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A tethered catalysis, two-hybrid system to identify protein-protein interactions requiring post-translational modifications

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We have modified the yeast two-hybrid system to enable the detection of protein-protein interactions that require a specific post-translational modification, using the acetylation of histones and the phosphorylation of the carboxyl terminal domain (CTD) of RNA polymerase II as test modifications. In this tethered catalysis assay, constitutive modification of the protein to be screened for interactions is achieved by fusing it to its cognate modifying enzyme, with the physical linkage resulting in efficient catalysis. This catalysis maintains substrate modification even in the presence of antagonizing enzyme activities. A catalytically inactive mutant of the enzyme is fused to the substrate as a control such that the modification does not occur; this construct enables the rapid identification of modification-independent interactions. We identified proteins with links to chromatin functions that interact with acetylated histones, and proteins that participate in RNA polymerase II functions and in CTD phosphorylation regulation that interact preferentially with the phosphorylated CTD.

Protein-protein interactions are fundamental to biological processes. For the yeast *Saccharomyces cerevisiae,* thousands of interactions have already been detected among its 6,000 proteins[1]. However, because most of the currently available data were derived from studies of unmodified proteins, interactions that require specific post-translational modifications would likely have escaped detection. For example, 14-3-3 proteins, which have crucial functions in cell cycle control, apoptosis and metabolic regulation, interact with their target proteins often only when the latter are phosphorylated at specific serine or threonine residues[2]. The SH2 and PTB domains specifically recognize phosphotyrosines[3]. Histones that are acetylated or methylated at specific lysine residues recruit bromodomain- and chromodomain-containing proteins, respectively[4]. Conversely, post-translational modifications may inhibit protein-protein interactions. For example, the Silent Information Regulator protein Sir3 binds preferentially to unacetylated histones for transcriptional silencing; acetylation of histones prevents Sir3 recruitment and consequently leads to loss of silencing[5].

A variety of biochemical and genetic approaches are available to identify protein-protein interactions, with the yeast two-hybrid system[6] being the most widely used genetic assay. To identify interactions that are induced by specific post-translational modifications, previous strategies[7–9] have been based on ectopically overexpressing protein kinases in the two-hybrid hosts that normally lack such enzymes. This design is necessary to avoid cellular phosphatase activities. However, deleterious effects have been linked to overexpression of heterologous kinases[9, 10], possibly due to unregulated modification of host proteins. Furthermore, overproduction of native enzymes at a high level may also cause cellular growth arrest[11]. Taylor and colleagues[12] tested specific phosphotyrosine-dependent interactions of insulin receptor substrate 3 (IRS3) in a yeast two-hybrid assay by fusing IRS3 to the insulin receptor in the context of a DNA-binding domain hybrid. We have extended this approach, which we term tethered catalysis, to demonstrate that native yeast modifications can occur in this context, be maintained despite the presence of antagonizing enzymatic activities and lead to two-hybrid signals dependent on acetylation or phosphorylation. More importantly, we demonstrate that screens of an activation domain library or a high-throughput array can identify novel modification-dependent binding activities.

In the tethered catalysis approach, efficient and specific modification of the protein fused to the DNA-binding domain ('bait' protein) occurs via the inclusion of the modifying enzyme as part of this hybrid. In the first test case, we fused the N-terminal tail domains of histones H3 and H4 to the catalytic domain[13] of the prototypic histone acetyltransferase Gcn5 (**Fig. 1a**). We anticipated that the physical linkage between the enzyme and its cognate substrate would result in constitutive acetylation of the histone tail. As a control, the histone tail domains were also fused to a catalytically inactive mutant of Gcn5, F221A (ref. 14). A point mutation abolishes the enzymatic activity of Gcn5 *in vitro* and *in vivo*[14]. Each of these enzyme-substrate combinations was fused to the Gal4 DNA-binding domain (GDBD) and a trimeric hemagglutinin (HA) epitope tag.

