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Relative levels of RNA polII subunits differentially affect starvation response in budding yeast

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Abstract

The Rpb4/7 subcomplex of RNA polymerase II in *Saccharomyces cerevisiae* is known to play an important role in stress response and stress survival. These two proteins perform overlapping functions ensuring an appropriate cellular response through transcriptional regulation of gene expression. Rpb4 and Rpb7 also perform many cellular functions either together or independent of one another. Here, we show that Rpb4 and Rpb7 differently affect during the nutritional starvation response pathways of sporulation and pseudohyphae formation. Rpb4 enhances the cells' proficiency to sporulate but suppresses pseudohyphal growth. On the other hand, Rpb7 promotes pseudohyphal growth and suppresses sporulation in a dose-dependent manner. We present a model whereby the stoichiometry of Rpb4 and Rpb7 and their relative levels in the cell play a switch like role in establishing either sporulation or pseudohyphal gene expression.

Keywords: Stress response; RNA polII; Rpb4; Rpb7; Pseudohyphal growth; Sporulation

Eukaryotic cells respond to environmental changes by sensing, amplifying, and communicating them to the nucleus where these diverse signals are integrated and appropriate responses are triggered by changing the pattern of gene expression. The core RNA polymerase II (polII) is the enzyme responsible for the transcription of protein coding genes. In *Saccharomyces cerevisiae*, Rpb4 and Rpb7 subunits of polII form a two-subunit subcomplex [1], and homologous complexes have been reported in many different species [2–5]. The existence of Rpb4/7 like subcomplex has also been demonstrated in polI and polIII [6–8]. The *S. cerevisiae* polII Rpb4/7 sub-complex is known to be important in different stress responses and stress survival [9,10]. *RPB7* is essential in *S. cerevisiae* and *Schizosaccharomyces pombe* [11,12] and is structurally and functionally conserved across many eukaryotes [10,13]. *RPB4*, on the other hand, is not essential in *S. cerevisiae* under optimal growth conditions but *rpb4Δ* cells exhibit various conditional phenotypes such as growth defects at temperature extremes [14], pseudohyphal predisposition and defective sporulation [15], poor recovery from stationary phase, etc. Rpb4 is conserved from archaea to humans at the sequence level. However, ScRpb4 is unique amongst Rpb4 homologs in having highly charged, non-conserved regions separating the N- and C-terminal conserved regions, which may be involved in interaction with other factors [16]. It is suggested that one of the roles of Rpb4 as an interacting partner of Rpb7 is to stabilize the inherent weak interaction of Rpb7 with the rest of the polymerase. Such a hypothesis is supported by lack of Rpb7 in polII purified from *rpb4Δ* strain [17] and the fact that the overexpression of *RPB7* in *rpb4Δ* null strain can partially rescue the conditional phenotypes of *rpb4Δ* strains [15,18,19]. In *S. cerevisiae*, Rpb4 mediates mRNA export during stress [20]. Rpb7 interacts with a multitude of proteins other than

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Rpb4 including transcriptional regulators as well as some structural proteins. The Rpb7 homolog from *C. albicans* has been shown to genetically interact with Ess1, a protein which modulates Rpb1 CTD conformation [21].

Saccharomyces cerevisiae cells exposed to severe starvation of carbon and nitrogen undergo sporulation while under mild nutritional starvation exhibit pseudohyphal growth [22]. Both these stress responses are very sensitive to environmental cues. At the molecular level, sporulation is brought about by a complex and well-coordinated pattern of gene expression involving cell surface molecules, signal transduction pathways, and transcriptional regulators [23]. Similarly, pseudohyphae formation in response to starvation is mediated by multiple pathways including the MAPK cascade involved in mating and the cAMP-dependent protein kinase A. Both these pathways converge on *FLO11*, a transcriptional activator of pseudohyphae formation [24]. Earlier, our group had shown that *rpb4Δ/rpb4Δ* strains are defective in sporulation and are predisposed to form pseudohyphal growth. Overexpression of *RPB7* in an *rpb4Δ/rpb4Δ* rescues the sporulation defect partially while the pseudohyphal growth is enhanced [15]. In an attempt to further understand the function of Rpb4 and Rpb7 in these two stress response pathways, we carried out differential expression studies of Rpb4 and Rpb7; effectively changing the stoichiometry of the subcomplex and its effect on these two stress responses. We find that *S. cerevisiae* strains predisposed to pseudohyphae formation are poor in sporulation suggesting that yeast cells that adopt one stress response may indeed down-regulate the other at the transcriptional level. We propose that Rpb4 and Rpb7 play a switch like role for sporulation and pseudohyphae formation dependent on their relative levels.

