LETTERS

A putative stimulatory role for activator turnover in gene expression

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The ubiquitin–proteasome system (UPS) promotes the destruction of target proteins by attaching to them a ubiquitin chain that is recognized by the 26S proteasome[1]. The UPS influences most cellular processes, and its targets include transcriptional activators that are primary determinants of gene expression. Emerging evidence indicates that non-proteolytic functions of the UPS might stimulate transcriptional activity[2,3]. Here we show that the proteolysis of some transcriptional activators by the UPS can stimulate their function. We focused on the role of UPS-dependent proteolysis in the function of inducible transcriptional activators in yeast, and found that inhibition of the proteasome[4] reduced transcription of the targets of the activators Gcn4, Gal4 and Ino2/4. In addition, mutations in SCF^{Cdc4}, the ubiquitin ligase for Gcn4 (ref. 5), or mutations in ubiquitin that prevent degradation[6], also impaired the transcription of Gcn4 targets. These transcriptional defects were manifested despite the enhanced abundance of Gcn4 on cognate promoters. Proteasome inhibition also decreased the association of RNA polymerase II with Gcn4, Gal4 and Ino2/4 targets, as did mutations in SCF^{Cdc4} for Gcn4 targets. Expression of a stable phospho-site mutant of Gcn4 (ref. 7) or disruption of the kinases that target Gcn4 for turnover[5,7] alleviated the sensitivity of Gcn4 activity to defects in the UPS.

The UPS is a fundamental component of normal cell growth and proliferation. The UPS is also important for cancer cell growth, as highlighted by the recent approval of the proteasome inhibitor, Velcade, for the treatment of relapsed multiple myeloma[8]. Recent studies have investigated the mechanism of Velcade action by examining the transcriptional response to the drug in human and yeast cells[9,10]. These studies indicate that proteasome inhibition does not substantially alter bulk transcription of the genome in myeloma cells or in *Saccharomyces cerevisiae*. However, a group of genes are repressed by Velcade, including human growth and survival genes and yeast genes involved in the biosynthesis of amino acids[9,10]. These yeast genes are regulated by the b-ZIP transcriptional activator Gcn4, which promotes the expression of more than 500 genes[11]. Gcn4 is a target for UPS-mediated degradation[12] through the E3-ubiquitin ligase SCF^{Cdc4}. Ligases comprise the final, substrate recognition step of the ubiquitination cascade. SCF^{Cdc4} ubiquitinates and targets for proteolysis Gcn4 molecules that have been phosphorylated by the cyclin-dependent kinases (CDKs) Srb10 and Pho85 (refs 5, 7). We have attempted to explain the role of the UPS in the function of Gcn4 and other activators.

To assess the impact of proteolysis on Gcn4 function, we treated a yeast strain (*pdr5Δ*) that is sensitive to proteasome inhibitors[10] with the Velcade analogue MG132 (ref. 4). Reverse transcriptase-mediated polymerase chain reaction (RT–PCR) analyses confirmed that, as for Velcade[10], treatment with MG132 substantially reduced the transcription of several Gcn4 targets, including *HIS4* and *CPA2*, in minimal medium, in comparison with dimethylsulphoxide (DMSO) alone (Fig. 1a and Supplementary Fig. S1a). Similar results were obtained when Gcn4 was highly induced by amino acid starvation (Fig. 1a, −Leu; see Supplementary Information for discussion of media). These effects were largely dependent on Gcn4, because we observed similar decreases in the transcription of a reporter driven exclusively by six Gcn4-binding sites[13] (Fig. 1a, *GCRE6–LacZ*).