We tested whether the histone-Gcn5 chimeras auto-acetylated *in vivo*. The fusion proteins were immunoprecipitated from yeast extracts by an anti-HA antibody, followed by western blot analysis

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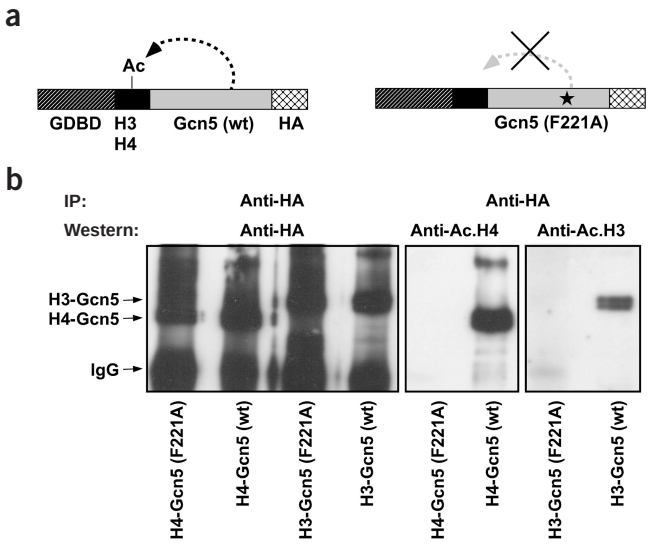

Figure 1 Acetylation of H3 and H4 when tethered to the catalytic domain of wild-type Gcn5. (**a**) Schematic drawing of the tethered catalysis histone baits. GDBD, Gal4 DNA binding domain. Ac, acetylation. Wt, wild type. (**b**) H3 and H4 acetylation shown by immunoprecipitation/western blot analyses. The fusion proteins were immunoprecipitated by an anti-HA antibody followed by western blot detection using antibodies against HA (left), acetylated H3 at Lys9 and Lys 14 (right) or acetylated H4 at Lys8 (middle). The doublets of each of the protein bands were results of gel artifacts.

with antibodies against HA (to quantify the amount of protein), or against the acetylated H3 or H4 (to assess the acetylation status). Both histones, when fused to the wild-type Gcn5, were acetylated, whereas no obvious acetylation of H3 or H4 was detected with the mutant Gcn5 fusion, even though the yeast produces the endogenous wild-type Gcn5 (**Fig. 1b**). There are about a dozen histone acetyltransferases and deacetylases in yeast[15]. We conclude that the acetylation of the chimeric H3 and H4 was predominantly carried out by the tethered Gcn5 catalytic domain, and that the autocatalysis observed from the H3-Gcn5 and H4-Gcn5 fusions is dominant over the antagonizing deacetylases.

We also tested whether tethered catalysis occurred in the yeast Ras recruitment system[16] or in *Escherichia coli*. We found that in both cases, histone tails were quantitatively acetylated by the linked Gcn5 but not by the F221A mutant (data not shown). In addition, both Gal4 and LexA DNA-binding domain fusions yielded comparable autoacetylation of H3 and H4 (data not shown). Thus, this tethered catalysis strategy is likely compatible with most two-hybrid genetic approaches.

We next performed two-hybrid screens using both an activation domain library and a high-throughput array. For the library screen, a yeast strain bearing the H3-Gcn5 construct was transformed with a yeast genomic library fused to the Gal4 activation domain[17]. The reporter strain carries the *ADE2* gene under the control of the *GAL2* promoter. Activation domain fusion plasmid DNA ('prey') was isolated from Ade$^+$ transformants (indicating activation of the two-hybrid reporter gene) and retransformed into yeast strains bearing one of the following DNA-binding domain and HA hybrids: H3-Gcn5(wild type), H3-Gcn5(F221A), H4-Gcn5(wild type), H4-Gcn5(F221A), Gcn5(wild type), or Gcn5(F221A). This panel of histone and Gcn5 fusion baits provides a comparison of the specificity of the interactions.

