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RESEARCH ARTICLE

S. pombe TORC1 activates the ubiquitin-proteasomal degradation of the meiotic regulator Mei2 in cooperation with Pat1 kinase

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ABSTRACT

Target of rapamycin (TOR) kinase regulates cell metabolism and growth, acting as a subunit of two multi-protein complexes, TORC1 and TORC2. Known TORC substrates are either kinases or general factors involved in growth control. Here, we show that fission yeast TORC1, which promotes vegetative growth and suppresses sexual development, can phosphorylate Mei2 (a specific factor involved in switching the cell fate) *in vitro*. Alanine substitutions at the nine Mei2 phosphorylation sites stabilize the protein and promote mating and meiosis *in vivo*. We found that Mei2 is polyubiquitylated *in vivo* in a TORC1-dependent manner. Based on these data, we propose that TORC1 contributes to the suppression of sexual development by phosphorylating Mei2, in addition to controlling the cellular metabolic status.

KEY WORDS: Fission yeast, Mating, Meiosis, Proteasome, TOR kinase, Mei2

INTRODUCTION

Target of rapamycin (TOR) is a highly conserved serine/threonine kinase, which is thought to regulate various growth-related functions in response to environmental changes (Wullschleger et al., 2006). TOR forms two types of multi-protein complexes, TORC1 and TORC2. In mammalian cells, a single catalytic subunit, mTOR, is present in both TORC1 and TORC2 (mTORC1 and mTORC2). In yeast species, TORC1 and TORC2 have a similar subunit composition to mTORCs, but they carry different TOR proteins as the catalytic subunit (Wullschleger et al., 2006).

Despite the physiological importance of TORCs, only a limited number of TORC substrates have been identified to date. S6K1, an AGC family protein kinase, and 4E-BP1, an eIF4E-binding protein, are the best-characterized substrates of mTORC1. mTORC1 regulates translation through these molecules. Although not as well characterized as S6K1 or 4E-BP1, additional proteins, including Maf1, a repressor of RNA polymerase III, and Atg13, an autophagy-related protein that acts as a conserved regulator of Atg1 kinase, are emerging as direct targets of TORC1 in mammals and yeast. TORC1 appears to promote cell growth and cell cycle progression through the phosphorylation of these substrates (Wullschleger et al., 2006; Laplante and Sabatini, 2012). An even smaller number of targets are known for TORC2, and these include mammalian Akt and yeast Ypk2.

In fission yeast, *Schizosaccharomyces pombe*, TORC1, containing Tor2 as its catalytic subunit, and TORC2, containing a Tor1 catalytic subunit, fulfill opposing functions with respect to the promotion of G1 arrest and sexual development (Kawai et al., 2001; Weisman and Choder, 2001; Alvarez and Moreno, 2006; Uritani et al., 2006; Matsuo et al., 2007; Weisman et al., 2007) (reviewed in Otsubo and Yamamato, 2008). TORC1 is active during vegetative growth and regulates sexual development negatively, apparently by transmitting signals from a sufficient nitrogen supply (Matsuo et al., 2007). TORC1 is known to phosphorylate Psk1, a fission yeast homolog of S6K1 (Nakashima et al., 2012), but the effectors of TORC1 that function in the suppression of sexual development remain largely unidentified.

An RNA-binding protein, Mei2, governs the entry into meiosis in fission yeast (Watanabe and Yamamoto, 1994; Watanabe et al., 1997; Yamashita et al., 1998). Mei2 has the ability to switch off the mitotic cell cycle and promote meiosis, enabling cells to stably express meiosis-specific mRNAs (Harigaya et al., 2006). In addition to eliciting meiosis, Mei2 participates in earlier steps of sexual development, namely G1 arrest and mating, with its loss causing a partial deficiency in these stages (Watanabe and Yamamoto, 1994) (supplementary material Fig. S1A). We have previously reported a physical interaction between Mei2 and Mip1 (Shinozaki-Yabana et al., 2000), which later turned out to be the fission yeast ortholog of mammalian Raptor, an essential component of mTORC1 (Hara et al., 2002; Kim et al., 2002; Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007). A physical interaction has been also found between Mei2 and Tor2 (Alvarez and Moreno, 2006). These observations suggest an intimate relationship between Mei2 and TORC1. In this study, we demonstrate that Mei2 is a novel target of TORC1.

RESULTS

Mei2 is stabilized in mutants of TORC1

We observed that cells of the temperature-sensitive *tor2* mutant (*tor2-ts6*), which are de-repressed for sexual development at the restrictive temperature (Matsuo et al., 2007), tended to accumulate Mei2 protein at a high temperature (Fig. 1A). To examine its stability, we tagged Mei2 with three copies of the hemagglutinin epitope (Mei2–3HA) and expressed it under the control of the thiamine-repressible promoter in wild-type and *tor2-ts6* cells. In wild-type cells, Mei2 disappeared rapidly following the addition of thiamine and cycloheximide to repress transcription and translation (Fig. 1B), as was reported previously (Kitamura et al., 2001). By contrast, Mei2 was retained more stably in *tor2-ts6* cells (Fig. 1B). Mei2 protein was also accumulated in the temperature-sensitive *mip1* mutant (Fig. 1C). These results suggested that active Tor2 (TORC1) could destabilize Mei2.

