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Casein kinase 2 inhibits HomolD-directed transcription by Rrn7 in *Schizosaccharomyces pombe*

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In *Schizosaccharomyces pombe*, ribosomal protein gene (RPG) promoters contain a TATA analogue element called the HomolD box. The HomolD-binding protein Rrn7 forms a complex with the RNA polymerase II machinery. Despite the importance of ribosome biogenesis to cell survival, the mechanisms involved in the regulation of transcription of eukaryotic RPGs are unknown. In this study, we identified Rrn7 as a new substrate of the pleiotropic casein kinase 2 (CK2), which is a regulator of basal transcription. Recombinant Rrn7 from *S. pombe*, which is often used as a model organism for studying eukaryotic transcription, interacted with CK2 *in vitro* and *in vivo*. Furthermore, CK2-mediated phosphorylation of Rrn7 inhibited its HomolD-directed transcriptional activity and ability to bind to an oligonucleotide containing a HomolD box *in vitro*. Mutation of Rrn7 at Thr67 abolished these effects, indicating that this residue is a critical CK2 phosphorylation site. Finally, Rrn7 interacted with the regulatory subunit of CK2 *in vivo*, inhibition of CK2 *in vivo* potentiated ribosomal protein gene transcription, and chromatin immunoprecipitation analyses identified that the catalytic subunit of CK2 was associated with the *rpk5* gene promoter in *S. pombe*. Taken together, these data suggest that CK2 inhibits ribosomal protein gene transcription in *S. pombe* via phosphorylation of Rrn7 at Thr67.

Introduction

Eukaryotic ribosome biogenesis is a highly coordinated process that requires three different RNA polymerases (*pol*). Each *pol* transcribes a specific set of genes that encode components of the ribosome; whereas *pol* III synthesizes the small 5S rRNA, *pol* I synthesizes the large 45S pre-rRNA precursor from a single gene (rDNA) and *pol* II transcribes ribosomal protein genes (RPGs). The abundance of ribosomes within a cell depends upon the availability of their components; therefore, regulation of rRNA and ribosomal protein synthesis impact the status of ribosome biogenesis. Several factors can modulate rRNA synthesis through rDNA transcription, such as the cell growth rate, chromatin structure, rDNA copy number, nutritional status, growth factors and chemical stresses [1–6]. Studies in human cells have revealed that selectivity factor 1, which binds to the rDNA gene promoter and promotes recruitment of *pol* I [7], is regulated by casein kinase 2 (CK2), extracellular signal-regulated kinase and mTOR [8–11]. CK2 modulates the interaction between selectivity factor 1 and upstream binding factor through upstream binding

Abbreviations
ChIP, chromatin immunoprecipitation; CK2, casein kinase 2; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; *pol*, RNA polymerase; rDNA, ribosomal gene; RPG, ribosomal protein gene; TBB, 4,5,6,7-tetrabromobenzotriazole.

factor phosphorylation [9,12,13], and rRNA synthesis is promoted by AKT in association with c-Myc and mTORC1 in human cancer-derived cells [14]. Although the regulation of rDNA transcription has been well characterized, the regulation of RPG transcription is poorly understood.

In *Saccharomyces cerevisiae*, Ifh1, a member of the Rap1 recognition complex that binds to the *rDNA* promoter region, also interacts with CK2 within the CURI complex, which is associated with rRNA processing and RPG transcription [15,16]. However, this mechanism is not conserved in most other organisms. In *Schizosaccharomyces pombe*, a *S. cerevisiae*-related yeast often used as a model of eukaryotic transcription, RPGs are present in multiple copies and have different promoter elements to those of other *pol* II promoters, the majority of which have a TATA box, an initiator and other elements found in all metazoans. By contrast, *S. pombe* RPGs have a conserved element in their promoter regions termed the HomolD box [17]. The family of RPGs that encodes the ribosomal L8 protein (*rpk5*, *rpkd4*, *rpk37*) and analysis of the *S. pombe* proteome showed that L8 expression is regulated by the cell cycle, as well as stress factors such as H_2O_2, metals and heat [18,19]. In addition, the HomolD-binding protein Rrn7 is a component of the yeast *pol* I core factor complex [20], suggesting a possible coordination between the *pol* I and *pol* II machineries.

CK2, a pleiotropic serine/threonine kinase, plays an important role in a number of cellular processes, including the control of gene expression. Several CK2 targets are components of the *pol* I, *pol* II and *pol* III basal transcription machineries [8,11,12,21,22], and prediction of protein interactions in *S. pombe* suggested a putative interaction between Rrn7 and the regulatory subunit of CK2 (CK2β) [23]. Therefore, the aim of this study was to determine whether phosphorylation of Rrn7 by CK2 affects RPG transcription, using the K5 gene (*rpk5*) promoter from *S. pombe* [20] as a model. *In vitro* and *in vivo* analyses revealed that Rrn7 interacts with CK2 subunits and that CK2 represses the transcription of RPGs directed by the HomolD box via phosphorylation of Rrn7. Furthermore, inhibition of CK2-potentiated RPG transcription *in vivo* and the catalytic CK2α subunit was found to be associated with HomolD-containing promoters. These results represent a new mechanism of regulation of RPG transcription in *S. pombe* and show that CK2 is present in both *pol* I- and *pol* II-associated complexes, suggesting a possible link between the transcription mechanisms employed by each complex.

Results

Rrn7 is a substrate of CK2 and interacts with both CK2 subunits

To determine whether Rrn7 is a CK2 substrate, recombinant N-terminally His-tagged CK2 subunits from *S. pombe* were cloned, expressed in *Escherichia coli*, and then purified and characterized. The recombinant catalytic subunit, CK2α, was able to phosphorylate casein *in vitro* and this activity was enhanced by the addition of increasing amounts of the regulatory subunit, CK2β (Fig. 1A). Similarly, recombinant CK2α was able to phosphorylate Rrn7 *in vitro* and the addition of CK2β stimulated this phosphorylation further (Fig. 1B). However, when the assay was performed in the presence of heparin (2 $\mu g \cdot mL^{-1}$) or 4,5,6,7-tetrabromobenzotriazole (TBB; 100 μM), which are both CK2 inhibitors [24,25], Rrn7 phosphorylation was abolished completely (Fig. 1C). Similarly, the addition of a *Xenopus laevis* anti-CK2α serum (2 μg), which was able to detect the recombinant *S. pombe* CK2α protein in an immunoblot (Fig. 1D), also abolished Rrn7 phosphorylation. The specificity of the phosphorylation was confirmed by the addition of an anti-fibrillarin Ig (2 μg), which did not affect Rrn7 phosphorylation (Fig. 1C).

Next, pull-down assays were performed to determine whether Rrn7 interacts with the CK2 subunits. The recombinant CK2α and CK2β proteins contained a His tag; therefore, recombinant *S. pombe* Rrn7 containing an N-terminal glutathione *S*-transferase (GST) tag was used in these experiments. After incubation of GST-tagged Rrn7 with recombinant CK2α and CK2β, immunoblots were performed using anti-His and anti-GST sera. The results indicated that both CK2 subunits interacted with Rrn7 *in vitro* (Fig. 2A,B). To determine whether CK2 also interacts with Rrn7 *in vivo*, protein extracts were prepared from a *S. pombe* strain expressing TAP-tagged Rrn7 and immunoprecipitation studies were performed using IgG–Sepharose. Rrn7 and CK2β were both detected in the eluates, confirming that CK2 interacts with Rrn7 *in vivo* (Fig. 2C). Neither Rrn7 nor CK2β were detected in the eluates from an IgG–Sepharose column incubated with protein extracts prepared from a yeast wild-type strain (data not shown).

