Sirtuin-independent effects of nicotinamide on lifespan extension from calorie restriction in yeast

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Summary

Two models have been proposed for how calorie restriction (CR) enhances replicative longevity in yeast: (i) suppression of rDNA recombination through activation of the sirtuin protein deacetylase Sir2 or (ii) decreased activity of the nutrient-responsive kinases Sch9 and TOR. We report here that CR increases lifespan independently of all Sir2-family proteins in yeast. Furthermore, we demonstrate that nicotinamide, an inhibitor of Sir2-mediated deacetylation, interferes with lifespan extension from CR, but does so independent of Sir2, Hst1, Hst2, and Hst4. We also find that 5 mM nicotinamide, a concentration sufficient to inhibit other sirtuins, does not phenocopy deletion of *HST3*. Thus, we propose that lifespan extension by CR is independent of sirtuins and that nicotinamide has sirtuin-independent effects on lifespan extension by CR. Key words: calorie restriction; nicotinamide; replicative aging; Sir2; yeast.

Introduction

Calorie restriction (CR) increases lifespan in multiple organisms, including yeast, worms, flies, and mammals. Despite several decades of study, however, the molecular mechanisms by which CR slows aging remain largely unknown. CR results in several physiological changes that may contribute to its effects on longevity, including decreased activity of nutrient and growth factor responsive pathways, increased resistance to a variety of stresses, altered translation and ribosome biogenesis, and increased autophagy (Weindruch & Walford, 1988; Masoro, 2005). It has also been suggested that CR increases the activity of Sir2-family protein deacetylases (sirtuins) in yeast, flies, and mammals (Guarente & Picard, 2005).

Sir2 is a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase (Imai *et al.*, 2000; Landry *et al.*, 2000; Smith *et al.*, 2000) that promotes transcriptional silencing at the rDNA, the silent mating (HM) loci, and near telomeres (Rine & Herskowitz, 1987; Aparicio *et al.*, 1991; Bryk *et al.*, 1997; Smith & Boeke, 1997). In addition to Sir2, yeast have four sirtuin proteins, Hst1–4, all of which are reported to deacetylate histone tails *in vitro* (Buck *et al.*, 2004). Hst1 is the yeast sirtuin most similar in sequence to Sir2, and overexpression of *HST1* can partially suppress the loss of silencing at HM loci in a Sir2 mutant (Brachmann *et al.*, 1995). It has also been reported that over-expression of *HST2* can partially compensate for the rDNA recombination defect of *sir2Δ* cells (Lamming *et al.*, 2005). No direct link between Hst3 or Hst4 and Sir2 has been described.

The role of Sir2 in yeast aging is thought to be limited to its ability to inhibit formation of extrachromosomal rDNA circles (ERC) (Kaeberlein *et al.*, 1999) by repressing rDNA recombination (Gottlieb & Esposito, 1989). ERCs are one factor that limits the replicative lifespan of a yeast cell, defined as the number of daughter cells produced by a given mother cell (Sinclair & Guarente, 1997). ERC formation is the result of homologous recombination between adjacent rDNA repeats, an event enhanced by the replication fork block protein, Fob1 (Defossez *et al.*, 1999), and antagonized by Sir2 (Kaeberlein *et al.*, 1999). Consistent with a link between ERCs and replicative lifespan, deletion of Fob1 or overexpression of Sir2 increases lifespan by 30–40%, and deletion of *SIR2* shortens lifespan by about 50% (Kaeberlein *et al.*, 1999).

Calorie restriction, accomplished through a reduction in the glucose concentration of the media, also increases replicative lifespan in yeast by 20–40% (Lin *et al.*, 2000, 2002; Kaeberlein *et al.*, 2002). CR fails to increase the short lifespan of *sir2Δ* cells (Lin *et al.*, 2000, 2002; Kaeberlein *et al.*, 2002, 2004), which led to the hypothesis that CR slows aging by activating Sir2 and, thus, decreasing ERC accumulation (Lin *et al.*, 2000). Lin *et al.* (2002, 2004) have proposed that CR activates Sir2 by increasing respiration, which results in decreased levels of NADH, an inhibitor of Sir2. Anderson *et al.* (2003) have proposed an alternative model whereby CR results in decreased levels of nicotinamide, a product of the Sir2 deacetylation reaction and an inhibitor of Sir2 activity.

More recently, we have challenged these models by reporting that CR increases lifespan in a Sir2-independent manner and that CR fails to result in detectable activation of Sir2 *in vivo* (Kaeberlein *et al.*, 2004, 2005a,c). Paradoxically, addition of the Sir2-inhibitor nicotinamide to the media partially blocks lifespan extension from CR in *sir2Δ fob1Δ* cells (Kaeberlein *et al.*, 2005a; Lamming *et al.*, 2005). Because Sir2 is absent from these cells, this suggested the possibility that lifespan extension from CR

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might be partially mediated by a nicotinamide-sensitive protein other than Sir2. We have explored this possibility by testing whether the Hst proteins, individually and in combination, are required for Sir2-independent lifespan extension by CR. We report here evidence that lifespan extension from CR in yeast, and the effects of nicotinamide on this lifespan extension, is not mediated by any of the yeast sirtuins either individually or in combination.

Results

To begin examining a possible role for Hst1–4 in aging, we determined the effect of individually deleting each *HST* gene on mother cell lifespan. Deletion of *HST1* (*P* = 0.81), *HST2* (*P* = 0.77), or *HST4* (*P* = 0.71) had no significant effect on lifespan, indicating that these genes do not function to determine the longevity of wild-type cells. Deletion of *HST3* shortened lifespan (*P* = 2.9×10^{-6}), although not to the same extent as deletion of *SIR2* (*P* < 10^{-13} for *sir2*Δ vs. wild-type; *P* = 3.9×10^{-5} for *sir2*Δ vs. *hst3*Δ Fig. 1A; Table 1). This observation is consistent with the idea that *HST3* might play a role in regulating aging; however, short lifespan is a relatively nonspecific phenotype, as approximately 20% of viable single-gene deletion mutants are short-lived in yeast (Kaeberlein *et al.*, 2005c).

Our finding that deletion of *HST2* had no effect on lifespan is in contrast to a prior report that deletion of *HST2* shortens lifespan (Lamming *et al.*, 2005). We considered the possibility that strain-specific effects could account for this difference, as our experiments were performed in the BY4742 background (Fig. 1A) (Kaeberlein *et al.*, 2005b), while the prior study used W303AR5 for some experiments (Lamming *et al.*, 2005). Strain-specificity cannot explain this discrepancy, however, as we also found no significant effect on lifespan as a result of *HST2* deletion in W303AR5 (*P* = 0.54, Fig. 1B). Also in contrast to the prior study (Lamming *et al.*, 2005), we observed that deletion of *HST2* slightly decreased rDNA recombination, as measured by loss of the *ADE2* marker inserted into the rDNA in W303AR5 (Fig. 1C). Our data are similar to those of Perrod *et al.* (2001) who also observed a slight decrease in rDNA recombination in *hst2*Δ cells. Thus, we conclude that *HST2* plays no role in determining longevity in yeast under standard growth conditions in either BY4742 or W303AR5, and that Hst2 does not repress rDNA recombination or ERC formation in W303AR5.