Materials and methods

Yeast strains. The *S. cerevisiae* strains used in this study were (i) SYD1001 (MLY97): *MAT a/α, ura3-52/ura3-52 Δleu2::hisG/Δleu2::hisG* [25], (ii) SYD2002: *Mat a/α*; *his3Δ1/his3Δ1*; *leu2Δ0/leu2Δ0*, *lys2Δ0/LYS2*; *MET15/met15Δ0*; *ura3Δ0/ura3Δ0*; *YJL140w::kanMX4/YJL140w::kanMX4*, and (iii) SYD2001: *Mat a/α*; *his3Δ1/his3Δ1*; *leu2Δ0/leu2Δ0*, *lys2Δ0/LYS2*; *MET15/met15Δ0*; *ura3Δ0/ura3Δ0*. These strains were transformed with the plasmid constructs listed in Table 1 to generate the appropriate strains assayed for various phenotypes in this study. Yeast transformations were done using a modified lithium acetate method, which does not involve heat shock treatment of yeast cells [26]. All plasmid manipulations were done in *Escherichia coli* strain DH5α {supE44ΔlacU169 (ϕ 80lacZΔM15) hsdR17recA1endA1gyrA96thi-1relA1} using standard protocols [27].

Assay conditions and media composition. Strains were routinely maintained on synthetic drop-out (SD) plates (2% dextrose or 2% galactose, 2% agar) supplemented with the required amino acids. Sporulation and pseudohyphal growth were assayed as described previously [13].

Construction of plasmids. The plasmids used in the study are listed in Table 1. The construction of *RPB4*, *RPB7* gene in 2μ plasmid and *PHD1* open reading frame under P_{GAL1} has been described previously [6,15]. The open reading frames of *RPB4* and *RPB7* were cloned under the P_{GAL1} in pPS2 as *Bam*HI–*Xho*I fragments to generate pPS40 and pPS41, respectively. *RPB7* gene was cloned in pPS2, pPS4, and pPS5 to generate pRA56, pBP244, and pSP186, respectively. *RPB7* open reading frame was cloned as *Bam*HI–*Sal*I fragment under P_{GAL1} to generate pNS170.

Total RNA isolation and RT-PCR. Total RNA was isolated from all the strains by hot-phenol method involving liquid nitrogen. RNA samples were quantified by measuring $A_{260\,nm}$. Five micrograms of total RNA isolated from each strain was reverse transcribed with M-MuLV RT (MBI Fermentas Inc.) using oligo(dT) primer and PCR was done for RPB4 using RPB4 (f) and RPB4 (r), and for RPB7 using RPB7 (f) and RPB7 (r). The mRNA levels of RPB4 and RPB7 levels were normalized against ACTIN1, which was amplified using ACT1 (f) and ACT1 (r). The PCR products were run on 1.2% agarose gels.

Total protein extraction, antibodies, and Western blot analysis. Protein extracts were prepared by glass bead lysis method. Protein samples were electrophoresed in 12.5% SDS–PAGE, transferred to Immobilon™-P Transfer Membrane, and probed with rabbit polyclonal 1° antibodies raised against ScRpb7 and anti Rpb4 polyclonal antibodies raised against *Dictyostelium discoideum* Rpb4. The blots were probed with HRP-conjugated anti-rabbit and anti-mouse 2° antibodies, respectively, and developed using Western Lighting™ Chemiluminescence Reagent *plus* and X-ray film reagents.