Furthermore, purified TAP–Rrn7 from the *S. pombe* extracts prepared in the presence of phosphatase inhibitors was detected by immunoblotting with an anti-(phospho-protein) Ig (Fig. 2D, lanes 1 and 3), indicating that Rrn7 is also phosphorylated *in vivo*, and alkaline phosphatase treatments abolished this phosphorylation (Fig. 2D, lane 2).

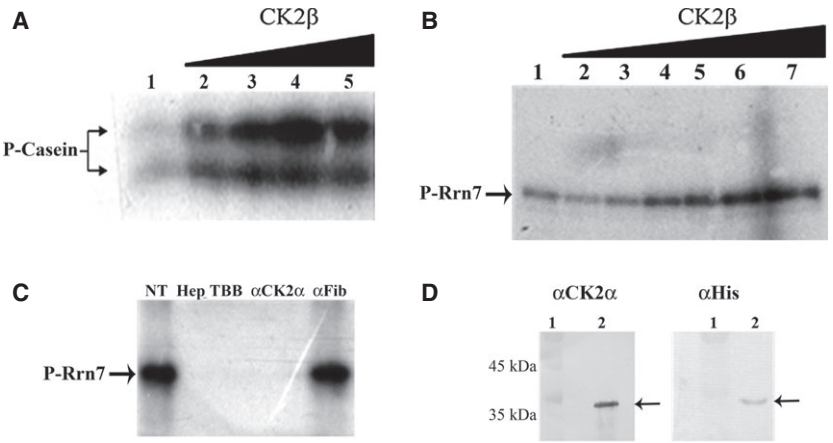

Fig. 1. Rrn7 is a substrate of protein kinase CK2. (A) *In vitro* phosphorylation assay. Casein (10 μg) was incubated with [^{32}P]ATP[γP] and 20 pmol of recombinant CK2α in the absence (lane 1) or presence of 5, 10, 15 or 25 pmol of recombinant CK2β (lanes 2–5, respectively). The arrows indicate phosphorylated casein (P-Casein). (B) Recombinant His–Rrn7 from *S. pombe* (10 pmol) was incubated with [^{32}P]ATP[γP] and 20 pmol of recombinant CK2α in the absence (lane 1) or presence of 5, 10, 15, 20 or 25 pmol of recombinant CK2β (lanes 2–6, respectively). The arrow indicates phosphorylated Rrn7. (C) *In vitro* phosphorylation assay performed with 10 pmol of recombinant Rrn7 and 20 pmol of CK2 holoenzyme in the absence (NT) or presence of 2 μg·mL^{-1} heparin (Hep), 100 μM TBB, 2 μg of anti-CK2α Ig (αCK2α), or 2 μg of anti-fibrillarin (αFib). (D) Immunoblot analysis of recombinant CK2α from *S. pombe* detected using an anti-CK2α Ig (1 : 7000) or anti-His Ig (1 : 500). In both panels, lane 1 is the molecular mass size marker and lane 2 corresponds to 20 pmol of recombinant CK2α. The band sizes are in agreement with the molecular mass of the CK2 subunits (39.53 kDa, www.pombase.org). The arrows indicate the protein band.

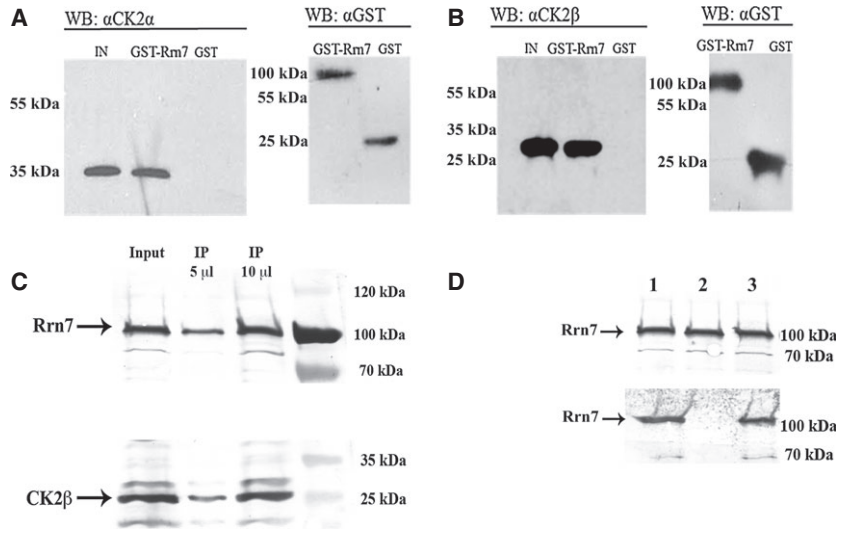

Fig. 2. Rrn7 interacts with CK2 subunits *in vitro* and *in vivo*. (A,B) Pull-down assays. Recombinant GST–Rrn7 (20 pmol) or GST alone was immobilized in a resin and then incubated with 20 pmol of CK2α (A) or CK2β (B) and analyzed by immunoblotting using an anti-His Ig (left) or anti-GST Ig (right). IN, input. (C) Regulatory subunit of CK2 and Rrn7 interaction was analyzed by coimmunoprecipitation (Co-IP) using IgG–Sepharose to precipitate TAP–Rrn7. Precipitated material was analyzed by immunoblotting using anti-Rrn7 Ig (upper) or anti-CK2β Ig (lower). (D) Purified TAP–Rrn7 fractions incubated with buffer (lane 1), alkaline phosphatase (lane 2) or neither (lane 3) were analyzed by immunoblotting using anti-Rrn7 Ig (upper) or anti-(Ser/Thr phospho-protein) serum (lower). The arrow indicates Rrn7 protein band.

HomolD box-directed transcription is inhibited by CK2 *in vitro*

To investigate the regulation of HomolD-directed transcription by CK2, *in vitro* transcription assays were performed by incubating whole-cell extracts of *S. pombe* with a number of different promoters. First, the HomolD-containing promoter of the *rpk5* gene was used. Second, because mutations in the HomolD box

render it inactive [20], $TATA^{+}Inr^{+}$ Adenovirus major late (Ad-ML) transcription promoter and the $TATA^{-}Inr^{+}$ promoter of the *S. pombe nmt1* gene were used as specificity controls, to test whether the effect of CK2 over HomolD-directed transcription is not due to an unspecific phosphorylation of basal machinery proteins. CK2 activity in the whole-cell extracts was modulated by adding inhibitors or recombinant CK2α and CK2β subunits in the presence of ATP. The addition of increasing amounts of CK2 holoenzyme (5–25 pmol) reduced the level of transcription of the HomolD-containing construct in a dose-dependent manner (Fig. 3A). Specifically, the addition of 5 pmol of CK2 inhibited almost 50% of HomolD-directed transcription, whereas the addition of 25 pmol inhibited transcription by ~ 90%. To corroborate these observations, *in vitro* transcription assays were performed in the presence of increasing amounts of TBB (12.5–100 μM) or an anti-CK2α Ig (0.8–3.2 μg). The addition of TBB increased transcription by the HomolD-containing promoter in a dose-dependent manner (Fig. 3B); in fact, transcription rose to approximately threefold that of nontreated whole-cell extracts in the presence of 100 μM TBB. Similar results were observed following the addition of the anti-CK2α Ig (Fig. 3C); transcription was potentiated by approximately sevenfold in the presence of the highest dose of the antibody.

