Interaction of Gcn4 with target gene chromatin is modulated by proteasome function

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ABSTRACT **The ubiquitin–proteasome system (UPS) influences gene transcription in multiple ways. One way in which the UPS affects transcription centers on transcriptional activators, the function of which can be stimulated by components of the UPS that also trigger their destruction. Activation of transcription by the yeast activator Gcn4, for example, is attenuated by mutations in the ubiquitin ligase that mediates Gcn4 ubiquitylation or by inhibition of the proteasome, leading to the idea that ubiquitin-mediated proteolysis of Gcn4 is required for its activity. Here we probe the steps in Gcn4 activity that are perturbed by disruption of the UPS. We show that the ubiquitylation machinery and the proteasome control different steps in Gcn4 function and that proteasome activity is required for the ability of Gcn4 to bind to its target genes in the context of chromatin. Curiously, the effect of proteasome inhibition on Gcn4 activity is suppressed by mutations in the ubiquitin-selective chaperone Cdc48, revealing that proteolysis per se is not required for Gcn4 activity. Our data highlight the role of Cdc48 in controlling promoter occupancy by Gcn4 and support a model in which ubiquitylation of activators—not their destruction—is important for function.**

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INTRODUCTION

Regulated proteolysis by the ubiquitin (Ub)–proteasome system (UPS) is crucial for a myriad of processes, including control of gene transcription (Geng *et al.*, 2012). In many cases, actions of the UPS in transcription follow the canonical paradigm in which ubiquitin ligases and the proteasome inhibit protein activity by reducing intracellular levels of target proteins. In the case of transcriptional activators, however, a counterintuitive scenario can play out in which components of the UPS stimulate the function of the activators they destroy. Transcription factors such as Gal4 (Muratani *et al.*, 2005; Collins *et al.*, 2009), Gcn4 (Lipford *et al.*, 2005), and nuclear hormone receptors (Perissi *et al.*, 2004), for example, have been shown to require their cognate Ub-ligases—and/or proteasomal proteolysis—for full activity, suggesting that Ub-mediated turnover of activator proteins promotes their function.

Precisely how ubiquitin-mediated destruction of an activator stimulates its activity is unclear. To account for the concept of how destruction of a protein could make it more active, we (Geng *et al.*, 2012) and others (Lipford *et al.*, 2005) hypothesized that the requirement for the UPS in transcription reflects the need for activators to cycle on chromatin to drive multiple rounds of transcriptional activation. We proposed that activator ubiquitylation occurs on promoter DNAs after transcription has initiated and that the modified activator remains bound to chromatin in an inactive configuration that must be cleared by the proteasome in order for subsequent rounds of activation to occur. This model assumes that activator ubiquitylation and proteolysis converge on a single mechanistic step in the activation process and that there is no difference between blocking activator ubiquitylation and inhibiting activator destruction. Moreover, this model was developed at a time when little was known about the role of factors that lie between ubiquitylation and proteolysis, such as complexes containing the Ub-dependent chaperone Cdc48 (Meyer *et al.*, 2012), which extract ubiquitylated substrates from protein complexes for proteasomal destruction. The potential effect of Cdc48 on activator function is particularly intriguing, as Cdc48 was recently shown to mediate proteolysis-independent stripping of a Ub-fusion activator from its target DNA sites in vivo (Ndoja *et al.*, 2014).

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Abbreviations used: ChIP, chromatin immunoprecipitation; RT-qPCR, reverse-transcription quantitative PCR; Ub, ubiquitin; UPS, ubiquitin–proteasome system.

The purpose of this study was to challenge the assumption that ubiquitylation and proteolysis target the same step in activator function and ask how Cdc48 features in the control of a native *Saccharomyces cerevisiae* transcriptional activator, Gcn4. Gcn4 is induced in response to amino acid starvation and drives the expression of genes encoding amino acid biosynthetic enzymes (Hinnebusch, 2005). Like a majority of transcriptional activators, Gcn4 carries an overlapping transcriptional activation domain and degron (Geng *et al.*, 2012) and is targeted for Ub-mediated proteolysis by the SCF^{Cdc4} Ub ligase (Chi *et al.*, 2001). Of importance, genetic inhibition of SCF^{Cdc4} or inhibition of the proteasome reduces Gcn4 activity (Lipford *et al.*, 2005), making Gcn4 an excellent focal point for our studies.

Here we confirm that full activity of Gcn4 is dependent on both SCF^{Cdc4} and the proteasome but show that disrupting the function of each affects Gcn4 activity in different ways. We also show that inhibiting the proteasome blocks the ability of Gcn4 to bind target genes in vivo and that this phenotype is suppressed by mutations in Cdc48. These observations reveal that proteasomal proteolysis per se is not required for the function of Gcn4 and highlight the importance of Cdc48 in controlling the activity of a native yeast activator.