From about 30,000 transformants, 3 out of 40 Ade$^+$ transformants showed specific interactions with H3-Gcn5(wild type). One of these was Rpm2 (**Fig. 2a**), a multifunctional, essential protein[18]. Although first identified as the protein component of a nonessential mitochondrial RNAse P complex, Rpm2 copurified with the nuclear protein kinase, Hrr25, implicated in DNA damage repair[19]. The two-hybrid interaction specificity test showed that Rpm2 strongly prefers acetylated H3 (**Fig. 2a**). These results thus link Rpm2 to chromatin metabolism. The other plasmids (data not shown) encoded Cin5/Hal6/Yap4 and the intergenic region between the *MCA1* and *LIP5* genes, which is likely an artifact (see **Supplementary Table 1** online for details).

In the array approach[20], a yeast strain bearing either the acetylated H3 or H4 Gcn5 hybrid was mated against an array of yeast strains of the opposite mating type bearing ~6,000 activation domain–open reading frame fusions. After diploid selection, the *GAL1-HIS3* and *GAL2-ADE2* reporters were used to identify two-hybrid positive colonies. Array screens were done in duplicate for each DNA-binding domain hybrid and only two-hybrid interactions that scored in both duplicates were considered to represent putative acetylated histone-binding proteins. Consistent with the previous findings that the promoter-tethered Gcn5 is sufficient to activate transcription[21], all baits containing the wild-type Gcn5 catalytic domain activated transcription and resulted in varying degrees of resistance to 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the His3 enzyme (data not shown). Intriguingly, we noticed a gradient of transcriptional

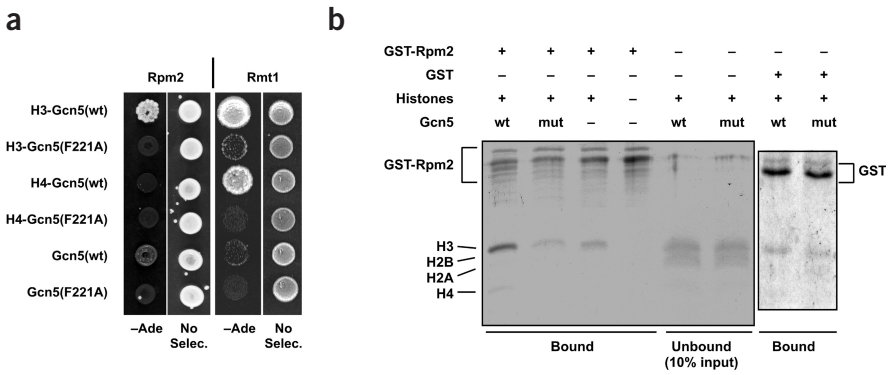

Figure 2 Rpm2 and Rmt1 bind acetylated histones. (**a**) Rpm2 and Rmt1 display different two-hybrid interaction specificities for acetylated histones. Yeast cells transformed with the indicated combinations of bait and prey constructs were grown to early log phase and equal numbers of cells were spotted to –Ade (Synthetic complete medium (SC), –leucine, –tryptophan, –Ade) and no selection (SC, –leucine, – tryptophan) plates (no selec.). Pictures were taken after 4 (Rmt1) and 7 (Rpm2) d of incubation at 30 °C. Note that the Gcn5 (wild type) alone causes weak Ade$^+$ growth after longer incubation. (**b**) Confirmation of the acetylated H3-Rpm2 interaction by GST pulldown assays. Treatment of chicken histones with the wild-type Gcn5 resulted in hyperacetylation of H3 and moderate acetylation of H2B and H4 (data not shown). Rpm2 is able to capture H3 and, to a lesser degree, H4 from the wild-type Gcn5-treated histone samples. Compared with the GST alone controls, the untreated or mock-acetylated histone H3 was retained only slightly by Rpm2 protein. Such weaker retention may reflect the background level of acetylation of chicken histones. The position of the Rpm2 protein is marked by the bracket on the left; the multiple bands were likely due to premature translational termination (data not shown). GST alone controls are shown on the right.

