Fission yeast Tor2 promotes cell growth and represses cell differentiation

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Summary

The fission yeast *Schizosaccharomyces pombe* is an excellent model system in which to study the coordination of cell growth and cell differentiation. In the presence of nutrients, fission yeast cells grow and divide; in the absence of nutrients, they stop growing and undergo cell differentiation. The molecular mechanisms underlying this response are not fully understood. Here, we demonstrate that Tor2, a fission yeast member of the TOR protein kinase family, is central to controlling the switch between cell growth and cell differentiation in response to nutrient availability. Tor2 controls cell growth and ribosome biogenesis by regulating ribosomal protein gene expression. We have found that Tor2 has an additional function in repressing sexual differentiation. Tor2 overexpression strongly represses mating, meiosis and sporulation efficiency, whereas Tor2 inactivation has the opposite effect, leading to cell differentiation, regardless of the nutritional conditions. This newly revealed function of Tor2 appears to operate by interfering with the functions of the transcription factor Ste11 and the meiosis-promoting RNA-binding protein Mei2. Thus, our data reveal a unique regulatory function of the Tor pathway – ensuring that growth and cell differentiation become mutually exclusive and that the choice between them depends on environmental conditions.

Supplementary material available online at
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Key words: Tor, Cell growth, Cell differentiation, Fission yeast

Introduction

The fission yeast *S. pombe* differentiates into ascospores under conditions of nutrient limitation. In the presence of nutrients, fission yeast cells reproduce asexually by means of the mitotic cell cycle. Upon nutritional starvation, they arrest the cell cycle in G1 and undergo sexual differentiation to give four resistant haploid spores that remain dormant until they encounter favourable growth conditions. Therefore, fission yeast cells have a system that carefully times the start of sexual development when nutrients become limiting. The molecular mechanisms that monitor the environmental conditions are still not clear.

TOR protein kinases are highly conserved key regulators of cell growth in all eukaryotes, including worms, flies and plants, and function in response to changes in the environment, in particular nutrient availability and cellular energy status (Chiu et al., 1994; Long et al., 2002; Menand et al., 2002; Oldham et al., 2000; Sabatini et al., 1994). Cell growth is regulated by TOR at multiple levels, including protein translation, ribosome biogenesis, gene transcription, nutrient permease expression, protein degradation and autophagy (reviewed by Fingar and Blenis, 2004; Jacinto and Hall, 2003; Wullschleger et al., 2006). *S. pombe* has two TOR homologues, *tor1+* and *tor2+*, which share 52% overall identity at the protein level. There is a requirement for *tor1+* under conditions of nutrient starvation, extreme temperatures, and osmotic or oxidative stress conditions, whereas *tor2+* is an essential gene under all circumstances (Kawai et al., 2001; Weisman and Choder, 2001).

Here, we demonstrate that Tor2 is a central controller of cell growth by sensing the nutritional conditions in fission yeast. We generated a temperature-sensitive allele of *tor2+*, named *tor2-51*, which caused growth arrest and decreased ribosome biogenesis at the restrictive temperature. Furthermore, at the restrictive temperature, homothallic h^{90} *tor2-51* arrested the cell cycle in G1 and underwent mating and meiosis, regardless of the nutritional conditions. By contrast, overexpression of Tor2 inhibited sexual differentiation when cells were nitrogen depleted. We also demonstrate that in vivo Tor2 exists in complex with Ste11 and Mei2, two essential regulators of different phases of sexual differentiation. These results provide the first evidence that Tor2 functions as a nutritional sensor in fission yeast.

Results

Isolation of *tor2-51*, a temperature-sensitive allele of fission yeast *tor2+*

Although fission yeast *tor2+* is essential for growth (Weisman and Choder, 2001), its cellular function has never been determined. To explore the *tor2+* function, we used a marker-switch approach (MacIver et al., 2003) to select a fission yeast mutant with a temperature-sensitive allele of *tor2+*, designated *tor2-51*. This mutant was temperature sensitive for growth and grew slowly even at 30°C (Fig. 1A). The *tor2+/tor2-51* heterozygous diploid grew normally at all temperatures tested (Fig. 1A), indicating that *tor2-51* is a recessive, loss-of-function mutation.

Incubation of *tor2-51* at the restrictive temperature resulted

in small cells that stopped dividing, both of which are hallmarks of the quiescent state (Fig. 1B). When these cells were released to the permissive temperature (25°C), they grew to the same density as the wild-type controls (Fig. 1C), suggesting that the *tor2-51* mutant phenotype is reversible.

Fission yeast Tor2 regulates transcription of ribosomal protein genes

The TOR pathway controls cellular functions necessary for cell growth in *S. cerevisiae* and higher eukaryotes (reviewed by Fingar and Blenis, 2004; Jacinto and Hall, 2003; Wullschleger et al., 2006). A key component of the control of cell growth is the regulation of ribosome biogenesis, not only because ribosome-dependent translation is directly required for growth, but also because ribosome biogenesis is a major consumer of cellular energy (Warner, 1999). In *S. cerevisiae*, the TOR pathway controls ribosomal protein gene transcription, contributing to an increase in the protein biosynthetic capacity of cells in response to increased nutrient availability (Powers and Walter, 1999).

To test whether fission yeast Tor2 is involved in growth regulation, we analysed ribosomal protein gene transcription during a temperature shift of the wild-type (*tor2+*) control and the *tor2-51* mutant from the permissive to the restrictive temperature. For this purpose, total RNA was extracted and probed with two cDNAs encoding the small and large ribosomal subunit proteins Rps1102 and Rpl1701, respectively. As shown in Fig. 2A, *tor2-51* cells showed a progressive downregulation of both gene transcripts at the restrictive temperature compared with the wild-type (*tor2+*) control, suggesting that Tor2 controls ribosome biogenesis in fission yeast by promoting the transcription of ribosomal protein genes.