Because nicotinamide partially blocks lifespan extension by CR through a Sir2-independent mechanism (Kaeberlein *et al.*, 2005a), we wished to examine the possibility that Sir2-independent lifespan extension from CR might be mediated by activation of one of the Sir2 paralogs. If this were the case, we reasoned that deletion of the appropriate sirtuin would abrogate lifespan extension by CR in cells lacking both Sir2 and Fob1. We therefore deleted *HST1*, *HST2*, *HST3*, or *HST4* in a *sir2*Δ *fob1*Δ background, and determined the lifespan of the resulting triple mutant cells on 2% and 0.05% glucose. Growth on 0.05% glucose significantly increased the lifespan of *fob1*Δ *sir2*Δ *hst1*Δ (*P* = 4.7×10^{-4}; Fig. 2A), *fob1*Δ *sir2*Δ *hst2*Δ
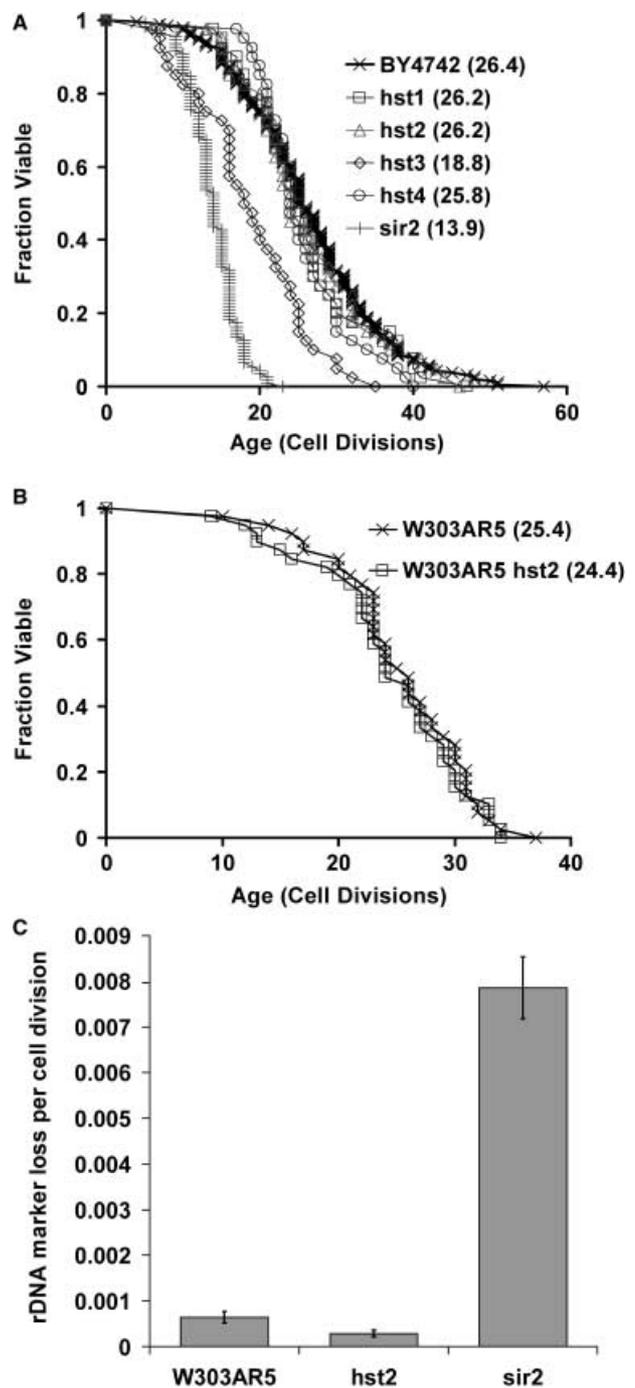

Fig. 1 Hst2 plays no role in replicative aging of BY4742 or W303AR5 mother cells. (A) Replicative lifespan analysis of single-sirtuin deletion strains in BY4742. Mean lifespans ± standard error were: BY4742 26.4 ± 0.7, *hst1*Δ 26.2 ± 1.2, *hst2*Δ 26.2 ± 1.3, *hst3*Δ 18.8 ± 1.2, *hst4*Δ 25.8 ± 0.7, *sir2*Δ 13.9 ± 0.3. Compared to wild-type, *hst3*Δ (*P* = 2.9×10^{-6}) and *sir2*Δ (*P* < 10^{-13}) were significantly short-lived, while *hst1*Δ (*P* = 0.81), *hst2*Δ (*P* = 0.77) and *hst4*Δ (*P* = 0.71) had a median lifespan not significantly different than wild-type. (B) Deletion of *HST2* has no effect on lifespan in W303AR5. Mean lifespans ± standard error were: W303AR5 25.4 ± 0.9, *hst2*Δ 24.4 ± 1.0. *hst2*Δ mother cells had a median lifespan not significantly different than W303AR5 wild-type cells (*P* = 0.54). (C) Deletion of *HST2* modestly decreases rDNA recombination rate in W303AR5, as measured by loss of an *ADE2* marker inserted into the rDNA. At least 20 000 colonies were assayed for each genotype. Error bars are standard error of the mean.

Table 1 Replicative lifespans of different sirtuin mutants in response to calorie restriction or nicotinamide

Genotype	2% Glu	0.5% Glu	0.05% Glu	0.005% Glu	2% Glu5 mM Nic	2% Glu25 mM Nic	0.05%Glu5 mM Nic
BY4742	26.1 ± 0.3	28.8 ± 0.9	31.2 ± 1.0	23.1 ± 1.0	13.0 ± 0.6	8.2 ± 0.8	
BY4742 *sir2Δ*	13.4 ± 0.3				12.0 ± 0.7		
BY4742 *hst1Δ*	26.2 ± 1.2						
BY4742 *hst2Δ*	26.2 ± 1.3						
BY4742 *hst3Δ*	17.5 ± 0.7				4.3 ± 0.5	4.2 ± 0.6	
BY4742 *hst4Δ*	25.4 ± 0.7				13.2 ± 0.8	7.1 ± 0.8	
BY4742 *hst3Δ hst4Δ*	5.6 ± 0.3				3.7 ± 0.3	4.0 ± 0.5	
BY4742 *fob1Δ sir2Δ*	27.0 ± 1.2	30.7 ± 1.7	43.9 ± 3.0	41.0 ± 2.6	27.6 ± 1.1*		34.2 ± 1.4*
BY4742 *fob1Δ sir2Δ hst1Δ*	26.4 ± 1.5		40.1 ± 2.7				
BY4742 *fob1Δ sir2Δ hst2Δ*	29.0 ± 1.6		45.9 ± 2.4				
BY4742 *fob1Δ sir2Δ hst3Δ*	16.7 ± 1.4		30.1 ± 2.9				
BY4742 *fob1Δ sir2Δ hst4Δ*	22.5 ± 1.5		46.1 ± 2.1				
BY4742 *fob1Δ sir2Δ hst1Δ hst2Δ*	27.7 ± 1.5	31.3 ± 1.8	42.1 ± 2.3	43.6 ± 2.4			
BY4742 *fob1Δ sir2Δ hst1Δ hst2Δ hst4Δ*	27.8 ± 1.0		33.9 ± 1.1		25.5 ± 1.1		26.7 ± 1.0
BY4742 *fob1Δ sir2Δ hst1Δ hst2Δ hst3Δ hst4Δ*	6.9 ± 0.6		6.5 ± 0.8				
W303AR5	25.4 ± 0.9						
W303 AR5 *hst2Δ*	24.4 ± 1.0						