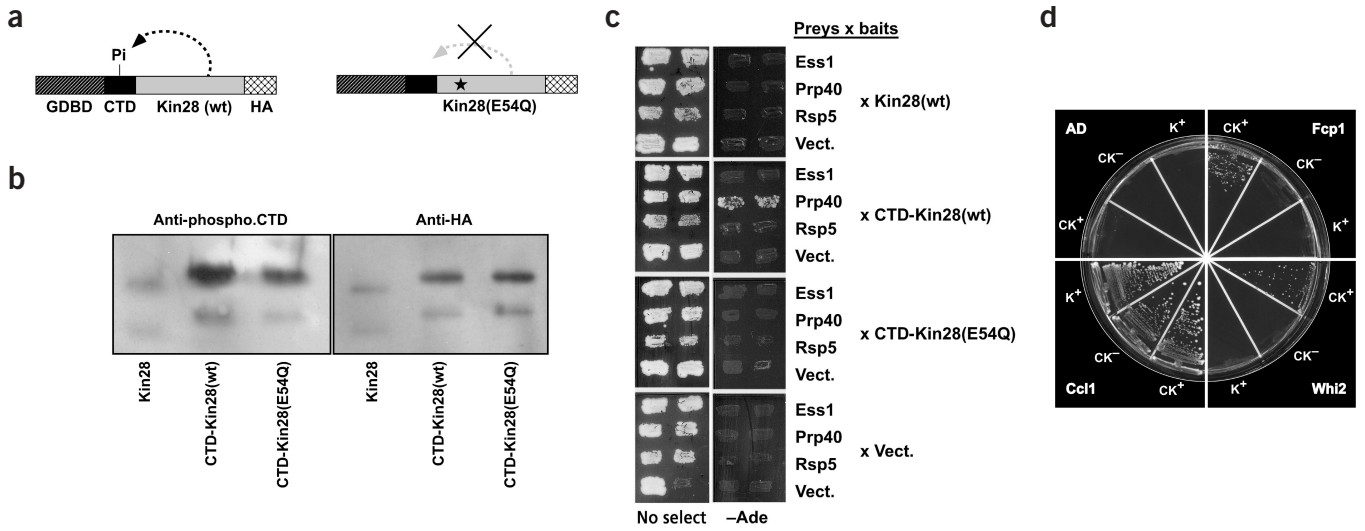

Figure 3 Autophosphorylation of CTD-Kin28 chimera. (**a**) Schematic drawing of the fusion proteins. Three tandem copies of CTD (YSPTSPS) were included in the constructs used for screening. Ser5 (underlined) is the predominant site of phosphorylation by Kin28. A longer repeat (ten copies) was used only for the affinity comparison (**Supplementary Figure 1** online). (**b**) Phosphorylation of the CTD occurs with the tethered wild-type Kin28 but not with the E54Q catalytically inactive Kin28. The three GDBD fusion proteins were expressed in bacteria and analyzed by western blot analyses. The antibody against the Ser5-phosphorylated CTD also showed weak nonspecific binding to the bait protein without the CTD (first lane). (**c**) The phosphorylation-dependent interaction between Prp40 and phosphorylated CTD can be detected in the yeast two-hybrid context. All three proteins were expressed from multicopy vectors. A weaker interaction was seen when AD-PRP40 was on a low-copy ARS/CEN plasmid (not shown). Yeast cells from two independent colonies were grown in the absence of adenine to test for two-hybrid interactions. No selection, SC, –leucine, –tryptophan. (**d**) Fcp1 and Whi2 are phospho-CTD-interacting proteins identified by the high-throughput yeast two-hybrid array screens. Candidate and control strains were streaked onto a plate containing 10 mM 3-AT and incubated for 7 d to compare their abilities to interact with CTD and Kin28 (also see **Supplementary Figure 1** online for the complete comparison). Ccl1 is a known Kin28-interacting protein whose association with Kin28 is not affected by the enzymatic activity of Kin28, or the presence of the CTD. In contrast, Fcp1 (a phospho-CTD phosphatase) and Whi2 interact only with the CTD fused to the wild-type Kin28, suggesting that these two proteins associate preferentially with phosphorylated CTD. CK^+, CTD-Kin28(wild type); CK^-, CTD-Kin28(E54Q); K^+, Kin28(wild type) alone.