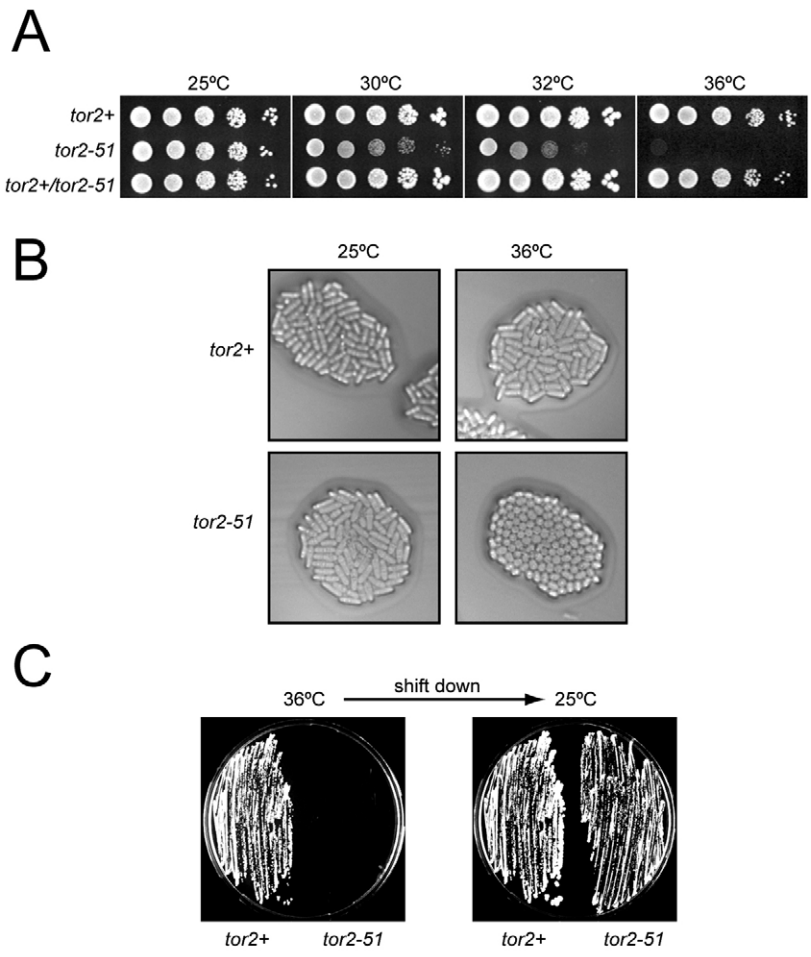


Fig. 1. Isolation of a temperature-sensitive allele of *tor2+*. (A) Wild-type (*tor2+*), *tor2*^{ts} (*tor2-51*) and the heterozygous diploid (*tor2+/tor2-51*) strains were spotted onto yeast extract (YES) medium at 1:10 dilutions. The plates were incubated at different temperatures (25, 30, 32 and 36°C). (B) Nomarski photomicrographs of wild-type (*tor2+*) and *tor2*^{ts} (*tor2-51*) colony cells plated on YES medium after incubation overnight at 25°C, transferred to the indicated temperatures and then incubated for 12 hours. (C) Wild-type (*tor2+*) and *tor2*^{ts} (*tor2-51*) cells were plated on YES medium and incubated at 36°C for three days (left panel). The same plate was then incubated at 25°C for a further three days (right panel). Photographs of the plates are shown.

Fission yeast Tor2 is a component of the TORC1 growth-controlling complex

In the budding yeast *S. cerevisiae*, Tor proteins are found in two different multi-protein complexes, TORC1 and TORC2. The TORC1 complex contains Lst8, Kog1, Tco89 and either Tor1 or Tor2. TORC1 regulates cell growth by coupling transcription, ribosome biogenesis, translation initiation, nutrient uptake and autophagy to the abundance and quality of available nutrients. By contrast, the TORC2 complex, which contains Lst8, Bit61, Avo1, Avo2, Avo3 and Tor2 (but not Tor1), mediates the spatial control of cell growth by polarising the actin cytoskeleton, and hence the secretory pathway, towards the bud or growth site (Loewith et al., 2002; Reinke et al., 2004; Wedaman et al., 2003).

KOG1, *LST8* and *AVO3* are conserved from budding yeast to humans, as is the interaction with *TOR* in the multiprotein complexes TORC1 and TORC2 (Jacinto et al., 2004; Kim et al., 2002; Kim et al., 2003; Sarbassov et al., 2004). However, little is known about the TOR complexes in *S. pombe*. The *KOG1* orthologue in *S. pombe*, *mip1+*, is an essential gene. Mip1 binds the RNA-binding protein Mei2 and the transcription factor Ste11, and it is involved in nutrient-responsive sexual development (Shinozaki-Yabana et al., 2000). The *LST8* orthologue in *S. pombe*, *wat1+*/*pop3+*, has been implicated in the control of cell morphology and microtubule integrity (Kemp et al., 1997; Ochotorena et al., 2001). By contrast, the *AVO3* orthologue in *S. pombe*, *ste20+*/*ste16+*, is dispensable for proliferation but is required for G1 arrest and for maintaining viability following nitrogen starvation (Maekawa et al., 1998).

We wondered whether fission yeast Tor1 and Tor2 were able to bind Mip1, Pop3 or Ste20, which would suggest a conservation in the formation and identity of the TORC1 or TORC2 complexes. To address this question, cells

expressing a chromosomal copy of HA-tagged *tor1+* or *tor2+* alleles were mated with cells expressing a chromosomal copy of myc-tagged *mip1+* or *ste20+* alleles to generate the four possible pair-wise combinations. In addition, we also generated cells expressing HA-tagged *tor1+* or *tor2+* in combination with myc-tagged *pop3+*. Co-immunoprecipitations were performed to determine whether Tor1 and Tor2 associate with the various myc-tagged proteins. As shown in Fig. 2B, Tor1 co-immunoprecipitated with Ste20 but not with Mip1, and Ste20 co-immunoprecipitated with Tor1 but not with Tor2. Furthermore, Tor2 co-immunoprecipitated with Mip1 but not with Ste20, and Mip1 co-immunoprecipitated with Tor2 but not with Tor1. However, Pop3 co-immunoprecipitated with both Tor1 and Tor2 (Fig. 2C).

These results suggest the presence of two TOR complexes in fission yeast. The composition of the complex containing Tor1, Pop3 and Ste20 most closely resembles that of TORC2 complexes in other species. The composition of the complex containing at least Tor2, Pop3 and Mip1 most closely resembles that of the TORC1 complexes. Thus, in spite of their nomenclature, fission yeast Tor1 might be more functionally orthologous to the budding yeast Tor2 whereas fission yeast Tor2 might be more functionally orthologous to budding yeast Tor1. The absence of TORC1-like complexes that contain Tor1 could explain why, in contrast to their budding yeast counterparts, fission yeast Tor1 and Tor2 show no redundancy of function.