Calorie restriction has been defined as media glucose concentration less than 2%. All data shown as mean lifespan ± standard error. The shown values are based on data pooled for all experiments shown in Figs 1–7. *Based on data from Kaeberlein *et al.* (2005a).
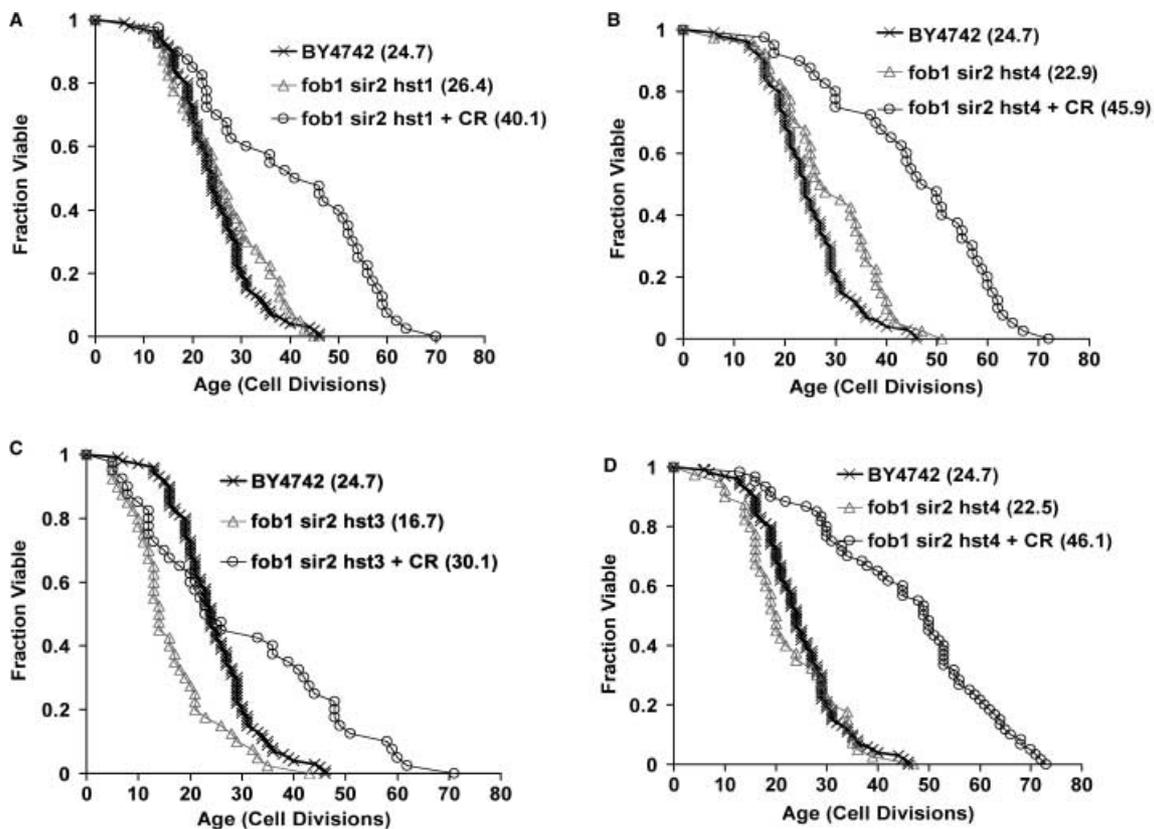
Fig. 2 Sir2-independent replicative lifespan extension by calorie restriction is not mediated by any one sirtuin. (A) Calorie restriction (0.05% glucose) significantly increases the replicative lifespan of cells lacking both Sir2 and Hst1 ($P = 4.7 \times 10^{-4}$). Mean lifespans ± standard error were: BY4742 24.7 ± 0.8, *fob1Δ sir2Δ hst1Δ* 26.4 ± 1.5, *fob1Δ sir2Δ hst1Δ* + CR 40.1 ± 2.7. (B) Calorie restriction (0.05% glucose) significantly increases the replicative lifespan of BY4742 cells lacking both Sir2 and Hst2 ($P = 1.5 \times 10^{-6}$). Mean lifespans ± standard error were: BY4742 24.7 ± 0.8, *fob1Δ sir2Δ hst2Δ* 29.0 ± 1.6, *fob1Δ sir2Δ hst2Δ* + CR 45.9 ± 2.4. (C) Calorie restriction (0.05% glucose) significantly increases the replicative lifespan of BY4742 cells lacking both Sir2 and Hst3 ($P = 1.8 \times 10^{-3}$). Mean lifespans ± standard error were: BY4742 24.7 ± 0.8, *fob1Δ sir2Δ hst3Δ* 16.7 ± 1.4, *fob1Δ sir2Δ hst3Δ* + CR 30.1 ± 2.9. (D) Calorie restriction (0.05% glucose) significantly increases the replicative lifespan of BY4742 cells lacking both Sir2 and Hst4 ($P = 2.4 \times 10^{-10}$). Mean lifespans ± standard error were: BY4742 24.7 ± 0.8, *fob1Δ sir2Δ hst3Δ* 22.5 ± 1.5, *fob1Δ sir2Δ hst3Δ* + CR 46.1 ± 2.4.
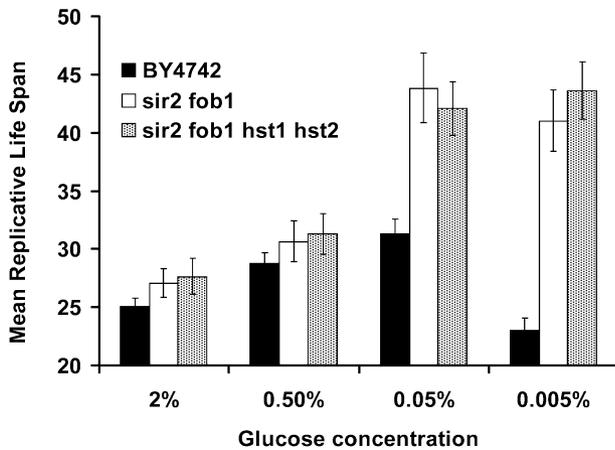
Fig. 3 Sir2-independent lifespan extension from calorie restriction does not involve Hst1 or Hst2 or both. Calorie restriction significantly increases the lifespan of BY4742 wild-type cells at 0.5% ($P = 1.9 \times 10^{-3}$) or 0.05% ($P = 1.3 \times 10^{-5}$), but not 0.005% ($P = 0.78$) glucose. Calorie restriction increases the lifespan of *fob1Δ sir2Δ* cells at 0.5% ($P = 0.03$), 0.05% ($P = 1.4 \times 10^{-4}$), or 0.005% ($P = 2.2 \times 10^{-4}$) glucose. Calorie restriction increases the lifespan of *fob1Δ sir2Δ hst1Δ hst2Δ* cells at cells 0.5% ($P = 0.01$), 0.05% ($P = 1.2 \times 10^{-5}$), or 0.005% ($P = 2.5 \times 10^{-6}$) glucose. Error bars are standard error of the mean.