RESULTS AND DISCUSSION

Full induction of Gcn4 target genes depends on SCF^{Cdc4} and the proteasome

A previous study (Lipford *et al.*, 2005) reported that activation of Gcn4 target genes is reduced by mutations in the Cdc34 and Cdc4 components of the SCF^{Cdc4} Ub ligase (Chi *et al.*, 2001), as well as by inhibition of proteasomal proteolysis. To confirm these findings, we monitored the effects of SCF^{Cdc4} or proteasome perturbation on activation of Gcn4 target genes by sulfometuron methyl (SM), an agent that induces Gcn4 synthesis by blocking branched-chain amino acid production (Jia *et al.*, 2000). Three sets of experiments were performed. First, we asked whether temperature-sensitive mutations in Cdc34 *or* Cdc4 (Patton *et al.*, 1998) affect expression of the Gcn4 target gene *ARG1*. Here we found that shifting yeast to the restrictive temperature of 37°C reduced the expression of *ARG1* in both the *cdc34-2* (Figure 1A) and *cdc4-1* (Figure 1B) strains. Second, we used the "anchor-away" technique (Haruki *et al.*, 2008) to exclude Cdc34 from the nucleus via a rapamycin-induced interaction with a subunit of the ribosome. Here, treatment of the anchored Cdc34 strain with rapamycin reduced the expression of *ARG1* (Figure 1C) to a level comparable to that observed in the *cdc34-2* strain. Finally, we combined point mutations in the tryptic and caspase sites of the proteasome (Heinemeyer *et al.*, 1997) with MG132-mediated inhibition of the chymotryptic site (Howard *et al.*, 2012) to probe the effect of 20S proteasome inhibition on Gcn4 activity. Here, inhibition of proteasome activity blocked induction of *ARG1* (Figure 1D), as well as of *ARG4* (Figure 1E) and *HIS4* (Figure 1F). Induction of Gcn4 protein by SM was not blocked by proteasome inhibition (Figure 1G), showing that this blockade is not at the level of Gcn4 synthesis. We did note, however, that proteasome inhibition promoted the accumulation of high–molecular weight Gcn4 species in total cell lysates, consistent with an increase in the level of ubiquitin-Gcn4 (Chi *et al.*, 2001) or SUMO-Gcn4 (Rosonina *et al.*, 2012) conjugates, or a combination of both. On the basis of these results, we conclude that induction of Gcn4 target genes is sensitive to mutations in SCF^{Cdc4} and chemical-genetic inhibition of proteasomal proteolysis.

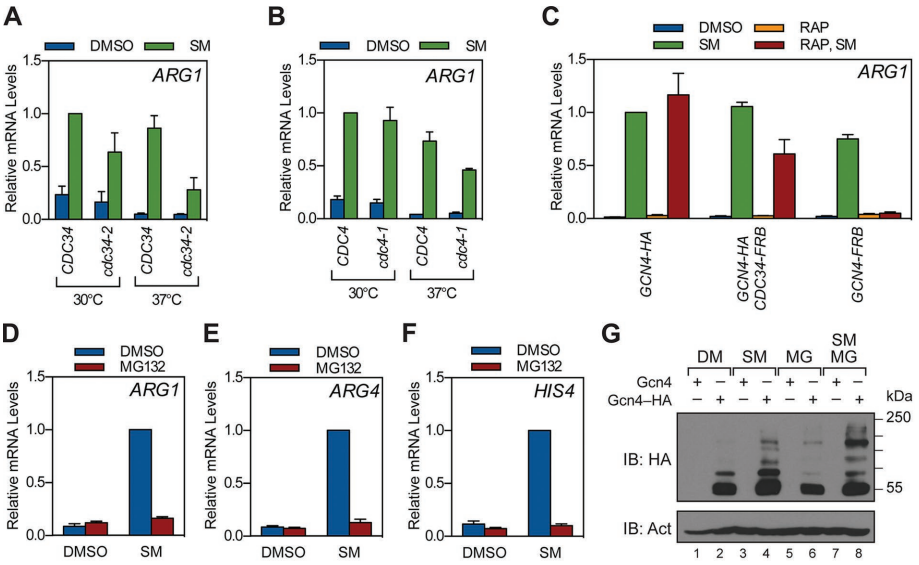

FIGURE 1: Stimulation of Gcn4 target genes by SCF^{Cdc4} and the proteasome. (A) *CDC34* (W303-1a) and *cdc34-2* (MT670) yeast were grown to log phase at 30°C in minimal medium and then shifted to 37°C or maintained at 30°C for 1 h as indicated. Strains were then treated with SM or DMSO for 1.5 h, at which time RNA was collected and *ARG1* mRNA levels quantified by RT-qPCR. Relative mRNA levels for *ARG1* were normalized to the *CDC34* strain treated with SM at 30°C. $n = 3$. (B) As in A, except using *CDC4* (W303-1a) and *cdc4-1* (MT668) strains. $n = 3$. (C) Anchor-away strains expressing HA-tagged Gcn4 (GHY139), FRB-tagged Cdc34 (GHY149), or FRB-tagged Gcn4 (GHY145) were grown to log phase in minimal medium, treated with either DMSO or rapamycin for 1 h, and then further treated with either DMSO or SM for 1.5 h. RNA was collected, and *ARG1* mRNA levels were measured by RT-qPCR, as in A. Relative mRNA levels for *ARG1* were normalized to the *GCN4-HA* strain treated with SM at 30°C. $n = 3$. (D–F) Yeast bearing the *pup1–T30A pre3–T20A* mutations (GHY010) were grown to log phase in minimal medium and treated with either DMSO or MG132 for 1 h. Strains were then treated with SM or DMSO for 1.5 h, at which time RNA was collected and *ARG1* (D), *ARG4* (E), and *HIS4* (F) mRNA levels quantified by RT-qPCR. Relative mRNA levels were then normalized to the SM-induced, DMSO-treated sample for each gene. $n = 4$. Error bars represent SEM. (G) Yeast expressing either native Gcn4 (GHY010) or HA-tagged Gcn4 (GHY025) were grown to log phase in minimal medium and treated with DMSO or MG132 for 1 h. Strains were then treated with SM or DMSO for 1.5 h, at which time protein was extracted, resolved by SDS–PAGE, and subject to Western blotting with antibodies against the HA epitope or β-actin (Act).