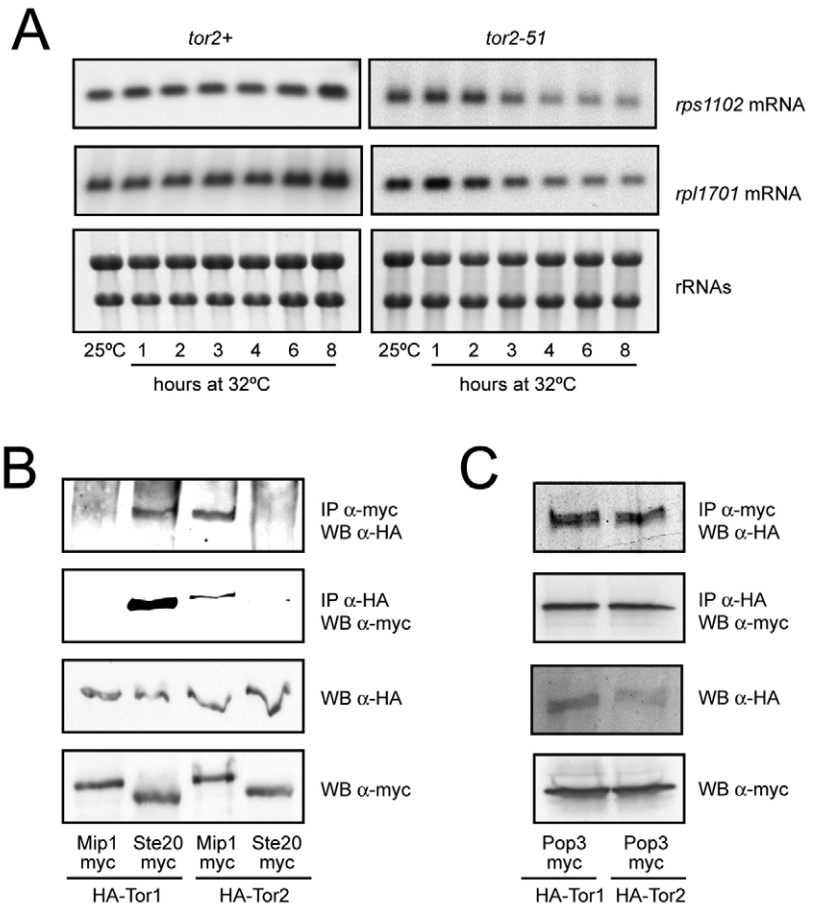


Fig. 2. Tor2 promotes cell growth and is part of the TORC1 complex. (A) Wild-type (*tor2+*) and *tor2-51* cells were grown in EMM to mid-log phase at 25°C, shifted to the restrictive temperature of 32°C, and samples were taken at the indicated times. RNA was extracted and northern blot performed and hybridised using probes against the ribosomal protein genes *rps1102* and *rpl1701*. The rRNA in each sample, stained with Methylene Blue, is shown to verify equal loading of RNA in each lane. (B,C) Crude extracts were prepared from cells exponentially growing in rich medium. The cells expressed tagged proteins, Mip1-myc, Ste20-myc or Pop3-myc, and HA-Tor1 or HA-Tor2, as indicated. The extracts were immunoprecipitated (IP) with the indicated antibodies (α, anti-) and the precipitates were subjected to SDS-PAGE and examined by western blot (WB). Immunoblots of the crude extracts are also shown.

Inactivation of *tor2+* induces a phenotype similar to nitrogen starvation

Fission yeast cells arrest the mitotic cell cycle in G1 upon nutrient starvation and, if cells of the opposite mating type are present, they undergo sexual development, conjugation and meiosis. Such stationary-phase cells are smaller than actively growing cells and remain viable for several weeks (Fantes and Nurse, 1977; Su et al., 1996).

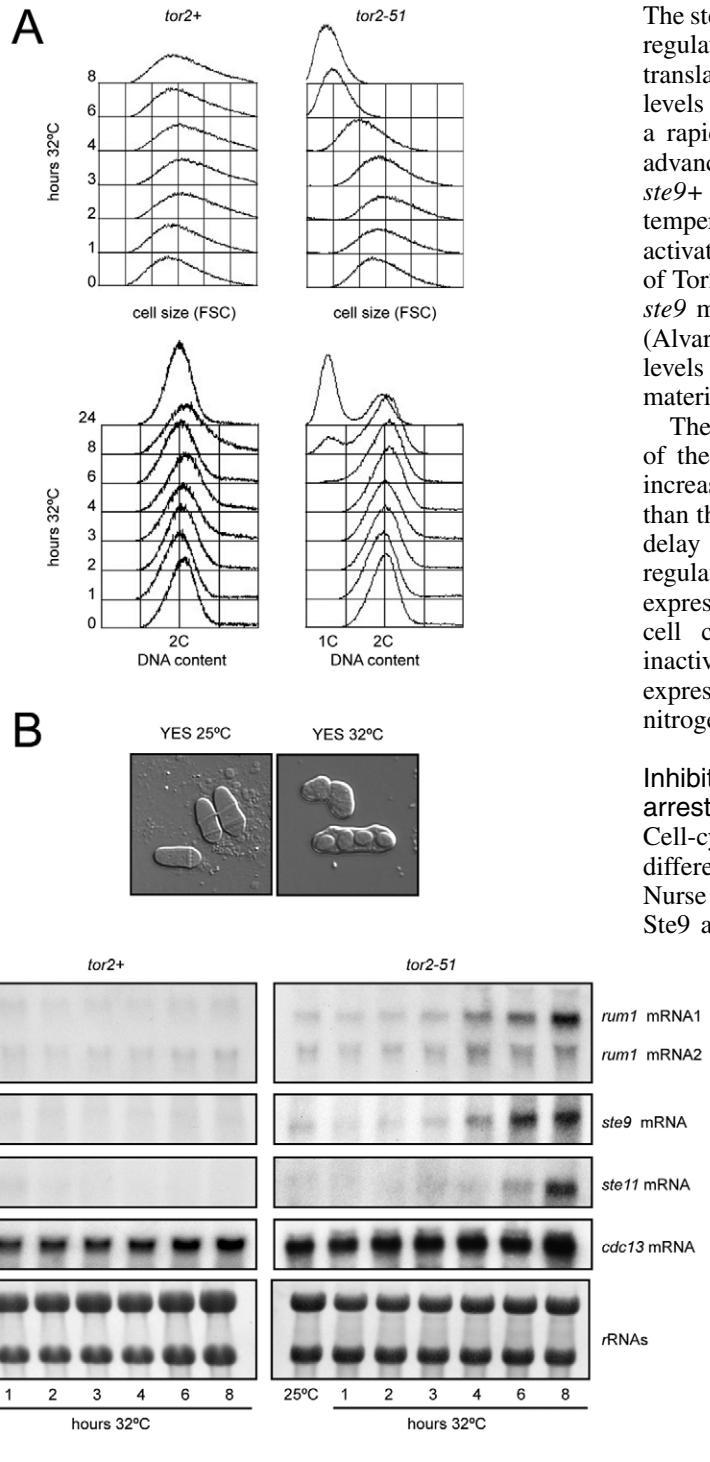
In *S. cerevisiae*, the TORC1 complex controls early G1 progression in response to nutrient availability, since cells treated with rapamycin or depleted for both Tor kinases arrest in G1, mimicking nutrient deprivation (Barbet et al., 1996; Beck and Hall, 1999; Di Como and Arndt, 1996; Zaragoza et al., 1998). By contrast, fission yeast *tor1+* plays a positive role in G1 arrest and sexual development (Weisman and Choder, 2001) because *tor1+* is required for cells to enter the G1 arrest in the stationary phase, which is a prerequisite for subsequent conjugation and meiosis.

To determine the effect of Tor2 inactivation in cell-cycle regulation, we used flow cytometric analysis to measure the cell size (measured by forward scatter) and DNA content of the *tor2+* and *tor2-51* mutant cells after a temperature shift from 25°C to 32°C in rich medium. As shown in Fig. 3A, *tor2-51* cells became smaller compared with *tor2+* cells, and began to accumulate with a 1C DNA content about 6 hours after Tor2 inactivation, eventually arresting in G1. Moreover, when the *tor2-51* mutation was introduced into a mating-competent homothallic h^{90} haploid strain, the cells conjugated and underwent meiosis and sporulation at the restrictive temperature in rich medium (Fig. 3B). By contrast, the wild-type controls do not undergo meiosis and sporulation under these conditions. These data show that Tor2 inactivation is sufficient to induce cell differentiation, mimicking the cell response to nitrogen depletion.

Tor2 inactivation induces the expression of cell-cycle inhibitors and genes required for the nitrogen starvation response

In fission yeast, nitrogen starvation promotes arrest in the G1

phase of the cell cycle (Egel and Egel-Mitani, 1974; Nurse and Bissett, 1981) and leads to the induction of the *ste11*+ gene, thereby allowing expression of the genes necessary to set up the pheromone communication system (Yamamoto, 1996). G1 arrest is triggered by downregulation of the cyclin-dependent kinase Cdc2/Cdc13 complex, and is mainly accomplished by the accumulation of the Rum1 and Ste9/Srw1 proteins, which inhibit Cdc2 activity and cause APC-mediated degradation of Cdc13 cyclin, respectively (Blanco et al., 2000; Correa-Bordes and Nurse, 1995; Kominami et al., 1998; Yamaguchi et al., 2000).



