Phosphorylation of the TOR ATP binding domain by AGC kinase constitutes a novel mode of TOR inhibition

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TOR (target of rapamycin) signaling coordinates cell growth, metabolism, and cell division through tight control of signaling via two complexes, TORC1 and TORC2. Here, we show that fission yeast TOR kinases and mTOR are phosphorylated on an evolutionarily conserved residue of their ATP-binding domain. The Gad8 kinase (AKT homologue) phosphorylates fission yeast Tor1 at this threonine (T1972) to reduce activity. A T1972A mutation that blocked phosphorylation increased Tor1 activity and stress resistance. Nitrogen starvation of fission yeast inhibited TOR signaling to arrest cell cycle progression in G1 phase and promoted sexual differentiation. Starvation and a Gad8/T1972-dependent decrease in Tor1 (TORC2) activity was essential for efficient cell cycle arrest and differentiation. Experiments in human cell lines recapitulated these yeast observations, as mTOR was phosphorylated on T2173 in an AKT-dependent manner. In addition, a T2173A mutation increased mTOR activity. Thus, TOR kinase activity can be reduced through AGC kinase–controlled phosphorylation to generate physiologically significant changes in TOR signaling.

Introduction

The eukaryotic target of rapamycin (TOR) protein kinases are highly conserved and couple cell growth and division with environmental cues (Wullschleger et al., 2006; Loewith and Hall, 2011). TOR kinases form at least two different complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2), which have distinct cellular functions. TORC1 incorporates Raptor, whereas Raptor is replaced by Rictor in TORC2 (Wullschleger et al., 2006). The molecular mechanisms by which the tight control of TOR signaling in response to environmental cues is exerted are not fully understood. In mammals, mTORC1 promotes cell growth, cell cycle progression, and cell proliferation. mTORC1 is activated by the Rheb GTPase, which, in turn, is inhibited by the TSC1–TSC2 complex (Long et al., 2005; Smith et al., 2005). mTORC1 activity can also be regulated in a variety of TSC1/2-independent ways. In response to changes in amino acid levels, mTORC1 activation is controlled on lysosomes by Rag GTPases (Sancak et al., 2008). mTORC2 controls metabolism, the cytoskeleton, and cell survival after exposure to stress (Sparks and Guertin, 2010). mTORC2 also controls the differentiation of nTreg cells and pre-T cells (Chang et al., 2012; Lee et al., 2012). The control of mTORC2 activity is less well understood, although mTOR autophosphorylation on serine 2481 is predominantly associated with mTORC2 (Copp et al., 2009).

In contrast to mammals, the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have two TOR kinases. In *S. pombe*, somewhat confusingly, Tor2 is the main component of TORC1, whereas Tor1 is the main component of TORC2 (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007). Like its mammalian counterpart mTORC2, *S. pombe* TORC2 is required for cytoskeletal organization, cell differentiation, and survival after stress (Tatebe and Shiozaki, 2010). Because fission yeast TORC2 phosphorylates the AGC kinase Gad8, and Gad8 is also required for survival after stress and for cell differentiation (Matsuo et al., 2003), Gad8 has been proposed to represent a homologue of mTORC2-controlled AKT1. In summary, conservation of mTORC1 and mTORC2 functions along with the regulation of TORC1 by Rheb and TSC1/2

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Abbreviation used in this paper: TOR, target of rapamycin.

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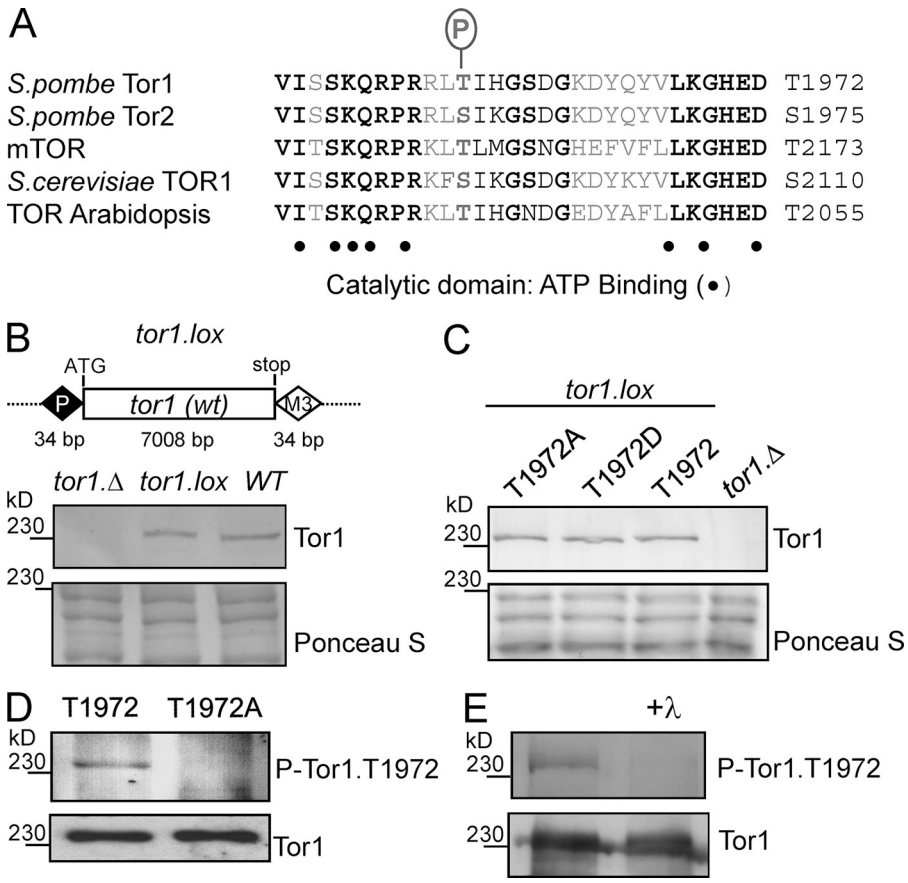


