New roles of the fission yeast eIF2α kinases Hri1 and Gcn2 in response
to nutritional stress

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Running title: eIF2α kinases response to stress

Keywords: eIF2α kinases, translational control, stress response, cell cycle, Schizosaccharomyces pombe

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SUMMARY

In fission yeast, three distinct eukaryotic initiation factor 2α (eIF2α) kinases (Hri1, Hri2 and Gcn2), regulate protein synthesis in response to various environmental stresses. Thus, Gcn2 is activated early after exposure to hydrogen peroxide (H₂O₂) and methyl methanesulfonate (MMS), whereas Hri2 is the primary activated eIF2α kinase in response to heat shock. The function of Hri1 is still not completely understood. It is also known, that the MAPK Sty1 negatively regulates Gcn2 and Hri2 activities under oxidative stress. In this study, we demonstrate that Hri1 is mainly activated, and its expression up-regulated, during transition from exponential growth to the stationary phase in response to nutritional limitation. Accordingly, both Hri1 and Gcn2, but not Hri2, are activated upon nitrogen source deprivation. In contrast, Hri2 is stimulated early during glucose starvation. We also found that Gcn2 is implicated in nitrogen starvation-induced growth arrest in the cell cycle G1 phase as well as in the non-selective protein degradation process caused upon this particular cellular stress. Moreover, Gcn2, but not Hri1 or Hri2, is essential for survival of cells growing in minimal medium, upon oxidative stress or glucose limitation. We further show that eIF2α phosphorylation at serine 52 by the eIF2α kinases is necessary for efficient cell cycle arrest in the G1 phase, for the consequent protein degradation and for sexual differentiation, under nitrogen starvation. Therefore, the eIF2α kinase signalling pathway modulates G1 phase cell cycle arrest, cell survival and mating under nutritional stress in the fission yeast Schizosaccharomyces pombe.
INTRODUCTION

In eukaryotic cells, the reversible phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) is a well-characterized mechanism of translational control in response to a wide variety of cellular stresses (de Haro et al., 1996; Dever, 2002). Four mammalian protein kinases (HRI, PKR, GCN2 and PERK), which inhibit translation initiation by phosphorylating eIF2α on Ser-51, have been identified. They are regulated independently in response to various different cellular stresses (Dever, 2002; Proud, 2005). In the yeast Saccharomyces cerevisiae, Gcn2 is the sole eIF2α kinase (Dever, 2002), however, in the fission yeast Schizosaccharomyces pombe, besides Gcn2, two additional eIF2α kinases related to mammalian HRI, called Hri1 and Hri2, phosphorylate eIF2α at the same residue (serine 52 in S. pombe). Hri1 and Hri2 showed a differential activation pattern in response to cellular stresses (Zhan et al., 2004). More recently, it has been reported that, upon distinct cellular stresses, phosphorylation of eIF2α constitutes a very early response and that a particular eIF2α kinase is activated in response to each stress stimuli. Thus, Hri2 responds to heat shock, whereas Gcn2 is activated early after exposure to hydrogen peroxide (H₂O₂) and methyl methanesulfonate (MMS) in S. pombe (Berlanga et al., 2010).

It is well known that eIF2α phosphorylation can regulate both gene-specific and general translation. Thus, the eIF2α kinases phosphorylate eIF2α, resulting in reduced general translation and increased translation of the transcription factors, Gcn4 in Saccharomyces cerevisiae and ATF4 in mammals, which in turn activate expression of their target genes involved in the stress response (Harding et al., 2000; Hinnebusch, 1997).

Despite the finding that phosphorylation of eIF2α is a general response to cellular stress it is well known that, in S. pombe, cellular responses to various environmental stresses are regulated primarily through the stress- and mitogen-activated protein kinase (SAPK/MAPK) Sty1. Sty1, also known as Spc1 and Phh1, is required for, and can be activated by different types of stress conditions, including oxidative stress and glucose or nitrogen starvation (Vivancos et al., 2006). Upon stress activation, Sty1 reversibly accumulates in the nucleus, where it stimulates gene expression via the Atf1 transcription factor. Thus, in response to stress stimuli, Sty1 is required for the
transcriptional regulation of a large set of genes which constitute the core environmental stress response (CESR) (Chen et al., 2003). For the majority of these genes, regulation is also dependent on Atf1 (Shiozaki and Russell, 1996; Wilkinson et al., 1996). Atf1, a heterodimeric transcription factor, is bound to stress genes promoters before activation, and only induces transcription of those genes once Atf1 becomes phosphorylated (Chen et al., 2008). In a previous report, it was suggested that downstream components of the Sty1 MAPK pathway can contribute both positively and negatively to the modulation of the eIF2α kinase pathway (Dunand-Sauthier et al., 2005). More recently, it has been reported that, the activated Sty1 negatively regulates Gcn2 and Hri2 activities under oxidative stress, but not upon heat shock or genotoxic stress caused by MMS, in fission yeast (Berlanga et al., 2010).