Opposing results to those obtained for the HomolD-containing promoter were obtained when the *in vitro* transcription assays were performed with the $TATA^{-}Inr^{+}$ promoter [26]. Specifically, the addition of 25 pmol CK2 potentiated the transcriptional activity of this promoter by 11-fold (Fig. 4A) and its activity was inhibited by TBB and the anti-CK2α Ig (Fig. 4B, C). By contrast, CK2 had no effect on transcription by the $TATA^{+}Inr^{+}$ promoter derived from the Ad-ML promoter (Fig. 4D) and its transcriptional activity was also unaffected by TBB and anti-CK2α Ig (Fig. 4E,F). However, transcription was inhibited slightly in the presence of the highest dose of TBB, possibly by unspecific mechanisms. Identical results were observed with the $TATA^{+}Inr^{+}$ yeast *nmt1*-derived promoter (not shown). Taken together, these results indicate that the negative effect of CK2 on transcription requires the HomolD box and is not due to phosphorylation of basal machinery proteins that would affect transcription in a nonspecific manner.

To determine whether the effect of CK2 on transcription of the HomolD-containing promoter was due to Rrn7 phosphorylation, *in vitro* transcription assays were performed using whole-cell extracts of the *S. pombe* strain expressing TAP-tagged Rrn7 that were depleted of TAP–Rrn7 using IgG Sepharose and then supplemented with or without increasing amounts of recombinant Rrn7 that had previously been incubated with CK2 in a standard phosphorylation assay in the presence or absence of nonradioactive ATP. We determined that Rrn7 is phosphorylated in almost 80% in an *in vitro* transcription assay by measurement of [^{32}P]ATP[γP] incorporation (data not shown). In the absence of recombinant Rrn7, transcriptional activity of the Ad-ML promoter ($TATA^{+}Inr^{+}$) was observed, but no detectable transcripts were generated when the assay was performed with the HomolD-containing

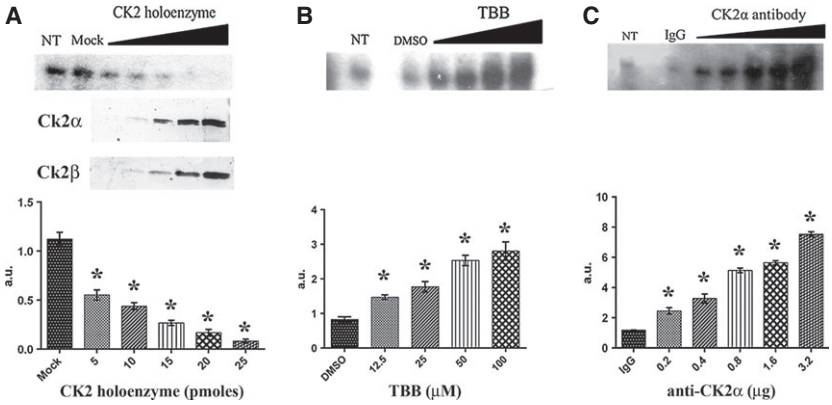

Fig. 3. Phosphorylation of Rrn7 by CK2 attenuates HomolD-directed transcription in *S. pombe*. (A–C) *In vitro* transcription assays using *S. pombe* whole-cell extracts and a HomolD-containing template with a downstream G-less cassette. The assays were performed with 1 mM ATP in the absence or presence of increasing amounts of CK2 holoenzyme (5, 10, 15, 20 or 25 pmol) (A), TBB (12.5, 25, 50 or 100 μM) (B), or an anti-CK2α Ig (0.2, 0.4, 0.8, 1.6 or 3.2 μg) (C). Control experiments were performed with ATP and phosphorylation buffer (A; Mock), DMSO (B), IgG (C) or none of these components (NT). Immunoblot of CK2 subunits are showed in the middle portion of (A). The graphs in the lower panels show quantifications of the transcripts, in which the signals were normalized to that of the corresponding NT control. The signal intensities are expressed in arbitrary units (a.u.) and are represented as the mean ± SD ($n = 3$). *$P < 0.05$.

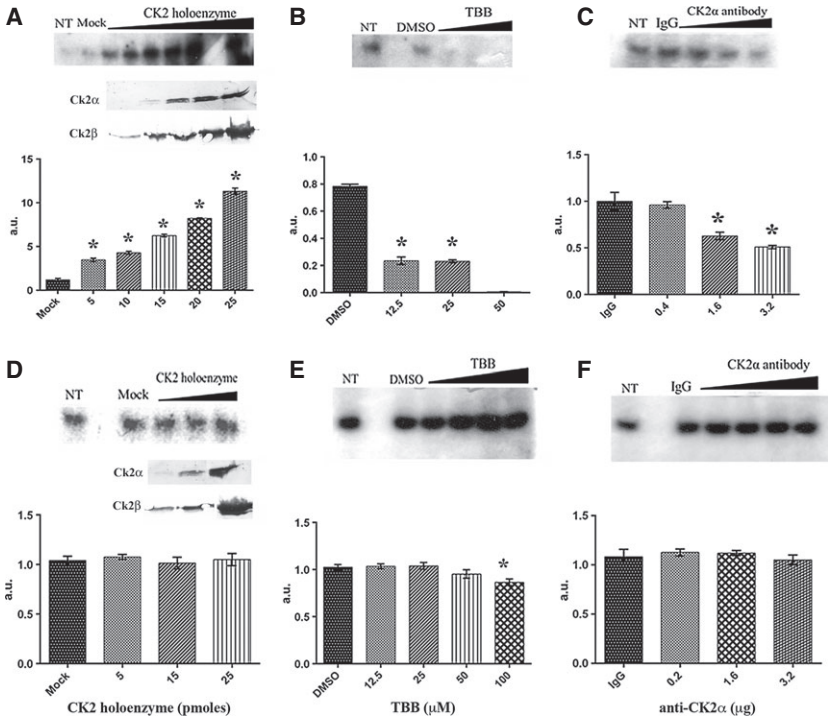

Fig. 4. CK2 potentiates Inr-directed transcription but does not affect TATA-directed transcription in *S. pombe*. (A–C) *In vitro* transcription assays using *S. pombe* whole-cell extracts and a TATA^{-}Inr^{+} template, derived from the *nmt1* gene, with a downstream G-less cassette. The assays were performed with 4 mM ATP in the absence or presence of increasing amounts of CK2 holoenzyme (5, 10, 15, 20 or 25 pmol) (A), TBB (12.5, 25, or 50 μM) (B), or an anti-CK2α Ig (0.4, 1.6 or 3.2 μg) (C). Control experiments were performed with ATP and phosphorylation buffer (A; Mock), DMSO (B) or IgG (C), or none of these components (NT). (D–F) *In vitro* transcription assay using *S. pombe* whole-cell extracts and a TATA^{+}Inr^{+} template, derived from Ad-MLP, with a downstream G-less cassette. The assays were performed with 4 mM ATP in the absence or presence of increasing amounts of CK2 holoenzyme (5, 15 or 25 pmol) (A), TBB (12.5, 25, 50 or 100 μM) (B) or an anti-CK2α Ig (0.2, 1.6 or 3.2 μg) (C). Control experiments were performed with ATP and phosphorylation buffer (A; Mock), DMSO (B) or IgG (C), or none of these components (NT). Immunoblot of CK2 subunits are showed in the middle panels of (A) and (D). The plots in the lower panels show quantifications of the transcripts, in which the signals were normalized to that of the corresponding NT control. The signal intensities are expressed in arbitrary units (a.u.) and are represented as the mean ± SD ($n = 3$). *$P < 0.05$.

