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Fission yeast TORC1 prevents eIF2α phosphorylation in response to nitrogen and amino acids via Gcn2 kinase

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Summary
Serine 51 phosphorylation of the eukaryotic initiation factor-2α (eIF2α) is an important mechanism involved in blocking general protein synthesis in response to diverse types of stress. In fission yeast, three kinases (Hri1, Hri2 and Gcn2) can phosphorylate eIF2α at serine 51. In this study, we show that Tor2, as part of the TORC1 complex, prevents the phosphorylation of eIF2α in cells growing in the presence of nitrogen and amino acids. Inhibition of TORC1, either by rapamycin treatment, mutation of Tor2 or nitrogen deprivation, induces Gcn2-dependent phosphorylation of eIF2α.

Key words: S. pombe, TOR, Rapamycin, Gcn2, eIF2α

Introduction
In response to diverse environmental stresses, the phosphorylation of eukaryotic initiation factor-2α (eIF2α) induces a programme of gene expression that mitigates cellular injury. Several protein kinases phosphorylate eIF2α at serine 51, leading to the inhibition of eIF2B activity, that in turn causes a reduction in general protein synthesis and an enhancement of the translation of specific mRNAs encoding for proteins that mediate the stress. In mammalian cells, four eIF2α kinases (HRI, GCN2, PEK/Perk and PKR) inhibit translation initiation through the phosphorylation of eIF2α in response to different types of stress (Dever, 2002; Proud, 2005). In Saccharomyces cerevisiae, Gcn2p is activated upon nutrient limitation (amino acids, purine and glucose), but also by high concentrations of sodium, rapamycin and methyl methanesulfonate (Cherkasova and Hinnebusch, 2003; Narasimhan et al., 2004; Valenzuela et al., 2001; Yang et al., 2000). In the fission yeast Schizosaccharomyces pombe, besides Gcn2 two additional eIF2α kinases related to mammalian HRI, called Hri1 and Hri2, phosphorylate eIF2α at serine 51 (serine 52 in S. pombe). Hri1 and Hri2 show a differential activation pattern in response to cellular stress, including heat shock, arsenite or cadmium (Zhan et al., 2004; Berlanga et al., 2010).

When supplied with enough nutrients, fission yeast cells activate protein translation, promoting cellular growth and proliferation and inhibiting sexual differentiation. The target of the rapamycin (TOR) signaling pathway plays a central role in the regulation of these processes. TOR, a serine/threonine protein kinase conserved from yeasts to mammals, exists in two different complexes, namely TORC1 and TORC2 (Loewith et al., 2002; Sarbassov et al., 2004). TORC1 contains Raptor and regulates cell growth positively by promoting anabolic processes, such as protein synthesis (Finager et al., 2002; Hay and Sonenberg, 2004), and by inhibiting catabolic processes, such as autophagy (Blommaart et al., 1995; Noda and Ohsumi, 1998; Shigemitsu et al., 1999). In contrast, TORC2, which contains Rictor, regulates Akt and also affects the actin cytoskeleton (Jacinto et al., 2004; Sarbassov et al., 2005). In mammalian cells, mTOR is a critical player in the TSC1–TSC2/Rheb/mTOR signaling pathway, which regulates cell growth in response to growth factors, nutrients and energy conditions. Unlike higher eukaryotes, which contain a single TOR protein, S. pombe and S. cerevisiae have two: Tor1 and Tor2. In contrast to S. cerevisiae, the TSC1–TSC2/Rheb/TOR pathway is conserved in S. pombe, providing an excellent model to study TOR pathway regulation. In S. pombe, Tor2 forms part of the TORC1 complex and is essential for cell growth and the repression of sexual differentiation, meiosis and sporulation (Alvarez and Moreno, 2006; Matsuo et al., 2007; Uritani et al., 2006; Weisman et al., 2007), whereas Tor1 is not essential for growth and is included in the TORC2 complex (Alvarez and Moreno, 2006; Hayashi et al., 2007; Matsuo et al., 2007).

The α subunit of the eukaryotic initiation factor-2α (eIF2α) and TOR play important roles in the translation response to nutritional variation. In S. cerevisiae, the inhibition of TOR leads to the activation of Gcn2 kinase, and eIF2α phosphorylation down-regulates general translation initiation (Cherkasova and Hinnebusch, 2003; Kubota et al., 2003). We therefore examined a possible relationship between TOR and eIF2α in S. pombe and obtained evidence that Tor2, as part of the TORC1 complex, prevents the Gcn2-mediated phosphorylation of eIF2α in response to the presence of nitrogen and amino acids in the medium.

