

# Fission yeast Tor1 functions as part of TORC1 to control mitotic entry through the stress MAPK pathway following nutrient stress

Sonya Hartmuth and Janni Petersen\*

University of Manchester, Faculty of Life Sciences, C.4255 Michael Smith Building, Oxford Road, Manchester M13 9PT, UK

\*Author for correspondence (e-mail: Janni.Petersen@manchester.ac.uk)

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## Summary

TOR signalling coordinates growth and division to control cell size. Inhibition of *Schizosaccharomyces pombe* Tor1, in response to a reduction in the quality of the nitrogen source (nutrient stress), promotes mitotic onset through activation of the mitogen-activated protein kinase (MAPK) Sty1 (also known as Spc1). Here we show that 'nutrient starvation' (complete withdrawal of nitrogen or leucine) blocks mitotic commitment by altering Sty1 signalling and that different degrees of Sty1 activation determine these differences in mitotic commitment decisions. Mammals contain one TOR kinase, whereas yeasts contain two. In each case, they comprise two distinct complexes: TORC1 and TORC2. We find that nutrient-stress-induced control of mitotic onset, through Tor1, is regulated through changes in TORC1 signalling. In minimal medium, Tor1 interacts with the TORC1 component Mip1 (raptor), and

overexpression of *tor1*<sup>+</sup> generates growth defects reminiscent of TORC1 mutants. Strains lacking the TORC2-specific components Sin1 and Ste20 (riCTOR) still advance mitotic onset in response to nutrient stress. By contrast, Mip1 and the downstream effector Gad8 (a S6K kinase homologue), like Tor1, are essential for nutrient stress to advance mitotic onset. We conclude that *S. pombe* Tor1 and Tor2 can both act in TORC1. However, it is the inhibition of Tor1 as part of TORC1 that promotes mitosis following nutrient stress.

Supplementary material available online at  
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Key words: MAPK, Mitotic entry, Nutrient stress, Pyp2, TOR, TORC1

## Introduction

Cells divide when a critical cell size is reached, and this size at division is modulated by changes in nutrient availability (Dolznig et al., 2004; Fantes and Nurse, 1977; Jorgensen and Tyers, 2004; Kim et al., 2002; Mitchison, 2003). The fission yeast *Schizosaccharomyces pombe* is an ideal model system for investigation of 'size control'. This is because the rod-shaped cells grow by elongation and undergo mitosis and division at a defined length in steady-state growth conditions. Thus, measurement of cell length at septation gives an accurate read-out of size control (Mitchison, 2003). In all eukaryotes, stress-activated MAP kinase response pathways (SAMRPs) play an important role in modulating intercellular signalling to entrain a range of processes with changes in the environment. Higher eukaryotes and budding yeast have several such SAMRPs, each of which responds to different stresses. By contrast, in *S. pombe* one main SAMRP is activated in response to a variety of external stimuli to activate the MAPK Sty1 (also known as Spc1) (Toone and Jones, 2004).

TOR (target of rapamycin) is a highly conserved protein kinase that controls cell growth and cell size in response to nutrients in many eukaryotes (Wullschlegel et al., 2006). Mammalian cells contain one TOR kinase, whereas both budding and fission yeasts contain two, of which only *tor2* is essential (Alvarez and Moreno, 2006; Hayashi et al., 2007; Uritani et al., 2006; Weisman et al., 2007). In all cell types, TOR kinases function in two distinct complexes; TOR complex 1 (TORC1) and TOR complex 2 (TORC2). Of these two complexes, it is the activity of TORC1 alone that is thought to be sensitive to rapamycin and regulated by

nutrients (Jacinto et al., 2004; Loewith et al., 2002). In fission yeast, the TORCs have mainly been studied in cells grown in rich media. Growth in this medium differs from the limited conditions cells encounter in their natural environment. Fission yeast Tor1 was shown to be part of the TORC2 complex (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007).

We previously established that inhibition of fission yeast Tor1 activity, through either small molecule inhibition with rapamycin or simply shifting cells from a rich to a poor nitrogen source, advanced the timing of mitotic commitment to reduce cell size at division (Petersen and Nurse, 2007). Here, we will refer to this change in nutrient availability as 'nutrient stress'. Similar observations have also been reported in *Drosophila*, where cell numbers increase following mild rapamycin treatment of cell cultures and wings (Wu et al., 2007). This conservation extends to humans as rapamycin-induced inhibition of mTOR in proliferating mammalian cells similarly reduced cell size at division (Fingar et al., 2002). Furthermore, Mammalian HEK293 cells advance mitotic commitment following modest starvation due to confluent growth. The subsequent addition of fresh serum to release this starvation delays cell division to increase cell size at division (Kim et al., 2002). This behaviour is completely mimicked in the response of fission yeast to modest changes in available nutrients (Fantes and Nurse, 1977). Therefore, a role for TOR signalling in the control of cell size at mitotic commitment appears to be generally conserved. In fission yeast, this nutrient-induced reduction in cell size at the onset of mitosis is regulated through a drop in Tor1 activity, which negatively regulates the MAPK phosphatase Pyp2. This

downregulation of Pyp2, boosts the basal level of MAPK Sty1 signalling that, in turn, drives the recruitment of Polo kinase to the spindle pole bodies (SPBs) to promote mitosis (Petersen and Hagan, 2005; Petersen and Nurse, 2007). Therefore, by modulating MAPK regulation of Polo recruitment to the SPBs, cells can either advance or delay mitotic commitment in response to changes in their environment. The control of mitotic commitment through SAMRP in fission yeast (Shiozaki and Russell, 1995) is conserved in mammalian cells and *Xenopus* oocytes as, in both systems, inhibition of MAPK Erk1 activation delays mitotic onset, whereas activation of another MAPK, p38, blocks mitotic commitment (Liu et al., 2004; Mikhailov et al., 2005; Wang et al., 2007).

Interestingly, two recent studies in *Saccharomyces cerevisiae* and mammalian cells reported a TOR-dependent block of mitotic commitment in response to changes in available nutrients (Nakashima et al., 2008; Smith and Proud, 2008). In both studies, cells were exposed to an acute withdrawal of a nitrogen source, which we will refer to here as 'nutrient starvation'. Thus, at first glance, fission yeast appear to regulate mitotic commitment in a distinct and different way to that by which *S. cerevisiae* and mammalian cells regulate mitotic commitment following a change in nutrient availability. However, the acute nutrient starvation used in the experiments to address the nutrient response in budding yeast and mammalian cells is likely to severely stress the cells. It is currently unclear whether MAPK signalling was affected by these alterations in nutrient provision in these two systems. In fact, the differences between the way by which cells control mitotic commitment in response to nutrient stress and those used in response to nutrient starvation are not known in any system.

Here we report that, just like *S. cerevisiae* and mammalian cells, fission yeast cells block mitotic commitment in response to nutrient starvation. Furthermore, we establish that the decisions to block, or to advance, mitosis following nutrient starvation or nutrient stress, respectively, are regulated by invoking different levels of MAPK signalling. Finally, we show that the Tor1-controlled advance in mitotic onset following nutrient stress is mediated by changes in TORC1, and not TORC2, signalling.