promoter (Fig. 5A, upper, lanes 1 and 2). Although the addition of phosphorylated Rrn7 rescued transcription by the HomolD-containing promoter to some extent (Fig. 5A, lower left and upper, lanes 4–6), the addition of nonphosphorylated Rrn7 rescued its transcriptional activity substantially (Fig. 5A, lower right and upper, lanes 7–10). The promotional effect of nonphosphorylated Rrn7 on transcriptional activity of the HomolD-containing promoter reached saturation between 2.5 and 5 pmol of Rrn7 (Fig. 5A, lower right). These results suggest that CK2 negatively regulates HomolD-directed transcription via Rrn7 phosphorylation *in vitro*.

Mutation of Rrn7 at Thr67 abolishes its phosphorylation by CK2

Knowledge of the specific site(s) at which Rrn7 is phosphorylated by CK2 is relevant to understanding the negative effect of this modification on the transcriptional and DNA-binding activities of Rrn7; therefore, a search for putative phosphorylation sites was performed using the GROUP-BASED PREDICTION SYSTEM software package [27]. Ser138, Ser141, Ser143, Ser144, Ser146, Thr67 and Thr139 presented high scores, indicating that these sites are potential CK2 targets. Consequently, Rrn7 mutants in which Thr67 (T67A–Rrn7) or Ser141 and Ser143 (S (141,143)A–Rrn7) were replaced by alanine were generated. In addition, a triple mutant in which all three of these residues were replaced by alanine (TM–Rrn7) was also generated. *In vitro* phosphorylation assays performed with the wild-type and mutant Rrn7 proteins showed that T67A–Rrn7 and TM–Rrn7 were insensitive to CK2 phosphorylation but S (141,143)A–Rrn7 was still phosphorylated (Fig. 5B), indicating that Thr67 is a CK2 phosphorylation site in this protein.

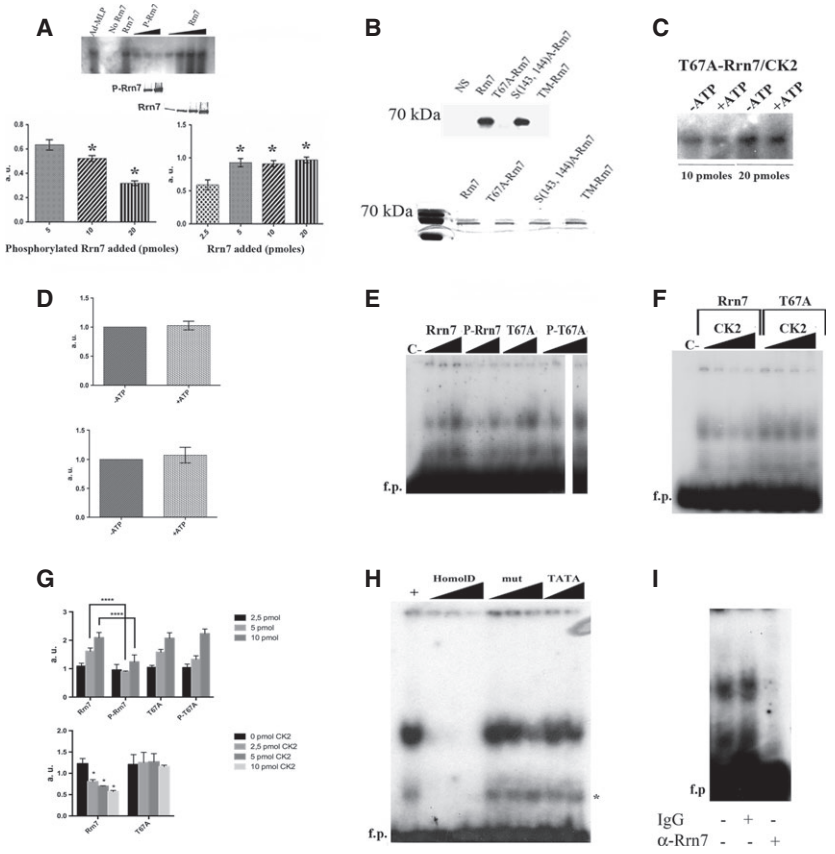

Fig. 5. Phosphorylation of threonine 67 in Rrn7 by CK2 affects DNA-binding activity and transcriptional function of Rrn7. (A) *In vitro* transcription assays using a HomolD-containing template and TAP–Rrn7-depleted *S. pombe* extracts that were supplemented with or without increasing amounts of recombinant nonphosphorylated or phosphorylated Rrn7 (P-Rrn7). Lane 1, Ad-ML promoter: control $TATA^{+}Inr^{+}$ template (instead of the HomolD-containing template) without recombinant Rrn7. Lane 2, No-Rrn7: Rrn7-depleted extract without recombinant Rrn7. Lane 3, Rrn7: Rrn7-depleted extract with 20 pmol of recombinant Rrn7. Lanes 4–6, P-Rrn7: Rrn7-depleted extract with increasing amounts (5, 10 and 20 pmol) of purified P-Rrn7. Lanes 7–10, Rrn7: Rrn7-depleted extract with increasing amounts of purified nonphosphorylated Rrn7. Data are given in arbitrary units (a.u.) as the mean ± SD ($n = 3$). $*P < 0.05$. (B) Autoradiography of *in vitro* phosphorylation assays in which 10 pmol of recombinant Rrn7, T67A–Rrn7, S(141,143)A–Rrn7, TM–Rrn7, or no substrate (NS) were incubated with 20 pmol of CK2 holoenzyme. The lower panel shows the amount of protein used in the assay, as determined by SDS/PAGE and Coomassie Brilliant Blue staining. Free probe is indicated (f.p.). (C) *In vitro* transcription assays performed with TAP–Rrn7-depleted *S. pombe* whole-cell extracts supplemented with 10 or 20 pmol of T67A–Rrn7 that was previously incubated with CK2 in the absence or presence of ATP. (D) Quantification of the signal intensities of *in vitro* transcription assays performed with TAP–Rrn7-depleted *S. pombe* whole-cell extracts supplemented with 10 pmol (upper) or 20 pmol (lower) of T67A–Rrn7 that was previously incubated with CK2 and with or without ATP. Data are represented in arbitrary units (a.u.) as the mean ± SD ($n = 3$). The signals of the transcripts formed in the presence of ATP were normalized to those in the absence of ATP. (E) EMSA analyses of the effect of CK2-mediated phosphorylation on the DNA-binding activity of Rrn7. Rrn7 and T67A mutated protein (2.5, 5 and 10 pmol) were incubated with CK2 holoenzyme (10 pmol) in the absence or presence of 1 mM ATP. Phosphorylated (P-Rrn7 and P-T67A) and nonphosphorylated proteins (Rrn7 and T67A) were then incubated with a radiolabeled probe containing a HomolD box. C-, negative control performed with BSA instead of Rrn7. (F) EMSA analysis of the effect of increasing amounts of CK2. Rrn7 and T67A mutated proteins (10 pmol) were incubated with CK2 holoenzyme (0, 2.5, 5 and 10 pmol) in the presence of 1 mM ATP. Phosphorylated proteins (P-Rrn7 and P-T67A) were then incubated with a radiolabeled probe containing a HomolD box. C-, negative control performed with BSA instead of Rrn7. (G) Quantifications of results showed in (C) and (E). The graphs show the intensity of the DNA–P-Rrn7 complex. Data are represented in arbitrary units (a.u.) as the mean ± SD ($n = 3$). $*P < 0.05$ or $****P < 0.001$. (H) EMSA analysis of the effect of nonlabeled probe containing HomolD box (left) and the addition of anti-Rrn7 to the assays (right). Rrn7 (10 pmol) was incubated with radiolabeled HomolD probe and unlabeled HomolD probe (HomolD; 10-, 50- and 100-fold molar excess), unlabeled mutated HomolD probe (mut; 10-, 50- and 100-fold molar excess) and unlabeled TATA probe (TATA; 50- and 100-fold molar excess). Secondary shift complex is indicated with *. (I) EMSA analysis of the effect of anti-Rrn7 on the DNA-binding activity of Rrn7. Rrn7 was incubated with 2 μg of antibody (IgG or anti-Rrn7). The result of a negative control assay without antibodies is indicated. f.p., free probe.

