

The fission yeast Rpb4 subunit of RNA polymerase II plays a specialized role in cell separation

Nimisha Sharma · Samuel Marguerat · Surbhi Mehta ·
Stephen Watt · Jürg Bähler

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Abstract RNA polymerase II is a complex of 12 subunits, Rpb1 to Rpb12, whose specific roles are only partly understood. Rpb4 is essential in mammals and fission yeast, but not in budding yeast. To learn more about the roles of Rpb4, we expressed the *rpb4* gene under the control of regulatable promoters of different strength in fission yeast. We demonstrate that below a critical level of transcription, Rpb4 affects cellular growth proportional to its expression levels: cells expressing lower levels of *rpb4* grew slower compared to cells expressing higher levels. Lowered *rpb4* expression did not affect cell survival under several stress conditions, but it caused specific defects in cell separation similar to *sep* mutants. Microarray analysis revealed that lowered *rpb4* expression causes a global reduction in gene expression, but the transcript levels of a distinct subset of genes were particularly responsive to changes in *rpb4* expression. These genes show some overlap with those regulated by the Sep1-Ace2 transcriptional cascade required for cell separation. Most notably, the

gene expression signature of cells with lowered *rpb4* expression was highly similar to those of *mcs6*, *pmh1*, *sep10* and *sep15* mutants. *Mcs6* and *Pmh1* encode orthologs of metazoan TFIID-associated cyclin-dependent kinase (CDK)-activating kinase (Cdk7-cyclin H-Mat1), while *Sep10* and *Sep15* encode mediator components. Our results suggest that Rpb4, along with some other general transcription factors, plays a specialized role in a transcriptional pathway that controls the cell cycle-regulated transcription of a specific subset of genes involved in cell division.

Keywords Transcription · *S. pombe* · Cell division · Growth

Introduction

RNA polymerase II (pol II) transcribes the coding genes, and its activity is the main target for transcriptional control in all eukaryotes. The pol II enzyme consists of 12 subunits, which are highly conserved during evolution. The specific functions of the smaller subunits such as Rpb4 are relatively poorly understood, and they can show differences between organisms. For example, Rpb4 is essential in mammals and fission yeast (*Schizosaccharomyces pombe*), but not in budding yeast (*Saccharomyces cerevisiae*). Here we use fission yeast as a model to learn more about particular roles of Rpb4 in genome-wide transcription.

Much of our current understanding of the mechanism of transcription and the transcription machinery comes from studies using budding yeast. The distantly related fission yeast provides a valuable complementary model system to dissect the functions of different

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N. Sharma · S. Mehta
University School of Biotechnology,
G.G.S. Indraprastha University, Kashmere Gate,
Delhi 110006, India

N. Sharma · S. Marguerat · S. Watt · J. Bähler (✉)
Cancer Research UK Fission Yeast Functional Genomics
Group, Wellcome Trust Sanger Institute, Hinxton,
Cambridge CB10 1HH, UK
e-mail: jurg@sanger.ac.uk

components of the transcriptional apparatus, because its transcription mechanism is more similar to that of mammals in some respects. For example, the initiation of transcription in both mammalian cells and *S. pombe* occur ~30 bp downstream of the TATA box, whereas in *S. cerevisiae* this distance can vary between 40 and 120 bp (Li et al. 1994). Moreover, transcriptional initiation from mammalian promoters introduced into *S. pombe* occurs at the same site as in mammalian cells (Toyama and Okayama 1990).

As in other eukaryotes, *S. pombe* pol II is a multi-subunit enzyme containing the 12 subunits Rpb1 to Rpb12. The four core subunits, Rpb1, Rpb2, Rpb3 and Rpb11, are homologous to the β' , β , α' and α subunits, respectively, of the eubacterial RNA polymerase. Five subunits, Rpb5, 6, 8, 10 and 12, are shared by the three eukaryotic RNA polymerases I, II and III, whereas Rpb4, Rpb7 and Rpb9 are subunits specific to pol II (Mitsuzawa and Ishihama 2004). The functions of most of these smaller subunits are not well characterized.

Rpb4 and Rpb7 form a conserved heterodimer complex in archaeobacteria, yeast, plants and humans (Choder 2004). Crystallographic studies of pol II and biochemical data provide evidence for a role of the Rpb4/7 heterodimer in transcription initiation and RNA binding in *S. cerevisiae* (Armache et al. 2005; Bushnell and Kornberg 2003; Edwards et al. 1991; Orlicky et al. 2001). *S. pombe* Rpb7 interacts with an RNA-binding protein, implying a role in the coupling of RNA processing to transcription, and it associates with glyceraldehyde-3-phosphate dehydrogenase and actin (Mitsuzawa et al. 2003, 2005).