In the fission yeast *Schizosaccharomyces pombe*, depletion of nitrogen from culture medium has been shown to effectively induce G1 phase cell cycle arrest and subsequent sexual differentiation. This nitrogen starvation response is also mediated by the target of rapamycin (TOR) pathway involving Tor1 and Tor2 (Otsubo, 2008; Weisman et al., 2007). Loss of Tor2 activity has a similar effect to that of nitrogen withdrawal and promotes sexual differentiation, whereas cells lacking Tor1 are incapable of G1 arrest and are therefore sterile (Alvarez and Moreno, 2006; Matsuo et al., 2007; Weisman et al., 2007). The coupling of growth to cell cycle progression involves the Sty1 pathway working through Polo kinase Plo1 recruitment to the spindle pole bodies (Petersen and Hagan, 2005). Moreover, nutrient-induced activation of mitotic onset has been suggested to occur due to changes in Tor1 signalling through Gcn2 by modulating Pyp2 levels, which then leads to an increase in Sty1 activity, resulting in subsequent Plo1 phosphorylation and Cdc2 activation (Petersen and Nurse, 2007). On the other hand, initiation of mitosis is advanced or delayed depending on the activity of Sty1 MAPK. Thus, mitotic onset is delayed when Sty1 activity is absent or very high, whereas intermediate activity of this kinase promotes mitosis, and cells divide at smaller size (Hartmuth and Petersen, 2009).

Previous studies showed that ultraviolet (UV) irradiation of fission yeast cells in G1 phase induced a transient delay in S-phase entry (Nilssen et al., 2003). This UV-induced G1-S checkpoint was totally dependent on the Gcn2 kinase and, additionally, UV light led to activation of Gcn2, which in turn resulted in phosphorylation of eIF2α. However, it was not clear whether eIF2α phosphorylation was required for the
checkpoint (Tvegard et al., 2007). Also, it was previously observed that entry into the S phase was delayed in response to oxidative damage by hydrogen peroxide and alkylation by MMS (Krohn et al., 2008). Both of these stress situations strongly induced eIF2α phosphorylation (Berlanga et al., 2010; Krohn et al., 2008). In this case, a tight correlation was found between eIF2α phosphorylation and the presence of a G1-phase delay suggesting that eIF2α phosphorylation was required for checkpoint induction (Krohn et al., 2008).

In *S. pombe*, the MAP kinase Sty1 pathway participates in the maintenance of viability of starved cells (Kronstad et al., 1998). Thus, during transition to stationary phase in glucose-limiting conditions, Sty1 becomes activated and triggers a transcriptional stress programme, whereas such activation does not occur under glucose-rich conditions (Zuin et al., 2010; Zuin et al., 2005). Then, the Sty1 activation at the onset of stationary phase is required for both stress-resistance acquisition and lifespan extension under calorie restriction (Zuin et al., 2010).

In a previous report, we showed that, upon distinct cellular stresses, phosphorylation of eIF2α constituted a very early response. Thus, Hri2 responded to heat shock, whereas Gcn2 was activated early after exposure to H₂O₂ and MMS in *S. pombe* (Berlanga et al., 2010). Here, we further investigate the participation of each eIF2α kinase in the fission yeast translational programme in response to nutritional stress. We show that eIF2α phosphorylation is strongly induced by stationary phase entry stress. Our results indicate for the first time that Hri1 is activated by both nitrogen starvation and stationary phase entry stresses, whereas Hri2 is activated in response to glucose limitation. Finally, we establish that Gcn2 and eIF2α phosphorylation are involved in the modulation of G1 phase cell cycle arrest, cell survival and mating under nutritional stress in the fission yeast *Schizosaccharomyces pombe*.

**RESULTS**

**Hri1 is activated at the onset of stationary phase**

Phosphorylation, as well as nuclear accumulation, of Sty1 and Sty1-dependent gene expression at the transition between exponential and stationary growth was previously reported (Zuin et al., 2010; Zuin et al., 2005). We wanted to investigate the possible contribution of the eIF2α kinase pathway when the culture reached high
density and reduced growth. We observed that stationary phase entry stress strongly 
induces eIF2α phosphorylation in S. pombe cells (Fig. 1A). To characterize the 
contribution of each eIF2α kinase in early stationary phase cultures, we studied 
different strains, including a wild-type strain and strains devoid of two of the three 
eIF2α kinase genes (thereby expressing only Hri1, Hri2 or Gcn2). There was a 
significant reduction of eIF2α phosphorylation in cells expressing only Gcn2 or Hri2 
during the late exponential and stationary phases, compared to wild-type cells. 
However, a significant increase of eIF2α phosphorylation was detected at stationary 
phase exclusively in the strain containing Hri1 as the only eIF2α kinase and in wild-type 
cells (Fig. 1A). Measurements of cell growth in rich medium, YES, indicated that the 
growth rate in cells expressing only Gcn2 or Hri2 was identical to that of wild-type 
cells, while the strain containing only Hri1 in fact gave a modest decrease of growth 
(Fig. 1B). Note that all cultures reached around 4 units of optical density at 595 nm at 
the onset of stationary phase when Hri1 became fully active (Fig. 1A).

We next examined whether the induction of eIF2α phosphorylation observed at 
the onset of stationary phase is accompanied by changes at the level of transcription and 
translation of the distinct eIF2α kinases in S. pombe. For that, further experiments were 
performed using cells of strains expressing HA-tagged Hri1, Hri2 and Gcn2. We 
analyzed the protein levels in cells by immunoprecipitation and immunoblotting with 
anti-HA antibodies. As shown in Fig. 1C, the expression of all three eIF2α kinases was 
strongly induced during transition to stationary phase, in good correlation with the 
significant induction of eIF2α phosphorylation in cells at that point of growth. 
Moreover, the immunoprecipitated endogenous HA-tagged Hri1, Hri2 and Gcn2 
exhibited increased autokinase activity in vitro, which mostly correlated with the 
increase in the amount of proteins present in the immune complexes (Fig. 1C). We 
conclude that during transition from exponential growth to the stationary phase the 
expression of all three eIF2α kinases is up-regulated; however the significant induction 
of eIF2α phosphorylation observed in response to this stress condition is mainly Hri1-
dependent.