activation potency (Gcn5 > H4-Gcn5 > H3-Gcn5), which is also consistent with the observations that histone N-terminal tails counteract transcriptional activation[22], and that different histone tails display different degrees of transcriptional repression[23]. Thus, more stringent selection (*e.g.*, higher concentrations of 3-AT, or ability to confer the Ade^+ phenotype as used in the library screen) was applied to identify acetylated histone-binding proteins, which may favor the detection of stronger interactions. Six positives were identified: Rmt1/Hmt1 (H3 and H4), Cac1/Rlf2 (H3), Exo84 (H3), Yap6 (H4), Cbf1 (H4) and Rgm1 (H4). Rmt1 is an arginine-specific methyltransferase whose activity is important for nuclear-cytoplasmic shuttling of mRNA-binding proteins[24]. Rmt1 is linked to chromatin function in that it specifically methylates the arginine residue at position 3 of the histone H4 N-terminal tail[25]. Most of the other proteins identified in the array screens possess chromatin or transcription-related functions (see **Supplementary Table 1** online for description and references).

The acetylated histone-binding specificity of Rmt1 was also tested against the panel of histone/Gcn5 baits (**Fig. 2a**). Strong Ade^+ growth was seen with the acetylated H3 or H4 bait, and relatively weak Ade^+ growth with the H3-Gcn5(F221A) bait (**Fig. 2a**). These growth differences indicate that Rmt1 binds acetylated H3 and H4 strongly and may have some affinity for hypoacetylated H3.

To biochemically confirm an acetylation-dependent interaction, we conducted glutathione S-transferase (GST) pull-down experiments on Rpm2 (**Fig. 2b**). The Rpm2 fragment identified in the two-hybrid screen (encoding amino acids 324–871) was cloned into a bacterial GST expression vector. The purified GST-Rpm2 or GST alone was immobilized on a glutathione matrix and incubated with chicken histones pretreated with acetyl coenzyme A and wild type or a catalytically inactive Gcn5. Pretreatment with the wild-type Gcn5 caused efficient acetylation of H3 and modest acetylation of H4 and H2B (not shown). Compared with the GST alone control, Rpm2 efficiently captured the wild-type Gcn5-treated H3 (**Fig. 2b**), consistent with the two-hybrid results. A small amount of H4 in the wild-type Gcn5-treated sample was also retained by Rpm2, which may be a result of a weak interaction between Rpm2 and (acetylated) H4 that was not detected in the two-hybrid assays. Similar GST pulldown results were also observed when Yap6 and Rmt1 were used to bind the acetylated histones (data not shown).

Certain acetylated histone species recruit bromodomain-containing proteins[4]. However, none of the yeast bromodomain-containing proteins was identified in our array screens. We tested specifically whether the bromodomains from two such proteins, the yeast Bdf1 and the human PCAF, interacted with the H3- or H4-Gcn5 bait. Only moderate activation of the reporter *GAL7-lacZ* was observed (data not shown). This relatively weak interaction likely reflects the reported dissociation constant (8.4 ± 0.2 μM) between the Bdf1 double bromodomains and a hyperacetylated H4 *in vitro*[26]. In comparison, the apparent K_M value of recombinant Rmt1 arginine methyltransferase has been calculated to be 0.05 ± 0.02 μM when a monoacetylated histone H4 peptide was used as the substrate for methylation reactions (M.-H. Kuo, unpublished data).

To examine the general applicability of the tethered catalysis yeast two-hybrid system, we tested interactions dependent on phosphorylation. We fused three tandem repeats of the heptapeptide CTD of the largest subunit of RNA polymerase II to either the wild type or a

catalytically inactive mutant (Glu54 to Gln) of the Kin28 protein kinase (**Fig. 3a**). Kin28 is one of several protein kinases capable of CTD phosphorylation, which results in the recruitment of factors regulating transcriptional elongation and pre-mRNA processing[27]. As RNA polymerase II contains from 26 (yeast) to 52 (mammals) repeats of CTD, the fusion used here with only three repeats may preferentially identify strong interactions.

