Requirement of NAD and *SIR2* for Life-Span Extension by Calorie Restriction in *Saccharomyces cerevisiae*

Su-Ju Lin,* Pierre-Antoine Defossez,* Leonard Guarente†

Calorie restriction extends life-span in a wide variety of organisms. Although it has been suggested that calorie restriction may work by reducing the levels of reactive oxygen species produced during respiration, the mechanism by which this regimen slows aging is uncertain. Here, we mimicked calorie restriction in yeast by physiological or genetic means and showed a substantial extension in life-span. This extension was not observed in strains mutant for *SIR2* (which encodes the silencing protein Sir2p) or *NPT1* (a gene in a pathway in the synthesis of NAD, the oxidized form of nicotinamide adenine dinucleotide). These findings suggest that the increased longevity induced by calorie restriction requires the activation of Sir2p by NAD.

Aging in budding yeast is measured by the number of mother cell divisions before senescence. Genetic studies have linked aging in this organism to the *SIR* (silent information regulator) genes, which mediate genomic silencing at telomeres, mating type loci, and the repeated ribosomal DNA (rDNA) (*1*). Sir2p determines life-span in a dose-dependent manner by creating silenced rDNA chromatin, thereby repressing recombination and the generation of toxic rDNA circles (*2, 3*). Silencing is triggered by the deacetylation of certain lysines in the NH_2-termini of histones H3 and H4 (*4*). Sir2p has a NAD-dependent histone deacetylase activity (*5*) that is conserved in Sir2p homologs (*5–7*).

Glucose enters yeast cells via highly regulated glucose-sensing transporters (Hxtp) (*8*) and is then phosphorylated by hexokinases (Hxk1p, Hxk2p, and Glk1p) to generate glucose-6-phosphate (G-6-P) (*9*). To study the effect of calorie restriction (CR) in yeast (*10*), we first determined whether life-span could be extended by limiting glucose availability. Yeast cells grown on 0.5% glucose media exhibited a longer life-span than cells grown on 2% glucose media (Fig. 1A). Limiting the glucose availability by mutating *HXK2* also significantly extended the life-span (Fig. 1B).

Glucose activates the yeast cAMP (adenosine 3′,5′-monophosphate)-dependent protein kinase (PKA) pathway. Major components of this pathway include guanosine triphosphate/guanosine diphosphate (GTP/GDP)–binding proteins (Ras1p and Ras2p), a GTP-GDP exchange factor (Cdc25p), GTP hydrolysis factors (Ira1p and Ira2p), the adenylate cyclase (Cdc35p/Cyr1p), phosphodiesterases (Pde1p and Pde2p) that catalyze the hydrolysis of cAMP, a PKA regulatory subunit (Bcy1p), and a PKA catalytic subunit (encoded by three functionally redundant genes, *TPK1*, *TPK2*, and *TPK3*) (*11–13*). We therefore tested whether limiting flow through this pathway by mutating its components would mimic the life-span extension effect of low glucose concentration. Mutations in *CDC35*, *CDC25*, *TPK1*, *TPK2*, and *TPK3*, all of which reduce the activity of PKA, lengthened life-span, whereas a mutation in *PDE2*, which increases PKA activity, shortened life-span (Table 1). Growth in 0.5% glucose did not further extend the life-span of long-lived *cdc25-10* mutants, which suggests that low glucose concentration and low PKA activity function in the same pathway to extend life-span (Fig. 1A). Because these effects occurred irrespective of strain background, all further experiments focused on one strain, PSY316.

GPA2 encodes a heterotrimeric GTP–binding protein (G protein) subunit that acts in parallel to Rasp to activate adenylate cyclase, and *GPR1* encodes a G protein–coupled receptor that associates with Gpa2p (*14, 15*). Similar to mutants in the cAMP-PKA pathway, the *gpr1*Δ and *gpa2*Δ mutants exhibited a longer life-span compared to the wild type (Fig. 1C). The above data show that reducing cAMP-PKA activity or reducing

Table 1. Longevity inversely correlates with PKA activity in multiple strain backgrounds. For each strain, life-span analysis was carried out at least twice independently on a cohort of more than 45 cells, and the values were averaged. SDs were less than 15% of the mean.