To determine whether Tor2 inactivation might induce the same molecular response to nitrogen starvation, we analysed the mRNA levels of the G1 arrest inducers *rum1*+ and *ste9*+, and also of the mating inducer *ste11*+, after shifting the wild-type (*tor2*+) and the *tor2-51* cell cultures to the restrictive temperature of 32°C in rich medium. The *rum1*+ mRNA and *ste9*+ mRNA levels increased after 4 hours of incubation at 32°C as shown in Fig. 3C, and were highly expressed after 6 hours, the time when cells with 1C DNA content began to accumulate in *tor2-51* cells (Fig. 3A). By contrast, *rum1*+ and *ste9*+ mRNAs levels remained low in wild-type control cells, indicative of their vegetative growth under these conditions. The steady-state levels of Rum1 and Ste9 expression are highly regulated at the level of transcription, mRNA stability, mRNA translation and protein stability, in order to guarantee low basal levels during vegetative growth in the presence of nutrients and a rapid increase after nitrogen deprivation, when mitosis is advanced and cells arrest in G1. The increase in *rum1*+ and *ste9*+ mRNA levels in the *tor2-51* mutant at the restrictive temperature seemed to be a consequence of transcription activation rather than mRNA stabilisation, because inactivation of Tor2 in cells expressing stable mutant versions of *rum1* and *ste9* mRNAs that lack the AU-rich elements in their 3′UTR (Alvarez et al., 2006; Daga et al., 2003) also led to increased levels of *rum1*+ or *ste9*+ mRNAs (Fig. S1, supplementary material).

The level of *ste11*+ mRNA also increased after incubation of the *tor2-51* mutant at the restrictive temperature but the increase in this message appeared at 8 hours, somewhat later than the increase in *rum1*+ and *ste9*+ mRNAs (Fig. 3C). This delay was not unexpected because pheromone-induced genes regulated by Ste11 are not required for G1 arrest and are expressed only once the cells have entered the G1 phase of the cell cycle (Stern and Nurse, 1998). We conclude that inactivation of Tor2 induces differentiation by upregulating the expression of the same set of genes as those expressed under nitrogen starvation.

Inhibition of cell growth is independent of cell-cycle arrest after Tor2 inactivation

Cell-cycle arrest in pre-Start G1 is a prerequisite for sexual differentiation in fission yeast (Egel and Egel-Mitani, 1974; Nurse and Bissett, 1981). In response to nitrogen starvation, Ste9 acts in collaboration with Rum1 to inactivate the Cdc2

Fig. 3. Inactivation of Tor2 mimics nitrogen starvation. (A) Flow cytometry analysis of wild-type (*tor2*+) and *tor2-51* cells at the indicated times after shifting a culture to the restrictive temperature of 32°C. The top panel represents cell size (measured as forward scatter, FSC) and the lower panel represents the DNA content. (B) Nomarski photomicrographs of h^{90} *tor2-51* cells in YES medium at the same density after incubation at 25°C or 32°C for 24 hours. (C) Wild-type (*tor2*+) and *tor2-51* cells were grown in EMM to mid-exponential phase at 25°C, then shifted to the restrictive temperature of 32°C, and samples were taken at the indicated times. RNA was extracted and northern blot performed and hybridised using probes against the indicated genes. *cdc13* mRNA and ribosomal (rRNA) levels were used as loading controls.

complexes and to target the Cdc13 cyclin for degradation during G1 (Blanco et al., 2000; Yamaguchi et al., 2000). Indeed, Cdc13 is stabilised in mutants deleted for *rum1+* or *ste9+*, which are sterile owing to an inability to arrest in G1 (Kominami et al., 1998; Yamaguchi et al., 1997).

To determine the relevance of G1 arrest for the Tor2 inactivation cell response, we deleted *rum1+* or *ste9+* genes in the *tor2* conditional strain h^{90} *tor2-51* and analysed its phenotype at the restrictive temperature. As shown in Fig. 4A, in the absence of *rum1+* or *ste9+*, h^{90} *tor2-51* cells did not arrest the cell cycle in G1 after 8 hours at the restrictive temperature and no asci were observed after 24 hours of incubation at 32°C. However, these cells were unable to proliferate; they underwent cell-cycle arrest in G2 and were smaller than the wild-type cells (Fig. 4A). This inhibition of cell growth seems to be a consequence of a decrease in ribosome biogenesis, as seen from the observed reduction in the transcription of the ribosomal protein gene *rps1102+* in *tor2-51* cells at the restrictive temperature, regardless of the presence or not of *rum1+* and *ste9+* (Fig. 4B).

These data show that cell-cycle arrest is necessary for cell differentiation after Tor2 inactivation. However, inhibition of cell growth occurs independently of the cell-cycle arrest during sexual differentiation.

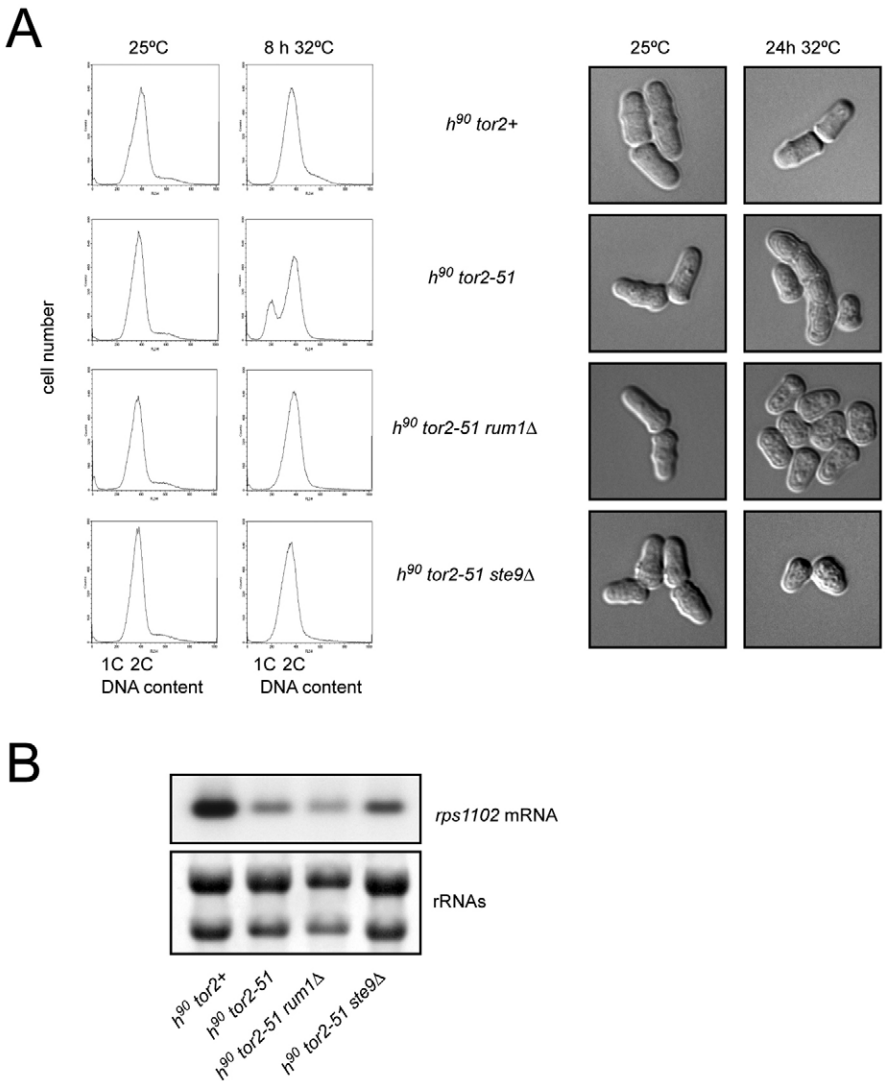


Fig. 4. Rum1 and Ste9 are required for G1 arrest but not for growth inhibition after Tor2 inactivation. (A) Flow cytometry analysis and Nomarski photomicrographs of h^{90} *tor2+*, h^{90} *tor2-51*, h^{90} *tor2-51 rum1Δ* and h^{90} *tor2-51 ste9Δ* cells incubated at 25°C or at 32°C for 8 hours and 24 hours, as shown. (B) The indicated strains were grown in EMM at 32°C for 24 hours. RNA was extracted and northern blot was performed and hybridised using probes against the ribosomal protein gene *rps1102*. The rRNA in each sample, stained with Methylene Blue, is shown to verify equal loading of RNA in each lane.

