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A screen for genes involved in respiration control and longevity in *Schizosaccharomyces pombe*

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We present results showing that glucose signaling has proaging effects in the yeast *Schizosaccharomyces pombe*. Deletion of the receptor that senses extracellular glucose (Git3) increases the life span of *S. pombe*, while constitutive activation of the Gα subunit acting downstream of this receptor (Gpa2) shortens its life span. The latter mutant is also impaired for growth under respiration conditions. We have used this phenotype in a selection strategy to identify genes that when overexpressed can rescue the respiratory defect of constitutively active Gα subunit mutants. Here, we report an extended version of the work we presented at the IABG meeting and the results of this screen. This strategy allowed us to isolate four genes: *psp1*$^+$/*moc1*$^+$, *cka1*$^+$, *adh1*$^+$, and *rpb10*$^+$. Interestingly, the overexpression of these genes was also capable of increasing the chronological life span of wild-type yeast cells.

Keywords: longevity; yeast; *Schizosaccharomyces pombe*; chronological life span; aging; *psp1*

Aging research has greatly benefited from contributions of discoveries in simple organisms like yeast and invertebrates. The results of these studies in model organisms often can be extrapolated to mammals.[1–3] *Saccharomyces cerevisiae* has been the favorite yeast in aging studies. More recently the fission yeast *Schizosaccharomyces pombe* also was shown to be a reliable yeast model to study aging.[4,5] Yeast life span can be measured through two methods. One consists of counting the number of divisions a single mother cell undergoes before death and is referred to as replicative aging.[3,6] The other one measures the time a population survives during its vegetative state once nutrients are depleted (stationary phase) and is referred to as chronological aging.[1,7] The latter is considered a model to study the aging of somatic cells of multicellular organisms, whereas replicative aging evokes the aging of dividing cells.[8]

Nutrient signaling pathways through the kinases protein kinase A (*PKA*)/Tor/Sch9 regulate chronological aging in *S. cerevisiae*.[9–11] Studies in *S. pombe* suggest the universality of the regulation of yeast aging, particularly for the role of nutrient-sensing pathways. Indeed, our laboratory showed that nutrient detection involving the kinases encoded by *pka1*$^+$ and *sck2*$^+$, the homologues of PKA active subunits and Sch9 in *S. cerevisiae*, is involved in the control of aging.[5] Furthermore, we demonstrated that these pathways act on different mechanisms due to their additive effects on longevity. More recent studies by other groups have confirmed our results.[12,13] Subsequently, we analyzed in more detail the proaging effect of glucose, focusing on the role of its signaling through the glucose membrane receptor Git3p coupled to the G protein Gpa2, which acts upstream of Pka1.[14] The loss of the glucose signal due to deletion of Git3p partially mimics the effect of increasing longevity by reducing glucose in the medium. Moreover, similar proaging effects of glucose can be obtained by constitutive activation of Git3/PKA signaling via a mutation in *gpa2*$^+$, the Gα active subunit of the G protein coupled to Git3p.[14] A point mutation in *gpa2*^{R176H}, with arginine 176 changed to histidine, confers constitutive activation of adenyl cyclase *git2*$^+$/*cyr1*$^+$ and a high level of cAMP synthesis that in turn releases Pka1p from the Cgs1p regulatory subunit.[15,16] Finally, we

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observed that the effects of glucose signaling on longevity through Git3/PKA are predominant over those due to metabolism of this sugar, as these effects are maintained in the absence of glucose metabolism in a knockout background for the hexokinases encoded by $hxk1^+$ and $hxk2^+$, the first enzymes of glycolysis.

A link between mitochondria (respiration) and longevity has been proposed for budding yeast.[17–19] Long-lived glucose-restricted *S. cerevisiae* displays a tubular network of mitochondria, compared to a punctuate morphology in short-lived yeast grown in a high glucose concentration.[20] We found the same differences in mitochondrial morphology when we compared short-lived Gpa2 constitutive active cells with wild-type *S. pombe* cells (unpublished data). Moreover, we observed that mitochondrial dysfunction correlated with a short life span in *S. pombe*. Interestingly, the $gpa2^{R176H}$ mutant also exhibits impaired mitochondrial regulation and higher production of reactive oxygen species.[14] Therefore, we thought that the $gpa2^{R176H}$ mutant might offer a convenient model system to isolate genes linked to aging in *S. pombe*.