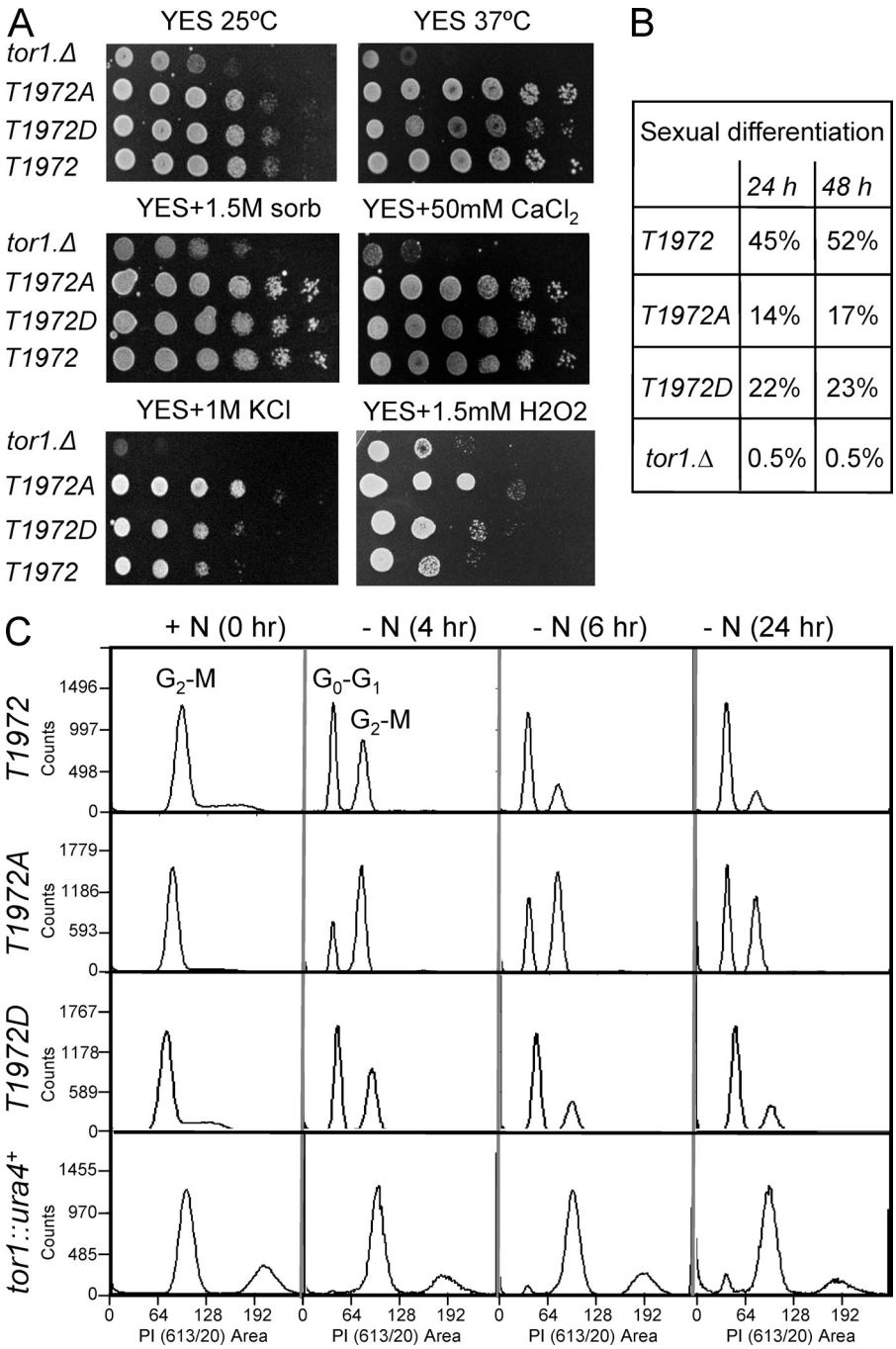
Figure 1. **Tor1.T1972 is phosphorylated.** (A) Tor1.T1972 is evolutionarily conserved. (B) Schematic of the endogenous *tor1* locus in the *tor1.lox* allele and protein levels. (C) Tor1 protein levels in *T1972A* and *T1972D tor1.lox* mutants. (D and E) P-Tor1.T1972 antibodies are phosphorylation specific. (E) Tor1 was immunoprecipitated and dephosphorylated with lambda phosphatase.

(Matsumoto et al., 2002; van Slegtenhorst et al., 2004; Uritani et al., 2006; Urano et al., 2007) to make fission yeast an attractive model organism in which to study TOR signaling.

Results and discussion

To gain further insight into the regulation of TOR kinase activity and signaling, we generated Tor1-specific antibodies with which we could characterize the wild-type molecule (Fig. S1 A). To map phosphorylation sites on Tor1, the kinase was purified from wild-type cells by immunoprecipitation followed by tandem mass spectrometry. This identified a novel and conserved phosphorylation site threonine 1972 within the ATP binding site of the kinase domain (Fig. 1 A and Fig. S1 C). The structure of mTOR has recently been solved (Yang et al., 2013). T2173, the threonine in mTOR that equates to T1972 of fission yeast, is found next to the ATP binding L2185 (Fig. S1 D). To assess the significance of phosphorylation on *S. pombe* Tor1 T1972, we mutated the threonine to either alanine to block signaling or aspartic acid in an attempt to mimic constitutive phosphorylation. We used a *cre* recombinase–mediated cassette exchange (RMCE; Watson et al., 2008) approach to replace the native kinase with each mutant (Fig. 1 B). The RMCE *tor1*$^{+}$ wild-type strain, in which the sequence of Tor1 remains unchanged (*tor1.lox*), has Tor1 protein levels and phenotypes that are indistinguishable from wild-type controls (Fig. 1 B and Fig. S2, A–D). Tor1 protein levels were also indistinguishable from wild type in *tor1.T1972A* and *tor1.T1972D* (Fig. 1 C). To study the dynamics of T1972 phosphorylation, we generated antibodies that recognized T1972-phosphorylated Tor1. Protein extracts from wild-type and *tor1* deleted strains established that these antibodies recognized phosphorylated Tor1 kinase. The phospho-specific signal was abolished in the *tor1.T1972A* mutant or removed upon treatment of Tor1 immunoprecipitates with lambda phosphatase (Fig. 1, D and E).

We next assessed whether blocking Tor1 T1972 phosphorylation altered the TORC2 control of sexual differentiation and cell survival after exposure to stress. *tor1.T1972A* cells displayed increased resistance to both oxidative (H_2O_2) and salt (KCl) stresses (Fig. 2 A). Nitrogen starvation of fission yeast arrests cell cycle progression in G1 phase and induces sexual differentiation, both of which rely on Tor1 (TORC2) activity (Fig. 2, B and C; Weisman and Choder, 2001). *tor1.T1972A* mutants failed to efficiently arrest in G1 (Fig. 2 C) and displayed a reduced differentiation capacity when starved of nitrogen (Fig. 2 B). Tor1 kinase activity is reduced by nitrogen stress (Petersen and Nurse, 2007); however, TORC2 deletion mutants completely fail to differentiate. It is therefore likely that a basal level of TORC2 signaling is required for differentiation but that the general level of TORC2 activity that is associated with proliferating cells has to be depressed to allow efficient cell differentiation. The enhancement of stress resistance and reduced efficiency of sexual differentiation suggest that blocking T1972 phosphorylation elevates Tor1 activity. To test this prediction, we used in vivo and in vitro assays to monitor Tor1 kinase activity. Tor1 (TORC2) mediates phosphorylation of Gad8.S546



Sexual differentiation	24 h	48 h
T1972	45%	52%
T1972A	14%	17%
T1972D	22%	23%
tor1.Δ	0.5%	0.5%

Figure 2. **Tor1.T1972 is required for Tor1 function.** (A) Stress response of *tor1* mutants. (B) Sexual differentiation efficiency assay. (C) *tor1.T1972A* mutants failed to efficiently arrest in G1 when starved for nitrogen. The data shown are from a single representative experiment out of two repeats.

