Lifespan extension by calorie restriction relies on the Sty1 MAP kinase stress pathway

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Either calorie restriction, loss-of-function of the nutrient-dependent PKA or TOR/SCH9 pathways, or activation of stress defences improves longevity in different eukaryotes. However, the molecular links between glucose depletion, nutrient-dependent pathways and stress responses are unknown. Here, we show that either calorie restriction or inactivation of nutrient-dependent pathways induces lifespan extension in fission yeast, and that such effect is dependent on the activation of the stress-dependent Sty1 mitogen-activated protein (MAP) kinase. 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. Deletion of the genes coding for the SCH9-homologue, Sck2 or the Pka1 kinases, or mutations leading to constitutive activation of the Sty1 stress pathway increase lifespan under glucose-rich conditions, and importantly such beneficial effects depend ultimately on Sty1. Furthermore, cells lacking Pka1 display enhanced oxygen consumption and Sty1 activation under glucose-rich conditions. We conclude that calorie restriction favours oxidative metabolism, reactive oxygen species production and Sty1 MAP kinase activation, and this stress pathway favours lifespan extension.

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Introduction

The molecular mechanisms that regulate cellular aging have been shown to be partially conserved throughout evolution, and studies with the yeast *Saccharomyces cerevisiae* have made it possible to identify signalling pathways and media

conditions, which regulate fitness and life extension (for a review, see Kaeblerlein *et al.*, 2007). In particular, chronological aging in the budding yeast has been defined as the viability of non-dividing cells after transition to the stationary phase, mimicking the aging process of non-proliferating cells in pluricellular organisms.

The aging process can be regulated both with dietary interventions and genetically (for a review, see Bishop and Guarente, 2007). Several studies have shown a direct link between environmental nutrients and longevity. Thus, reducing glucose from the culture media can increase the chronological lifespan in yeast (Reverter-Branchat *et al.*, 2004). This situation has been referred to as dietary or calorie restriction. Several genome-wide screenings have recently made it possible to start unravelling the molecular mechanisms that underlie longevity promotion by calorie restriction. In budding yeast, two nutrient-responsive pathways, led by SCH9 (a component of the TOR pathway) and protein kinase A, seem to coordinate the cellular response to altered glucose and nitrogen levels (Fabrizio *et al.*, 2001; Powers *et al.*, 2006; Wei *et al.*, 2008). Chemical or genetic inhibition of either of these two pathways leads to chronological lifespan extension, even under glucose-rich conditions. Similar results have recently been reported in *Schizosaccharomyces pombe* (Weisman and Choder, 2001; Roux *et al.*, 2006, 2009). However, the downstream effectors that trigger the required biological changes have not been clearly defined.

Another genetic and environmental intervention traditionally linked to life extension is the activation of adaptive, defensive anti-stress responses (Kaeblerlein *et al.*, 2007; Gems and Partridge, 2008; Morimoto, 2008). These cellular programmes, triggered upon environmental stressors, could through gene expression prepare cells against the molecular damage associated to aging. Thus, reactive oxygen species (ROS) have been repeatedly hypothesized to trigger cellular responses meant to enhance stress defences and probably extend lifespan (Kharade *et al.*, 2005; Schulz *et al.*, 2007). However, oxidative stress had also been postulated to be the main cause of the molecular damage associated to aging (for a review, see Muller *et al.*, 2007). Therefore, ROS formation and accumulation have been connected to either fitness promotion and lifespan or to systemic molecular damage and reduced life expectancy.

The Pka1 pathway has been repeatedly isolated in genetic screens as essential for entry and maintenance of viability at stationary phase in the fission yeast (Byrne and Hoffman, 1993; Nocero *et al.*, 1994; Yamamoto, 1996; Stiefel *et al.*, 2004; Wang *et al.*, 2005). During growth in the presence of glucose, the Pka1 kinase phosphorylates and inactivates the transcription factor Rst2 (Higuchi *et al.*, 2002). On nutrient starvation, the levels of cyclic AMP decrease and allow the interaction of Cgs1, the regulatory subunit of protein kinase A, with the catalytic subunit Pka1, leading to the inactivation of the kinase (for a review, see Hoffman, 2005). Hypophosphorylated Rst2 accumulates at the nucleus and triggers transcription of

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genes such as *fbp1*, coding for the gluconeogenic enzyme fructose-1,6-bisphosphatase (Hoffman and Winston, 1991; Higuchi *et al*, 2002). Expression of *fbp1* and other PKA-dependent genes in the presence of glucose and/or nitrogen can be genetically de-repressed by deletion of the *pkal* gene (Hoffman and Winston, 1991), whereas cells lacking *Cgs1* cannot induce the expression of these genes on nutrient starvation (Wu and McLeod, 1995).