Tor2 overexpression inhibits sexual differentiation and mating

The data reported above suggested that Tor2 might inhibit cell differentiation independently of its role in cell growth induction. To test this hypothesis, we analysed the effects of increasing Tor2 levels in the cell. Tor2 was overexpressed using the thiamine-repressible *nmt1+* promoter (Maundrell, 1993) introduced by gene-replacement into the *tor2+* locus in a homothallic h^{90} haploid strain (Bähler et al., 1998). These cells grew normally and had an identical morphology and cell size to those of the control cells (data not shown). As expected, when h^{90} wild-type cells were grown in MEA medium, they mated and underwent meiosis; however, h^{90} cells overexpressing Tor2 were impaired for sexual differentiation after nitrogen deprivation, as shown by the reduction in iodine staining and in the number of spores generated (Fig. 5A).

As previously mentioned, cell-cycle arrest is one of the prerequisites for sexual differentiation (Egel and Egel-Mitani, 1974; Nurse and Bissett, 1981). Accordingly, we wondered whether Tor2 overexpression might lead to a sterility phenotype as a result of impaired G1 arrest after nitrogen deprivation. To test this possibility, *tor2+* or *nmt-tor2* cells were pre-incubated in minimal medium in the presence or absence of thiamine for 18 hours. The cells were then nitrogen depleted and analysed for their DNA content by flow cytometry. As shown in Fig. 5B, cells overexpressing Tor2 were able to arrest in G1 after nitrogen deprivation, even in the absence of thiamine, when mating was almost totally impaired (Fig. 5A).

When fission yeast cells are nitrogen starved, G2 cells are advanced into mitosis and the newborn G1 cells are smaller, increasing their probability to arrest in G1. As shown in Fig. 5C, Tor2-overexpressing cells had the same cell size (measured by forward scatter) as the wild-type cells and, like the control wild-type cells, they became smaller after nitrogen deprivation. Therefore, Tor2 overexpression represses sexual differentiation without affecting entry into the stationary phase upon nitrogen deprivation.

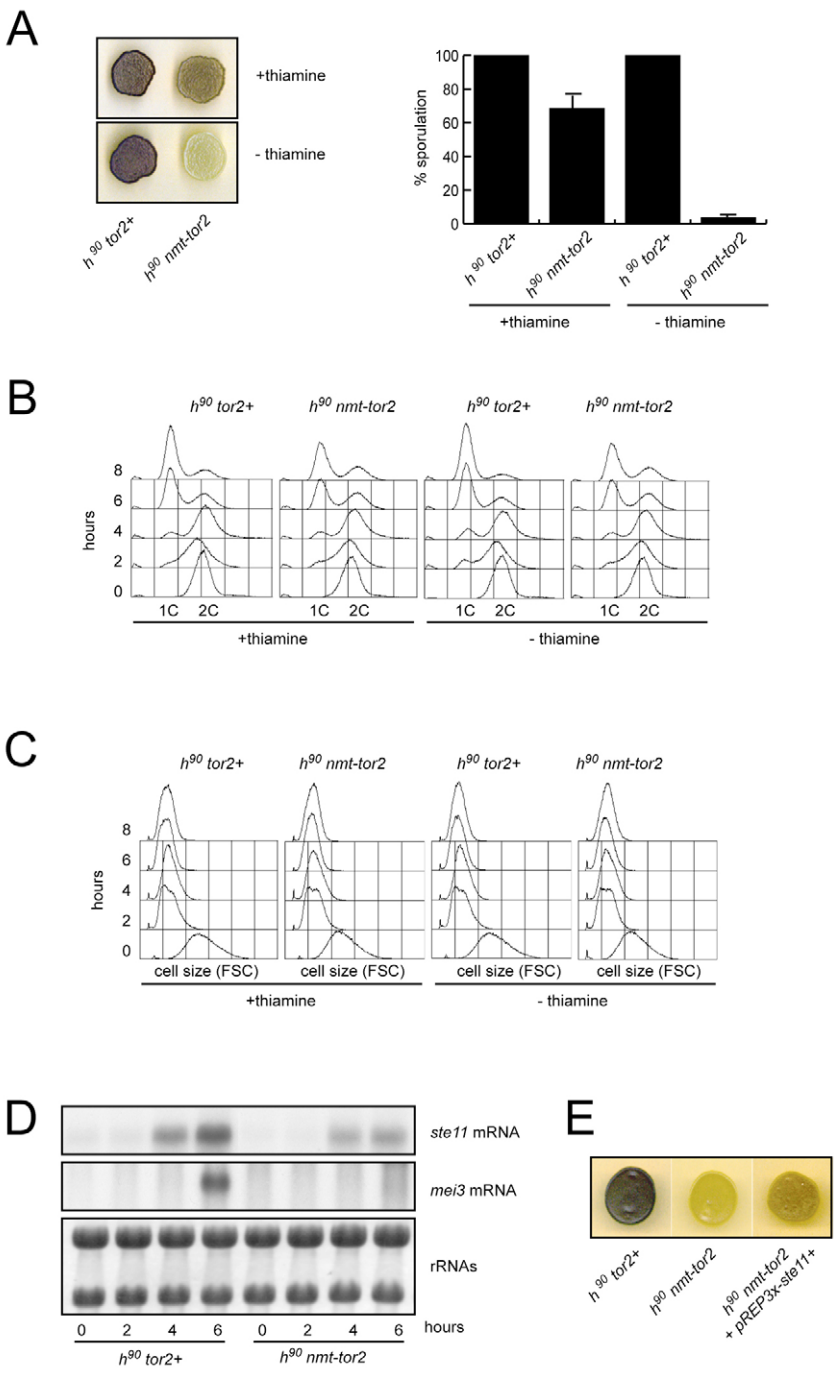


Fig. 5. Overexpression of Tor2 inhibits sexual differentiation but not entry into the stationary phase. (A) h^{90} *tor2+* and h^{90} *nmt-tor2+* cells were grown in the presence or absence of thiamine for 18 hours, and then spotted onto malt extract plates with or without thiamine. These plates were incubated for 48 hours at 25°C and then stained with iodine vapour (left panel). Alternatively, a sample from each spot was resuspended in water at the same optical density and treated overnight with glusulase. Equal volumes were then plated on YES medium and the number of colonies was counted and represented as a plot (right panel). (B) Flow cytometry analysis of h^{90} *tor2+* and h^{90} *nmt-tor2+* cells after nitrogen starvation. Cells were grown to mid-exponential phase in minimal medium with or without thiamine for 18 hours to induce Tor2 overexpression. These cells were washed several times in minimal medium lacking nitrogen, and were nitrogen starved in the same medium in the presence or absence of thiamine. Samples were taken at the indicated times after nitrogen depletion. (C) Cell size distribution determined by flow cytometry analysis (measured as forward scatter, FSC) of the h^{90} *tor2+* and h^{90} *nmt-tor2+* cells as in (B). (D) h^{90} *tor2+* and h^{90} *nmt-tor2+* cells were grown to mid-exponential phase in minimal medium plus thiamine, then washed several times in minimal medium lacking thiamine prior to incubation in the same medium for 18 hours. They were then washed several times in minimal medium lacking thiamine and nitrogen, and nitrogen starved in the same medium for the indicated times. Samples were collected from which RNA was extracted, and a northern blot was performed and hybridised with probes against *ste11+* and *mei2+* genes. As a loading control, the amount of rRNA in each sample was estimated from its staining with Methylene Blue. (E) h^{90} *tor2+*, h^{90} *nmt-tor2+* and h^{90} *nmt-tor2+ pREP3x-ste11+* cells were grown to mid-exponential phase in the absence of thiamine for 18 hours, and then spotted onto malt extract plates. These plates were incubated for 48 hours at 25°C and then stained with iodine vapour.