(Fig. S2 E; Matsuo et al., 2003) and so represents an accurate in vivo readout of Tor1 activity. This phosphorylation event is analogous to mTORC2 phosphorylation of AKT1 serine 473 (Oh and Jacinto, 2011). Gad8.S546 phosphorylation was elevated when phosphorylation of T1972 was blocked (Fig. 3 A). This enhancement of S546 phosphorylation was accompanied by an increase in the proportion of Gad8 whose migration through SDS PAGE was delayed (Fig. 3 A). Gad8 is phosphorylated at the conserved activating T-loop residue threonine 387 (AKT1 T308) by the essential Ksg1 kinase (PDK1 homologue; Matsuo et al., 2003). Gad8.T387 phosphorylation is therefore TORC2 independent and can be used as a control to ensure that the elevation of S546 phosphorylation when Tor1.T1972 phosphorylation was blocked was not simply the consequence of an enhancement of Gad8 phosphorylation in general. We therefore generated antibodies that recognized Gad8.T387 phosphorylation (Fig. S2 F). Gad8.T387 phosphorylation was abolished when the *ksg1.208* temperature-sensitive mutant was inactivated at the restricted temperature (37°C; Fig. S2 F), confirming published data that shows Gad8.T387 phosphorylation by Ksg1 (Matsuo et al., 2003). Consistently, Gad8.T387 phosphorylation was unchanged in the Tor1.T1972A mutant (Fig. 3 A). The increased activity of Tor1.1972A in an in vitro kinase assay supports the in vivo Gad8.S546 data and indicates that T1972 phosphorylation must indeed depress TORC2 kinase activity (Fig. 3 B). The small Rab GTPase Ryh1 is essential for

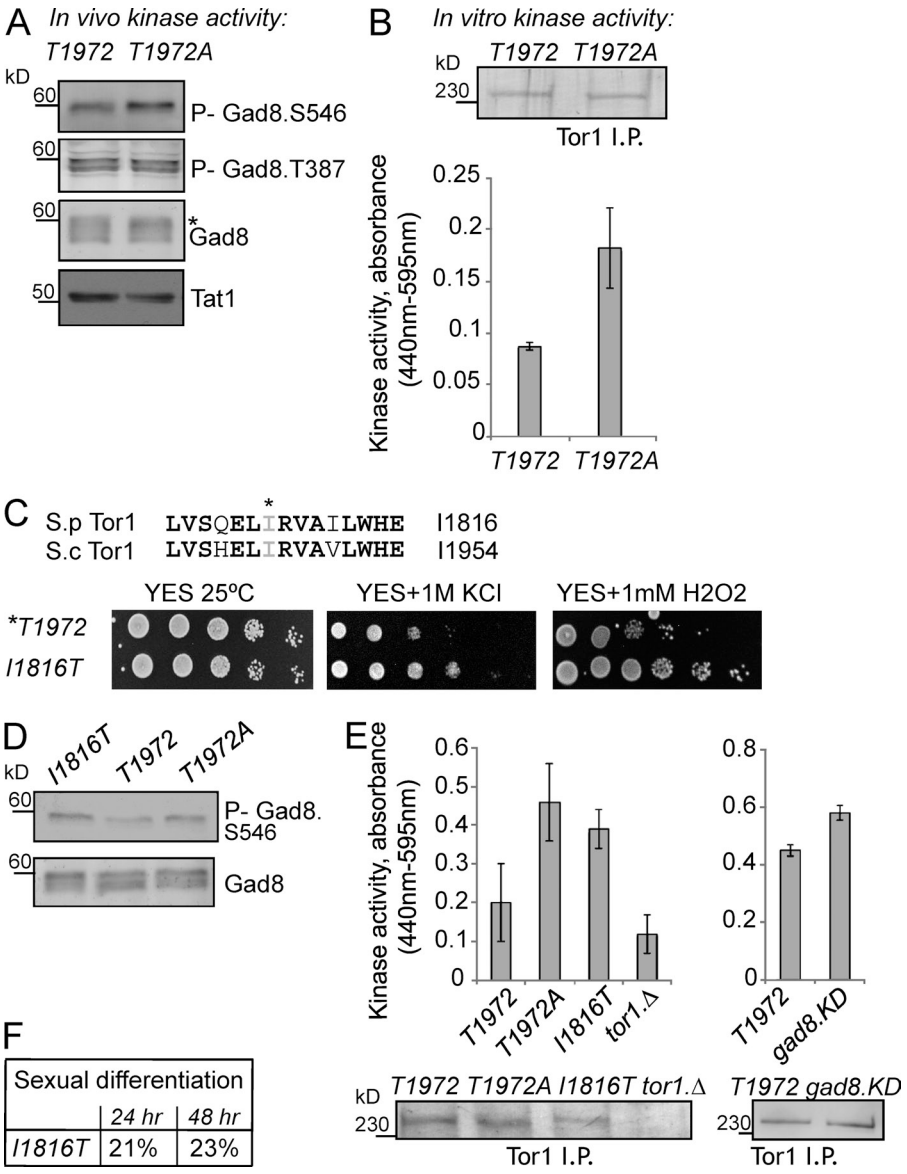


Figure 3. **Lack of Tor1.T1972 phosphorylation increases Tor1 kinase activity.** (A) In vivo Tor1 activities assessed by phosphorylation of the Tor1-specific substrate Gad8.S546. The asterisk indicates slower migrating Gad8 bands. (B) Immunoprecipitated Tor1.T1972A has a higher kinase activity than wild-type Tor1. (C) Sequence alignment of Tor1.I1816 and stress responses (asterisks) 1 M KCl of wild type is also shown in Fig. 2 A. (D–F) Tor1 I1816T has increased Tor1 activity. (D) Levels of Gad8.S546 phosphorylation and total protein levels. (E) Tor1 was immunoprecipitated and kinase activity was measured. (F) Tor1 I1816T has reduced sexual differentiation efficiency. Error bars indicate standard deviation.

full TORC2 activity (Tatebe et al., 2010). TORC2 regulation in Tor1.1972A still required Ryh1, as the depressed level of Tor1 activity in *ryh1.Δ* was not restored to wild-type levels by the increased activity of Tor1.1972A even when combined with deletion of the Ryh1 regulators *sat1*$^+$ and *sat4*$^+$ (Fig. S3 A).

To consolidate the link between the elevation of kinase activity and both the stress resistance and reduced differentiation of *tor1.T1972A* cells, we sought ways to independently increase Tor1 activity. This would allow us to separately test the impact of elevated Tor1 activity upon stress resistance and differentiation. It has previously been established that the *S. cerevisiae* Tor1.I1954T mutant has increased kinase activity (Reinke et al., 2006). Sequence alignments reveal that I1954 is conserved in fission yeast Tor1 (Fig. 3 C). We therefore generated the analogous *tor1.I1816T* mutation and assessed its kinase activity and stress/differentiation phenotypes. Both the in vivo and in vitro assays of Tor1 kinase activity revealed that *tor1.I1816T* mutant cells had increased activity. Importantly, this mutation also enhanced stress resistance and reduced the efficiency of sexual differentiation (Fig. 3, C–F). When we combined the *tor1.I1816T* and *tor1.T1972A* mutations in the same gene, the activity of the double mutant was that of the Tor1.I1816T mutant (Fig. S3, B and C), which suggests that the Tor1.I1816T mutant may already have fully phosphorylated and activated Gad8 kinase. In summary, these two independent mutations at two distinct sites independently elevate kinase activity to enhance stress resistance and reduce sexual cell differentiation. We therefore conclude that phosphorylation of T1972 of Tor1 depresses the kinase activity of the TORC2 complex.