Proteasome inhibition disrupts the ability of Gcn4 to engage target gene chromatin

We next used chromatin immunoprecipitation (ChIP) to quantify the effect of Cdc4 or proteasome inactivation on binding of hemagglutinin (HA) epitope–tagged Gcn4 to target gene promoters. Consistent with a previous study (Lipford *et al.*, 2005), Gcn4 binding to *ARG1* was not different in the *cdc4-1* strain versus its control strain at the restrictive temperature (Figure 2A). When we examined the effect of proteasome inhibition, however, we discovered that binding of Gcn4 to the *ARG1* UAS was disrupted by proteasome inhibition (Figure 2B). The failure of Gcn4 to bind chromatin was

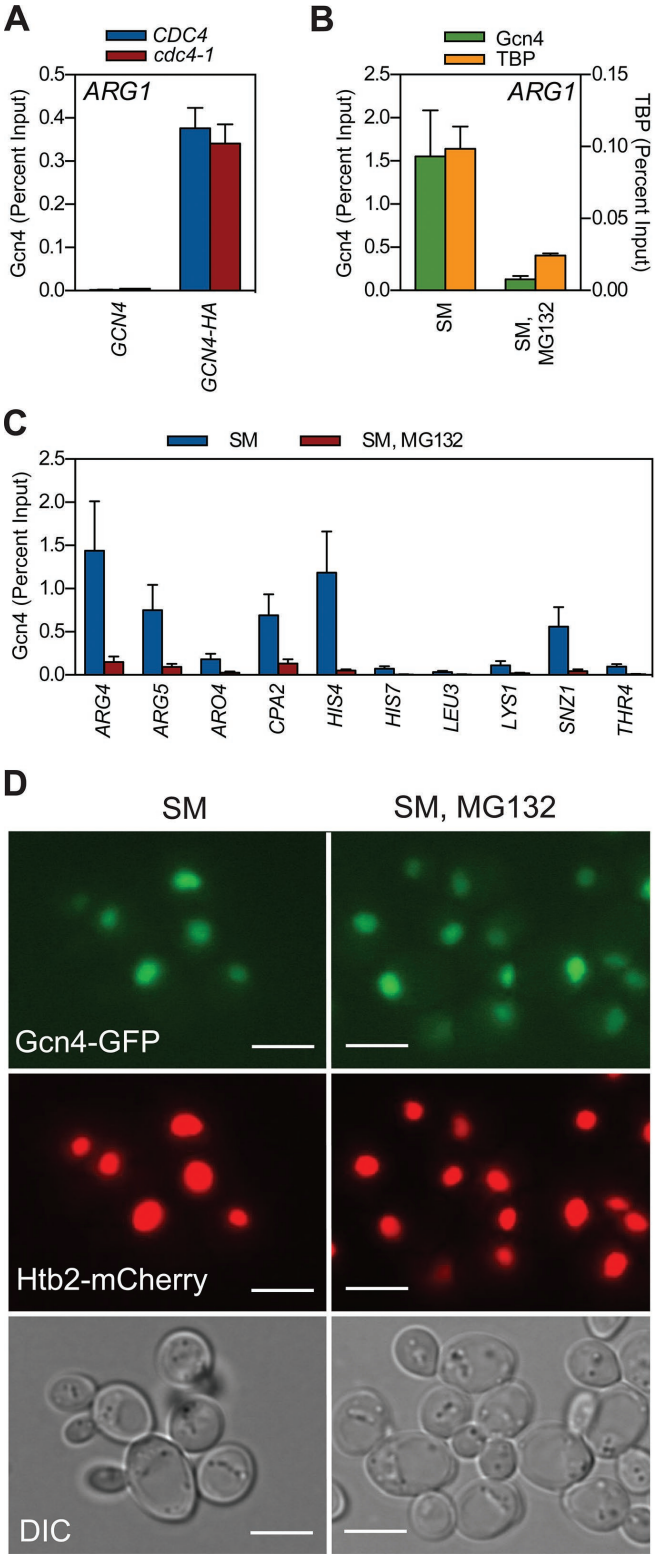

FIGURE 2: Inhibiting the proteasome impedes the ability of Gcn4 to bind target gene chromatin. (A) *CDC4* (W303-1a), *CDC4 GCN4-HA* (GHY107), *cdc4-1* (MT668), and *cdc4-1 GCN4-HA* (GHY107) strains were grown to log phase at 30°C in minimal medium, shifted to the restrictive temperature of 37°C for 1 h, and then induced with SM for an additional 1.5 h. At this time, ChIP was performed with an antibody against the HA-epitope tag. Coprecipitating *ARG1* promoter DNA was quantified by qPCR, expressed relative to the percentage of input DNA. $n = 3$. (B) *GCN4-HA* (GHY025) yeast were grown to log phase at 30°C in minimal medium, treated with either DMSO or MG132 for 1 h, and induced with SM for 1.5 h. ChIP was performed using antibodies against the HA-epitope tag or TBP. Coprecipitating *ARG1* promoter DNA was quantified by qPCR. $n = 3$. (C) As in B, except that coprecipitating DNAs from the anti-HA ChIP were quantified by qPCR, using primer pairs that amplify Gcn4-binding sites in the indicated genes. $n = 3$. Error bars represent SEM. (D) *GCN4-GFP HTB2-mCherry* (GHY339) yeast were grown to log phase at 30°C in minimal medium, treated with either DMSO or MG132 for 1 h, and induced with SM for 1.5 h. Samples were imaged using either fluorescence (top) or differential interference contrast microscopy (bottom). Scale bars, 5 μm.

accompanied by loss of binding of the TATA box–binding protein TBP to the *ARG1* TATA box (Figure 2B) and was not restricted to *ARG1*, as we observed the same phenomenon at UAS elements in another 10 Gcn4 target genes (Figure 2C). Of importance, there was no obvious alteration in the cellular distribution of Gcn4 in response to proteasome inhibition (Figure 2D and Supplemental Figure S1), demonstrating that Gcn4 remained in the nucleus under these conditions but was unable to associate with target gene chromatin as measured by ChIP.