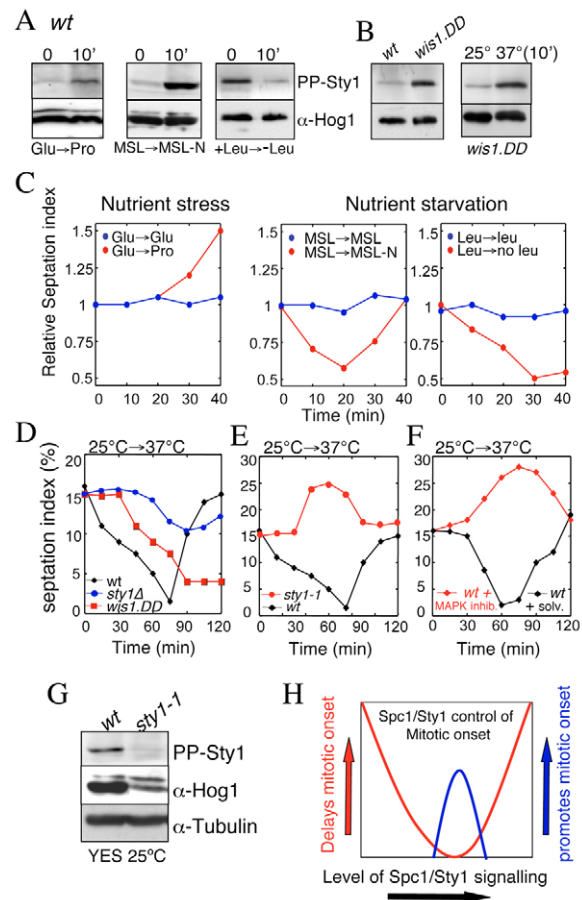
## Results

### Nutrient stress advances mitotic onset, whereas nutrient starvation transiently blocks it

In fission yeast, mitotic commitment is advanced in response to a change from a rich to a poor nitrogen source – 'nutrient stress' (Fantes and Nurse, 1977). This acceleration of mitotic onset is regulated through a Tor1-controlled increase in MAPK activity following the shift into poor medium (Petersen and Nurse, 2007). Importantly, MAPK signalling is essential for the timing of mitotic commitment in fission yeast as *sty1Δ* cells are delayed at mitotic onset, whereas MAPK activation through the constitutive activation of the MAP kinase kinase (MAPKK) Wis1 (*wis1.DD*) advances mitosis to promote proliferation at reduced cell length (Shiozaki and Russell, 1995; Shiozaki et al., 1998). Two recent studies in *S. cerevisiae* and mammalian cells reported a TOR-dependent block of mitotic commitment in response to nutrient starvation (Nakashima et al., 2008; Smith and Proud, 2008). In *S. cerevisiae*, nitrogen starvation was used to change nutrient availability, whereas leucine withdrawal was used in the mammalian cells. The addition of leucine to prototroph cells is thought to reduce the endogenous leucine biosynthesis within the recipient cells (Ulm et al., 1972). Thus, acute removal of an external supply of leucine is likely to transiently starve cells for leucine. In other words, both the addition and removal of

leucine is likely to stress the cells and so modify MAPK signalling. We therefore examined whether the cellular responses to 'nutrient stress' in fission yeast is different to that of 'nutrient starvation'.

To monitor Sty1 activity, we followed the phosphorylation status of the conserved MAPK TXY activation motif because full Sty1 activity requires phosphorylation on T171 and Y173 (Gaits et al., 1998). As previously shown, shifting wild-type cells from glutamate-based (good) to proline-based (poor) medium 'nutrient stress' advances mitotic entry, as demonstrated by the increased cell division (Fig. 1C). This nutrient stress led to an increase in MAPK



**Fig. 1.** Changes in MAPK activity modulate entry into mitosis. (A) Wild-type cells were filtered into proline or leucine or ammonia-free medium. (B) Wild-type and *wis1.DD* cells were grown in YES at 25°C, and *wis1.DD* were shifted to 37°C for 10 minutes before cells were collected by filtration (A,B). Blots were probed with PTPY antibodies against phospho-p38 MAPK, an activating phosphorylation and re-probed with antibody against hog1 to detect total Sty1 levels [anti-hog1 recognises fission yeast Sty1 (see supplementary material Fig. S2B)]; a minor nonspecific band is occasionally seen above the main Sty1 band]. (C) Early exponential prototroph wild-type cells were filtered, washed and resuspended in the indicated media to change the nutrient status (to proline, to no leucine or to no nitrogen – red graphs) plus appropriate control media (blue graphs). The division septa were stained with calcofluor to count 500 cells/time-point. The septation index shown indicated the septation relative to the unstressed starting culture. (D,E) Early exponential cells of the indicated genotype grown in YES at 25°C were shifted to 37°C. (F) Early exponential wild-type cells grown in YES at 25°C were treated with 100 μM total concentration of MAPK inhibitor or the same volume of DMSO and simultaneously shifted to 37°C. (G) Wild-type and *sty1-1* cells were grown in YES at 25°C and cells were collected by filtration. Blots were probed with PTPY antibodies against phospho-p38 MAPK, an activating phosphorylation and re-probed with antibody against hog1 to detect total Sty1 levels and anti-tubulin as a loading control. (H) A schematic showing the proposed effect of Sty1 signalling on mitotic entry.

activity (Petersen and Nurse, 2007) (Fig. 1A). In parallel, wild-type cells were shifted from the minimal medium MSL, into MSL minus the nitrogen source – ‘nutrient starvation’. In addition, prototroph wild-type *leu*<sup>+</sup> cells that had been grown to early exponential phase in glutamate medium that contained leucine were filtered and resuspended at the same density in glutamate media either with the same level of leucine as the original culture or in medium from which leucine was absent. Following both leucine and nitrogen withdrawal, the acute nutrient starvation transiently blocked mitotic commitment (Fig. 1C). Therefore, similar to what has been reported for budding yeast and mammalian cells, acute starvation by the withdrawal of the nitrogen source or removal of the amino acid leucine transiently blocks mitotic commitment in fission yeast. Importantly, this differs from the cellular response to a modest change in nitrogen quality as that nutrient stress response promotes mitotic onset (Fig. 1C). Both nitrogen starvation and leucine withdrawal altered Sty1 activity (Fig. 1A). In response to leucine withdrawal, the level of T171 and Y173 phosphorylation on Sty1 was reduced; however, as previously shown, acute nitrogen starvation promoted a striking rise in Sty1 phosphorylation (Fig. 1A) (Shiozaki and Russell, 1996).

#### Different degrees of Sty1 activation have opposing impacts upon mitotic commitment

Interestingly, the level of Sty1 activation following acute nitrogen starvation was significantly higher than the Sty1 activation following nutrient stress (Fig. 1A). We hypothesised that this difference in activation levels could provide an explanation for the observed differences in the cellular response to nutrient stress versus nutrient starvation. In turn, this would also explain the apparent differences between responses to nutrient availability in different model systems.