The sequence that immediately surrounds T1972 is a perfect match for an AGC kinase consensus phosphorylation site (Fig. 4 A; Pearson and Kemp, 1991), which suggests that Gad8 could be the kinase responsible for regulating TORC2 activity via Tor1.T1972 phosphorylation. Comparison of T1972 phosphorylation levels in cell extracts prepared from *gad8.Δ*, *gad8.kd*, or *psk1.Δ* (another AGC kinase) revealed that Tor1.T1972 phosphorylation did indeed require Gad8 activity (Fig. 4 B). We therefore purified Gad8 along with the catalytically inactive Gad8.kd

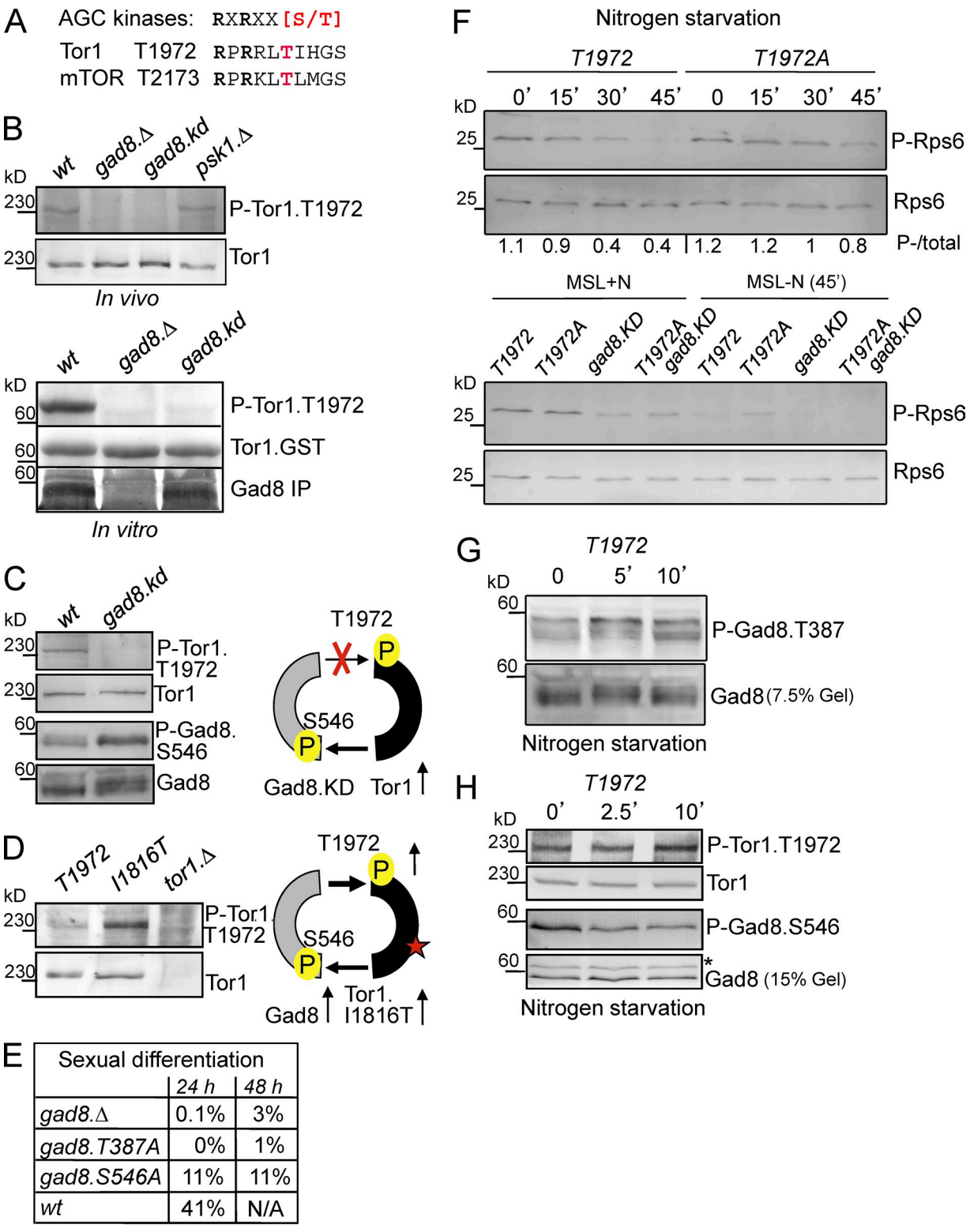


Figure 4. **The AGC kinase Gad8 phosphorylates Tor1.T1972.** (A) Tor1.T1972 resembles an AGC kinase phosphorylation motif. (B) Tor1.T1972 phosphorylation requires Gad8 activity. (top) In vivo assay. (bottom) In vitro kinase assay. (C) Lack of Tor1.T1972 phosphorylation increases Tor1 activity. (C, left) Tor1 activity was determined by P-Gad8.S546 antibodies. (C, right) Schematic; failure to phosphorylate Tor1 on T1972 increases Tor1 activity toward Gad8.S546. (D) Tor1.I1816T, a constitutively active Tor1 mutant, has higher levels of Tor1.T1972 phosphorylation. (D, right) Schematic; constitutively active Tor1 (Tor1.I1816T) activates Gad8 to promote Tor1.T1972 phosphorylation. (E) Sexual differentiation efficiency. (F–H) Nitrogen starvation enhanced Gad8 activity toward T1972 to reduce Tor1 activity. (F) Nitrogen starvation–induced inhibition of Tor1 activity is delayed in *tor1.T1972A* mutant. (G and H) Transient increase in Gad8.T387 phosphorylation is followed by an increase in Tor1.T1972 phosphorylation to reduce Gad8.S546 phosphorylation. The asterisk indicates a background band.

