

# Nicotinamide Riboside Promotes Sir2 Silencing and Extends Lifespan via Nrk and Urh1/Pnp1/Meu1 Pathways to NAD<sup>+</sup>

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## SUMMARY

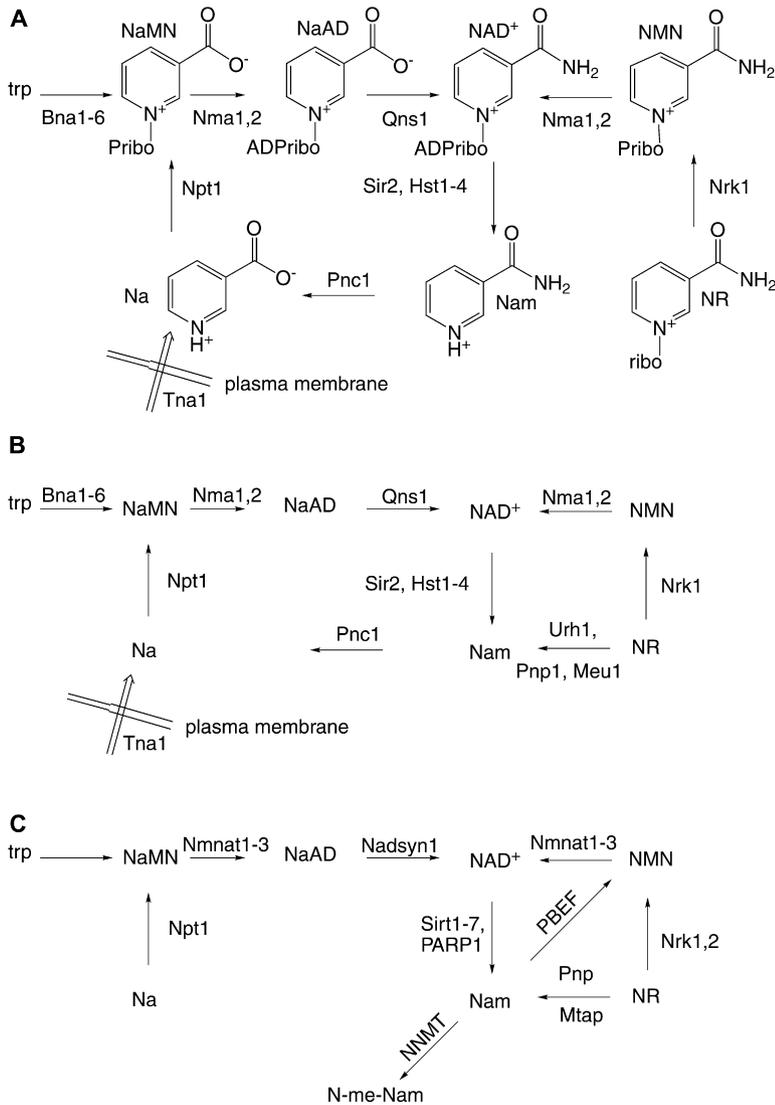
Although NAD<sup>+</sup> biosynthesis is required for Sir2 functions and replicative lifespan in yeast, alterations in NAD<sup>+</sup> precursors have been reported to accelerate aging but not to extend lifespan. In eukaryotes, nicotinamide riboside is a newly discovered NAD<sup>+</sup> precursor that is converted to nicotinamide mononucleotide by specific nicotinamide riboside kinases, Nrk1 and Nrk2. In this study, we discovered that exogenous nicotinamide riboside promotes Sir2-dependent repression of recombination, improves gene silencing, and extends lifespan without calorie restriction. The mechanism of action of nicotinamide riboside is totally dependent on increased net NAD<sup>+</sup> synthesis through two pathways, the Nrk1 pathway and the Urh1/Pnp1/Meu1 pathway, which is Nrk1 independent. Additionally, the two nicotinamide riboside salvage pathways contribute to NAD<sup>+</sup> metabolism in the absence of nicotinamide-riboside supplementation. Thus, like calorie restriction in the mouse, nicotinamide riboside elevates NAD<sup>+</sup> and increases Sir2 function.

## INTRODUCTION

In wild-type yeast cells, whose replicative lifespan is limited by accumulation of extrachromosomal rDNA circles (ERCs) (Sinclair and Guarente, 1997), Sir2 activity as an NAD<sup>+</sup>-dependent protein lysine deacetylase promotes replicative lifespan (Kaeberlein et al., 1999; Imai et al., 2000; Lin et al., 2000). Calorie restriction (CR), the most powerful known intervention to extend lifespan in vertebrates, extends lifespan in wild-type yeast cells in a Sir2- and NAD<sup>+</sup>-dependent manner (Lin et al., 2000). Although ERCs are a species-specific aging mechanism and ERC-independent and Sir2-independent processes for replicative-lifespan extension clearly exist (Kaeberlein

et al., 2004), overexpression experiments in worms (Tissenbaum and Guarente, 2001) and flies (Rogina and Helfand, 2004) have demonstrated conserved roles for Sir2-related enzymes (Sirtuins) in animal longevity.

Because of the central roles of NAD<sup>+</sup> and its reduced and phosphorylated derivatives in metabolism (Belenky et al., 2007), and the conserved functions of Sirtuins in longevity, much attention has been paid to the mechanisms of NAD<sup>+</sup>-dependent regulation of Sir2 in CR-mediated alterations of metabolism and lifespan. Two leading mechanisms from yeast molecular biology are that CR promotes clearance of Sir2-inhibiting nicotinamide (Nam) (Anderson et al., 2003a) or a decrease in NADH (Lin et al., 2004), thereby increasing Sir2 function. It was also proposed that CR might increase the level of the Sir2 substrate, NAD<sup>+</sup> (Guarente, 2000). It has now been shown that in livers of mice fasted for one day, NAD<sup>+</sup> levels and Sirt1 protein levels are increased, leading to deacetylation of PGC-1 $\alpha$  and a consequent gluconeogenic transcriptional program (Rodgers et al., 2005). Additionally, in a murine model of Alzheimer's disease expressing the Swedish allele of human A $\beta$  precursor (Hsiao et al., 1996), CR almost completely prevents amyloid neuropathology coincident with increased Sirt1 protein expression in the neocortex, increased brain NAD<sup>+</sup> levels, and reduced brain Nam (Qin et al., 2006). Thus, a CR-mimetic small-molecule intervention to improve Sirt1 function would have considerable potential in metabolic and neuroprotective therapeutic areas. Indeed, evidence has recently been presented that high-dose resveratrol improves health, longevity (Baur et al., 2006), and muscle performance (Lagauge et al., 2006) in overfed mice through a mechanism that appears to involve Sirt1 activation. Although a nutritional approach of increasing Sir2 activity and longevity would be attractive, this has only been achieved by engineered gene overexpression in yeast (Anderson et al., 2002) because nicotinic acid fails to extend lifespan and Nam shortens lifespan (Bitterman et al., 2002; Gallo et al., 2004). Moreover, extra copies of *NPT1* were reported to activate Sir2 and extend lifespan in a manner mimicking CR but without increasing NAD<sup>+</sup> levels (Anderson et al., 2002). In fact, although now supported by animal data



**Figure 1. Schematized Intracellular NAD<sup>+</sup> Synthesis in Eukaryotes**

(A) In yeast, NAD<sup>+</sup> is synthesized via a de novo biosynthetic pathway from trp and three salvage pathways from the vitamin precursors, nicotinic acid, Nam, and NR (Bieganowski and Brenner, 2004).