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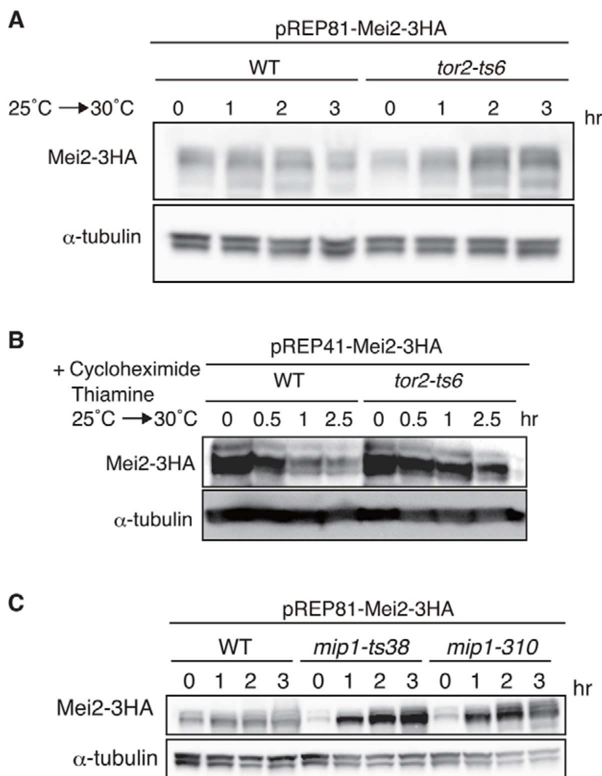

Fig. 1. Mei2 is destabilized by TORC1. (A) Wild-type (WT) or *tor2-ts6* cells carrying pREP81-*mei2-3HA* were grown in minimal medium at 25˚C, shifted to 30˚C, and sampled at 1-h intervals. The levels of Mei2–3HA were detected by immunoblotting with an anti-HA antibody. α-tubulin is shown as a loading control. (B) Half-lives of Mei2 in the wild-type (JZ127) and *tor2-ts6* (JV789) strains. Cells expressing HA-tagged Mei2 from the pREP41 vector were shifted from 25˚C to 30˚C, and thiamine and cycloheximide were added to the culture. Cells were sampled at the indicated intervals and Mei2–3HA was detected by immunoblotting with an anti-HA antibody. (C) Cells of wild-type (JT984) and two temperature-sensitive *mip1* strains, *mip1-ts38* (JT985) and *mip1-310* (JT986), which carried pREP81-*mei2-3HA*, were grown in minimal medium at 25˚C, shifted to 36˚C, and sampled at 1-h intervals. The levels of Mei2–3HA were detected by immunoblotting with an anti-HA antibody.

Genetic interactions between Mei2 and Tor2

We next analyzed genetic interactions between *tor2* and *mei2*. Although the *tor2-ts6* mutant underwent extensive mating under nutrient-rich conditions at the restrictive temperature (Matsuo et al., 2007) (Fig. 2A), this mating was much reduced when combined with deletion of *mei2* (Fig. 2A). By contrast, the sterility of an activated *tor2* mutant, *tor2-s65*, which was isolated in our screen for sterile *tor2* mutants (Urano et al., 2007), was suppressed by the overexpression of wild-type *mei2* (Fig. 2B). This suppression was also observed with another activated *tor2* mutant, *tor2-s69* (supplementary material Fig. S1B). Furthermore, overexpression of *mei2* induced mating more efficiently in the *tor2-ts6* mutant than in the wild-type strain (Fig. 2C). These results suggest that Tor2 and Mei2 might interact and modulate the function of Mei2 to promote mating.

TORC1 phosphorylates Mei2 *in vitro*

To investigate whether Tor2 could directly phosphorylate Mei2, we performed an *in vitro* Tor2 phosphorylation assay. FLAG-tagged Tor2 was immunoprecipitated from fission yeast cell extracts using an anti-FLAG antibody. We used the same immunoprecipitation conditions as described in our previous study, in which other components of TORC1 were co-precipitated (Matsuo et al., 2007). The kinase preparation was then mixed with a GST–Mei2 fusion protein, which harbors Mei2 residues 30–750 and was shown previously to be functional (Watanabe et al., 1997), and incubated with radioactive ATP. Tor2 phosphorylated GST–Mei2 efficiently in this system, whereas a kinase-dead form of Tor2 (Tor2KD), used as a negative control, displayed only marginal phosphorylation toward GST–Mei2 (Fig. 3A).

Multiple Ser/Thr sites of Mei2 are phosphorylated by TORC1 *in vitro*

To identify the amino acid residues of Mei2 that are phosphorylated by Tor2, we carried out mass spectrometric analysis of recombinant GST–Mei2 that had been subjected to *in vitro* phosphorylation. We identified nine phosphorylated residues in this analysis – S34, S35, S39, S43, T44, T231, T339, S343 and S694 (Fig. 3B; supplementary material Fig. S2A). Given these results, we constructed a plasmid that expressed GST–Mei2-9A, in which all of the nine phosphorylation sites were replaced with alanine. GST–Mei2-9A, which carries Mei2 residues 1–750, was barely phosphorylated by Tor2 *in vitro*, indicating that Tor2 principally phosphorylates the nine identified Ser/Thr residues (Fig. 3C). It has been shown previously that Pat1 kinase phosphorylates Mei2 on S438 and T527 during the mitotic cell cycle (Watanabe et al., 1997). The above results indicated that these residues are not preferentially phosphorylated by Tor2. Consistently, Tor2 phosphorylated GST–Mei2-SATA, in which the two sites phosphorylated by Pat1 were replaced with alanine, as efficiently as it phosphorylated wild-type Mei2 (Fig. 3D; supplementary material Fig. S2B).