Strain background (*27*)	Genotype	PKA activity	Average life-span
PSY316	Wild type		21.8
	cdc25-10	↓	32.0
	*pde2*Δ	↑	16.2
W303	Wild type		20.8
	cdc25-10	↓	26.2
A364A	Wild type		21.4
	cdc35-1	↓	37.3
SGY	*tpk1*Δ *TPK2* *tpk3*Δ		21.4
	*tpk1*Δ *tpk2-63* *tpk3*Δ	↓	26.5

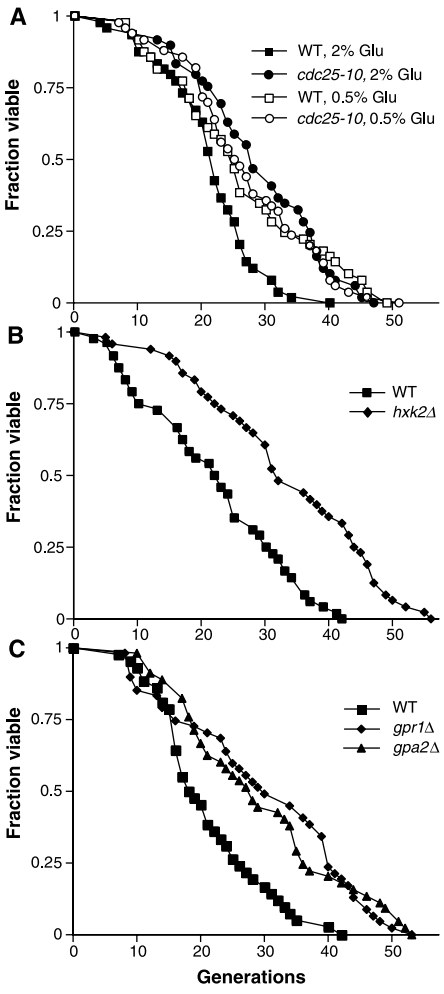

Fig. 1. Limitation of glucose availability or attenuation of glucose signaling extends life-span. (**A**) Longevity is increased by growth on low-glucose medium or by attenuation of cAMP-PKA signaling. Average life-spans (*26*) in 2% glucose (number of generations): PSY316 (wild type, WT), 21.2; *cdc25-10* (*27*), 28.5; in 0.5% glucose, WT, 26.2; *cdc25-10*, 27.4. (**B**) Deletion of hexokinase gene *HXK2* extends life-span (*28*). Average life-spans: WT, 21.8; *hxk2*Δ, 33.3. (**C**) Deletion of the glucose-sensing genes *GPR1* or *GPA2* extends life-span. Average life-spans: WT, 21.0; *gpr1*Δ, 29.7; *gpa2*Δ, 29.3.

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glucose concentration extends life-span and so provides a model for CR in yeast.

Studies in model systems have shown a correlation between enhanced stress resistance and extended life-span. It thus seemed possible that the *cdc25-10* mutation extends life-span by activating the stress response pathway. Indeed, two transcription factors, encoded by *MSN2* and *MSN4*, mediate stress-induced gene expression and are repressed by the cAMP-PKA pathway (*16*). We thus tested whether the extended life-span of a *cdc25-10* mutant was due to an increase in Msn2p and Msn4p activity. Fewer than 2% of the wild-type cells survived a 20-min heat shock at 50°C, whereas 75% of the *cdc25-10* cells survived the same treatment (Fig. 2A). Deletion of the *MSN2* and *MSN4* genes largely reduced the heat shock resistance of *cdc25-10* cells (Fig. 2A). However, the *msn2*Δ *msn4*Δ cells exhibited a life-span close to that of the wild type; moreover, the *cdc25-10 msn2*Δ *msn4*Δ cells showed the same long life-span as *cdc25-10* cells (Fig. 2B). These results indicate that life-span extension by the *cdc25-10* mutation is not mediated by *MSN2* and *MSN4* and therefore does not require the increase in the expression of stress resistance genes.

Does CR require the function of Sir2p to extend life-span? First, we examined the life-span of *cdc25-10* mutants in a *sir2*Δ background. The *sir2*Δ mutation shortened life-span, as reported (*3*), and no life-span extension was provoked by addition of the *cdc25-10* mutation (Fig. 3A). However, it could be argued that the short-lived *cdc25-10 sir2*Δ mutant dies from hyperaccumulation of rDNA circles before the benefit of CR occurs. To further address whether Sir2p is required for CR-induced longevity, we introduced the *fob1*Δ mutation into *CDC25 sir2*Δ and *cdc25-10 sir2*Δ mutants. Fob1p is required for most rDNA recombination and the generation of rDNA circles (*17*), and its elimination suppresses hyperrecombination in the rDNA and the short life-span of *sir2*Δ mutants (*3*). The *fob1*Δ mutation extended the life-span of the *sir2*Δ mutant to an approximately wild-type level, and this extension was not further increased by the *cdc25-10* mutation. Hence, Sir2p is likely required for CR-mediated life-span extension.