Results

Effect of relative levels of Rpb4 and Rpb7 on sporulation

Saccharomyces cerevisiae rpb4Δ/rpb4Δ cells are defective in sporulation and this defect can be completely rescued by ectopic expression of a wild-type copy of *RPB4* [15]. In this

Table 1
List of plasmids used in the study

Plasmid	Description	References
pPS2	YCplac111; *CEN4*, *LEU2*	[29]
pPS4	YCplac33; *CEN4*, *URA3*	[29]
pPS5	YEplac181; *2μ*, *LEU2*	[29]
pPS7	YEplac195; *2μ*, *URA3*	[29]
pPS12	pYES2; *2μ*, *URA3*, PGAL1	Invitrogen
pPS25	pRP725; *2μ*, *LEU2*, PRPB4, *RPB4*	[6]
pPS26	pRP721; *2μ*, *LEU2*, PRPB7, *RPB7*	[6]
pPS40	*2μ*, *URA3*, PGAL1, *RPB4*; *RPB4* ORF under P_{GAL1} in pYES2	This work
pPS41	*2μ*, *URA3*, PGAL1, *RPB7*; *RPB7* ORF under P_{GAL1} in pYES2	This work
pRA56	*CEN4*, *LEU2*, PRPB7, *RPB7*; *RPB7* gene in YEplac111	This work
pBP128	*2μ*, *URA3*, PGAL1, *PHD1*; *PHD1* ORF under P_{GAL1} in pYES2	[15]
pNS170	*2μ*, *LEU2*, PGAL1, *RPB7*; *RPB7* ORF under P_{GAL1}	This work
pSP186	*2μ*, *URA3*, PRPB7, *RPB7*; *RPB7* gene in YEplac181	This work
pBP244	*CEN4*, *URA3*, PRPB7, *RPB7*; *RPB7* gene in YEplac33	This work

study, we have used an *rpb4Δ/rpb4Δ* strain which has a severe sporulation defect (Fig. 1). In order to understand the role of Rpb4 and its interacting partner Rpb7 in sporulation, we have checked the effect of different levels of Rpb4 and Rpb7 on sporulation. We observed that an increase in the level of Rpb4 results in the increase in the fraction of sporulating cells. As shown in Fig. 1, while the *rpb4Δ/rpb4Δ* do not sporulate, the percentage of sporulating cells increases with the concomitant increase in the level of Rpb4 in the cell. When *RPB4* gene is expressed under a 2μ copy plasmid, the percentage of sporulation is more than the wild-type cells and even higher when the *RPB4* ORF is expressed under the strong P_{GAL1} promoter. We show that the actual level of sporulation correlates well with the level of *RPB4* mRNA (Fig. 3A) and the amount of Rpb4 protein present in the cell (Fig. 3B). These results show a direct correlation of sporulation on Rpb4 and its level in the cell. At each level of Rpb4, we observed a decrease in sporulation with increasing levels of Rpb7 (Fig. 1) showing that Rpb7 has a detrimental effect on the level of sporulation in a dose-dependent manner. The degree of the inhibition of sporulation by Rpb7 correlates with the levels of *RPB7* mRNA (Fig. 3A) and Rpb7 protein (Fig. 3C).

Effect of relative levels of Rpb4 and Rpb7 on pseudohyphal growth

Saccharomyces cerevisiae rpb4Δ/rpb4Δ cells are predisposed to undergo pseudohyphal growth even under nutrient rich medium [13,15]. We believe that such a predisposition is exclusively dependent on the absence of Rpb4 alone because the corresponding *RPB4*$^{+}$ cells of this strain background are not predisposed to this phenotype even under optimally starved conditions that favor pseudohyphal growth (Fig. 2). The wild-type cells are morphologically indistinguishable from those grown under nutrient rich conditions. Earlier we have shown that overexpression of Sc*RPB7* and its orthologs in an *rpb4Δ/rpb4Δ* strain exaggerates the pseudohyphal growth [13,15]. We observed that even a small increment in the level Rpb7 (which was obtained by transforming a *CEN4* copy of *RPB7* gene in *rpb4Δ/rpb4Δ*) can exaggerate the pseudohyphal growth in *rpb4Δ/rpb4Δ* (Fig. 2C). With an increase in the level of Rpb7, the extent of exaggeration in the pseudohyphal growth increases in a dose-dependent manner (Figs. 2D, E, and 3C). However, such an exaggeration of pseudohyphal growth is not readily discernible in *RPB4*$^{+}$ cells even when Rpb7 is present in high amount (data not shown). Nevertheless, qualitatively, we observe that cells expressing higher levels of Rpb7 are more elongated. These results show that pseudohyphal growth is directly dependent on Rpb7 but inversely to Rpb4. Moreover, Rpb4 seems to play a dominant role over Rpb7 in the pseudohyphal growth. Its presence could mask the otherwise positive effect of Rpb7.