However, in *S. pombe* most genetic screenings suggest that not only the cyclic AMP-dependent Pka1 pathway but also the mitogen-activated protein (MAP) kinase Sty1 pathway participates in the maintenance of viability of starved cells (for a review, see Kronstad *et al*, 1998). Furthermore, several reports indicate that these two pathways antagonistically regulate several processes such as mating and adaptation to nutrient starvation (Stettler *et al*, 1996; Neely and Hoffman, 2000). Sty1, also known as Spc1 and Phh1, is required for, and can be activated by different types of stress conditions, including hydrogen peroxide (H_2O_2) and glucose or nitrogen starvation (for a review, see Vivancos *et al*, 2006). On any type of stress, the Sty1 kinase is phosphorylated by Wis1 and translocated to the nucleus, where it activates a battery of genes in an Atf1-dependent manner (Shiozaki and Russell, 1995, 1996; Takeda *et al*, 1995; Wilkinson *et al*, 1996). Atf1, a heterodimeric transcription factor, is bound to stress promoters before activation, and only induces transcription of the stress genes once Sty1 becomes phosphorylated (Chen *et al*, 2008). One of the genes activated by the Atf1 transcription factor in a stress- and Sty1-dependent manner is *atf1* (Chen *et al*, 2008); however, over-expression of Atf1 *per se* is not sufficient to induce the stress response (Sanzo *et al*, 2008), what implies that a phosphorylation event downstream of the Sty1 kinase is required for gene induction. In fact, on stress-mediated Sty1 activation, both Atf1 phosphorylation (as determined by a shift in electrophoretic mobility) and enhanced Atf1 protein levels can be detected in protein extracts (Lawrence *et al*, 2007; Sanzo *et al*, 2008). The com-

mon environmental stress response triggered by Sty1 and Atf1 is intended to allow survival of cells on stress imposition, as a number of proteins facilitate repair of the damage and promote adaptation to or decrease in the insult. However, the role of Sty1 in adaptation to starvation is not known.

We show here that either low-glucose media or inactivation of the nutrient-dependent Pka1 pathway promotes life extension in fission yeast by inducing (in order of self-activating events): high respiratory rates, elevated ROS production, activation of the MAP kinase Sty1 pathway and expression of survival genes, which will promote elongation of lifespan. Our results show for the first time that activation of an MAP kinase by calorie restriction participates in life-span extension.

Results

We have reported earlier that Sty1 is activated at the onset of stationary phase: the MAP kinase is phosphorylated, accumulates in the nucleus and promotes the transcription of stress-dependent genes (Zuin *et al*, 2005). As shown in Supplementary Figure 1A, Sty1 (WT + pRep41x or $\Delta sty1$ + *psty1*) is required to maintain viability of cultures 3 days after reaching stationary phase. Expression of a kinase-dead Sty1 mutant is not sufficient to enable the cells to survive at stationary phase (Supplementary Figure 1A, $\Delta sty1$ + pSty1.K49R).

To find out how the media composition affects the fitness of *S. pombe* cultures at stationary phase, we grew yeast in the two most commonly used glucose-containing laboratory media, MM (also known as defined medium or synthetic minimal medium, containing 2% glucose) and YE (also known as rich or complex medium, with 3% glucose) (Alfa *et al*, 1993). Unlike what happened in MM, the viability of wild-type cells grown in YE-3% glucose was severely compromised at days 3–5 after reaching the stationary phase (Figure 1A, WT). The absence of Sty1 decreased the lifespan of cells grown in MM, but barely impaired the already

Figure 1 Sty1-dependent lifespan promotion only occurs on calorie restriction. (A) Growth in minimal media (MM; 2% glucose), but not in complex media (YE; 3% glucose), induces life extension in a Sty1-dependent manner. Strains 972 (WT) and AV18 ($\Delta sty1$) were grown in standard MM or YE-3% glucose media. Serial dilutions of logarithmic (Log) or stationary phase (Day 6) cultures were spotted onto YE plates. (B) Concentrations of glucose below 1% in YE media induce chronological lifespan extension. Strain 972 (WT) was grown in MM, or in YE with the indicated concentrations of glucose (Glu). Serial dilutions of logarithmic (Log) and stationary phase (Days 4 and 5) cultures were spotted onto YE plates. (C) Life extension by low glucose concentrations is dependent on Sty1. Survival percentages of wild-type strain 972 (WT) and AV18 ($\Delta sty1$) are indicated in the graphs.

compromised viability of yeast cultures grown in YE (Figure 1A, $\Delta sty1$). To test the hypothesis that carbon source availability was the critical differential factor between YE and MM, we grew wild-type and $\Delta sty1$ cells in YE media supplemented with different concentrations of glucose. The Sty1-dependent improvement of lifespan by growth in MM could be similarly accomplished when yeast cells were grown in YE media with concentrations of glucose below 1% (Figure 1B and C). We concluded that growth in MM or in YE-1% glucose, conditions which in *S. pombe* could be defined as calorie restriction, extends the lifespan of fission yeast in an Sty1-dependent manner.

We then analysed glucose consumption and Sty1 activation during the growth of *S. pombe* in YE-1% versus YE-4% glucose media. As observed in Figure 2A, the glucose was exhausted in cultures grown in YE-1% glucose at the time at which the maximum optical density at 600 nm (OD_{600}) was reached, whereas the concentration of glucose was consider-

ably higher (around 0.7%) when the YE-4% glucose media cultures reach the plateau of stationary phase. We analysed Sty1 phosphorylation (Figure 2B) at different points of the growth curves (A-E for YE-1% glucose culture and A'-E' for YE-4% glucose culture; Figure 2A), and determined that such phosphorylation was significantly weaker for the YE-4% glucose culture. Similarly, Sty1 phosphorylation and Sty1-dependent gene response occurred when cells were grown in MM, and it was significantly weaker when cells were grown in YE-3% glucose (Figure 2C-E). We concluded that Sty1 only becomes fully activated at the onset of stationary phase when cells are grown in YE-1% glucose (Figure 2A and B) or in MM (Figure 2C-E), but neither in YE-4% (Figure 2A and B) nor in YE-3% glucose (Figure 2C-E).