Because deacetylation by Sir2p requires NAD, we investigated the role of NAD synthesis in CR-induced life-span extension. In eukaryotes, NAD is synthesized via two major pathways (*18*). In one pathway, NAD originates from the recycling of nicotinic acid by nicotinic acid phosphoribosyl transferase (NPRTase). In yeast, NPRTase is encoded by *NPT1* (*19*). In the other pathway, NAD is generated by quinolinic acid phosphoribosyl transferase (QPRTase) from quinolinic acid. Although yeast QPRTase has not yet been definitively identified, it is likely to be encoded by the *YFR047c* gene, which has 26% identity to the *Escherichia coli* QPRTase. For clarity, we named this open reading frame *QPT1*. To analyze these genetic loci, we deleted *NPT1* and *QPT1* in isogenic strains of opposite mating type by replacement with the *kan*^{r} (kanamycin resistance) gene (*20*). When these two mutants were crossed and the diploid sporulated, kanamycin-resistant segregants of two types appeared. The first

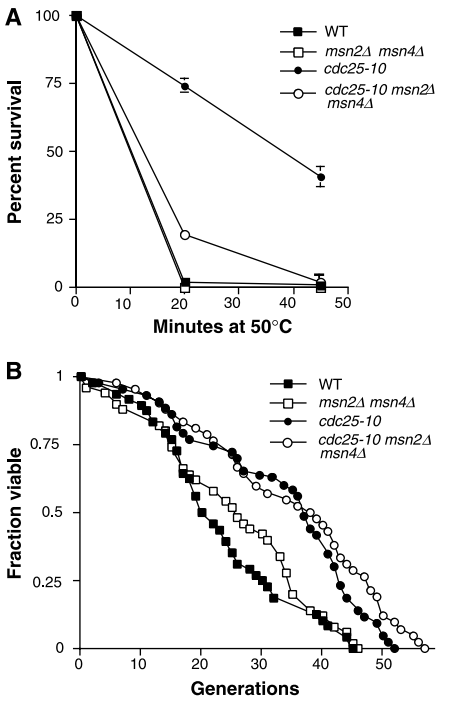

Fig. 2. Life-span extension in the *cdc25-10* strain is not due to increased stress resistance. (**A**) *MSN2* and *MSN4* are required for heat-shock resistance. Cells of the indicated genotypes were preincubated at 37°C for 2 hours, then shifted to 50°C for the indicated time. Survival rates were calculated by determining the plating efficiency versus non–heat-shocked cells (set at 100%). The experiment was carried out twice. SDs were less than 5% of the mean for all data points. (**B**) *MSN2* and *MSN4* are not required for extension in life-span. Average life-spans: WT, 23.3; *msn2*Δ *msn4*Δ, 25.4; *cdc25-10*, 33.4; *cdc25-10 msn2*Δ *msn4*Δ, 35.0.