($P = 1.5 \times 10^{-6}$; Fig. 2B), *fob1Δ sir2Δ hst3Δ* ($P = 1.8 \times 10^{-3}$; Fig. 2C), or *fob1Δ sir2Δ hst4Δ* ($P = 2.4 \times 10^{-10}$; Fig. 2D) mother cells, demonstrating that lifespan extension by CR is not solely mediated by any one of the yeast Sir2 homologs, either alone or redundantly with Sir2.

Although our findings indicate that Sir2-independent lifespan extension is not the result of activation of any one Hst protein, Lamming *et al.* (2005) reported that both Hst2 and Hst1 can partially compensate for Sir2 function at the rDNA in *fob1Δ* cells in the BY4742 strain background. We have since reported, however, that CR significantly increases the lifespan of BY4742 *fob1Δ sir2Δ hst1Δ hst2Δ* cells (Kaeberlein *et al.*, 2006). Indeed, CR increased the lifespan of *fob1Δ sir2Δ hst1Δ hst2Δ* cells to the same extent as *fob1Δ sir2Δ* cells at three different levels of restriction: 0.5%, 0.05%, or 0.005% glucose (Fig. 3). Importantly at all reduced glucose levels tested, CR significantly increased the lifespan of *sir2Δ fob1Δ hst1Δ hst2Δ* cells (0.5% glucose, $P = 0.01$; 0.05% glucose, $P = 1.2 \times 10^{-3}$; 0.005% glucose, $P = 2.5 \times 10^{-6}$). Thus, we conclude that CR increases lifespan independently of Sir2, Hst1, and Hst2, and that the theory that different mechanisms of lifespan extension occur at different glucose levels (Lamming *et al.*, 2006) cannot account for Sir2, Hst1, Hst2-independent lifespan extension by CR.

We next wished to determine whether CR could increase the lifespan of a strain lacking all five yeast sirtuins along with *FOB1*. Unfortunately, the lifespan of *fob1Δ sir2Δ hst1Δ hst2Δ hst3Δ hst4Δ* mother cells was extremely short ($P = 3.4 \times 10^{-10}$; Fig. 4A), indicating that complete absence of sirtuin activity is detrimental for cellular function. Although CR fails to increase the lifespan of *fob1Δ sir2Δ hst1Δ hst2Δ hst3Δ hst4Δ* cells, the severe lifespan defect precludes interpretation of this result. In addition to dra-

matically shortening lifespan, deletion of all five yeast sirtuins also resulted in markedly slower growth relative to wild-type or *fob1Δ sir2Δ* cells (Fig. 4B). Slow growth has been previously reported for *hst3Δ hst4Δ* double mutant cells (Starai *et al.*, 2003), causing us to wonder whether the extremely short lifespan of the *fob1Δ sir2Δ hst1Δ hst2Δ hst3Δ hst4Δ* mother cells could be attributed to simultaneous loss of *HST3* and *HST4*. Consistent with this idea, *hst3Δ hst4Δ* double mutants had a lifespan defect comparable to *fob1Δ sir2Δ hst1Δ hst2Δ hst3Δ hst4Δ* cells (Fig. 4C) and a growth rate substantially slower than wild-type cells (Fig. 4D).

All five yeast sirtuins are thought to carry out a similar NAD-dependent deacetylation reaction (Buck *et al.*, 2004). It is therefore surprising that growth on 5 mM nicotinamide, which is sufficient to inhibit Sir2 *in vivo* (Bitterman *et al.*, 2002), fails to phenocopy the severe lifespan defect of *hst3Δ hst4Δ* cells. This could be explained if either Hst3 or Hst4 (or both) is not inhibited *in vivo* by 5 mM nicotinamide. In order to further examine this possibility, we created a diploid strain that is doubly hetero-zygous for deletion of *HST3* and *HST4* and dissected tetrads onto either YPD or YPD supplemented with 5 mM nicotinamide. When tetrads were dissected onto YPD, only *hst3Δ hst4Δ* double mutant spore clones showed slow growth (Fig. 5A). In contrast, *hst3Δ* spore clones, and not *hst4Δ* or wild-type clones, grew slowly when dissected on YPD + 5 mM nicoti-namide (Fig. 5B). Consistent with the effect on colony size, addition of 5 mM nicotinamide to the media dramatically short-ened the lifespan of *hst3Δ* cells to the level of *hst3Δ hst4Δ* cells in the absence of nicotinamide (Fig. 4C), but only short-ened the lifespan of *hst4Δ* cells to the same level as *sir2Δ* cells (Fig. 4D).

The inability of 5 mM nicotinamide to phenocopy deletion of *HST3* in an *hst4Δ* background could be explained if Hst3 is more resistant to nicotinamide-inhibition *in vivo* than other sirtuins. Consistent with this idea, addition of 25 mM nicotinamide dra-matically shortened the lifespans of wild-type ($P = 1.9 \times 10^{-13}$) cells to an extent comparable to (although still slightly greater than, $P = 0.02$) *hst3Δ* or *hst3Δ hst4Δ* cells at that nicotinamide concentration (Fig. 6A). Growth on 25 mM nicotinamide similarly shortened the lifespan of *hst4Δ* ($P = 2.0 \times 10^{-13}$) mutant cells, resulting in a median lifespan not significantly different than *hst3Δ hst4Δ* cells ($P = 0.33$). Growth rate was also reduced in the presence of 25 mM nicotinamide, indicating that 25 mM nicotinamide is sufficient to phenocopy deletion of both *HST3* and *HST4* (Fig. 6B). While it is not possible to rule out a non-sirtuin-related defect caused by extremely high levels of nicotinamide, these data suggest that Hst3 is inhibited (although perhaps not completely) by addition of 25 mM, but not 5 mM, nicotinamide to the growth media.