We conducted western blot analyses to determine whether Kin28 phosphorylated the fused CTD. Kin28 phosphorylated Ser5 of the fused CTD, but the Kin28(E54Q) mutant failed to do so (**Fig. 3b**). The phosphorylated CTD is known to bind and recruit RNA processing and nuclear proteins involved in promoter clearance, primary transcript processing, transcriptional elongation and termination[27]. The Ser5-phosphorylated CTD bait was tested for its ability to interact with three proteins, Ess1, Prp40 and Rsp5, that have been biochemically shown to bind phosphorylated CTD[27]. Of the three, Prp40 associated specifically with CTD-Kin28(wild type), but not with CTD-Kin28(E54Q) or Kin28 alone (**Fig. 3c**), indicating that at least some phosphorylation-dependent CTD interactions can be detected with this approach. The lack of apparent two-hybrid interaction between the CTD-Kin28 and Ess1 or Rsp5 may be due to differences in affinity.

We next screened the array to identify phospho-CTD-interacting proteins. Candidates that met our phosphorylation-dependent selection criteria included Fcp1, Whi2 (**Fig. 3d**) and several other proteins with roles in controlling transcription (see **Supplementary Table 2** online for the complete list and references), consistent with the functions of CTD phosphorylation. Fcp1 is a phosphatase acting on the phosphorylated CTD[28], and thus is a highly plausible phosphorylation-dependent interactor. Ccl1 is a cyclin partner for Kin28 that is known to interact with Kin28 by two hybrid screening[29] and thus does not require the presence of the CTD (**Fig. 3d**).

There are several useful features to this methodology. First, substrate modifications are very efficient and constitutive. Kinetically, an enzymatic reaction approaches V_{max} if all enzyme molecules are engaged in the enzyme-substrate complex. In the tethered catalysis context, every molecule of the enzyme is juxtaposed stoichiometrically to its substrate. It is thus conceivable that the rate of tethered catalysis approaches V_{max}. Second, Kin28 and many other protein kinases rely on selective cofactors for substrate selection. By artificially tethering the enzyme to the desired substrate, we effectively bypass the need for concomitant overexpression of the regulator Ccl1 in order for Kin28 to modify the CTD bait *in vivo*. Third, both histone acetylation and CTD phosphorylation are reversible modifications naturally occurring in yeast. Thus, the host organism chosen for two-hybrid screening is not restricted to one that lacks the particular modification system to avoid opposing enzyme activities[7–9]. Fourth, many protein-modifying enzymes contain well-defined catalytic domains (e.g., Kin28) or cofactor binding motifs (e.g., Gcn5)in which minimal targeted mutations are sufficient to quantitatively abolish the enzymatic activity without causing dramatic conformational changes of the enzyme. Therefore, the inclusion of a catalytically inactive mutant as a counter-screen appears to be feasible in many cases. Alternatively, a targeted mutation introduced to the modification site of the bait may provide an equivalent counterscreen control. Finally, this method is amenable to a high-throughput format for systematic analyses of biological pathways that are dependent on proteins with specific post-translational modification states.

METHODS

Yeast methods. The activation domain library screen was based on reference 17 and provided generously by P. Kaufman (UC Berkeley). The high-throughput screening was based on reference 24. Standard yeast techniques and media were used[30].

Plasmid construction. Plasmid sequences used in this study have been deposited to GenBank (see below). The detailed description for plasmid construction is available upon request. Relevant features of plasmids used in this study are the following: pAB8, trimeric HA insertion to pODB2; pDG1, H3-Gcn5 (wild type, residues 18–252) inserted between GDBD and HA; pDG2, same as pDG1 except that the Gcn5 possesses the F221A mutation; pDG3, same as pDG1 except that H4 (residues 1–20) replaces H3 (residues 1–59); pDG4, the F221A version of pDG3; pDG28 and 29, same as pDG1 and 2 except that the H3 tail was deleted; pMK498, GDBD-Kin28 (wild type, residues 1–306)-HA; pMK500, GDBG-3xCTD-Kin28(wild type)-HA; pMK502, GDBD-3xCTD-Kin28(E54Q)-HA. pMK531TEV, 532TEV and 534TEV are identical to pMK498, 500 and 502, respectively, except that the entire bait open reading frames were inserted into the *Nco*I-*Hin*dIII sites of the bacterial expression vector pET28b (Novagen) for CTD phosphorylation assays, and that the TEV protease cleavage site was engineered immediately downstream of the GDBD.