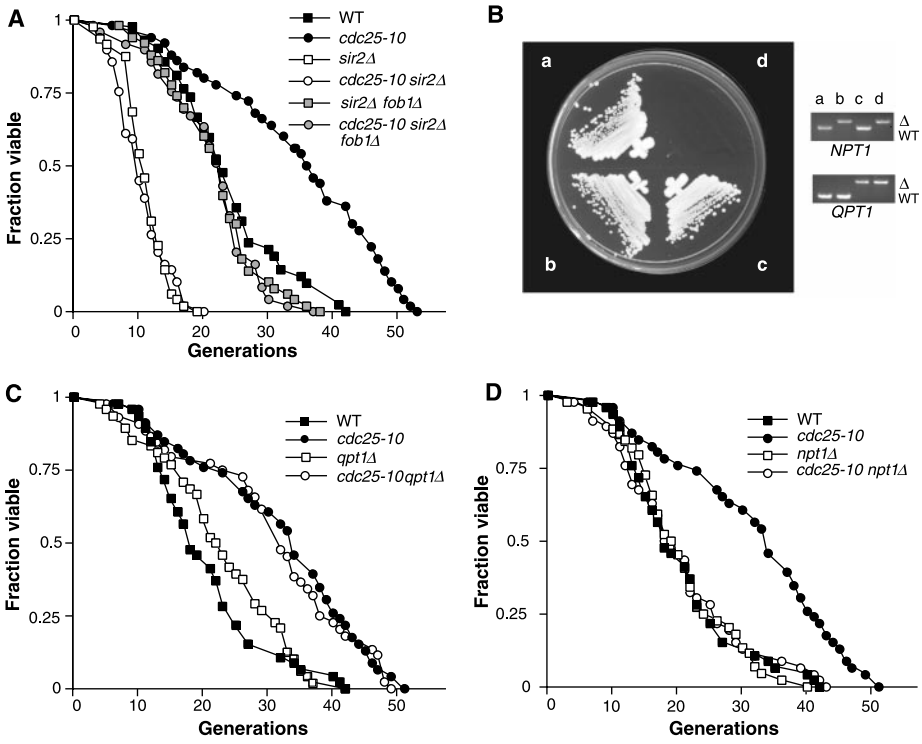

Fig. 3. Life-span extension by the *cdc25-10* mutation requires the presence of Sir2p and Npt1p, an enzyme in a pathway of NAD synthesis. (**A**) *SIR2* is required for extended life-span. Average life-spans: WT, 23.1; *cdc25-10*, 34.4; *sir2*Δ, 11.1; *cdc25-10 sir2*Δ, 10.4. The short life-span of a *sir2*Δ strain is suppressed by *FOB1* deletion, but the life-span of the *sir2*Δ *fob1*Δ mutant is not further extended by the *cdc25-10* mutation. Average life-spans: *sir2*Δ *fob1*Δ, 21.7; *cdc25-10 sir2*Δ *fob1*Δ, 21. (**B**) Deletions of *NPT1* and *QPT1* have a synthetic effect on cell growth. Four segregants of a typical tetratype tetrad derived from a *NPT1/npt1*Δ, *QPT1/qpt1*Δ diploid were streaked on YPD and grown for 2 days at 30°C (left). After 4 days, small colonies of the double mutant could be observed (*21*). The genotypes of these segregants were determined by colony PCR (right) using oligonucleotides flanking *NPT1* (top) or *QPT1* (bottom). (**C**) *QPT1* is not required for extended life-span. Average life-spans: WT, 20.5; *cdc25-10*, 31.8; *qpt1*Δ, 22.6; *cdc25-10 qpt1*Δ, 30.8. (**D**) *NPT1* is required for extended life-span. Average life-spans: WT, 20.5; *cdc25-10*, 31.8; *npt1*Δ, 20.3; *cdc25-10 npt1*Δ, 20.4.

class grew like the parental strains, and all 35 colonies tested were shown by colony polymerase chain reaction (PCR) to bear single deletions of either *NPT1* or *QPT1* (Fig. 3B). The second class grew extremely slowly, and all four colonies were shown to bear deletions of both genes (Fig. 3B). Thus, *NPT1* and *QPT1* function in two redundant pathways of NAD synthesis in yeast.

We next assayed the effect of *qpt1*Δ and *npt1*Δ mutations on life-span with or without CR. Deletion of either *QPT1* or *NPT1* had little effect on the life-span of *CDC25* cells (Fig. 3, C and D). In addition, the *qpt1*Δ mutation did not affect the long life-span of *cdc25-10* mutants (Fig. 3C). However, deletion of *NPT1* prevented the *cdc25-10* mutation from extending life-span (Fig. 3D), indicating that *NPT1*, but not *QPT1*, plays an essential role in life-span extension by CR.

What is the molecular basis of life-span extension by CR? Previous studies suggest that a decrease in rDNA transcription reduces DNA double-strand breaks, thereby repressing rDNA recombination and circle formation (*3*). The fact that the *fob1*Δ mutation did not further extend the life-span of the *cdc25-10* mutant (*21*) suggested that CR might function by this mechanism. To measure the recombination rate in the rDNA, we plated cells with *ADE2* integrated in the rDNA on yeast extract, peptone, and dextrose (YPD) media and then determined the percentage of half-sectored colonies, which indicates marker loss by recombination at the first cell division. The *cdc25-10* mutation reduced the rate of recombination by a factor of ~8 and also reduced levels of rDNA circles in seven-generation-old cells by a factor of ~3 (Fig. 4, A and B); these results suggest that CR extends life-span by reducing rDNA recombination and rDNA circles. This effect on recombination is likely due to increased silencing by Sir2p, because silencing of a telomeric *ADE2* gene is also increased by the *cdc25-10* mutation (*21*).