We first examined whether MAPK signalling was required for the nutrient starvation block of mitotic commitment. When *sty1Δ* cells were shifted into nitrogen-free medium or were subjected to a depletion of leucine from their growth medium, we no longer observed the block to mitotic onset seen in wild-type cells (supplementary material Fig. S1A). Thus, in a similar manner to the essential role for Sty1 MAPK signalling in Tor1-controlled advanced mitotic onset following nutrient stress (Petersen and Nurse, 2007), Sty1 MAPK signalling is also essential to block mitotic entry following nutrient starvation. Next, we asked whether changes in Sty1 activity could influence the regulation of mitotic commitment. As shown previously, cells with constitutively active Wis1 MAPKK (*wis1.DD*) have increased Sty1 activity (Fig. 1B) and so advance mitotic commitment to promote proliferation at reduced cell length (Shiozaki and Russell, 1995; Shiozaki et al., 1998). As also shown previously, exposure of *wis1.DD* cells to heat stress induces a further activation of Sty1 (Fig. 1B) because the interaction between the Sty1 inhibitory phosphatase Pyp1 and Sty1 is inhibited, leading to strong activation of the MAPK (Nguyen and Shiozaki, 1999). Heat stress activates Sty1 (supplementary material Fig. S2A) and transiently blocks mitosis and cell division of wild-type cells (Nurse, 1975) (Fig. 1D). We asked therefore whether the increase in Sty1 activity seen following heat shift of *wis1.DD* would reverse the initial advancement in mitotic entry exhibited by these cells in steady state and instead lead to a block in mitosis. When *wis1.DD* cells were shifted from 25°C to 37°C, the heat stress blocked mitosis and cell division (Fig. 1D), with a slight delay compared with wild-type cells. A heat shift of *sty1Δ* cells demonstrated that the

block in mitotic entry and cell division following heat stress was MAPK dependent (Fig. 1D). Therefore, the heat-stress-induced increase in MAPK activity of *wis1.DD* cells now blocked mitotic onset until the stress had been dealt with.

The observation that the intermediate levels of Sty1 activity of *wis1.DD* mutants promoted mitotic entry in unperturbed cell cycles but that further activation following heat stress blocked mitosis predicts that the control of mitotic commitment is acutely sensitive to changes in the level of Sty1 MAPK activity. If correct, this predicts that the level of MAPK signalling regulates the decision to advance or block the onset of mitosis. Thus, a change in Sty1 activity would alter the fate of mitotic entry following heat stress. To test this hypothesis, we took advantage of the *sty1-1* mutant (Millar et al., 1995), which exhibits compromised Sty1 signalling as cells are elongated at division (Millar et al., 1995). This mutation leads to a dramatic reduction in the amount of Sty1 available to participate in signal transduction (Fig. 1G). In sharp contrast to wild-type cells, heat shift of *sty1-1* mutants was insufficient to block mitosis, but instead advanced mitosis onset was observed (Fig. 1E). Thus, the inability of the *sty1-1* allele to induce a full response now mimics the type of milder stress response associated with nutrient stress and thus promotes mitotic commitment. To corroborate this finding, we took advantage of a MAPK inhibitor (JNK inhibitor II) to further address whether altered MAPK signalling could alter mitotic commitment. Addition of the drug to cells growing in mid-log phase in steady state had no impact upon mitotic commitment and cell division of *sty1Δ* cells, whereas both processes were delayed by drug treatment of wild-type cells (supplementary material Fig. S3A). Thus, in a manner similar to that by which *sty1Δ* cells are delayed at mitotic onset, treatment with a MAPK inhibitor delays mitotic commitment.

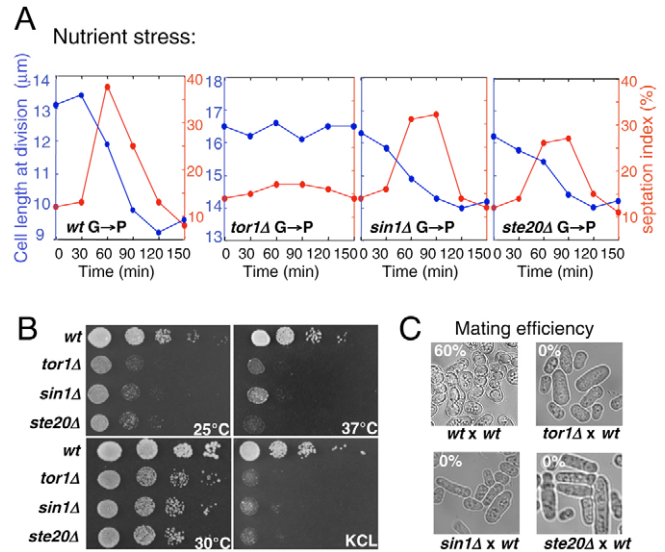
We next determined whether treatment of wild-type cells with JNK inhibitor II, like *sty1-1* mutant cells, would reduce Sty1 signalling following heat shifts and reverse a block in mitotic commitment to an advanced mitotic commitment. When wild-type cells were treated with JNK inhibitor II, mitosis was no longer blocked in response to heat shifts – instead, mitotic commitment was advanced (Fig. 1F). Advanced mitotic commitment following the combination of a heat shift and JNK inhibitor II treatment was also observed in *pmk1Δ* cells that lacked the MAPK Pmk1. However, it was not seen in cells deleted for *sty1Δ*, indicating that the drug is impacting upon Sty1 in this response (supplementary material Fig. S3B). Interestingly, the addition of the JNK inhibitor II to *sty1-1* mutant cells that are subjected to a heat shift to further inhibit the MAPK signalling in these cells led to a response that mimicked that of *sty1Δ* cells – the heat-shift-induced advanced mitotic onset was abolished (supplementary material Fig. S3B). Together, these results are consistent with the view that the impact of MAPK signalling upon mitotic onset is dictated by the level of MAPK signalling invoked by the manipulation. In other words, a basal level of MAPK activity is required to promote mitotic onset; therefore, a reduction in MAPK activity will transiently block mitotic onset. Strong activation of MAPK signalling similarly transiently blocks mitotic onset, to avoid segregation of damaged chromosomes, arising from damage if mitosis and cell division were to immediately follow the severe stress (summarised in Fig. 1H). Both nitrogen starvation and leucine withdrawal resulted in altered Sty1 phosphorylation (Fig. 1A). Both manipulations lead to changes in Sty1-activating phosphorylation and are consistent with the transient delay in mitotic onset observed following the nutrient starvations.

Following an initial nutrient-starvation-induced block in mitotic entry, fission yeast cells advance mitotic onset through Wee1. Complete nitrogen starvation is a signal for fission yeast cells to exit the cell cycle and undergo sexual differentiation and mating. The response of fission yeast cells to nitrogen starvation is biphasic (Fig. 1C; supplementary material Fig. S1A). Initially, mitotic entry is blocked; however, this is followed by an acceleration of mitotic onset, resulting in approximately two fast rounds of cell division before the cells undergo mating. We found that the initial block in mitotic onset following nitrogen starvation requires a kinase, *Srk1*, which is also required to block mitosis following other severe stresses (Lopez-Aviles et al., 2005) (supplementary material Fig. S1A). By stark contrast, *Srk1* was not required to advance mitotic onset following nutrient stress (supplementary material Fig. S1B). Combined with our previous demonstration that nutrient stress advancement in mitotic commitment is regulated through the *Plc1* kinase (Petersen and Nurse, 2007) (supplementary material Fig. S1B), this establishes that, while nutrient stress and nutrient starvation responses both utilise *Sty1* MAP kinase signalling, the downstream effector is distinct in each case.