Results
Tor2 impairs the phosphorylation of eIF2α in the presence of nitrogen
Fission yeast eIF2α is phosphorylated by three different eIF2α kinases, Gcn2, Hri1 and Hri2, in response to different types of
cellular stress (Zhan et al., 2004) in order to regulate protein translation and adapt to the new cellular environment. Tor2 is a key regulator of the switch between cell division and cell differentiation, mainly by sensing nitrogen availability (Alvarez and Moreno, 2006; Matsuo et al., 2007; Uritani et al., 2006; Weisman et al., 2007). A loss of Tor2 activity mimics nitrogen starvation and the cells stop growth and undergo cell cycle arrest in G1 and sexual differentiation.

Since phosphorylation of eIF2α is a key aspect in the regulation of protein translation to adjust the cell to the new environment, and since the TOR pathway also regulates different processes to adapt the cell to nitrogen starvation, we analyzed whether there might be a cross-talk between these two major pathways in fission yeast. In budding yeast, addition of rapamycin (a Tor inhibitor) or nitrogen starvation induces phosphorylation of eIF2α (Cherkasova and Hinnebusch, 2003; Kubota et al., 2003). Previous reports in fission yeast have described that rapamycin does not induce eIF2α phosphorylation in S. pombe (Nakashima et al., 2010). In our hands rapamycin was able to induce eIF2α phosphorylation at serine 52 in fission yeast (Fig. 1A). This phosphorylation was specific of the serine 52 residue because it was absent in cells expressing an eIF2α-Ser52 to Ala52 mutant allele (Fig. 1A). Moreover, eIF2α Ser52 phosphorylation was coincidental with the disappearance of phosphorylated 40S ribosomal S6 protein (Rps6), indicating low Tor2 activity (Nakashima et al., 2010). Therefore, we conclude that like in budding yeast, addition of rapamycin to exponentially growing nitrogen-starved cells induces phosphorylation of serine 52 in eIF2α.

Next, we analyzed whether eIF2α phosphorylation was regulated by the presence of nitrogen. To accomplish this, we collected wild-type cells exponentially growing and starved for nitrogen and we observed that eIF2α became phosphorylated in nitrogen-starved cells (Fig. 1B). Like rapamycin, nitrogen starvation produces a decrease in Tor2 activity, as measured by the phosphorylation of Rps6 (Fig. 1A,B). Since a lack of Tor2 has been reported to mimic nitrogen starvation, we used a temperature-sensitive allele of the tor2 gene, tor2-51, to analyze the phosphorylation of eIF2α after Tor2 inactivation at the restrictive temperature. As shown in Fig. 1C, eIF2α also became phosphorylated after Tor2 inactivation. Rps6 phosphorylation was used as a control of Tor2 activity (Nakashima et al., 2010) to show that eIF2α phosphorylation takes place when Tor2 activity drops. Thus nitrogen starvation or Tor2 inactivation, either by addition of rapamycin or by mutation, lead to eIF2α Ser52 phosphorylation, suggesting that nitrogen prevents eIF2α phosphorylation via the Tor2 pathway.

Tor2 impairs the phosphorylation of eIF2α in the presence of amino acids

In fission yeast, Tor2 activity increases in response to amino acids. In the presence of amino acids, the Gtr1/Gtr2 complex interacts and activates the TORC1 complex at the vacuole membrane (Valbuena et al., 2012). Mutants in the Gtr1/Gtr2 complex showed reduced Tor2 activity and were unable to respond to the presence of amino acids in the medium (Valbuena et al., 2012). In order to test whether eIF2α phosphorylation would be decreased by the presence of amino acids (due to high Tor2 activity), we collected wild-type, gtr1A and gtr2A mutant cells growing in minimal medium in the presence and in the absence of amino acids, and eIF2α phosphorylation was measured. Wild-type cells growing in minimal medium with amino acids showed a higher degree of Rps6 phosphorylation (indicating high Tor2 activity) and a lower degree of eIF2α phosphorylation than in the absence of amino acids (Fig. 1D). The mutants (gtr1A and gtr2A) were unable to sense the presence of amino acids, and no increase in Tor2 activity was observed (measured as Rps6 phosphorylation), as has been described previously (Valbuena et al., 2012). Moreover, we observed constitutive eIF2α phosphorylation in the gtr1A and the gtr2A mutants, indicating that these cells were unable to respond to the addition of amino acids. Therefore, the presence of amino acids in the medium seems to impair eIF2α phosphorylation via the Gtr1/Gtr2-Tor2 pathway.