Characterization of autoacetylation and autophosphorylation. We collected 3×10^9 yeast cells from mid-log phase cultures by centrifugation (10,000*g*, 1 min, 4 °C). Cell suspension (in 500 µl buffer containing 300 mM sorbitol, 10 mM Tris-HCl, pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM PMSF and 1× complete protease inhibitor (Roche)) was mixed vigorously with 1–2 g of acid-washed glass beads, 450–650 µm in diameter, (Sigma) in a bead beater (BioSpec) four times for 1 min each with ice-water chilling for at least 1 min between each agitation. The cell lysate was collected and adjusted to 1% Triton X-100 (vol/vol), and vortexed for 30 s. The lysate was clarified (21,000*g*, 15 min at 4 °C). Immunoprecipitation was conducted with 0.8 µg of an anti-HA monoclonal antibody (clone 3F10, Roche) in 200 µg of total yeast protein. The volume was adjusted to 200 µl with 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1% NP-40 (vol/vol), 1 mM PMSF and 1× complete protease inhibitors. Reactions were rotated at 4 °C overnight, followed by the addition of 30 µl protein G Sepharose 4 Fastflow 1:1 slurry (Sigma) and rotated at 4 °C for 2 h. The beads were pelleted by pulse-spin in a microcentrifuge and washed six times with the binding buffer (1 ml each). The final immune complex was boiled in 1× SDS-PAGE loading dye and resolved and blotted for western blot analyses. For western blot detection, the 12CA5 clone of anti-HA Ab (1:2,000) or anti-acetylated H3 (Lys9/14, 1:2,500) Ab was used.

The CTD phosphorylation was conducted using bacterially expressed proteins. Strong induction of the proteins was necessary for the H14 antibody (Covance) to detect Ser5 phosphorylation. This is likely due to the low copy number of CTD in the current bait constructs. pMK531, 532 and 534 were transformed into BL21 codon plus strain and treated with 1 mM IPTG for induction for 4 h at 37 °C . The total cellular proteins were resolved by SDS-PAGE and analyzed by western blot assays with the H14 antibody (1:500 dilution).

GST pulldown. Chicken histones were prepared as described[25]. 180 µg of total histones were acetylated in a 500 µl reaction containing ~100 ng of recombinant Gcn5, 15 µM unlabeled acetyl coenzyme A, 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl. The reaction was carried out at 30 °C for 1 h. The His-tagged Gcn5 was removed by absorption with Ni-NTA agarose beads (Qiagen) before the treated histones were precipitated with 20% trichloroacetic acid (vol/vol).

GST-Rpm2 fusion was induced in the BL21(DE3) host cells with 1 mM IPTG (37 °C for 4 h). Cells from each 100-ml culture were concentrated in 10 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM PMSF, 1× complete protease inhibitor cocktail) and lysed by French press. Cell lysates were spun at 20,000*g* for 15 min. Each 200 µl lysate was incubated with 30 µl swollen glutathione Sepharose beads (Sigma) (4 °C, 2 h). 1 ml lysis buffer was used to wash the beads (three times for 10 min each). We added 8 µg of treated or mock-treated chicken histones to the immobilized fusion proteins. Proteins after the final wash were boiled in the SDS-PAGE loading buffer and resolved in 15% (wt/vol) polyacrylamide gels, followed by Coomassie blue staining.

Accession numbers. pAB8, AY647980; pDG1, AY647981; pDG2, AY647982; pDG3, AY647983; pDG4, AY647984; pMK498, AY647985; pMK500, AY647986; pMK502, AY647987.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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