(B) As shown in this study, Urh1, Pnp1, and Meu1 constitute a second salvage pathway for NR utilization, which is merged into Nam and nicotinic-acid salvage.

(C) The presumed vertebrate intracellular pathways for NAD<sup>+</sup> synthesis are shown. Vertebrate intracellular NAD<sup>+</sup> metabolism differs from fungal NAD<sup>+</sup> metabolism by the addition of PARP as an NAD<sup>+</sup> consumer, the use of Nam PRTase (PBEF) instead of nicotinamidase (Pnc1), and the encoding of Nam N-methyltransferase (NNMT) activity.

(Rodgers et al., 2005; Qin et al., 2006), the idea that Sir2 might be activated by increased NAD<sup>+</sup> (Guarente, 2000) seems to have been set aside in light of the fact that at least four studies did not observe increased NAD<sup>+</sup> either in CR conditions or when a biosynthetic gene was overexpressed (Lin et al., 2001; Anderson et al., 2002; Anderson et al., 2003b; Lin et al., 2004).

Our investigation of NAD<sup>+</sup> synthesis continues to indicate that not all of the metabolic steps for the molecule discovered 100 years ago as cozymase (Harden and Young, 1905) have been identified genetically, even though tryptophan, nicotinic acid, and Nam were identified as biosynthetic precursors long ago (Krehl et al., 1945). Accordingly, the lack of a complete set of genes controlling NAD<sup>+</sup> metabolism may be hampering mechanistic analysis of Sir2-dependent and NAD<sup>+</sup>-dependent functions in all eukaryotic models under study. Recently, we discovered that nicotinamide riboside (NR), a natural product present in milk, is an NAD<sup>+</sup> precursor that is con-

verted to NAD<sup>+</sup> through the action of conserved eukaryotic NR kinases including yeast and human Nrk1 and human Nrk2 (Bieganowski and Brenner, 2004). As shown in Figure 1A, the de novo pathway from tryptophan, the nicotinic-acid import/salvage pathway, and the yeast Nam salvage pathway all depend on the action of glutamine-dependent NAD<sup>+</sup> synthetase Qns1 (Bieganowski et al., 2003). Thus, provision of extracellular NR defined a novel biosynthetic pathway to NAD<sup>+</sup>, a pathway that was shown to be Qns1 independent and Nrk1 dependent (Bieganowski and Brenner, 2004).

Although NR rescued the growth of yeast cells containing *qns1* gene deletions, it was not clear whether exogenous NR would contribute to the NAD<sup>+</sup> pool in wild-type cells, whether the Nrk1 pathway to NAD<sup>+</sup> promotes Sir2 functions including longevity, and whether there is an Nrk1-independent route to NAD<sup>+</sup>. Here, we show that exogenous NR promotes Sir2-dependent repression of recombination, gene silencing, and extension of longevity through the Nrk1

pathway of NAD<sup>+</sup> synthesis in yeast *npt1* mutants. We discover a second and Nrk1-independent pathway for NR utilization initiated by Uhr1, Pnp1, and Meu1 and establish that NR salvage pathways are important components of NAD<sup>+</sup> metabolism in the presence and the absence of exogenous NR. We establish that although yeast cells relying on de novo NAD<sup>+</sup> biosynthesis grow well, they perform Sir2-dependent gene silencing poorly and age rapidly. Finally, we show that NR improves gene silencing and longevity in glucose-replete wild-type cells by a mechanism that depends on NAD<sup>+</sup> synthesis through the Nrk1 pathway and the Uhr1/Pnp1/Meu1 pathway.

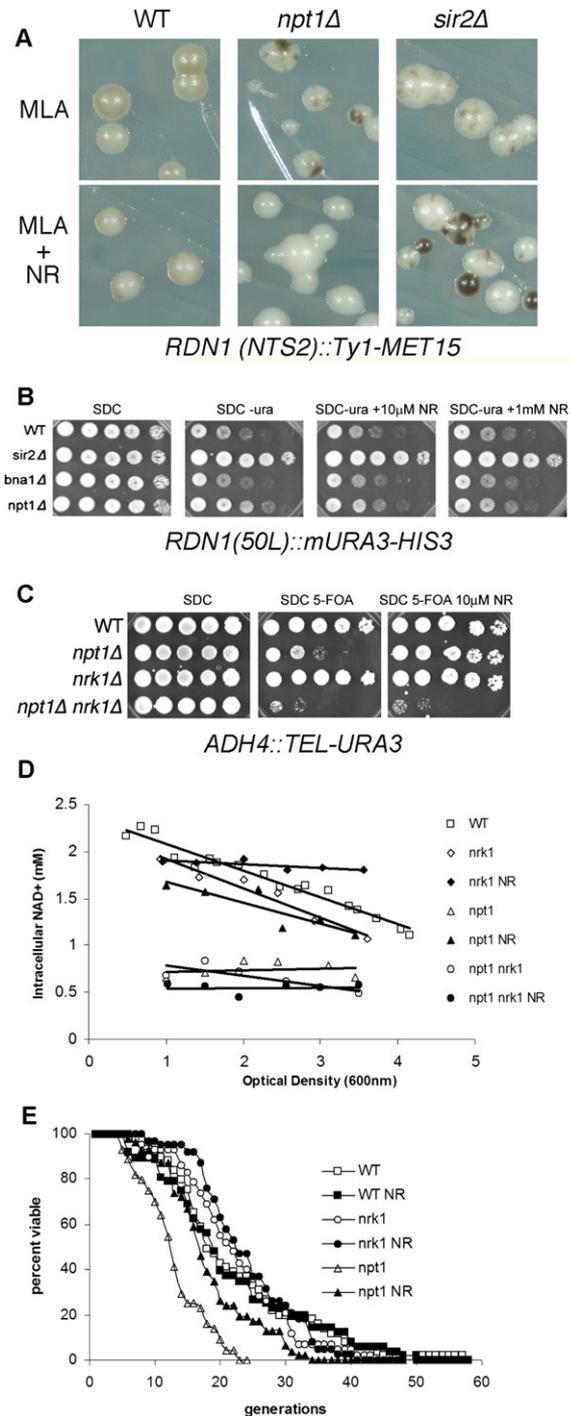
## RESULTS

### NR Increases Sir2-Dependent Repression of Recombination, Gene Silencing, and Replicative Lifespan via Nrk1-Dependent NAD<sup>+</sup> Synthesis