A classical marker of fitness on entry of microbial cultures into the stationary phase is the exhibition of enhanced resistance to a variety of stress conditions such as heat shock (Nystrom, 2004). As shown in Figure 3, stationary

Figure 2 Sty1 activation at stationary phase only occurs on calorie restriction. (A) Growth curves and glucose concentrations of YE-1% (calorie restriction) and YE-4% glucose (glucose-rich) wild-type cultures. Wild-type strain (972) was grown in both media; OD_{600} (triangles) and glucose concentration (circles) were recorded at the times indicated in hours. (B) Sty1 is barely activated at stationary phase in cells grown in YE-4% glucose media. At the time points indicated in (A) (A-E for growth in YE-1% and A'-E' for YE-4% cultures), cells were collected, TCA protein extracts were prepared and proteins were analysed by western blot using anti-p38-P (Sty1-P, top panels) or anti-Sty1 antibodies as a loading control (Sty1, bottom panels). As a control of activated Sty1, logarithmic cultures of wild-type cells were treated with 1 mM H_2O_2 for 10 min (H). (C) Growth curves and glucose concentrations of MM (calorie restriction) and YE-3% glucose (glucose-rich) wild-type cultures. OD_{600} and glucose concentrations were recorded as indicated in (A). (D) Sty1 phosphorylation is barely observed in YE-3% glucose culture. Levels of activated Sty1 (Sty1-P) were determined as indicated in (B). (E) Sty1-dependent gene transcription is not activated at stationary phase in cells grown in YE-3% glucose media. RNA from the same time points as in (D) were obtained and hybridized with probes against *atf1*, *gpx1*, *cta1* and *fbp1*.

phase wild-type cells grown in YE-1% glucose media can survive a severe heat shock (2 h at 48°C). This development of stress resistance is not accomplished on growth in YE-4% glucose media or in the absence of Sty1 (Figure 3).

We tested whether life promotion and gain-of-stress resistance on calorie restriction depend on the activation of the main effector of Sty1, the transcription factor Atf1. Activation of Atf1 is essential to trigger the transcriptional anti-stress programme (Chen *et al*, 2008) (Figure 4A). As observed in Figure 4, cells lacking Atf1 are defective in life extension (Figure 4B and C) and gain-of-stress resistance (Figure 4D) by calorie restriction, and over-expression of the transcription factor is not sufficient to promote lifespan extension in cells lacking Sty1 (Supplementary Figure 1B), as reported earlier for the development of stress resistance (Sanzo *et al*, 2008). We concluded that activation of Atf1 by Sty1 is required for life extension by calorie restriction.

To further confirm that Sty1 activation, and not only the presence of Sty1, at the onset of stationary phase is

required for both stress-resistance acquisition and lifespan extension under calorie restriction, we constructed a strain harbouring an analogue-sensitive mutant of Sty1, Sty1.T97A (Gegan *et al*, 2007) (Figure 5). The threonine-to-alanine modification in the ATP-binding pocket of the kinase renders a protein with wild-type properties, until the ATP analogue 1NM-PP1 is added to the growth media. This inhibitory analogue can only bind to this mutant kinase, allowing specific inactivation of the modified kinase *in vivo* (Gegan *et al*, 2007). As shown in Figure 5A, addition of 1NM-PP1 specifically blocked the catalytic activity of the mutant MAP kinase, Sty1.T97A, and inhibited the H₂O₂-dependent activation (both phosphorylation and accumulation) of Atf1. When the inhibitory analogue was added to YE-1% cultures before the stationary phase-dependent Sty1 activation (at an OD₆₀₀ of 2), we significantly inhibited Atf1 activation (Figure 5B), impaired the gain-of-heat-shock resistance (Figure 5C) and decreased the viability of stationary phase cultures (Figure 5D).

Figure 3 Heat shock resistance of stationary phase cells is calorie restriction dependent and Sty1 dependent. Strains 972 (WT) and AV18 (Δ sty1) were grown in YE-1% or YE-4% glucose media. At logarithmic phase (Log), 48 h (Day 2) and 72 h (Day 3) after reaching stationary phase, liquid cultures were heat shocked at 48°C during 2 h or left untreated (Unt.). Serial dilutions of the cultures were spotted onto YE plates.

Figure 4 Activation of the transcription factor Atf1 by Sty1 is required for life extension by calorie restriction. (A) Scheme of the stress-, Sty1-, Atf1-dependent activation of gene expression. (B) Lifespan of cells lacking Atf1. Strains 972 (WT), AV18 (Δ sty1) and AV15 (Δ atf1) were grown in YE-1% glucose media. At the logarithmic phase (Log) or several days after reaching stationary phase (Day 3 and Day 5), serial dilutions of the cultures were plated onto YE plates. (C) Life extension by low glucose concentrations is dependent on Atf1. Survival percentages of strains as in (B) are indicated in the graphs. (D) Heat shock resistance of stationary phase cells is calorie restriction dependent and Atf1 dependent. Strains as in (B) were heat shocked and analysed as described in Figure 3.