Furthermore, the results of *in vitro* transcription assays performed with TAP–Rrn7-depleted *S. pombe* whole-cell extracts supplemented with T67A–Rrn7 that had previously been incubated with CK2 in the presence or absence of ATP *in vitro* showed that the transcript levels were independent of the Rrn7 phosphorylation status (Fig. 5C,D). These results indicate that mutation of Thr67 also abolished the inhibitory effect of CK2 on Rrn7-mediated HomolD-directed transcription.

CK2 inhibits the DNA-binding activity of Rrn7

Chemical modification of transcription factors affects their ability to regulate gene expression [28]. For example, phosphorylation modulates the DNA-binding ability and protein–protein interactions of several transcription factors in eukaryotic cells [29]. To determine whether CK2-mediated phosphorylation of Rrn7 affects its ability to bind to the HomolD box, recombinant Rrn7 was incubated with CK2 in the presence or absence of ATP. DNA-binding activities of the phosphorylated and nonphosphorylated proteins were analyzed by electrophoretic mobility shift assays (EMSA), using a radiolabeled HomolD box-containing probe and increasing amounts of either phosphorylated or nonphosphorylated Rrn7. Previously, we calculated that ~ 80% of Rrn7 protein was phosphorylated by CK2, as measured by the incorporation of [^{32}P]ATP[γP] (data not shown). Retardation of the DNA probe occurred in the presence of both phosphorylated and nonphosphorylated Rrn7; however, the intensity of the shifted band generated using phosphorylated Rrn7 was lower than that of the shifted band generated by nonphosphorylated Rrn7 (Fig. 5E,G, upper). A T67A–Rrn7 mutant resulted in DNA-binding activity that was similar to that of wild-type Rrn7 irrespective of whether it was incubated with CK2 in the absence of ATP or CK2 in the presence of ATP (Fig. 5E). These data indicated that phosphorylation inhibited the HomolD-binding activity of Rrn7, and that mutation of Thr67 abolished this inhibitory effect.

Another experiment was performed using Rrn7 or T67A–Rrn7 incubated in the presence of increasing amounts of CK2. The DNA-binding activity of wild-type Rrn7, but not that of T67A–Rrn7, was inhibited by almost 50% when 10 pmol of CK2 holoenzyme was added to the assays (Fig. 5F,G, lower). To confirm the specificity of the EMSA bands in Fig. 5(E,F; the higher and lower bands), EMSA assays were performed with recombinant Rrn7 in the presence of an unlabeled HomolD-box probe or an anti-Rrn7 Ig (Fig. 5H,I). The band shifts were abolished by the unlabeled HomolD-box probe but not appreciably by a TATA-containing probe (Fig. 5H). The anti-Rrn7 Ig abolished DNA-binding activity (Fig. 5I). We did not observe a supershift of the DNA-binding complex in the presence of the antibody because anti-Rrn7 is a polyclonal antiserum that could recognize the Rrn7 DNA-binding site.

Inhibition of CK2 potentiates HomolD-directed transcription *in vivo*

To determine the effects of inhibiting CK2 on RPG transcription *in vivo*, yeast cultures (A_{600} = 0.5) were incubated with 100 μM TBB for 3 h and the expression levels of the *rpk37* and *rpk5* HomolD-containing genes were examined by RT-PCR. The expression levels of these genes were normalized to those of actin, which was not affected. Yeast cells incubated with TBB had higher *rpk5* and *rpk37* mRNA levels than untreated or DMSO-treated controls (1.6- and 2.6-fold of induction respectively, Fig. 6A,B). These data confirm that HomolD-directed transcription is inhibited by CK2 *in vivo*.

CK2 is associated with HomolD box-containing promoters *in vivo*

To determine the biological significance of the Rrn7–CK2 association, the presence of CK2 at HomolD-containing promoters was examined using chromatin immunoprecipitation analyses of *S. pombe* cultures. Immunoprecipitation was performed using an anti-CK2α Ig or an anti-GST Ig as a control and the resulting material was amplified by PCR using gene-specific primers for the core promoter region of *rpk5* (including the HomolD box), the *rDNA* promoter as a positive control and the ORF of *act1* as an internal control. Unlike *act1*, both the *rDNA* promoter and the HomolD-containing *rpk5* promoter were detected in the DNA that was immunoprecipitated with the anti-CK2α Ig (Fig. 6C,D). As expected, the anti-GST Ig failed to immunoprecipitate any of the DNAs probed. These data indicate that CK2α is associated with HomolD-containing promoters *in vivo*.

Discussion

In this study, the biological significance of phosphorylation of Rrn7 by CK2 in *S. pombe* was determined by two approaches. First, Rrn7 was evaluated as a CK2 substrate *in vitro* and the effect of CK2 on the DNA-binding activity and functional role of Rrn7 in