TORC1-dependent *in vivo* phosphorylation of Mei2

As expected, Mei2 protein recovered from growing cells was phosphorylated, showing a mobility shift after phosphatase treatment (Fig. 4A). We next examined whether TORC1 participated in this *in vivo* phosphorylation of Mei2. It was difficult, however, to detect an obvious mobility shift between the Mei2 protein derived from wild-type cells and that derived from *tor2-ts6* cells, when they were extracted under the native conditions as in Fig. 1A, partly owing to their instability. Therefore, we extracted Mei2 protein under denaturing conditions (see Materials and Methods). We also tried to load the protein samples in equal amounts for ease of comparison. Consequently, the Mei2 protein recovered from *tor2-ts6* cells revealed faster migration than that recovered from wild-type cells, even at low temperature (Fig. 4B). Mei2-9A recovered from wild-type cells also migrated faster, indicating that the nine sites are likely to be phosphorylated *in vivo*. Unexpectedly, however, the mobility of Mei2 protein from *tor2-ts6* cells slowed after a longer incubation at 30℃ (3 h). This might be due to phosphorylation of Mei2 by some kinase(s) activated under starved conditions, as a reduction in Tor2 activity mimics nitrogen starvation (Alvarez and Moreno, 2006; Uritani et al., 2006; Matsuo et al., 2007; Weisman et al., 2007). In support of this idea, phosphatase treatment of Mei2-9A-SATA recovered from growing cells indicated that it was still phosphorylated, suggesting the presence of a kinase(s) other than Tor2 and Pat1 that could phosphorylate Mei2 (supplementary material Fig. S2C). We also examined Mei2N, carrying only residues 1–428, which contains eight of the nine Tor2 phosphorylation sites but lacks the Pat1 sites. A similar

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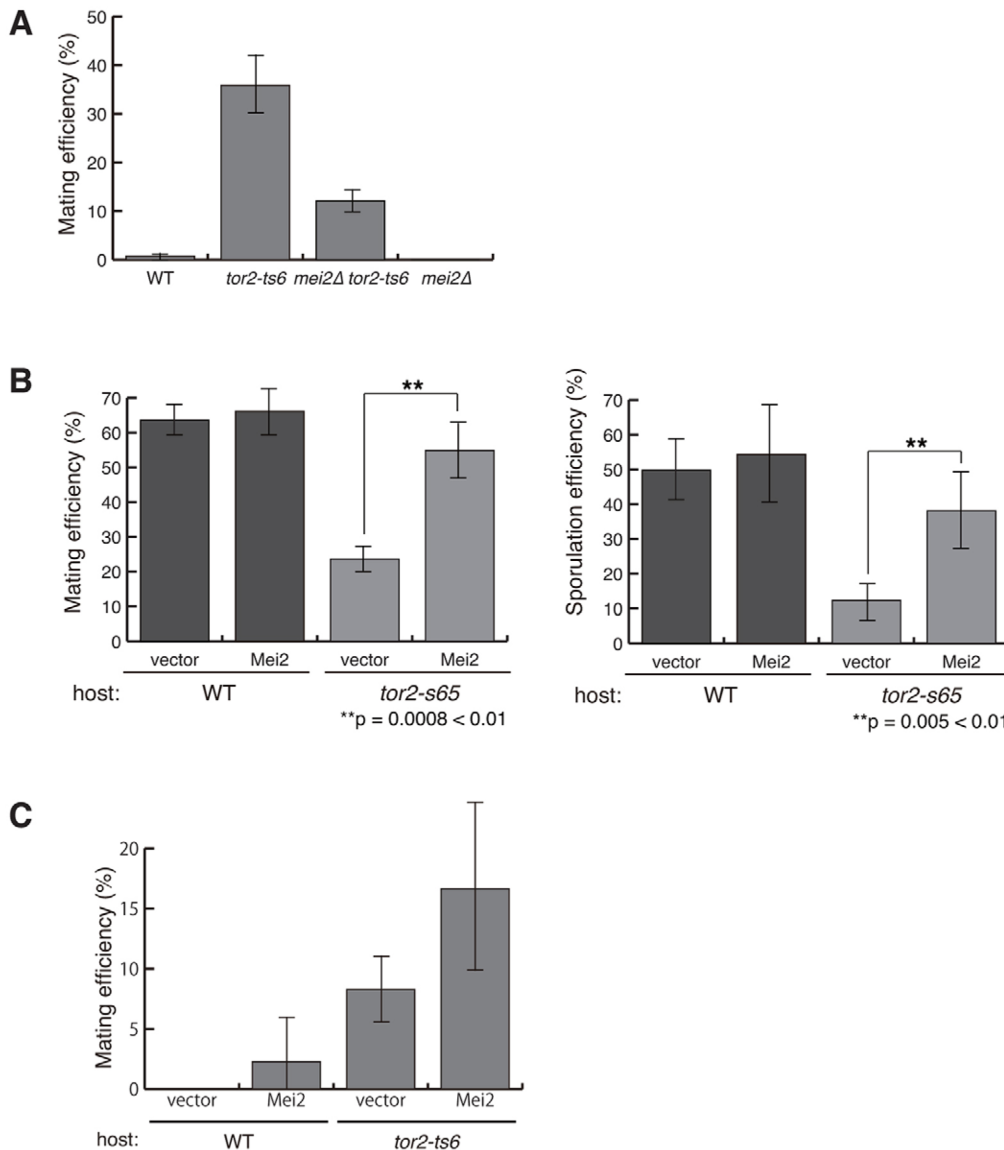