Our finding that Gcn4 fails to associate with its cognate UAS elements when the proteasome is inhibited is at odds with a report by Lipford *et al.* (2005), who showed that Myc epitope–tagged Gcn4 robustly binds its target genes in the presence of MG132. This discrepancy raises the possibility that differential epitope tagging of Gcn4 is responsible for the disagreement between these studies. We therefore repeated our experiments with untagged Gcn4 using a polyclonal anti-Gcn4 antibody and with Myc-tagged Gcn4, as used in the Lipford *et al.* (2005) study. For untagged Gcn4 (Supplemental Figure S2A), we observed that proteasome inhibition reduced the level of Gcn4 binding at the *ARG1* UAS to that observed in the uninduced state, mirroring the effect of proteasome inhibition on *ARG1* gene induction (Figure 1D) and what we observed for HA-tagged Gcn4 (Figure 2B). When we examined Myc-tagged Gcn4, however, we found that proteasome inhibition had little if any effect on its binding to the *ARG1* UAS (Supplemental Figure S2B). These results are consistent with the Lipford *et al.* (2005) study but also demonstrate that Myc tagging of Gcn4 changes the behavior of the protein—as measured by ChIP—in a way that does not recapitulate that of the native Gcn4 protein.

In sum, the contrasting effects of Cdc4 versus proteasome disruption on the ability of Gcn4 to bind DNA in vivo suggest that ubiquitylation and proteolysis influence distinct steps in Gcn4 function and that the profound effects of proteasome inhibition on Gcn4 target gene induction result from a failure of Gcn4 to stably bind chromatin when proteasome function is blocked.

Proteasome inhibition does not act via the ArgR repressor or by modulating nucleosome occupancy

The simplest explanation for how proteasome inhibition disables the ability of Gcn4 to bind chromatin is that it induces a state on chromatin that is refractory to Gcn4 binding by either inducing an unstable repressor protein or perhaps altering nucleosome positioning. To address these possibilities, we first asked whether the ArgR repressor complex—which binds to DNA elements in the *ARG* promoters and inhibits their transcription (Messenguy *et al.*, 1991)—mediates the effects of proteasome inhibition we observe. We found, however, that deletion of the gene encoding the Arg80 subunit of this complex (Dubois *et al.*, 1987) had no detectable effect

on the sensitivity of *ARG1* to proteasome inhibition (Figure 3A), excluding the possibility that accumulation of this known repressor complex inhibits Gcn4 when proteasome function is blocked.

We next performed nucleosome scanning analysis (Sekinger *et al.*, 2005) to ask whether proteasome inhibition alters nucleosome occupancy across the *ARG1* (Figure 3B) and *HIS4* (Figure 3C) genes. Consistent with a previous report (Crisucci and Arndt, 2012), we found that Gcn4-binding sites in the *ARG1* (Figure 3B) and *HIS4* (Figure 3C) promoters lack nucleosomes and that SM induction reduces the density of nucleosomes within the transcribed portions of *ARG1* and *HIS4*, as expected when their transcription is induced. Proteasome inhibition increased the levels of nucleosomes across the *ARG1* and *HIS4* open reading frames—again consistent with a decrease in transcriptional activity—but had no detectable effect on nucleosome position or density across the "nucleosome-free" region bound by Gcn4. Of interest, nucleosome density at the *HIS4*-adjacent gene *BIK1*, which is not regulated by Gcn4, is markedly increased in response to MG132, demonstrating that proteasome function is involved in mediating some aspect of nucleosome dynamics that is yet to be described. In terms of Gcn4, however, these data exclude the notion that accumulation of nucleosomes in response to proteasome inhibition mediates the failure of Gcn4 to access its cognate sites on chromatin.

Mutation of Cdc48 suppresses the effects of proteasome inhibition on Gcn4 activity

Our data support the concept that ubiquitylation and proteolysis play distinct roles in controlling Gcn4 activity. One possibility is that ubiquitylation of chromatin-bound Gcn4 stimulates a step in the activation process but that once Gcn4 is ubiquitylated, it is unable to stably associate with chromatin. In this scenario, proteasome inhibition could force Gcn4 to accumulate off chromatin via the accrual of ubiquitylated Gcn4 species. Indeed, direct analysis of Gcn4–Ub conjugates via the His-Ub method (Yaglom *et al.*, 1996) revealed that proteasome inhibition promotes a striking increase in the level of ubiquitylated Gcn4 protein (Figure 4A), suggesting that accumulation of Ub conjugates could underlie the defects in Gcn4 function we observe when the proteasome is inhibited.

We attempted to challenge this notion by producing a form of Gcn4 that cannot be ubiquitylated. We engineered alanine-substitution mutations in Gcn4 (Supplemental Figure S3A) that block phosphorylation events required for ubiquitylation by SCF^{Cdc4} (Chi *et al.*, 2001). Unfortunately, this form of Gcn4 still accumulated a single Ub-conjugated form in response to proteasome inhibition (Supplemental Figure S3B) and remained sensitive to proteasome inhibition (Supplemental Figure S3, C and D). We also constructed a version of Gcn4 in which all 23 lysine residues are simultaneously mutated to arginine (K0). Although this mutation blocked the ubiquitylation of Gcn4 (Supplemental Figure S3E), we were unable to detect binding of K0 Gcn4 to the UAS of *ARG1* by ChIP (Supplemental Figure S3F) and thus did not pursue this mutant further.