**Fig. 1. Tor2 regulates eIF2α phosphorylation.**

(A) Wild-type and eIF2α-S52A cells were grown exponentially at 25°C in YES medium and treated with 220 nM rapamycin added from a 500x stock solution of 110 μM in DMSO. Cells treated with the same volume of DMSO (vehicle) were used as controls. Samples were taken at 0, 30 and 60 minutes after the addition of rapamycin. (B) Wild-type cells were grown exponentially at 25°C in EMM and transferred to EMM without nitrogen (NH4Cl). Samples were taken at 0 and 60 minutes. (C) Wild-type and tor2-51 cells were grown exponentially at 25°C in YES medium and shifted from 25°C to 32°C to inactivate Tor2. Cells were collected at the indicated times. (D) Wild-type, gtr1A and gtr2A cells were grown exponentially at 30°C in EMM and transferred to EMM (−) or to EMM supplemented with amino acids (+) for 1 hour. In all cases, cell extracts were prepared and subjected to SDS-PAGE to detect Ser52-phosphorylated eIF2α (eIF2α-P) and phosphorylated Rps6 (Rps6-P), as a measure of Tor2 activity. Tubulin and eIF2α were used as loading controls.
Gcn2 is the main eIF2α kinase that is activated after Tor2 activation

The differential activation of eIF2α kinases in response to several cellular stresses in *S. pombe* has been described (Zhan et al., 2004; Berlanga et al., 2010). Gcn2 is activated in response to a nutrient downshift (from rich to minimal medium), elevated NaCl levels, or oxidative stress (Zhan et al., 2004). Hri2 is the main eIF2α kinase activated in response to exposure to heat shock, arsenite, or cadmium, and Hri1 appears mainly to provide a supportive role, together with its paralogue Hri2 (Zhan et al., 2004).

In order to investigate the contribution of each eIF2α kinase to eIF2α phosphorylation after Tor2 inactivation, we compared wild-type and *tor2-51* mutant cells with mutants lacking the different eIF2α kinases in a *tor2-51* background. Cells were grown exponentially at 25°C and Tor2 was inactivated by shifting the cells to the restrictive temperature of 32°C. As shown in Fig. 2, deletion of *gcn2* significantly reduced the phosphorylation of eIF2α, indicating that Gcn2 is the main kinase that phosphorylates eIF2α after Tor2 inactivation.

The TORC1-A complex inhibits eIF2α phosphorylation

Tor proteins exist in two distinct complexes, TORC1 and TORC2, with different physiological functions (Loewith et al., 2002; Sarbassov et al., 2004). TORC1 contains Raptor and TORC2 contains Rictor. In *S. pombe*, Tor2 together with Mip1 (the Raptor orthologue) form part of TORC1, whereas Tor1 together with Ste20 (the Rictor orthologue) form part of TORC2. Moreover, Tor1 also interacts with the TORC1 component Mip1 (Hartmuth and Petersen, 2009). However, Tor1 and Tor2 are unlikely to be interchangeable in the essential TORC1 complex, suggesting that two distinct TORC1 complexes co-exist: TORC1-A containing Tor2, and TORC1-B containing Tor1 (Hartmuth and Petersen, 2009). To distinguish which complex – TORC1-A, TORC1-B or TORC2 – was involved in regulating the phosphorylation of eIF2α, we deleted *tor1*, which forms part of TORC1-B and TORC2, and *ste20*, which forms part only of TORC2, in a *tor2-51* background. Inactivation of Tor2 in a *tor1* or *ste20* mutant background did not lead to any differences in eIF2α phosphorylation (Fig. 3A), indicating that neither TORC1-B nor TORC2 are involved in eIF2α phosphorylation, and pointing to TORC1-A as the only complex regulating the phosphorylation of eIF2α.

When fission yeast cells are exposed to a nutritional downshift from minimal medium with glutamate, as the nitrogen source, to minimal medium with proline, Tor1 activates mitotic onset through Gcn2, which leads to an increase in the activity of the MAP kinase Sty1, without the involvement of eIF2α phosphorylation (Petersen and Nurse, 2007; Hartmuth and Petersen, 2009). We therefore explored whether the connection between the Tor1-Gcn2-Sty1 pathway and mitotic onset was related or not with Tor2-Gcn2-eIF2α and the regulation of protein translation. To test whether Sty1 was regulating eIF2α phosphorylation after Tor2 inactivation, we deleted *sty1* in a *tor2-51* thermosensitive strain. The phosphorylation levels of eIF2α observed after Tor2 inactivation was similar, regardless of the presence or not of Sty1 (Fig. 3B), suggesting that the MAP kinase Sty1 is not involved in this pathway.