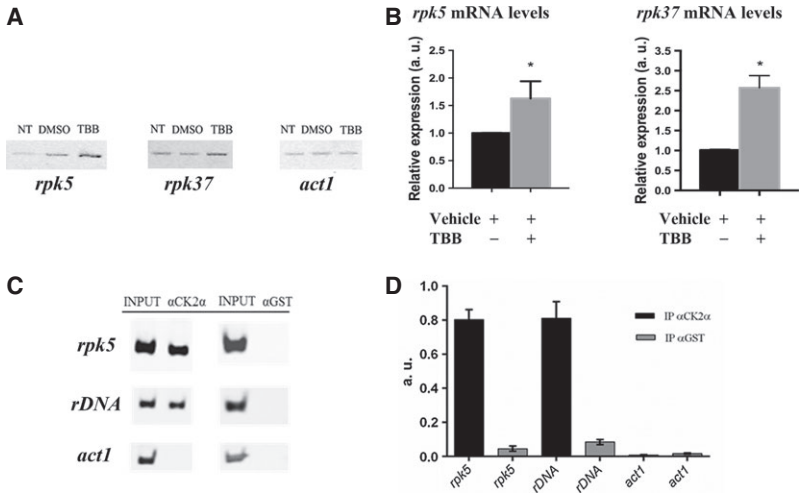

Fig. 6. CK2 modulates HomolD-directed transcription and is associated with HomolD box-containing promoters *in vivo*. (A) Representative qRT-PCR analyses of *rpk5, rpk37* and *act1* in *S. pombe* cultures that were untreated (NT) or incubated for 3 h with 100 μM of TBB or DMSO (Vehicle). (B) Quantification of the data presented in (A). The expression levels of *rpk5* and *rpk37* were normalized to those of *act1*. Data are expressed as arbitrary units (a.u.) as the mean ± SD ($n = 3$). *$P < 0.05$. (C) Representative ChIP analyses of cross-linked sonicated extracts from a wild-type *S. pombe* strain (972 h^{-s}). Immunoprecipitation was performed using an anti-CK2α or anti-GST serum (control). The co-precipitated DNA was amplified by qPCR using gene-specific primers for the HomolD box-containing core promoter of *rpk5, rDNA* as a positive control, and the ORF of *act1* as an internal control. (D) Quantification of the signal intensities shown in (C). The signals were normalized to that of the input. Data are represented in arbitrary units (a.u.) as the mean ± SD of at least $n = 3$ independent experiments.

HomolD-directed transcription was examined. Second, the association of CK2 with HomolD-containing promoters and the effect of CK2 inhibition on HomolD-directed transcription *in vivo* were determined. Also, *in vivo* findings of phosphorylated TAP–Rrn7 and an association between Rrn7 and CK2β served as support data for our discoveries. To our knowledge, the results presented here are the first evidence that RPG transcription in *S. pombe* is negatively regulated via phosphorylation of the HomolD-binding protein Rrn7 by CK2 and that CK2α is associated with HomolD-containing promoters *in vivo*. The results also demonstrate that Thr67 is a crucial CK2 phosphorylation site in Rrn7. Although phosphorylation of other predicted CK2 target sites (Ser138, Ser141, Ser143, Ser144 and Ser146, as well as Thr139) was not detectable under the conditions used here, we do not exclude the possibility that they are also involved in CK2-mediated regulation of Rrn7 activity. It is possible that these residues, which are located in an acid tract, are not exposed in the Rrn7 protein and a change in the conformational state of Rrn7 may be required to enable their modification.

In vivo assays identified the presence of the CK2 holoenzyme at RPG promoters, suggesting that CK2 forms part of the transcriptional complex that binds to the HomolD box, a phenomenon that has been described for other gene promoters, such as the human *rDNA* promoter [10]. The close proximity of CK2 to its potential substrates may explain how CK2 affects transcription through chemical modification of members of the preinitiation complex. The data presented here indicate that Rrn7 is a CK2 substrate and that phosphorylation of this transcription factor leads to a decrease in HomolD-directed transcription of RPGs both *in vitro* and *in vivo*. The effect of CK2 on HomolD-directed transcription via Rrn7 could be explained by a phosphorylation-induced conformational change in Rrn7, which may underlie the reduced DNA-binding affinity observed here. In support of this result, inhibitory effects of CK2 on the DNA-binding activity of several other transcription factors, such as SSRP1, PRH/Hhex, cMax and Sp1 [30–32] in human, and general transcription factors such as TATA-binding protein in *S. cerevisiae* and *S. pombe* [33], have been reported. Phosphorylation of Rrn7 could also enhance protein–protein interactions with other members of the *pol* II machinery, sequestering them and/or itself from the preinitiation complex and resulting in lower transcriptional activity.

The biological implication of the effect of CK2 on RPG transcription is unknown. CK2 expression is related to rapid cell proliferation rates, during which there is high demand for protein synthesis [34]. In metazoans such as rat, chicken, human and *Caenorhabditis elegans*, CK2 mRNA and protein expression and activity are particularly high in organs during the

early stages of development, compared with their adult counterparts [35]. CK2 is also more active in tumor tissues that have rapid proliferation rates than in normal tissues [5,6]. Furthermore, proliferation demands an increment of protein synthesis, which correlates with an increase in ribosomal biogenesis [36] and requires extremely fine regulation of the synthesis of the required amounts of proteins and rRNAs. Inhibition of RPG transcription by CK2 may be part of a regulatory network of ribosomal protein synthesis that also regulates rRNA expression, although the contribution of Rrn7 phosphorylation to the complete process is unknown.

CK2 can have opposing effects on rRNA transcription, depending on the availability of substrates. Studies performed in HeLa cells [12] and *S. cerevisiae* [13] have shown that phosphorylation of upstream binding factor potentiates transcription because of increased binding of this factor to the *pol* I complex. In addition, phosphorylation of TAF_I110, a human selectivity factor 1 protein, reduces its affinity for DNA, leading to a decrease in formation of the preinitiation complex and subsequent inhibition of transcription. The data presented here suggest that the effect of TAF_I110 phosphorylation is similar to that observed here for Rrn7 phosphorylation.

Additional experiments are required to determine the effect of Rrn7 phosphorylation on preinitiation complex assembly, and to investigate the biological role of CK2-mediated regulation of RPG transcription in *S. pombe* and whether this mechanism is conserved in other organisms.

Experimental procedures

Purification of recombinant proteins

The complete ORF of *S. pombe* Rrn7 (Database ID: NM_001022047.2) was cloned into the pQE-30 vector (containing N-terminal 6× His) (Qiagen, Hilden, Germany) and the pGEX-2T vector (containing an N-terminal GST tag) (GE Healthcare, USA). The coding sequences of *S. pombe cka1* (CK2α; Database ID: NM_001019073.2) and *ckb1* (CK2β; Database ID: NM_001020034.2) were cloned into the pET15b vector (containing an N-terminal His tag) (Novagen, Madison, WI, USA). The recombinant proteins were expressed in *E. coli* BL21 (DE3) or M15 cells and induction was performed by incubating the cells at 37 °C with 0.5 mM isopropyl 1-thio-β-D-galactoside until they reached A_{600} = 0.8, at which point the cells were sonicated. His-tagged proteins were purified as described previously [37]. GST-fusion proteins, as well as GST from the empty vector, were also purified as described previously [38].

In vitro phosphorylation

Reactions were performed with 1 μCi of [^{32}P]ATP[γP] in a final volume of 25 μL, under standard conditions with some modifications [37]. Recombinant CK2α was incubated for 10 min at room temperature in the presence or absence of recombinant CK2β, heparin (2 $\mu g \cdot mL^{-1}$, Sigma-Aldrich, Steinheim, Germany), TBB (100 μM, Sigma-Aldrich), *X. laevis* anti-CK2α Ig (2 μg, Abcam, Cambridge, UK) or yeast anti-fibrillarin Ig (2 μg, Abcam). Then, 10 μg of total casein or 10 pmol of recombinant Rrn7 were added to the mixtures. In assays without radioisotope, the reaction mixes contained 4 mM ATP. These mixtures were incubated at 30 °C for 30 min and then each reaction was stopped by adding Laemmli buffer. The samples were analyzed by 11% SDS/PAGE followed by autoradiography. Total casein was prepared from a commercial stock (Sigma-Aldrich).