Fig. 2. Genetic interaction between *tor2* and *mei2*. (A) Comparison of mating efficiencies in nutrient-rich medium. Cells of the wild-type (WT, JY476), *tor2-ts6* (JV302), *tor2-ts6 mei2Δ* (JV789) and *mei2Δ* (JZ417) strains were grown in liquid YE medium at 25˚C to exponential phase and were then cultured at 30˚C for 24 h. The mating efficiency of each strain was scored under the microscope. For each measurement, >300 cells were examined. (B) Overexpression of Mei2 rescues the mating and sporulation deficiency of the *tor2*-activated mutant. Homothallic haploid cells, either wild type (JY878) or *tor2* activated (*tor2-s65*; JT163), were transformed with the plasmids pREP41 or pREP41-*mei2*. Transformants were grown on SSA medium at 30˚C for 3 days. Their mating efficiency (left) and sporulation efficiency (right) were scored under the microscope. Sporulation efficiency was calculated by using the following equation: (2×zygotic asci)/(2×zygotes+unmated cells). For each measurement, >400 cells were examined. (C) Overexpression of Mei2 enhances ectopic sexual development in the *tor2-ts6* mutant strain. Homothallic *tor2-ts6* cells (JV789) and control wild-type cells (JZ127) were transformed with the plasmids pREP1 or pREP1-*mei2*. Transformants were cultured in MM liquid medium at 25˚C to exponential phase, shifted to 30˚C and incubated for 24 h. The mating efficiency of each strain was scored under the microscope. For each measurement, >400 cells were examined. Data show the mean±s.d.; $**P<0.01$ (Welch's *t*-test).

mobility shift was observed with Mei2N prepared from *tor2-ts6* cells, although the shift was smaller compared with that of full-length Mei2 (supplementary material Fig. S2D). These results indicate that Mei2 is phosphorylated *in vivo* at least in a TORC1-dependent manner, if not directly by TORC1.

Nonphosphorylatable Mei2 mutants efficiently induce sexual development

We constructed a *mei2* mutant strain in which the nine Tor2 phosphorylation sites were replaced with alanine. However, substitution of alanine for S694 was found to result in a sporulation deficiency (supplementary material Fig. S3A). Replacement of S694 with aspartic acid or glutamic acid also compromised the function of Mei2 (data not shown), suggesting that this residue should be serine to allow Mei2 to function properly. Hence, we decided to characterize the phenotype of a *mei2-8A* mutant, in which all the phosphorylation sites except S694 were replaced with alanine. The *mei2-8A* strain initiated sexual development more frequently than its wild-type counterpart under nutrient-rich conditions, in a similar fashion to the *tor2-ts* mutants at high temperature (Fig. 4C) (Matsuo et al., 2007). A series of alanine substitution mutants altered at one or more of the eight Tor2 phosphorylation sites (S694 was excluded from this analysis) revealed an increase in the mating and sporulation frequency to nearly the same degree (supplementary material Fig. S3B), suggesting that all of the Tor2 target sites must be phosphorylated to repress sexual development effectively. Furthermore, the overexpression of the *mei2-8A* allele could suppress the mating deficiency in the *tor2* active mutant more efficiently than the overexpression of wild-type *mei2* (supplementary material Fig. S1B). These results indicate that Tor2, and hence TORC1, contributes to the suppression of sexual development during vegetative growth through the phosphorylation of Mei2.

Alanine substitutions at the phosphorylation sites stabilize Mei2

To examine the effect of the Tor2-dependent phosphorylation of Mei2, we analyzed the stability of Mei2-9A in wild-type cells, as was performed in Fig. 1B. The mutant Mei2 protein was more stable than the wild-type (Fig. 5A). Mei2-8A was also more

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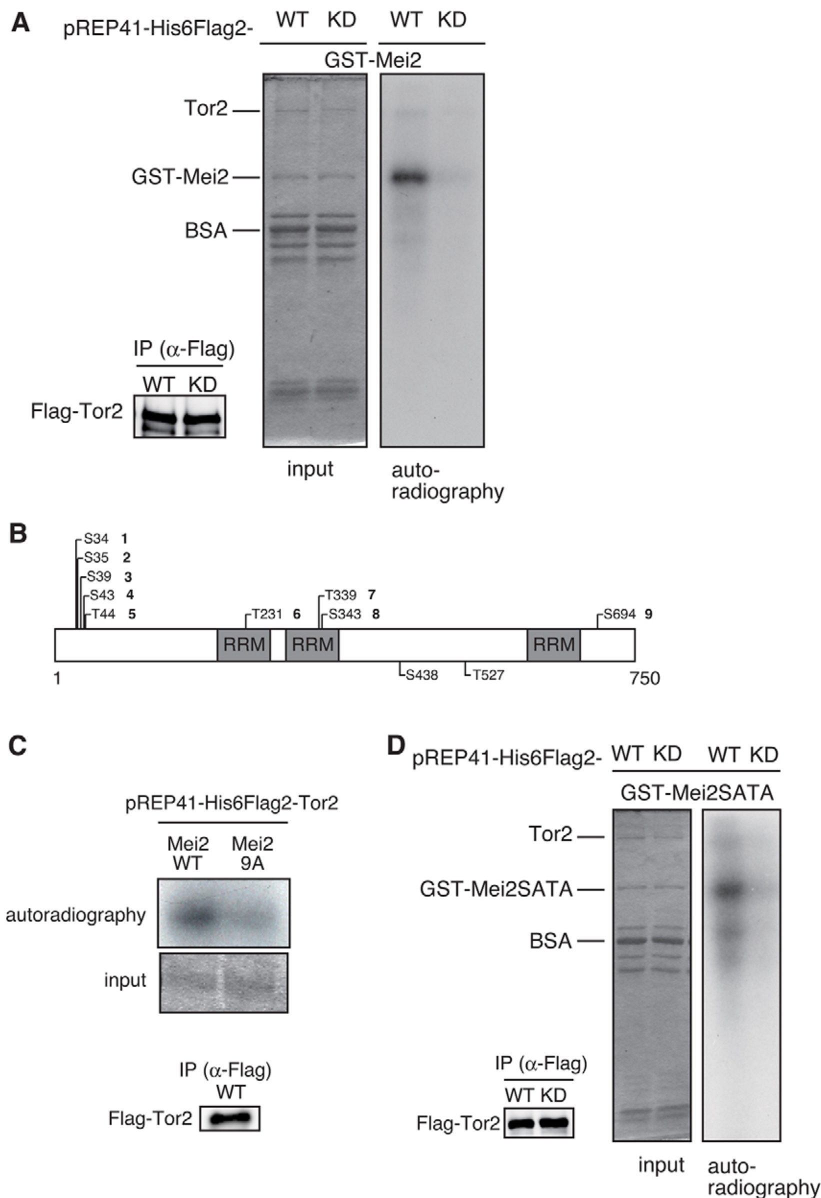