Screen for cDNA allowing recovery of respiration and longevity of the $gpa2^{R176H}$ mutant

In order to find targets acting positively on longevity downstream of the Git3/PKA signal, we took advantage of the phenotype exhibited by the constitutive active mutant $gpa2^{R176H}$ to screen for cDNAs which, when overexpressed, rescue respiration and promote longevity in this strain. We used an *S. pombe* cDNA library in a plasmid with a strong promoter (*nmt1* promoter in pREP3X) to transform $gpa2^{R176H}$ cells. We screened first for recovered growth on respiratory medium containing glycerol and then second for a longer life span (Fig. 1).

The first step consisted of the dilution and the shift of a culture of the $gpa2^{R176H}$ mutant previously transformed with the cDNA library from a liquid medium containing 2% glucose to a solid medium containing 2% glycerol and 0.1% ethanol (Fig. 2). The same procedure was carried out with the $gpa2^{R176H}$ strain as a control, in order to ascertain that this strain without the plasmid did not grow in this medium (data not shown). Several thousand clones from the library grew in the respiration medium, and of these 100 were selected. These clones were used to individually inoculate new cultures containing glucose. After 21 days of incubation in stationary phase, samples were diluted and spotted on plates as 10-fold serial dilutions (Fig. 3A). We were surprised to see that among the 100 clones tested, 53 clones had a longer life span than the wild-type (SP14000) and $gpa2^{R176H}$ (RWP1) controls (Fig. 3A). Among the 53 clones with a longer life span, 33 were chosen to be tested again for longevity using colony-forming unit (*CFU*) counting on plates, which is a more stringent and precise assay.[5] Only 14 of 33 showed a substantial increase of life span based on CFU and were kept for further investigation. The plasmids from these clones were recovered and sequenced. Among 14 clones, 4 entire open reading frames were identified (Table 1), but the others were incomplete and rejected.

Overexpressed genes increase life span in wild-type cells

The four genes identified as aging regulators in *S. pombe* were:

1. $psp1^+/sds23^+/moc1^+$, a nonessential gene that is required for long-term survival in stationary phase.[21] Psp1 is phosphorylated in stationary phase by the cyclin-dependent kinase complex Cdc2/Cdc13.[21]
2. casein kinase $cka1^+$, an orthologue of the α-subunit of casein kinase II in mammals.[22] Its function is essential in yeast but remains to be determined.
3. $adh1^+$, encoding the alcohol dehydrogenase that converts acetaldehyde to ethanol.[23]
4. $rpb10^+$, the corresponding protein is an essential small subunit shared by RNA polymerases I, II, and III.[24] This gene is conserved throughout eukaryotes and has been proposed to be involved in the assembly or integrity of RNA polymerases.

These genes were amplified from genomic DNA and cloned into another expression vector containing the same promoter as the one used for the cDNA library (*nmt1*). Measurement of life span of these strains by CFU counting revealed that they were all long lived compared to the strain with the empty vector (Fig. 3B). Because the overexpression