Nitrogen deprivation activates Hri1 and Gcn2, whereas glucose limitation activates 
Hri2
The possible triggers of the eIF2α kinase stress pathway upon initiation of the stationary phase should be the decreases in culture glucose or nitrogen source concentration. Previously, it has been reported that nitrogen depletion leads to the induction of phosphorylation of Ser52 in eIF2α (Nakashima et al., 2010). Then, we decided to study which eIF2α kinase was required for the response to these two nutritional stresses, by using mutant cells expressing only one of the three eIF2α kinase genes. We examined the eIF2α phosphorylation levels in response to either glucose limitation (Fig. 2A) or nitrogen starvation (Fig. 2B) in both wild-type and the previously mentioned mutant cells. As shown in Fig. 2A, we detected a dramatic increase in the eIF2α phosphorylation levels within 15 min in wild-type cells cultured in glucose-limited conditions. A similar pattern of eIF2α phosphorylation was only observed in the strain expressing Hri2. However, deletion of Hri2 significantly reduces the early activation of eIF2α phosphorylation in glucose-limited conditions. At longer times, there was an increase of eIF2α phosphorylation in cells expressing Hri1 or Gcn2. In fact, a small increase in eIF2α phosphorylation levels was found in Hri1-expressing cells, whereas with the strain containing only Gcn2, the analysis revealed a marked, but transient increase in the eIF2α phosphorylation levels (Fig. 2A). Therefore, it is clear that Hri2 is the primary eIF2α kinase activated in response to glucose limitation. Cells lacking Hri2 exhibit a delayed and transient activation of Gcn2 and a modest induction of Hri1 activity.

In parallel, we studied other nutritional limitation such as nitrogen source (NH₄Cl) deprivation. To examine the role of each eIF2α kinase in response to nitrogen starvation, wild-type *S. pombe* log-phase cells and the mutant strains used in Fig. 2A were transferred to nitrogen-free medium for different periods of time. In this case, a marked increase on eIF2α phosphorylation was also observed in wild-type cells within 30 min. However, in contrast to the situation in glucose-limited medium, a similar pattern of eIF2α phosphorylation was only detected in the strain expressing Hri1, whereas the Gcn2-expressing cells showed a slightly lower and transient effect. Moreover, no eIF2α phosphorylation was detected in the strain expressing only Hri2 (Fig. 2B). Therefore, it is clear that Hri1 is the primary eIF2α kinase activated in response to nitrogen starvation. These findings demonstrate what, to our knowledge, is the first example of Hri1 function in *S. pombe* cells.
Sty1 negatively regulates eIF2α kinase activity under nitrogen starvation

It was reported earlier that Sty1 was required for the arrest of cell growth under the stress conditions produced by nitrogen starvation (Shiozaki and Russell, 1996) and activated at the onset of stationary phase (Zuin et al., 2005). In addition, we have previously determined that the activated MAPK Sty1 negatively regulates Gcn2 and Hri2 activities under oxidative stress (Berlanga et al., 2010). To investigate the possible role of the SAPK pathway in modulating eIF2α kinase activation in response to nitrogen starvation, further experiments were performed using mutants in different components of these two pathways, including a wild-type strain, strains devoid of two of the three eIF2α kinase genes, and the triple mutants in which there was additional disruption in sty1 gene.

As described before, in response to nitrogen starvation both wild-type cells and mutants expressing either Gcn2 or Hri1 showed an increased level of eIF2α phosphorylation within 30 min. Moreover, eIF2α phosphorylation was not increased in the strains expressing only Hri2 (Fig. 2B and Fig. 3A). Interestingly, at longer periods of stress treatment there was a significant induction of Sty1 phosphorylation only in cells expressing Gcn2, coincident with a marked reduction of eIF2α phosphorylation (Fig. 3A,B). We also examined eIF2α phosphorylation levels in response to nitrogen starvation in both wild-type cells and in mutants in which the SAPK pathway was disrupted (Δsty1). When we eliminated Sty1 in these wild-type and mutant backgrounds, the analysis revealed an increase in the eIF2α phosphorylation levels in comparison to those of the Sty1-expressing cells, at all time points examined after exposure to nitrogen starvation conditions. In particular, when we eliminated Sty1 in the strain expressing only Hri2, we observed that a significant induction of eIF2α phosphorylation took place either in the absence or in the presence of such stress (Fig. 3B). Our findings suggest a model in which the SAPK pathway functions to negatively regulate eIF2α kinase activity whereas Gcn2 appears to stimulate Sty1 activation observed under conditions of nitrogen starvation stress in fission yeast. We do not yet know the molecular mechanism of these functional interactions.

Gcn2 is involved in the arrest of cells in G1 phase induced by nitrogen deprivation

It was previously reported that the experimental depletion of nitrogen from the culture medium of S. pombe induced G1 phase cell cycle arrest and a bulk protein
degradation (Nakashima et al., 2006). The finding that both Hri1 and Gcn2 are activated upon nitrogen source deprivation (Fig. 2B) raises the hypothesis that either Hri1 or Gcn2 could be a regulator of the cell cycle in *S. pombe*, such that it would collaborate in the arrest of cells in G1 and in the induction of protein degradation, which is required for conjugation and, ultimately, sexual differentiation. To address this notion, we estimated the DNA content of cells by flow cytometry to analyze the response of cells lacking different eIF2α kinases to nitrogen starvation compared with that of wild-type strain. In agreement with the hypothesis, all cells lacking Gcn2 exhibited a delay in nitrogen deprivation-induced G1 arrest (Fig. 4A, four right-hand columns) whereas both wild-type and cells containing only Gcn2 generated a significant percentage of cells with 1C DNA content after 4 hours (Fig. 4A, two left-hand columns). This indicates that Gcn2, but not Hri1, is implicated in an efficient nitrogen starvation-induced G1 arrest. Additionally, note that the Δhri2 Δgcn2 mutant showed more delay than the Δgcn2 mutant in the G1 arrest, suggesting that Hri2 also has a minor role in the nitrogen deprivation-induced G1 arrest. It is of interest to note that *gcn2*-disruption mutants, which showed a delayed entry in the G1 phase, also showed a delay in the previously described protein degradation process triggered during nitrogen starvation (Nakashima et al., 2006) (Fig. 4B and supplementary material Fig. S1).

