

Regulation of chronological aging in *Schizosaccharomyces pombe* by the protein kinases Pka1 and Sck2

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Summary

Budding yeast shows a progressive decline in viability after entering stationary phase, a phenomenon known as chronological aging. We show here that the fission yeast *Schizosaccharomyces pombe* also undergoes chronological aging and that the process is regulated by genes controlling two related nutrient signalling pathways. The first pathway includes the serine/threonine cAMP-activated protein kinase Pka1 and the second pathway comprises the serine/threonine kinase Sck2, a homologue of *Saccharomyces cerevisiae* SCH9. A double mutant for *pka1* and *sck2* displayed an additive effect on prolonging the fission yeast lifespan, suggesting that these genes regulate related but independent pathways. These long-lived mutants also accumulated less reactive oxygen species and had a delayed initiation of apoptosis compared with wild-type cells. We also found that strains carrying *pka1* deletion but not those with *sck2* deletion gained resistance to oxidative stress due to exposure to H₂O₂ or menadione. On the other hand, the additional increase in lifespan shown by the $\Delta pka1\Delta sck2$ double-mutant strain correlated with an increased resistance to both oxidative stress and heat shock. These results underscore the importance of nutrient signalling pathways and reactive oxygen species on organismal lifespan and establish *S. pombe* as a new model organism to study the molecular mechanisms underlying aging.

Key words: cAMP/PKA pathway; fission yeast; lifespan; ROS; SCH9; yeast genetics.

Introduction

The viability of individual yeast cells decreases with the number of cell generations (replicative aging) or with the time they spend in stationary phase (chronological aging). Replicative

aging is linked to exponential growth and measures the number of daughters a single mother cell is able to generate before its death. Replicative aging in yeast has been associated with the accumulation of rDNA circles in the nucleolus of old cells (Sinclair & Guarente, 1997). Overexpression of the NAD⁺-dependent deacetylases Sir2 and Hst2 extends the replicative potential of yeast cells and reduces the accumulation of rDNA circles (Kaeberlein *et al.*, 1999; Lamming *et al.*, 2005). In addition, caloric restriction, a condition that extends lifespan in a variety of species including flies, worms and mice also extends replicative lifespan (Lin *et al.*, 2000). Chronological aging relates to the time a yeast population can survive in stationary phase (Fabrizio *et al.*, 2001). Most chronological lifespan studies in yeast were performed in postdiauxic stage, which is characterized by a high degree of cell metabolism and respiration. It has been proposed that the loss of viability observed in this stage is mainly due to ethanol production (Fabrizio *et al.*, 2005), toxicity induced by reactive oxygen species (ROS), and loss of mitochondrial function (Longo & Fabrizio, 2002). More importantly, it has been established that death in postdiauxic cultures is not the result of simple starvation. First, individual yeast cells die at a high rate when their glycogen content is still high (Longo *et al.*, 1997). Second, yeast cells survive better in water than in synthetic complete medium (SCM), and switching from SCM to water diminishes cell death (Longo *et al.*, 1997). Third, cell-cycle mutants that arrest growth in the presence of nutrients also lose viability (Motizuki *et al.*, 1995). Finally some genetic mutations extend lifespan without changing metabolic rates (Fabrizio & Longo, 2003).

Although the processes that limit lifespan during replicative and chronological aging may differ, it is known that passage through the stationary phase accelerates replicative aging (Ashrafi *et al.*, 1999). Moreover, disabling mutations in the gene coding for the protein kinase SCH9 increases both replicative and chronological lifespan in yeast, suggesting a common link between the two processes (Kaeberlein *et al.*, 2005).

Previous studies have found that deletion of the genes *SCH9*, *CYR1* and *RAS2* enhances chronological lifespan of the budding yeast (Fabrizio *et al.*, 2001; Fabrizio & Longo, 2003). The encoded proteins mediate glucose signalling; this pathway stimulates cell proliferation and glycolysis, but decreases stress resistance (Longo, 2003). *CYR1* is an adenylate cyclase (Matsumoto *et al.*, 1983) and *RAS2* a small GTPase (Powers *et al.*, 1984), and both belong to the cAMP/PKA signal transduction pathway. Increased chronological lifespan due to deletion of these genes requires the stress response transcription factors Msn2 and Msn4, and the ROS-detoxifying enzyme Sod2 (Fabrizio *et al.*, 2003). *SCH9* encodes a serine/threonine protein kinase and is a high-copy suppressor of the growth defect caused by deletion of the PKA signalling

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pathway (Toda *et al.*, 1988), suggesting that it controls a redundant or parallel signalling pathway to PKA. Sch9 pro-aging functions seem to be conserved since its *Caenorhabditis elegans* homologue, AKT, also decreases longevity acting downstream of the insulin-like receptor DAF2 (Kenyon *et al.*, 1993; Hekimi & Guarente, 2003).

Saccharomyces cerevisiae has been shown to be a successful model to study aging. However, aging in budding yeast is influenced by the formation of ribosomal DNA circles, a process controlled by the histone deacetylase SIR2. These studies have generated a considerable interest in the possibility that SIR2 also increase longevity in mammals. Intriguingly, the increase in chronological lifespan in budding yeast due to mutations in PKA pathway and in Sch9, or caloric restriction can be largely extended in a strain deleted for SIR2 (Fabrizio *et al.*, 2005). Hence, SIR2 mediates paradoxical pro-aging and anti-aging effects. We reasoned that studying aging in another species of yeast might help in identifying universal aging pathways. To develop such a model we have chosen the fission yeast *Schizosaccharomyces pombe*. Several cellular processes like cell division, cell-cycle regulation and signalling pathways in *S. pombe* are closer to mammalian cells than the same pathways in *S. cerevisiae* (Sipiczki *et al.*, 2004). This striking similarity to mammalian cells constitutes a compelling reason to develop *S. pombe* as a model system to study aging. Like the budding yeast, the fission yeast also offers a convenient genetic system that makes it relatively straightforward to isolate mutants for a given phenotype. Replicative aging of *S. pombe* has already been measured by Barker & Walmsley (1999), but no long-lived mutant of this sort has been isolated as yet.

In budding yeast, mutations extending longevity commonly affect the glucose-sensing signalling pathway. In *S. pombe*, this pathway regulates growth through the activation of the cAMP-dependent protein kinase A (Hoffman & Winston, 1991; Maeda *et al.*, 1994). Most of the genes of this pathway have been dubbed *git* (glucose insensitive transcription). The *git2*⁺ gene, also called *cyr1*⁺, codes for the adenylate cyclase (Hoffman & Winston, 1991); *git6*⁺/*pka1*⁺ encodes the catalytic subunit of PKA (Byrne & Hoffman, 1993); *git8*⁺/*gpa2*⁺ encodes a G α subunit (Isshiki *et al.*, 1992; Nocero *et al.*, 1994) and *git5*⁺ encodes a G β subunit (Landry *et al.*, 2000). The G proteins Git8 and Git5 transduce glucose signalling from a GPCR named *Git3*⁺ (Welton & Hoffman, 2000). There are two *S. pombe* homologues of the budding yeast *SCH9* gene: *sck1*⁺ and *sck2*⁺ (Fujita & Yamamoto, 1998). Overexpression of *sck1*⁺ or *sck2*⁺ suppresses the defects associated with *git* mutants in the cAMP/PKA pathway of *S. pombe* (Jin *et al.*, 1995; Fujita & Yamamoto, 1998). The deletion of both *sck1*⁺ and *sck2*⁺ has no obvious phenotype, but results in less efficient spore germination (Fujita & Yamamoto, 1998).