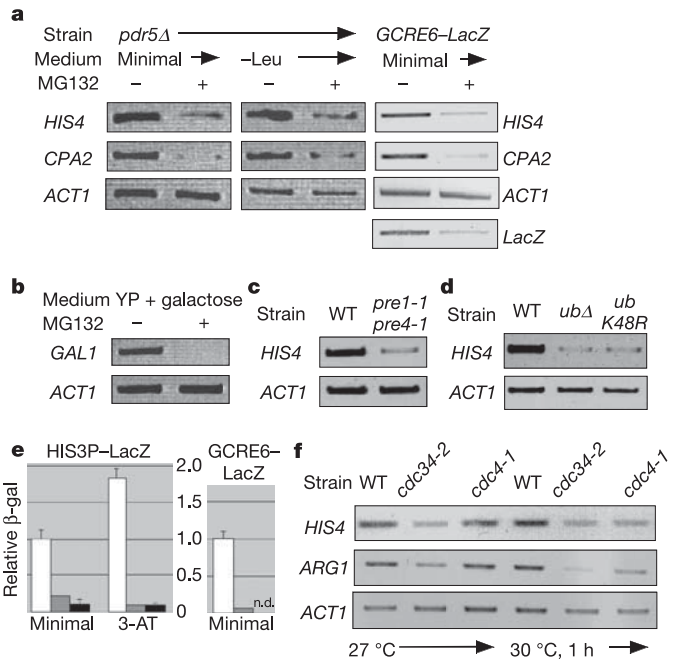

Figure 1 | UPS-dependent proteolysis positively regulates inducible transcriptional activators. a, Indicated strains were grown in minimal or starvation (−Leu) medium, treated with MG132 (50 μM) or DMSO, and processed for RT–PCR of the indicated transcripts. *pdr5Δ* enables the uptake of MG132 into yeast. **b**, A *pdr5Δ* strain was induced with galactose, treated with MG132 and prepared for RT–PCR of *GAL1* and *ACT1*. **c**, wild-type (WT) and *pre1-1*, *pre4-1* strains were grown in minimal medium at 27 °C and processed for RT–PCR of *HIS4* and *ACT1*. **d**, Conditionally expressed ubiquitin was depleted from strains while expression from a complementing plasmid encoding either WT ubiquitin, no ubiquitin (Δ) or K48R-ubiquitin was induced (see Supplementary Information). Samples were prepared for RT–PCR as above. **e**, WT (white bars), *cdc34-2* (grey bars) and *gcn4Δ* (black bars) strains expressing LacZ from the *HIS3* or GCRE6 promoter were grown in minimal or starvation medium (3-AT) at 30 °C and then processed to test β-galactosidase (β-gal) activity. Standard deviations are from three replicates. n.d., not detectable. **f**, WT, *cdc34-2* and *cdc4-1* strains were grown at 27 °C or shifted to 30 °C for 1 h. RT–PCR was performed for *HIS4*, *ARG1* and *ACT1*.

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We also tested the proteasome dependence of two regulons (GAL and INO) that might have eluded the Velcade microarray analysis because they were not expressed under the growth conditions that were employed. MG132 substantially decreased induction of the Gal4 target, *GAL1*, on the addition of galactose (Fig. 1b). Similarly, on the removal of inositol, transcription of *INO1*, a target of the activators Ino2 and Ino4, was largely abrogated by MG132 (Supplementary Fig. S1b). In all cases treatment with MG132 did not affect the constitutive transcription of *ACT1*.

These findings indicate that the 26S proteasome might promote the function of some inducible transcriptional activators. Focusing on Gcn4, we tested additional components of the UPS for their impact on transcription. A strain (*pre1-1* and *pre4-1*) mutated for the peptidase activity of the proteasome showed reduced transcription of *HIS4* in comparison with a wild-type (WT) strain (Fig. 1c)[14]. We also tested transcription of *HIS4* in strains that conditionally express alternative versions of ubiquitin[6]. When endogenous ubiquitin was depleted in the presence of a vector plasmid (UbΔ) or depleted in a cell expressing K48R ubiquitin, *HIS4* messenger RNA levels were sharply decreased in comparison with a depleted strain that expressed WT ubiquitin (Fig. 1d). Again, the *ACT1* mRNA remained constant. These findings confirm that ubiquitination and proteolysis are important for Gcn4 function. Importantly, because the K48R mutant cannot form chains that target substrates to the proteasome, these findings also indicate that, in contrast to the regulation proposed for Gal4–VP16 (ref. 15) and c-Myc[16,17], mono-ubiquitination might not be able to sustain Gcn4 activity.

We next examined the impact of Cdc34–SCF^{Cdc4}, the specific E2–E3 ubiquitin ligase for Gcn4 (refs 5, 7), on activator function. Gcn4-dependent expression of β-galactosidase was evaluated in WT, temperature-sensitive *cdc34-2*, and *gcn4Δ* strains. After growth at the semi-permissive temperature of 30 °C, the *cdc34-2* strain exhibited a fourfold decrease (relative to WT) in reporter expressed from the *HIS3* promoter (HIS3P) in minimal medium and a roughly 15-fold reduction on starvation by 3-aminotriazole (3-AT, ref. 11) (Fig. 1e). Expression from GCRE6–LacZ was also compromised about 10-fold in *cdc34-2* (Fig. 1e). In addition, expression from both promoters was extinguished in *gcn4Δ* cells under all conditions (Fig. 1e). RT–PCR analysis was then used to assess effects on endogenous targets. Gcn4-dependent transcription of *HIS4* and *ARG1* was defective in both *cdc34-2* and *cdc4-1* (a thermosensitive allele of the SCF F-box protein Cdc4) strains at 30 °C (Fig. 1f). In contrast, *ACT1* transcript levels in all strains were similar. These results point to a stimulatory role for Cdc34–SCF^{Cdc4} in Gcn4-mediated transcription.