Earlier studies established that the increase in longevity observed in calorie-restricted yeast cells depends not only on Sir2 but also on Npt1, the nicotinic acid phosphoribosyltransferase (PRTase) (Lin et al., 2000). Similarly, Npt1 has been shown to be required for optimal Sir2-dependent gene-silencing activities (Smith et al., 2000; Anderson et al., 2002; Sandmeier et al., 2002). As shown in Figure 2A, we reproduced the requirement for Sir2 (Gottlieb and Esposito, 1989) and Npt1 (Smith et al., 2000) in repressing recombination between repeated ribosomal RNA genes. Addition of 10  $\mu$ M NR was sufficient for repressing ribosomal gene recombination in the *npt1* strain. To determine whether NR represses recombination via a Sir2-dependent mechanism, we tested whether the hyper-recombinational phenotype of *sir2* could be reduced by an addition of 10  $\mu$ M NR. As shown in Figure 2A, NR was without benefit to the *sir2* mutant, establishing that the target of NR function depends on Sir2.

Repression of recombination within the ribosomal-gene array has the limitation of being a yeast-specific function of Sir2. Because regulation of gene expression by virtue of protein lysine deacetylase activity is the more universal function of Sirtuins (Sinclair and Guarente, 2006), we treated wild-type, *npt1* mutant, and *sir2* mutant yeast cells containing a modified *URA3* gene (*mURA3*) integrated at the ribosomal-gene array (Buck et al., 2002) with increasing concentrations of NR. As shown in Figure 2B, *npt1* mutants exhibited moderately poor silencing of ribosomal-gene-integrated *mURA3*, and this silencing was improved by addition of 10  $\mu$ M NR. However, *sir2* mutants failed to silence the rDNA-integrated *mURA3* construct, thus resulting in aberrant Ura3 expression not improved by 10  $\mu$ M or 1 mM NR.

In view of the subtle, Sir2-dependent defect in rDNA silencing, exhibited by *npt1*, that was corrected by NR, we turned to two robust assays in which NR might benefit cells, namely subtelomeric silencing and replicative lifespan. Sir2 has well-established roles in silencing genes at subtelomeric loci (Smith et al., 2000) and promoting the longevity of wild-type yeast cells (Kaerberlein et al.,



**Figure 2. Support of Sir2 Functions by Nrk1-Dependent NR Conversion to NAD<sup>+</sup>**

The hyper-recombination (A) and ribosomal-array gene-silencing (B) phenotypes of *npt1* but not *sir2* are suppressed by NR. Correction of the telomeric-gene-silencing phenotype (C) and low NAD<sup>+</sup> content (D) of *npt1* mutants by NR depends on Nrk1. (E) shows that NR extends the lifespan of yeast *npt1* mutants on YPD (2% glucose) media.

1999), whose replicative lifespan is limited by the accumulation of ERCs (Sinclair and Guarente, 1997). As shown in Figure 2C, the *npt1* mutant suffers a severe silencing defect such that it expresses a telomere-integrated *URA3* construct, resulting in 5-fluoroorotic acid (5-FOA) sensitivity. This phenotype was suppressed by addition of 10  $\mu$ M NR. To address whether the mechanism of NR suppression of *npt1* depends on NAD<sup>+</sup> biosynthesis, we introduced an *nrk1* mutation into telomeric-silencing reporter strains that are wild-type and mutant for *npt1*. Although the *nrk1* mutation on its own did not produce a defect in telomeric silencing, this mutation produced a synthetic reduction in telomeric silencing with *npt1* and completely eliminated the ability to be suppressed by NR.

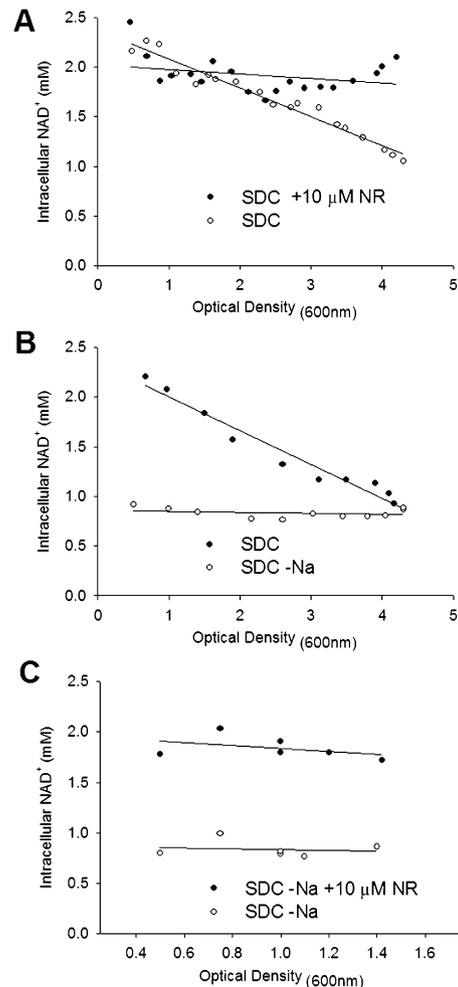
As shown in Figure 2D, *npt1* mutation causes a severe defect in intracellular NAD<sup>+</sup> levels, and this defect is made somewhat worse by deletion of *nrk1*. We grew cultures in synthetic dextrose complete (SDC) media and in SDC supplemented with 10  $\mu$ M NR and followed cellular NAD<sup>+</sup> concentration by performing acid extractions of nucleotides over the course of growth of liquid cultures. By using mole amounts of extracted NAD<sup>+</sup> to calculate intracellular concentrations, we determined that NR corrects the deficit in NAD<sup>+</sup> of an *npt1* strain. Moreover, in precise agreement with the Nrk1 dependence of NR as a silencing factor for the *npt1* mutant, Nrk1 is required for the *npt1* strain to incorporate NR into NAD<sup>+</sup>. In the *npt1 nrk1* mutant, NR can neither elevate NAD<sup>+</sup> levels (Figure 2D) nor improve telomeric silencing (Figure 2C).