Fig. 3. Mei2 is phosphorylated by TORC1 *in vitro*. (A) *In vitro* phosphorylation of Mei2 by Tor2. Flag-tagged wild-type Tor2 (WT) or kinase-dead Tor2 (KD), immuno-purified (IP) with an anti-FLAG antibody (left panel), was incubated with GST–Mei2 in the presence of [γ-^{32}P]-ATP and separated by gel electrophoresis. The gel was then dried and autoradiographed (right panel). Coomassie Brilliant Blue staining of the gel is also shown (middle panel). (B) Phosphorylation sites on Mei2 for Tor2 (nine upper residues) and Pat1 (S438 and T527). Gray boxes indicate RNA-recognition motifs (RRMs). (C) *In vitro* phosphorylation of Mei2-9A was performed as in A, except that full-length Mei2 (1–750) was used instead of Mei2 (30–750). (D) *In vitro* phosphorylation of Mei2-SATA, examined as described for A.

stable (supplementary material Fig. S3C,D). It has been shown that Mei2 protein phosphorylated by Pat1 undergoes degradation through the ubiquitin-proteasome pathway, involving Ubc2 and Ubr1 (Kitamura et al., 2001). We confirmed that wild-type Mei2 protein in growing cells, presumably phosphorylated by both Pat1 and Tor2 kinases, became more stable in the *mts2* mutant, which is defective in the 19S proteasome (supplementary material Fig. S4A). This observation implies that the phosphorylation of Mei2 by Tor2 accelerates its degradation through the previously identified ubiquitin-proteasome pathway (Kitamura et al., 2001). Indeed, polyubiquitylation of Mei2 was decreased greatly by the *mei2-9A* mutation (Fig. 5B). Mei2-8A was also less polyubiquitylated than wild-type Mei2 (supplementary material Fig. S4B). Furthermore, *mts2* mutant cells initiated sexual development readily under nutrient-rich conditions (supplementary material Fig. S4C). These observations suggest that the proteasomal degradation of Mei2 is important for the repression of sexual development. Although Mei2-SATA, in which the Pat1 phosphorylation sites are replaced with alanine, was shown previously to be more stable than wild-type Mei2 in *tor2*$^{+}$ cells (Kitamura et al., 2001) (Fig. 6A), its stability was comparable to that of wild-type Mei2 in the *tor2-ts6* mutant cells at high temperature (Fig. 6B). This might indicate that the phosphorylation of Mei2 by both TORC1 and Pat1 kinase is necessary to induce proteasomal degradation of the protein.

DISCUSSION

This study has shown that TORC1 phosphorylates Mei2 *in vitro* and is also likely to do so *in vivo*. Alanine substitution at the identified phosphorylation sites stabilized Mei2 protein and enhanced sexual differentiation under nutrient-rich conditions. The TORC1-dependent phosphorylation of Mei2, in conjunction with the phosphorylation mediated by Pat1 kinase, accelerated its degradation through the ubiquitin-proteasome pathway. These observations led us to propose that TORC1 participates in the repression of sexual differentiation during vegetative growth through phosphorylation of Mei2. However, because the enhanced mating of *tor2-ts* cells on nutrient-rich medium was not completely blocked by the *mei2* deletion (Fig. 2A), it is likely

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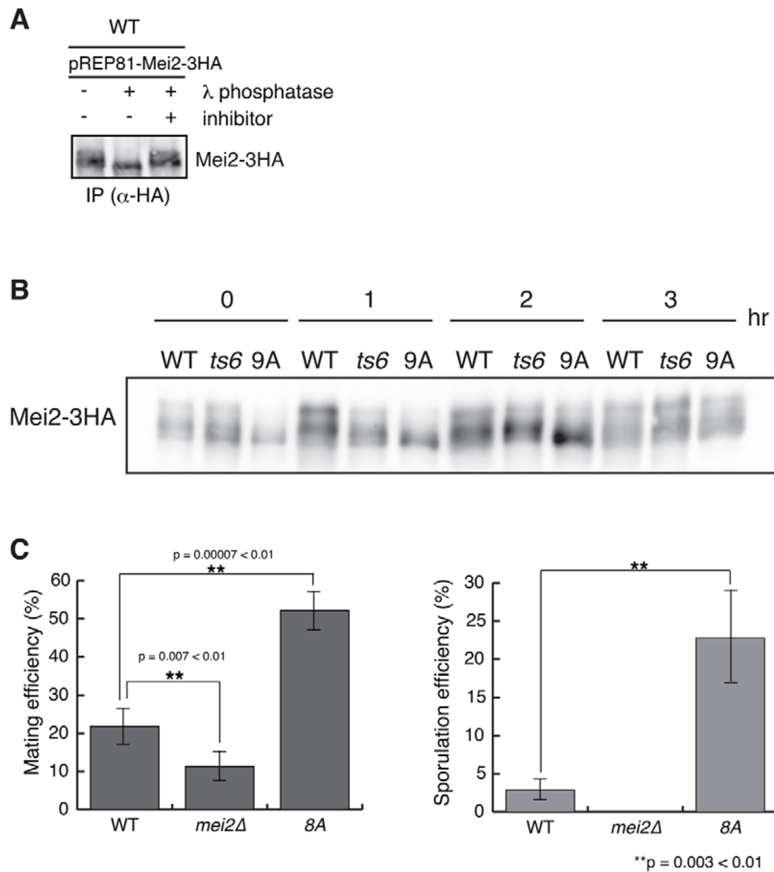