To explore the molecular basis of the stimulation of Gcn4 function by the UPS, we examined the effect that proteasome inhibition has on the abundance and ubiquitination of Gcn4. Western blotting showed that MG132 increased the abundance of chromosomally encoded Gcn4–Myc9 and led to the appearance of a high-molecular-mass ladder (Fig. 2a, lanes 1 and 2). Immunoprecipitation and western analysis with anti-ubiquitin antibodies confirmed that this ladder was ubiquitinated Gcn4 (Fig. 2a, lanes 5, 6, 9 and 10). Because transcription mediated by Gcn4 was strongly repressed by MG132 (Fig. 1), ubiquitination was presumably insufficient to sustain Gcn4 activity. To confirm the specificity of Gcn4 ubiquitination, we repeated the analysis with a *gcn4–3T2S* strain. Gcn4–3T2S lacks five phosphorylation sites, is no longer phosphorylated by Srb10 or Pho85 nor ubiquitinated by SCF^{Cdc4} *in vitro*, and is stabilized[7]. As predicted, the *3T2S* strain had much lower levels of ubiquitinated Gcn4 (Fig. 2a, lanes 3, 4, 7, 8, 11 and 12).

We next investigated promoter occupancy by Gcn4 and Gal4 and recruitment of RNA polymerase II (polII) to target genes. Chromatin immunoprecipitation (ChIP) assays[18] revealed a substantial increase in the association of Gcn4–Myc9 with the *HIS4* promoter in cells treated with MG132 (Fig. 2b, 9E10). Similar experiments showed little change in levels of TAP-tagged Gal4 at the *GAL1* promoter on treatment with MG132 (Fig. 2c, IgG). The galactose-dependent