Figure 5 Strain AZ107, expressing an analogue-sensitive mutant Sty1, shows shorter viability at stationary phase on addition of the inhibitory ATP analogue 1NM-PP1. **(A)** Addition of 1NM-PP1 inhibits phosphorylation of the Sty1-dependent transcription factor Atf1 on H₂O₂ treatment. Strain AZ107, harbouring the *sty1.T97A* mutation at its genomic locus, was grown in YE-1% glucose. At an OD₆₀₀ of 0.2, 10 μ M 1NM-PP1 was added (1NM-PP1) or not to the cultures for 2 h. Then, when indicated, a 15 min treatment with 1 mM H₂O₂ was performed (H₂O₂) or not (Unt.). The phosphorylation of Sty1 (Sty1-P) and the accumulation/phosphorylation of Atf1 (Atf1 and Atf1-P) were determined from TCA extracts using anti-p38-P and anti-Atf1 antibodies. Anti-Sty1 antibody was used as a loading control (Sty1). **(B)** Addition of 1NM-PP1 to strain AZ107 inhibits calorie restriction-dependent phosphorylation of Atf1 at stationary phase. Cultures of strain AZ107 (*sty1.T97A*), grown in YE-1% glucose media to an OD₆₀₀ of 2, were left untreated or were treated with 10 μ M of 1NM-PP1. Cells were collected at the indicated OD₆₀₀. The accumulation and phosphorylation of Atf1 were determined as in **(A)**. **(C)** Inactivation of Sty1 by 1NM-PP1 impairs heat shock resistance acquisition. Two days (Day 2) after reaching stationary phase, cultures from **(B)** were heat shocked (48°C, 2 h) or not (Unt.), and viability was determined by sequential spotting. **(D)** Inactivation of Sty1 by 1NM-PP1 decreases cell viability during stationary phase. At the logarithmic phase (Log) or several days after reaching stationary phase (Day 3 and Day 5), viability of cultures from **(B)** was assayed by sequential spotting.

Figure 6 Oxygen consumption and H₂O₂ levels of *S. pombe* cells are higher in cells grown in YE-1% than in YE-4% glucose media. **(A)** Oxygen consumption along the growth curve is higher in YE-1% than in YE-4% glucose cultures. Wild-type strain (972) was grown in YE-1% or YE-4% glucose media; OD₆₀₀ (squares) and oxygen consumption (circles) were recorded at different times during the growth curve. **(B)** Relative intracellular H₂O₂ levels of cells grown in YE-1% and YE-4% glucose media. Wild-type strain (972) was grown in YE-1% and YE-4% glucose media to reach the relative OD₆₀₀ indicated on the right y axis (% OD₆₀₀ max). At the indicated time points from the starting OD₆₀₀ (around 5% of the maximum OD₆₀₀ of each culture), cells were incubated with the redox-sensitive dye dihydrorhodamine 123 (DHR123) and with the permeability-dependent dye propidium iodide (PI), and the fluorescence of live cells was analysed by flow cytometry, as described in 'Materials and methods'. The DHR123 green fluorescence was normalized to the PI red fluorescence and to the cell size (left y axis: Relative DHR123/IP/cell size), and all the values are referred to that of YE-4% glucose cultures at 2 h, with an assigned value of 1; s.e.m. of three replicates are indicated.

Oxygen consumption, as an indicator of respiratory rates, is consistently higher in YE-1% glucose than in YE-4% glucose cultures all along the growth curve (Figure 6A), as

it is in MM (Supplementary Figure 2), suggesting that ROS production and ROS steady-state levels are constitutively higher in cells grown under calorie restriction. To verify this

hypothesis, we used the redox-sensitive fluorescent dye dihydrorhodamine 123 (DHR123). DHR123 is an uncharged and non-fluorescent ROS indicator, which can passively diffuse across membranes and then can become oxidized in the cytoplasm to yield cationic rhodamine 123, which localizes in the mitochondria and exhibits green fluorescence. As permeability to extracellular dyes could vary depending on the growth phase, the intensity of the DHR123-dependent green fluorescence was normalized to the red fluorescent values of propidium iodide (PI), which we used as an indicator of the permeability to extracellular dyes. All along the growth curve, the levels of intracellular ROS production were significantly higher for cells growing in YE-1% than in YE-4% glucose (Figure 6B).

To further test our proposal that ROS accumulation triggers Sty1 activation on calorie restriction, we added anti-oxidants to the cultures only during the transition to stationary phase. Most of the anti-oxidants that we tested were not useful in preventing Sty1 activation, as they were also useless to facilitate the aerobic growth of redox-deficient strains such as those lacking superoxide dismutase or thioredoxin peroxidase (our unpublished data). Only the anti-oxidant DL- α -tocopherol seemed to be able to inhibit phosphorylation of Sty1 on calorie restriction; when so, this intervention also

impaired lifespan promotion (Supplementary Figure 3). Therefore, we propose that calorie restriction promotes lifespan through the ROS-dependent activation of the Sty1 pathway.

We analysed the effect of the inactivation of the Pka1 or Sck2 kinases on lifespan in fission yeast. Deletion of the *pka1* or *sck2* genes had little, if any, effect on survival under calorie restriction, neither for YE-1% glucose cultures (Supplementary Figure 4A) nor for MM cultures (Supplementary Figure 4B). However, we observed that genetic inactivation of the Pka1 or Sck2 kinases leads to life extension of *S. pombe* cells under glucose-rich conditions (both in YE-4%, Figure 7A and in YE-3% glucose, Figure 7B) as reported earlier (Roux *et al*, 2006, 2009). As we have shown here that life extension on calorie restriction relies on the MAP kinase Sty1, we tested whether the beneficial effects of Sck2 or Pka1 inactivation on lifespan in rich media could depend on the Sty1 stress pathway. As shown in Figure 7A and B, stationary phase viability of the glucose-rich cultures of the double mutants $\Delta pka1 \Delta sty1$ or $\Delta sck2 \Delta sty1$ was even lower to that of a wild-type strain, indicating that inactivation of the Pka1 or Sck2 kinases prolongs the lifespan in an Sty1-dependent manner. As we have shown that life extension on calorie restriction is dependent on ROS-mediated activation of