mutant from fission yeast and used recombinant Tor1 as a substrate for in vitro kinase assays. Wild-type Gad8 but not the kinase-dead mutant could phosphorylate T1972 of Tor1 in vitro (Fig. 4 B). A prediction of the Gad8 control of Tor1.T1972 phosphorylation is that mutant strains in which Gad8 activity is reduced should have correspondingly increased Tor1 activity. As mentioned earlier, Gad8.S546 phosphorylation is a direct readout of TORC2 (Tor1) activity analogous to mTORC2 phosphorylation of AKT1 serine 473. Furthermore, Gad8 is, in fact, the only direct TORC2 substrate identified in fission yeast to date. Therefore, somewhat confusingly, in *gad8*-deficient mutants, the in vivo Tor1 activity is actually monitored by phosphorylation of Gad8 itself. Thus, to examine the impact of reduced Gad8 catalytic activity (*gad8.kd*) upon Tor1 activation in vivo, we monitored Gad8.S546 phosphorylation. Strikingly, there was a clear increase in Tor1-dependent phosphorylation of serine 546 in the Gad8 kinase-dead mutant, indicating that Gad8 phosphorylation of T1972 does indeed depress Tor1 kinase activity (Fig. 4 C). Consistently, in vitro assays of Tor1 kinase activity also revealed that *gad8.kd* kinase-dead mutant cells had increased activity (Fig. 3 E). To determine whether enhancement of Gad8 activity would increase phosphorylation of Tor1 on T1972 in vivo, we explored the ability of the active *tor1.I1816T* mutation (Fig. 3, C–F) to increase Gad8 activity and in turn Tor1 T1972 phosphorylation. *tor1.I1816T* cells displayed both higher levels of Gad8.S546 phosphorylation (Fig. 3 D) and elevated Tor1 T1972 phosphorylation (Fig. 4 D), supporting the view that enhanced Gad8 activity promoted Tor1. T1972 phosphorylation. In addition, we also found that environmental stimulation of Gad8 activity in wild-type cells also enhanced Tor1.T1972 phosphorylation (see "Conclusions").

To further characterize the environmental control of Tor1 and Gad8 activities, we exposed cells to nitrogen starvation. As discussed earlier, nitrogen starvation arrests cell cycle progression in G1 and induces sexual differentiation. The activities of both Gad8 and Tor1 are required for these processes (Figs. 2 B and 4 E; Weisman and Choder, 2001; Matsuo et al., 2003). When the *tor1.T1972A* strain was starved of nitrogen, cells failed to undergo efficient differentiation (Fig. 2 B). The phosphorylation of the ribosomal protein S6 (Rps6) is mainly controlled by Tor2 (TORC1; Nakashima et al., 2010); however, the activities of both Gad8 and Tor1 are also required for full phosphorylation (Du et al., 2012). As previously observed, wild-type cells starved of nitrogen showed decreased levels of Rps6 phosphorylation (Nakashima et al., 2010). This nitrogen-controlled reduction in Rps6 phosphorylation was delayed in the active *tor1.T1972A* mutant, and this delay in Rps6 dephosphorylation was Gad8 dependent (Fig. 4 F). In summary, a delay in the inhibition of the *tor1.T1972A* kinase activity leads to a failure to efficiently execute differentiation.

As mentioned earlier, the main phosphorylation site that activates Gad8 is the conserved T-loop threonine 387 (AKT1 T308) that is phosphorylated by Ksg1. Consequently, a *gad8. T387A* mutant is inactive (Fig. 4 E and Fig. S3D). To assess the impact of nitrogen starvation upon Gad8 activation, T387 phosphorylation was monitored as cells were starved of nitrogen. A rapid increase in Ksg1-dependent activation of Gad8 (Fig. 4 G) was seen within 5 min of starvation. This increased Gad8 activity enhanced Tor1.T1972 phosphorylation (Fig. 4 H). Monitoring Gad8.S546 phosphorylation as a readout of Tor1 kinase activity confirmed that this increase in phosphorylation of Tor1 on T1972 was accompanied by a decrease in Tor1 activity (Fig. 4 H). Importantly, the ability to regulate Gad8 T387 phosphorylation was essential for the environmentally induced decline in Tor1-controlled phosphorylation of Gad8 on S546, because high S546 phosphorylation levels were maintained in a *gad8.T387D* mutant (Fig. S3 E). A complete loss of Gad8 activity blocked differentiation (Fig. 4 E). Interestingly, however, a decline in Gad8 S546 phosphorylation did not completely compromise Gad8 activity, as revealed by the reduced sexual differentiation and modest heat sensitivity phenotypes of the *gad8.S546A* mutants (Fig. 4 E and Fig. S3 D). This TORC2 control of Gad8 activity is highly reminiscent of studies in mammalian cells where mTORC2 control of AKT1 phosphorylation enhances its kinase activity but is not absolutely essential for activity (Jacinto et al., 2006; Oh and Jacinto, 2011). Phosphorylation of T1972 by Gad8 is unlikely to be the only mechanism by which Tor1 kinase is inhibited in response to nitrogen starvation because *tor1.T1972A* mutant cells still underwent sexual differentiation (albeit with very reduced efficiency; Fig. 2 B). Together, our results suggest that cells respond to nitrogen starvation by an initial activation of Gad8 through Ksg1. In addition to Ksg1 control of Gad8, this initial nitrogen-induced activation of Gad8 could also be regulated through currently uncharacterized phosphorylation sites, as Gad8 appears to be heavily phosphorylated (Fig. S3 F). After Gad8 activity has been elevated, it inhibits Tor1 activity to allow efficient cell differentiation, as summarized in Fig. 5 K. Whether any further substrates join Gad8 in TORC2-dependent regulation to promote or facilitate efficient cell differentiation remains to be established.

Tor1 T1972 is evolutionarily conserved (Fig. 1 A). As a first step to assess whether phosphorylation of this residue represents a universal mode of TOR kinase control, we raised phospho-specific antibodies that would recognize the homologous residue S1975 in Tor2, the other fission yeast TOR kinase. Protein extracts from wild-type and *tor2* temperature-sensitive mutants established that these antibodies did indeed recognize phosphorylated Tor2 kinase (Fig. 5, A and B). In vivo experiments with protein extracts from Gad8 kinase-dead mutants as well as in vitro kinase assays established that Tor2.S1975 phosphorylation joins Tor1.T1972 in also being controlled by Gad8 (Fig. 5, C and D). The role of Gad8 phosphorylation of Tor2 has yet to be determined.

To extend these observations across the species barrier, we exploited the high level of homology surrounding this threonine (Fig.1A) and used the yeast Tor2.S1975 phospho-specific antibodies on the human mTOR kinase. mTOR T2173 was phosphorylated in a manner that correlated with the nutritional status of the cells as phosphorylation was lost after starvation and promoted after serum restimulation (Fig. 5, E and F). Interestingly, like its fission yeast counterpart Gad8, the activity of the mammalian AKT kinase is required for this phosphorylation (Fig. 5 F). We next assessed whether blocking mTOR T2173 phosphorylation also altered mTOR activity. Transfection and expression of mTOR in HeLa cells proved highly problematic (not depicted), prompting us to express mTOR and mTOR.