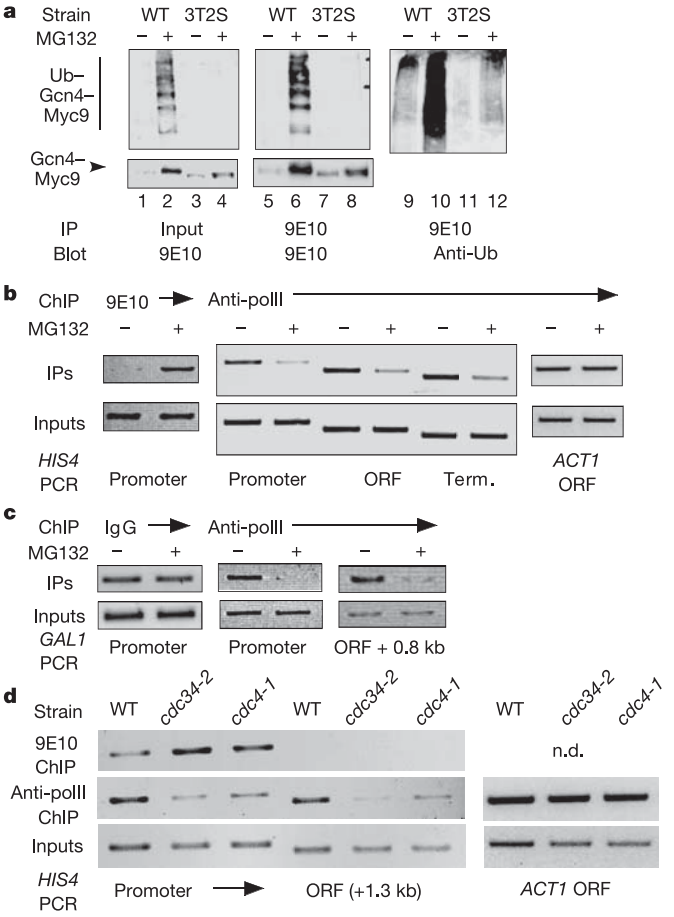

Figure 2 | Defects in the UPS lead to the accumulation of ubiquitinated Gcn4 and impair the association of RNA polymerase II with Gcn4 and Gal4 targets. a, *pdr5Δ* strains expressing Gcn4–Myc9 (WT) or Gcn4–3T2S–Myc9 (3T2S) were grown in minimal medium and treated with MG132 or DMSO. Immunoprecipitations (IPs) were performed with 9E10 antibodies recognizing Myc9, and subsequent western blots of the input (lanes 1–4) or immunoprecipitation (IP; lanes 5–12) samples were probed with 9E10 (lanes 1–8) or anti-Ub antibodies (lanes 9–12). **b**, A WT strain, as above, was processed for ChIP analysis with 9E10 and anti-polII antibodies. PCR was performed to amplify the promoter, ORF and terminator regions of *HIS4* and the ORF of *ACT1*. **c**, ChIP analysis of Gal4–TAP and polII was performed with galactose-induced strains treated with MG132 or DMSO. The promoter and ORF regions of *GAL1* were amplified. IgG was used to retrieve Gal4–TAP. **d**, ChIP analysis of Gcn4–Myc9 and polII was performed, as in **b**, with WT, *cdc34-2* and *cdc4-1* strains that were grown at 27 °C, then shifted to 30 °C for 1 h. kb, kilobases; n.d., not done.

decrease in promoter-bound Gal80–TAP was also unaffected by MG132 (Supplementary Fig. S3). When the ChIP was performed with polII, MG132 reduced the signal for the promoter, open reading frame (ORF) and terminator regions of the Gcn4 target *HIS4*, whereas the signal for the *ACT1* ORF was unaffected (Fig. 2b, polII). MG132 also decreased the polII ChIP signal for the *GAL1* promoter and ORF (Fig. 2c, polII) and the *INO1* promoter (data not shown).

The ChIP analysis was extended to *cdc34-2* and *cdc4-1* strains. At 30 °C, more Gcn4–Myc9 (Fig. 2d, 9E10) but less polII was associated with the *HIS4* promoter in the SCF mutants than in the WT. Recruitment of polII to *ACT1* was unaffected in the SCF mutants. Results from all the ChIP analyses closely parallel the transcription results and imply that the UPS is important for sustaining the interaction of RNA polymerase II with the targets of some activators despite the increased accumulation of activator (for Gcn4) at the promoter.