Fig. 4. Phosphorylation of Mei2 is regulated by TORC1 *in vivo* and alanine substitutions at the phosphorylation sites promote sexual development. (A) Mei2 is phosphorylated *in vivo*. Immunoprecipitated HA-tagged Mei2 in the wild-type (WT) strain (JY839) carrying pREP81-*mei2-3HA* was treated with λ-phosphatase in the presence or absence of its inhibitor.
(B) Comparison of the mobility of Mei2 protein recovered from the wild-type (JZ127) and *tor2-ts6* (JV789) strains. Mei2-9A was also recovered from the wild-type (JZ127) strain. Cells expressing HA-tagged Mei2 or Mei2-9A from the pREP81 vector were sampled at the indicated intervals after the shift from 25˚C to 30˚C. At each time-point, equal amounts of Mei2 protein were loaded. (C) Comparison of the mating and sporulation efficiencies of the wild-type (JY450), *mei2Δ* (JZ127) and *mei2-8A* (JT921) strains. Cells of each strain were incubated on minimal medium at 30˚C for two days, and the mating efficiency (left) and the sporulation efficiency (right) were scored under the microscope. The sporulation efficiency was calculated as in Fig. 2B. In each case, >400 cells were examined. Data show the mean±s.d.; $^{**}P<0.01$ (Welch's *t*-test).

that TORC1 has some other target(s), phosphorylation of which would also contribute to the repression of untimely mating during vegetative growth.

We found that nine residues of Mei2 were phosphorylated by TORC1. Alanine substitution at one target site or at a combination of sites resulted in de-repression of sexual development, although alanine substitution at the eight sites excluding S694, which was indispensable for Mei2 function, was most effective. These observations suggest that TORC1 must maintain a high activity to phosphorylate all of the target sites on Mei2 during vegetative growth, and presumably each Mei2 target site is a good substrate for TORC1. It will be intriguing to investigate whether the latter is indeed the case, because it has been suggested that the quality of a phosphorylation site as a TORC1 substrate can be a key determinant of how downstream effectors respond in the signaling pathway (Kang et al., 2013).

Mei2 has been characterized in considerable detail as the master regulator of meiosis in fission yeast (Harigaya et al., 2006; Harigaya and Yamamoto, 2007; Yamamoto, 2010). It remains puzzling, however, how Mei2 participates in the earlier steps of sexual differentiation, i.e. G1 arrest and mating, even though it is not absolutely necessary for these steps. One possible explanation

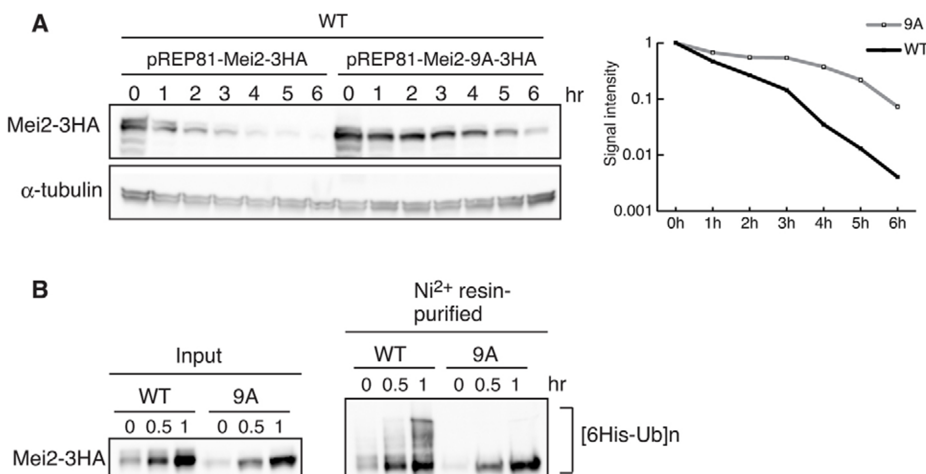

Fig. 5. Tor2 negatively regulates sexual development through the ubiquitin-proteasomal degradation of Mei2. (A) Stability of Mei2-9A. The half-life of Mei2-9A was examined as described in Fig. 1B, together with that of wild-type (WT) Mei2, for comparison. Quantitative calibration of the protein levels is plotted on a single logarithmic scale (right). (B) Mei2-9A was less polyubiquitylated than wild-type Mei2. 6His-ubiquitin was expressed in *mei2Δ mts2* cells carrying a pREP81-derived plasmid that expressed Mei2–3HA or Mei2-9A–3HA. Cells were sampled at the indicated intervals after shifting from 25˚C to 36˚C, ubiquitylated proteins were purified with Ni^{2+}–NTA beads and immunoblotted with an anti-HA antibody.