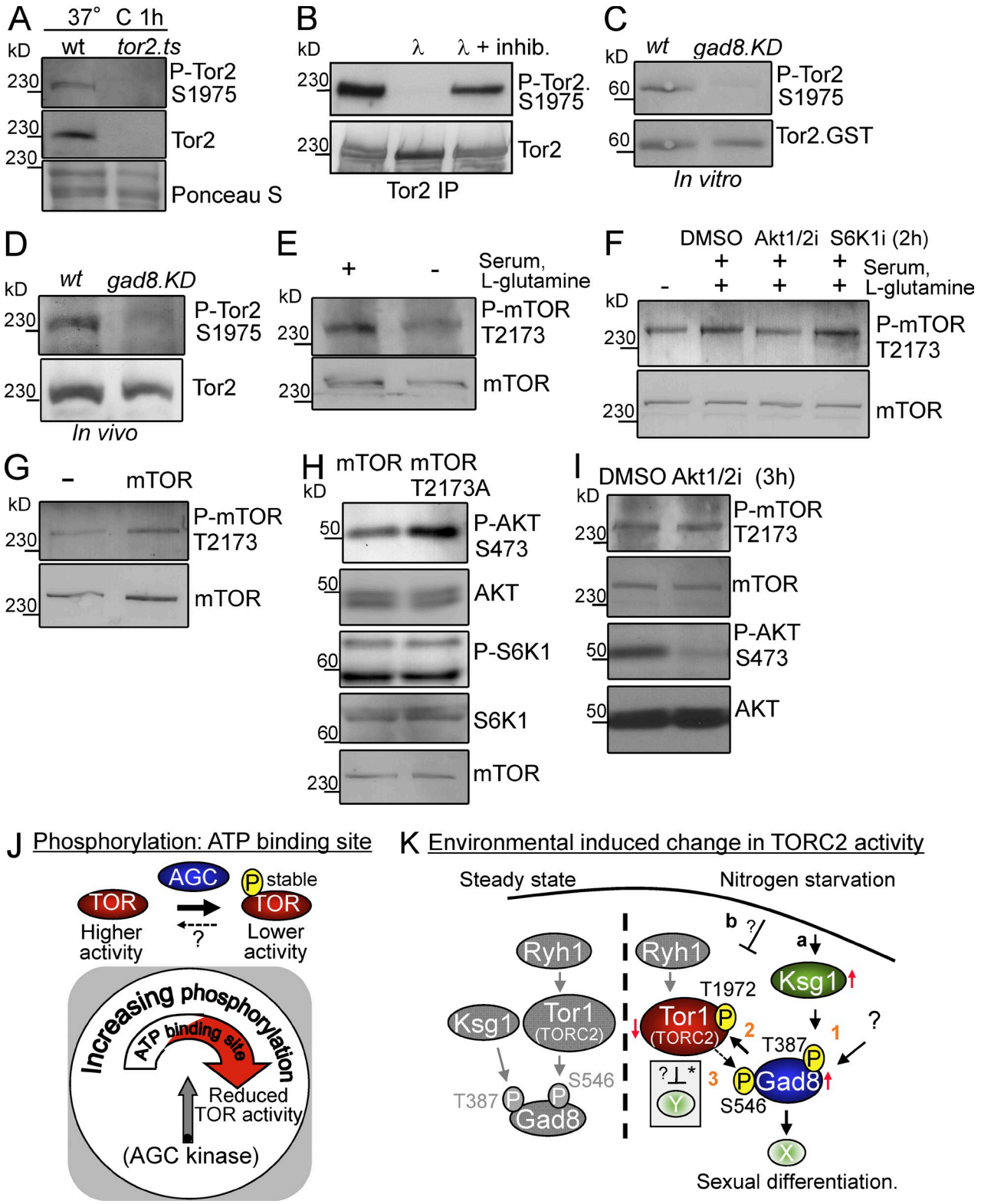


Figure 5. **Tor1 phosphorylation at the ATP binding domain is evolutionarily conserved.** (A) Tor2 is phosphorylated on serine 1975. (B) Specificity of P-Tor2.S1975 antibodies. (C and D) Tor2.T1975 phosphorylation requires Gad8 activity. (C) In vitro kinase assay. (D) In vivo cell extracts. (E–I) mTOR T2173 is phosphorylated. (E and F) Phosphorylation of mTOR T2173 in HeLa cells is reduced after starvation (E) and promoted after serum restimulation (F). mTOR T2173 rephosphorylation was abolished by treatment with the AKT inhibitor, but not the S6K1 inhibitor. (G) Transfection and expression of mTOR in A375 melanoma cells. (H) Blocking mTOR T2173 phosphorylation altered mTOR activity. (I) mTOR T2173 phosphorylation is stable in serum-rich conditions (this level of AKT inhibition abolished rephosphorylation of unphosphorylated molecules; see F). (J) Phosphorylation of the TOR ATP-binding domain by an AGC kinase is stable and reduces TOR activity. (K) See main text for details. After nitrogen starvation, Gad8 is activated (1) and in turn phosphorylates Tor1 on T1972 (2) to reduce Tor1 (TORC2) activity (3) to allow efficient differentiation. Further TORC2 substrates (asterisk) may join Gad8 in facilitating efficient differentiation.

Table 1. **Strains used in this paper**

Strain number	Genotype	Reference
JP3	*h−*	Laboratory stock
JP350	*h+*	Laboratory stock
JP543	*h− psk1:ura4+ leu1.32 ura4.d18*	Bimbó et al., 2005
JP598	*gad8::ura4+ ura4.d18*	gad8::ura4+ from Bimbó et al., 2005
JP624	*h+ tor2.51:ura4+ ura4.d18*	Alvarez and Moreno, 2006
JP1822	*h+ ksg1.208*	Matsuo et al., 2003
JP1293	*h− tor1::loxUra4+ ura4.d18 leu1.32*	This study
JP1294	*h+ nmt1.HA.tor1*	Alvarez and Moreno, 2006
JP1295	*h− pku70::KanMx gad8::ura4+ ura4.d18 leu1.32*	This study
JP1308	*h− tor1::loxUra4+ ura4.d18*	This study
JP1411	*h+ gad8.S546A*	This study
JP1364	*h− tor1.lox*	This study
JP1365	*h+ tor1.lox*	This study
JP1421	*h− gad8.T387A*	This study
JP1511	*h gad8.K259R*	This study
JP1560	*h− tor1.T1972A*	This study
JP1561	*h+ tor1.T1972A*	This study
JP1563	*h+ tor1.I1816T*	This study
JP1570	*h− tor1.T1972D*	This study
JP1571	*h+ tor1.T1972D*	This study
JP2194	*tor1.T1972A gad8.K259R*	This study
JP2205	*h− sat1::kanMX*	This study
JP2207	*h− sat4::kanMX*	This study
JP2208	*h− ryh1::kanMX*	This study
JP2215	*h+ sat1::kanMX tor1.T1972A*	This study
JP2232	*h− sat4::kanMX tor1.T1972A*	This study
JP2233	*h+ ryh1::KanMX tor1.T1972A*	This study
JP2230	*h− gad8::ura4+ ura4.d18 leu1.32 pRep1.gad8*	This study
JP2334	*h+ tor1.I1816T,T1972A*	This study

T2173A in A375 melanoma cells that tolerated a modest level of mTOR overexpression (Fig. 5 G). These experiments in mammalian cells recapitulated our observation in fission yeast. Increased phosphorylation of the mTORC2 substrate AKT1 when mTOR.T2173A was expressed at identical levels to wild-type mTOR indicated that blocking phosphorylation on T2173 enhanced mTOR kinase activity (Fig. 5 H).