To establish *S. pombe* as a model organism to study aging, we studied the lifespan in stationary phase of wild-type *S. pombe* and mutant strains for *pka1*, *sck1* and *sck2*. As in the budding yeast, we found that the cAMP/PKA pathway controls chronological aging in fission yeast. Of the two homologues of

SCH9, we found that only *sck2*⁺ regulates aging, and the pathway in which it belongs seems to be independent of the cAMP/PKA pathway. Long-lived *S. pombe* mutants maintained their viability for longer times and accumulated less ROS and caspase activity compared to wild-type cells. Surprisingly, of Δ *pka1* and Δ *sck2*, only *pka1* deletion mutants were more resistant to oxidative stress compared to the wild type, while only the double mutant Δ *pka1 Δ *sck2* displayed a significant increased resistance to heat stress. Hence, by reporting the first long-lived mutants of *S. pombe*, we introduce here this organism as a model for the study of aging.*

Results

Chronological aging of *Schizosaccharomyces pombe*

As an initial step, we wished to characterize the chronological aging of *S. pombe* by analysing the survival of a wild-type strain in stationary phase. As shown previously in *S. cerevisiae*, the content of the growth medium greatly affects chronological lifespan (Fabrizio & Longo, 2003). We chose to carry out the experiment in SCM containing 2% glucose because in budding yeast this condition is not associated with growth during the stationary phase (Fabrizio & Longo, 2003). Chronological aging in this medium is not simply starvation because growth of a wild-type strain in minimum medium (MM) resulted in approximately threefold longer survival than in SCM (data not shown). We measured cell survival from the early stationary phase, 10 h after the cultures ceased to grow, until the time when 99.9% of the cells were dead. Survival was scored by counting colony-forming units (CFU) from aliquots taken at different time points that were then diluted and plated on yeast extract completed medium (YEC; Moreno *et al.* 1991). We established that after 4 \pm 1 days, 50% of the wild-type *S. pombe* population grown in SCM was able to give rise to colonies on plates (Fig. 1). After 15 \pm 2 days, 99.9% of this population was replicatively dead. To verify that mutations in the genetic markers present in the wild-type reference strain (*leu1-32*, *ura4-D18* and *ade6-M210*) would not influence survival, a prototrophic strain without these markers was tested in this assay. It was found that the latter had the same lifespan as the auxotrophic population (Supplementary Fig. S1A,B), suggesting that these mutations have no effect on longevity. Furthermore, we occasionally noticed regrowth during stationary phase when using MM (data not shown) as it was described in low caloric conditions with baker yeast, possibly due to a better adapted subpopulation (Fabrizio & Longo, 2003). The use of SCM prevents the cells from entering such a state of regrowth.

While CFU counting provides information on the number of cells that can resume growth, such measurement does not reflect the viability of the cells in culture. To follow the viability of the yeast population, we used the fluorescent dye Phloxin B. This marker is passively taken up by cells and is actively excreted only by 'metabolically active', i.e. living cells (Supplementary Fig. S3). Thus, Phloxin B stains dead cells, which can

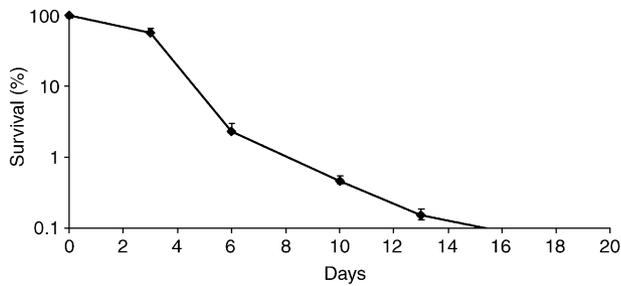


Fig. 1 Chronological lifespan of wild-type *Schizosaccharomyces pombe*. Survival of auxotrophic strain cultured in synthetic complete medium (SCM) 2% glucose as a carbon source and estimated by CFU (colony-forming units) counting. Y-axis is shown in logarithmic scale (see Supplementary Fig. S1B for nonlogarithmic scale).

be readily counted under the fluorescence microscope or by using flow cytometry (Fig. 2A,B). By using Phloxin B in wild-type yeast cultures, it was found that the proportion of metabolically dead cells was lower than the number of dead cells estimated from the CFU counting (Fig. 2C). Therefore, the lifespan measured using Phloxin B was considerably longer than the one measured by CFU (Table 1). For example, after 6 days in stationary phase, around 60% of the population was metabolically alive but only 2.3% of these cells were able to form colonies. Considering metabolic activity, 46 days were necessary to attain the threshold of 99.9% mortality, against 15 days for CFU counting (data not shown). These results show that CFU counting informs about the proportion of replicatively active cells in a population and underestimates the number of metabolically active cells, i.e. living cells estimated by Phloxin B staining.

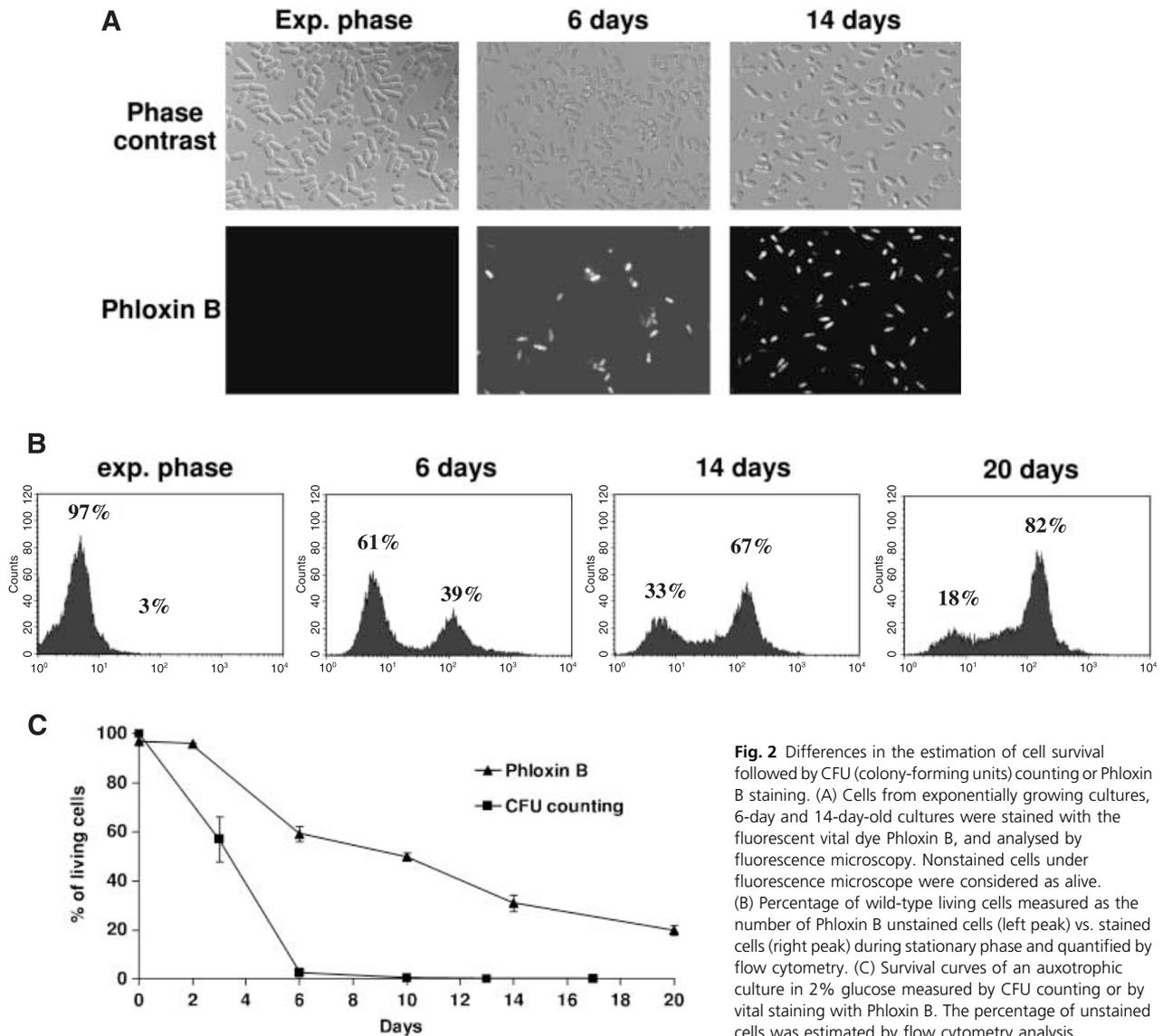


Fig. 2 Differences in the estimation of cell survival followed by CFU (colony-forming units) counting or Phloxin B staining. (A) Cells from exponentially growing cultures, 6-day and 14-day-old cultures were stained with the fluorescent vital dye Phloxin B, and analysed by fluorescence microscopy. Nonstained cells under fluorescence microscope were considered as alive. (B) Percentage of wild-type living cells measured as the number of Phloxin B unstained cells (left peak) vs. stained cells (right peak) during stationary phase and quantified by flow cytometry. (C) Survival curves of an auxotrophic culture in 2% glucose measured by CFU counting or by vital staining with Phloxin B. The percentage of unstained cells was estimated by flow cytometry analysis.