Instead, we considered the possibility that the Gcn4–Ub conjugates that accumulate in response to proteasome inhibition are removed from chromatin by Cdc48, as previously reported for a Ub-fusion activator (Ndoja *et al.*, 2014). Coimmunoprecipitation assays demonstrated that Gcn4 interacts with Cdc48 (Figure 4B) and that this interaction is disrupted by the K0 mutation in Gcn4, which disrupts ubiquitylation (Figure 4B). Moreover, a temperature-sensitive mutant of *CDC48* (*cdc48-3*; Sato and Hampton, 2006), assayed at the semipermissive temperature, suppressed the effects of proteasome inhibition on transcription at both *ARG1* and *HIS4* (Figure 4, C and D), as well as restored binding of Gcn4 to these genes

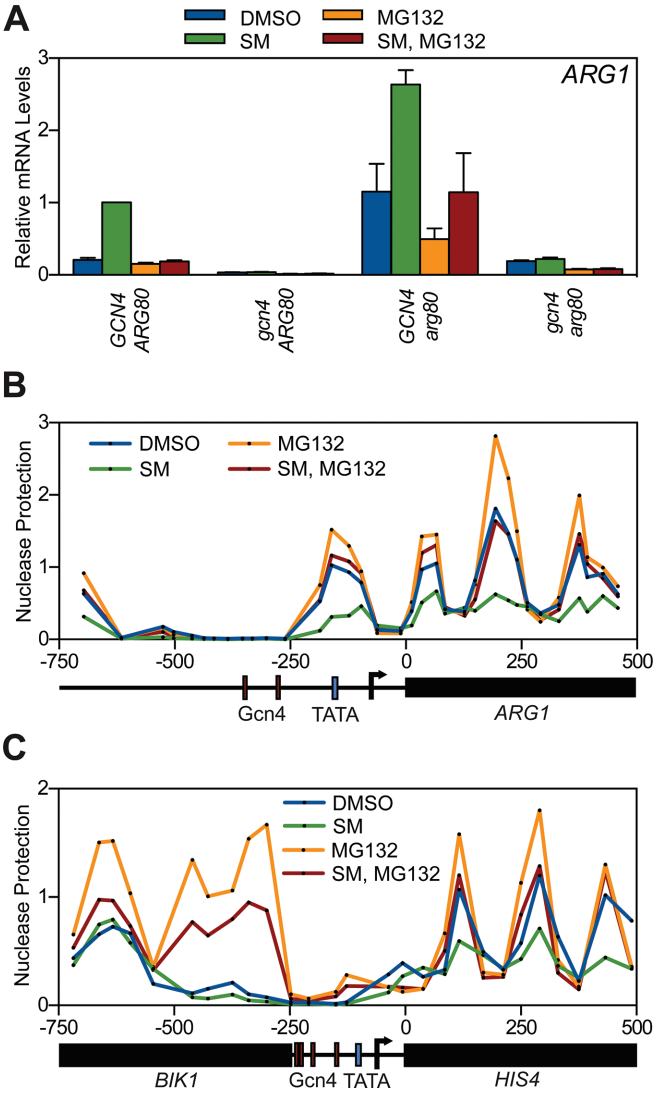

FIGURE 3: The effect of proteasome inhibition on Gcn4 activity is not mediated via the ArgR repressor or changes in nucleosome occupancy. (A) *GCN4 ARG80* (GHY010), *gcn4 ARG80* (GHY004), *GCN4 arg80* (GHY081), and *gcn4 arg80* (GHY079) strains were grown to log phase in minimal medium and treated with DMSO or MG132. After 1 h, strains were treated with DMSO or SM for 1.5 h and RNA harvested, and *ARG1* mRNA levels were quantified by RT-qPCR. Each qPCR was normalized to the SM-treated *GCN4 ARG80* sample. Error bars represent SEM. $n = 4$. (B) *GCN4* yeast (GHY010) were grown to log phase at 30°C in minimal medium, treated with DMSO or MG132, and induced with SM or a DMSO control for an additional 1.5 h, and nucleosome occupancy was mapped by MNase digestion, coupled with tiled primer sets spanning *ARG1*. qPCR data were normalized to the signal from a *GAL1-10* promoter-localized nucleosome. Data points represent an average of two independent experiments. (C) As in B, except monitoring nucleosome positioning surrounding the *HIS4* locus. Data points represent an average of two independent experiments.

(Figure 4, E and F). Suppression of the effects of proteasome inhibition on Gcn4 activity in the *cdc48-3* strain is due to the mutations in *CDC48*, as expression of wild-type Cdc48 in this strain background restored proteasome sensitivity (Figure 4G). The ability of mutations in Cdc48 to suppress the effects of proteasome inhibition supports the concept that proteasome inhibition drives the accumulation of