Conclusions

We conclude that TOR kinase activity can be inhibited through a universally conserved phosphorylation event within the ATP-binding domain. Interestingly, observations from both fission yeast and human cell lines suggest that this phosphorylation event is very stable. Tor1.T1972 phosphorylation persisted for several hours after Gad8 expression had been turned off (Fig. S3 G). Similarly, there was no change in mTOR.T2173 phosphorylation over a 3-h period after inhibition of AKT1 under serum-rich conditions (Fig. 5 I). Importantly, 2 h of AKT1 inhibition is sufficient to block phosphorylation after serum restimulation (Fig. 5 F). This may suggest that either the phosphatase that dephosphorylates this threonine residue in each organism has a very low activity or that there is no phosphatase for this site. Because AGC kinases are themselves activated by TOR, the stability of this TOR phosphorylation within the ATP binding domain ensures that the mutual regulation of the two kinases is not simply cancelled out (Fig. 5 J).

In summary, phosphorylation of this conserved threonine resembles a rheostat in which AGC kinases feed back and modify TOR activity (Fig. 5 J). Thus, depending on the nature of activation of the individual AGC kinase, we have uncovered a novel and conserved mode by which TOR activity can be downregulated in response to changes in environmental cues.

Materials and methods

Strains and cell cultures

Strains used in this study are listed in Table 1. The *ksg1.208* mutant was provided by M. Yamamoto (University of Tokyo, Tokyo, Japan). The psk1::ura4 and gad8::ura4 mutants were provided by M. Balasubramanian (Mechanobiology Institute, Singapore). The tor2.51 mutant was provided by S. Moreno (University of Salamanca, Salamanca, Spain). The sat1, sat4, and ryh1 delete strains were obtained from Bioneer. Unless otherwise specified, cells were cultured at 28°C in Edinburgh minimal media (EMM2; Fantes and Nurse, 1977) using 20 mM L-Glutamic acid (EMMG) or 5 g/liter NH_4Cl (EMM) as a nitrogen source (EMMG). Cells were grown exponentially for 48 h before being harvested at early exponential phase of 1.5 × 10^6 cells/ml.

To assay sexual differentiation, 4 × 10^6 cells were mixed with equal numbers of cells of the opposite mating types and the mixture spotted onto sporulating agar (SPA) before incubation at 30°C. Mating efficiency was determined at the indicated time by 2 × zygotes/[cells + (2 × zygotes)]. Nitrogen starvation was applied as follows. Cells were cultured at 28°C in minimal sporulating liquid media (MSL; Egel et al., 1994) to a density of 1.5 × 10^6 cells/ml and filtered into MSL minus nitrogen source.

For stress sensitivity spot test assays, cells were grown in yeast extract media (YES) to a cell density of 1.5 × 10^6 cells/ml. 10-fold dilution series starting with 5 × 10^4 cells were spotted on media as indicated.

Cell length and division ratio measurement

Cells were grown at 28°C in EMMG to 1.5 × 10^6 cells/ml and filtered into media using proline as nitrogen source (EMMP). Cells were harvested at the indicated time point, fixed with 3% formaldehyde, washed with PBS, and stained by calcofluor. Images of cells were obtained using a CoolSNAP HQ^2 CCD camera (Photometrics) and processed with ImageJ (National Institutes of Health). Cell length at division was measured (in each analysis, >200 cells were measured/counted).

Molecular manipulations and generation of single point mutations

The *Δtor1* construction. A cassette for *tor1* deletion was prepared by amplifying the *loxP-ura4-loxM3* region of the pAW1 plasmid by PCR (Watson et al., 2008) using the primers 5′-ATTGTGATGAATGCCTAAGTGGAAGAATTGAACACCGCGACTATTAGAAAGTCTATCGTTTCACTCGCTCTCTTTGATTCCGGATCCCCGGGTTAATTAA-3′ and 5′-CAGAAACGAGCGAATTTATAGACATAAATTAATAACAACACGAAAAAAATTATCATAATCTCAAAAAACAGAAAACATCAGAATTCGAGCTCGTTTAAAC-3′. This construct was used to replace the *tor1*$^+$ gene at the native locus with the *ura4*$^+$ gene with the *loxP* and *loxM3* flanking regions, respectively. To generate *tor1* point mutations, standard site-directed mutagenesis was used and the mutated *tor1* allele was cloned into the pAW8 plasmid (Watson et al., 2008). The recombinant plasmid was then used to replace the *ura4*$^+$ gene in JP1293 with *tor1.loxP* wild-type or mutant alleles after the *cre–lox* protocol (Watson et al., 2008). The resulting strains were backcrossed and prototroph progeny was selected. The presence of the *tor1.loxP* allele was verified by PCR. Thus, all *tor1* point mutations used in this study are integrated into the *tor1* locus, and they are all prototroph strains.

Gad8 overexpression. The Gad8 ORF was amplified, sequenced, and cloned into pREP1 (Basi et al., 1993). Cells overexpressing Gad8 were grown in EMMG, and Gad8 expression was inhibited by the addition of 15 µM thiamine. Standard site-directed mutagenesis was used to generate all *gad8* point mutations. The individual *gad8* alleles were integrated into the genomic *gad8* locus in JP1295 by FOA selection. *ryh1Δ*, *sat1Δ*, *and sat4Δ* deletions were generated by Bioneer.

Generation of the Tor1-GST fusion, substrate in the Gad8 kinase assay. Tor1 was amplified using following primers, 5′-CTCGGATCCATCTCGCATTTCCATCACACTTTCGAAG-3′ and 5′-ACGCGTCGACAAGTCTCCAATTAATCAAAGGGTCATAG-3′, and cloned into pET-41a(+) vector. Tor1-GST was expressed in *Escherichia coli* BL-21 cells.

Generation of mTOR.T2173A. Standard site-directed mutagenesis was performed, and the mutated mTOR allele was cloned into the pCMV-SPORT6 vector by replacing the wild-type mTOR allele.

Western blotting

TCA precipitation protocol was followed for total protein extracts (Caspari et al., 2000). The following dilutions of antibodies were used in this study: 1:100 anti-Tor1, 1:100 anti–P-Tor1.T1972, 1:100 anti–P-Tor2.S1975, 1:1,000 anti–P-Gad8.S546, 1:100 anti–P-Gad8.T387, 1:100 anti-Gad8 antibodies, 1:2,000 Phospho-(Ser/Thr) Akt substrate (PAS) antibody (Cell Signaling Technology), 1:100 p70 S6Kα (Santa Cruz Biotechnology, Inc.), 1:100 P-S6Kα.T389 (Cell Signaling Technology), and 1:1,000 S6 antibody (Abcam). 1:100 mTOR antibodies were from EMD Millipore. 1:100 AKT and 1:100 P-AKT.S473 antibodies were from Cell Signaling Technology. Anti–p-Tor1.T1972, anti–P-Gad8.S546, and anti–P-Gad8.T378 were all generated by Eurogentec. Anti–P-Tor2.S1975 antibodies were used to detect both *S. pombe* Tor2 and mTOR phosphorylation and were generated by Eurogentec. Anti-Tor1 antibodies were raised against the unique Tor1 sequence (aa 2,231–2,274). Alkaline phosphatase– or peroxidase (only for loading control in Figs. 1 D, 3 A, and 5 I)-coupled secondary antibodies were used for all blots followed by direct detection with NBT/BCIP (VWR International) substrates on polyvinylidene fluoride membranes.