Table 1 Survival at day 10 estimated by CFU (colony-forming units) counting or phloxin B staining. Survival is given as the percentage of total living cells at day 0 (fold factor of survival of a given mutant compared to wild-type)

Strain	CFU (%)		Phloxin B (%)	
WT	2.3	–	59	–
$\Delta sck2$	32	(13.9)	80	(1.35)
$\Delta pka1$	31.5	(13.7)	89.5	(1.5)
$\Delta pka1\Delta sck2$	67	(29)	94.7	(1.6)

The cAMP/Pka1 pathway controls longevity in stationary phase

The cAMP/PKA1 pathway controls chronological lifespan in *S. cerevisiae* (Longo, 1999; Fabrizio *et al.*, 2001). We advanced that mutations in genes regulating the cAMP/PKA pathway will increase longevity in *S. pombe* as well. The cAMP/Pka1 pathway of *S. pombe* is required for growth and exit from stationary phase (Jin *et al.*, 1995; Fujita & Yamamoto, 1998). The unique PKA catalytic subunit known to date in *S. pombe* is coded by *pka1*⁺, and cells deleted for this gene are viable (Maeda *et al.*, 1994). The $\Delta pka1$ cells were analysed for their ability to survive in stationary phase. The survival of $\Delta pka1$ estimated by CFU counting was found to be threefold longer than wild type (Fig. 3A). The $\Delta pka1$ mutant also stayed metabolically active for longer time compared to wild type. For example, after 6 days, 89% of the population of this mutant was still alive compared to 59% for wild-type culture (Fig. 3C and Table 1). Therefore, there seems to be a good correlation between the ability of yeast cells to stay metabolically active (Phloxin B positive) in stationary phase and their ability to resume cell proliferation after being replated on rich medium.

Deletion of *sck2* increases longevity in stationary phase

To investigate in more detail chronological aging in *S. pombe*, we focused on the two homologues of the budding yeast *SCH9* gene, *sck1*⁺ and *sck2*⁺. The $\Delta sck1$ and $\Delta sck2$ deletion mutants were grown until stationary phase and their chronological longevities were analysed. The *sck1* knockout had the same survival as the wild-type reference strain SP14000 (Fig. 3A). However, the *sck2* knockout exhibited a lifespan threefold longer than the wild-type strain while the double mutant $\Delta sck1\Delta sck2$ displayed the same survival as the $\Delta sck2$ strain (Fig. 3A), indicating that *sck1*⁺ has no pro-aging effect. Phloxin B staining of $\Delta sck2$ was followed during stationary phase and showed that dead cells appeared later in this mutant (Fig. 3C). Neither $\Delta sck2$ nor $\Delta sck1\Delta sck2$ exhibit a significant delay in their generation doubling time (Fujita & Yamamoto, 1998) as observed in long-lived *SCH9* mutant strains of *S. cerevisiae* (Toda *et al.*, 1988). Therefore, the increased longevity of *sck2* mutants in *S. pombe* is not a consequence of slow growth. Altogether, these results suggest that *sck2*⁺ is the functional homologue of *SCH9* since they both have a similar effect on the control of aging.

We found that both *pka1*⁺ and *sck2*⁺ control longevity. Next we wanted to investigate whether they act in the same pathway. In *S. cerevisiae*, the PKA1 and SCH9 kinases control separate but partially redundant signal transduction pathways (Roosen *et al.*, 2005). Indeed, overexpression of one of these kinases compensates for the loss of function of the other (Toda *et al.*, 1988). A recent study has shown that they have common target genes, which explains the redundancy of their function (Roosen *et al.*, 2005). Interestingly, the double deletion $\Delta pka1\Delta sck2$ showed an increase in lifespan compared with the single mutants $\Delta pka1$ and $\Delta sck2$ (Fig. 3B). This result suggests that the deletion of both cAMP/Pka1 and Sck2 pathways has an additive effect on longevity.

Cell-cycle arrest of wild-type and mutant strains during stationary phase

The longer the cells spend time in stationary phase, the lower chance they have to reverse their cell-cycle arrest and resume cell division when the nutritive environment becomes favourable. We observed that deletion of *sck2* or *pka1* extends chronological lifespan enabling these mutants to prolong the period where re-entrance into the cell cycle is possible. Therefore, we investigated if these mutants may have a different cell-cycle arrest. Most *S. pombe* cells in logarithmic phase show a typical G2 (2 N) DNA content. Furthermore, it has been previously reported that in stationary phase, fission yeast stops its cell cycle either in G1 (N) or in G2 (2 N) and that the survival of cells arrested in G1 is the same as that of cells arrested in G2 (Costello *et al.*, 1986). Using flow cytometry, we found that wild-type and mutants displayed no difference in their cell-cycle profile, neither in exponential phase nor in stationary phase (Fig. 4). Interestingly, after 6 days the $\Delta pka1$ mutant displayed an increased number of polyploid cells (4 N) similar to wild-type cells during log phase, whereas $\Delta sck2$ displayed a much lower peak. This probably represents binuclear cells that have completed S phase in each nucleus but have not finished cytokinesis. Together, our data suggest that long-term survival in stationary phase does not depend on this kind of cell-cycle arrest (G1 or G2 arrest) since long-lived mutants have cell-cycle profiles identical to wild-type.

Lower accumulation of ROS and decreased apoptosis in $\Delta pka1$, $\Delta sck2$, $\Delta pka1\Delta sck2$ long-lived mutants

One of the main causes of cellular damage are the reactive oxygen species (ROS) produced by cellular metabolism. ROS accumulate proportionally with age and mutations. Therefore, increasing the antioxidant capacity of the cells lengthens the mean lifespan in several species (Finkel & Holbrook, 2000). In order to better understand the cause for the longer lifespan of *pka1* and *sck2* deletions mutants, the accumulation of ROS was investigated in these strains during stationary phase using dihydrorhodamine 123 (DHR123), a free-radical fluorescent probe (Fig. 5A). The results confirmed a low concentration of ROS in