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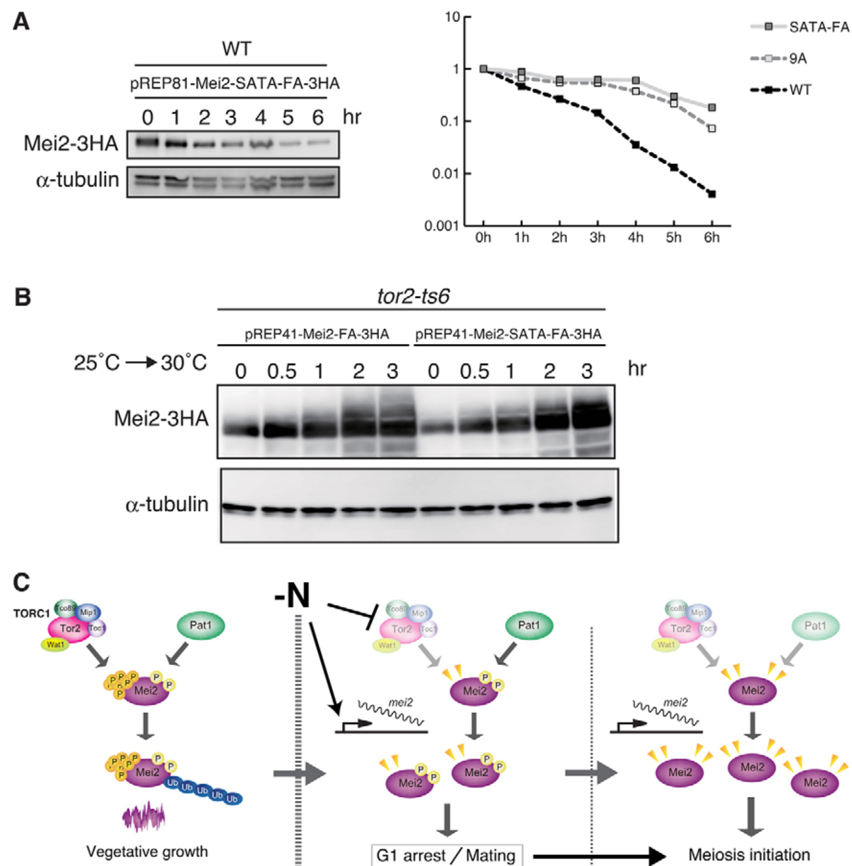

Fig. 6. Stepwise activation of Mei2 through inactivation of Tor2 and Pat1. (A) Stability of Mei2-SATA. The half-life of Mei2-SATA-FA protein was examined as described in Fig. 1B. Quantitative calibration of the protein levels is plotted on a single logarithmic scale (right). For comparison, wild-type (WT) Mei2 and Mei2-9A shown in Fig. 5A are re-plotted. (B) *tor2-ts6* cells carrying pREP41-mei2-FA or pREP41-mei2-SATA-FA were grown in minimal medium at 25˚C and shifted to 30˚C. Mei2–3HA was then assayed by immunoblotting. (C) A provisional model for the role of Mei2 in switching the cells from vegetative growth to mating and meiosis. We propose that Mei2 proteins that experience reduced phosphorylation by TORC1 under conditions of nitrogen starvation (−N) but that are still phosphorylated by Pat1 kinase can stimulate the mating process. Mei2 that is no longer phosphorylated by Pat1 promotes meiosis without mating, as we have demonstrated previously. P, phosphorylation; Ub, ubiquitylation.

has emerged from our recent study; activated Mei2 was found to enhance the expression of *ste11* through a positive-feedback loop involving the stress-responsive MAP kinase pathway (Sukegawa et al., 2011). Ste11 is a major transcription factor controlling the switch from cellular proliferation to sexual differentiation (Sugimoto et al., 1991; Otsubo and Yamamoto, 2012). Thus, lack of the full expression of *ste11* in the absence of Mei2 function might result in lower mating efficiency.

Nutrient starvation induces sexual development in fission yeast cells. Both TORC1 and Pat1 kinase phosphorylate Mei2 and cooperate to destabilize it under conditions of rich nutrition. However, the phenotypes of the mutants defective in the respective kinases are largely different. Cells defective in *tor2*, encoding the catalytic subunit of TORC1, initiate the whole program of sexual development, including mating, whereas cells defective in *pat1* skip mating and start meiosis directly (Iino and Yamamoto, 1985; Nurse, 1985; Matsuo et al., 2007). Considering all of the above observations, we propose the following stepwise activation of Mei2 as a plausible model (Fig. 6C). During vegetative growth, both TORC1 and Pat1 phosphorylate Mei2 to destabilize and inactivate it. Upon nitrogen starvation, TORC1 is downregulated and Mei2 becomes stable. Partially dephosphorylated Mei2 that accumulates at this stage might promote mating. Enhanced production of Mei2 under nitrogen starvation might also aid the accumulation of dephosphorylated Mei2 protein (Watanabe et al., 1988; Sugimoto et al., 1991). Following mating, Pat1 kinase is inactivated in zygotes (McLeod and Beach, 1988; Li and McLeod, 1996), and fully dephosphorylated Mei2 acquires the capacity to elicit meiosis. Consistent with this model, ectopic expression of Mei2-8A-SATA resulted in the induction of meiosis without mating (data not shown).

Although Mei2 appears to be an unusual substrate of TORC1, as it regulates specific switching rather than general translation or cell metabolism, there is a report that *Arabidopsis* AtRaptor1B, a Raptor homolog, binds to a Mei2-like protein, AML1 (Anderson and Hanson, 2005). Hence, it will be intriguing to determine whether AML1 is a substrate of *Arabidopsis* TORC1. Furthermore, it will also be of interest to determine whether this phosphorylation event has any role in linking environmental conditions to cellular development or in promoting ubiquitin-proteasomal degradation.