Figure 7 Loss-of-function of the glucose-dependent Pka1 kinase triggers enhanced respiratory rates, activation of the Sty1 pathway and life extension at high glucose concentrations. (A) Lack of Sck2 and Pka1 kinases promotes stationary phase cell survival under glucose-rich conditions in a Sty1-dependent manner. Strains 972 (WT), MC22 ($\Delta pka1$), MC24 ($\Delta pka1 \Delta sty1$), MC25 ($\Delta sck2$) and MC27 ($\Delta sck2 \Delta sty1$) were grown in YE-4% media. Serial dilutions of the logarithmic (Log) and stationary phase (Day 5) cultures were spotted onto YE plates. (B) Survival percentages of strains as in (A) grown in YE-3% glucose media are indicated in the graph. Similar results were obtained with three different biological replicates. (C) Oxygen consumption of wild-type and *pka1* cells. Oxygen consumption of strains 972 (WT), grown in YE-1% and YE-4% glucose media, and MC22 ($\Delta pka1$), grown in YE-4%, was determined from logarithmic cultures at low OD₆₀₀ (10–30% of the maximum OD₆₀₀); s.e.m. of seven replicates are indicated. Significant difference between wild-type and *pka1* cells respiration in YE-4% glucose was determined by the Student's *t*-test. **P* < 0.05. (D) *pka1* cells show enhanced levels of phosphorylated Sty1 and increased protein levels of Atf1. Strains as in (C) were grown in YE-4% media. As a control of activated Sty1, wild-type cells at the logarithmic phase were treated with 1 mM H₂O₂ for 10 min (H₂O₂), or were left untreated (Unt.). Western blot analysis of TCA extracts is shown, using anti-p38-P (Sty1-P, top panel), anti-Atf1 (Atf1, middle panel) or anti-Sty1 antibodies (Sty1, bottom panel). (E) Sty1-dependent gene transcription is constitutively activated in cells lacking Pka1. RNA from strains and conditions as in (D) was obtained, and probed with *gpx1* (activated by Sty1 and Atf1) or *fbp1* (repressed by Pka1). *rRNA* are shown as a loading control. As a control of activated Sty1, wild-type cells were treated (WT H₂O₂) or not (Unt.) with 1 mM H₂O₂ for 30 min.

Sty1, we first wanted to test whether there is a de-repression of respiration in the $\Delta pka1$ and/or $\Delta sck2$ mutants grown in glucose-rich media. We compared the oxygen consumption levels of wild-type, $\Delta pka1$ and $\Delta sck2$ cells grown in YE-4% glucose media. Cells lacking Sck2 had respiratory rate levels similar to wild-type cells (data not shown). However, cells lacking Pka1 consumed 1.4-fold the amount of oxygen of wild-type cells grown in the same glucose-rich media, YE-4% glucose (Figure 7C). In fact, respiratory rates of cells lacking Pka1 grown in YE-4% glucose media were even higher than those of wild-type cultures under calorie restriction conditions (both in YE-1% glucose, Figure 7C, and in MM, Supplementary Figure 4C). As described in 'Introduction', Sty1 becomes phosphorylated when exposed to environmental stresses, and the active kinase phosphorylates and induces accumulation of the Atf1 transcription factor, which triggers the transcription of stress genes such as *gpx1* (Vivancos *et al*, 2006; Lawrence *et al*, 2007) (Figure 4A). As observed in Figure 7D and E, Sty1 phosphorylation, Atf1 protein levels and *gpx1* transcript correlate with the oxygen consumption levels, being higher in cells lacking Pka1 than in wild-type cells. Therefore, inactivation of the Pka1 kinase by gene deletion triggers de-repression of respiration and constitutive Sty1 activation.

We hypothesized that high respiratory rates, either by calorie restriction or by loss-of-function of the Pka1 kinase, would promote extended lifespan through enhanced ROS production causing Sty1 MAP kinase activation. We tested this by determining the viability of stationary phase YE-4% glucose cultures of strains displaying constitutive activation of the Sty1 MAP kinase. Strains lacking the Sty1-phosphatase Pyp1, or expressing a mutant MAP kinase kinase, Wis1DD, have been reported to express a constitutively activated Sty1 MAP kinase (Shiozaki and Russell, 1995; Samejima *et al*, 1997; Shiozaki *et al*, 1998) (Figure 8A). As observed in Figure 8B and C, the viability of the YE-3% glucose cultures of these strains is not lost at day 6, and is comparable

to that observed for wild-type cells on calorie restriction. Same results were observed in YE-4% glucose media (data not shown). Constitutively active Sty1, however, has no further beneficial effects on the viability of calorie restricted cultures, neither in YE-1% glucose cultures (Supplementary Figure 5A) nor in MM cultures (Supplementary Figure 5B).

Discussion

Chronological aging in eukaryotic cells has been traditionally linked to the nutrient-dependent PKA and TOR-SCH9 pathways. We show here that fission yeast can be used as a model of chronological aging, because either calorie restriction or the inactivation of the SCH9 homologue Sck2 or the Pka1 kinases leads to life extension under glucose-rich conditions. An essential conclusion from this work is that such beneficial effects are fully dependent on the Sty1 response pathway. Thus, all the pathways convey on a single stress-activated MAP kinase, its activation being essential for extended lifespan. The stress-dependent Sty1 pathway can be activated at stationary phase either by calorie restriction or by genetic manipulation of components of the Sty1 kinase pathways, such as its kinase Wis1 or its phosphatase Pyp1 (Supplementary Figure 6). Such activation not only contributes to the development of stress resistance of chronologically aged cultures, but also promotes viability.