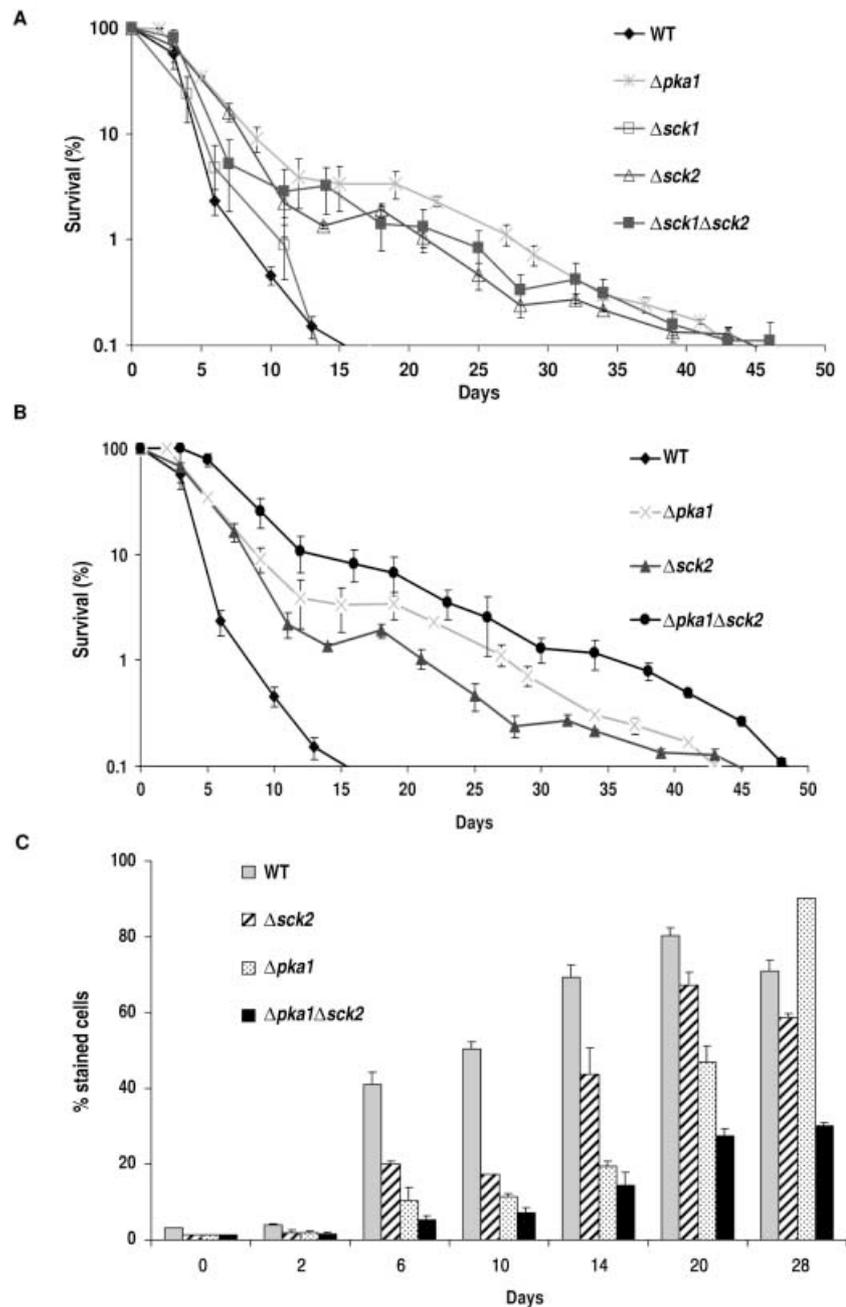


Fig. 3 Deletions of *sck2*⁺ and *pka1*⁺ increase survival in stationary phase. (A) Survival of WT, $\Delta pka1$, $\Delta sck1$, $\Delta sck2$ and $\Delta sck1\Delta sck2$ strains was evaluated by CFU (colony-forming units) counting. Y-axis is shown in logarithmic scale (see Supplementary Fig. S2A for nonlogarithmic scale). (B) Survival of WT, $\Delta pka1$, $\Delta sck2$ and $\Delta pka1\Delta sck2$ strains evaluated by CFU counting. Y-axis is shown in logarithmic scale (see Supplementary Fig. S2B for nonlogarithmic scale). (C) Percentage of dead cells measured by Phloxin B staining of WT, $\Delta sck2$, $\Delta pka1$ and $\Delta pka1\Delta sck2$ strains. Stained cells were considered as dead and were quantified by flow cytometry.

long-lived $\Delta pka1$ and $\Delta sck2$ mutants compared with wild-type cells (Fig. 5B), while the double deletion of *pka1* and *sck2* displayed an even lower accumulation of ROS during stationary phase.

Recent studies have reported that unicellular organisms like the budding and fission yeasts can undergo an apoptosis-like phenomenon triggered in response to stresses such as low concentrations of acetic acid, hydrogen peroxide, high concentration of mating pheromone, nutrients and DNA-damage conditions (Burhans *et al.*, 2003; Zhang, 2003). In higher eukaryotes, apoptosis is mediated by a family of proteases known as caspases and *S. pombe* possesses a similar enzyme, the metacaspase Pca1p (Low *et al.*, 2005). Moreover, typical

apoptotic markers such as activated caspase are observed in old yeast cells accumulating ROS (Madedo *et al.*, 2002). Therefore, long-lived mutants that display lower intracellular levels of ROS are expected to enter later in apoptosis. We tested this hypothesis with the *pka1* and *sck2* mutants and measured the number of cells with induced caspase activity by using a fluorescent probe. With this assay, we found that the percentage of cells exhibiting caspase activation increased much more rapidly for the wild-type strain than for the long-lived mutants $\Delta sck2$, $\Delta pka1$ and $\Delta pka1\Delta sck2$ (Fig. 6A,B). Furthermore, the less these strains accumulated ROS (Fig. 5B), the less they triggered the apoptosis-like program. Curiously, the number of wild-type and

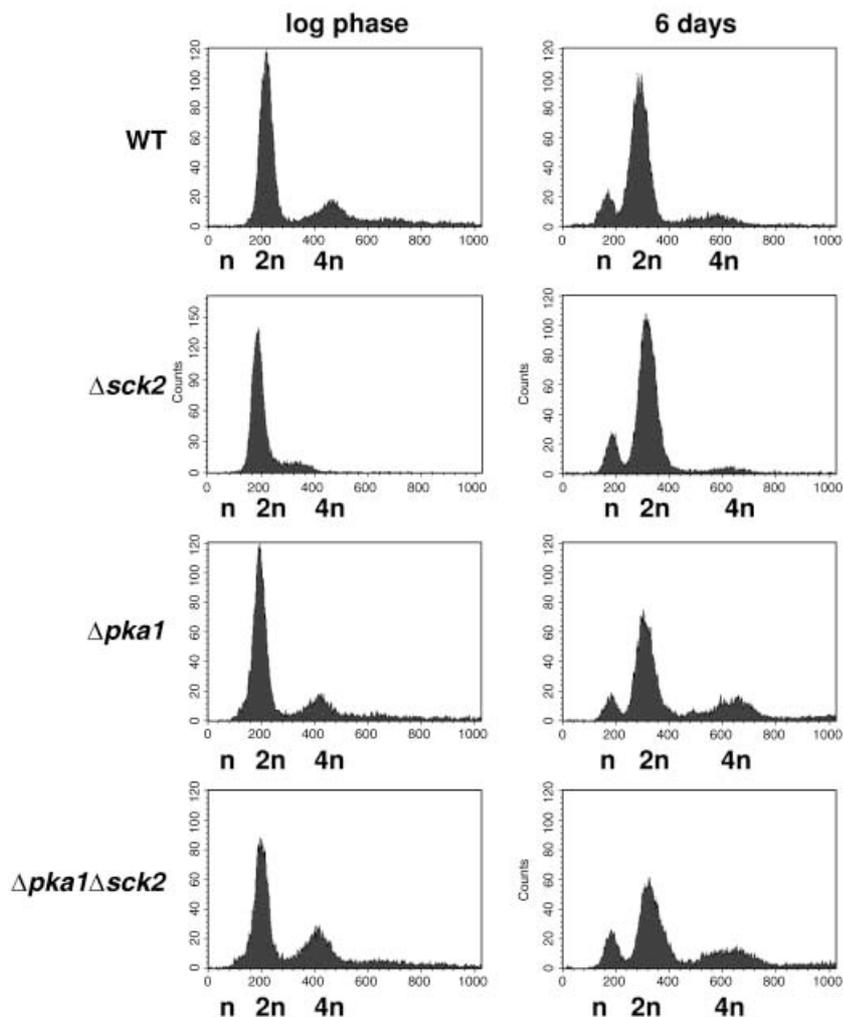


Fig. 4 Wild-type, $\Delta sck2$, $\Delta pka1$ and $\Delta pka1\Delta sck2$ strains in log phase and stationary phase show no major differences in their cell-cycle arrest. Wild-type and mutant strains were fixed with ethanol, and stained with propidium iodide and quantified by flow cytometry. The N, 2 N, and 4 N peaks are indicated.

