{2+}-induced dissociation of Met30 from the core SCF complex, recent evidence suggests that oxidative stress specifically impairs the interaction between Cul3 and Keap1 (Zhang *et al*, 2004). As a consequence, Nrf2 is stabilized by Cd^{2+} and other oxidative stresses, and thereby able to drive transcription of stress response genes (Stewart *et al*, 2003). While the mechanism whereby oxidative stress inhibits the Keap1–Cul3 interaction is not precisely known, several cysteine residues on Keap1 are critical for the degradation of Nrf2 (Zhang *et al*, 2004), perhaps serving as sites of modification for oxidative metabolites (Levonen *et al*, 2004) and/or disulfide bridges (Wakabayashi *et al*, 2004). In addition, phosphorylation of Nrf2 appears to impair its interaction with Keap1 (Huang *et al*, 2002). Whether or not Cd^{2+} or other oxidative stresses lead to similar modifications on Met30 and/or Met4 remains to be determined. The striking parallels between the Nrf2–Keap1 and Met4–Met30 systems should afford further cross-species insights into the oxidative stress response.

Materials and methods

Yeast culture

Saccharomyces cerevisiae strains used in this study are listed in Table I. Minimal B medium is a synthetic medium that lacks organic and inorganic sulfur sources (Cherest and Surdin-Kerjan, 1992). Unless indicated, cells were grown in the presence of 0.2 mM DL-homocysteine as a sulfur source, and amino acids needed to complement the auxotrophic requirements for each strain were

Table I Yeast strains

Strain	Genotype	Source
CC769-7D	*MATα, ade2, his3, leu2, trp1, ura3, met28::LEU2*	Kuras *et al* (1997)
CC845-1A	*MATa, ade2, his3, leu2, trp1, ura3, met31::LEU2, met32::URA3*	This study
CC845-1C	*MATa, ade2, his3, leu2, trp1, ura3, met32::URA3*	This study
CC849-1B	*MATa, ade2, his3, leu2, trp1, ura3, met4::TRP1*	Rouillon *et al* (2000)
CC867-1C	*MATα, ade2, his3, leu2, trp1, ura3, met31::LEU2*	Blaiseau *et al* (1997)
CC932-6D	*MATa, ade2, his3, leu2, trp1, ura3, met4::GAL1-MET4*	Patton *et al* (2000)
CC932-8B	*MATa, ade2, his3, leu2, ura3, met4::GAL1-MET4, met30::LEU2*	Patton *et al* (2000)
CD233	*MATα, his3, leu2, trp1, ura3, met4::^{HA3}MET4*	Kuras *et al* (2002)
CD240	*MATα, his3, leu2, trp1, ura3, met4::GFP-MET4*	Kuras *et al* (2002)
CD269	*MATα, his3, leu2, trp1, ura3, met4::^{HA3}MET4, sua7::SUA7^{9MYC}::TRP1*	Kuras *et al* (2002)
CYS37	*MATa, his3, leu2, trp1, ura3::pGAL1-GFPmut3-Sic1::URA3*	This study
MT1885	*MATa, met4::GAL1-MET4*	This study
MT3341	*MATα, met4::GAL1-MET4, cdc53-1*	This study
W303-1A	*MATa, ade2, his3, leu2, trp1, ura3,*	R Rothstein

added (i.e., drop-in medium). YPD rich medium contains 0.5% yeast extract (Difco), 0.5% bacto-peptone (Difco) and 3% glucose. Transformation was by the lithium acetate method (Gietz *et al*, 1992). To test the specificity of the Cd^{2+} response, yeast cells were exposed to other heavy metals including cobalt, copper, manganese, mercury, silver and zinc. The heavy metals were used at concentrations that were shown to be toxic to yeast cells (Thorvaldsen *et al*, 1993; Ciriolo *et al*, 1994; Blackwell *et al*, 1998; Li and Kaplan, 1998).

Fluorescence microscopy and cytometry

GFP-Met4 fusion protein signals were monitored in living cells on a Nikon Eclipse fluorescence microscope using an Omega XF116 filter. All images were collected with a Princeton CCD camera using identical settings and analyzed with the Meta-Imaging V4.5 software (Universal Imaging Corporation, Downingtown, PA). Nuclei were stained using the dye HOECHST no. 33342 (Sigma), which was added at 1 μg/ml to the culture 20 min prior to imaging. Levels of the GFP-Sic1 fusion protein were assessed by quantitative fluorescence of live cells using a Beckton Dickinson FacsScalibur flow cytometer.

Chromatin immunoprecipitation and RNA analysis

Crosslinked chromatin preparation and immunoprecipitation (ChIP) was performed as described previously (Kuras *et al*, 2002) using the following antibodies: mouse monoclonal anti-HA antibody F-7 from Santa Cruz, mouse monoclonal anti-Myc antibody PL14 from StressGen, and a rabbit polyclonal Met4 antiserum produced against full-length Met4 produced in insect cells. Immunoprecipitated and total DNA samples were analyzed by quantitative PCR in the presence of [α-^{32}P]dATP. Linearity of the PCR reaction was established in multiple independent dilutions of each sample. PCR products were separated on an 8% TBE polyacrylamide gel and quantified on a PhosphorImager (Molecular Dynamics). The occupancy level at a given promoter was defined as the ratio of immunoprecipitated DNA over total DNA for each PCR product. The occupancy level at *MET25* was arbitrarily set to 100 and all other values were represented relative to this standard. For Northern analysis, total RNA was extracted by the hot phenol method, separated and probed as described (Rouillon *et al*, 2000).

Protein analysis

Total proteins were extracted either by a TCA procedure (Rouillon *et al*, 2000) or by glass bead lysis (Kuras *et al*, 2002). For co-immunoprecipitation, total protein was extracted by glass bead lysis in buffer containing 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 250 mM NaCl, 50 mM Tris–HCl (pH 7.5), 10 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1 mM DTT, 5 mM NEM, 1 mM PMSF, 1 μg/ml leupeptin and pepstatin. A 1 μl portion of crude anti-Met4 antiserum was added to 5 mg of extract, incubated on ice for 1 h, bound to 30 μl of 50% protein A bead slurry for 1 h at 4°C and washed. Samples were separated on a 7.5% acrylamide gel, transferred to a PVDF membrane and probed with anti-Met4 polyclonal antibody (1:200 dilution) followed by peroxidase-conjugated anti-rabbit secondary antibody (1:10 000 dilution) and detected by SuperSignal West Pico chemiluminescent substrate (Pierce). A GFP-Sic1 protein fusion was detected with anti-GFP antibody. Equal loading was established by detection with a lysyl-tRNA synthetase antibody.

In vitro ubiquitylation assays

Recombinant proteins were produced in insect cells by baculovirus coinfection and used for *in vitro* ubiquitination assays as described (Skowyra *et al*, 1997). A ^{Myc}Cdc53–Rbx1^{Myc}–Skp1^{FLAG}–^{GST}Met30 complex was affinity purified from infected insect cell lysate on anti-FLAG-M2 agarose resin (Sigma). Recombinant $^{His6-HA}$Met4 produced in insect cells was affinity purified on nickel resin and treated with lambda phosphatase. Immobilized SCF^{Met30} was washed three times with 1 ml of ubiquitination buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 2 mM ATP, 50 μM DTT), incubated with ^{His6}Uba1 (E1), ^{His6}Cdc34 (E2), ubiquitin (Sigma) or methylated ubiquitin (Affiniti) and Met4 in 10 μl ubiquitylation buffer at 30°C for 1 h. Products were separated by SDS–PAGE and immunoblotted with anti-HA (12CA5) antibody.

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