Pseudohyphal strains are compromised for sporulation

The above observations suggest that *S. cerevisiae* strains, which are proficient in undergoing sporulation, are poor in pseudohyphal growth and that the pseudohyphal strains are compromised for sporulation. At the molecular level, it seems that *S. cerevisiae* cells down-regulate genes in one of the two stress response pathways while the other is operational. It also means that Rpb4: Rpb7 ratios lie at the center of this regulatory switch, or the balance between sporulation and pseudohyphae formation. To test this model, we studied the effect of pseudohyphal transcriptional activator, *PHD1* on Σ1278b, a wild-type strain which shows pseudohyphal growth under appropriate condition. As expected, under nitrogen-starved condition, wild-type Σ1278b strain shows pseudohyphal growth. Overexpression of *PHD1* in this background

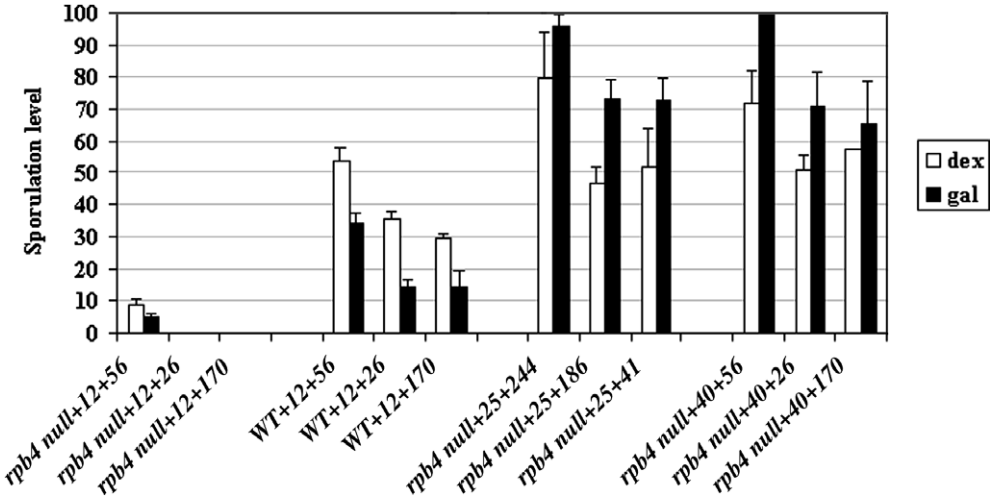

Fig. 1. Effect of relative levels of Rpb4 and Rpb7 on sporulation: *rpb4Δ/rpb4Δ* cells sporulate poorly. Increase in the level of Rpb4 leads to an increase in the level of sporulation. At each level of Rpb4 an increase in the level of Rpb7 results in a decrease in sporulation in a dose-dependent manner.