$\Delta sck2$ cells displaying caspase activity decreased after 20 days. Since viability is low by this time, a caspase-independent mechanism of cell death may be acting late in stationary phase. Alternatively, cells well advanced in the execution of apoptosis may not display caspase activity anymore, and hence will be counted as a negative.

Longevity correlates with resistance to heat-shock and oxidative stress with $\Delta pka1$ mutant but not with $\Delta sck2$ mutant in *S. pombe*

Previous studies showed that most chronological long-lived mutants of *S. cerevisiae* show high oxidative stress or heat resistance (Fabrizio & Longo, 2003). In agreement, long-living *pka1* and *sck2* mutants of *S. pombe*, but not *sck1* mutants, showed low levels of ROS. In order to find out whether *pka1* and *sck2* mutants control lifespan in *S. pombe* by increasing stress resistance, we performed several stress resistance assays. To carry out these tests, wild-type or mutant yeasts were grown to stationary phase and 3 days later the cells were submitted to hydrogen peroxide, menadione or heat shock. The survival of these stationary phase cells after treatment was measured by drop tests

on solid rich medium. We noticed that wild-type *S. pombe* is resistant to high concentrations of hydrogen peroxide and menadione (Fig. 7). In fact, a 45-min treatment in 1 M H_2O_2 was necessary to kill a significant proportion of cells, whereas only 30 min in 100 mM H_2O_2 was sufficient to kill wild-type *S. cerevisiae* (Fabrizio et al., 2001). Likewise, a 0.75 M menadione treatment for 3 h had to be applied to our strains, while only 20 μ M for 1 h of the same superoxide/ H_2O_2 generating agent was enough to reduce *S. cerevisiae* viability (Fabrizio et al., 2001). For $\Delta sck1$, $\Delta sck2$, $\Delta sck1\Delta sck2$ and $\Delta pka1\Delta sck2$ mutant strains we did not score any difference in the sensitivity to H_2O_2 during stationary phase when compared to wild type (Fig. 7A). We did observe a moderate increase in resistance to H_2O_2 in the *pka1* mutant strain as compared to the reference strain. This trend was clearly enhanced when we tested the sensitivity to another pro-oxidant, menadione. Figure 7A shows that $\Delta pka1$ and $\Delta pka1\Delta sck2$ mutant strains grew better after treatment in menadione compared with wild-type.

The relationship between resistance to heat shock and longevity seems to be more complex. After 3 days in stationary phase, $\Delta pka1$, $\Delta sck2$, $\Delta sck1\Delta sck2$ and $\Delta pka1\Delta sck2$ mutants

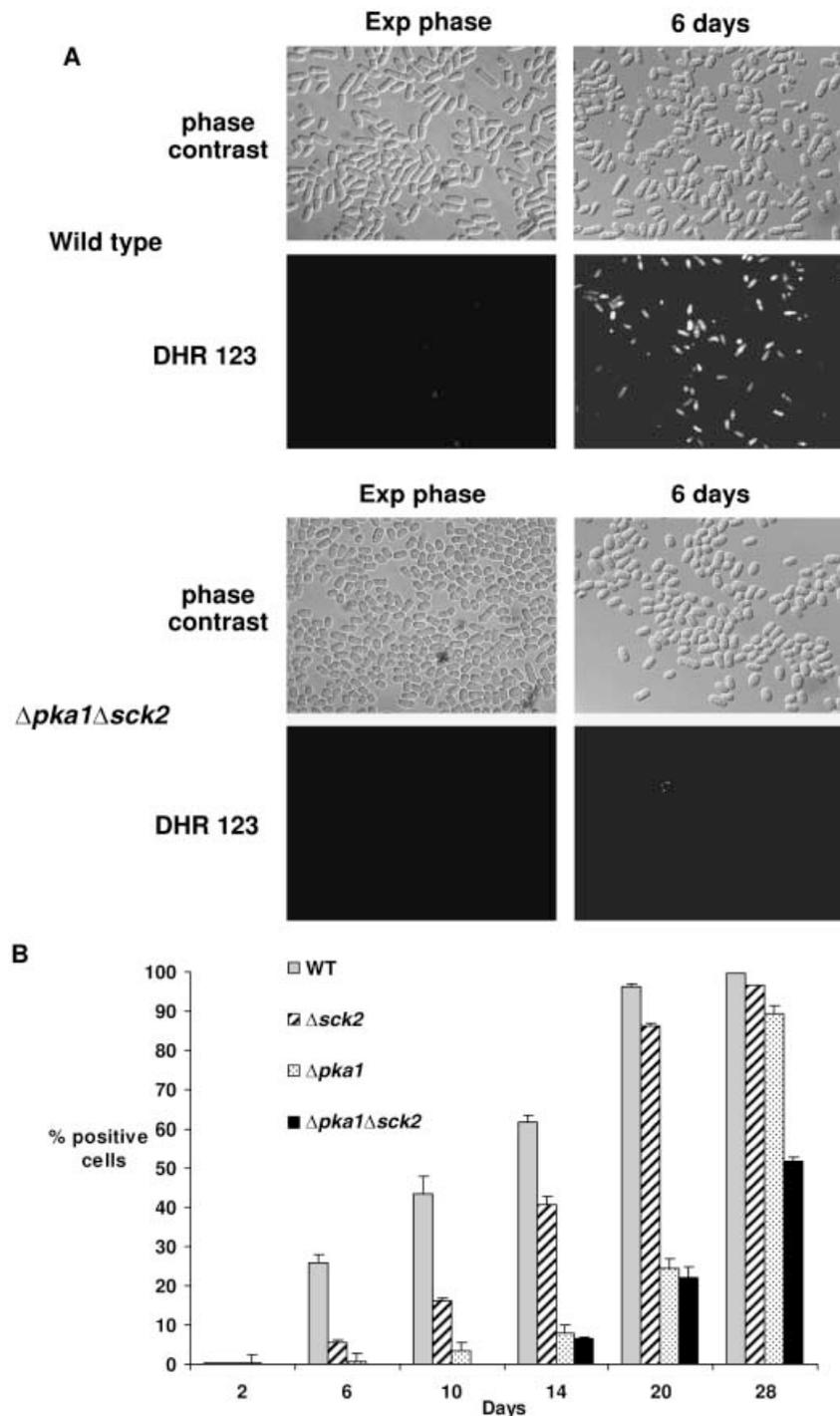


Fig. 5 Strains $\Delta pka1$, $\Delta sck2$, $\Delta pka1\Delta sck2$ show slower accumulation of reactive oxygen species (ROS) during stationary phase as compared to wild type. (A) Wild-type and $\Delta pka1\Delta sck2$ strains were incubated with the ROS probe DHR123 and cells were observed under fluorescence microscopy. (B) Percentages of ROS accumulating cells are displayed for wild-type, $\Delta sck2$, $\Delta pka1$ and $\Delta pka1\Delta sck2$ strains. At least 400 cells per count were examined and the average of DHR123-positive cells from two cultures was calculated.

displayed a moderate resistance compared to wild-type and the $\Delta sck1$ mutant strain (Fig. 7B). Surprisingly, the double mutant $\Delta pka1\Delta sck2$ showed a much higher resistance to heat shock in comparison to wild type.

Discussion

We have shown that *S. pombe* cells progressively lose their viability after entering the stationary phase, a phenomenon known

as chronological aging in *S. cerevisiae* (Fabrizio & Longo, 2003). We found that chronological aging in *S. pombe* was accelerated by glucose signalling because cells bearing mutations in genes controlling this pathway such as *pka1* and *sck2* lived longer. Since glucose also accelerates aging in *S. cerevisiae*, our results indicate a conserved mechanism of lifespan reduction operating in yeast during the stationary phase.