**Cells lacking phosphorylation at serine 52 (eIF2αS52A) are defective in nitrogen starvation-induced G1 arrest, size shortening and mating**

It is well known that *S. pombe* wild-type cells in the absence of nitrogen source divide twice, producing short and round cells, and arrest at a temporal G1 phase followed by meiosis or entry into quiescent G0 phase depending on the presence or absence of mating (Yanagida, 2009). Moreover, for several mutants of kinases, including MAPK Sty1, this cell shortening does not normally occur and, thus, mitotic onset is clearly delayed and cells keep their rod shape (Petersen and Nurse, 2007; Sajiki et al., 2009). We have shown before that Gcn2 is involved in nitrogen starvation-induced growth arrest in G1 phase together with a pronounced protein degradation process. We further wanted to investigate whether eIF2α phosphorylation contributed or not to the modulation of the cell cycle observed in wild-type and Gcn2-expressing cells when grown under nitrogen starvation. To address this question, we used again flow cytometry to analyze the response of an eIF2αS52A strain to nitrogen starvation.
compared with that of wild-type strain. Interestingly, eIF2αS52A cells exhibited a very strong delay in the nitrogen deprivation-induced G1 arrest, thus, as shown in Fig. 5A, wild-type cells began to accumulate with 1C DNA content about 4 hours after nitrogen starvation, whereas in the eIF2αS52A mutant no G1-arrested cells were observed within 6 hours. In good agreement with this result, the eIF2αS52A strain showed also a significant decrease in protein degradation during nitrogen starvation (Fig. 5B).

As described previously, nitrogen source deficiency induced cell size shortening of wild-type cells. In contrast, the eIF2αS52A mutant showed no change in cell shape even 24 hours after shifting to a medium without nitrogen but underwent two rounds of divisions (Fig. 5C). This phenotype of long rod-shape mutant cells resembled that of the styl1 and wis1 deletion mutants (Sajiki et al., 2009). Other mutants (cdc2, cdc13 and ssp1) also revealed rod-shaped cells (Yanagida et al., 2011). In addition, it was well established that the Sty1-Atf1 pathway became active upon nitrogen deprivation and had an important role in the initiation of mating and meiosis programmes through regulation of Ste11 levels, a master regulator of the mating pathway (Sugimoto et al., 1991). In order to test the relative importance of the MAPK Sty1 and eIF2α kinase pathways for sexual differentiation, we analyzed the mating efficiency of homothallic h⁹⁰ S. pombe cells in wild-type, Δstyl and eIF2αS52A backgrounds. The mating efficiency of mutant cells, expressed as the percentage of cells undergoing mating, was very similar and approximately fourfold lower compared with that of otherwise-wild-type isogenic cells after 48 hours of mating (Fig. 5D). We thus conclude that in the fission yeast S. pombe, depletion of nitrogen from culture medium induces G1 arrest and subsequent sexual differentiation in an eIF2α serine52 (eIF2αS52)-dependent way.

The presence of Gcn2 and eIF2αS52 is involved in cell viability under oxidative stress and glucose limitation conditions

We reported previously that Sty1, but not the eIF2α kinases, is essential for survival of cells growing on rich medium under oxidative stress. Furthermore, in contrast to oxidative stress, neither Sty1 nor eIF2α kinases are essential for survival under MMS-induced DNA damage stress conditions (Berlanga et al., 2010). Now, we wanted to investigate whether eIF2α phosphorylation contributed to the loss of viability of cells growing in a synthetic minimal medium (EMM) under several stress conditions. As shown in Fig. 6A, elimination of the MAPK Sty1 caused high sensitivity to
hydrogen peroxide. Notably, all cells lacking gcna grown in EMM were severely compromised; however, none of the gcna-expressing mutants exhibited any difference in viability compared to wild-type cells under oxidative stress. When we grew wild-type and mutant cells in minimal medium containing 0.1% glucose, the respective viability of each strain was the same as with hydrogen peroxide (Fig. 6A and supplementary material Fig. S2A), except for Δstyl strain, which showed a limited sensitivity to glucose limitation. Thus, both Sty1 and Gcn2 play a role in cell survival under oxidative stress; only Gcn2 is essential for cell survival under glucose limitation; and neither SAPK nor eIF2α kinase pathways do appear to play any role in cell survival under conditions of genotoxic stress caused by MMS in S. pombe, when compared with a sensitive Δrad3 strain (Perez-Hidalgo et al., 2008) (Fig. 6A and supplementary material Fig. S2B).