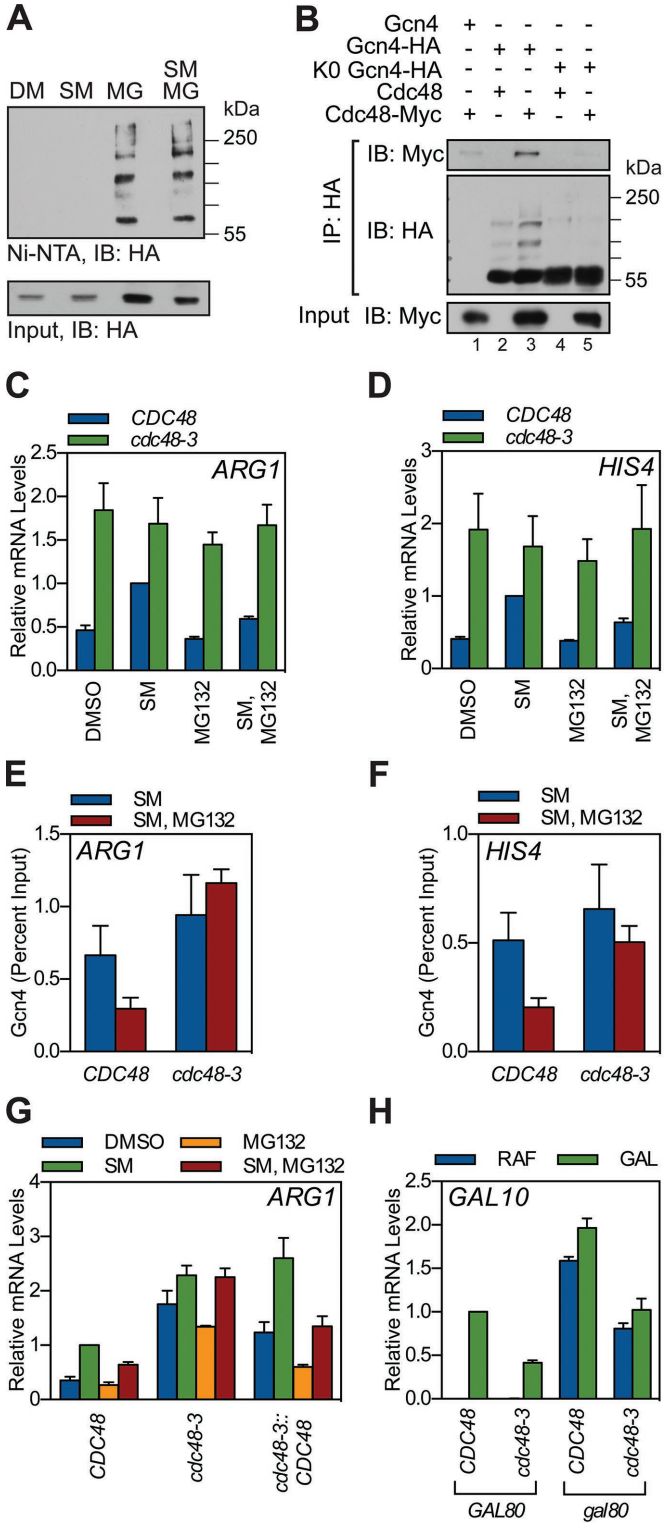

FIGURE 4: Mutation of Cdc48 suppresses the effect of proteasome inhibition on Gcn4 activity. (A) *GCN4-HA* (GHY356) yeast carrying a copper-inducible His-Ub expression plasmid (pUB221) were grown to log phase in minimal medium and treated with $CuSO_4$ and DMSO or MG132 for 1 h. Yeast were induced with SM or DMSO for an additional 1.5 h, at which time protein lysates were collected under denaturing conditions. Ubiquitin-conjugates were captured by nickel-resin (Ni-NTA) chromatography, resolved by SDS–PAGE, and probed for HA-tagged Gcn4 protein by Western blotting. A sample of the input material to the nickel resin was also probed for HA-tagged Gcn4. IB, immunoblot. (B) *GCN4 CDC48-MYC* (GHY285), *GCN4-HA CDC48* (GHY025), *GCN4-HA CDC48-MYC* (GHY287), *K0-GCN4-HA CDC48* (GHY124), and *K0-GCN4-HA CDC48-MYC* (GHY293) yeast were grown to log phase at 30°C in minimal medium and treated with DMSO or MG132. After 1 h, Gcn4 was induced with SM for 1.5 h. Protein lysates were collected and Gcn4-HA immunoprecipitated (IP) via an anti-HA antibody, and IPs were probed with antibodies against the Myc-epitope (Cdc48) and HA-epitope (Gcn4) tags. A sample of the input material to the IP was also probed for Myc-tagged Cdc48. (C, D). *CDC48* (RHY2455) and *cdc48-3* (RHY2457) strains were grown to log phase at 30°C in minimal medium and treated with DMSO or MG132. After 1 h, strains were treated with DMSO or SM for 1.5 h, RNA harvested, and processed as described to measure mRNA levels from the *ARG1* (C) and *HIS4* (D) loci. $n = 4$. (E, F). *CDC48 GCN4-HA* (GHY116) and *cdc48-3 GCN4-HA* (GHY118) strains were grown to log phase at 30°C in minimal medium, treated with either DMSO or MG132 for 1 h, and induced with SM for 1.5 h. ChIP was then performed using antibodies against the HA-epitope tag. Coprecipitating *ARG1* (E) or *HIS4* (F) promoter DNAs were quantified by qPCR, expressed relative to the percentage of input DNA. $n = 3$. (G) *CDC48* (RHY2455), *cdc48-3* (RHY2457), and *cdc48-3::CDC48* (GHY279) strains were grown and treated as described in C and RNA harvested, and RT-qPCR was performed to quantify *ARG1* transcripts. $n = 3$. (H) *CDC48 GAL80* (RHY2455), *cdc48-3 GAL80* (RHY2457), *CDC48 gal80* (GHY304), and *cdc48-3 gal80* (GHY305) yeast were grown in raffinose medium and treated with water or 2% galactose for 1.5 h. RNA was collected and used for RT-qPCR for *GAL10*. Each qPCR was internally normalized to *ACT1* and then normalized to the galactose-treated *CDC48 GAL80* sample. Error bars represent SEM. $n = 3$.