Studying chronological aging in *S. pombe* is not simply an extension of similar efforts in *S. cerevisiae*. The physiology of *S.*

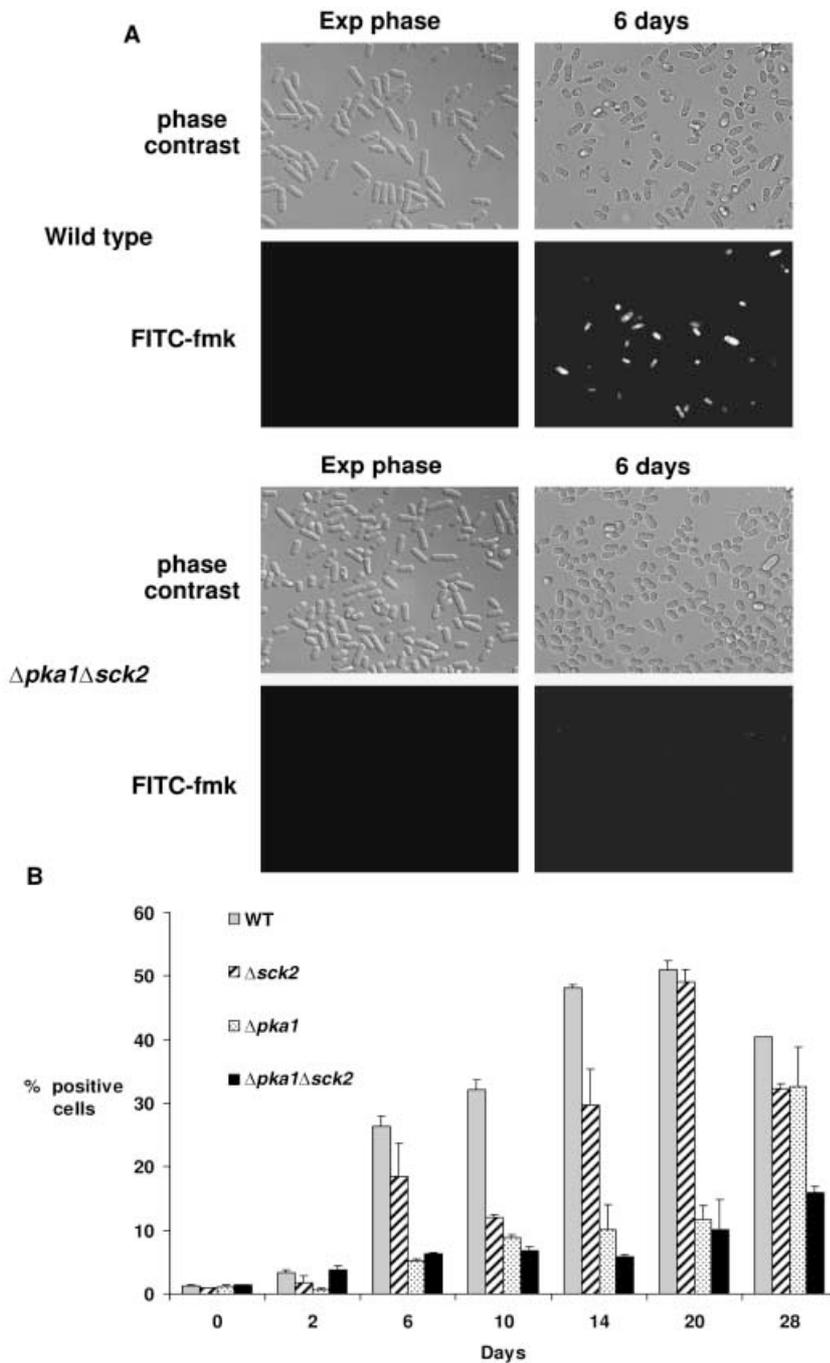


Fig. 6 Caspase induction during aging of $\Delta sck2$, $\Delta pka1$ and $\Delta pka1\Delta sck2$ strains is delayed compared to wild type. (A) Wild-type and $\Delta pka1\Delta sck2$ strains were stained with an apoptotic marker (FITC-VAD-fmk) during exponential and stationary phase, and observed by fluorescence microscopy. (B) Quantification of caspase-positive cells was done by flow cytometry as described in the Experimental procedures. The average measurements of two independent cultures is presented.

pombe is considered closer to higher eukaryotes and our results started to reveal differences in chronological aging between fission and budding yeast. For example, although the cAMP/PKA pathway controls longevity in both species, no mutants of the PKA holoenzyme (composed of the subunits Tpk1, Tpk2 and Tpk3, and the regulatory subunit Bcy1p) have been identified to increase lifespan in budding yeast. We show here, for the first time, that a deletion of *pka1*, coding for the PKA catalytic subunit in *S. pombe*, increases longevity. Furthermore, although mutations in a single gene coding for the protein kinase SCH9

extends lifespan in the budding yeast, *S. pombe* possesses two *SCH9* homologues, *sck1*⁺ and *sck2*⁺ (Fujita & Yamamoto, 1998). We discovered that of the two, *sck2*⁺ is the longevity regulator in *S. pombe*. Since Sck1p and Sck2p have similar structures, studying the differences in activators and effectors of these two proteins should shed considerable light on the pathways controlling aging in yeast.

Global expression studies have shown that *sck1*⁺ and *sck2*⁺ are expressed in *S. pombe* (Chen et al., 2003). Surprisingly, their expressions are regulated in opposite ways under different

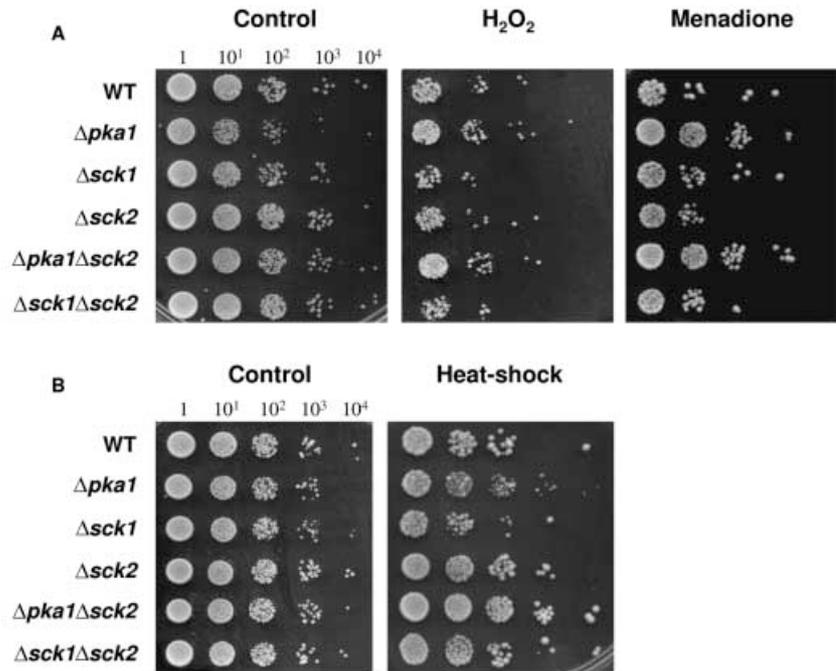


Fig. 7 Stress resistance of long-lived mutants in stationary phase. All strains were treated by short exposure to high concentration of oxidative stress: H₂O₂ (1 M) and menadione (0.75 M) (A) or heat shock (B). Dilutions were then spotted onto rich media to measure survival.

stress conditions such as oxidative stress, heat, osmolarity stress or cadmium treatment. In these conditions *sck1*⁺ is up-regulated whereas *sck2*⁺ is down-regulated (Chen *et al.*, 2003). Taken together, this expression data and our results suggest, that under certain stress conditions, *S. pombe* has evolved pathways to reduce Sck2 expression but in stationary phase there is still sufficient Sck2 activity to reduce viability.