Pull down

Each experiment used 20 μL of slurry glutathione agarose resin (Sigma-Aldrich) in a 1.5-mL microcentrifuge tube. The resin was washed twice with 6 vol of pull-down buffer (40 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% NP-40, and 1 $mg \cdot mL^{-1}$ BSA) and then collected by centrifugation at 1000 ***g*** for 2 min at 4 °C. GST–Rrn7 or GST (50 pmol) was then added to the resin and the mixture was incubated for 15 min at 4 °C with rotation. The resin was collected by centrifugation and washed three times with 25 vol of pull-down buffer. CK2α and CK2β (50 pmol) were added to the resin containing immobilized GST–Rrn7 or GST and the mixtures were incubated for 30 min at 4 °C in a rotator incubator. Next, each resin was washed five times with 25 vol of washing buffer (pull-down buffer without BSA). The bound proteins were eluted by adding 2 vol of elution buffer (washing buffer supplemented with 20 mM reduced glutathione). The mixtures were incubated for 15 min at 4 °C in a rotator incubator and then centrifuged at 1000 ***g*** for 2 min at 4 °C. The supernatants were mixed with Laemmli buffer and analyzed by immunoblotting using an anti-(His tag) Ig (Invitrogen, Carlsbad, CA, USA) for recombinant CK2α and CK2β (1 : 700), or an anti-GST Ig (Abcam) for GST and GST-Rrn7 (1 : 7000).

Production of anti-Rrn7 and anti-CK2α serum

Anti-Rrn7 sera were produced commercially (Biomatik, Cambridge, ON, Canada), using as an immunogen the peptide YVESCLKTGIHNQD. *Xenopus laevis* anti-CK2α Ig was obtained from rabbits injected subcutaneously with 1 mg of recombinant protein (full-length CK2α) plus complete Freund's adjuvant. Twenty-one days after the first inoculation, same rabbits were injected subcutaneously with

0.5 mg of protein with incomplete Freund's adjuvant, and 1 week later another dose of 1 mg of protein in $NaCl/P_i$ was injected intraperitoneally. The serum of the inoculated rabbits was obtained and then IgG antibodies were purified with protein A–agarose resin (Invitrogen) for immunoblotting and transcription assays.

Co-immunoprecipitation

Forty-five milligrams of Rrn7–TAP extracts were semi-purified in 3 mL of P11 resin (Whatman, Cambridge, UK), previously equilibrated in the following buffer: 20 mM Hepes, pH 8, 50 mM potassium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol. The resin was washed with the equilibrium buffer until there was no detectable protein in the eluate using the Bradford method. Then, proteins were eluted with 10 column volumes of ammonium sulfate gradient (0.1–1 M) prepared in equilibrium buffer. Protein fractions (500 μL) were collected and analyzed by western blot using anti-Rrn7 and anti-CK2β sera. Two fractions containing both proteins were mixed and dialyzed in transcription buffer (70 mM potassium glutamate, 20 mM Hepes, pH 7.9, 1 mM EGTA, 0.1 mM EDTA and 2.5 mM dithiothreitol). The dialyzed samples were loaded in a column packed with IgG Sepharose resin, equilibrated with transcription buffer. The resin was incubated for 2 h at 4 °C and then washed twice with 20 column volumes of transcription buffer. The bounded proteins were eluted adding 5 vol of 0.2 M glycine–HCl, pH 2.6. The eluate was neutralized with 0.1 column volumes of Tris 2 M, pH 8 and then mixed with 5× Laemmli buffer. The sample was loaded in a gradient SDS polyacrylamide gel (4–12%, Novex, Carlsbad, CA, USA). Immunoblotting was performed with anti-Rrn7 Ig (from rabbit), 0.5 $\mu g \cdot mL^{-1}$ of pure antibody and monoclonal anti-CK2β Ig (Calbiochem, New Orleans, LA, USA, 1 : 200).

Detection of phosphorylated Rrn7 *in vivo*

TAP–Rrn7 was purified as describes previously. The washing buffer was replaced by a stringent buffer (twice with 40 vol of 1 M potassium acetate, 0.1% NP-40, 0.1% Triton X-100) Elution was performed with 2 vol of elution buffer (0.2 M glycine–HCl, pH 2.6), separating three aliquots of equal volumes. One aliquot was incubated with alkaline phosphatase buffer alone, the second aliquot with 10 U alkaline phosphatase enzyme plus buffer and the last aliquot with transcription buffer. The mixes were incubated at room temperature for 30 min. The samples were loaded in gradient SDS polyacrylamide gel (4–12%) for immunoblotting. The membrane was blocked with 5% gelatin in NaCl/Tris at melting point for 1 h and analyzed with anti-(phospho-serine/threonine-tyrosine) Ig 1 : 500 (Abcam) or anti-Rrn7 Ig (rabbit), 0.5 $\mu g \cdot mL^{-1}$ of pure antibody.

In vitro transcription

In vitro transcription assays were performed as described previously [20]. Briefly, 0.5 μg of each promoter template (HomolD, Ad-MLP, nmt1 $TATA^{-}Inr^{+}$) was incubated with 30 μg of whole-cell extract from the *S. pombe* $972h^{-S}$ strain expressing TAP–Rrn7 or Rrn7-depleted whole-cell extract at room temperature for 20 min with or without anti-CK2α (0.2–3.2 μg), recombinant Rrn7 (wild-type and mutant T67A–Rrn7) previously phosphorylated or nonphosphorylated (5–20 pmol), CK2 holoenzyme (5–25 pmol) or TBB (12.5–100 μM). Control experiments were performed with ATP and phosphorylation buffer [37], DMSO, IgG (3.2 μg), or none of these components, as appropriate. The samples were analyzed in a 5% polyacrylamide gel supplemented with 6 M urea. The gel was dried and analyzed by autoradiography. The results were quantified and analyzed using PRISM v. 6.00 software (GraphPad Software, San Diego, CA, USA).

Purification of phosphorylated Rrn7

Recombinant Rrn7 (200 pmol) was incubated with an equal amount of CK2 holoenzyme in the presence or absence of ATP for 30 min at 30 °C. Each mixture (200 μL) was then loaded onto a 2 mL column containing Sepharose CL4B resin (Sigma-Aldrich) and eluted with phosphorylation buffer supplemented with 150 mM KCl. The eluates were analyzed using Bradford reagent (BioRad, Hercules, CA, USA) and two peaks were detected, the second of which corresponded to Rrn7. Quantified phosphorylated and nonphosphorylated Rrn7 were used in *in vitro* transcription assays, as described above.

Rrn7-depletion

Whole-cell extracts were prepared from an *S. pombe* strain expressing from TAP–Rrn7. An aliquot containing 1.2 mg of the whole-cell extract in reaction buffer (70 mM potassium glutamate, 20 mM Hepes, pH 7.9, 1 mM EGTA, 0.1 mM EDTA and 2.5 mM dithiothreitol) was incubated with 100 μL of IgG Sepharose (Sigma-Aldrich) that had previously been equilibrated with 10 vol of reaction buffer. Proteins that did not interact with the resin were collected and 4 μL were incubated with purified phosphorylated or nonphosphorylated Rrn7 in an *in vitro* transcription assay, as described above.