Gcn4–Ub conjugates that are prevented from stably binding chromatin by Cdc48.

In addition to suppressing defects associated with proteasome inhibition, we observed that the *cdc48-3* mutation increased levels of Gcn4 target gene activity in the absence of MG132 (Figure 4, C and D). This result contrasts with our report that Cdc48 stimulates the activity of the yeast activator Gal4 (Bonizec *et al.*, 2014). To confirm that Gcn4 and Gal4 are affected differently by the *cdc48-3* mutation, we examined Gal4 activity in the *cdc48-3* mutant strain; we also examined the effect of *GAL80* deletion in this context, which has been argued to be a point of Gal4 regulation by the UPS (Ang *et al.*, 2012). Supporting our previous study (Bonizec *et al.*, 2014), mutation of Cdc48 reduced activation of the *GAL10* locus (Figure 4H), whereas the *GAL80* deletion did not affect the sensitivity of *GAL10* transcription to the *cdc48*-3 mutation. Thus, despite many parallels in how Gcn4 and Gal4 are regulated by the UPS, the effect of Cdc48 on the function of these two activators is different. Further work will be required to determine the mechanistic basis for the differences between Gcn4 and Gal4 in terms of the role that Cdc48 plays in their function.

Conclusion

In this study, we confirmed the importance of an intact UPS to the ability of Gcn4 to activate transcription of select target genes but made the surprising discovery that ubiquitylation and proteasomal destruction appear to control distinct steps in the activation process. On one hand, disrupting the activity of the SCF^{Cdc4} Ub ligase reduces the expression of Gcn4 target genes via a process that is most likely downstream of target gene recognition by Gcn4, as previously suggested (Lipford *et al.*, 2005). On the other hand, inhibition of the proteasome inhibits Gcn4 target gene activation at a step that is apparently upstream of chromatin recognition by Gcn4 and in a manner that can be suppressed by a mutation in the Ub-selective

chaperone Cdc48. Although we have been unable to test whether the actions of SCF^{Cdc4} and the proteasome in this context are mediated directly on Gcn4, the simplest conclusion from these data is that ubiquitylation of Gcn4 stimulates two distinct processes, promoting the inherent transcriptional activity of the protein while at the same time limiting activation by triggering the Cdc48-mediated extraction of Gcn4 from target gene promoters. In this way, disruption of SCF^{Cdc4} reduces the level of Gcn4-target gene transcription without affecting Gcn4-promoter binding, whereas proteasome inhibition promotes the accumulation of ubiquitylated Gcn4, which is then either stripped off chromatin or prevented from stably binding once it dissociates (Supplemental Figure S4).

The foregoing scenario is thematically similar to the previously proposed "licensing" (Salghetti *et al.*, 2001) and "Ub-clock" (Wu *et al.*, 2007) models, in that activator ubiquitylation sets an inherent limitation on the functional lifetime of transcriptional activators but differs profoundly in the role of proteasomal proteolysis in this process. Indeed, the ability to suppress the effect of proteasome inhibition on Gcn4 activity by inactivation of Cdc48 strongly implies that proteasomal proteolysis per se cannot play a positive role in Gcn4-mediated transcriptional activation, irrespective of whether Gcn4 or some other factor is the relevant substrate. Instead, the effects of proteasome inhibition on Gcn4 must be indirect, perhaps a result of the accumulation of ubiquitylated Gcn4 species, as we propose. A revised view of how the proteasome features in transcriptional activation is more consistent with work from the Laney (Wilcox and Laney, 2009) and Yao (Ndoja *et al.*, 2014) laboratories, which pinpoint Cdc48-mediated extraction of ubiquitylated activators as a limiting point in the activation process, and implies that the widespread and intimate relationship between activation and destruction elements in transcription factors (Salghetti *et al.*, 2000; Geng *et al.*, 2012), if anything, reflects the functional importance of activator ubiquitylation over destruction.

MATERIALS AND METHODS

Yeast manipulations

Yeast strains are described in Supplemental Table S1. Gene deletions were performed via homologous recombination using PCR-amplified auxotrophic markers or antibiotic resistance genes from plasmids, as indicated. Epitope tagging of endogenous loci was performed similarly (Knop *et al.*, 1999; Sheff and Thorn, 2004). mCherry tagging of endogenous Htb2 was performed as described except using an mCherry-tagging cassette amplified from SWY5678 genomic DNA (Lord *et al.*, 2015). Strains carrying the *3T2S-GCN4* allele were generated through deletion of endogenous *GCN4* with the *URA3* cassette followed by insertion of PCR-amplified *3T2S-GCN4* through homologous recombination. PCR genotyping was used to confirm all genomic manipulations. Replacement of the *cdc48-3* allele for wild-type *CDC48* was performed through homologous recombination of a PCR fragment encoding wild-type *CDC48* and spanning the sites of the two mutations in *cdc48-3* (P257L, R387K). Colonies were selected for ability to grow at the restrictive temperature of 37°C, and restoration of the wild-type *CDC48* sequence was confirmed by PCR amplification of the locus and Sanger DNA sequencing. For anchor-away strains, epitope tagging of *GCN4* and deletion of *PDR5* were performed as described. *CDC34* and *GCN4* were tagged with FKBP12-rapamycin-binding domain (FRB) as described for epitope tagging except using an FRB-tagging construct (Haruki *et al.*, 2008). A high-efficiency yeast transformation protocol was used for genomic manipulations and introduction of plasmids (Gietz and Schiestl, 2007). Supplemental Table S2 lists primer sequences.