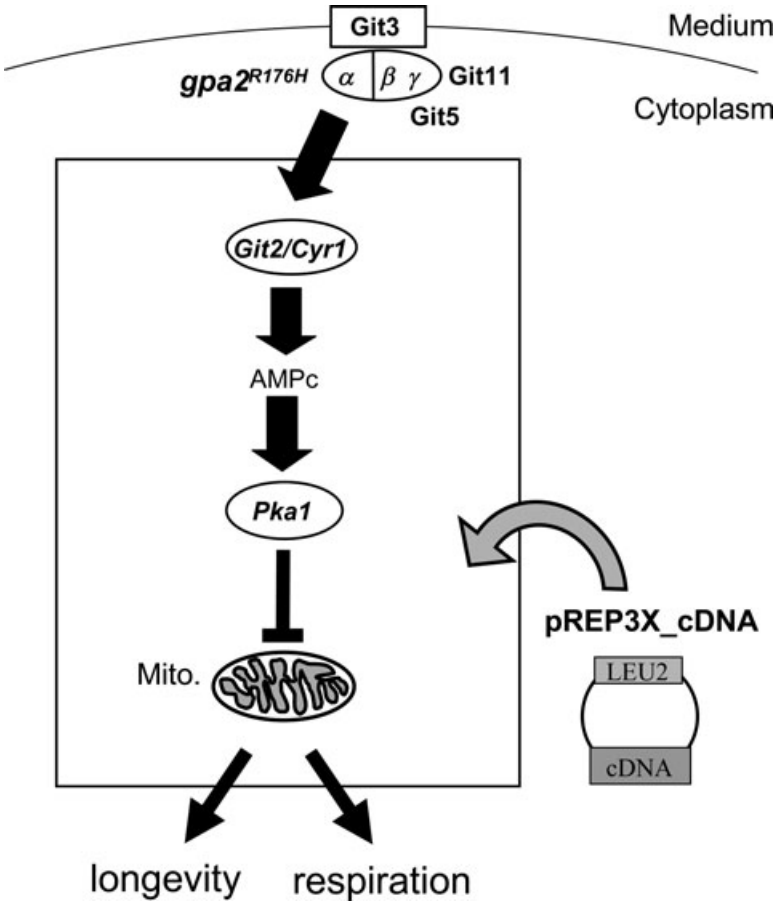

Figure 1. Strategy to screen for genes regulating longevity and respiration downstream of *Git3/gpa2*. The graphic shows the levels on which the candidate proteins isolated in the screen might act to rescue *gpa2^{R176H}* phenotypes. Mito., mitochondrion.

of *psp1*$^{+}$ induced the strongest life span extension, we chose to further characterize this gene.

Psp1 is required for regulation of mitochondrial respiration

In order to study in more detail the effect of *psp1*$^{+}$ overexpression on the short-lived strain *gpa2^{R176H}*, the plasmid carrying the *psp1*$^{+}$ open reading frame under the control of the strong promoter (pREP1) was reintroduced in *gpa2^{R176H}*. Life span was analyzed by CFU counting and compared to that of the same strain with a control vector and also that of the wild-type strain with the same plasmids (Fig. 3C). The resulting longevity curves confirmed that overexpression of this gene rescues the short life span of the *gpa2^{R176H}* strain. The extent of the life span extension by this gene is similar in both *gpa2^{R176H}* and wild-type strains. In other words, its life span is four times longer than the average longevity, which is defined as the day by which half of the population has died. The fact that the pR1_Psp1 plasmid could not increase the life span in *gpa2^{R176H}* mutant cells to the same extent as in the wild type suggests that the constitutive activation of Git3/PKA does not act only on Psp1.

S. pombe is a Crabtree-positive yeast, so the respiration rate rises in the presence of a low concentration of glucose.[14,25] The importance of Psp1 in the control of mitochondrial activity was suggested because its overexpression rescued growth in 2% glycerol–0.1% ethanol of the *gpa2^{R176H}* strain, which requires respiration. To verify the involvement of this gene in the control of mitochondrial