EMSA

Each binding reaction contained 20 mM Hepes, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 2% poly(ethylene glycol) 20K, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 50 μg BSA, 100 ng poly

(dI.dC) and 7.5 pmol phosphorylated or nonphosphorylated Rrn7, previously incubated with 10 pmol of CK2 holoenzyme or different amounts, according to the experiment. This mixture was incubated at room temperature for 5 min and then 20–40 ng (~ 20 000 c.p.m.) of an end-labeled HomolD box probe (containing the sequence in the *rpk5* gene) was added and the mixture was incubated for a further 10 min at 30 °C. The DNA–protein complexes were electrophoresed at 4 °C in a gel comprising 8% acrylamide, 0.2% bisacrylamide (39 : 1 ratio), 10% glycerol and 50 mM Tris borate (pH 8.3). The gel was pre-run for 30 min at 100 V and at 4 °C. After sample loading, the gel was run for a further 2 h under the same conditions. The gel was then dried and exposed to film for 1 day. Data were fitted and analyzed using PRISM v. 6.00 software. EMSA assays designed to test the effect of CK2-mediated phosphorylation of T67A–Rrn7 on its DNA-binding activity contained 2.5, 5 and 10 pmol of Rrn7 or T67A–Rrn7 and 10 pmol CK2 in the absence or presence of ATP. Competition assays were performed with the addition of 10-, 50- and 100-fold molar excess of unlabeled HomolD box probe, mutant HomolD box or TATA probe [20]. Supershifts assays were performed with the addition of 2 μg of anti-Rrn7 or IgG. Protein extracts and antibodies were previously incubated at 25 °C for 30 min before labeled probe incubation.

Rrn7 mutations

Mutations in the *S. pombe* Rrn7 ORF were generated by PCR using the primers listed in Table 1. The initial PCR was performed with *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA), primers containing the corresponding mutations (Table 1) and the pGEMT-Easy vector (Promega, Madison, WI, USA) containing the wild-type Rrn7 sequence as a template. The PCR products were separated in a 1% agarose gel and the appropriate bands were purified. The purified products were then used as the template in a second PCR, in which primers designed to amplify full-length Rrn7 (Rrn7-F and Rrn7-R; Table 1) and High Fidelity DNA *Taq* polymerase (Invitrogen) were used. The resulting PCR products were cloned into pGEMT-Easy, subcloned into the pET15b vector, and then analyzed by sequencing (Macrogen, Rockville, MD, USA).

Table 1. Primers used for PCR amplification of the Rrn7 mutants, *rpk5*, *rpk37* and *act1*. Mutated codons are shown as bold and underlined letters. For Rrn7-F and Rrn7-R, the bold letters indicate restriction sites for *Nde*I and *Bam*HI, respectively.

Primer	Sequence
T67A-F	CCATCTAGAC**GCT**GGAGATG
T67A-R	CATCTCC**AGC**GTCTAGATGG
S(141–143)-F	GCAA**GCA**GAA**GCA**TCAGATTCG
S(141–143)-R	CGAATCTGA**TGC**TTC**TGC**TTGC
Rrn7-F	**CATATG**GAAGGCAATTGGTTTGA
Rrn7-R	**GGATCC**TTAATCTTGATTGTGTATACC
rpk5-F	AAGACCCGTGTTAAGTTGCC
rpk5-R	AGCAACACCACGAGTACGAG
rpk37-F	TGTACACTGGCCAATTCGTT
rpk37-R	TCTTGCCAGTGTCAACATCA
act1-F	CGGTCGTGACTTGACTGACT
act1-R	TCAAGGGAGGAAGATTGAGC

Chromatin immunoprecipitation

Chromatin was isolated from a wild-type *S. pombe* strain (972 h^{-s}), as described previously [20]. For resin preclearing, 10.5 mL of immunoprecipitation solution (1% Triton X-100, 1 mM EDTA, 15 mM Tris/HCl pH 8, 150 mM NaCl) was added to 12 μL of protein A–agarose beads (General Electric, Pittsburgh, PA, USA). The resin was collected by centrifugation at 2500 ***g*** for 3 min at 4 °C, and the supernatant was discarded. The resin was incubated with 5 mL of immunoprecipitation solution supplemented with 0.05% BSA (Sigma-Aldrich) for 15 min at 4 °C, and centrifuged as described previously. The supernatant was discarded and the resin was mixed with the total volume of a previously purified chromatin fraction. The volume was adjusted to 2 mL with IP solution supplemented with the protease inhibitor 1X (Roche, Mannheim, Germany). The samples were incubated for 1 h at 4 °C and then centrifuged as previously described. The mixture (10%; input) was kept for further experiments and the remaining sample (precleared) was used for immunoprecipitation.

The resin was blocked with 1 mL of blocking solution (0.1% BSA and 0.1% sodium azide in TE) for 16 h at 4 °C. In parallel, 150 μL of the precleared chromatin preparation was incubated with 2 μg of anti-CK2α (Abcam) or anti-GST (Abcam) sera for 16 h at 4 °C. Later, the blocked resin was centrifuged at 1000 ***g*** for 3 min at 4 °C and then mixed with 150 μL of chromatin previously treated with antibody. The mixture was incubated for 3 h at 4 °C and centrifuged at 2500 ***g*** for 2 min at 4 °C. The resin was washed once with 1 mL of TSE 150 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 150 mM NaCl), 1 mL of TSE 500 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 500 mM NaCl), 1 mL of lithium/detergent (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris/HCl, pH 8.1) and twice with 1 mL of TE, using centrifugation between each step as previously described. The resin was resuspended in 250 μL of 1% SDS/0.1 M $NaHCO_3$ solution and incubated for 15 min at room temperature. After centrifugation, 200 μL of the supernatant was mixed with 8.3 μL of 5 M NaCl and then incubated for 16 h at 56 °C to reverse cross-linking. Then, 14 μg of proteinase K (Roche) was added to each immunoprecipitation and the mixture was incubated at 37 °C for 1 h. DNA was purified using the QIAquick PCR purification Kit 250

(Qiagen) and then analyzed by end-point PCR and qPCR, using Taq polymerase (Stratagene) and SensiMix SYBR Hi-ROX Kit (Bioline, London, UK) in a RotorGene 6000 Series (Corbett Research, Qiagen), respectively. The primers and PCR program used to amplify the *rpk5* gene core promoter, including the HomolD box, transcription start sites and the exon region that encodes the first 11 amino acids and the rDNA core promoter are detailed elsewhere [20]. As an internal control, primers designed to amplify parts of the ORF of the *act1* gene were used. PCR products were analyzed on a 5% polyacrylamide gel.

TBB treatment

Schizosaccharomyces pombe cultures were grown in YPD media at 30 °C until they reached an D_{600} of 0.5, at which point TBB was added at a final concentration of 100 μM (or an equal volume of DMSO as a control), and the cultures were incubated at 30 °C for a further 3 h. RNA was then extracted from each culture using the YeaStar RNA Kit (Zymo Research, Irvine, CA, USA) and cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). Amplification of *rpk5* and *rpk37* and qPCR were performed using the SensiMix SYBR Hi-ROX Kit (Bioline) in a RotorGene 6000 Series (Corbett Research) with the specific primers listed in Table 1. Fold changes in gene expression were determined by calculating $2^{-\Delta\Delta Ct}$.

Statistical analysis

All assays were repeated three times. Data were analyzed by two-way ANOVA and Dunnett's multiple comparison tests using PRISM v. 6.0 (GraphPad Software). $P < 0.05$ was considered significant.

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Author contributions

SMR and DAR designed and performed the most of the experiments, wrote and reviewed the manuscript. MM prepared recombinant proteins and helped in chromatin immunoprecipitation experiments. FU helped in *in vitro* transcription assays. VJM Analyzed experimental data and reviewed the manuscript. EM designed and performed the experiments; wrote and reviewed the manuscript.

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