Earlier, Npt1 function was shown to be required for the longevity benefit of CR (Lin et al., 2000), and increased *NPT1* copy number was shown to promote longevity without imposition of CR (Anderson et al., 2002). In Figure 2E, we show that the *npt1* mutant reduces longevity in (non-CR) YPD media from an average replicative lifespan of 20 generations to an average lifespan of 8.3 generations. Just as NR suppressed the ribosomal DNA recombination and silencing defects of *npt1*, addition of 10  $\mu$ M NR to YPD suppressed the short lifespan of the *npt1* mutant, thereby extending the average lifespan of this mutant to 16.1 generations. This is a unique result among NAD<sup>+</sup> precursors because nicotinic acid fails to extend lifespan and Nam shortens lifespan (Bitterman et al., 2002; Gallo et al., 2004).

### Exogenous NR Boosts NAD<sup>+</sup> Levels as Yeast Cells Deplete Nicotinic Acid

Although NR functions as an Nrk1-dependent silencing and longevity factor, careful examination of Figure 2D suggested that there are unanticipated complexities in NAD<sup>+</sup> metabolism and NR utilization. First, the intracellular NAD<sup>+</sup> concentration of a wild-type cell was not stable over the course of growth in SDC liquid culture. Second, addition of 10  $\mu$ M NR to the strain deficient in Nrk1 appeared to elevate NAD<sup>+</sup> levels over the course of growth in liquid culture (Figure 2D).

To dissect the basis for the instability in NAD<sup>+</sup> concentration and to determine how yeast cells containing intact



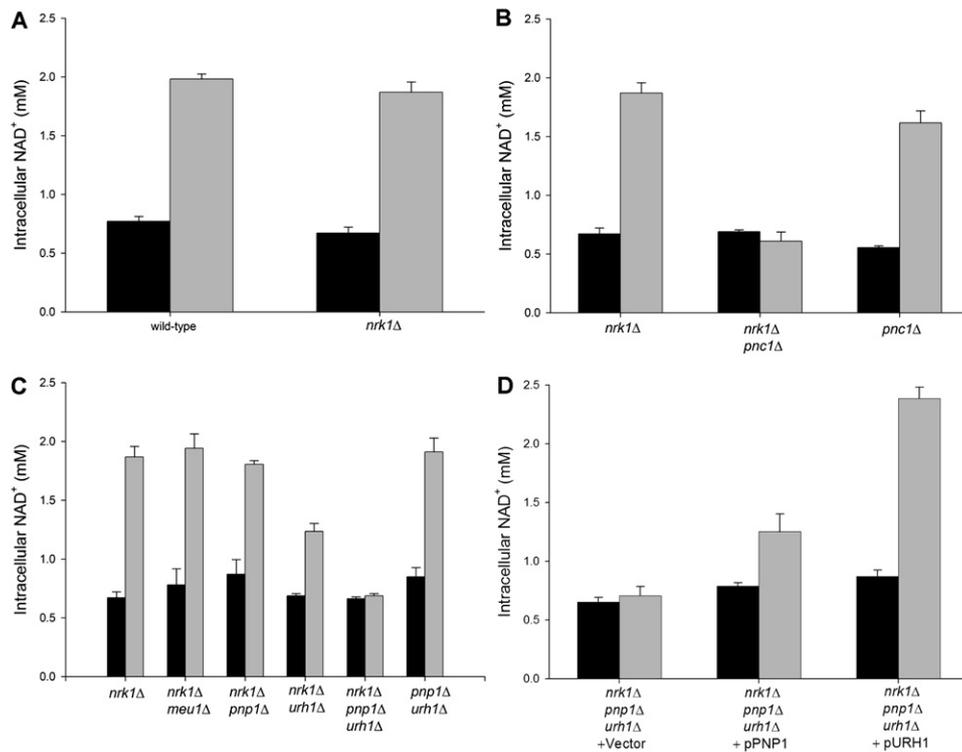
**Figure 3. Wild-Type Yeast Cells Convert Extracellular NR into Intracellular NAD<sup>+</sup>**

(A) Intracellular NAD<sup>+</sup> concentration is plotted as a function of the cell density of wild-type yeast cells grown in SDC media (shown in circles) or SDC supplemented with 10  $\mu$ M NR. Under these conditions, NR elevates intracellular NAD<sup>+</sup> as cells exceed an OD<sub>600 nm</sub> of 2.5.

(B) Intracellular NAD<sup>+</sup> concentration is followed as a function of cell density in wild-type cells grown in SDC with and without the 3  $\mu$ M nicotinic acid (Na) in yeast nitrogen base. Without Na, NAD<sup>+</sup> concentrations are stable throughout the culture.

(C) The amount of intracellular NAD<sup>+</sup> is more than doubled by addition of 10  $\mu$ M NR to Na-free SDC, and the high NAD<sup>+</sup> concentration is stable throughout the culture.

NAD<sup>+</sup> biosynthetic pathways incorporate NR into NAD<sup>+</sup>, we grew a wild-type yeast strain in SDC and in SDC supplemented with 10  $\mu$ M NR and followed cellular NAD<sup>+</sup> concentrations over the course of growth of liquid cultures. As shown in Figure 3A, the intracellular NAD<sup>+</sup> concentration of a wild-type yeast cell declines from greater than 2 mM early in the culture to 1.1 mM late in the culture. Inclusion of 10  $\mu$ M NR stems the progressive decline in intracellular NAD<sup>+</sup>, but not until an optical density (OD<sub>600 nm</sub>) of approximately 2.5 is reached.



**Figure 4. A Second NR Salvage Pathway Is Initiated by Urh1 and Pnp1**

Intracellular NAD<sup>+</sup> concentration was determined in yeast cells of varying genotype in Na-free SDC media (black bars) and in Na-free SDC media plus 10 μM NR (gray bars). Error bars represent 1 SD of the independently determined NAD<sup>+</sup> concentrations of different cultures.

(A) Surprisingly, *nrk1* cells have only a slight deficit in incorporation of NR into NAD<sup>+</sup>.

(B) Nrk1-independent NR salvage depends on Pnc1.

(C) Nrk1-independent NR salvage depends principally on Urh1 and secondarily on Pnp1.

(D) Nrk1-independent salvage is restored to a *nrk1 urh1 pnp1* mutant by the cloned *URH1* and *PNP1* genes.

Given the four known pathways to produce NAD<sup>+</sup> in yeast (Bieganowski and Brenner, 2004) and the Sirtuin-dependent degradative pathway to Nam (Tanner et al., 2000; Tanny and Moazed, 2001), we hypothesized that cells possess homeostatic mechanisms regulating NAD<sup>+</sup> levels such that NR is not utilized when another precursor remains abundant. Because SDC medium was formulated to contain tryptophan and nicotinic acid but not Nam or NR (Wickerham, 1946), it was straightforward to determine whether depletion of tryptophan or nicotinic acid was limiting for utilization of exogenous NR. We inoculated cells from stationary phase cultures into fresh SDC media and into SDC media lacking nicotinic acid and followed growth and intracellular NAD<sup>+</sup> levels. As shown in Figure 3B, whereas stationary cells diluted into SDC increased NAD<sup>+</sup> concentration to that of naive cells and then exhibited a decline in NAD<sup>+</sup>, cells diluted into SDC media lacking nicotinic acid grew well but with a constant low level of NAD<sup>+</sup> (~0.9 mM) from the beginning of the culture to the end.