The in vivo functions of Rpb4 have been characterized in *S. cerevisiae*, and several lines of evidence suggest that it plays an important role in the response and survival to stress (reviewed by Choder 2004). *S. cerevisiae* Rpb4 is also important for activated transcription from a subset of promoters and in carbon and energy metabolism at moderate temperatures (Pillai et al. 2001, 2003). Recent data show that it is required for the decay of a class of mRNAs whose products are involved in protein synthesis, and it interacts with subunits of the mRNA decay complex Pat1/Lsm1-7 that enhances decapping (Lotan et al. 2005).

We are only beginning to understand the role of the Rpb4 subunit in *S. pombe*: it binds to the TFIIF-interacting carboxyl-terminal domain (CTD) phosphatase Fcp1, and it has been proposed to play a role in the assembly of the Fcp1–pol II complex, thereby promoting CTD phosphorylation for the reutilization of pol II in a new cycle of transcription (Kimura et al. 2002). *S. pombe* Rpb4 exhibits features that resemble its budding yeast counterpart, while others place it closer to

its orthologs in multicellular eukaryotes. It is essential for viability, whereas in *S. cerevisiae* it is not required for cell growth under optimal conditions (Sakurai et al. 1999; Choder 2004). *S. pombe* Rpb4 contains 135 amino acids, placing it closer in size to its counterparts in humans (142 amino acids) and plants (138 amino acids) than to *S. cerevisiae* (221 amino acids). Stoichiometric amounts of Rpb4 associate with pol II in *S. pombe* and higher eukaryotes, while the fraction of pol II molecules containing Rpb4 in optimally growing *S. cerevisiae* cells is only about 20%. Finally, Rpb4 is more tightly associated with *S. pombe* pol II than with *S. cerevisiae* pol II (Sakurai et al. 1999).

Here, we provide evidence that Rpb4 influences the growth and gene expression of *S. pombe* cells in a dose-dependent fashion under optimal conditions. We show that lower levels of *rpb4* expression do not affect the survival of cells under several stress conditions. Our results imply a particularly important role for Rpb4 in the expression and regulation of a subset of genes involved in cell separation. The defects in cell growth and separation as well as the gene expression signatures associated with low levels of *rpb4* expression are similar to the phenotypes and expression signatures of a range of transcriptional regulatory mutants with defects in cell separation.

Materials and methods

Strains and molecular genetic methods

The *S. pombe* strains used in this study are listed in Table 1. JB22 was the parental haploid strain used to generate the strains expressing *rpb4* under the P3*nmt1*, P41*nmt1* and P81*nmt1* promoters (Basi et al. 1993). The strains were constructed by PCR using the pFA6a-kanMX6-P3/P41/P81*nmt1* constructs as described (Bähler et al. 1998), and the kanamycin-resistant transformants were selected on KsNoT media (Linder et al. 2002). Recombinant DNA methods were performed as described (Sambrook et al. 1989) and standard methodology and media for the manipulation of *S. pombe* were used (Moreno et al. 1991).

Table 1 Strains used in this study

Strain	Relevant genotype	Source
JB22	972 <i>h</i> ⁻	Leupold (1970)
JB394	kanMX6- <i>p3nmt1-rpb4 h</i> ⁻	This study
JB395	KanMX6- <i>p41nmt1-rpb4 h</i> ⁻	This study
JB396	kanMX6- <i>p81nmt1-rpb4 h</i> ⁻	This study

Growth experiments

Pre-cultures for the growth curves were made by inoculating the respective strains in Edinburgh Minimal Medium (EMM) without thiamine or with 15 μM thiamine and growing the cultures for ~ 17 h at 32°C with shaking. Subsequently, the pre-culture was diluted into 50 ml of fresh EMM medium at an $\text{OD}_{600} = 0.05\text{--}0.1$ and allowed to grow at 32°C with shaking. Samples were removed at various timepoints thereafter for measuring ODs at 600 nm, microscopic examination, and microarray hybridization experiments. The low OD measurements in cells expressing *rpb4* under the P41*nmt1* and P81*nmt1* promoters is not caused by the phenotype, because mutants with similar phenotypes such as *sep1* show strong increases in OD during growth (unpublished data).

Quantitative RT-PCR experiments

To measure *rpb4* expression, the strains expressing *rpb4* under the three promoters were grown for 24 h in EMM medium at 32°C (promoter on) or cultivated for a further 21 h in the presence of 15 μM thiamine (promoter off). RNA was isolated by phenol extraction, purified using the RNeasy kit (Qiagen), and treated with RNase-free DNase (Roche). The reverse transcription reactions were performed using Superscript III (Invitrogen). Real-time PCR was performed using the Brilliant[®] SYBR[®] Green QPCR Core Reagent Kit (Stratagene) on an Mx3000P QPCR system (Stratagene) with primers specific for *rpb4* or *fbal* (control) (sequences available on request). Arbitrary expression units were calculated using a standard curve made from serial dilutions of fission yeast genomic DNA.