Is the inhibition of CR from 5 mM nicotinamide mediated by a sirtuin-dependent or sirtuin-independent mechanism? Cells lacking all yeast sirtuins were extremely short lived, but *fob1Δ sir2Δ hst1Δ hst2Δ hst4Δ* quintuple mutant cells lacking all of the 5 mM nicotinamide-inhibited sirtuins, were only modestly shorter-lived than wild-type cells (Fig. 7). Interestingly, CR still
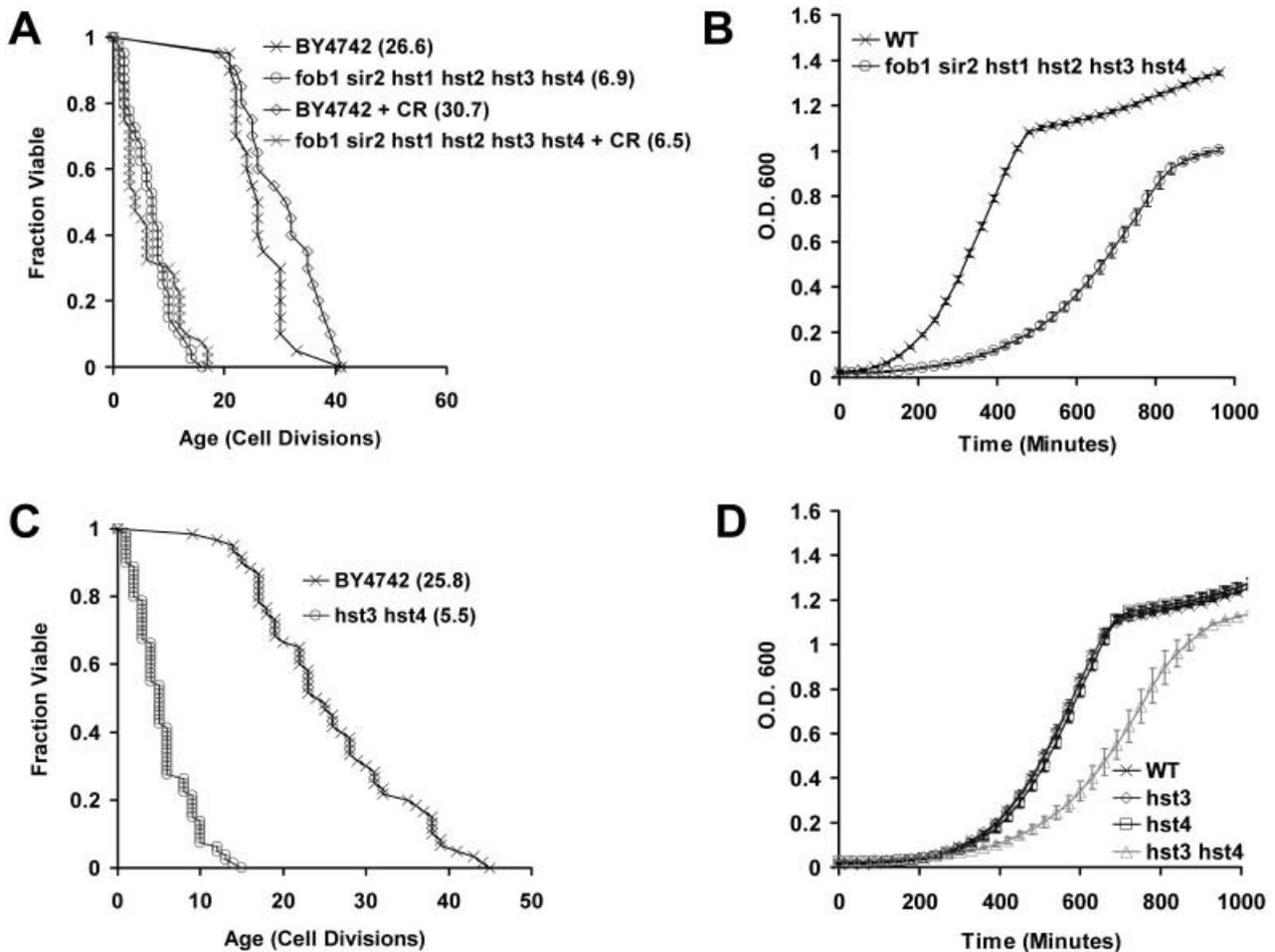
Fig. 4 Deletion of both HST3 and HST4 results in slow growth and extremely short lifespan. BY4742 cells lacking all 5 sirtuins are (A) extremely short-lived ($P = 3.4 \times 10^{-10}$). Mean lifespans ± standard error were: BY4742 26.6 ± 1.1, *fob1Δ sir2Δ hst1Δ hst2Δ hst3Δ hst4Δ* 6.9 ± 0.6, BY4742 + CR 30.7 ± 1.5, *fob1Δ sir2Δ hst1Δ hst2Δ hst3Δ hst4Δ* + CR 6.5 ± 0.8. (B) BY4742 cells lacking all 5 sirtuins are slow growing. (C) BY4742 cells lacking only *HST3* and *HST4* are extremely short-lived ($P < 10^{-13}$). Mean lifespans ± standard error were: BY4742 25.8 ± 1.1, *hst3Δ hst4Δ* 5.5 ± 0.4. (D) BY4742 *hst3Δ hst4Δ* cells are slow growing.

significantly increased the lifespan of *fob1Δ sir2Δ hst1Δ hst2Δ hst4Δ* mother cells ($P = 2.1 \times 10^{-4}$). Addition of 5 mM nicotinamide to the media interfered with this lifespan extension ($P = 6.0 \times 10^{-6}$) even though the only sirtuin present, Hst3, is apparently not inhibited by nicotinamide at this drug concentration. This finding is consistent with the hypothesis that nicotinamide influences lifespan through both sirtuin-dependent and sirtuin-independent mechanisms, and that nicotinamide can influence sirtuin-independent lifespan extension from CR in yeast.

Discussion

The question of whether sirtuins play a role in lifespan extension by CR remains a controversial topic. It has been proposed that CR activates Sir2 in yeast (Lin *et al.*, 2000) and that this activation results in lifespan extension (Guarente, 2005). Significant data have accumulated, however, which is inconsistent with this idea. First, the lifespan extension in response to CR is greater in cells lacking Sir2 than in wild-type cells, as long as ERC levels