Importantly, the accelerated mitotic onset following nutrient starvation (the second response) still occurs in *sty1Δ* cells (supplementary material Fig. S1). *sty1Δ* cells actually underwent mitotic onset earlier than wild-type cells after removal of the nitrogen source; therefore, *Sty1* signalling is not required for the second response to nitrogen-starvation-advanced mitotic onset. Nutrient-stress-induced acceleration of mitotic onset is dependent on the *Wee1* kinase, an inhibitor of the mitotic *Cdc2* kinase (Fantès and Nurse, 1978; Nurse, 1990). We therefore asked whether *Wee1* was required for the second response to removal of the nitrogen source (acceleration of mitotic onset). When *wee1.50* cells were shifted into nitrogen-free medium, the cells, similar to wild-type cells, also slowed down mitotic entry. However, in contrast to the response of wild-type cells, we no longer observed a subsequent acceleration of mitosis (supplementary material Fig. S1A). This indicates that *Wee1* controls the advanced mitotic entry following nitrogen starvation and that this response does not rely on changes in *Sty1* MAPK signalling. Therefore, this second response to nitrogen starvation serves as a very useful tool to address whether mutants defective in *Tor1*-controlled nutrient-stress-induced mitotic onset are generally unable to advance mitotic entry.

#### TORC2-specific components are not essential for nutrient-stress-induced mitotic onset

We previously established that *Tor1* was essential for advancement of mitotic onset following nutrient stress and that *Tor1* kinase activity is reduced upon nutrient stress (Petersen and Nurse, 2007). *tor1Δ* cells did not advance mitotic commitment in response to nutrient stress and maintained their cell length at mitotic onset throughout the experiment (Fig. 2A). This is not a general defect in the ability to advance mitotic onset as *tor1Δ* cells advance mitosis following nutrient starvation (Petersen and Nurse, 2007). In contrast to *tor1Δ*, wild-type cells advanced mitotic entry and reduced the cell size at which cells execute division following nutrient stress. Because *Tor1* has been shown to be part of TORC2 in rich medium (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007), we examined the contribution of TORC2 and its components *Sin1* and *Ste20* in the regulation of this G2–M control in greater detail. Cells lacking *Sin1* or *Ste20* were still able to accelerate mitotic commitment when shifted from glutamate to proline. In both *sin1Δ* and *ste20Δ* mutants, cell length at division was reduced and

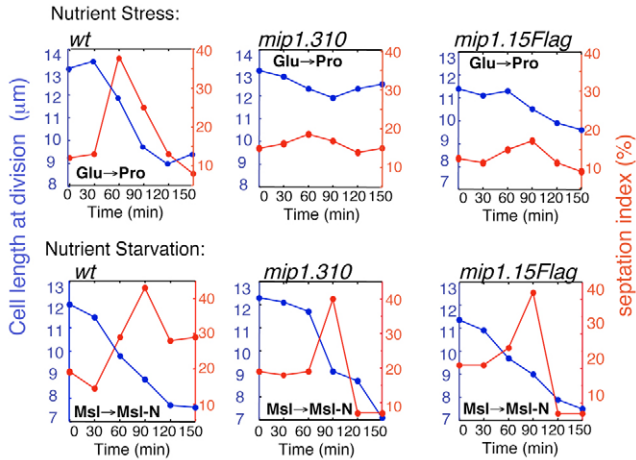


**Fig. 2.** Strains lacking the TORC2 components *Sin1* and *Ste20* advance mitotic onset in response to nutrient stress. (A) Left-hand Y-axes show cell length ( $\mu\text{m}$ ) at division (blue); right-hand Y-axes show frequency of dividing cells (red). Cells grown in glutamate were collected by filtration and resuspended in proline. The cell length of *sin1Δ* and *ste20Δ* mutants at 120 minutes was significantly different ( $P=0.05$ ) from that of *tor1Δ* cells at 120 minutes as determined by a Student's *t*-test. (B) Exponentially growing cells were spotted on YES or YES plus 0.4 M KCL and incubated at 30°C or as indicated. (C) Equal volumes of *wt h<sup>+</sup>*, *tor1Δ*, *sin1Δ* and *ste20Δ* cells were mixed with *wt h<sup>-</sup>* cells (or *wt h<sup>+</sup>* as control; data not shown) and spotted on SPA. Mating efficiencies are indicated in percentages.

accompanied by an increase in dividing cells (Fig. 2A). Therefore, in contrast to *Tor1*, the TORC2 components *Sin1* and *Ste20* are not required to regulate mitotic entry in response to nutrient stress. Thus, even though all three TORC2 components, *Tor1*, *Sin1* and *Ste20*, as previously shown, are essential under temperature – and osmotic – stress conditions and are required for mating (Fig. 2B,C) (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007; Uritani et al., 2006; Weisman and Choder, 2001; Weisman et al., 2007), they differ with respect to their function in the regulation of mitotic commitment in response to nutrient stress. A major problem with mutants in TORC2 is the rapid accumulation of secondary suppressor mutations; however, our mutants are sterile and sensitive to stress – so therefore all are free of suppressors, confirming that the response of the cells in Fig. 2 can be interpreted in terms of the impact of the loss of *Sin1* and *Ste20*. We conclude that the role of *Tor1* (Petersen and Nurse, 2007) in the nutrient stress response is independent of the role it plays as part of the TORC2 complex in response to different manipulations.

#### Cells defective in the TORC1 component *Mip1* fail to advance mitosis in response to nutrient stress

Having established that mutants in the TORC2 components *Sin1* and *Ste20* were able to advance mitotic onset in response to nutrient stress (Fig. 2A), we asked whether mutants in the TORC1-specific component *Mip1* (raptor) played a role in mitotic control following nutrient stress. Interestingly, we found that *mip1.310* and *mip1-15p-FLAG* mutants (Shinozaki-Yabana et al., 2000) mimicked *tor1Δ* in being compromised in the nutrient-stress-induced mitotic onset (Fig. 3). Importantly, the *mip1* mutants were still able to accelerate mitosis, when exposed to acute nutrient starvation (Fig. 3). This



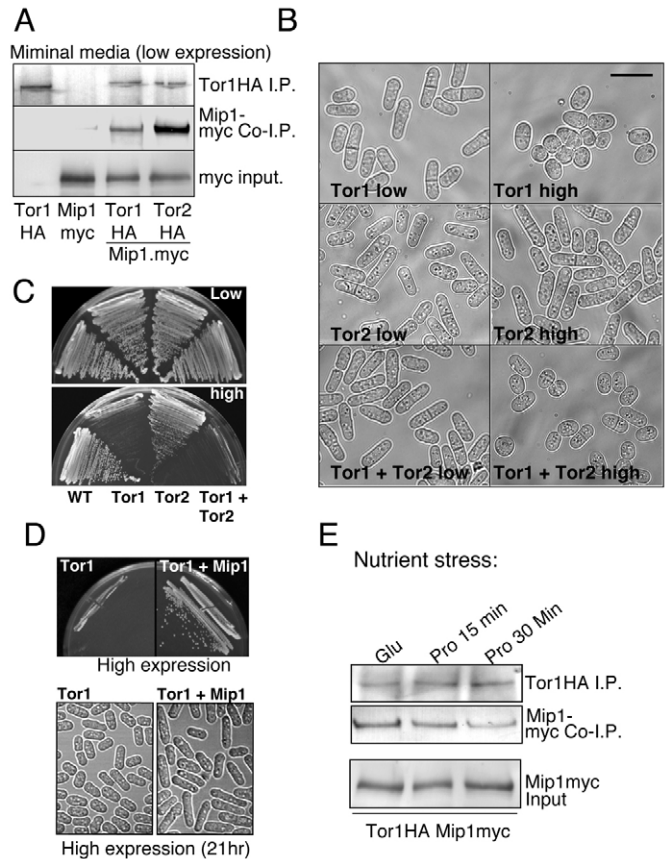
**Fig. 3.** Mutants in the TORC1 component Mip1 fail to advance mitotic onset in response to nutrient stress. Left-hand y-axes show cell length (μm) at division (blue); right-hand Y-axes show frequency of dividing cells (red). Cells grown in glutamate or MSL at 28°C were collected by filtration and resuspended in proline or MSL minus ammonia. The division septa were stained with calcofluor to count 500 cells/time-point (wild-type graphs from Fig. 2 are shown for comparison).