MATERIALS AND METHODS

Yeast strains, media, plasmids, genetic methods and mutants

The *Schizosaccharomyces pombe* strains used in this study are listed in supplementary material Table S1. Yeast media YE, SD, MM and SSA were used for routine culture of *S. pombe* strains (Gutz et al., 1974; Moreno et al., 1991). Vectors carrying the thiamine-repressible *nmt1* promoter or its derivatives have been described previously (Maundrell, 1990; Basi et al., 1993). General genetic procedures for *S. pombe* have also been described previously (Gutz et al., 1974). Transformants of *S. pombe* were generated using a lithium acetate method (Okazaki et al., 1990). Alanine substitution alleles of *mei2* were created using the PrimeSTAR Mutagenesis Basal Kit (TaKaRa). Cells of JZ127 (h^{90} *mei2::ura4*$^{+}$) were transformed with linear DNA fragments carrying a mutated *mei2* allele, and transformants were selected on media containing fluoroorotic acid.

Measurement of protein stability

Strains carrying a plasmid expressing Mei2–3HA or its derivative from a weakened thiamine-repressible *nmt1* promoter (pREP41 or 81) were grown in minimal medium MM for 14 h in the absence of thiamine. Thiamine (5 µM) and cycloheximide (100 µg/ml) were then added to

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stop transcription and translation, and cell extracts were prepared at specific intervals and subjected to immunoblotting analysis. When analyzing Mei2-SATA *in vivo*, the protein was given an additional FA mutation (Fig. 6B) to suppress its ability to induce ectopic meiosis, as described previously (Watanabe et al., 1997).

Immunochemistry

Harvested cells were disrupted with glass beads in buffer B [50 mM Tris-HCl pH 7.6, 150 mM KCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol, 0.2% NP-40, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 15 mM p-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Complete Mini EDTA-free; Roche)]. Mouse monoclonal antibodies against tubulin (TAT-1; a gift from Keith Gull, University of Oxford, Oxford, UK), HA (12CA5, Sigma), GFP (Roche) and FLAG (M2 Sigma) were used to detect target proteins. Horseradish-peroxidase-conjugated sheep anti-mouse-IgG (GE Healthcare) and a chemiluminescence system (ECL, GE Healthcare) were used to visualize bound antibodies. For phosphatase treatment, immunoprecipitated Mei2–GFP was incubated with 400 U of λ-phosphatase (BioLabs) at 30℃ for 20 min, either with or without an inhibitor mixture (10 mM EGTA, 10 mM Na_3VO_4, 20 mM β-glycerophosphate, 15 mM p-nitrophenyl phosphate). For the detection of mobility shift of Mei2 *in vivo*, harvested cells were disrupted with glass beads in 20% trichloroacetic acid. The amounts of Mei2 protein were determined by measuring the HA signal intensity.

Detection of Mei2 ubiquitylation

The proteasome-defective *mts2* strain (JT922) was transformed with either pREP81-*mei2-3HA*, pREP81-*mei2-9A-3HA* or pREP81-*mei2-8A-3HA*, together with a plasmid that expresses 6His–ubiquitin from the *nmt1* promoter (pREP2-6His-Ubi). The cells were then cultured at 25℃ in the absence of thiamine and were shifted to 36℃ for 1 h. Cell extracts were prepared, and the ubiquitylated fraction was purified by using Ni^{2+}–NTA beads as described previously (Shiozaki and Russell, 1997). Precipitated proteins were separated by electrophoresis and immunoblotted with an anti-HA antibody to detect Mei2–3HA ubiquitylation.

In vitro kinase assay

The JY450 strain was transformed with either pREP41-*His6Flag2-tor2* or pREP41-*His6Flag2-tor2KD* (kinase dead allele; D2140A). Tor2 or Tor2KD was immunoprecipitated with an anti-FLAG antibody as described previously for the analysis of TORC1 (Matsuo et al., 2007). Precipitates were washed three times with buffer B with no protease inhibitors, and then twice with buffer Ktor (20 mM HEPES-KOH pH 7.5, 1 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 15 mM p-nitrophenyl phosphate, 10 mM $MnCl_2$). Bacterially purified GST-fusion proteins carrying either wild-type Mei2 residues 30–750, which retain normal Mei2 function (Watanabe et al., 1997), full-length Mei2 (1–750) or Mei2 (1–750) with alanine substitutions at TORC1 or Pat1 kinase phosphorylation sites (Mei2-SATA) were prepared as substrates. An *in vitro* kinase assay was carried out as follows: 2 μg (Fig. 3A,D) or 1 μg (Fig. 3C; supplementary material Fig. S2B) of GST–Mei2 was mixed with immunoprecipitated Tor2 or Tor2KD in buffer Ktor supplemented with 2 μg of bovine serum albumin (BSA). The reaction was initiated by adding 5 μCi [γ-^{32}P]-ATP together with 25 μM cold ATP, and terminated by adding SDS-PAGE buffer after a 35-min incubation at 32℃.

Fluorescence microscopy

To visualize Mei2–GFP, strains to be examined were observed under a fluorescence microscope (Axioplan 2; Carl Zeiss), and images were recorded using a chilled CCD camera (Quantix; Photometrics) and the MetaMorph software (Universal Imaging).

Mass spectrometry

GST–Mei2 phosphorylated by Tor2 *in vitro* was prepared as described above and analyzed by mass spectrometry undertaken by Filgen. The data were also analyzed by Filgen using ProteinPilot (AB SCIEX).

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Competing interests

The authors declare no competing interests.

Author contributions

Y.O., A.Y. and H.O. performed the experiments; Y.O., A.Y. and M.Y. designed the experiments and analyzed the results; Y.O., A.Y. and M.Y. wrote the manuscript and all authors read it.

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Supplementary material

Supplementary material available online at
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