MM and YE are the two most common glucose-containing laboratory media for growth of *S. pombe* (Alfa *et al*, 1993). We had already shown that, unlike what has been described for the budding yeast, *S. pombe* oxidative phosphorylation is functioning in cells growing in glucose-containing MM and, to a lesser extent, in YE-3% glucose, and the differences in respiratory rates between these two media suggested that cells grown in MM have higher levels of intracellular ROS when compared with cells grown in YE-3% glucose (Zuin *et al*, 2008). We have shown here that growth in MM is beneficial for lifespan extension in a Sty1-dependent manner.

Figure 8 Constitutively active Sty1 is sufficient to promote life extension of cells grown under glucose-rich conditions. (A) Scheme of the regulation of Sty1 phosphorylation. (B) Strains 972 (WT), AZ103 ($\Delta pyp1$), AZ104 ($\Delta pyp1 \Delta sty1$), AZ98 (*wis1DD*) and AZ102 (*wis1DD \Delta sty1*) were grown in YE-3% glucose media. Serial dilutions of logarithmic (Log) and stationary phase (Day 6) cultures were spotted onto YE plates. (C) Survival percentages of strains as in (B) are indicated in the graph. Similar results were obtained with three different biological replicates.

To show that calorie restriction, and not other differences between MM and YE media, are enhancing lifespan by activating Sty1, we have also shown here that growth in YE supplemented with concentrations of glucose equal or below 1% yields cell cultures with elongated viability. In this second model of calorie restriction, the final density of the cultures varies, what may have an effect itself on life expectancy, but that is not the case for the YE-3% versus MM model. Therefore, we believe that the two models of calorie restriction described in this work complement each other.

It was soon reported that the MAP kinase is required for survival under diverse stress conditions. However, cells lacking Sty1 show an elongated morphology even in the absence of stress, and it has been proposed that this pathway may have a role in cell cycle regulation (Shiozaki and Russell, 1995; Lopez-Aviles *et al*, 2005). However, cells lacking Atf1, which do not display any phenotype in the absence of stress (Shiozaki and Russell, 1996), are also required for cell survival at stationary phase (Figure 4). Furthermore, a strain carrying an analogue-sensitive Sty1 protein displays defects at stationary phase after the addition of the inhibitory ATP analogue just before the transition to stationary phase (Figure 5). All together, these results indicate that Sty1 activation on calorie restriction is required to trigger an Atf1-dependent change in the gene expression programme.

It is worth pointing out that Sty1 is a global stress response regulator, and that the pathway can be activated by different types of stimuli. Thus, extracellular stress conditions, such as osmotic stress, heat or cold shock, H₂O₂, glucose or nitrogen starvation, an excess of toxic cations or heavy metals can induce Sty1 phosphorylation (Degols *et al*, 1996; Shiozaki and Russell, 1996; Samejima *et al*, 1997; Buck *et al*, 2001; Soto *et al*, 2002; Madrid *et al*, 2004). Whether Sty1 activation at the transition to stationary phase is a direct consequence of glucose depletion, cAMP production or increased levels of H₂O₂ remains to be determined. Our experiments suggest, however, that conditions shown to promote respiration during logarithmic growth (such as calorie restriction or genetic inactivation of the Pka1 pathway) trigger ROS production, Sty1 activation and lifespan extension. Confirmation of the signalling role of ROS in Sty1 activation under calorie restriction conditions has not become trivial. The use of the redox-sensitive dye DHR123 has proved useful as an indicator of the intracellular redox state in *S. pombe*; the dye could be added directly to the cultures, and without further manipulation the intracellular fluorescence was analysed by flow cytometry. Our experiments demonstrate that cells growing in YE-1% glucose display enhanced ROS production (up to two-fold) than cells grown in YE-4% glucose (Figure 6B), all along the growth curve. A recent publication (Roux *et al*, 2009) reported that growth under high glucose conditions enhanced ROS levels at stationary phase in fission yeast, when compared with cultures grown under calorie restriction, as determined using DHR123. In fact, we have also determined that protein carbonylation, as an indicator of oxidative stress, is preceding death of YE-4% glucose cultures at stationary phase (data not shown). We suspect, however, that the number of DHR123-positive cells in stationary phase correlates with the number of dead cells: the percentage of cells displaying heavy staining using any type of fluorescent dye (i.e. DHR123 or IP) correlates with the percentage of dead cells as determined by viable cell counting (Supplementary

Figure 7). In *S. cerevisiae*, it has been postulated that acetic acid, produced by cells as a by-product of fermentative metabolism, is the cause of death of glucose-rich media conditions, known to promote fermentation versus respiration (Burtner *et al*, 2009). We have tested such hypothesis in *S. pombe* cultures and have discarded acetic acid as a causal death agent (Supplementary Figure 8).

As hypothesized earlier by others, our results suggest that low caloric intake is a mildly stressful condition that provokes a survival response within the organism, helping it to survive adversity by altering metabolism and increasing the organism's defences against the causes of aging. Regarding these beneficial effects of ROS, several reports describe that moderate and controlled enhancement of ROS levels can participate in some differentiation programmes such as the regulation of haematopoietic cell fate (Owusu-Ansah and Banerjee, 2009) or inhibition of tumour initiation by induction of apoptosis in a p38 alpha-dependent manner (Dolado *et al*, 2007), or even the lifespan extension by ROS formation in *Caenorhabditis elegans* (Schulz *et al*, 2007).