To test whether the eIF2α phosphorylation is required for stress survival, we compared the phenotypes of cells lacking all three eIF2α kinases and the eIF2αS52A cells under oxidative stress and glucose limitation conditions. Interestingly, both cell types showed severely impaired survival, thus, an eIF2αS52A strain showed stress sensitivity similar to cells lacking Gcn2 or all three eIF2α kinases (Fig. 6B). As previously reported, a mutant expressing Gcn2 as the only eIF2α kinase was markedly less sensitive to both stresses, in fact, these cells showed a behavior with survival efficiencies closer to those of the wild-type cells (Fig. 6B). These results support the notion that in S. pombe, the presence of Gcn2 and eIF2αS52 is essential for cell survival under oxidative stress and glucose limitation conditions. Additional preliminary experiments (supplementary material Fig. S2C) suggest that eIF2α phosphorylation could contribute to the lifespan extension induced by calorie restriction which is dependent of the MAP kinase Sty1 (Zuin et al., 2010).

**DISCUSSION**

The results presented here provide important insights into the translational responses of fission yeast to nutritional stresses. It is well known that eIF2α phosphorylation can promote changes in gene expression through preferential translation of stress response genes (Harding et al., 2000; Hinnebusch, 1997).
Moreover, three protein kinases (Hri1, Hri2 and Gcn2) could be responsible for the phosphorylation of eIF2α in *Schizosaccharomyces pombe* and a differential activation of these eIF2α kinases has been found in response to distinct cellular stresses (Berlanga et al., 2010; Zhan et al., 2004; Zhan et al., 2002). Thus, Gcn2 is activated early in response to H$_2$O$_2$ whereas Hri2 is activated early under exposure to high temperatures (Berlanga et al., 2010). In addition, the UV-induced phosphorylation of eIF2α is dependent on Gcn2 (Tvegard et al., 2007).

In this paper, we show that eIF2α phosphorylation is strongly induced when cells reached stationary phase. Our results demonstrate for the first time that Hri1 is mainly activated, and its expression up-regulated, at the onset of the stationary phase of cell growth. It is known that the transition from exponential to stationary growth is caused by nutrient starvation, mainly by the decreases in glucose or nitrogen source concentrations. Our experiments demonstrate that after exposure to both stresses, elevated levels of eIF2α phosphorylation were observed. Again, we found a differential activation of the distinct eIF2α kinases in response to nutrient starvation. Thus, Hri2 is the primary eIF2α kinase activated upon glucose starvation, whereas Hri1 and Gcn2 are mainly involved in the response to nitrogen depletion. These findings represent what is to our knowledge the first example of Hri1 function in fission yeast. Previous reports showed that during transition to the stationary phase, Sty1 also becomes activated and triggers a transcriptional stress programme (Zuin et al., 2010; Zuin et al., 2005). Moreover, Sty1 is required for, and can be activated by different stress conditions, including hydrogen peroxide and glucose or nitrogen starvation (Vivancos et al., 2006). Previous findings indicate that Sty1 negatively regulates Gcn2 and Hri2 kinases under conditions of oxidative stress (Berlanga et al., 2010). Now, our studies also suggest a possible relationship between SAPK and eIF2α kinase pathways, demonstrating for the first time that the MAPK Sty1 negatively regulates eIF2α kinase activity under conditions of nitrogen starvation. On the other hand, our observations indicate that only in the strains showing an early and transient activation of Gcn2 occurs an increase of Sty1 phosphorylation under nitrogen starvation at late times of stress treatment. Note that such Sty1 activation was accompanied with a significant decrease of the Gcn2-mediated eIF2α phosphorylation. Our results agree with a previous report suggesting that Gcn2, as downstream effector of TOR signalling, regulates the Pyp2 phosphatase that in turn modulates Sty1 activity, when a cell culture was shifted from a good
nitrogen source (glutamate) to a poor nitrogen source (proline) (Petersen and Nurse, 2007). Nevertheless, the mechanistic insights of such an observation remain to be clarified.

Previous reports showed that Tor1 is required for the response to nutrient starvation leading to sexual differentiation. Thus, under nitrogen starvation, tor1 cells promote two rounds of cell division with a decrease in their cell length. The resultant small cells are arrested at the G1 phase before initiation of sexual development whereas cells lacking Tor1 are incapable of G1 arrest and are therefore almost sterile (Kawai et al., 2001). Moreover, it has been suggested that this nutrient-induced activation onset is brought about by changes in Tor1 signalling 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). Very recently, it has been shown 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 (Petersen and Nurse, 2007; Valbuena et al., 2012). Additionally, UV-irradiation of fission yeast cells in the G1 phase induced a transient delay in S-phase entry that is totally dependent of the Gcn2 kinase (Krohn et al., 2008; Tvegard et al., 2007). Our data are consistent with those results but further indicate that cells lacking eIF2αS52 phosphorylation are defective in nitrogen starvation-induced G1 arrest, size shortening and mating. We have shown that Gcn2 is implicated in the proper arrest of cells in G1 phase and we have also observed that cells lacking Gcn2 show a lower level, or a delay, in the protein degradation induced by nitrogen starvation previously described (Mukaiyama et al., 2009; Nakashima et al., 2006). It should be noted that, although both Hri1 and Gcn2 are activated upon nitrogen source deprivation, just Gcn2 appears to be required for the proper arrest of cells in the G1 phase. In fact, we detected a differential activation of these two eIF2α kinases. Thus, cells expressing Hri1 showed a moderate increase in the level of eIF2α phosphorylation within 30 min, which was sustained for at least 120 min, whereas in the Gcn2-expressing cells, the significant peak of eIF2α phosphorylation, observed between 30 and 60 min after nitrogen starvation, decreased rapidly at subsequent time points. Interestingly, this dramatic decrease in eIF2α phosphorylation observed in Gcn2-expressing cells is coincident with a significant Sty1 activation. Perhaps, the transient induction of the catalytic activity of Gcn2 and the subsequent
Sty1 activation could promote the cell cycle arrest in the G1 phase under nitrogen starvation conditions.