Because nicotinic acid is not a necessary supplement for yeast cells with an intact de novo pathway, we eliminated the complication of nicotinic-acid-dependent changes in NAD<sup>+</sup> concentration. Thus, we grew wild-type cells in SDC media without nicotinic acid and examined

the effect on NAD<sup>+</sup> levels of supplementation with 10 μM NR. As shown in Figure 3C, wild-type cells in nicotinic-acid-free media have a stable NAD<sup>+</sup> level that is doubled by addition of 10 μM NR. Thus, NR is not only a compound that rescues the otherwise lethal *qns1* deletion (Bieganowski and Brenner, 2004) or the phenotypes of Sir2-limited *npt1* mutants (Figure 2) but also a vitamin that elevates intracellular NAD<sup>+</sup> levels by ~1 mM in wild-type cells, although its contribution to NAD<sup>+</sup> accumulation depends on depletion of nicotinic acid.

#### Nrk1-Dependent and Nrk1-Independent Salvage of NR

Although all known functions of NR depend on the presence of the *NRK1* gene or its human homologs *NRK1* and *NRK2*, the increased levels of NAD<sup>+</sup> in *nrk1* cells grown with NR (Figure 2D) led us to ask whether there might be an Nrk-independent pathway for NR utilization. Accordingly, we grew wild-type and *nrk1* mutant yeast cells in nicotinic-acid-free media in the absence or presence of 10 μM NR. Surprisingly, as judged by incorporation of NR into NAD<sup>+</sup>, both wild-type and *nrk1* mutants substantially increase NAD<sup>+</sup> levels when provided with 10 μM NR in the culture media (Figure 4A). In nicotinic-acid-free

media, wild-type cells increase intracellular NAD<sup>+</sup> from  $0.77 \pm 0.04$  mM to  $1.98 \pm 0.04$  mM with addition of 10  $\mu$ M NR, whereas the *nrk1* mutant cells increase intracellular NAD<sup>+</sup> level from  $0.67 \pm 0.05$  mM to  $1.87 \pm 0.09$  mM with addition of 10  $\mu$ M NR. This experiment suggested the existence of an Nrk1-independent pathway for salvage of NR to NAD<sup>+</sup>.

### Nrk1-Independent Salvage of NR Requires Nicotinamidase

Because Nrk1 is required for NR to support the growth of *qns1* mutants and to improve gene silencing in *npt1* mutants, the presence of an Nrk1-independent NR utilization pathway suggested that Nrk1-independent utilization of NR produces metabolites that require Npt1 and Qns1 for maturation. We hypothesized that a second pathway for salvage of NR is initiated by an enzyme or enzyme converting NR to Nam. Thus, we deleted the *PNC1* gene encoding nicotinamidase (Ghislain et al., 2002) from wild-type cells and from an *nrk1* mutant strain and measured the ability of these strains to incorporate NR into NAD<sup>+</sup> on nicotinic-acid-free media. As shown in Figure 4B, whereas the *pnc1* mutant exhibits an increase in intracellular NAD<sup>+</sup> from  $0.55 \pm 0.02$  mM to  $1.62 \pm 0.10$  mM with addition of 10  $\mu$ M NR, the *nrk1 pnc1* double mutant is incapable of incorporating supplemented NR into NAD<sup>+</sup> ( $0.69 \pm 0.02$  mM to  $0.61 \pm 0.08$  mM with addition of 10  $\mu$ M NR). Thus, NRK1-independent salvage of NR requires the nicotinamidase, Pnc1.

Formally, nicotinamidase might act at either of two points in an Nrk1-independent NR salvage pathway. The pathway might be initiated by a nucleoside-splitting activity that produces Nam, which would be converted to nicotinic acid by Pnc1. Alternatively, although there is no precedent for nicotinamidase acting on a nucleoside, Pnc1 might convert NR into nicotinic-acid riboside, which would be converted to nicotinic acid by a nucleoside-splitting activity. We reasoned that if Nrk1-independent salvage is initiated by a nucleoside-splitting activity that produces Nam, the silencing ability of an *nrk1 pnc1* mutant would be impaired by NR, because of accumulation of Sir2-inhibiting Nam (Bitterman et al., 2002). To test this hypothesis, we generated a series of strains containing a telomeric *URA3* reporter and performed gene-silencing assays on SDC lacking nicotinic acid and SDC lacking nicotinic acid, supplemented with 10  $\mu$ M NR. As reported earlier on standard SDC media (Gallo et al., 2004), *pnc1* exhibited poor telomeric silencing on media without nicotinic acid or Nam (Figure S1 in the Supplemental Data available online). Although NR improved telomeric *URA3* silencing of wild-type, *nrk1* mutant, and *pnc1* mutant strains, addition of NR exacerbated the silencing defect of the *nrk1 pnc1* double-mutant strain (Figure S1).

### The Second NR Salvage Pathway Requires Urh1 and Pnp1

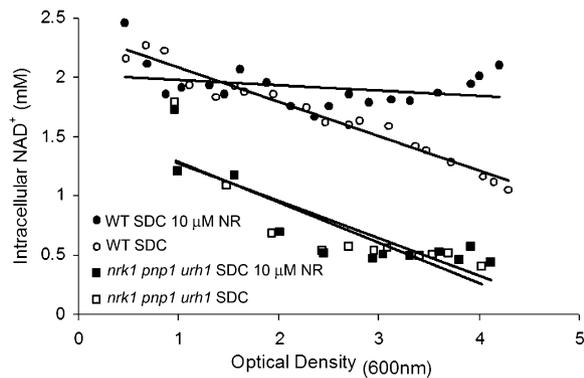
The glycosidic bond between the Nam and the ribosyl moieties of NR corresponds to the bond in NAD<sup>+</sup> cleaved

by Sirtuins, poly(ADPribose)polymerases (PARPs), and cADPribose synthases. However, because there are no PARPs or cADPribose synthases in yeast, and Sirtuins are highly specific for NAD<sup>+</sup>, we hypothesized that NR is cleaved to Nam by a nucleosidase. We identified one candidate nucleoside hydrolase and two candidate nucleoside phosphorylases in the yeast genome. These enzymes are mechanistically distinct in that nucleoside hydrolases use water to break the glycosidic linkage, thus producing the base plus ribose, whereas nucleoside phosphorylases use phosphate to break the same bond, thus producing the base plus ribose-1-phosphate.