response is similar to the response of *tor1Δ* strains to nutrient starvation. These data established that Tor1 and Mip1 but not the TORC2 components Sin1 and Ste20 regulate mitotic commitment specifically in response to the modest change in nitrogen quality of nutrient stress.

#### Tor1 interacts with the TORC1 component Mip1

In cells grown in rich media, Tor1 has been shown to be part of TORC2 and Tor2 to be part of the TORC1 complex (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007). The ability of cells lacking the TORC2 components Ste20 and Sin1 to accelerate mitotic commitment following nutrient stress, along with the inability of mutants deficient in the TORC1 component Mip1 to do so, raises the interesting possibility that Tor1 might exert its role in control of mitotic onset as part of TORC1. We therefore asked whether Tor1 could physically interact with Mip1 and so could indeed be part of TORC1. During growth in minimal media, that most closely mimics the limited conditions cells naturally encounter, Tor1 interacted with Mip1 in immunoprecipitation assays (Fig. 4A). Interestingly, nutrient stress reduced the extent of this association of Mip1 with Tor1 (Fig. 4E).

In fission yeast, only *tor2* is essential (Alvarez and Moreno, 2006; Hayashi et al., 2007; Uritani et al., 2006; Weisman et al., 2007). Elevating levels of Tor1 by de-repression of a thiamine-repressible *nmt1* promoter generated a surprising growth defect that was highly reminiscent of that of *tor2* mutants (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007; Uritani et al., 2006; Weisman and Choder, 2001; Weisman et al., 2007) – the cells were small, round and stopped growth. By contrast, cells with lower Tor1 levels grew normally in minimal media (Fig. 4B,C). *tor1* is not essential. When considered in conjunction with the ability of Tor1 to bind to the TORC1 component Mip1, when expressed at low levels, the overexpression phenotype could suggest that high levels of Tor1 compete with Tor2 for binding of TORC1 components and so disrupt Tor2-specific functions. To test this possibility, we overexpressed Mip1 at the same time as overexpressing Tor1 to see



**Fig. 4.** Tor1 interacts with the TORC1 components Mip1 (Raptor) in minimal media, and elevation of Tor1 expression inhibits growth. (A) Glutamate-grown cells expressing low levels (thiamine was added) of Tor1 and Tor2 were harvested for anti-HA-dependent immunoprecipitations. (B) Cells were grown in glutamate media for 14 hours with (low expression) or without (high expression) addition of thiamine. (C) Cells were grown on EMMG plates with (low expression) or without (high expression) inclusion of thiamine. (D) Cells were grown on EMMG plates with high expression of Tor1 and Tor1 plus Mip1 (upper), or cells were grown in EMMG to mid-log with 21 hours of high expression of Tor1 or Tor1 plus Mip1. (E) Glutamate-grown cells expressing low levels (thiamine was added) of Tor1 were filtered into proline and harvested for anti-HA-dependent immunoprecipitations.

whether these elevated levels of Mip1 would titrate out the excess Tor1 and so reduce its impact upon Tor2-specific complexes. The increased Mip1 expression rescued the growth defect induced by Tor1 overexpression (Fig. 4D). In addition, we were able to coimmunoprecipitate more Mip1 from cells expressing elevated levels of Tor1 (supplementary material Fig. S4A). Cells with high Tor2 levels had no apparent growth defect (Fig. 4B,C). These data suggest that Tor1, like Tor2, binds to TORC1 components in cells grown in minimal medium.

Tor2 inhibition induces cell cycle exit and sexual differentiation by upregulating Ste11-transcribed genes that are normally induced by nitrogen starvation and mating pheromones (Matsuo et al., 2007). We find that blocking cell cycle exit and sexual differentiation by deletion of *ste11Δ* is not sufficient to rescue the *tor2Δ*-dependent growth defect in *ste11Δ tor2Δ* double mutants (data not shown), indicating that Tor1 is unable to complement the essential role of Tor2 in growth control. Together, our data suggest that both Tor1 and Tor2 are TORC1 components but that Tor1 and Tor2 are unlikely to be interchangeable in the essential TORC1.

### The S6K homologue Gad8 is essential for nutrient-stress-induced advancement of mitotic onset

In mammalian cells, the S6K kinase has been shown to be a downstream effector of the TORC1 complex (Kim et al., 2002). In fission yeast, Tor1-dependent phosphorylation of Gad8 (S6K homologue) has been demonstrated previously (Matsuo et al., 2003). We asked, therefore, whether Gad8 had a role in nutrient-induced advancement of mitotic commitment. Cells deleted for *gad8* (Matsuo et al., 2003) failed to advance mitotic commitment in response to nutrient stress, and maintained a constant cell length at mitotic onset throughout the experiment (Fig. 5A). By contrast, just like *tor1* and *mip1* mutants, *gad8Δ* cells retained the ability to accelerate mitosis following nutrient starvation (Fig. 5A). Thus, the response of *gad8* mutants to nutrient stress is highly reminiscent of that of *tor1* and *mip1* mutants, suggesting that they function in the same signalling pathway to advance mitotic entry.

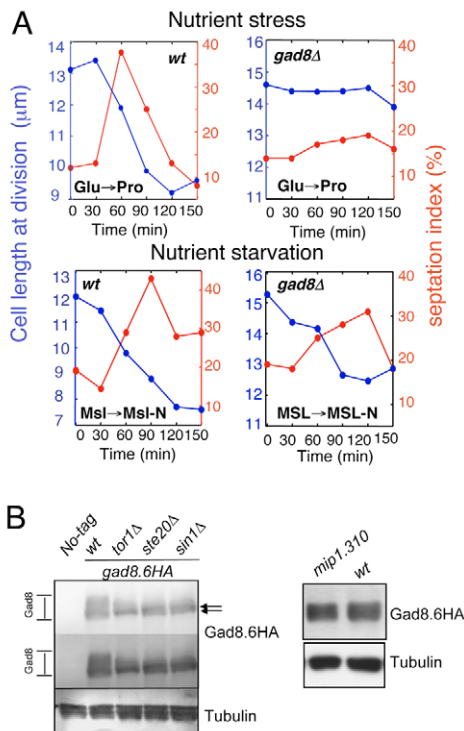
Previous studies in fission yeast indicated that Gad8 is an AKT1 homologue and a target of Tor1 bound to TORC2 that regulates mating (Ikeda et al., 2008; Matsuo et al., 2003). In BLAST alignments, Gad8 shows higher sequence identity to S6K (50%) than to the AKT1 kinase (46%). However, AKT1 kinase is also a key regulator of mTOR signalling (Wullschieger et al., 2006). Therefore, Gad8 is likely to be the fission yeast functional equivalent of both S6K and AKT1 kinases. The Sck1 kinase is a homologue of Gad8 and budding yeast SCH9, which is a major target of TORC1