Since the cAMP/Pka1 pathway controls chronological lifespan in *S. pombe*, what could be the downstream targets of this pathway? One possible target is Rst2p, which is known to be controlled by Pka1p and inhibited by the presence of glucose (Higuchi *et al.*, 2002). This transcription factor has been shown to bind the stress response element STREP that resembles the *S. cerevisiae* stress response element STRE (Marchler *et al.*, 1993). The expression of genes with a STREP motif in their promoter region is enhanced after glucose starvation (Hoffman & Winston, 1991). Our study showed that mutants without active cAMP/Pka1 pathway accumulated less ROS, suggesting that enzymes involved in ROS generation and/or degradation may be under the control of the cAMP/Pka1 pathway. Since this mutant is more resistant to hydrogen peroxide and menadione, we currently favour the idea that they act by suppressing ROS accumulation. Yet, no antioxidant protein gene is known to have a STREP motif and to be regulated by Rst2/Pka1. In *S. pombe* antioxidant genes are under the control of the Atf1 transcription factor (Shiozaki & Russel, 1996; Wilkinson *et al.*, 1996) and the Pap1 transcription factor (Toone *et al.*, 1998). While a direct connection between Pka1p and Pap1p is not clear, a link between the cAMP/Pka1 and Wis1/Spc1/Atf1 pathways has already been suggested (Stettler *et al.*, 1996). Moreover, nutritional limitation is known to activate the kinase Spc1, an element of the Wis1

MAP kinase cascade that is upstream of Atf1 (Shiozaki *et al.*, 1997). Our results support the possibility of a crossed regulation between these two pathways.

While both *pka1*⁺ and *sck2*⁺ affected *S. pombe* longevity separately, the double deletion mutant Δ*pka1*Δ*sck2* showed an additive effect promoting a better survival than the two single deletion mutants Δ*pka1* and Δ*sck2*. This additive effect was not only visible at the level of cell viability, but also on ROS accumulation, caspase activation and surprisingly on resistance to heat shock. These results suggest that either both factors act on the same gene targets and have an additive effect, or they act on different genes with complementary function. Indeed, Roosen and collaborators (2005) have shown in *S. cerevisiae* that PKA and Sch9p act through separate signalling cascades, and affect synergistically or oppositely common target genes. A similar pattern can be envisaged in *S. pombe* since *sck2*⁺ is a high-copy suppressor of *pka1* and both affect longevity, suggesting partially overlapping functions.

While the increased lifespan of Δ*pka1* and Δ*sck2* mutant strains correlated with low accumulation of ROS in stationary phase, strains carrying *pka1* deletion but not those with *sck2* deletion gained resistance to oxidative stress such as H₂O₂ and menadione (Fig. 7). On the other hand, the additional increase in lifespan shown by the Δ*pka1*Δ*sck2* double mutant strain correlated with an increased resistance to both oxidative stress and heat shock. Although several models may explain these correlations, the results are consistent with the idea that Sck2 controls a pathway of ROS production but not resistance to ROS. In contrast, Pka1p may control the pathways of ROS resistance or elimination as described in *S. cerevisiae* (Fabrizio *et al.*, 2003). Combined, the effects of these two deletions may increase

Table 2 Genotypes of strains used in this study

Strain	Genotype	Origin
SP14078 (prototrophic WT)	<i>h</i> ⁻	C. Hoffman
SP14000 (auxotrophic WT)	<i>h</i> ⁻ <i>ade6-M210 ura4-D18 leu1-32</i>	This study
SP14073	<i>h</i> ⁻ <i>ade6-M210 leu1-32 ura4-D18 sck1::neo</i> ^R	This study
SP14086	<i>h</i> ⁻ <i>ade6-M210 leu1-32 ura4-D18 his7-366 sck2::LEU2</i>	This study
SP14094	<i>h</i> ⁻ <i>ade6-M210 leu1-32 ura4-D18 his7-366 sck1::neo</i> ^R <i>sck2::LEU2</i>	This study
CHP453	<i>h</i> ⁻ <i>leu1-32 ura4 -D18 his7-366 pka1::ura4</i> ⁺	C. Hoffman
SP14089	<i>h</i> ⁻ <i>ade6-M216 leu1-32 ura4-D18 his7-366 pka1::ura4</i> ⁺ <i>sck2::LEU2</i>	This study
JX770	<i>h</i> ⁻ <i>ade6-M216 leu1-32 ura4-D18 his7-366 pka1::ura4</i> ⁺ <i>sck1::his7</i> ⁺ <i>sck2::LEU2</i>	M. Yamamoto

WT, wild type.

lifespan by both decreasing ROS production and increasing ROS resistance. Of note, Perez-Campo and colleagues (1998) have reported that longevity among different species negatively correlates with the amount of ROS produced by the mitochondria. Our results are consistent with that possibility and suggest that studying chronological aging in *S. pombe* may help to identify genes controlling ROS production.

We also observed that a large proportion of metabolically active (Phloxin B positive) cells in stationary phase were unable to resume growth when plated on complete solid medium. It is possible that these cells may have accumulated too many oxidative damages on their DNA and proteins and will ultimately undergo apoptosis. Alternatively, these cells are already dead but cannot take up or retain Phloxin B. We exclude the latter possibility, because when we killed the cells using heat or sodium azide, all of them were stained by Phloxin B (Supplementary Fig. S3).

It has been proposed that yeast cells commit suicidal apoptosis in stationary phase to keep a small fraction of cells alive and able to resume growth when nutrients become available (Fabrizio *et al.*, 2004; Herker *et al.*, 2004). According to this interpretation, the pro-aging functions of Pka1 and SCH9/Sck2 were selected for the sake of the population. Alternatively, these proteins may be important for logarithmic growth while their effects on reducing fitness in stationary phase have not been neutralized by natural selection. The latter argument may be valid if yeasts living in stationary phase do not significantly contribute to subsequent generations. Although *S. pombe* and *S. cerevisiae* diverged several million years ago, *pka1*⁺ and *SCH9/sck2*⁺ have a pro-aging effect in both species. This conservation is consistent with the idea of a selective value for aging and death in stationary phase. It is intriguing that there are mechanisms in *S. pombe* to down-regulate *sck2*⁺ expression during certain stresses but these mechanisms do not seem to fully operate during the stationary phase. Again, this supports the idea that aging in stationary phase has a selectable value in yeast. Decoding the molecular components of the conserved aging pathway in *S. pombe* may shed some light on the aging process of higher eukaryotes, including humans. Proof that this is indeed the case is well illustrated by our results showing that mutations in *pka1*⁺ and *sck2*⁺ extend lifespan in *S. pombe*, and reduce the accumulation of

ROS and the activation of caspase in these cells. Both ROS and caspase-mediated cell death have been implicated in the functional declines associated with aging in mammals (Balaban *et al.*, 2005) and ROS production negatively correlates with species lifespan (Perez-Campo *et al.*, 1998). Hence, we propose to add *S. pombe* to the list of model organisms for the study of aging.

Experimental procedures

Strains and media

Schizosaccharomyces pombe strains used in this study are listed in Table 2. All disruption strains were obtained by mating JX770 with a wild-type strain and different combinations of disruptions were isolated on selective media, except for *sck1* disruption in SP14073, which is detailed below. Cultures were grown in synthetic complete medium (SCM) and MM (Edinburgh minimal medium; Moreno *et al.*, 1991) with 2% glucose. SCM is supplemented with 111 mg L⁻¹ of amino acids and a fourfold excess of the supplements that strains do not synthesize (Ade, Ura, Leu and His). Solid rich medium (YEC), was made of YE 5 g L⁻¹ supplemented with 111 mg L⁻¹ of adenine, uracil, leucine, histidine. Liquid cultures were grown at 30 °C with shaking at 250 rotations per minute.