We have shown here that oxygen consumption is exacerbated in cells lacking the Pka1 kinase under glucose-rich conditions, which leads to constitutive activation of the MAP kinase Sty1. The way oxidative phosphorylation is repressed by the Pka1 kinase during logarithmic growth in a glucose-dependent manner has not been reported. In the case of *S. cerevisiae*, the protein kinase A Tpk2 seems to inhibit respiratory growth through the negative regulation of iron uptake genes (Robertson *et al*, 2000). We have not detected any upregulation of the Sty1 pathway, nor enhanced oxygen consumption, in logarithmic cultures of *sck2* cells. However, the *S. cerevisiae* Sck2 homolog, SCH9, has been reported to negatively regulate chronological lifespan by inhibiting respiration (Lavoie and Whiteway, 2008), and we have not discarded that cells lacking Sck2 may have a subtle de-repression of respiration under glucose-rich conditions only at the transition to stationary phase.

Recently, a role for p38 MAP kinase in the induction of senescence has been proposed (for reviews, see Davis *et al*, 2007; Han and Sun, 2007; Coulthard *et al*, 2009; Maruyama *et al*, 2009). Cellular senescence is a state of irreversible growth arrest shown by normally proliferating cells as a result of telomere shortening, a process known as replicative senescence. However, other unrelated stimuli such as activation of the Ras oncogene or inappropriate culture conditions have been used to induce transformation and isolate senescence cells with arrested growth; these cells display phenotypes indistinguishable from those of cells undergoing replicative senescence. In all three cases of induced senescence, the main role of p38 activation seems to be that of a tumour suppressor, driving normally proliferating cells towards a premature and irreversible cell cycle arrest known as senescence by inducing the expression of multiple cell cycle inhibitors (Lee *et al*, 2009; Wong *et al*, 2009). As senescent cells never re-enter the cell cycle, cellular senescence appears to prevent malignant transformation, but an excessive accumulation of senescent cells attenuates the integrity and normal function of tissues, leading to age-related diseases. Therefore, drug-induced or genetic inactivation of the p38 kinase pathway in these models of fully proliferating cells has been reported to alleviate some of the senescence-associated phenotypes (Kang *et al*, 2005; Davis *et al*, 2006;

Wong *et al*, 2009), but it also obviously fails to inhibit proliferation. As we have described in this report for the role of the MAP kinase Sty1 in the induction of the chronological aging genetic programme, the mammalian p38 kinase has a key function on the onset of senescence. However, a premature exit from the proliferation programme seems to be the main role of p38 at senescence, whereas Sty1 induces a non-premature adaptation programme of already nutritionally arrested cells to long periods of starvation by triggering a global anti-stress, defence programme.

In summary, dietary or genetic interventions aimed to enhance respiration and moderate ROS production may extend lifespan in an MAP kinase Sty1-dependent manner, which raises the possibility that p38, its mammalian orthologue, might have a similar role in higher eukaryotes controlling not only senescence but also aging. Our results show that respiration-induced oxidative stress causes an adaptive response promoting endogenous stress defence capacity and life extension in an MAP kinase-dependent manner. Therefore, interventions aimed at decreasing ROS formation may not necessarily promote longevity and may rather reduce lifespan in eukaryotes. Accordingly with this observation, it has been recently reported that administration of anti-oxidants may abrogate the beneficial effects of physical exercise in healthy humans, through inhibition of the ROS-mediated activation of endogenous anti-oxidant capacity (Ristow *et al*, 2009).

Materials and methods

Growth conditions, yeast strains and plasmids

Cells were grown in YE medium (also known as complex medium, with 3% glucose) or in MM medium (also known as synthetic minimal medium or defined medium; contains 2% glucose). When indicated, YE medium was supplemented with various glucose concentrations. Culture media were prepared as described elsewhere (Alfa *et al*, 1993). We used the wild-type strain 972 (h^-) (Leupold, 1970), AV18 (h^- *sty1::kanMX6*) (Zuin *et al*, 2005), and AV15 (h^- *atf1::kanMX6*) (Zuin *et al*, 2005). Strain KS2088 [h^- *sty1-HA6His(ura4⁺) wis1DD::12Myc(ura4⁺) leu1-32 ura4-D18*] was kindly provided by MA Rodríguez-Gabriel and Paul Russell (Shiozaki *et al*, 1998). *S. pombe* strain AZ98 [h^- *wis1DD::12myc(ura4⁺) ura4-D18*] was selected after crossing strain KS2088 with JA364 (h^- *ura4-D18*, our laboratory stock). To construct AZ103 (h^- *pyp1::kanMX6*), we transformed strain 972 with a linear fragment containing *pyp1::kanMX6* as described earlier (Castillo *et al*, 2003). To generate MC22 (h^- *pka1::ura4⁺ura4-D18 leu1-32*) and MC25 (h^- *skc2::ura4⁺ura4-D18 leu1-32*), we transformed strain PN513 (h^- *leu1-32 ura4-D18*) with a linear fragment containing open reading frame (ORF)::*ura4⁺*; the *ura4⁺* cassette was designed to substitute codons 227–319 and 266–368 of the *pka1* and *skc2* ORFs, respectively. To construct the double mutant strains AZ102 [h^- *wis1DD::12myc(ura4⁺) sty1::natMX6 ura4-D18*], AZ104 (h^- *pyp1::kanMX6 sty1::natMX6*), MC24 (h^- *pka1::ura4⁺sty1::natMX6 ura4-D18 leu1-32*), MC27 (h^- *skc2::ura4⁺sty1::natMX6 ura4-D18 leu1-32*) and AZ108 (h^- *atf1::kanMX6 sty1::natMX6*), we transformed AZ98, AZ103, MC22, MC25 and AV15, respectively, with linear fragments containing *sty1::natMX6*, as described earlier (Moldon *et al*, 2008). The ATP analogue-sensitive strain AZ107 (h^+ *sty1.T97A ura4-D18*) was derived from AZ105 (h^+ *sty1::ura4⁺ura4-D18*) as described before (Gegan *et al*, 2007). To specifically inhibit Sty1 kinase activity, strain AZ107 was incubated with 10 μ M of the ATP analogue 1NM-PP1 (Calbiochem), which was added to the YE-1% glucose cultures at OD₆₀₀ of 0.2 or 2, as indicated in the figure legend (Figure 5). To express a catalytically dead Sty1 kinase (Sty1.K49R), plasmid *psty1* (pRep.41-*sty1HA-6His*; Millar *et al*, 1995) was mutagenized using QuikChangeSite-Directed Mutagenesis kit (Stratagene) following manufacturer's instructions. NT224 strain (h^+ *leu1-32 ura4-D18*