Stress response assays

Serial dilutions (1/10) of *rpb4* strains generated in our study were spotted on EMM plates with or without 15 μM thiamine. The plates were supplemented with 0.5 mM hydrogen peroxide or different concentrations of sorbitol (1, 2, 3 and 4 M) to test the response to oxidative and osmotic stress, respectively. These plates were incubated at 32°C for 2 days and photographed. The effect of temperature stress was tested by incubating the strains at 36°C for 2 days.

Microscopy

Unfixed cells were observed at 2-h intervals for 25 h by light microscopy using a Zeiss AxioScope fluorescence microscope set up for differential interference

contrast (DIC) with a 40 \times objective and Axiovision digital imaging system. To stain the nuclei, cells were spread onto microscopic glass slides. Subsequently, the cells were fixed by heating at 70°C for 1 min and DAPI (4',6' diamidino-2-phenylindole) was added at a final concentration of 1 $\mu\text{g}/\text{ml}$. To view the division septa, calcofluor was added to cultures at a final concentration of 5 mg/ml and the cultures were incubated in the dark at room temperature for 5 min. The cells were washed twice with phosphate buffer saline, and 5 μl of cell suspension was spotted onto microscopic glass slides. DAPI- and calcofluor-treated cells were visualized under a Zeiss AxioScope microscope as above.

Microarray experiments

To compare the gene expression profiles as a function of *rpb4* expression, cells were grown in the presence of thiamine to OD_{600} of 0.2, and 50 ml of culture was centrifuged. The remaining culture was washed four to five times with EMM and subsequently grown in EMM medium without thiamine for 17 h and centrifuged. To identify transcripts affected by lowered *rpb4* expression, the desired strains were grown in the presence of thiamine for 21 and 23 h and harvested. A wild-type parental strain grown in the presence of thiamine served as a reference. Although thiamine leads only to minimal expression changes (Jenkins et al. 2005), this experimental design corrects for effects caused by the presence of thiamine. Pellets were frozen in liquid nitrogen and kept at -70°C until further processing. RNA was isolated by phenol extraction and purified by the RNeasy kit (Qiagen). cDNA probes were prepared with Superscript (Invitrogen) and labeled with Cy3 or Cy5. Detailed methods were as described (Lyne et al. 2003; http://www.sanger.ac.uk/PostGenomics/S_pombe). Microarrays were scanned with a Genepix 4000B scanner and analyzed with Genepix software (Axon Instruments). Data filtering, normalization and quality control were performed as described (Lyne et al. 2003). The data are based on two to three independent biological repeats with dye swaps. To quantify global effects on gene expression, we repeated microarray analysis spiked with external control RNAs. The expression data were normalized using *Bacillus subtilis* spikes added in equal quantities to the RNA samples before labeling (Lee et al. 2005). Each microarray contains 720 control elements for the spikes, which are spread across the complete grid of the array in a spatially even manner. Clustering and visualization were done with GeneSpring. Gene annotations were taken from the *S. pombe* GeneDB database (www.genedb.org/

genedb/pombe/index.jsp). Overlaps between different gene lists were calculated using the hypergeometric distribution. All normalized data sets are available from our website: http://www.sanger.ac.uk/PostGenomics/S_pombe.

Results

Dosage-dependent effect of Rpb4 on cell growth

Rpb4 is an essential subunit of *S. pombe* pol II. To elucidate its in vivo function(s), we constructed haploid strains expressing *rpb4* from its normal chromosomal location using the thiamine-regulated *nmt1* promoter. Three different versions of this promoter were used: the wild-type P3*nmt1* promoter has the highest level of promoter activity and maintains substantial basal levels of expression even under repressed conditions; the two derivatives, P41*nmt1* (medium strength) and P81*nmt1* (weakest strength) have reduced levels of activity due to mutations in the TATA box (Basi et al. 1993).

We performed quantitative PCR of cells expressing *rpb4* under the three promoters, both in the absence (*nmt1* promoter ‘on’) or presence (*nmt1* promoter ‘off’) of thiamine to verify changing levels of *rpb4* mRNA (Fig. 1). Expression levels of *rpb4*