Disruption of *sck1*⁺

The two flanking regions of *sck1*⁺ were cloned into the pDRIVE vector (QIAGEN, Mississauga, Canada) after their amplification by PCR from plasmid pBC1 (Jin *et al.*, 1995) and using the pair of primers: 5'FLSCK1 for (5'-CGGGTACCCTCTGTTTCGATACTCC-3'), 5'FLS CK1rev (5'-GTAACACCAGACGAGGA-3'); and 3'FLSCK1-sense (5'-GCGCTCGAGTAAGCTTGCTCCAATC-3'), 3'FLSCK1-antisense (5'-TGCTCTAGATCATGCAAAC AACAGG-3'). Then pDRIVE-5'FLsck1 (bearing the 5' flanking region) was digested with *KpnI* and *SphI*, and pDRIVE-3'FLsck1 (bearing the 3' flanking region) with *XhoI* and *XbaI*. First, 5'FLsck1 was ligated to pDRIVE-Neo^R linearised by *KpnI* and *SphI* digestion. Subsequently, 3'FLsck1 was ligated to pDRIVE-5'FLsck1-*neo*^R cut with *XhoI* and *XbaI* to obtain pDRIVE-*sck1::neo*^R. The deletion cassette was removed from pDRIVE-*sck1::neo*^R by *KpnI* and *XbaI* double digestion, and

Table 3 Maximum optical density (OD) reached by different strains in this study. The measurement has been done at the first point of survival curve representing 100% in the conditions described in the Experimental procedures

Strain	Name	Maximum OD*
SP14000	WT	10.0 ± 0.3
SP14073	$\Delta sck1$	9.6 ± 0.7
SP14086	$\Delta sck2$	10.7 ± 0.5
SP14094	$\Delta sck1\Delta sck2$	9.9 ± 0.3
CHP453	$\Delta pka1$	10.6 ± 0.6
SP14089	$\Delta pka1\Delta sck2$	11.7 ± 0.6

*(n = 3). WT, wild type.

used to transform SP14000 strain using a PEG/Li protocol (Elble, 1992). Transformants were selected on MM supplemented with adenine, uracil, leucine and G418. The knockout of *sck1+* was validated by PCR and Southern blot analysis.

Survival in stationary phase

Yeast strains were streaked on MM and grown for 5 days at 30 °C. From these plates we inoculated a preculture on SMC and grew it until it reached OD₅₉₅ 3–4. Then, this preculture served to start a second preculture that was cultured until OD₅₉₅ 5–6. This latter preculture served to inoculate a 200 mL culture at initial OD₅₉₅ 0.2–0.4. This culture was grown until the end of exponential phase when OD₅₉₅ stopped increasing and reached the maximum density (Table 3). Ten hours after this point, the culture was separated into three independent 50-mL cultures in 250-mL flasks (keeping a medium/volume ratio of 1 : 5 as described in Fabrizio & Longo, 2003). Next, a first aliquot was diluted and plated on complete yeast extract solid medium. After 10 days, the total number of colonies was counted, with this number representing 100% survival and day 0 of the curve (see Supplementary Tables S1 and S2). The following measurements were done every 3–5 days. The two mutant strains, CHP453 and SP14089, have a slightly slower growth rate than the wild type as described by Fujita & Yamamoto (1998). To compensate for these differences, we adjusted the initial quantity of cells used to start cultures with these strains.

Cell survival was also followed using the Phloxin B fluorescent dye. Around 1.4×10^7 cells were collected and 5 mg L⁻¹ of Phloxin B were added to the medium. After 2 h incubation at 30 °C with shaking, the cells were washed twice in 1× PBS pH 7.4 and used for fluorescence microscopy or flow cytometry analyses. Controls with exponentially growing cells, and cells killed by heat (65 °C for 1 h) or NaN₃ (0.1% for 2 h), showed that Phloxin B stains only dead cells (Supplementary Fig. S3). For all microscopic analysis, we used a fluorescence inverted microscope Nikon TE2000U (Nikon, Mississauga, Canada). Images were acquired using a motion-picture camera CCD CoolSnapFX 12 (Photometrics, Tucson, AZ, USA) bit and treated with UIC Metamorph software (Molecular Devices Corporation, Downington, PA, USA).

In vivo staining of caspase activity

Cultures were harvested in exponential phase or stationary phase. Samples of 10^7 cells were washed once in 1 mL 1× PBS pH 7.4 and resuspended in 150 µL of 1× PBS containing 10 µM FITC-VAD-fmk (CaspACE, Promega, Madison, WI, USA). After incubation for 20 min at 30 °C, cells were washed in 1 mL 1× PBS, resuspended in 100 µL 1× PBS and analysed by FACS or under the fluorescence microscope. The percentage of apoptotic cells was determined using flow cytometry.

Flow cytometry analysis

Cells were stained with Phloxin B or FITC-VAD-fmk as described above. Flow cytometry analysis was performed using FACS Calibur (Becton Dickinson Biosciences, Mississauga, Canada) on 10 000 cells. Emission from the argon LASER was at 488 nm; emission settings were 515–545 nm (filter FL1) or 560–600 nm (filter FL2), respectively, for FITC-VAD-fmk and Phloxin B staining. The percentage of positive stained cells was determined as the population of fluorescent cells with a higher fluorescent intensity than an unstained negative control. Two independent cultures were analysed.

Cultures were treated with propidium iodide in order to stain DNA and determine the quantity of DNA in cells. Cells were fixed and stained following the protocol of Sazer & Sherwood (1990), with 2 h incubation at 37 °C with RNase A. Samples containing 10 000 cells were analysed by flow cytometry using FL2-A detection line.

In vivo staining of ROS accumulation

Samples of 100 µL ($1-2 \times 10^7$ cells) of culture were collected and incubated with 30 µM dihydrorhodamine123 for 30 min at 30 °C. Cells were applied on a poly lysine pretreated slide, washed by 1× PBS pH 7.4 and observed under fluorescence microscopy. Background fluorescence was manually removed using the UIC Metamorph software and at least 400 cells of two independent cultures were counted to determine the percentage of positively stained cells.

Stress resistance in stationary phase

Cells were cultured for 3 days in stationary phase as described above. After washing with water, the cell concentration was adjusted to OD₅₉₅ of 0.5. Next, we added H₂O₂ (1 M final concentration) and incubated for 1 h at 30 °C, or menadione (0.75 M final concentration) and incubated for 3 h at 30 °C. Cells were pelleted and washed two times with 1 mL of water. Serial dilutions were spotted onto YE rich medium and incubated for 4 days at 30 °C. For heat treatment, equal amounts of cells (OD₅₉₅ of 0.5) were heat shocked for 45 min at 51 °C. Serial dilutions were dropped onto YE rich medium and incubated for 5 days at 30 °C.

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Supplementary material

The following supplementary material is available for this article

Experimental procedures

Wild-type SP14000 cells were cultivated in minimum medium (MM) and collected during exponential phase or after 24 h in stationary phase. They were submitted to heat shock for 1 h at 65 °C or 2 h in 0.1% NaN₃. Then, cells were stained by Phloxin B and analysed as described above.

Fig. S1 Chronological lifespan of wild-type *Schizosaccharomyces pombe* and control in different background strains. (A) Survival of reference strains containing genetic markers (auxotrophic SP14000) or not (prototrophic SP14078). Cells were cultured in synthetic complete medium with 2% glucose. (B) Same curves as in (A) but presented with linear Y-axis.

Fig. S2 (A) Survival of wild-type, $\Delta pka1$, $\Delta sck1$, $\Delta sck2$ and $\Delta sck1\Delta sck2$ strains was evaluated by colony-forming units (CFU) counting. (B) Survival of wild-type, $\Delta pka1$, $\Delta sck2$ and $\Delta pka1\Delta sck2$ strains evaluated by CFU counting.

Fig. S3 Phloxin B staining and fluorescence microscopy analysis of yeast cells killed by heat or sodium azide. Dead cells were all (100%) stained by Phloxin B, whereas less than 0.5% of exponential phase cells were stained.

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