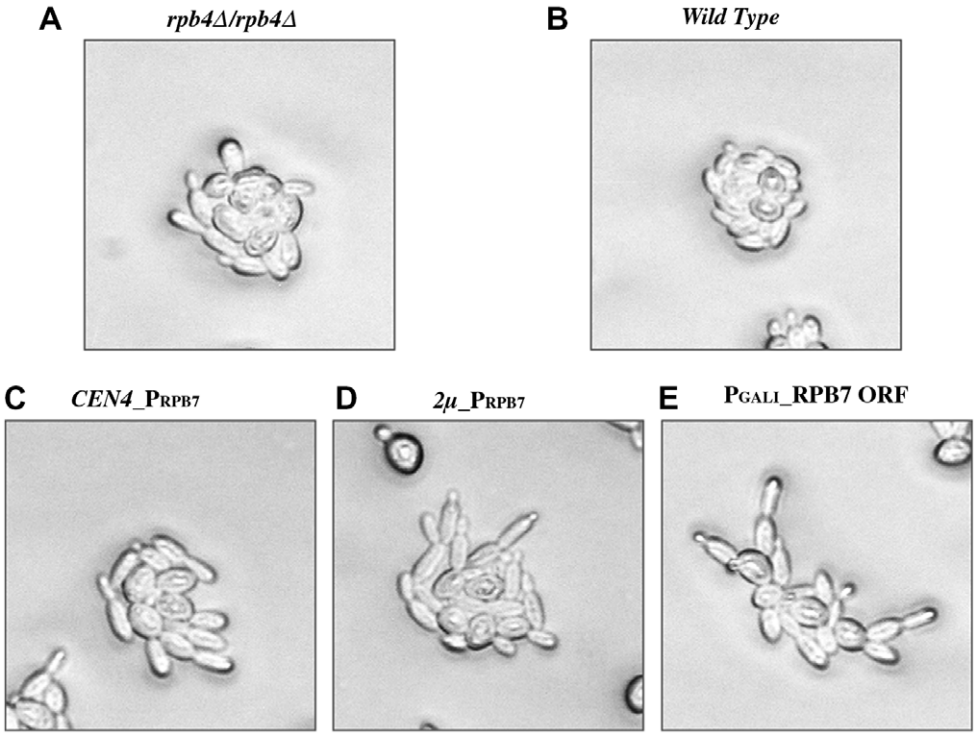

Fig. 2. Effect of relative levels of Rpb4 and Rpb7 on pseudohyphal growth: *rpb4Δ/ rpb4Δ* are predisposed to pseudohyphae formation (A) as compared to the wild-type (B). With increasing levels of Rpb7 in *rpb4Δ/ rpb4Δ*, exaggeration of pseudohyphae formation is enhanced in a dose-dependent manner (C–E).