Urh1 was reported as a uridine- and cytidine-specific nucleoside hydrolase that is without vertebrate homologs and that plays a role in salvaging pyrimidine bases (Mitterbauer et al., 2002; Kurtz et al., 2002). Meu1 was reported as methylthioadenosine phosphorylase, involved in the first step of methionine salvage and repression of ornithine decarboxylase activity in yeast and human cells (Subhi et al., 2003). Pnp1 was identified as the yeast homolog of bovine and human purine nucleoside phosphorylase (Pnp), which is involved in purine nucleoside degradation and salvage of purine bases (Lecoq et al., 2001). Inhibition of human Pnp has been proposed as a means to control T cell proliferative disorders (Schramm, 2002). Although in vitro reports dating back more than 50 years state that NR can be phosphorylated by bovine Pnp (Rowen and Kornberg, 1951; Wielgus-Kutrowska et al., 1997), there are other reports that NR phosphorylase is a specific enzyme, distinct from Pnp (Grossman and Kaplan, 1958; Imai and Anderson, 1987). Prior to this study, there has never been an in vivo system in which the potential roles of hydrolysis or phosphorolysis of NR could be evaluated in NAD<sup>+</sup> metabolism and the identity of the responsible enzymes determined by genetic criteria.

Deletions of each gene encoding a candidate NR hydrolase or phosphorylase were introduced into the *nrk1* background in order to test whether the mutant gene of interest reduced Nrk1-independent incorporation of NR into NAD<sup>+</sup>. As shown in Figure 4C, assays of NAD<sup>+</sup> accumulation revealed that *meu1* had no detectable effect on NR utilization. In contrast, *urh1* deletion reduced the NR-dependent increase in NAD<sup>+</sup> of an *nrk1* mutant by 55%, and deletion of *pnp1* produced a mild but reproducible 22% reduction in the ability of an *nrk1* mutant to incorporate NR into NAD<sup>+</sup>. Moreover, when *pnp1* deletion was introduced into the *nrk1 urh1* strain, virtually the entire 1.2 mM Nrk1-independent increase in NAD<sup>+</sup> concentration was eliminated. Finally, just as the extent of NR incorporation into NAD<sup>+</sup> on nicotinic-acid-free media is nearly unaltered by *nrk1* deletion (Figure 4A), the extent of NR utilization in a *urh1 pnp1* disruption strain (Figure 4C) is not strikingly altered. This indicates that, in nicotinic-acid-free media, cells can use either the Nrk1 pathway or the Urh1/Pnp1 pathway to incorporate NR into NAD<sup>+</sup>.

The genes encoding Urh1 and Pnp1 were cloned into centromeric plasmids under the control of their native promoters. As shown in Figure 4D, upon transformation into



**Figure 5. NR Salvage Enzymes Contribute to NAD<sup>+</sup> Biosynthesis in the Absence of Supplemented NR**

Intracellular NAD<sup>+</sup> concentration was determined in wild-type and *nrk1 urh1 pnp1* strains in SDC media without and with 10  $\mu$ M NR supplementation. Surprisingly, strains lacking Nrk1, Urh1, and Pnp1 have a substantially lower ( $\sim$ 0.8 mM) NAD<sup>+</sup> concentration than wild-type, suggesting that NR is a normal cellular metabolite.

the *nrk1 urh1 pnp1* strain, the plasmid carrying *URH1* restored 127% of the Nrk1-independent NR utilization ability of an *nrk1* strain, consistent with moderate overexpression. As expected for a minor source of NR utilization under moderate overexpression, the plasmid carrying *PNP1* restored 69% of the Nrk1-independent NR utilization ability of an *nrk1* strain. Thus, Urh1 and Pnp1 individually and collectively function as NR to Nam catabolic enzymes in vivo for Nrk1-independent synthesis of NAD<sup>+</sup> from NR. Although Urh1 and Pnp1 break down NR, the assays of Figure 4 establish that Urh1 and Pnp1 initiate from NR a Pnc1-linked NAD<sup>+</sup> synthesis pathway that functions independently of the Nrk1 pathway of NAD<sup>+</sup> synthesis.

#### NR Salvage Enzymes Are Required to Maintain NAD<sup>+</sup> Levels in the Absence of NR Supplementation

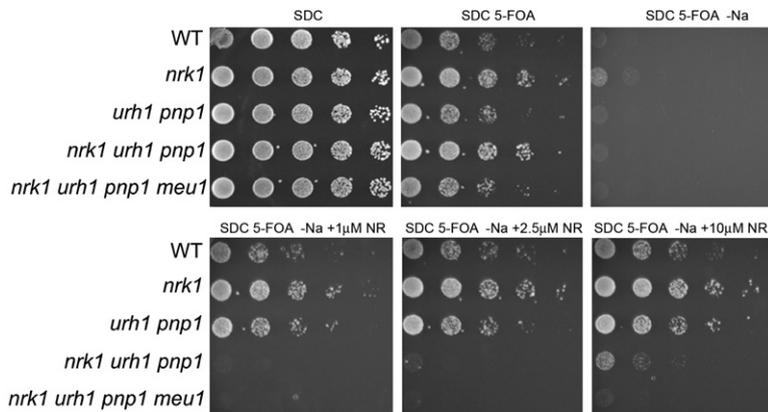
Myristyl nicotinic acid (MNA) is a synthetic prodrug form of nicotinic acid, designed to be made available to cells by an esterase (Catz et al., 2005). Although one would expect efficacy of MNA to be limited by expression of the esterase, one would not term the esterase an authentic NAD<sup>+</sup> biosynthetic enzyme because MNA is neither an endogenous metabolite nor a naturally identified vitamin. To address whether the identified NR salvage pathways represent authentic and endogenous participants in NAD<sup>+</sup> metabolism, we grew wild-type and *nrk1 urh1 pnp1* mutant strains in SDC medium without and with addition of 10  $\mu$ M NR. Although the requirement for Nrk1, Urh1, and Pnp1 in utilization of exogenous NR was demonstrated in Figure 4, Figure 5 illustrates that these enzymes play a highly significant role in maintaining NAD<sup>+</sup> levels in the absence of supplemented NR. Thus, NR has activities both as a vitamin (Bieganski and Brenner, 2004) and as a metabolite that depend on Nrk1-dependent and Urh1/Pnp1-dependent salvage.

Genetically validated steps in yeast NAD<sup>+</sup> metabolism are depicted in Figure 1B, and the presumed vertebrate intracellular NAD<sup>+</sup> biosynthetic pathways are depicted in Figure 1C. In addition to Sirtuins as NAD<sup>+</sup>-consuming enzymes, vertebrate cells express NAD<sup>+</sup>-consuming PARP and ADP-ribose transferase activities and exhibit an entirely different approach to Nam salvage. Rather than converting Nam to nicotinic acid, vertebrate cells convert Nam to NMN with a Nam PRTase (Rongvaux et al., 2002). This would suggest that in vertebrate cells, conversion of NR to NMN could either be accomplished in one step, via the Nrk pathway, or in two steps, via expression of a nucleoside phosphorylase and Nam PRTase. A competing methyltransferase-specific reaction is catalyzed by Nam *N*-methyltransferase (Aksoy et al., 1994), which removes rather than salvages Nam (Figure 1C).