Discussion

In this paper we present evidence that inhibition of Tor2 in fission yeast, either by rapamycin treatment or mutation, induces the phosphorylation of the α subunit of the eukaryotic initiation factor-2 on the highly conserved serine 52. Phosphorylation of eIF2α on serine 52 (serine 51 in budding yeast and mammals) constitutes an evolutionary conserved mechanism to inhibit general protein translation (Dever, 2002). We also show that the main protein kinase responsible for this phosphorylation is Gcn2, the primary sensor of amino acid starvation in budding yeast (Hinnebusch, 2005; Fig. 4). Our result contradicts previous reports by Petersen and Nurse (Petersen and Nurse, 2007) and Nakashima et al. (Nakashima et al., 2010) showing that rapamycin addition did not affect eIF2α phosphorylation.
The main players of this pathway downstream of the TOR pathway (Cherkasova and Hinnebusch, 2003; Yan et al., 2006). The main players of this pathway downstream of the TOR pathway (Cherkasova and Hinnebusch, 2003; Yan et al., 2006). Recently it has been shown that binding of rapamycin to TORC1 dissociation of Tap42-2A from TORC1 (Yan et al., 2006) causing dephosphorylation of serine 577 in Gcn2 by interacting and retaining the Tap42-2A phosphatase in membrane structures. Treatment with rapamycin or nutrient deprivation induces the inactive by phosphorylation on serine 577 (Cherkasova and Hinnebusch, 2003; Narasimhan et al., 2004; Valenzuela et al., 2001).

In exponentially growing budding yeast cells Gcn2 is kept inactive by phosphorylation on serine 577 (Cherkasova and Hinnebusch, 2003; Kubota et al., 2003). Active TORC1 prevents phosphorylation of eIF2α-dependent phosphorylation of eIF2α takes place, blocking general protein synthesis.

used YES medium in our experiments, whereas in previous publications rapamycin was added to minimal medium, this difference could account for the discrepancy considering that Tor signalling is altered by the nutritional environment. This result is in full agreement with previous reports in S. cerevisiae stating that TOR prevents the Gcn2-dependent phosphorylation of eIF2α (Cherkasova and Hinnebusch, 2003; Narasimhan et al., 2004; Valenzuela et al., 2001).

In exponentially growing budding yeast cells Gcn2 is kept inactive by phosphorylation on serine 577 (Cherkasova and Hinnebusch, 2003; Kubota et al., 2003). Active TORC1 prevents dephosphorylation of serine 577 in Gcn2 by interacting and retaining the Tap42-2A phosphatase in membrane structures. Treatment with rapamycin or nutrient deprivation induces the dissociation of Tap42-2A from TORC1 (Yan et al., 2006) causing the release of active Tap42-2A phosphatase to the cytoplasm. Recently it has been shown that binding of rapamycin to TORC1 induces the activation of Rho1 GTPase, which in turn binds to TORC1 and releases Tap42-2A (Yan et al., 2012). Tap42-2A phosphatase will dephosphorylate Gcn2 and many factors downstream of the TOR pathway (Cherkasova and Hinnebusch, 2003; Yan et al., 2006). The main players of this pathway (TORC1, Gcn2, eIF2α, Tap42-2A and Rho1) are conserved in S. pombe and a number of eukaryotes.

Protein extraction and western blots
Protein extracts were obtained using trichloroacetic acid (TCA) extraction, as described previously (Foiani et al., 1995). Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in fission yeast (Foiani et al., 1994). For Western blots, 75-100 μg of total protein extract were run on 15% SDS-PAGE, transferred to a nitrocellulose filter (Amersham), and probed with rabbit anti-Phospho-eIF2α (Ser51) (Cell Signaling Technology), rabbit anti-eIF2α (Santa Cruz Biotechnology), for the phosphorylation of Rps6 rabbit anti-Phospho-Ser-Thr (Akt Substrate (Cell Signaling Technology) and mouse anti-tubulin (a gift from Dr Keith Gull, Sir William Dunn School of Pathology, University of Oxford, UK) primary antibodies and, as secondary antibodies, NA 931, anti-mouse IgG, Horseradish Peroxidase (Amersham). Immunoblots were developed using the enhanced chemiluminescence procedure (ECL kit, Amersham).

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**Table S1. Fission yeast strains**

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<td>Prof. Paul Nurse</td>
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