in budding yeast (Urban et al., 2007). Sck1 is not required for nutrient stress control of mitotic onset (data not shown); thus, Gad8 is likely to be a key target of Tor1 required for nutrient-stress-controlled mitosis. In western blots, Gad8 migration is slowed by phosphorylation, and this reduced migration is altered in *tor1Δ*, *sin1Δ* and *ste20Δ* strains (Ikeda et al., 2008; Matsuo et al., 2003). Because the TORC2 components *sin1Δ* and *ste20Δ* but not *tor1Δ* accelerate mitotic onset in response to nutrient stress, we re-examined the migration of Gad8 following precipitation of total cellular protein with TCA and separation on a low-percentage acrylamide gel in *tor1Δ*, *sin1Δ* and *ste20Δ* mutant backgrounds. Migration of Gad8 was retarded to a greater degree in *sin1Δ* and *ste20Δ* than in *tor1Δ* cells (Fig. 5B), suggesting that a degree of Gad8 modification is maintained in *sin1Δ* and *ste20Δ*, that was not present in *tor1Δ* cells. Importantly, as previously reported, all three strains showed significantly lower levels of Gad8 modification than wild-type cells (Ikeda et al., 2008; Matsuo et al., 2003), suggesting that TORC2 is responsible for the majority of Gad8 modification, and that the TORC1-specific modification is moderate. Because the TORC1 component *mip1.310* was defective in advancing mitotic onset in response to nutrient stress (Fig. 3), we also examined Gad8 protein migration in *mip1.310* mutant cells. A minor reduction in Gad8 protein modification was observed (Fig. 5B). These data support the view that Gad8 is a downstream target of both Tor1 bound to TORC2 and of Tor1 bound to TORC1.

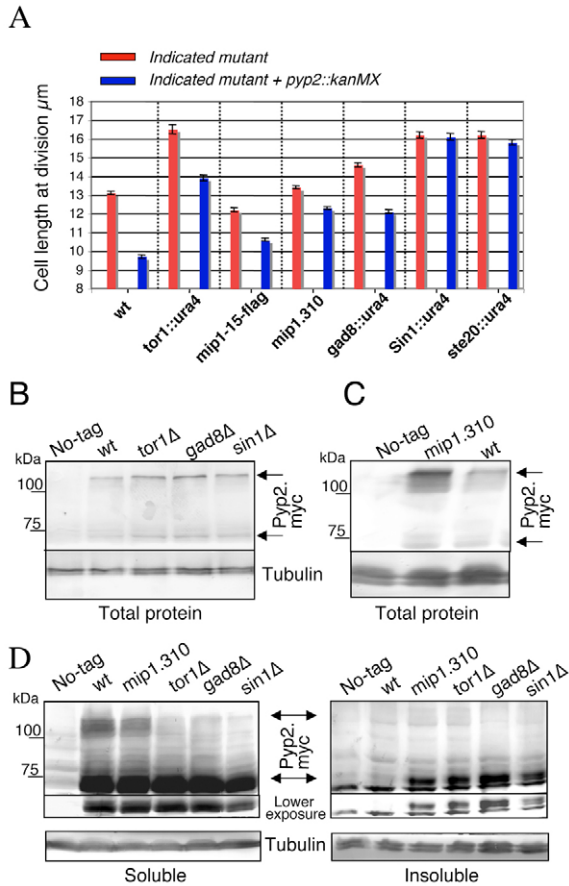
### Deletion of *pyp2* advanced mitotic onset in mutants that compromise the function of TORC1 but not TORC2

We previously established that Tor1 controls nutrient-stress-induced mitotic onset by negatively regulating the MAPK phosphatase Pyp2 (Petersen and Nurse, 2007). This downregulation of Pyp2 enhances MAPK Sty1 signalling that promotes the recruitment of Polo kinase to the SPBs to drive mitotic commitment (Petersen and Hagan, 2005; Petersen and Nurse, 2007). Interestingly, a recent study in mammalian cells demonstrated a posttranslational regulation of the ERK phosphatase DUSP6/MKP3 by the mTOR pathway (Bermudez et al., 2008). Therefore, the Tor1-dependent control of MAPK signalling through regulation of a MAPK phosphatase appears to be conserved in human cells. Fission yeast *tor1Δ* mutants are elongated, indicating that they are delayed at the G2–M boundary (Weisman and Choder, 2001). If this delay arises from deregulation of Pyp2, it should be reduced by simultaneous removal of this putative target, Pyp2. Significantly, we found that deletion of *pyp2* in the *tor1Δ* mutant background did indeed reduce cell length at division (Fig. 6; supplementary material Table S1). Furthermore, deletion of *pyp2* in *mip1* mutants also reduced the cell length at division (Fig. 6; supplementary material Table S1). Consistent with a role for Gad8 in nutrient-stress-induced mitotic onset, the elongated cell length at division of *gad8Δ* mutants was also decreased by the additional deletion of *pyp2* (Fig. 6; supplementary material Table S1). In stark contrast, however, deletion of *pyp2* did not compensate for the cell cycle delay of either a *sin1Δ* or *ste20Δ* mutants.

As the removal of *pyp2* reduced the delay in mitotic commitment arising from mutation of *tor1*, *mip1* and *gad8*, it was likely that an increase in the basal levels of Pyp2 protein could account for a decreased MAPK activity, which would in turn delay mitotic onset. Alternatively, it could be due to an inability of the mutant cells to regulate Pyp2 activity correctly. To differentiate between these possibilities, we determined by immunoblotting the basal level of Pyp2 in cultures of wild-type and *tor1Δ*, *gad8Δ*, *sin1Δ* and *mip1.310*



**Fig. 5.** Gad8, a S6K kinase homologue, is essential for advancement of mitotic onset in response to nutrient stress. (A) Left-hand y-axis shows cell length (μm) at division (blue); right-hand Y-axis shows frequency of dividing cells (red). Cells grown in glutamate or MSL were collected by filtration and resuspended proline or into MSL minus ammonia (wild-type graphs from Fig. 2 are shown for comparison). (B) Western blot of TCA-extracted total protein from early-exponential glutamate-grown cells of the indicated genotype. The protein extracts were run on a 6% gel and Gad8 was detected by antibodies against HA.