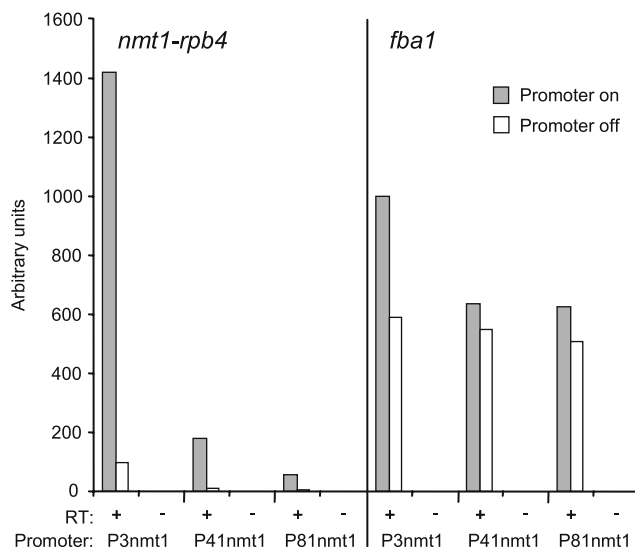


Fig. 1 Expression levels of *rpb4* mRNA when transcribed from different promoter constructs. Cells expressing *rpb4* from the P3*nmt1*, P41*nmt1* or P81*nmt1* promoter were grown for 24 h in EMM medium at 32°C (promoter on; gray bars) or cultivated for a further 21 h in the presence of thiamine (promoter off; white bars). *rpb4* (left) and *fba1* (right; control) mRNA levels from the same cells were measured by quantitative RT-PCR. The PCR was performed on reverse transcription reactions, which were carried out in the presence (RT+) or absence (RT-) of reverse transcriptase

showed a range of more than 1,000-fold in the different conditions, and the relative expression levels were as expected for the three promoters of different strength. The *rpb4* levels were especially low when expressed from the P41*nmt1* and P81*nmt1* promoters in the presence of thiamine. The *fba1* gene is highly expressed and was used as a control gene. Although it shows much less variation in expression levels than *rpb4*, there are some changes in accordance with *rpb4* levels (Fig. 1). This probably reflects effects on global transcription as a function of *rpb4* expression levels (see below).

To analyze effects of *rpb4* expression levels, the three strains were grown at 32°C either in the absence or presence of thiamine, and cell growth was monitored. In the absence of thiamine, all strains grew similar to each other and to the wild-type strain (Fig. 2a). This shows that *rpb4* expressed from the weak P81*nmt1* promoter under induced conditions was sufficient to sustain normal cell growth. When these strains were grown in the presence of thiamine, however, there was a direct correlation between residual promoter activity and growth rate: cells expressing *rpb4* from the P3*nmt1* promoter showed the same growth rate as wild-type cells, while cells expressing *rpb4* from the P41*nmt1* promoter and, even more so, from the P81*nmt1* promoter showed reduced growth rates (Fig. 2b). Thus, in the presence of thiamine, the lowered Rpb4 levels become limiting for cell growth, and residual growth directly reflects basal expression levels from the different promoter constructs.

Effect of Rpb4 on cell growth under stress conditions

Rpb4 is required for stress response in *S. cerevisiae* (Choder 2004). We therefore, determined if Rpb4 played a similar role under stress conditions in *S. pombe*. Cells expressing varying levels of *rpb4* were subjected to temperature stress (36°C), osmotic stress (1 M sorbitol) or oxidative stress (0.5 mM hydrogen peroxide). We found that neither in the presence nor absence of thiamine, the cells exhibited any increased stress sensitivity compared to control cells when grown under the different stress conditions (data not shown). Similar results were obtained when cells were grown in up to 2 M sorbitol. No significant growth of either the wild-type or Rpb4 expressing cells was observed in the presence or absence of thiamine at 3 and 4 M concentrations of sorbitol. These observations indicate that low levels of Rpb4 do not affect the survival of cells under any of the tested stress conditions.

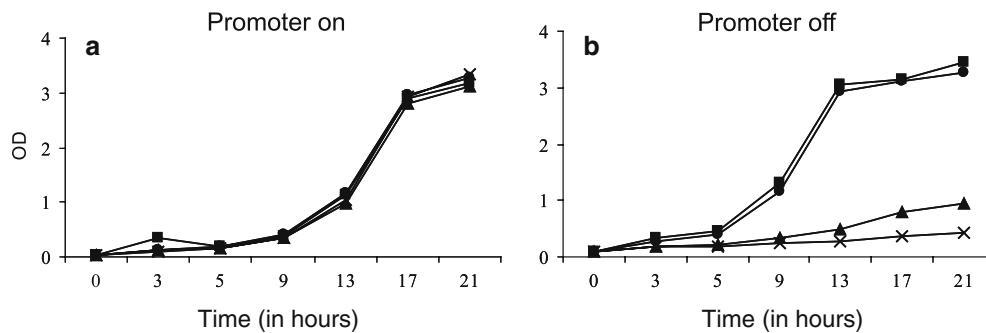


Fig. 2 Effect of Rpb4 on cell growth. Wild-type cells (filled circle) and cells expressing *rpb4* from either the P3*nmt1* (filled square), P41*nmt1* (filled triangle) or P81*nmt1* (crosses) promoter were grown in EMM medium at 32°C. OD₆₀₀ was measured at the indi-

cated times. **a** In the absence of thiamine, all strains grew at similar rates. **b** In the presence of thiamine, the growth rates were dependent on the residual levels of *rpb4* being expressed from the different *nmt1* promoters

Repression of *rpb4* transcription causes defects in cell separation

We next examined whether different levels of *rpb4* expression would influence specific cellular processes. Cells expressing *rpb4* from any of the three *nmt1* promoters in the absence of thiamine did not lead to any obvious phenotypes even after 25 h of growth in medium without thiamine (Fig. 3a).