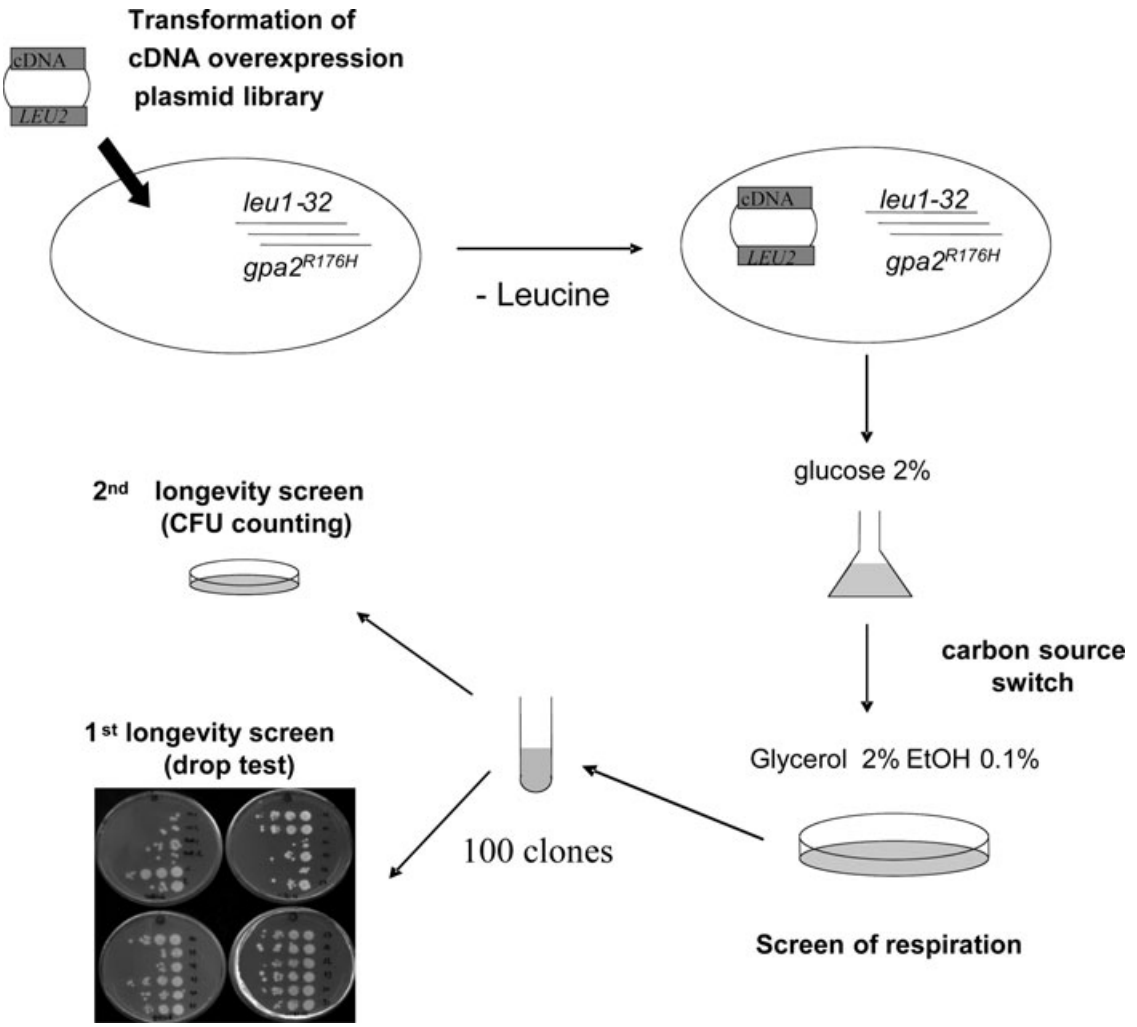

Figure 2. Protocol to screen genes that rescue growth on glycerol and the longevity of the *gpa2*^{R176H} mutant. The screen consisted of the transformation of an overexpression cDNA plasmid library in strain *gpa2*^{R176H}, which is unable to grow on glycerol due to constitutive activation of the Git3/Gpa2/PKA pathway. *gpa2*^{R176H} (RWP1 from M. Yamamoto[34]) was transformed with a pREP3X library of cDNA (from C. Norbury[35]). A total of 200,000 transformants were grown to an optical density of 1 in synthetic complete medium with adenin and uracil (*SCM AU*) (see Refs. 5 and 14 for condition details) with 2% glucose, washed, serially diluted, and spread on plates containing essential minimal medium AU with 2% glycerol and 0.1% ethanol (respiratory medium). After 15 days, 100 colonies were selected. As a control, untransformed RWP1 was treated the same way, and it showed no growth on respiratory medium. The 100 clones were assayed for survival in 4 mL of SCM AU with 2% glucose after 21 days of incubation. At this point, they were spotted on yeast extract–sucrose plates as 10-fold serial dilutions (see Fig. 3A). The 32 clones with the longer life span were chosen for a second longevity test in 4 mL of SCM with 2% glucose in a CFU counting assay.[5] Two measurements were taken, at days 7 and 14. Among 32 clones, 14 displayed survival significantly greater than RWP1 at both time points. Plasmids were recovered and amplified in *Escherichia coli* as described in a previous study[36] and sequenced.