Ste11 is a transcription factor that activates its own transcription (Kunitomo et al., 2000) and that of many genes that are essential for conjugation in fission yeast (Sugimoto et al., 1991). We compared the mRNA levels of *ste11+* and its target gene *mei3+* after nitrogen starvation in wild-type (h^{90} *tor2+*) cells with cells overexpressing *tor2+* (h^{90} *nmt-tor2*) by northern blot. As shown in Fig. 5D, *ste11+* mRNA induction was reduced and *mei3+* mRNA levels were not detectable in cells overexpressing Tor2, suggesting that Tor2 overexpression was impairing Ste11 function. However, as shown in Fig. 5E, Ste11 overexpression only partially rescued the impaired sexual differentiation of the h^{90} Tor2-overexpressing strain, as measured by iodine staining, suggesting that Tor2 overexpression might affect other aspects of meiotic differentiation in addition to Ste11 function.

Tor2 impairs meiosis by interfering with the function of Mei2

Meiosis is an essential step for the sexual reproduction of eukaryotes. Fission yeast haploid cells must mate to form zygotes before undergoing meiosis; however, diploid cells initiate meiosis directly after G1 arrest upon nitrogen starvation. To study whether Tor2 has a role in specifically inhibiting meiosis, we examined the sporulation efficiency of

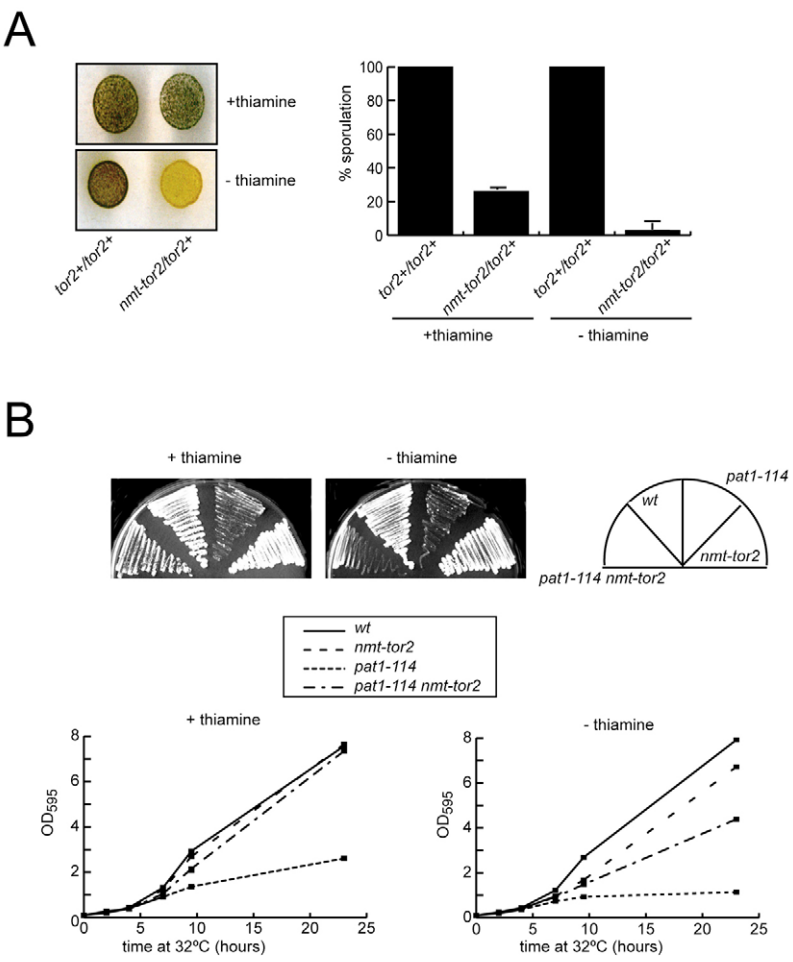


Fig. 6. Tor2 interferes with Mei2 function. (A) *h+/h– tor2+/tor2+* and *h+/h– nmt-tor2/tor2+* diploid cells were grown in the presence or absence of thiamine for 18 hours, and were then spotted onto malt extract plates with or without thiamine. Plates were incubated for 48 hours at 25°C and then stained with iodine vapour (left panel). Alternatively, a sample from each spot was resuspended in water at the same optical density and treated overnight with glusulase. Equal volumes were then plated on YES medium and the number of colonies was counted and represented as a plot (right panel). (B) The indicated strains were plated on minimal medium or minimal medium supplemented with thiamine and incubated at 32°C for three days. Photographs of the plates are shown. Lower panel, the indicated strains were grown at 25°C in minimal medium in the presence or absence of thiamine for 18 hours to mid-exponential phase (OD_{595}=0.8-1), diluted to OD_{595}=0.1, and then incubated in the same medium at 32°C. Growth curves at 32°C are shown. (C) Crude extracts were prepared from exponentially growing cells expressing tagged proteins, as indicated. They were subjected to IgG pull-down (TAP pull-down) with IgG-Sepharose beads. HA-Tor2 was detected by western blot (WB). Immunoblots of the crude extracts are also shown.

an *h+/h– nmt-tor2/tor2+* diploid strain after nitrogen deprivation in the absence or presence of thiamine. As shown in Fig. 6A, Tor2 overexpression impaired meiosis of the diploid strain, as shown by a reduction in iodine staining and a reduction in the number of spores generated.

Mei2 is an RNA-binding protein that is an essential regulator of meiosis (Bresch et al., 1968; Shimoda et al., 1987; Watanabe and Yamamoto, 1994). The Pat1 protein kinase inhibits Mei2 by mediating its phosphorylation during the mitotic cell cycle (Beach et al., 1985; Watanabe et al., 1997). The temperature-sensitive *pat1-114* mutant stops dividing and initiates meiosis at the restrictive temperature because inactivation of Pat1 kinase leads to a loss of inhibitory phosphorylation of Mei2. To test whether Tor2 was able to inhibit meiosis, we overexpressed Tor2 (*nmt-tor2*) in the *pat1-114* mutant and analysed the ability of these cells to undergo meiosis at the restrictive temperature. As shown in Fig. 6B, upper panel, overexpression of Tor2 suppressed the meiosis induced by Pat1 inactivation and instead allowed cell proliferation. To confirm these data, we analysed the proliferation rates of the different strains at 32°C in the presence or absence of thiamine after pre-incubation under the same conditions at 25°C. As shown in Fig. 6B, lower panel, Tor2 overexpression rescued the *pat1-114* growth defect at the restrictive temperature of 32°C. These data indicate that Tor2 overexpression interferes with the onset of meiosis, probably by inhibiting Mei2 itself or one of its downstream effectors.