In thiamine-containing medium, however, cells expressing *rpb4* from the weak P81*nmt1* promoter became more elongated than the wild-type cells after 19 h of growth (Fig. 3b). Moreover, after continued incubation in the same medium for another 2 h (21 h with thiamine), cells also showed defects in cell separation, which became more evident after an additional 4 h of incubation (25 h with thiamine) (Fig. 3b). Most cells were elongated with either a single or multiple septa, and some cells also displayed bent morphologies and/or growth by branching. Cells expressing *rpb4* from the medium strength P41*nmt1* promoter displayed no obvious cell separation defects until 23 h of growth in the presence of thiamine. Similar defects as described above, however, became apparent after 23 h of growth in thiamine (data not shown). In contrast, cells expressing *rpb4* from the strong P3*nmt1* promoter did not exhibit any aberrations in morphology or division after growth in thiamine-containing medium for 25 h (Fig. 3b) and continued to look like wild-type cells even at 40 h of growth in thiamine-containing medium (data not shown). Thus, the basal level of expression from the strongest promoter is sufficient to prevent the cell separation defects seen with the weaker promoters.

We investigated these phenotypes in more detail for cells expressing *rpb4* under the P81*nmt1* promoter by staining the division septa and cell nuclei (Fig. 3c).

Several nuclei were present separated from each other by either a single septum or multiple, aberrant septa. Some cells also contained large deposits of septal material at random positions (Fig. 3c). We conclude that lowered *rpb4* expression levels lead to filamentous growth with multiple cells remaining attached to each other. Similar phenotypes to those of cells expressing *rpb4* under the P81*nmt1* promoter have been observed in several different mutants (see Discussion); for example, deletions in two transcription factor genes, *sep1* and *ace2*, also lead to defects in cell separation (Fig. 3d; Bähler 2005). These findings raise the possibility that Rpb4 plays a specific role in cell separation after cytokinesis.

Low levels of *rpb4* transcripts most strongly affect expression of genes involved in septation

We used *S. pombe* whole-genome microarrays to investigate the effects on gene expression caused by low levels of *rpb4*. Wild-type cells and cells expressing *rpb4* under the different regulatable promoters were grown in the presence and absence of thiamine followed by RNA extraction. Cells expressing *rpb4* under the P3*nmt1* promoter did not show any significant differences in gene expression profiles when compared with wild-type cells, either in the presence or absence of thiamine (data not shown), consistent with our earlier observations that these cells show normal growth and phenotypes. If *rpb4* was expressed under the weakest promoter in the presence of thiamine, however, gene expression changed substantially. As down-regulation of *rpb4* is expected to have a global effect on transcription, we used external spikes for normalization (Materials and methods). This revealed that mean transcript levels were 26–36% lower compared to wild-type cells