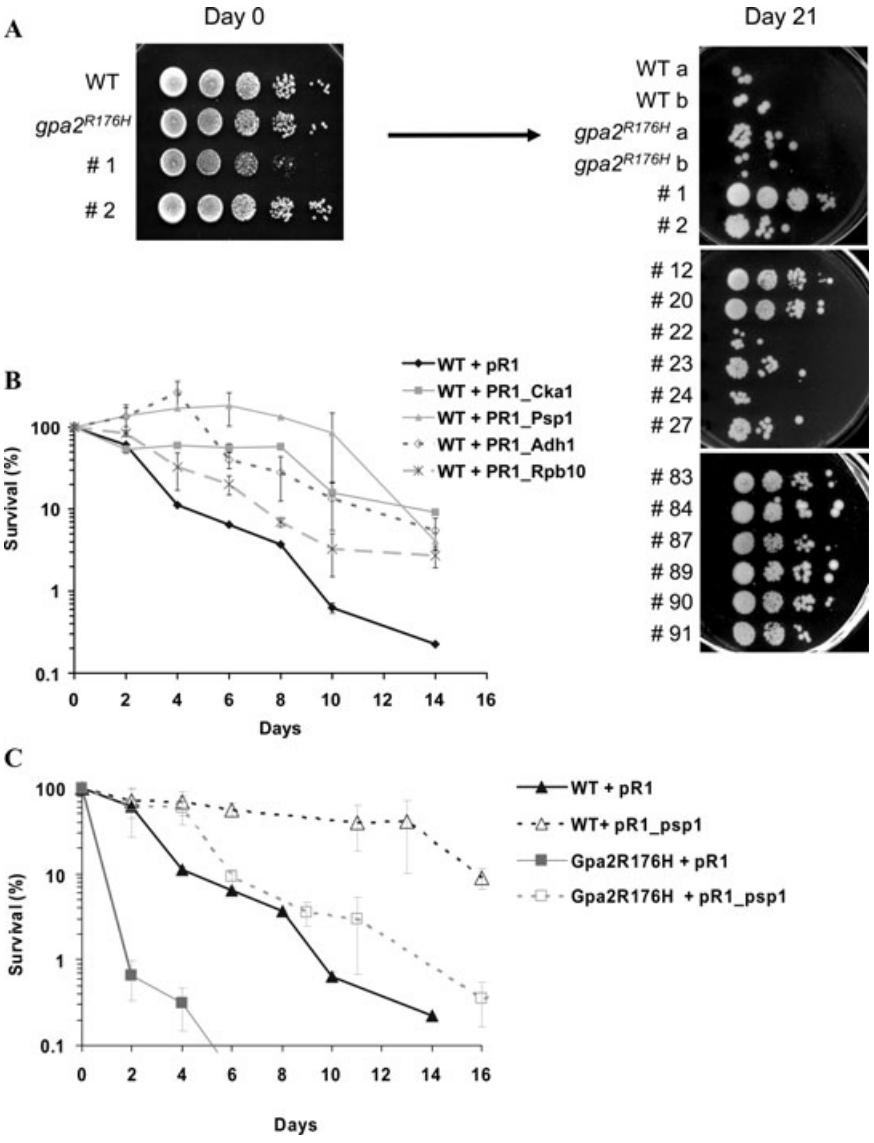

Figure 3. Isolated genes that increase life span in the *gpa2*^{R176H} mutant and wild type (*WT*) when overexpressed. (**A**) Representative results of survival at day 21 in stationary phase during the first longevity test carried out with serial dilutions. Controls are WT and *gpa2*^{R176H}. Numbers refer to clones chosen from the plates with glycerol. (**B**) The four genes isolated were recloned in an overexpression plasmid, transformed into the WT strain, and tested for longevity. *pR1*, plasmid pREP1 with the corresponding open reading frame. (**C**) Characterization of *psp1*$^{+}$ function in longevity. Survival assay results are shown for the wild type with pREP1_Psp1 plasmid or control vector and *gpa2*^{R176H} with pREP1_Psp1 plasmid or control vector. Results are averages of two cultures.