As Tor2 appears to inhibit cell differentiation by interfering with the functions of Ste11 and Mei2, we explored whether fission yeast Tor2 was able to interact in vivo with Ste11 and Mei2. To address this question, cells co-expressing epitope-tagged versions of Tor2 (HA-Tor2) and Ste11 (Ste11-TAP) or Mei2 (Mei2-TAP) were generated. TAP-epitope-tagged proteins were pulled down from yeast extracts using IgG-Sepharose beads and the presence of HA-tagged Tor2 in the pellets was examined by western blot. As shown in Fig. 6C, Tor2 was pulled down together with Ste11 and also with Mei2. These results indicate that in vivo Tor2 is a component of complexes containing these two important effectors of cell differentiation in fission yeast. It is therefore likely that Tor2 repression of Ste11 and

Mei2 is mediated through its interactions with the same complexes.

Discussion

Two Tor proteins with different functions in fission yeast

There are two Tor proteins in fission yeast – Tor1 and Tor2 – that seem to perform different functions. Tor1 is a non-essential protein and is required under special conditions, such as nutritional starvation, high pH, high temperatures, and osmotic and oxidative stress (Kawai et al., 2001; Weisman and Choder, 2001). Here, we show that Tor2, an essential protein, is required to promote cell growth by controlling ribosome biogenesis. We also show that the fission yeast Tor proteins, like their budding yeast counterparts, associate with two different multi-protein complexes, TORC1 and TORC2 (Loewith et al., 2002; Wedaman et al., 2003). Tor1 interacts specifically with the Avo3/Rictor orthologue Ste20, which is part of the TORC2 complex, whereas Tor2 interacts with Mip1, the fission yeast orthologue of Kog1 in *S. cerevisiae* or Raptor in animal cells, and is part of the TORC1 complex. In *S. cerevisiae*, the TORC1 complex regulates cell growth and might include either Tor1 or Tor2. In *S. pombe*, TORC1, the Tor complex that regulates cell growth, contains at least Tor2, Pop3 and Mip1 (but not Tor1), providing an explanation as to why Tor2 is the only Tor protein essential for growth in fission yeast; by contrast, in budding yeast, there is some overlap in function between Tor1 and Tor2.

A new function for Tor2 as a repressor of sexual differentiation

In addition to its role in promoting cell growth, fission yeast Tor2 acts as a repressor of sexual differentiation. Inactivation of Tor2, using the temperature-sensitive mutant allele *tor2-51*, resulted in small G1-arrested cells; these cells were able to mate, and underwent meiosis and sporulation in the presence of nutrients. Therefore, inactivation of Tor2 is sufficient to induce cell differentiation, mimicking the cell response to nitrogen starvation. Consistent with this idea, inactivation of Tor2 induces the expression of at least two genes required for G1 arrest, *rum1+* and *ste9+*, which are the fission yeast orthologues to *SIC1* and *HCT1/CDH1* in budding yeast, respectively. Tor2 inactivation also induces the expression of *ste11+*, a transcription factor that is in turn required to induce the expression of other genes necessary for conjugation and meiosis (Yamamoto, 1996). Therefore, inactivation of Tor2 appears to induce cell differentiation by upregulating the expression of the same set of genes as those expressed during nitrogen depletion. By contrast, overexpression of Tor2 inhibits sexual differentiation, mating and meiosis. Cells overexpressing Tor2 respond normally to nitrogen starvation by stopping cell growth and undergoing cell-cycle arrest in G1; however, they were unable to undergo mating and meiosis. This inhibition seems to occur through interference with the activity of the Ste11 transcription factor because *ste11* and *mei3* mRNA levels increased in the *tor2-51* mutant at the restrictive temperature and their induction after nitrogen depletion was impaired following overexpression of Tor2. Moreover, in vivo Ste11 is a component of complexes that also contain Tor2.

Consistent with the idea that Tor2 overexpression inhibits mating and meiosis through interference with Ste11 activity, these effects can be partially reversed by increased Ste11 levels. However, the fact that rescue is incomplete might be linked to the finding that Tor2 overexpression also inhibits the function of Mei2, a protein required to induce meiosis in fission yeast. In this respect, it is interesting to note that Mip1, another member of the TORC1 complex and the fission yeast orthologue of *KOG1* in *S. cerevisiae* and Raptor in mammalian cells, has also been shown to interact with Ste11 and Mei2 (Shinozaki-Yabana et al., 2000). Therefore, Tor2 seems to inhibit sexual differentiation at two levels: before mating, by inhibiting Ste11; and before meiosis, by interfering with Mei2. It is tempting to speculate that the Tor2 kinase could regulate the function of Ste11 and Mei2 by phosphorylation.

In fission yeast, other nutrient signalling pathways, such as the protein kinase A (PKA) and the Sty1/Spc1 mitogen-activated protein (MAP) kinase pathways, have already been shown to regulate sexual differentiation, by controlling the transcription of *ste11+*. PKA is a negative regulator of Ste11, through the phosphorylation and inhibition of the transcription factor Rst2 that binds to the upstream region of *ste11+* and seems to be central for its transcription (Higuchi et al., 2002; Kunitomo et al., 2000), Spc1/Sty1 is a positive regulator of *ste11+* gene expression that is likely to function by activating the Atf1 transcription factor, which is also required for *ste11+* transcription (Shiozaki and Russell, 1996; Takeda et al., 1995). Interestingly, in budding yeast, PKA and TOR pathways converge at the level of Rim15 and Fhl1 transcription factors in the regulation of entry into G0 and ribosomal protein gene expression, respectively (Martin et al., 2004; Pedruzzi et al., 2003). For future work, it would be interesting to study the possible interactions between PKA, Sty1/Spc1 MAP kinase and Tor2 pathways in fission yeast.

A conserved Tsc1-Tsc2/Rheb/Tor pathway from fission yeast to mammalian cells

In mammalian cells and *Drosophila*, the Tsc1-Tsc2/Rhb1/Tor pathway controls cell growth (reviewed by Findlay et al., 2005; Martin and Hall, 2005; Sarbassov et al., 2005). The tumour suppressor tuberous sclerosis complex Tsc1-Tsc2 forms a heterodimer that integrates many signals and regulates TORC1 activity (Gao et al., 2002; Tapon et al., 2001). Tsc2 acts as a GTPase-activating protein (GAP) for the small GTPase Rheb (Garami et al., 2003; Saucedo et al., 2003; Stocker et al., 2003; Tee et al., 2003; Zhang et al., 2003), which has been shown both to activate and bind directly to the TORC1 complex (Long et al., 2005). Orthologues of Tsc1 and Tsc2 are absent in budding yeast, but all the proteins in this pathway are present in fission yeast (Mach et al., 2000; Matsumoto et al., 2002). In *S. pombe*, inactivation of *tsc1+* or *tsc2+*, or the presence of hyperactive forms of Rhb1 encoding the fission yeast Rheb GTPase orthologue, represses the expression of genes that are normally induced under nitrogen starvation, such as *mei2+* or *fnx1+*, and results in sterility (Matsumoto et al., 2002; Nakase et al., 2006; Urano et al., 2005). These effects are similar to what we observed when Tor2 was overexpressed. Furthermore, we have also observed that Tor2 inactivation rescues the *tsc1* or *tsc2* deletion sterility phenotype (B.A. and S.M., unpublished data), whereas mutants in *rhb1* arrest cell growth and cell division with a phenotype similar to *tor2-51* mutants (Mach et al., 2000). Since fission yeast Rhb1 has also been reported to interact with and to activate Tor2 in a GTP-