Large-scale Tor1 immunoprecipitation for mass spectrometry analysis

20 liters of fission yeast culture at 3 × 10^6 cells/ml was harvested and resuspended in 20% TCA. Cells were disrupted using a 6870 freezer mill (SPEX) in liquid nitrogen. After washing with 0.1% TCA, the sample was resuspended in sample buffer (80 mM Tris, pH 7.5, 5 mM DTT, and 5 mM EDTA) plus 2% SDS with 3 min of boiling. 4.5 volumes of sample buffer plus 1% Triton X-100 were added to the supernatant. The mix was centrifuged at 10,000 *g* for 5 min. IP buffer (0.5% Doc, 50 mM NaF, 0.2 mM Na_3VO_4, 20 mM sodium-β-glycerophosphate, 1 mM PMSF, and protease inhibitors) was added to the supernatant. The Tor1 kinase was immunoprecipitated on protein G Dynabeads for 30 min at 4°C. The beads were then washed six times with sample buffer plus inhibitors, then heated to 80°C for 10 min before electrophoreses.

Tor1 for phospho-site mapping

Large-scale Tor1 immunoprecipitations were run on a NuPAGE Bis-Tris 4–12% SDS-PAGE gel (Invitrogen). The Tor1 Coomassie-stained band was excised and digested with 20 ng of sequencing-grade trypsin (Sigma-Aldrich), 400 ng LysN (Associates of Cape Cod), or 350 ng Elastase (EMD Millipore) in 100 µl of 40-mM ammonium bicarbonate, 9% (vol/vol) acetonitrile at 37°C for 18 h. The peptides were separated using a nanoACQUITY UPLC system (Waters) using a Waters nanoACQUITY BEH C18 column (75 µm inner diameter, 1.7 µm, 25 cm) with a gradient of 1–25% (vol/vol) acetonitrile, 0.1% formic acid over 30 min at a flow rate of 400 nl/min. The LTQ-Orbitrap XL mass spectrometer was operated in parallel data-dependent mode where the MS survey scan was performed at a nominal resolution of 60,000 (at m/z 400) resolution in the Orbitrap analyzer in an m/z range of 400–2,000. The top six precursors were selected for collision-induced dissociation in the LTQ at normalized collision energy of 35% using multistage activation at m/z 98.0, 49.0, and 32.7 D.

Gad8 in vitro kinase assay

Tor1-GST was purified as follows. BL-21 cells' expression of Tor1-GST was disrupted with lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EGTA, 10 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail). The supernatant was incubated with Glutathione Sepharose for 2 h at 4°C. The Sepharose was washed 10× with lysis buffer and Tor1-GST was eluted with elution buffer (as lysis buffer but using 50 mM Tris, pH 9.6, plus 6 mg/ml glutathione). An in vitro kinase assay was performed as described previously (Matsuo et al., 2003). In brief, Gad8 was immunoprecipitated in IP buffer (50 mM Tris, pH 7.6, 150 mM KCl, 5 mM EDTA, 1 mM EDTA, 10% glycerol, 0.2% NP-40, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 15 mM *p*-nitrophenyl phosphate [PNPP], 1 mM PMSF, and protease inhibitors) and resuspended into kinase assay buffer (20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and 15 mM PNPP). It was mixed with 3 µg of Tor1-GST and 25 µM of ATP, incubated at 32°C for 30 min. The reaction was stopped by 5 min of boiling with 2× loading buffer.

Tor1 in vitro kinase assay

S. pombe cells were harvested from early exponential phase cultures of 1.5 × 10^6 cells/ml. Tor1 immunoprecipitation was performed according to Alarcon et al. (1999). In vitro Tor1 kinase assays were performed using the K-LISA mTOR Activity kit (EMD Millipore) according to Ikai et al. (2011).

Cell culture media, cell starvation, and restimulation

A375 and HeLa cells were cultured in DMEM (no. D6429; Sigma-Aldrich)/0.5% penicillin-streptomycin (Sigma-Aldrich)/10% FBS "Gold" (PAA Laboratories) or Heat Inactivated serum (Sigma-Aldrich), respectively. For starvation experiments, cells were seeded into 6-well plates at a density of 2 × 10^5 cells/ml and cultured overnight. The next day, the growth medium was aspirated, cells were washed twice with the starvation medium (DMEM without serum and L-glutamine; no. D6171; Sigma-Aldrich), and cells were then incubated in the starvation medium. 24 h later, the starvation medium was replaced by DMEM supplemented with serum, L-glutamine, and solvent (DMSO); 3 µM Akt1/2 inhibitor (Sigma-Aldrich); or 30 µM PF-4708671 (S6K1 inhibitor; EMD Millipore), and cultures were incubated for a further 2 or 3 h, as indicated. To ensure that the inhibitors had a chance to affect their targets before cells were restimulated, inhibitors (or solvent) were also added to the appropriate cultures half an hour before refeeding the cells with the rich medium. The AKT1/2i stabilizes an inactive conformation that cannot become phosphorylated to be activated.

Transfection and cell lysis

Exponentially growing A375P cells were seeded into 6-well plates at a density of 10^5 cells/ml and cultured overnight. The next day, cells were transfected by Attractene (QIAGEN) with an empty vector (pCDNA3) or the pCMV-SPORT6 vector containing mTOR (Thermo Fisher Scientific) or mTOR.T2173A. 24 h after transfection, cells were rinsed with PBS and lysed with lysis buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromphenol blue, and 1.25% β-Mercaptoethanol), and protein expression levels were detected by immunoblotting.

Online supplemental material

Fig. S1 shows Tor1- and Tor2-specific antibodies; a product ion spectrum and identification of the site of phosphorylation of Tor1 on T1972; and a model showing that mTOR T2173 is adjacent to the ATP binding residue L2185. Fig. S2 shows that endogenous *tor1.lox* phenocopies *tor1*$^+$, and that anti–P-Gad8T387 is phospho-specific and regulated by Ksg1. Fig. S3

shows that TORC2 regulation in Tor1.1972A still required Ryh1. Activity of the *tor1.I1816T.T1972A* mutant is also shown. The figure shows that Gad8.S546 phosphorylation is not essential for Gad8 activity, that starvation does not down-regulate Gad8.S546 phosphorylation in the *gad8.T387D* mutant, that Gad8 is phosphorylated on multiple residues, and that Tor1.T1972 phosphorylation is stable. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201305103/DC1.

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