are kept low through deletion of *FOB1* (Kaeberlein *et al.*, 2004). Second, CR increases lifespan additively with either overexpression of Sir2 or deletion of *FOB1* (Kaeberlein *et al.*, 2004). Third, *in vivo* Sir2 activity is not increased by CR, as measured by transcriptional silencing of a subtelomeric marker gene (Kaeberlein *et al.*, 2005a,c). Fourth, respiration is not required for lifespan extension by CR (Kaeberlein *et al.*, 2005a), which is inconsistent with a previous model (Lin *et al.*, 2004) for how Sir2 might be activated by CR. Fifth, the ability of nicotinamide to prevent lifespan extension by CR in cells with reduced ERC accumulation is not due to Sir2 inhibition (Kaeberlein *et al.*, 2005a). Here we provide evidence suggesting that CR in yeast is independent of the entire sirtuin family of proteins by showing that none of the yeast paralogs of Sir2 are required for lifespan extension by CR, either alone or in combination with Sir2. Furthermore, we find evidence that 5 mM nicotinamide is not sufficient to inhibit Hst3 *in vivo*, and the effect of nicotinamide on lifespan extension by CR at this concentration is independent of the four remaining sirtuins.
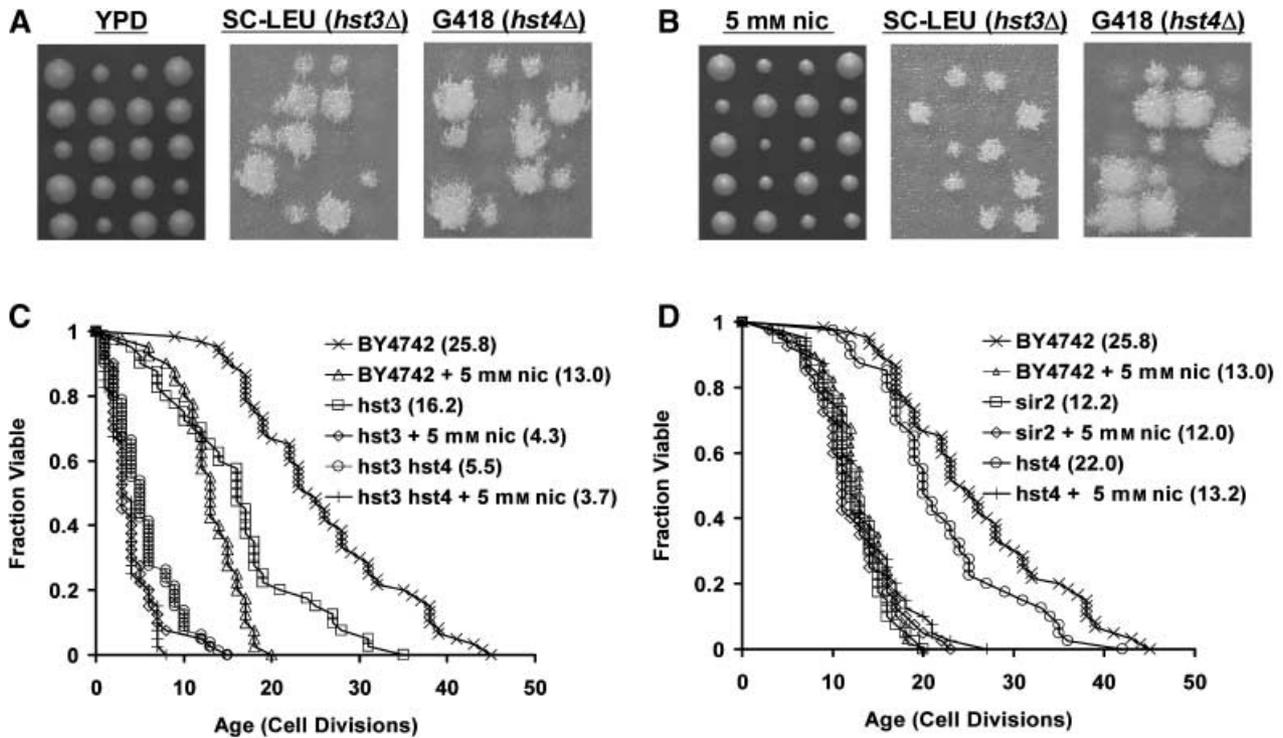
Fig. 5 Hst3 is not inhibited by 5 mM nicotinamide *in vivo*. Dissection of spores from a BY4743 diploid doubly heterozygous for deletion of *HST3* and *HST4* onto (A) YPD or (B) YPD +5 mM nicotinamide. The *hst3*Δ allele is marked with *LEU2* and the *hst4*Δ allele is marked with G418 resistance. *hst3*Δ *hst4*Δ spore clones are slow growing on either YPD or YPD + nicotinamide. *hst3*Δ spore clones are slow growing on YPD +5 mM nicotinamide. Wild-type and *hst4*Δ spore clones are not substantially slower growing on either YPD or YPD + 5 mM nicotinamide. (C) The lifespan of BY4742 *hst3*Δ cells on 5 mM nicotinamide is not significantly different than the lifespan of *hst3*Δ *hst4*Δ double mutant cells on 5 mM nicotinamide ($P = 0.74$). Mean lifespans ± standard error were: BY4742 25.8 ± 1.1, BY4742 + nic 13.0 ± 0.6, *hst3*Δ 16.2 ± 1.3, *hst3*Δ + nic 4.3 ± 0.5, *hst3*Δ *hst4*Δ 5.5 ± 0.4, *hst3*Δ *hst4*Δ + nic 3.7 ± 0.3. (D) Growth on 5 mM nicotinamide shortens the lifespan of *hst4*Δ cells only to the level of a *sir2*Δ mutant. The lifespan of *hst4*Δ cells on 5 mM nicotinamide is not significantly different than the lifespan of *sir2*Δ cells in the absence ($P = 0.6$) or presence ($P = 0.3$) of nicotinamide. Mean lifespans ± standard error were: BY4742 25.8 ± 1.1, BY4742 + nic 13.0 ± 0.6, *sir2*Δ 12.0 ± 0.6, *sir2*Δ + nic 12.0 ± 0.7, *hst4*Δ 22.0 ± 0.6, *hst4*Δ + nic 13.2 ± 0.8.
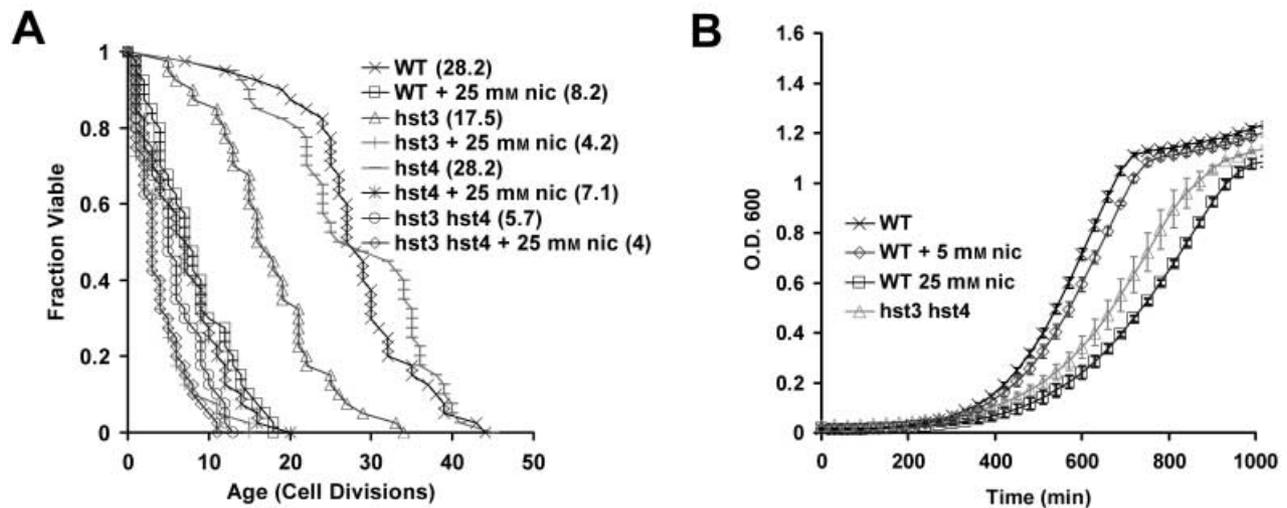
Fig. 6 Hst3 is inhibited by 25 mM nicotinamide *in vivo*. (A) Growth on media with 25 mM nicotinamide dramatically shortens the lifespan of BY4742 wild-type ($P = 1.9\ 10^{-13}$), *hst3*Δ ($P = 1.0\ 10^{-12}$), and *hst4*Δ ($P = 2.0 \times 10^{-13}$) cells. Mean lifespans ± standard error were: BY4742 28.2 ± 1.2, BY4742 +25 mM nicotinamide 8.2 ± 0.8, *hst3*Δ 17.5 ± 1.1, *hst3*Δ +25 mM nicotinamide 4.2 ± 0.6, *hst4*Δ 28.2 ± 1.5, *hst4*Δ + 25 mM nicotinamide 7.1 ± 0.8, *hst3*Δ *hst4*Δ 5.7 ± 0.6, *hst3*Δ *hst4*Δ + 25 mM nicotinamide 4.0 ± 0.5. (B) Addition of 25 mM nicotinamide to the media slows growth rate similar to deletion of both *HST3* and *HST4*.
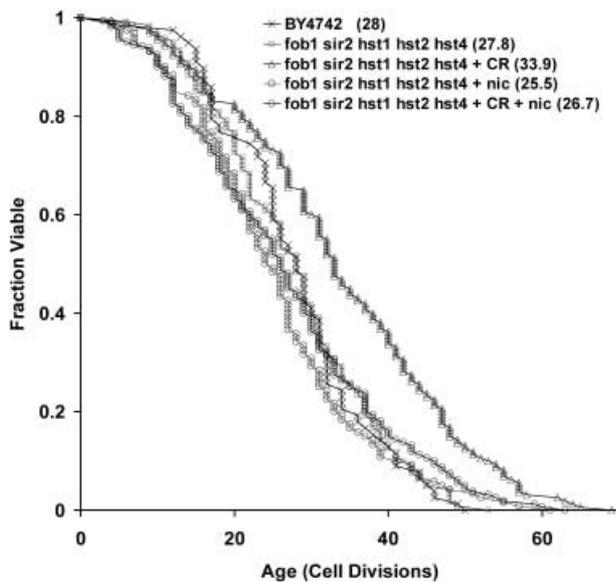
Fig. 7 Effect of 5 mM nicotinamide on lifespan extension from calorie restriction in cells lacking Sir2, Hst1, Hst2, and Hst4. Calorie restriction significantly increases the lifespan of BY4742 *fob1Δ sir2Δ hst1Δ hst2Δ hst4Δ* cells ($P = 2.1 \times 10^{-4}$), and 5 mM nicotinamide interferes with this lifespan extension ($P = 6.0 \times 10^{-6}$). Mean lifespans ± standard error were: BY4742 28.0 ± 1.1, *fob1Δ sir2Δ hst1Δ hst2Δ hst4Δ* 27.8 ± 1.0, *fob1Δ sir2Δ hst1Δ hst2Δ hst4Δ* + CR 33.9 ± 1.1, *fob1Δ sir2Δ hst1Δ hst2Δ hst4Δ* + nic 25.5 ± 1.1, *fob1Δ sir2Δ hst1Δ hst2Δ hst4Δ* + CR + nic 26.7 ± 1.0.