#### Either Nicotinic Acid or NR and an Intact Salvage Pathway Are Required for Telomeric Gene Silencing

In Figure 2, we showed that the Sir2-limited phenotypes of *npt1* mutants are suppressed by exogenous NR in a manner that depends on Nrk1. Because the Nrk1-independent NR salvage pathway depends on Npt1, it was not possible to use that system to determine whether Urh1/Pnp1-dependent salvage of NR also promotes Sir2-dependent silencing and longevity functions. In Figure 6, we examined the silencing of a telomeric *URA3* reporter gene in strains varying in NR salvage enzymes as a function of media supplementation. On media containing nicotinic acid, all strains were proficient in silencing the reporter gene as demonstrated by formation of 5-fluoroorotic-acid-resistant colonies. However, on media without nicotinic acid, each of the strains, including wild-type, was strikingly defective in silencing the reporter gene. This result is similar to that observed in *Candida glabrata* in which subtelomeric adhesin gene expression is derepressed in the absence of nicotinic acid (Domergue et al., 2005). However, the absence of silencing in nicotinic-acid-deprived *C. glabrata* was attributed to lack of de novo NAD<sup>+</sup> synthesis. In contrast, our study demonstrates the failure of yeast strains, which grow and thrive with de novo NAD<sup>+</sup> synthesis, to support telomeric silencing without the addition of a vitamin.

On media containing 10  $\mu$ M NR, telomeric silencing by the wild-type and *nrk1* and *urh1 pnp1* strains was fully restored, apparently because NR is utilized through the Nrk1 pathway in the *urh1 pnp1* strain, the Urh1/Pnp1 pathway in the *nrk1* strain, and both NR salvage pathways in the wild-type strain. We were surprised to observe that NR supplementation enabled some telomeric silencing in the *nrk1 urh1 pnp1* strain in which virtually all incorporation of NR was eliminated (Figures 4C and 6). However, by deleting *meu1* in the *nrk1 urh1 pnp1* telomeric-silencing reporter strain, we obtained a strain in which NR provided no improvement in silencing. By titrating NR into the series of strains, we showed that the *nrk1 urh1 pnp1* mutant, whose incorporation of NR into NAD<sup>+</sup> is virtually undetectable (Figure 4C), increases telomeric silencing at



**Figure 6. NR Improves Telomeric Gene Silencing through Both Salvage Pathways**

Whereas all strains were effective in telomeric gene silencing on media containing nicotinic acid, all strains, including wild-type, are grossly defective in telomeric gene silencing on Na-free media as judged by 5-FOA sensitivity. Possession of either or both NR salvage pathways is sufficient for restoring telomeric gene silencing with 1  $\mu$ M NR. Surprisingly, at 2.5  $\mu$ M NR and greater, deletion of *meu1* was required to completely shut down the silencing benefit of NR even though the *nrk1 urh1 pnp1* strain appeared to be completely deficient in NR utilization (Figure 4C).

concentrations of NR greater than 2.5  $\mu$ M, whereas strains of genotype *nrk1* and *urh1 pnp1* utilizes NR at 1  $\mu$ M. Thus, with the sensitive assay of telomeric gene silencing, the data indicate that *Meu1* contributes to *Nrk1*-independent NR salvage in a minor way. Moreover, although *S. cerevisiae* differs from *C. glabrata* by synthesizing  $NAD^+$  de novo and produces a stable intracellular  $NAD^+$  concentration of  $\sim$ 0.9 mM without vitamin supplementation (Figure 2), *S. cerevisiae* requires either nicotinic acid or NR and an intact salvage pathway for efficient telomeric silencing (Figure 6).

### NR Is a Longevity Factor

Strains varying in NR salvage were analyzed for replicative lifespan on synthetic media without nicotinic acid in the absence and the presence of NR. As shown in Figure 7 and Table 1, on synthetic, 2% glucose media containing tryptophan and no vitamin, the mean lifespans of strains were similar. When strains were supplemented with 10  $\mu$ M NR, the mean lifespans of wild-type, *nrk1* mutant, and *urh1 pnp1* mutant strains were extended by 10.6, 4.3, and 5.3 generations, respectively. In contrast, the mean lifespan of genotype *nrk1 urh1 pnp1*, which is severely deficient in NR utilization, was extended by only 1.2 generations by NR, and the mean lifespan of genotype *nrk1 urh1 pnp1 meu1* was not extended at all. The data summarized in Table 1 indicate that yeast cells on YPD media are substantially longer lived than those depending on de novo  $NAD^+$  synthesis; that YPD extends lifespan in a manner depending on *Npt1*; and that NR extends lifespan in a manner that is totally dependent on NR salvage pathways. Whereas either NR salvage pathway is sufficient for efficient NR-dependent telomeric silencing (Figure 6), both pathways are required for the full benefit of NR in extending lifespan (Figure 7 and Table 1).