Moreover, our data indicate that during nitrogen starvation, a large part of the wild-type cells became arrested at the G1 phase; in contrast, in the eIF2αS52A strain, most cells remained at G2 throughout a large period of starvation, although finally became arrested at the G1 phase. Also, whereas the wild-type cells became shorter, in the eIF2αS52A strain, the cell length remained constant during nitrogen starvation. In good agreement with these results, we observed that the mating efficiency of mutant cells was very similar to that of the Sty1-deficient strain but about fourfold lower compared with that of wild-type cells. Therefore, we believe that Gcn2-mediated eIF2α phosphorylation plays an important role in the response to nitrogen starvation.

It is noteworthy that, under nitrogen starvation, the phenotype showed in the eIF2S52A strain is very similar to that previously reported in cells depleted of Cdc2, Cdc13 or Ssp1 (Yanagida et al., 2011). Thus, it is well known that, under nitrogen source starvation, cell size determination is considerably modulated, and cell size shortening occurs for wild-type cells. For several mutants of kinases including Tor1, Sty1, Wis1, Cdc2, Cdc13 and Ssp1, this cell shortening does not normally occur (Kawai et al., 2001; Petersen and Nurse, 2007; Yanagida et al., 2011). Thus, resulting long rod-shaped sty1 and wis1 mutant cells displayed an abnormally expanded nucleus, whereas other mutants (cdc2, cdc13 and ssp1) also revealed rod-shaped cells, but maintained a normal-sized nucleus (Yanagida et al., 2011). On the other hand, previous studies revealed that eIF2α phosphorylation can promote changes in gene expression through preferential translation of stress response genes. Thus, both GCN4 and ATF4 mRNAs are preferentially translated in response to phosphorylation of eIF2α by a mechanism involving upstream open reading frames (uORFs) (Harding et al., 2000; Hinnebusch, 1997). It is thus plausible to speculate that Cdc2, Cdc13 or Ssp1 may be potential targets for eIF2α phosphorylation translational regulation by the eIF2α kinase pathway in S. pombe.

We have proposed a regulatory coordination between the SAPK and eIF2α kinase pathways and we found that Gcn2 activation leads to an increase in Sty1 activity under nitrogen starvation conditions. In addition, it was previously reported that TOR signalling modulates mitotic onset through the stress MAPK pathway (Petersen and Nurse, 2007). Therefore, we believe that the Gcn2-mediated eIF2α phosphorylation
could be involved in the tight coordination between the Tor1 and Sty1 pathways for the nutrient-induced activation of mitotic onset. Whereas activation of Gcn2 during nitrogen starvation could be explained by the highly probable increase of uncharged tRNA levels, as a consequence of low amino acid availability, the mechanisms involved in the activation of eIF2α kinases under other stress situations are still unknown.

We have shown that the eIF2αS52A mutant was unable to promote cell cycle arrest and to initiate sexual differentiation upon nitrogen starvation and failed to enter the quiescent state. On the other hand, hydrogen peroxide induces a Gcn2-dependent delay in the progression from the G1 to the S phase. It appears that the duration of the delay of entry into S phase correlates with the duration of an increase in the level of eIF2α phosphorylation (Krohn et al., 2008). In good agreement with these data, we found here that both Gcn2 and eIF2α.S52 are essential for cell survival under oxidative stress and glucose limitation conditions, probably due to their involvement in the proper arrest of cell cycle. Thus, both gcn2-disrupted and eIF2αS52A cells lost viability compared to wild-type cells under both types of stress conditions. To our surprise, Hri2, the primary eIF2α kinase activated in response to glucose limitation did not play any role in cell survival under this stress condition. Thus, the Δhri1 Δhri2 mutant showed similar survival efficiency compared to wild-type cells. It is possible that Gcn2, but not Hri1 and Hri2, targets other proteins that affect cell viability under glucose limitation and participate in cell cycle arrest upon nitrogen starvation. In addition, eIF2α phosphorylation does not seem to be required for the divisions per se after the shift to nitrogen deficiency, but is required for the proper arrest of cell growth under the stress conditions promoted by nitrogen starvation and for the development of characteristics of quiescent cells e.g. round cell shape, small nucleus and 1C DNA. The details of how Gcn2 plays such role and how its major known activity, eIF2α phosphorylation, contributes to cell cycle delay remain to be explored.

MATERIALS AND METHODS

Yeast strains and growth conditions

The strains of S. pombe used in this study are listed in Table 1. Yeast media and general methods were as described (Moreno et al., 1991). S. pombe strains were grown
at 32ºC until they reached logarithmic phase in yeast extract plus supplements medium (YES, Bio 101) with all 20 amino acids (225 g/ml), or at 30ºC in Edinburgh minimal medium (EMM). The addition of the amino acids to the YES medium assures minimal phosphorylation of eIF2α in non-stressed conditions.

Stress assays

For stationary phase conditions strains were grown at 32ºC in YES medium until they reached 0.5, 1, 2, 3 and 4 units of optical density at 595 nm (OD595).

Cells were subjected to stress when they reached logarithmic phase, OD595 of 0.6 for YES medium cultures or OD595 of 0.25 for EMM cultures. Cells were washed twice and re-suspended in YES without glucose (YES-G) to produce glucose starvation or in EMM without NH4Cl (EMM-N) to induce nitrogen starvation. At the end of the stress treatment cells were collected by centrifugation at 3,000 x g for 3 min and frozen immediately on dry ice. Cell pellets were resuspended in lysis buffer (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1% (v/v) Triton-X100, 25 μg/ml of DNase, protease and phosphatase inhibitors cocktails [Complete® and Phostop®, Roche]) and broken in the presence of acid-washed glass beads (Sigma) by using a FastPrep®-24 (MP Biomedicals), in four cycles of 32 seconds at a speed of 6 m/s. The lysates were clarified by centrifugation at 12,000 x g at 4ºC for 15 min and stored at 70ºC. Protein determination was performed by using a Bio-Rad protein assay according to the manufacturer’s instructions.