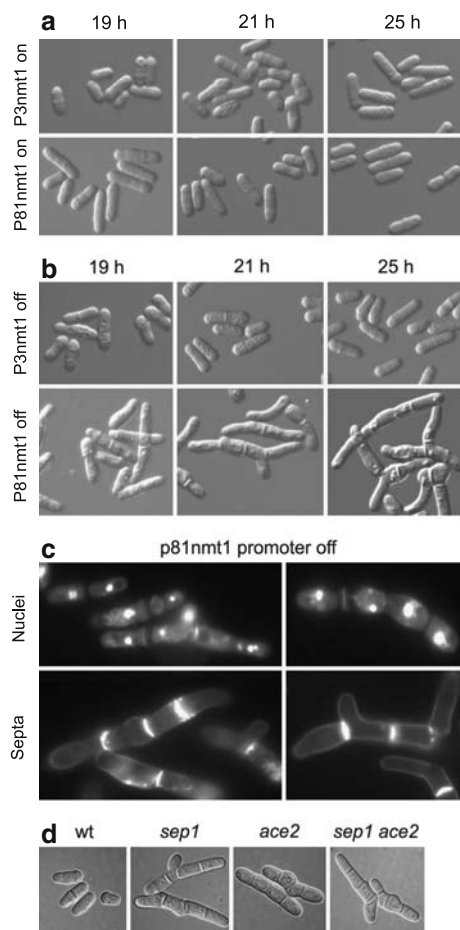


Fig. 3 Rpb4 affects cell separation and morphology. Cells expressing *rpb4* from either the P3nmt1 or P81nmt1 promoter were analyzed by microscopy. **a** Cells grown in EMM in the absence of thiamine (promoter ‘on’) at 32°C appear like normal, wild-type cells. **b** When grown in the presence of thiamine (promoter ‘off’), cells expressing *rpb4* from the P3nmt1 promoter appear normal, while those expressing *rpb4* under the P81nmt1 promoter display defects in cell morphology and septation. Multiple phenotypes including elongated cells with single or multiple septa and growth by branching are evident. **c** Cells expressing *rpb4* under the P81nmt1 promoter were analyzed by fluorescence microscopy in the presence of thiamine. DAPI staining revealed single nuclei in each cell compartment, while calcofluor staining showed septa (some of them aberrant) separating the cell compartments. **d** Control cells and mutants showing similar phenotypes as cells expressing *rpb4* under the P81nmt1 promoter cells. From left to right: wild-type cells, *sep1* deletion mutants, *ace2* deletion mutants and *sep1 ace2* double deletion mutants

grown under the same conditions (Fig. 4a). The expression levels of *rpb4* were two- to threefold lower under these conditions compared to the levels under its own promoter. Thus, lowered *rpb4* expression leads to reduced overall transcript levels.

On top of this genome-wide down-regulation of transcription, 96 transcripts were at least 1.5-fold upregulated in three of the four samples tested, while 150 genes were at least threefold downregulated in

three of the four samples tested upon lowering *rpb4* expression (Supplementary Tables S1 and S2). These genes were categorized according to their functions (Supplementary Table S3). The majority of the genes whose expression levels were affected by *rpb4*, encoded proteins involved in metabolism and transport. Many of the upregulated transcripts in cells with lowered *rpb4* expression levels overlapped with genes upregulated during different environmental stresses (Chen et al. 2003) and nitrogen starvation (Mata et al. 2002) ($P \sim 1 \times 10^{-13}$ to 1×10^{-36}). This probably reflects an indirect effect caused by the defects in these cells. A substantial number of the downregulated genes in the category of cell cycle and cell division played specific roles in cytokinesis and cell separation. For example, the two genes *eng1* and *agn1* encode β -glucanase and α -glucanase, respectively; Eng1 degrades the primary division septum between the new ends of daughter cells and Agn1 hydrolyzes the old cell wall surrounding the septum leading to full separation of daughter cells (Martín-Cuadrado et al. 2003; Dekker et al. 2004). The genes *adg1* and *adg3* have also been shown to be involved in cell separation (Alonso-Nunez et al. 2005), and *rng3* encodes a protein involved in formation of contractile ring during cytokinesis (Wong et al. 2000).

Both the lists of genes being upregulated and downregulated after *rpb4* down-regulation showed highly significant overlaps with lists of genes upregulated or downregulated, respectively, in *sep10*, *sep15*, *mcs6* and *pmh1* mutants ($P \sim 1 \times 10^{-16}$ to 1×10^{-47}) (Lee et al. 2005), whose genes encode different proteins involved in transcriptional regulation (see Discussion). Among the downregulated genes, the most pronounced overlap was observed with *sep10* and *sep15* mutants (Fig. 4b). The downregulated genes also showed some overlap with genes downregulated in *sep1* and *ace2* mutants ($P \sim 1 \times 10^{-14}$) (Rustici et al. 2004), which encode cell-cycle transcription factors. Figure 4c shows a cluster analysis of the genes most strongly regulated by *rpb4* repression and their expression levels in the various transcriptional mutant backgrounds. The *sep10*, *sep15*, *mcs6* and *pmh1* mutants show highly similar expression signatures to cells with compromised *rpb4* expression, while the overlap with *sep1* and *ace2* mutants is less evident. Intriguingly, if *rpb4* was overexpressed using the medium strength promoter, the expression levels of these genes were reversed, with downregulated genes becoming upregulated and upregulated genes becoming downregulated (Fig. 4c). Thus, transcript levels of this gene set are particularly responsive to the transcript levels of *rpb4*.