We initially considered the possibility that one of the Sir2 paralogs might mediate lifespan extension by CR, based on the finding that Sir2-independent lifespan extension by CR is partially inhibited by 5 mM nicotinamide in the media (Kaeberlein *et al.*, 2005a; Lamming *et al.*, 2005). If this hypothesis were correct, then CR should fail to increase the lifespan of cells lacking both *SIR2* and the corresponding redundant paralog. This was not the case, however, as CR significantly increased the lifespan *fob1Δ sir2Δ* cells also deleted for *HST1*, *HST2*, *HST3,* or *HST4*. Thus, Sir2-independent lifespan extension by CR, and the effect of 5 mM nicotinamide on this lifespan extension, cannot be explained by activation or inhibition of any one Sir2 paralog.

In several instances, our findings differ from a prior report suggesting that Sir2-independent lifespan extension by CR is mediated by Hst2 (Lamming *et al.*, 2005). To the best of our knowledge, there are no substantial differences in the protocols we use for these assays compared to those used by Lamming *et al.* (2005) that would account for these differences. Although we typically use media containing 0.05% glucose for our CR protocol, differences in the glucose concentration used for CR cannot explain these discrepancies. At every level of CR tested, including the suboptimal (compare the magnitude of lifespan extension at 0.5% glucose vs. 0.05% glucose in Fig. 3) 0.5% glucose CR protocol used by Lamming *et al.* (2005), the magnitude of lifespan extension from CR in *fob1Δ sir2Δ hst1Δ hst2Δ* cells is not significantly different than the magnitude of lifespan extension seen in *fob1Δ sir2Δ* cells and is greater than the magnitude of extension in wild-type cells.

Based on the proposed mechanism for sirtuin-catalyzed protein deacetylation, it would be predicted that nicotinamide should inhibit all five yeast sirtuins *in vivo*. Surprisingly, deletion of all five sirtuins results in severe growth and lifespan defects not phenocopied by addition of 5 mM nicotinamide to the media. Treatment of *hst3Δ* cells with 5 mM nicotinamide does phenocopy the lifespan and growth defects caused by deletion of all five sirtuins, however, suggesting that 5 mM nicotinamide is sufficient to inhibit *SIR2*, *HST1*, *HST2*, and *HST4*. Sauve & Schramm (2003) have reported that nicotinamide inhibits Sir2 activity by favoring base exchange over deacetylation, and that nicotinamide is more effective against mouse Sir2α than yeast Sir2. It is therefore reasonable to suppose that different sirtuin paralogs within yeast could also have different sensitivities to nicotinamide inhibition and that Hst3 may be particularly resistant.

Alternatively, it is possible that the protein deacetylase activity of Hst3 is inhibited by 5 mM nicotinamide, but the growth and longevity phenotypes associated with deletion of *HST3* arise from a structural or alternative catalytic activity of the protein. This seems unlikely, however, as the synthetic defect observed between deletion of *HST3* and deletion of *HST4* suggests a redundant or parallel function for these two proteins, and nicotinamide inhibits *HST4*, as indicated by the slow growth and very short lifespan of *hst3Δ* cells grown on nicotinamide. Further arguing against a structural or alternative catalytic role for Hst3 is our finding that growth of wild-type cells in the presence of 25 mM nicotinamide is sufficient to largely recapitulate the phenotypes of *hst3Δ hst4Δ* double mutant cells. Thus, we propose that Hst3-mediated protein deacetylation is inhibited by nicotinamide when added to the media at levels higher than 25 mM, but is largely unaffected when nicotinamide is present in the media at 5 mM or lower. More detailed studies of the Hst3 enzyme will be necessary to determine whether this hypothesis is correct.