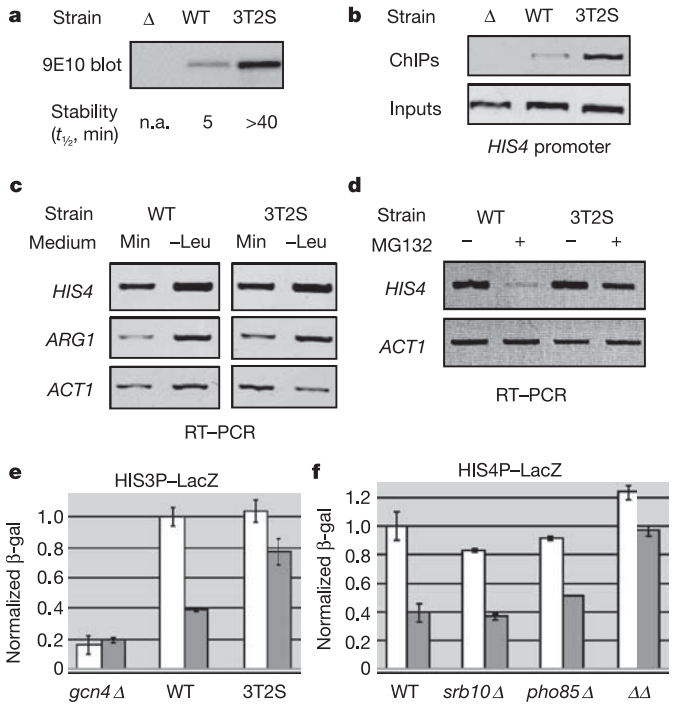

Figure 3 | The UPS has little effect on the activity of stable, non-phosphorylated versions of Gcn4. a, *GCN4* (WT), *gcn4–3T2S* or *gcn4Δ* strains were grown in minimal medium and processed for western blotting with 9E10 antibodies. Protein half-life data ($t_{1/2}$) were reported previously[7]. n.a., not applicable. **b**, ChIP analysis of the above strains was performed for the *HIS4* promoter. **c**, RT–PCR of the indicated transcripts were performed with WT and *gcn4–3T2S* strains grown in minimal (Min) or starvation (−Leu) medium. **d**, RT–PCR analysis of *HIS4* and *ACT1* was performed with WT and 3T2S strains in the presence and absence of MG132. **e**, *CDC34* (open bars) and *cdc34-2* (filled bars) strains with the HIS3P-LacZ reporter and expressing either WT, 3T2S or null (*gcn4Δ*) versions of *GCN4* were grown in minimal medium and processed for β-galactosidase (β-gal) activity. Standard deviations were calculated from three replicates. **f**, WT and *cdc34-2* strains with a HIS4P-LacZ reporter and harbouring WT or deleted versions of *SRB10* and/or *PHO85* were treated as in **e**.

To test whether turnover of the activator itself can promote function, we evaluated the stabilized Gcn4–3T2S (ref. 7). In minimal medium the levels of total protein (Fig. 3a) and promoter-associated Gcn4–Myc9 (Fig. 3b) were about twofold to threefold higher in the *gcn4–3T2S* strain. Despite such increases, *gcn4–3T2S* did not alter the expression of Gcn4 targets in minimal (Fig. 3c, SM +; Fig. 3e, *CDC34*) or starvation medium (Fig. 3c, −Leu), indicating a possible decrease in specific activity. Most importantly, *gcn4–3T2S* partly alleviated the deleterious effects of an impaired UPS on Gcn4-dependent transcription. For example, MG132 inhibited *HIS4* transcription in *gcn4–3T2S* much less than in *GCN4* (Fig. 3d). In addition, *cdc34-2* diminished HIS3P–LacZ expression only about 1.2-fold in *gcn4–3T2S*, in comparison with more than 2.5-fold in *GCN4* (Fig. 3e). The *cdc34-2* mutation had no impact on *gcn4Δ* (Fig. 3e). A similar epistatic relationship was seen on deletion of *SRB10* and *PHO85*, which stabilizes Gcn4 to a similar extent to the *gcn4–3T2S* mutation[7]. Deletion of both CDKs (ΔΔ) slightly increased LacZ expression (1.2-fold) and, as with *gcn4–3T2S*, *cdc34-2* only mildly affected expression (1.2-fold) in the ΔΔ strain (Fig. 3f). The suppression of *cdc34-2* required the deletion of both kinases, because *srb10Δ* and *pho85Δ* single mutants remained relatively sensitive to *cdc34-2* (Fig. 3f). We note that *gcn4–3T2S* is not completely refractory to UPS inhibition, indicating that, in addition to Gcn4, the UPS might also promote transcription through other factors. Nevertheless, these findings indicate that proteolysis of CDK-phosphorylated Gcn4 by means of the UPS might be important in sustaining maximal expression of Gcn4 targets and that, in the absence of phosphorylation, Gcn4 activity is less dependent on its turnover.

Components of the UPS have been posited to activate transcription by multiple mechanisms[2,3]. In numerous examples, including the activation of Gcn4 in response to ultraviolet radiation[19] and transcription of NF-κB (ref. 2) and oestrogen receptor targets[20], the UPS seems to mediate signalling upstream of the activator. As discussed in Supplementary Fig. S3, this mechanism probably does not account for our findings. Meanwhile, subunits of the 19S cap of the proteasome have been suggested to have a positive role in transcription that is independent of their proteolytic function[21,22]. In addition, it has been proposed that the ubiquitination of Gal4–VP16 and c-Myc transiently increases the activity of these factors before proteolysis[15–17]. Our results differ substantively from these examples, in that neither the 19S cap (whose activity is not known to be affected by inhibition of the 20S proteases[23]) nor ubiquitination was sufficient to achieve maximal transcription of Gcn4 targets (Figs 1d and 2a). Instead, we found that inhibition of the proteasome and genetic manipulations of the UPS, the CDKs for Gcn4, and Gcn4 itself all provided evidence that turnover of Gcn4 normally enhances its function.