RNA isolation and analysis

For Gcn4-based experiments, yeast were grown overnight in yeast extract/peptone/adenine/dextrose (YPAD), washed with sterile water, and diluted to an OD_{600} of 0.3 in minimal medium (0.67% yeast nitrogen base without amino acids and 2% dextrose, supplemented with amino acids as appropriate). Yeast were grown for 5 h and treated with 0.5 μg/ml SM (or dimethyl sulfoxide [DMSO] control) before RNA was harvested by the hot-phenol method (Leung *et al.*, 2011). If proteasome inhibition was part of the experiment, yeast were grown for 4 h and treated with 50 μM MG132 (or DMSO control) plus 0.004% SDS (Liu *et al.*, 2007) for 1 h before SM induction. For anchor-away experiments, yeast were grown for 4 h in minimal medium and treated with 1.0 μg/ml rapamycin (or DMSO control) for 1 h before SM induction. For Gal4-based experiments, yeast were grown overnight in YPAD, washed with sterile water, and diluted to an OD_{600} of 0.3 in CSM-RAF medium (0.67% yeast nitrogen base without amino acids, 2% raffinose) supplemented with CSM. After 5 h, cultures were induced with 2% galactose for 90 min before RNA was isolated. In all cases, mRNA levels were quantified by reverse-transcription quantitative PCR (RT-qPCR) as described (Leung *et al.*, 2011) and normalized to an *ACT1* primer pair. Supplemental Table S2 lists primer sequences.

Western blotting, coimmunoprecipitation, and ubiquitylation assays

For Western blotting, yeast pellets were resuspended in buffer A (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, 10 mM Imidazole) to a final OD_{600} of ~150, and proteins were extracted by bead beating. For each protein preparation, 20 μg was ethanol precipitated to remove guanidine, resolved by SDS–PAGE, and transferred to nitrocellulose membranes. Membranes were probed using the appropriate antibodies: anti-HA–horseradish peroxidase (HRP; 12013819001; Roche, Basel, Switzerland), anti-c-Myc-HRP (11814150001; Roche), or anti–β-actin (ab8224; Abcam, Cambridge, MA). For coimmunoprecipitation assays, cell pellets from 100-ml cultures were resuspended in 800 μl of yeast lysis buffer (10 mM Na_2HPO_4/NaH_2PO_4, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% NP-40, 50 mM NaF, 0.1 mM Na_3VO_4, 1× Roche Complete, EDTA-free Protease Inhibitor Cocktail; 0.4 mg/ml Pefabloc SC, 50 μM MG132, 2 mg/ml iodoacetamide, and 200 μM 1,10-phenanthroline). Cells were lysed by bead beating at 4°C for 40 s, followed by incubation in ice water for 2 min, for a total of five times. Cell lysate was collected and cleared by centrifugation. Immunoprecipitation reactions were performed using the anti-HA antibody 12CA5 as described (Daulny *et al.*, 2008). Ubiquitylation assays were performed using the His-tagged Ub method essentially as described (Daulny *et al.*, 2008). Copper-inducible, His-tagged Ub was expressed from the plasmid pUB221 (Yaglom *et al.*, 1996).

Chromatin immunoprecipitation

For each reaction, 100-ml cultures of yeast were processed as described for RNA isolation and analysis and cross-linked with 1% formaldehyde, and ChIP was performed as described (Geng and Tansey, 2012). The 12CA5 anti-HA antibody, 9E10 anti-Myc antibody, anti-Gcn4 antibody (sc-50443; Santa Cruz Biotechnology, Dallas, TX), or anti-TBP antibody (a gift from P. A. Weil, Vanderbilt University, Nashville, TN), was used to immunoprecipitate chromatin as indicated. Coprecipitating DNAs were quantified by qPCR using primer sets that amplify TATA box–proximal Gcn4-binding sites in each gene (for detection of Gcn4–promoter interaction) or the TATA box of the *ARG1* gene. In each case, signal is calculated as equivalent to percentage of the input to the ChIP reaction. Primer sequences are listed in Supplemental Table S2.

Fluorescence microscopy

Yeast cultures were processed as described for RNA isolation and analysis and transferred to a glass slide for imaging. Images were acquired with a standard microscope (BX50; Olympus, Center Valley, PA) equipped with a motorized stage (model 999000; Ludl), UPlanF1 100×/numerical aperture 1.30 oil immersion objective, and digital charge-coupled device camera (Orca-R2; Hamamatsu, Hamamatsu City, Japan). Image manipulations were performed using ImageJ software.

Micrococcal nuclease protection assay

The micrococcal nuclease (MNase) protection assay was performed as described (Crisucci and Arndt, 2012). Briefly, 185-ml yeast cultures were processed as described for RNA isolation and analysis and cross-linked with a final concentration of 2% formaldehyde while being shaken at 30°C for 30 min. Formaldehyde was quenched with glycine, cells collected, and spheroplasts prepared by Zymolyase treatment. Spheroplasts (300 µl) were treated with increasing concentrations of MNase (10107921001; Roche) at 0, 1.0, 2.5, 5.0, 10, or 20 U MNase at 37°C for 45 min on a nutator. Reactions were stopped by addition of SDS (1% final concentration) and EDTA (10 mM final concentration), DNA purified by phenol-chloroform extraction, and recovered by ethanol precipitation. The efficiency of each MNase digestion was determined using a qPCR primer set flanking a region in the *GAL1-10* upstream activating sequence (UAS) protected from MNase digestion by a nucleosome and a primer set flanking a region in the *GAL1-10* UAS not protected from MNase digestion. Samples with MNase digestion efficiency of ~95% were used for subsequent qPCR analysis using tiled primer sets. Primer sequences are listed in Supplemental Table S2.

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