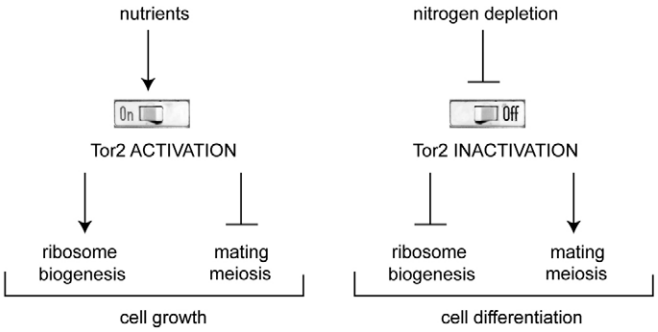


Fig. 7. Tor2-regulated pathways in fission yeast: a working model. In the presence of nutrients (nitrogen), Tor2 actively represses mating and meiosis, and promotes ribosome biogenesis and cell growth. When nitrogen is limited, Tor2 is inactivated, cells stop growing, and cell differentiation is switched on.

dependent manner (Urano et al., 2005), the Tsc1-2/Rhb1/Tor2 pathway might be involved in promoting cell growth and in repressing sexual differentiation.

Conclusion

We report here the characterisation of *tor2+* gene function in *S. pombe*. We observed that Tor2 regulates cell growth, controls ribosome biogenesis and associates with the Raptor homologue Mip1, forming the growth-controlling TORC1 complex that is conserved from *S. cerevisiae* to human cells (Kim et al., 2002; Loewith et al., 2002). Moreover, we show that Tor2 exists in complex with Ste11 and Mei2 and has an additional function in repressing sexual differentiation. Accordingly, Tor2 overexpression strongly represses meiosis and sporulation efficiency whereas Tor2 inactivation has the opposite effect, leading to G1 arrest and to cell differentiation, mating and meiosis, regardless of the nutritional conditions. Together, these findings lead us to propose that Tor2 is a key regulator of the switch between cell growth and cell differentiation in response to nutrient availability (Fig. 7).

Materials and Methods

Fission yeast strains and media

All *S. pombe* strains used in this study are listed in Table S1 in supplementary material. Standard methods were used for growth, transformation and genetic manipulations (Moreno et al., 1991). The *nmt1* promoter was introduced at the 5′UTR of *tor2+* in the strain *nmt-tor2+* by a PCR-based method (Bähler et al., 1998). The same method was used to generate the tagged versions of the genes *mip1-myc*, *ste20-myc*, *pop3-myc*, *nmt:HA-tor1* and *nmt:HA-tor2*. Except where specifically indicated, all experiments in liquid culture were carried out in Edinburgh minimal medium (EMM) containing the required supplements, starting with a cell density of 2-4×10^6 cells/ml that corresponds to the mid-exponential phase of growth. Temperature shift experiments were carried out using a water bath at 32°C.

Construction of a *tor2+* temperature-sensitive allele

To generate a temperature-sensitive allele of *tor2+*, we used the method described by MacIver et al. (MacIver et al., 2003). Briefly, the *ura4+* and *LEU2* markers were inserted in a *Not*I restriction site generated 336 bp 3′ to the *tor2+* gene to generate plasmids ptor2:ura4 and ptor2:LEU2, respectively. Sequences containing the region that encompasses the *LEU2* marker were excised and transformed into *h– ura4-d18 leu1-32* (S125) and *h+ ura4-d18 leu1-32* (S124) strains. Colony PCR was used to confirm that the integration had occurred at the right locus without rearrangements in 15 leu+ transformants. The strain *h+ tor2:LEU2 ura4-d18 leu1-32* (BA117) was then transformed with a mutagenised fragment, containing 2 kb of C-terminal region of the *tor2+* gene and the 3′ region with the *ura4+* marker, obtained from the plasmid ptor2:ura4, following a previously described method (Fromant et al., 1995). ura4+ leu1- transformants were selected at 25°C. Approximately 700 transformants were replicate plated and examined for their ability to grow at 36°C. One strain, *h+ tor2-51:ura4+ ura4-d18 leu1-32* (BA120) was found to be temperature sensitive. PCR and Southern blot analysis of the genomic DNA isolated from this clone confirmed the integration of the mutagenic cassette at the *tor2+* locus.

Mating efficiency after Tor2 induction

h^{90} *tor2+* and h^{90} *nmt-tor2+* cells were grown overnight to mid-exponential phase in EMM with and without 5 μg/ml thiamine, then spotted onto malt extract plates in the presence or absence of 5 μg/ml thiamine, and incubated for 48 hours at 25°C. The cells were resuspended in water to a concentration of 2.5×10^6 cells/ml and treated with glusulase (Sigma) for 24 hours to kill vegetative cells. The number of spores was determined by plating the same volume in yeast extract (YES) medium and counting the number of colonies generated.

Flow cytometry

Approximately 10^7 cells were collected by centrifugation, fixed in 70% cold ethanol and processed as described (Moreno et al., 1991). Flow cytometric analysis was performed on a Becton-Dickinson FACScan using cells stained with propidium iodide. Cell size measurements were made using the forward light scatter (FSC) data of the FACS.

RNA extraction and northern blots

Total RNA from cells was isolated by lysis with glass beads in the presence of phenol (Moreno et al., 1991) and 5-10 μg of each sample was resolved on a formaldehyde gel. Northern blotting was carried out using Gene ScreenPlus (NEN, Dupont), following the manufacturer's suggested protocols. DNA probes were labelled with [^{32}P]dCTPs using the Rediprime II Random Prime Labelling System kit (Amersham).

Protein extraction and western blots

Protein extracts were obtained using trichloroacetic acid (TCA) extraction, as described previously (Foiani et al., 1994). For western blots, 75-100 μg of total protein extract were run on 10%, 7.5% or 4% SDS-PAGE, transferred to a nitrocellulose filter and probed with monoclonal anti-HA (1:1000) or anti-myc (1:1000) antibodies. Goat anti-mouse HRP-conjugated antibody was used as the secondary antibody at a dilution of 1:2000. TAP-Ste11 and TAP-Mei2 were detected using a horseradish peroxidase (HRP)-conjugated anti-peroxidase antibody (Sigma). Immunoblots were developed using the enhanced chemiluminescence procedure (ECL kit; Amersham).

Co-immunoprecipitations and TAP pull-downs

Extracts from cells expressing the appropriately tagged proteins were prepared from 3×10^8 exponentially growing cells using HB buffer (Moreno et al., 1991). Cells extracts were cleared by centrifugation for 15 minutes at 4°C. For immunoprecipitations, an aliquot of total protein extract was incubated consecutively with either the monoclonal anti-HA.11 (Babco) or the monoclonal anti-myc.9E10 (Roche) antibodies for 4 hours at 4°C, and then with protein A-Sepharose (Pharmacia-Biotech) for 1 hour at 4°C in a rotating wheel. For TAP pull-downs, IgG-Sepharose (Amersham) was added to the protein extract and incubated for 1 hour at 4°C. For both immunoprecipitations and pull-downs, beads were collected by centrifugation, washed three times with HB buffer and resuspended in SDS-PAGE sample buffer for electrophoresis. The samples were boiled and loaded onto either 4%, 7.5% and 10% SDS-PAGE followed by western blot analysis as above.

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