Proteasome activity also seems to sustain inducible transcription mediated by Gal4 and Ino2/4. Interestingly, activation of promoter-associated Gal4 in galactose medium requires Srb10-dependent phosphorylation[24,25], and this activated form has a short half-life[24]. This indicates that Gal4 might be regulated by degradation in a manner similar to that of Gcn4. We previously proposed a model consistent with these current findings in which proteolysis is required to remove 'spent' activators and to reset the promoter[3]. The initial 'pioneer round(s)' of transcription would not involve the UPS, but subsequent rounds would be stimulated by turnover of the spent, promoter-bound activator to allow binding of a fresh molecule. This mechanism places Gcn4, Gal4 and Ino2/4 into a class of regulatory factors—including securin, p21 and p27—whose activity is required early in a process but whose subsequent turnover or removal promotes completion of the process or subsequent reaction cycles. We call this phenomenon 'activation by destruction' and believe that, given the diversity of the examples listed in Supplementary Table S2, it might represent a regulatory mechanism for a large class of factors and might be an important determinant of infection and disease.

METHODS

Yeast strains, growth conditions and extract preparation. A complete list of yeast strains used in this study is provided in Supplementary Table S1. All strains were derived from the S288C background, except RJD 2505 and RJD 3137–3141, which were derived from the W303 background. Strains were constructed and grown in accordance with standard protocols[26]. A description of the various media used in the study is given in Supplementary Information. MG132 (American Peptide) was added to cultures of *pdr5Δ* strains to a final concentration of 50 μM. All extracts were prepared by lysis with glass beads (Sigma) in a Fast Prep (Bio 101) device. Ubiquitin derivative analysis (Fig. 1d) is described in the Supplementary Methods.

RT–PCR analysis. mRNA was prepared using RNeasy kits (Qiagen). Total mRNA (200 ng) and 10 pmol of oligo(dT) were used to reverse-transcribe complementary DNA (Stratagene). One-tenth of the cDNA reaction was then used for 20–22 cycles of PCR and products were resolved on 2% agarose gels. Primer sequences are available from the authors on request.

β-Galactosidase assays. The HIS3P–LacZ, HIS4P–LacZ and GCRE–LacZ reporter constructs and the protocol to measure β-galactosidase activity were as described previously[27]. Reported activity was normalized to total extract protein as measured by bicinchoninic acid assay (Pierce). Relative activities are reported with average WT activity set to 1.

Western blots. Except as noted, extracts were prepared by immediate boiling of cell pellets in 2 × Laemmli SDS sample buffer followed by lysis with glass beads. Equal amounts of total protein (as judged by Coomassie staining) were resolved by SDS–PAGE. Blots were probed with 9E10 antibodies to recognize Gcn4–Myc9 or FK1 antibodies (Affiniti) to recognize ubiquitin. Horseradish peroxidase-coupled goat anti-mouse secondary antibodies (Bio-Rad) were used for detection.

Immunoprecipitations and detection of ubiquitinated Gcn4. DMSO-treated or MG132-treated cultures were treated with formaldehyde (final concentration 1%) for 20 min to trap ubiquitinated intermediates. Crosslinking was quenched and extracts were made in ChIP buffer[18]. A 10% sample of the extract (whole cell extract) was removed and boiled in SDS sample buffer. The remainder of the extract was incubated at 4 °C with 9E10 antibodies coupled to Protein A–Sepharose (Sigma) beads. Proteins were eluted and crosslinks were reversed by being boiled in SDS sample buffer. Proteins samples were then processed for western blotting. For FK1 (anti-ubiquitin) western blots, the nitrocellulose was boiled before incubation with the antibody.

ChIP assays. ChIP assays were performed as described[18]; 9E10 antibodies were used to immunoprecipitate chromatin fragments associated with Gcn4–Myc9 and antibodies against the carboxy-terminal domain of the largest subunit of RNA polymerase II (anti-polII; Covance) were used to immunoprecipitate fragments associated with polII. Protein G–Sepharose beads (Amersham Biosciences) were used to precipitate antibody–antigen complexes. Rabbit immunoglobulin (Ig) G–agarose (Sigma) was used to immunoprecipitate Gal4-associated fragments. Primer sequences are available from the authors on request.

Received 2 June; accepted 3 August 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank D. Finley, D. H. Wolf, R. Young, A. Hinnebusch, J. Shaw, B. Westermann, J. Nunnari and G. Braus for gifts of strains and reagents; B. Tansey for communicating results before publication; and J. Shaw, B. Westermann, J. Nunnari, S. Sadis, A. Ansari and the members of the Deshaies laboratory for comments and criticism. This work was supported in part by an NIH Research Project Grant to R.J.D. R.J.D. is an Investigator of the Howard Hughes Medical Institute. J.R.L. was supported by an NIH National Research Service Award and a Caltech-Amgen Postdoctoral Fellowship.

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