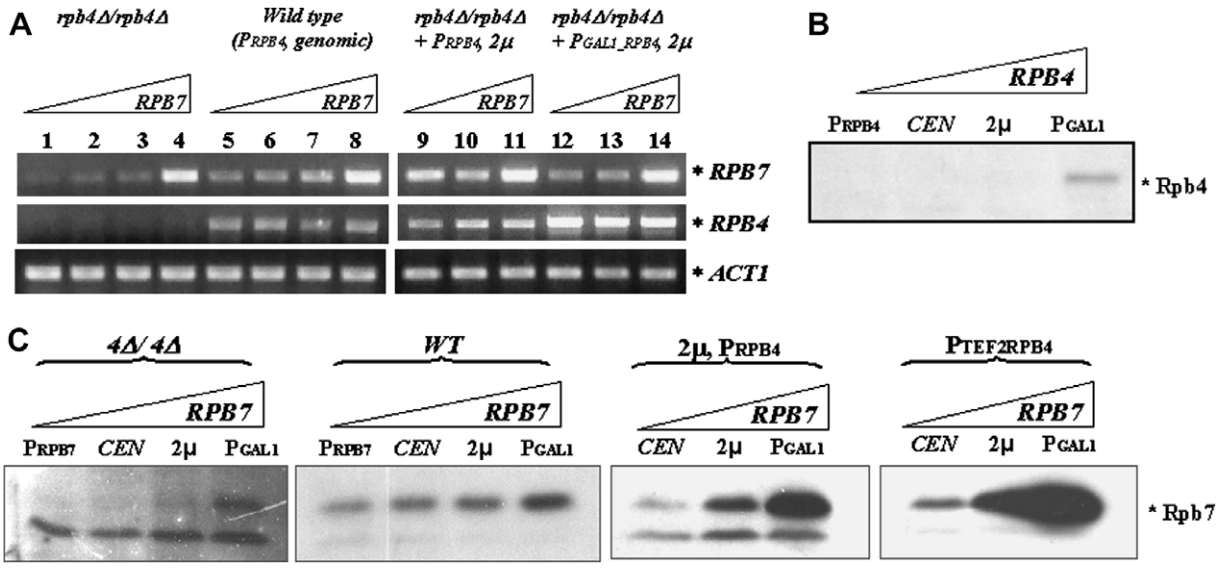

Fig. 3. mRNA and protein levels of *RPB4* and *RPB7*. The transcript levels (A) were determined by RT-PCR using ORF specific primers for *RPB4* and *RPB7* with A*CTIN1* as internal control. Rpb4 (B) and Rpb7 (C) protein levels were detected by Western blot using anti-Rpb4 raised against *D. discoideum* rpb4 and polyclonal antibodies raised against ScRpb7, respectively.

enhanced the pseudohyphal differentiation (Fig. 4A). Interestingly, we found that overexpression of *PHD1* reduced the sporulation levels of Σ1278b wild-type by ~30% (Fig. 4B). We have also carried out similar experiments in *rpb4Δ/rpb4Δ* cells in the same background and seen that the effect of *PHD1* overexpression on sporulation is not dependent on the presence or absence of Rpb4. Thus, overexpression of *PHD1* and *rpb4Δ* (which may be functionally equivalent to Rpb7 overexpression) favored pseudohyphae formation and reduced sporulation levels.

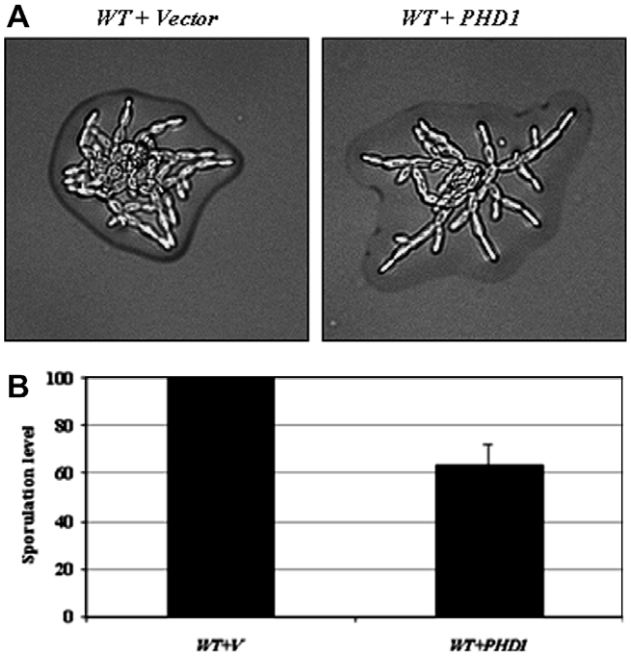

Fig. 4. Pseudohyphal strains are compromised for sporulation: Σ1278b wild-type strain shows pseudohyphae morphology in low nitrogen medium. Overexpression of *PHD1* enhances the pseudohyphae morphology (A). Overexpression of *PHD1* in Σ1278b wild-type strain reduces the sporulation level (B).