In order to induce cell cycle arrest in G1 phase, cells were cultivated in EMM and, when the culture reached an OD595 of 0.2, were shifted to EMM-N for different periods of time. Cells were harvested at 3, 4, 5, 6 and 24 hours after the shift, and processed to produce cell extracts, for western blot analysis, or to analyze their DNA content by flow cytometry.

For stress experiments on plates, the same number of cells (10⁶–10⁷) in 3 μl was spotted on EMM agar plates from cultures grown in EMM at the logarithmic phase (OD595 of 0.25). The spots were allowed to dry and the plates were incubated at 32ºC for 2–3 days. For oxidative stress, cells were grown in the presence of 0.75 mM H2O2; for low glucose conditions, in EMM containing 0.1% of glucose (EMM-G); and for genotoxic stress, in the presence of 0.02% MMS.

To induce mating, cells were cultivated in ME (Malte extract) agar plates at 25ºC for 48 hours. Tetrads, zygotes and cells were quantified by microscopic observation.
using a Neubauer chamber. The formula for calculating conjugation efficiency was $2Z/(2Z + A)$, where $Z$ is the number of zygotes plus asci formed and $A$ is the number of non-mating cells expressed as a percentage (Rodriguez-Sanchez et al., 2011). At least 200 cells were counted in each culture.

**Immunoprecipitation, electrophoresis, and immunoblotting**

For immunoprecipitation, cell lysates containing 1 mg of total protein were incubated for 16 hours at 4°C with 10 µg of anti-HA monoclonal antibody (Covance) and protein G-Sepharose (Pharmacia-Biotech). After extensive washing with lysis buffer, immune complexes were equilibrated in kinase buffer and their kinase activity was assayed (see below). Protein extracts (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). For immunoblots, the antibodies used were rabbit anti-eIF2α (sc-11386, Santa Cruz), rabbit anti-p38-P (Signalway Antibodies), rabbit anti-eIF2α-P (Cell Signaling), rabbit anti-cdc2-P (Cell Signaling), rabbit anti-Hog1 (sc-9079, Santa Cruz) and mouse anti-cdc2 (Abcam), followed by rabbit or mouse secondary antibody conjugated with horseradish peroxidase (Promega). After extensive washing, the immunoreactive bands were detected by enhanced chemiluminescence (ECL, GE Healthcare).

**In vitro phosphorylation assays**

The washed immune complexes containing HA-tagged Hri1, Hri2 and Gcn2 proteins were assayed for their ability to autophosphorylate as reported previously (Mendez and de Haro, 1994; Santoyo et al., 1997), with modifications as described. In a total volume of 20 µl, immune complexes were incubated for 30 min at 30°C in kinase buffer (20 mM Tris-HCl pH 7.6, 2.5 mM magnesium acetate, 0.25 mg/ml bovine serum albumin, 50 µM ATP) containing 3 µCi of $\gamma$-[$^{32}$P]ATP (3,000 Ci/mmol). Incubations were terminated by the addition of SDS-PAGE sample buffer. Proteins were resolved on a 9% SDS-PAGE and transferred to a PVDF membrane. Phosphoproteins present on the membrane were detected by autoradiography using Amersham Hyperfilm™ MP (GE Healthcare) films and an Amersham-Pharmacia Biotech intensifying screen. Then HA-tagged proteins were detected by immunoblotting using anti-HA antibody.

**Flow cytometry**
Flow cytometry was used to estimate the relative DNA content of fission yeast cells, and define the cell cycle stage of the population. Cells present in 1 ml of culture, growing under nitrogen starvation conditions, were collected by centrifugation at 1,500 x g for 3 min. Then cells were washed and incubated in ethanol at 4°C for at least 24 hours. After washing cells were resuspended in 50 mM sodium citrate pH 7.0 containing 250 mg/ml of RNase A (Roche). After 4 hours incubation at 37°C, propidium iodine (Sigma) was added to each sample to reach a concentration of 2.5 µg/ml. Cell preparations were sonicated for 20 seconds, in order to break cellular aggregates, before to be analyzed in a flow cytometer FACSCalibur (Becton Dickinson) and data analysis was performed using Cell Quest Pro and Flow Jo 6.4.1 software.

Optical microscopy

Cells were fixed in 70% ethanol for 24 hours and rehydrated in water. Small aliquots (3 µl) of the fixed cells were attached onto glass slides by heating at 70°C. Then cells were stained adding 3-4 µl of 1 µg/ml of 4,6-Diamidino-2-phenylindole (DAPI) in Mowiol (Calbiochem) and a glass coverslip was placed over them. A fluorescence microscope (Zeiss) with a 100x objective was used, and images were captured with a CCD Coolsnap FX color camera (Roper Scientific).