**Fig. 6.** Deletion of *pyp2* advances mitotic onset in mutants of TORC1 but not TORC2 components. (A) Average cell length ( $\pm$ s.e.m.) at division of mutants of the indicated genotype. (B,C) Western blot of total protein extracts from early-exponential glutamate-grown cells. The Pyp2 levels are detected with antibodies against the myc-tag, and tubulin is shown as a loading control. (D) Soluble/insoluble Pyp2 was isolated from glutamate-grown cells; tubulin is shown as loading control. Mutants in TORC2 components have less full-length Pyp2; however, increased insoluble Pyp2 is seen compared with that of wild-type cells.

mutants. In total extracts, the Pyp2 levels in *tor1 $\Delta$*  and *gad8 $\Delta$*  mutant strains were slightly higher than in wild-type cells (Fig. 6B), whereas Pyp2 levels in *mip1.310* mutants were significantly higher than in wild-type cells (Fig. 6C). When soluble and insoluble extracts were prepared, a Pyp2 cleavage product appeared on the blots (Fig. 6D). It appeared that the proteolytic activity giving rise to a faster-migrating form of Pyp2 was greater in extracts of TORC2 mutants because full-length Pyp2 was virtually undetectable in extracts from *tor1 $\Delta$*  and *sin1 $\Delta$* , and little full-length Pyp2 was observed in soluble extracts from *gad8 $\Delta$*  mutants (a substrate of TORC2). Thus, only the TORC1-specific mutant *mip1.310* show a marked increase in the levels of full-length Pyp2 in total extracts. However, in both *tor1 $\Delta$*  and *gad8 $\Delta$*  extracts, in addition to having slightly more full-length Pyp2, the level of the cleavage product is also increased, suggesting an initial elevation of Pyp2 levels (Fig. 6B). Furthermore, extracts from the TORC2-specific mutant *sin1 $\Delta$*  have lower levels of the Pyp2 cleavage product in all three extracts than *tor1 $\Delta$* , suggesting that the total amount of Pyp2 in *sin1 $\Delta$*  cells is less than that in *tor1 $\Delta$* . At present, it is unclear why TORC2 mutants have higher proteolytic activity.

Together, these findings are consistent with the view that TORC1 (Mip1 and Tor1) and Gad8 control mitotic onset by regulating the level of Pyp2 and thus control the Sty1 MAP kinase activity. By contrast, the delay in mitotic onset that leads to increased cell length at division in mutants of the TORC2 components Sin1 and Ste20 (Matsuo et al., 2007; Wilkinson et al., 1999) is due to a control of mitotic onset that is independent of MAPK phosphatase control. Thus, these data also support the view that Sin1 and Ste20 differ with respect to their function in regulation of mitotic commitment when compared with Tor1.

## Discussion

### MAPK control of mitotic onset

The control of mitotic commitment through stress MAPK kinase signalling in fission yeast (Shiozaki and Russell, 1995) is conserved in mammalian cells and *Xenopus* oocytes. In both metazoan systems, a block to Erk1 MAPK activation delays mitotic onset, indicating that, just like Sty1, Erk1 activity is required for mitotic entry. When mammalian cells are exposed to severe stress, another MAPK, p38, is activated. The function of p38 in this context resembles that of Sty1 as it blocks mitotic onset until the stress has been dealt with (Liu et al., 2004; Mikhailov et al., 2005; Wang et al., 2007). Fission yeast Sty1 might therefore be the functional homologue of both Erk1 and p38 MAPKs – that is, Sty1 either delays or advances mitotic onset depending upon the level of change in its activity (Fig. 1H).

Following osmotic stress and nutrient starvation, the Sty1-dependent block to G2–M commitment is regulated through phosphorylation of Srk1 kinase. Phosphorylated Srk1 blocks mitotic onset by inhibiting the activity of the Cdc25 phosphatase that is required to activate maturation-promoting factor (MPF) (Lopez-Aviles et al., 2005; Lopez-Aviles et al., 2008). Because Sty1 both promotes and delays mitotic onset, an extra layer of regulation is likely to be needed. This could arise from differential activation thresholds for the kinase to target phosphorylation of individual substrates. This model would predict that Srk1 would need a high threshold of activation before it becomes phosphorylated. Interestingly, the site in Srk1 that is phosphorylated by Sty1 is ARTP (Lopez-Aviles et al., 2008) (amino acid shown in bold indicates phosphorylation site), which differs from the optimal MAPK consensus sites PX(S/T)P and so might require a high level of Sty1 activity for phosphorylation and thus inhibition of mitosis.

The activation of Sty1 following the acute nitrogen starvation that promotes sexual differentiation initially blocks mitotic onset. This initial block is later overridden by a Wee1-dependent acceleration of mitotic entry (supplementary material Fig. S1A). Thus, following nitrogen starvation, Wee1 is regulated in a MAPK-independent way to promote mitotic entry. The Wee1-dependent acceleration of mitosis leads to two rapid rounds of cell division before cells undergo mating. As wild-type cells switch mating types during division, these extra rounds of division are thought to increase the chances that a cell will encounter a mating partner of the opposite mating type in their immediate environment (i.e. if no other is found, then the sister produced in the accelerated division). In budding yeast, the block in mitotic onset following nitrogen starvation is not followed by an acceleration of mitotic entry (Nakashima et al., 2008). Swe1 (the Wee1 homologue) is therefore unlikely to be regulated by a similar nitrogen-starvation-dependent mechanism. It is interesting to note that budding yeast cells do not need nitrogen starvation to induce mating. In this yeast, pheromone signalling and mating can occur in rich media (Duntze et al., 1970). The Wee1-

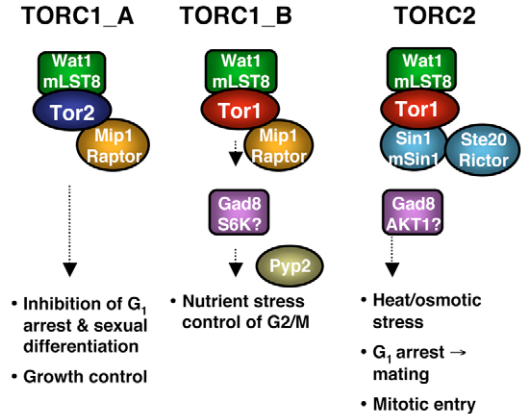
dependent acceleration of mitotic onset might therefore be a specific adaptation of fission yeast to increase the chances of survival in the form of a stress-resistant spore.

When comparing the effect of the environment upon mitotic commitment in different organisms, our findings suggests that monitoring the specific levels of MAPK activities associated with particular changes would be useful in other organisms too because mild alterations might accelerate mitosis, whereas more severe stresses transiently block it. For example, studies in *S. cerevisiae* and mammalian cells reported an mTOR-dependent block to mitotic commitment in a response that the authors referred to as nutrient stress – however, by our definition, their manipulation was a nutrient starvation response as it was in fact an acute withdrawal of nutrients (Nakashima et al., 2008; Smith and Proud, 2008). It would be interesting to monitor the degree of changes in MAPK signalling following their manipulations as similar acute starvations of nutrients from fission yeast cells also transiently block mitosis (Fig. 1C). Thus, only the modest changes in nitrogen quality in response to nutrient stress advance mitotic onset, whereas complete nutrient starvation transiently block mitosis. Significantly, it is this type of modest change in nutrient supply that is most likely to mimic the chronic fluctuations in nutrient supply experienced *in vivo*.

We previously reported that inhibition of fission yeast Tor1 promotes mitotic onset by controlling the activity of the stress MAPK pathway through regulation of its phosphatase Pyp2 (Petersen and Nurse, 2007). We have extended these observations of Tor1-dependent regulation of Pyp2 activity to incorporate Mip1 and the S6K homologue Gad8. The molecular mechanism behind TORC1 (Mip1 and Tor1) and Gad8 regulation of Pyp2 levels is unclear at present; however, future mapping of phosphorylation sites on Pyp2 might help to clarify this issue. Interestingly, inhibition of mTORC1 in human cancers also leads to activation of MAPK signalling as ERK phosphorylation is elevated (Carracedo et al., 2008). Furthermore, the mTOR pathway also regulates the posttranslational modulation of ERK phosphatase DUSP6/MKP3 (Bermudez et al., 2008). Therefore, the Tor1-dependent control of MAPK signalling through regulation of a MAPK phosphatase that we observed in fission yeast appears to be conserved in human cells.