activity, the strain with this gene knocked out (Δ*psp1*; received from M. Kawamukai) was grown in different concentrations of glucose. This strain lost its ability to upregulate its respiration rate when grown in low glucose (data not shown). Interestingly, this strain is also chronologically short lived, as described in a previous study.[21] The requirement of respiration for survival during stationary phase was already proposed in other studies in fission yeast.[14,26] Our result suggests that Psp1

Table 1. Summary of genes involved in life span regulation in the Git3/PKA pathway and potential downstream effectors

Gene	Function	Manipulation[a]	Effect on longevity[b]	Reference(s)
pka1	Ser/Thr kinase	Δ	+++	5, 12
git3	Ser/Thr kinase	Δ	+++	13
$gpa2^{R176H}$	Gα subunit	Mutation conferring constitutive activation	–	13, 15
psp1/sds23	APC regulator	oe	+++	This study
cka1	Kinase, Tor interactant	oe	++	This study
adh1	Alcohol dehydrogenase	oe	+	This study
rpb10	RNA polymerase subunit	oe	+	This study

[a] Δ indicates deletion of the corresponding gene, and oe indicates its overexpression on a plasmid.
[b] +, ++, and +++ refer to weak, average, and strong extension of life span, respectively; – refers to a decrease in life span.

participates in the transduction of the glucose signal from Git3/PKA to mitochondria.

Conclusion and perspectives

The signal from Git3/Gpa2 is activated by extracellular glucose and leads to the release of Pka1 kinase from its Cgs1 regulatory subunit and its transit to the nucleus (Fig. 4). This mechanism is cAMP dependent and is activated by Git2/Cyr1 activity. Our study shows a strong proaging effect of glucose signaling through the Git3 glucose membrane receptor and its associated Gpa2 G protein in fission yeast.[14] We found that this signaling effect on longevity is predominant on the metabolic effect of glucose. Indeed, this pathway still has proaging effects in cells not able to metabolize glucose because of a lack of hexokinase activity. The mechanism of the proaging effect of glucose signaling remains to be elucidated, but our data show strong correlations between glucose signaling, low respiration rate, and reactive oxygen species production. Hence, it seems that the Git3/PKA pathway points to the mitochondrion as a possible downstream target for aging control.

To find new proteins controlling mitochondrial activity and life span, we carried out a screen for genes acting on or downstream of the Git3/PKA pathway (Figs. 1 and 2). Our approach resulted in the isolation of four different genes (Table 1) that were also able to increase life span in wild-type cells (Fig. 2B).

The gene $psp1^+$, also called $sds23^+$ or $moc1^+$, was previously found to rescue the infertility defect caused by the constitutive activation of cAMP signaling after Cyr1 overexpression and is also known to be essential for viability in stationary phase.[21,27] A role in the control of proteolysis mediated by the cyclosome or anaphase-promoting complex (APC) has also been associated with Psp1/Sds23/Moc1 in fission yeast.[28] Interestingly, in budding yeast, the APC is known to regulate replicative aging and is required for normal chronological life span (CLS).[29] A recent study showed that PsP1/Sds23 is an inhibitor of PP2A-related phosphatase, which points toward a role of these phosphatases in the regulation of longevity.[30] Finally, we associated this gene with a novel function in the regulation of mitochondrial respiration. In future studies, it will be interesting to test if the APC is related to mitochondrial control (Fig. 4).