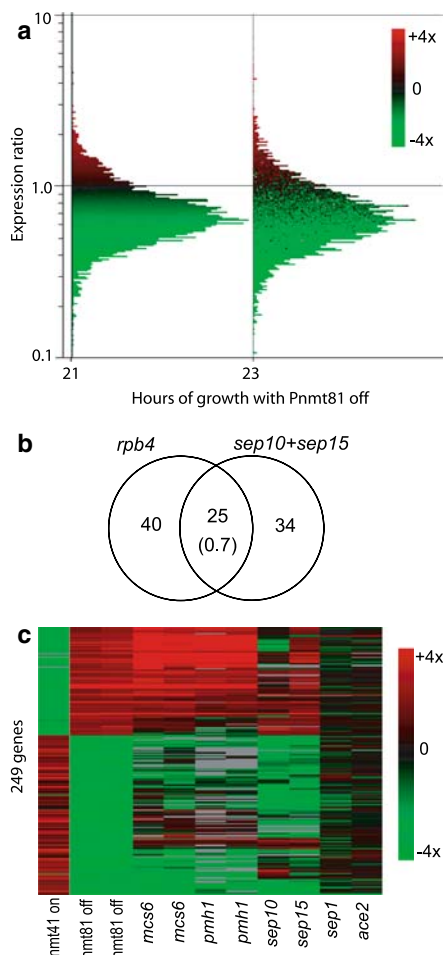


Fig. 4 Effects on global and specific gene expression as a function of *rpb4* levels. **a** Microarray experiments of cells expressing *rpb4* under the weakest promoter. The histograms show the distribution of gene expression ratios of P81nmt1-*rpb4* cells grown in the presence of thiamine for 21 and 23 h relative to wild-type cells grown in the presence of thiamine. Genes in both histograms are colored by their relative expression levels at 21 h as indicated at upper right. External spikes have been used for normalization, revealing a global down-regulation of transcript levels by 26% (21 h) to 36% (23 h) on average when *rpb4* expression is lowered. The averages of two independent repeats for each timepoint are shown. **b** Overlap between the genes downregulated when *rpb4* is expressed under the weakest promoter in the presence of thiamine and genes downregulated in both *sep10* and *sep15* mutants (Lee et al. 2005). The number in brackets represents the overlap expected by chance, given the sizes of the gene sets considered and the total number of 5,180 genes. The overlap is highly significant as calculated using the hypergeometric distribution ($P \sim 1 \times 10^{-30}$). **c** Hierarchical cluster analysis of 249 genes that are either 1.5-fold upregulated or threefold downregulated when *rpb4* is expressed under the weakest promoter in presence of thiamine. Horizontal rows represent the profiles of hierarchically clustered genes. Columns represent experiments of different mutant strains and different time-points for the same strain. The transcript levels for each strain (indicated at bottom) relative to levels in wild-type cells are color-coded as indicated at right, and missing data are shown in gray. The microarray data from *mcs6*, *pmh1*, *sep10* and *sep15* mutants are from Lee et al. (2005), and the data from *sep1* and *ace2* mutants are from Rustici et al. (2004)

Discussion

We have analyzed functions of the Rpb4 subunit of pol II in *S. pombe*. Despite the overall conservation in structure and function of pol II across species, the Rpb4 subunit presents a special case. *S. pombe* Rpb4 is similar to its orthologs in higher eukaryotes with respect to its structure and essential nature (Sakurai et al. 1999), but in contrast to its human ortholog, it can form stable hybrid dimers with *S. cerevisiae* Rpb7 (Sakurai et al. 1999) and can rescue the temperature-sensitive phenotype associated with the lack of *S. cerevisiae* Rpb4 (Shpakovski et al. 2000). Thus, *S. pombe* Rpb4 may have intermediate characteristics useful to bridge the knowledge from *S. cerevisiae* and multicellular eukaryotes.

We used regulatable gene expression constructs to study the in vivo roles of Rpb4. Under optimal conditions, *rpb4* influences the growth of cells in a dose-dependent manner when expression drops below a certain level using the regulatable promoters (Figs. 1, 2). In *S. cerevisiae*, deletion of the *RPB4* gene leads to slow cell growth under optimal conditions (Woychik and Young 1989; Choder 2004), while in *S. pombe* *rpb4* is essential (Sakurai et al. 1999). We found that low levels of *rpb4* expressed from the weakest promoter were sufficient for the cells to survive a range of different stress conditions including temperature stress. Accordingly, core stress genes were actually upregulated in *rpb4* mutants, and *S. pombe* Rpb4 is therefore unlikely to play any specialized role during environmental stress. *S. cerevisiae* Rpb4 is dispensable for oxidative and osmotic stress (Maillet et al. 1999), but unlike in *S. pombe*, it is required for survival of temperature stress (Woychik and Young 1989).