The observation that CR can increase the lifespan of cells lacking all four sirtuins inhibited by 5 mM nicotinamide, and that this lifespan extension is abrogated by 5 mM nicotinamide, suggests a mechanism of nicotinamide-inhibition that is independent of sirtuins. What might such a mechanism be? Nicotinamide potentially plays a role in multiple cellular processes, any one (or more) of which could be important for proper response to nutrient depletion. It may also be the case that addition of exogenous nicotinamide results in a nonspecific detrimental effect that becomes apparent in aged cells. For example, we have identified several single-gene deletion mutants that are slow growing or unable to grow on media supplemented with 5 mM nicotinamide, one class of which contains mutants defective for DNA repair (data not shown). It is known that mutation of DNA repair enzymes can result in lifespan defects ranging from moderate to severe (Park *et al.*, 1999; McVey *et al.*, 2001; Saffi *et al.*, 2001; Hoopes *et al.*, 2002; Kaeberlein *et al.*, 2005b), raising the possibility that nicotinamide might prevent extreme longevity by interfering with the response to DNA damage. Whether this effect is specific to the calorie-restricted state or nonspecific remains to be determined.

The findings reported here also have relevance for interpreting the role of nicotinamidase in longevity determination. Overexpression of the gene coding for nicotinamidase can partially suppress Sir2-inhibition by exogenous nicotinamide (Gallo *et al.*, 2004), and up-regulation of Pnc1 is reported to be required for lifespan extension by CR (Anderson *et al.*, 2003). It has been proposed that up-regulation of Pnc1 activates Sir2 through depletion of nicotinamide (Anderson *et al.*, 2003); however, our data suggest an alternative explanation. Because addition of exogenous nicotinamide blocks CR independently of sirtuins, it may be that increased Pnc1 activity could promote lifespan extension in response to CR by a sirtuin-independent mechanism, as well. It will be informative to determine whether overexpression of Pnc1, like CR, increases lifespan independently of Sir2.

The question of whether sirtuins play a role in lifespan extension by CR in yeast or any other organism remains unresolved (Kennedy *et al.*, 2005; Longo & Kennedy, 2006). We have presented data suggesting that CR acts through a pathway independent of the entire family of sirtuin enzymes to modulate yeast replicative lifespan. Recent work from Fabrizio *et al.* (2005), has demonstrated that Sir2 antagonizes yeast chronological lifespan extension in a genetic model of CR, deletion of the nutrient responsive kinase Sch9. In *Caenorhabditis elegans*, it has been reported that *sir-2.1* is partially required for lifespan extension from a genetic model of CR, mutation of *eat-2* (Wang & Tissenbaum, 2006); however, lifespan extension from CR using a reduced feeding protocol is completely independent of *sir-2.1* (Kaeberlein *et al*. in press). Furthermore, deletion of *sir-2.1* fails to shorten lifespan, and there is no evidence supporting a role for other *C. elegans* sirtuins in longevity determination or CR (Kaeberlein *et al*. in press). In flies, on the other hand, it is reported that lifespan extension from CR requires the Sir2 ortholog, dSir2 (Rogina & Helfand, 2004), and intriguing phenotypes have recently been reported for mice lacking individual sirtuin genes (Cheng *et al.*, 2003; Bordone *et al.*, 2005; Chen *et al.*, 2005; Lemieux *et al.*, 2005; Mostoslavsky *et al.*, 2006). It will be of particular interest to learn whether CR can increase the lifespan of these knockout mice and whether increased expression of sirtuins is sufficient to slow aging in mammals (Longo & Kennedy, 2006).

The importance of nicotinamide in longevity determination remains, for now, a yeast specific phenomenon. However, dietary nicotinamide has been implicated in human disease processes, including neurodegenerative diseases (Williams & Ramsden, 2005) and septic shock (Szabo, 2003). Interestingly, nicotinamide has also been reported to reverse aging phenotypes and delay senescence in human fibroblasts in culture (Matuoka *et al.*, 2001). Nicotinamide is likely to have a variety of nonsirtuin effects, such as its known ability to inhibit poly(ADP-ribose) polymerase (Szabo, 2003). Therefore, a better mechanistic understanding of the different cellular pathways affected by nicotinamide will help to clarify the effects of nicotinamide that are distinct from sirtuin inhibition, which may be of relevance in higher eukaryotes.

Experimental procedures

Strains and media

Unless otherwise stated, all yeast strains were derived from the parent strain for the haploid yeast open reading frame deletion collection (Winzeler *et al.*, 1999), BY4742 (Research Genetics) or from W303AR5 (Kaeberlein *et al.*, 1999). Gene disruptions were carried out by transforming yeast with PCR-amplified deletion constructs containing 45 nucleotides of homology to regions flanking the ORF to be deleted and either *HIS3*, *LEU2*, or *URA3* amplified from pRS403, pRS405, or pRS406 (Sikorski & Hieter, 1989), respectively. In each case, the entire open reading frame of the deleted gene was removed. All gene disruptions were verified by PCR. Media used for lifespan studies was YEP (2% bacto peptone, 1% yeast extract) supplemented with filter-sterilized glucose at the designated concentration. For nicotinamide supplementation experiments, nicotinamide was added to YEP from a 500-mM nicotinamide filter-sterilized stock solution to a final concentration of 5 mM just prior to pouring plates. Nicotinamide was obtained from Sigma (St Louis, MO, USA).

Replicative lifespan analysis

Replicative lifespan analysis was carried out as described (Kaeberlein, 2006). All lifespan experiments were performed 'blind' with strains coded such that the researchers performing the microdissection had no knowledge of the genotype for any particular strain. Unless otherwise stated, standard lifespan media was YEP + 2% glucose (YPD), and CR media was YEP +0.05% glucose. Wilcoxon *P*-values were calculated using the MATLAB rank sum function, and strains are stated to have a significant difference in lifespan for $P < 0.05$. A summary of lifespan data generated in this manuscript is provided in Table 1.

rDNA marker loss assays

Loss of the *ADE2* marker from W303AR5-derived cells was measured as described (Kaeberlein *et al.*, 1999). Cells were grown in YPD at 30 °C to a density of ~1×10^8 cells/mL, diluted back to approximately 1×10^5 cells/mL, and 100 μL of this dilution was plated onto YPD agar in 150 mm Petri dishes. Cells were allowed to form colonies at 30 °C, and then transferred to 4 °C for 3 days to allow for maximum color formation in *ade2* cells. For each plate, the number of half-sectored colonies was determined and the total number of colonies (excluding entirely red colonies) was determined.

Growth rate assays in liquid culture

Growth curves were generated using a Bioscreen C incubator/shaker/reader (Growth Curves USA, Piscataway, NJ). Cells were cultured overnight in YPD at 30 °C. For each strain analyzed, 5 μL of overnight culture was diluted into 145 μL of fresh YPD

in a Bioscreen C Honeycomb plate (Growth Curves USA). Cells were cultured in the Bioscreen C machine at 30 °C with continuous shaking and optical density (OD) at the Bioscreen C wideband wavelength (~600 nm) was determined every 30 min. At least six replicates were analyzed for each strain.

Growth rate (doubling time) was determined for each strain analyzed in the Bioscreen C machine from a best-fit linear regression equation to the natural logarithm of OD vs. time data. For consistency, only those data points corresponding to background normalized OD values 0.05 < OD < 0.4 were used to derive the linear regression equation. For each data set, the doubling was calculated as natural logarithm of 2 divided by the slope of the linear regression equation.

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