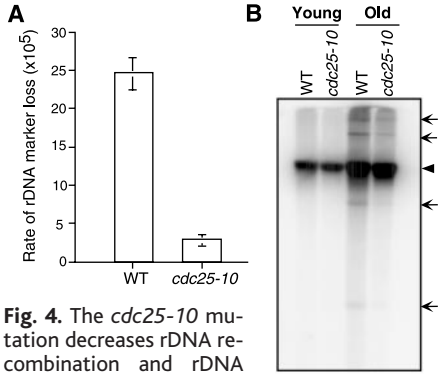

Fig. 4. The *cdc25-10* mutation decreases rDNA recombination and rDNA circle accumulation (*29*). (**A**) Ribosomal DNA recombination rates were determined by the rate of loss of *ADE2* in the rDNA of strain PSY316 (WT) at the first cell division (half-sectored colonies) (*17*). More than 40,000 colonies were examined for each strain. Results show average values and SDs for two experiments. (**B**) DNA from young (unsorted) and old (sorted seven-generation-old) cells was isolated as described (*17*). After Southern blotting, the membrane was hybridized to an rDNA probe. The chromosomal band is indicated by an arrowhead; rDNA circle species are denoted with arrows. The ratio of circle species to total rDNA in old cells, as quantitated by PhosphorImager, was 0.098 for the wild type and 0.028 for *cdc25-10*.

Our results show that lowering glucose concentration or mutating components of the cAMP-PKA pathway that senses glucose provides a model of CR and extends the life-span of yeast mother cells. This extension evidently does not require the intrinsic stress resistance of mutants in the PKA pathway. However, life-span extension by CR does require the NAD-dependent histone deacetylase, Sir2p, and one of the two major pathways of NAD synthesis. How might the activation of Sir2p by NAD be involved in life-span extension? We have shown that CR represses recombination in the rDNA, thereby slowing the formation of toxic rDNA circles. It is possible that a down-regulation of rDNA transcription may also directly contribute to the increase in life-span.

CR induces a variety of physiological changes in primates and rodents, including lowered serum glucose and cholesterol levels, an increase in insulin sensitivity, and a decrease in oxygen consumption and body temperature (*22–24*). In mouse skeletal muscle, CR induces transcriptional reprogramming toward energy metabolism, increased protein turnover, and decreased macromolecular damage (*25*). These findings are consistent with the conventional view that CR acts through a reduction in metabolic rate that lowers production of toxic reactive oxygen species (*22*). However, our findings suggest that the benefit to life-span may involve a more specific molecular mechanism, namely the activation of Sir2p by NAD. In yeast, this activation of Sir2p may extend life-span by increasing silencing and rDNA stability. In metazoans, as in yeast, Sir2p likely promotes genomic silencing because a mammalian homolog, mSir2α, is also an NAD-dependent histone deacetylase (*5*). It is possible that a maintenance of silencing is critical to longevity in metazoans, either by repressing genomic instability or by preventing inappropriate gene expression.

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26. All life-span analyses in this study were carried out on YPD plates with 2% glucose (unless otherwise stated) at least twice independently with more than 45 cells for each strain each time. Representative results are shown.
27. Strain PSY316 *MAT*α*ura3-52 leu2-3, 112 his3-*Δ*200 ade2-101 lys2-801 RDN1::ADE2* was made by P. Park (Guarente laboratory strain). W303AR *MAT***a** *ura3-1 leu2-3, 112 his3-11, 15 trp1-1 ade2-1 RDN1::ADE2 can1-100* has been described in (*3*). A364A *MAT***a** *ade1 ade2 ura1 his7 lys2 tyr1 gal1* and A364A *cdc35-1* (BR214-4a) were obtained from the Yeast Genetic Stock Center (University of California, Berkeley). SGY446 *MAT***a** *his3 leu2-3, 112 ura3-52 trp1 ade8 tpk1*Δ*::ADE8 tpk2-63 (ts) tpk3::TRP1* and SGY446 *TPK2* (SGY559) have been described in (*16*). Owing to the slow growth of *cdc35-1* and *tpk1*Δ *tpk2-63 tpk3*Δ mutants at 30°C, life-span analyses of A364A and SGY strains were carried out at 25°C. Strains bearing the *cdc25-10* temperature-sensitive mutation were made as described (*13*).
28. All gene deletions in this study were done by replacing the wild-type genes with the *kan*^{r} marker, as described (*20*), and were verified by PCR using oligonucleotides flanking the genes of interest.
29. Ribosomal DNA recombination and rDNA circle assays were carried out at 30°C, the temperature at which life-span assays were performed. At 25°C we did notice an increase in the life-span of *cdc25-10* cells without a detectable decrease in recombination (*21*), consistent with previous findings that life-span is a more sensitive assay than recombination for Sir2p function (*3*).
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