### TORC1 in fission yeast

We now provide evidence that the role of Tor1 in controlling mitotic entry in response to nutrient stress is independent of any participation in TORC2. We show that Tor1 interacts with the TORC1 component Mip1 and that the nutrient-stress-induced mitosis is only compromised in TORC1 mutants and not in mutants of TORC2. Our data suggest that Tor1 and Tor2 are unlikely to be interchangeable in the essential TORC1, suggesting that two distinct TORC1 subcomplexes exist – TORC1\_A, including Tor2, and TORC1\_B, including Tor1 (Fig. 7). Interestingly, two distinct TORC1 subcomplexes have also been described in *S. cerevisiae* (Xie et al., 2005). We previously reported that inhibition of Tor1 by both nutrient stress or rapamycin treatment promoted mitotic onset through Sty1 signalling (Petersen and Nurse, 2007). The existence of two TORC1 subcomplexes incorporating either Tor1 or Tor2 supports the view that, in fission yeast, like in all organisms studied to date, TORC1 complexes alone are inhibited by rapamycin. Mutants in the TORC2 components Sin1 and Ste20 are both delayed at the G2-M transition (Matsuo et al., 2007; Wilkinson et al., 1999), and this defect appears to be independent of the Pyp2 phosphatase and control of MAPK activity because deletion of *pyp2* does not compensate for the mitotic entry defect in *sin1Δ* and *ste20Δ* mutants



**Fig. 7.** TORCs in fission yeast. A cartoon showing the proposed TOR-containing complexes in fission yeast. Additional complexes, components and various other molecules upstream and downstream of these complexes are omitted to illustrate the key points better.

(Fig. 6A). The TORC2 mutants are also defective in nutrient-starvation-induced sexual differentiation, which suggests that acute nitrogen starvation is sensed by TORC2 and that TORC2 is essential for the G<sub>1</sub> arrest required for nutrient-starvation-induced sexual differentiation. Previously, it was suggested that Tor2 also interacts with TORC2 (Matsuo et al., 2007). We found that, in minimal glutamate media, Tor2 also interacts with Ste20 and that this interaction increased when *tor1* is deleted (supplementary material Fig. S4B). At present, it remains to be established whether Tor2 has an independent role as part of TORC2 as no biological function has been associated with Tor2 as part of TORC2.

Together, our data consolidate the view that Tor kinases perform different functions in different complexes and that TORCs can be further subdivided into distinct functional units that might be required to respond to different environmental cues. Future characterisation of TORC1 and its activation will further clarify the Pyp2- and MAPK-dependent regulation of mitotic entry in response to nutrient stress.

### Materials and Methods

#### Cell cultures and strains

Strains are listed in supplementary material Table S2. Cells were cultured at 28°C in yeast extract media (YES) (Moreno et al., 1991) or in EMM minimal media with either glutamic acid (EMM-G) or proline (EMM-P) (20 mM proline) as a nitrogen source (Fantes and Nurse, 1977). Thiamine (4 μM) was added to minimal media to repress the *nmt1* promoters. Nutritional shift or rapamycin experiments were performed as described previously (Petersen and Nurse, 2007). Non-starved starter cultures in YES were washed twice and grown exponentially in EMM-G for 40 hours. At a density of  $1.5 \times 10^6$  cells ml<sup>-1</sup>, cells were either filtered into EMM-P or rapamycin was added. Cellular responses to leucine starvation were studied following the removal of 300 mg/l leucine that was added to prototroph wild-type glutamate-grown cells. Cells were grown in MSL (Egel et al., 1994) and filtered into MSL minus nitrogen when studying the 'nitrogen starvation' response. For spot test and mating experiments, cultures were grown exponentially in YES to a density of  $2.5 \times 10^6$  cells ml<sup>-1</sup>. A 10-fold dilution series starting with  $5 \times 10^4$  cells was spotted on YES or YES plus 0.4 M KCL and incubated at 30°C or as indicated.  $4 \times 10^6$  cells of each culture were mixed with equal volumes of either JP3 *wt h<sup>-</sup>* or JP350 *wt h<sup>+</sup>* and spotted on SPA. Mating frequencies were determined after 30 hours incubation at 30°C by:  $2 \times \text{zygotes} / [\text{total cells} + (2 \times \text{zygotes})]$ . 100 μm/ml noursesthrin (Hentges et al., 2005) was added to media to select for pRep81NAT-Mip1.

#### Molecular genetics

Mip1 was cloned by PCR from genomic DNA and subcloned into pRep81Nat using *Nde1* and *Sac1* restriction sites included in the primers GGAATTCATATGAATGATAGAATTAGTGAAGTATCTGGTAGCTCAAGGG and GGCGAGCTCT-



TAAAACTCGTTCGGCGAATCCGTATGAATCTCGTTTTTGG; restriction sites are indicated in bold.

### Microscopy

Calcofluor staining of septa was performed as described previously (Moreno et al., 1991) and at least 500 cells were counted for each time-point. Images and cell length measurements were obtained using a Quantix camera (photometrics) with Metamorph software (Universal Imaging). More than 100 dividing cells per strain were measured for obtaining cell length data.

### Inhibitor treatment

Cell cultures, exponentially grown in YES at 25°C to a density of  $1.5 \times 10^6$  cells ml<sup>-1</sup>, were treated with 100 μM total concentration of MAPK inhibitor SP600125 (Calbiochem) or the same volume of DMSO and simultaneously shifted to 37°C where indicated. Samples were fixed with formaldehyde and calcofluor stained to determine septation indices.

### Biochemistry

Total protein extracts were prepared by TCA precipitation (Petersen and Hagan, 2005). For soluble/insoluble isolation of Pyp2, cells were lysed in (50 mM HEPES pH 8, 150 mM KCl, 0.5% Tween, 1 mM EDTA, 1 mM DTT plus protease inhibitors), lysates were spun at 13,100 g for 10 minutes and supernatant (soluble) and pellets (insoluble) were separated. Proteins were separated by SDS-PAGE and detected using the following antibodies: 1:250 46A anti-myc (Millipore); 1:100 12CA5 anti-HA; 1:1000 anti-PTPY phospho-p38 MAPK (Cell Signaling) to detect active Sty1; 1:500 anti-Hog1 y-215 (Santa Cruz Biotechnology) to detect Sty1; 1:1000 TAT anti-tubulin and alkaline-phosphatase- or horseradish-peroxidase-conjugated secondary antibodies. HA-dependent immunoprecipitations of Tor1 and Tor2 were performed using Protein G Dynal beads. Immunoprecipitation buffer was: 50 mM HEPES pH 7.5, 100 mM KCl, 0.1 mM EDTA, 1 mM DDT, 0.2% Tween 20 plus protease and phosphatase inhibitors. Immunoprecipitated complexes were washed five times in wash buffer (immunoprecipitation buffer plus 300 mM NaCl).

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