Discussion

Our observations suggest that in *S. cerevisiae*, Rpb4 and Rpb7 play contrasting roles during sporulation and pseudohyphal growth. The capacity to undergo sporulation is directly related to the level of Rpb4 in the cell. Absence of Rpb4 renders cells defective in sporulation and a gradual increase in the level of Rpb4 leads to a gradual increase in the percentage of sporulation. On the other hand, Rpb4 suppresses pseudohyphae formation. In contrast to Rpb4, Rpb7 promotes pseudohyphae formation but suppresses sporulation in a dose-dependent manner (at three different levels of Rpb4). We also show that other pseudohyphal predisposed strains are defective in sporulation and overexpression of a pseudohyphal transcriptional activator *PHD1* reduces the sporulation level. These observations allow us to hypothesize that strains, which are proficient to undergo sporulation, are poor in psudohyphal growth and that pseudohyphal strains are compromised for sporulation. Many mutants are known to affect both pseudohyphae formation and sporulation. *ras2* mutants sporulate in rich media but do not differentiate into pseudohyphae. Gpa2, the subunit of a heterotrimeric guanine nucleotide-binding protein, interacts with the regulatory domain of Ime2p, a protein kinase essential for sporulation. Under starvation conditions, Ime2p and Gpa2p get induced but Gpa2p, in addition to promoting the pseudohyphal pathway, prevents activity of Ime2p and thus inhibits precocious sporulation. When nutrients are completely exhausted, Ime2p is released from Gpa2p and induces sporulation. Moreover, overexpression of Ime2p inhibits pseudohyphal development and enables diploid cells to sporulate even in the presence of glucose or nitrogen. In contrast, overexpression of Gpa2p in cells overproducing Ime2p results in a drastic reduction in sporulation efficiency [28]. The authors attribute a switch function to Gpa2p in the "meiosis-pseudohyphal growth decision." Since these two phenotypes are complex stress responses involved in a large number of proteins, it is certainly possible that such MPGD making switches exist at multiple levels. On the other hand, Rpb4/7 may be mediating regulation of a novel pathway at transcriptional level. Here, we propose that Rpb4 and Rpb7 play a similar switch like role for sporulation and pseudohyphae formation at a different level of regulation. Mechanistically, Rpb4 and Rpb7 may achieve this regulation in different ways. First, these core polymerase subunits could achieve transcription of pseudohyphal or sporulation specific genes by directly interacting or hindering interactions with transcriptional regulators in these pathways in different manner. This is supported by the fact that the two proteins have been shown to functionally interact with different proteins [30]. Such observations allow us to predict that the two proteins are functionally different and could behave differently under many stress conditions. Although, Rpb7 has been demonstrated to rescue many of the phenotypes of *rpb4Δ* [13,19], the rescue of these phenotypes is partial and not complete. It is likely that Rpb7 acts as a partial suppressor of Rpb4 without actually curing the defect. While the conserved orthologs of Rpb7 can complement the lethality of *rpb7Δ* in *S. cerevisiae* [13], ScRpb4 is unable to complement this lethality.

Second, Rpb4 and Rpb7 can interfere in each other's function by virtue of their being interacting partners. Thus, by overexpressing one of these proteins we may have squelched away its interacting partner, thus preventing other interactions. Alternatively, holoenzyme composition may be one of the regulatory steps in stress responsive transcription in yeast. The stoichiometry of Rpb4 within the polymerase has been reported to change with the phase of growth [9]. We propose that Rpb4 and Rpb7 may be important for establishing different protein–protein interactions leading to the formation of different holoenzymes required for sporulation/pseudohyphal gene expression. By altering relative levels we may be shifting the dynamic equilibrium between the different forms of holoenzyme.

The mutually exclusive expression of genes involved in sporulation and pseudohyphae formation is intuitively appealing because it would enable cells under nutritional starvation to conserve their resources better and utilize them only for the stress response pathway that is appropriate under the prevailing conditions. Thus under severe carbon and nitrogen starvation, yeast cells undergo meiotic division and sporulation, and down-regulate genes required for pseudohyphae formation. We predict that based on results presented above, under mild nutritional starvation conducive for pseudohyphae formation, a majority of genes in sporulation are likely to be repressed at the tran-

scriptional level. Earlier our whole genome transcription profiling analysis indicated that many important genes involved in carbon metabolism and sporulation are affected in *rpb4Δ* strains as compared to wild-type cells [15]. Cells under such a defective transcriptional state are arrested in G1 phase as is evident from our electron microscopic and FACS analysis (data not shown) resulting in defective sporulation. Our data indicate that a higher level of Rpb4 makes the polymerase much more proficient in transcribing the sporulation specific genes resulting in higher level of sporulation. On the other hand, Rpb7 seems to have an antagonistic effect on sporulation.

Saccharomyces cerevisiae was recognized as an excellent genetic model system by virtue of its stable diploid and haploid states in the laboratory and well sporulating strains were selected during development of *S. cerevisia*e as a model genetic system. This we speculate might have led to not having the pseudohyphal phenotype in this yeast reported earlier on and almost all studies untill date have been carried out in Σ1278b background that exhibits excellent pseudohyphal growth but sporulates poorly. We have shown the absence of *RPB4* allows the pseudohyphal growth to be observed in other genetic backgrounds and reveal an important transcriptional level control.

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