sty1-1) transformed with *patf1* (pHA-*atf1.41x*) has been described earlier (Sanzo *et al*, 2008).

Stationary phase conditions and survival assays

Strains were grown at 30°C in MM or YE media until they reached stationary phase, at an approximate OD₆₀₀ of 5–9, depending on the strains and growth conditions. The same number of cells (10³–10) in 3 μ l was spotted on YE agar plates from cultures at the logarithmic phase (OD₆₀₀ of 0.5) or days after reaching the stationary phase. The spots were allowed to dry and the plates were incubated at 30°C for 2–4 days. When indicated (Supplementary Figure 3), the anti-oxidant DL- α -tocopherol acetate (Sigma) (0.1 or 0.4 mM) was added to late logarithmic cultures, and it was washed out 15 h after the cultures reached stationary phase, as we wanted to avoid the beneficial effects of anti-oxidants on the already arrested stationary phase cultures. When indicated (Figures 3, 4D and 5C), day 2 or day 3 stationary phase cultures of wild-type or mutant strains, growing in YE-1% or YE-4% glucose media, were heat shocked at 48°C during 2 h before being spotted.

Measurement of glucose concentration in the culture media

To measure the glucose concentration of a cell culture, supernatant was first obtained centrifuging 1 ml of culture to a maximum speed and then diluted 10-fold in milli Q water. This dilution was used to perform the measurement using a commercial spectrophotometric assay kit (Cromatest-Linear) following manufacturer's instructions.

Preparation of trichloroacetic acid extracts to detect Sty1 and Atf1

To detect Sty1 and Atf1 proteins, trichloroacetic acid (TCA) extracts were obtained as described before (Sanzo *et al*, 2008). Atf1 and Sty1 were immunodetected with polyclonal anti-Atf1 (Sanzo *et al*, 2008) or anti-Sty1 (Jara *et al*, 2007) antibodies, whereas phosphorylated Sty1 was detected with commercial anti-p38-P MAPK antibody (Cell Signaling).

RNA preparation for northern blot analysis

RNA from cells grown in MM or YE media to the OD₆₀₀ indicated in the figures was isolated and northern blot performed as described elsewhere (Vivancos *et al*, 2004). The blots were hybridized with labelled *atf1*, *gpx1*, *cta1* or *fbp1* ORFs. *rRNA* was used as a loading control.

Measurement of oxygen consumption

Oxygen consumption of approximately 10⁷ cells in 1 ml, collected from cultures at the indicated OD₆₀₀, was measured as described before (Zuin *et al*, 2008), but using a Hansatech Oxygraph (Hansatech), with readings being recorded during 15 min.

Measurement of intracellular H₂O₂ levels

Relative intracellular peroxide levels were determined using the redox-sensitive fluorescent probe DHR123 (Invitrogen). This dye produces green fluorescence in the presence of H₂O₂ in both living and dead cells. We used a second dye, PI (Sigma-Aldrich), as a redox-independent, permeability-dependent fluorescent probe. DHR123 stocks are sensitive to oxidation, and should be discarded if displaying colour before the assays. Strains were grown in YE-1% glucose or YE-4% glucose media to the OD₆₀₀ indicated in the figures. DHR123 and PI were added to 1 ml cell cultures at final concentrations of 30 and 4.5 μ M, from stock solutions of 5 mM in DMSO and 1.5 mM in ethanol, respectively. Incubation proceeded at 30°C in the dark for 30 min. Peroxide production (using DHR123) and dye permeability (using PI) were simultaneously analysed by flow cytometry, using an FACS Calibur (Becton Dickinson) at low flow. PI was monitored in channel FL3 (detecting red fluorescence), whereas DHR123 was monitored in channel FL1 (detecting green fluorescence). First of all, the percentage of dead cells, as shown in Supplementary Figure 7, was calculated by measuring the number of cells that displayed heavy staining for either DHR123 or PI; these heavily stained dead cells were discarded from further redox analysis. A total of 10 000 live cells for each sample were analysed for DHR123-dependent green fluorescence (as an indicator of intracellular H₂O₂ levels) and for IP-dependent red fluorescence (as an indicator of permeability). The absolute green fluorescence numbers were normalized to red fluorescence numbers and to cell size. Relative fluorescence values [mean $\pm$ standard error measurements (s.e.m.)] are indicated using the wild-type strain growing in

YE-4% glucose media at the lowest OD₆₀₀ as a reference (with an assigned value of 1) (Figure 6B).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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