ACKNOWLEDGEMENTS

We thank Paul Nurse, Sergio Moreno, Elena Hidalgo, Erik Boye and Miguel A. Rodríguez-Gabriel for the S. pombe strains. We thank Sandra López-Aviles, Sergio Moreno and Miguel A. Rodríguez-Gabriel for valuable comments on the manuscript and for technical advice. We also thank José Alcalde for excellent technical assistance. This work was supported in part by grants BFU2007-62987 (to C de H), and BFU2009-09469 (to JJB) from the DGICYT, a grant for young scientists from CIBERehd (to JJB), a grant from the Fundación Ramón Areces (to C de H), and by an institutional grant from the Fundación Ramón Areces to the Centro de Biología Molecular Severo Ochoa.
REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Hri1 is activated at the onset of the stationary phase of growth. (A) eIF2α phosphorylation increases at the onset of the stationary phase. Wild-type *S. pombe* cells or cells of strains lacking the different eIF2α kinases were maintained in culture until they reached the optical densities indicated in the figure. Phosphorylation of eIF2α was analyzed in cell extracts by immunoblot with phospho-specific antibodies, as described in Materials and Methods. (B) The growth of *S. pombe* cells used in A) was monitored for 24 hours by measuring OD at 595 nm. (C) Levels of Hri1, Hri2 and Gcn2 proteins and kinase activity increased at the onset of the stationary phase. *S. pombe* cells of strains expressing endogenous HA-tagged Hri1, Hri2 or Gcn2 were maintained in...
culture until they reached the optical densities indicated in the figure. Extracts from
these *S. pombe* cells were subjected to immunoprecipitation with anti-HA antibody. The
immune complexes were subjected to in vitro kinase assay. Then proteins were resolved
by SDS-PAGE and transferred to PDVF membranes. Phosphoproteins were evidenced
by autoradiography (upper panel). The presence of HA-tagged proteins in the immune
complexes was detected by immunoblot using anti-HA antibody (middle panel).
Phosphorylation of eIF2α was analyzed in cell extracts by immunoblot with phospho-
specific antibodies, and immunoblots of eIF2α and Cdc2 were used as loading controls
(lower panel). Shown are results representative of at least three independent
experiments.

**Figure 2.** Differential activation of the distinct eIF2α kinases in response to
nutrient starvation. (A) Hri2 is the primarily eIF2α kinase activated in response to
glucose starvation. (B) Hri1 and Gcn2 are involved in the response to nitrogen
depletion. Wild-type *S. pombe* cells or cells of strains lacking two of the three eIF2α
kinases, as indicated, were kept in the absence of glucose (A) or nitrogen (B) for
different periods of time. Phosphorylation of eIF2α was analyzed in the cell extracts by
immunoblot with phospho-specific antibodies, as described in Materials and Methods.
Shown are the duration of the stress (top), the names of the strains (top) and the
antibodies used for the immunoblot (left). Results are representative of at least three
independent experiments.

**Figure 3.** Effect of nitrogen starvation on eIF2α and Sty1 phosphorylation. (A)
Wild-type *S. pombe* cells or cells expressing one of the three eIF2α kinases, as
indicated, were subjected to nitrogen starvation for different periods of time. The
phosphorylation state of eIF2α and Sty1 (eIF2α-P and Sty1-P) were analyzed by
immunoblot. Immunoblots of eIF2α, Sty1 and Cdc2 were used as loading controls (B)
Wild-type cells or cells expressing one of the three eIF2α kinases, lacking or not Sty1,
were subjected to nitrogen starvation for different periods of time. Phosphorylation of
eIF2α and Sty1 were analyzed in the cell extracts by immunoblot with phospho-specific
antibodies. Shown are results representative of at least three independent experiments.

**Figure 4.** Gcn2 is involved in cell cycle arrest in G1 phase under nitrogen
starvation. Wild-type *S. pombe* cells or cells of strains lacking the different eIF2α
kinases, as indicated, were subjected to nitrogen starvation for different periods of time. (A) Cells were fixed in ethanol at the indicated times and the DNA content was quantified by flow cytometry analysis, as described in Materials and Methods. (B) Immunoblot analysis of the phosphorylation state of eIF2α and Cdc2 (eIF2α-P and Cdc2-P), and of the total amount of both proteins (eIF2α and Cdc2), in cell extracts. Results are representative of at least three independent experiments.

Figure 5. eIF2α phosphorylation during nitrogen starvation is involved in G1 arrest. Wild-type cells and cells of the strain eIF2αS52A were kept in the absence of nitrogen during the indicated time periods. (A) Flow cytometry analysis of DNA content in fixed cells, as described in figure 4. (B) Immunoblot analysis of eIF2α and Cdc2 phosphorylation, and of the total amount of these proteins, using specific antibodies. (C) Differential interphase contrast (DIC) and DAPI staining images of wild-type and eIF2αS52A fixed cells after growth in nitrogen starvation conditions for the indicated times. (D) eIF2α phosphorylation promotes mating capacity in fission yeast. Homothallic wild-type, Δsty1 and eIF2αS52A cells were plated in sporulation MEA medium and incubated for 48 hours at 25°C. Afterwards mating efficiency was calculated, as described in Materials and Methods. Results represent the mean ± standard deviation of four independent experiments.

Figure 6. Gcn2 and eIF2α phosphorylation promote cell survival under different stress situations. Serial dilutions of logarithmic phase growing cultures of wild-type S. pombe cells, cells lacking the different eIF2α kinases or cells of the Δsty1 and eIF2αS52A strains, were plated in minimal medium (EMM) and in minimal medium containing hydrogen peroxide (0.75 mM) or MMS (0.02%) and in low glucose minimal medium (EMM-G + 0.1% glucose), and grown for 72 hours at 32°C. (A) Cells lacking Gcn2 are more sensitive to the presence of hydrogen peroxide or low glucose conditions, but not to MMS exposure. (B) eIF2α phosphorylation improves cell viability during oxidative stress or low glucose conditions. Results are representative of at least three independent experiments.
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32P - Autoradiography

Western blot anti-HA

Cell extracts

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Cdc2
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