Fission yeast cells grown under low expression of *rpb4* were elongated and showed defects in cell separation as indicated by the accumulation of division septa, some of them highly aberrant (Fig. 3). Strains with cell separation defects similar to the ones described here include mutants in components of the exocyst complex (*sec6*, *sec8*, *sec10* and *exo70*), an anillin homolog (*mid2*), septins (*spn3*, *spn4*), an endo- α -1,3-glucanase (*agn1*), an endo- β -1,3-glucanase (*eng1*), calcineurin (*ppb1*), a MAP kinase and phosphatase (*pmk1*, *pmp1*), two transcription factors (*sep1*, *ace2*), and subunits of the mediator complex (*sep10*, *sep11* and *sep15*) (Sipiczki et al. 1993; Yoshida et al. 1994; Longtine et al. 1996; Toda et al. 1996; Sugiura et al. 1998; Grallert et al. 1999; Wang et al. 2002; Martín-Cuadrado et al. 2003; Spähr et al. 2003; Tasto et al. 2003; Dekker et al. 2004). In *S. cerevisiae*, haploid *rpb4* deletion mutants exhibit an axial budding pattern with normal yeast cell

morphology, but diploid *rpb4* mutants are more elongated than wild-type cells during nitrogen starvation and grow in a unipolar pattern, resembling pseudohyphae formation (Pillai et al. 2003).

Lowered expression of *rpb4* causes relatively modest reductions in global transcription, which probably leads to the reduced growth rates under these conditions (Fig. 4a). In addition, low *rpb4* transcription results in substantially lowered expression of a distinct subset of genes. Among the strongly affected genes were *eng1*, *agn1*, *adg1* and *adg3* with known functions in cell separation, which are targets of a transcriptional cascade regulated by the Sep1 and Ace2 transcription factors (Rustici et al. 2004; Alonso-Nunez et al. 2005). Accordingly, the genes affected by lowered *rpb4* expression significantly overlapped with genes affected in *sep1* and *ace2* mutants. Much stronger similarities, however, were evident with the gene expression signatures of several strains mutated in the *sep10*, *sep15*, *mcs6* and *pmh1* genes, which encode various components of the transcriptional machinery. Sep10 and Sep15 are essential components of the mediator complex of pol II, while Mcs6 and Pmh1 are components of a complex homologous to metazoan Cdk7-cyclinH-Mat1, a cyclin-dependent kinase-activating kinase (CAK) and a pol II CTD kinase associated with transcription factor IIH (Spåhr et al. 2001, 2003; Lee et al. 2005). The overlap in expression signatures between these four mutants and cells with lowered *rpb4* expression was evident for both upregulated and downregulated genes. These differentially regulated genes showed reverse regulation (upregulated genes became downregulated and vice versa) when *rpb4* was overexpressed from the medium strength promoter (Fig. 4c). Thus, the expression of this gene set is particularly responsive to *rpb4* expression levels and also depends on a range of other factors involved in general transcription. This could either reflect a specific transcriptional pathway involving Rpb4 and the other factors (either directly or indirectly via the control of a regulator), or the affected genes could be generally sensitive to transcriptional interference. In any case, these data show that the transcriptional pathway required for cell separation in fission yeast (Bähler 2005) is strongly affected when interfering with *rpb4* expression levels.

We speculate that the Rpb4/Rpb7 complex is involved in linking the transcriptional output to the metabolic status of the cell. The *rbp4* and *rpb7* transcripts are down-regulated when cells enter stationary phase (unpublished observations), Rpb7 interacts with the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Mitsuzawa et al. 2005), and global transcription is sensitive to *rpb4* expression levels. It is

possible that the transcriptional pathway required for cell separation is particularly sensitive for compromised transcription to adjust the growth mode to availability of nutrients: in abundant nutrients, global transcription is efficient and growth is best as single cells, while in limiting nutrients, global transcription and cell separation are compromised, and cells grow as multicellular pseudohyphae, which may allow a more efficient grazing for new nutrients as growth is directed. In the first description of *S. pombe*, multi-cellular pseudohyphae similar to those seen in *rpb4* mutants have been observed (Lindner 1893); the natural capacity to switch to multi-cellular growth might have been lost in the yeast strains cultured in the laboratory.

Taken together our data suggest that *S. pombe* Rpb4, besides its general role in transcription, performs a more specialized function in regulating a specific gene expression program required for cell separation at the end of the cell cycle. It is possible that this role is carried out by interacting with other general factors of the transcriptional machinery such as the mediator and CTD kinase as suggested by similarities in phenotypes and expression signatures in our study. Accordingly, a comparison of the X-ray crystallographic structure of the complete pol II, including the Rpb4/7 heterodimer, with an electron microscopic structure of the pol II-mediator complex, has indicated that Rpb4/7 may interact with the mediator (Bushnell and Kornberg 2003). Alternatively, the subset of genes affected by changes in *rpb4* expression (and by manipulating other general transcription factors) could reflect genes that are especially sensitive to transcriptional stress. This interpretation does not imply any specific gene expression program, although it could be used by the cell to adjust transcription to available nutrients. Given the conservation of Rpb4, it will be interesting to see whether the human ortholog plays a similar specialized role to transcribe particular subsets of genes.

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