The gene $adh1^+$ encodes the alcohol dehydrogenase. The activation of Adh2 has already been proposed to be a regulator of aging under the control of Sir2 in budding yeast, as ethanol is known to shorten survival in stationary phase.[31] However, the effect of $adh1^+$ overexpression on *S. pombe* longevity observed in this study was weak compared to that of its *S. cerevisiae* counterpart. In addition, the longer life span due to $adh1^+$ overexpression could be due to growth in stationary phase, as seen in Figure 3B.

We also isolated the casein kinase Cka1, whose function is essential in yeast.[22] The Cka1 protein

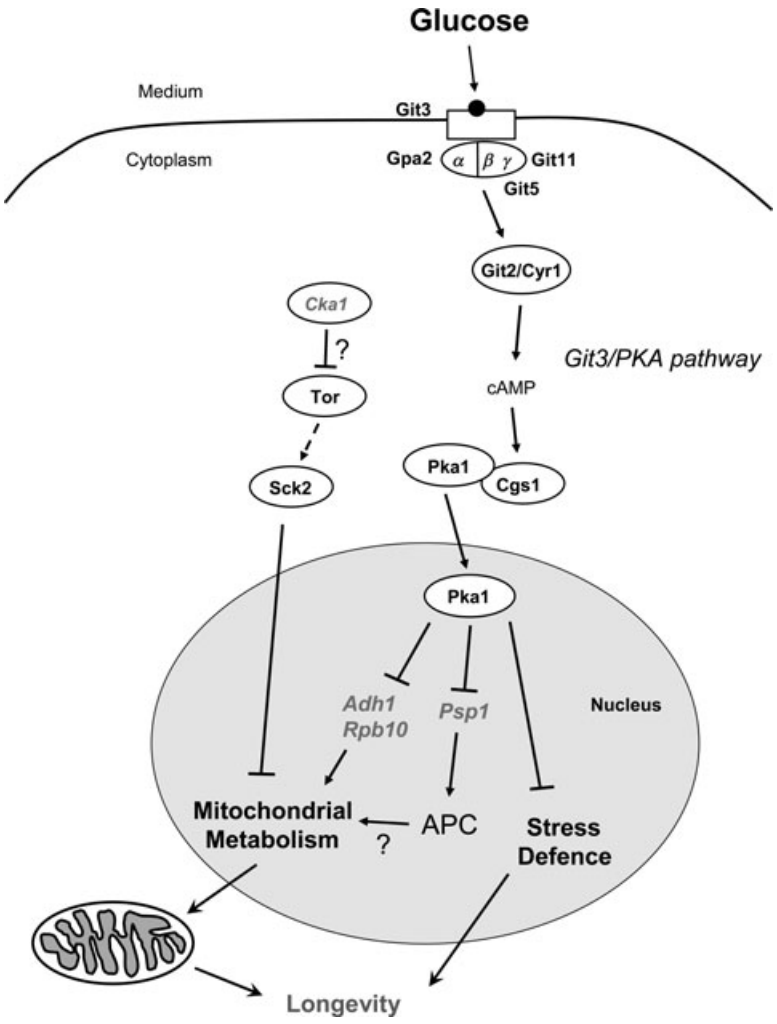

Figure 4. Model for the mechanisms responsible for aging regulation downstream of kinases Pka1/Sck2/Tor. The proteins isolated by screening for activation of respiration and longevity are represented in italics.

has been previously isolated as part of the Tor complex in fission yeast in mass spectrometry analyses.[32] Because Tor is known to be a central proaging kinase regulating Sch9 in yeast,[10,17] this led us to speculate that Cka1 could negatively regulate Tor activity in fission yeast (Fig. 4).

Finally, the *rpb10*$^{+}$ gene encodes an essential small subunit shared by RNA polymerases I, II, and III.[24] The role of this gene in longevity could be due to a global effect on gene expression, a feature that influences life span in yeast and invertebrates.[33] Further investigation is needed to address the precise role of these genes in the control of mitochondria and aging. However, our work demonstrates the power of *S. pombe* for aging research and anticipates future advances in the field.

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Conflicts of interest

The authors declare no conflicts of interest.

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