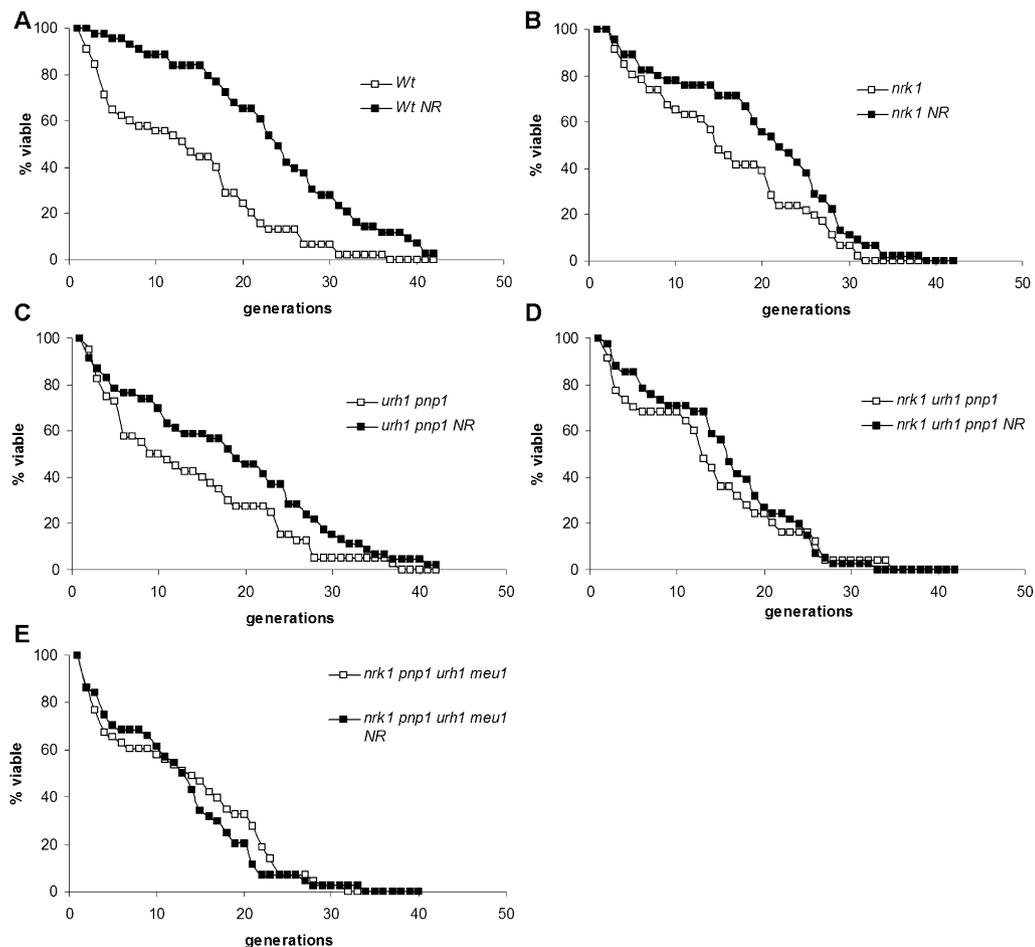
### DISCUSSION

Because *Npt1* and *Sir2* are required for the increase in longevity because of CR in wild-type yeast cells (Lin et al., 2000), there has been substantial interest in dissecting

the  $NAD^+$ -dependent mechanisms by which CR promotes *Sir2* function and in developing therapeutic strategies that are CR mimetic. Three mechanisms have been suggested. First, it was postulated that CR might increase  $NAD^+$  substrate levels (Guarente, 2000). Second, evidence was presented that CR and certain stresses act by lowering *Nam* inhibitor levels (Anderson et al., 2003a). Third, evidence was presented that CR decreases  $NADH$  levels (Lin et al., 2004). Although the mechanisms are not mutually exclusive and might be different in particular animal tissues, recent work in the mouse establishes that a one-day fast increases  $NAD^+$  in the liver (Rodgers et al., 2005) and that CR elevates  $NAD^+$  and reduces *Nam* in the brain (Qin et al., 2006). Because elevated  $NAD^+$  appears to mediate some of the beneficial effects of CR, we have focused on interventions that increase net  $NAD^+$  synthesis. To our knowledge, this is the first study to show that NR elevates  $NAD^+$  levels, promotes *Sir2* functions, and extends lifespan.

The experiments performed herein establish a second NR salvage pathway initiated in yeast cells by the products of the *URH1*, *PNP1*, and *MEU1* genes. Although yeast cells can double intracellular  $NAD^+$  levels with provision of either nicotinic acid or NR in the growth media, there are important differences between the salvage pathways of the two vitamins. As shown in Figure 2, nicotinic acid, which is almost universally used in yeast synthetic media though not required for *S. cerevisiae* growth, produces an intracellular  $NAD^+$  level that drops by more than 2-fold over the course of growth in liquid culture. NR stably increases intracellular  $NAD^+$  levels and extends lifespan via two distinct metabolic pathways.

The poor gene-silencing activities and rapid aging exhibited by strains grown under conditions in which intracellular  $NAD^+$  levels are  $\sim$ 1 mM is striking when one considers that the  $K_M(NAD^+)$  for yeast *Sir2* is reported to be 29  $\mu$ M (Borra et al., 2004), and virtually all  $K_M(NAD^+)$  values for Sirtuin reactions are between 5 and 500  $\mu$ M (Sauve et al., 2006). Regulation of Sirtuins in the cell by increase of  $NAD^+$  from  $\sim$ 1 mM toward 2 mM can be explained if most of the "first mM" of  $NAD^+$  is protein bound, such that there is a minimal amount of free  $NAD^+$ . Our data



**Figure 7. NR Extends Lifespan**

Yeast strains of (A) wild-type, (B) *nrk1*, (C) *urh1 pnp1*, (D) *nrk1 urh1 pnp1*, and (E) *nrk1 urh1 pnp1 meu1* genotypes were subjected to replicative-longevity assays on nicotinic-acid-free media without and with 10  $\mu$ M NR supplementation. NR extended average lifespan 10.6, 3, 3.1, and 1.2 generations, respectively, in the wild-type, *nrk1*, *urh1 pnp1*, and *nrk1 urh1 pnp1* strains. Extension of lifespan by NR was abolished in the *nrk1 urh1 pnp1 meu1* strain. Thus, NR functions as a longevity factor for wild-type yeast by increasing NAD<sup>+</sup> synthesis through both NR salvage pathways.

suggest that whereas the first mM of NAD<sup>+</sup> fulfills essential functions through NAD<sup>+</sup>-dependent oxidoreductases, the second mM fuels discretionary functions of improved

**Table 1. Average Replicative Lifespans of Yeast Strains as a Function of Media**

	YPD	YPD + NR	SDC – Na	SDC – Na + NR <sup>a</sup>
Wild-type	20.0	20.4	12.5	23.1
<i>npt1</i>	8.3	16.1		
<i>nrk1</i>	22.5	23.9	15.0	19.3
<i>urh1 pnp1</i>			12.5	17.8
<i>nrk1 urh1 pnp1</i>			12.3	13.5
<i>nrk1 urh1 pnp1 meu1</i>			12.4	11.7

<sup>a</sup> Na, nicotinic acid; NR, 10  $\mu$ M nicotinamide riboside.

gene silencing, reduced rDNA recombination, and increased lifespan.

Eukaryotic NR kinases are highly specific enzymes that appear to specialize in NAD<sup>+</sup> synthesis (Bieganowski and Brenner, 2004). In contrast, the second NR salvage pathway is initiated by multifunctional nucleoside catabolizing enzymes. Urh1, which has homologs in fungi and protista (Degano et al., 1996; Shi et al., 1999), is not only the major enzyme responsible for Nrk1-independent NR utilization but also the enzyme that *S. cerevisiae* uses to salvage pyrimidine bases from pyrimidine nucleosides (Mitterbauer et al., 2002; Kurtz et al., 2002). Urh1 homologs in protozoan parasites have been proposed as drug targets because *Leishmania* and *Crithidia* do not encode enzymes for de novo synthesis of purine nucleotides (Degano et al., 1996; Shi et al., 1999). If these enzymes are also involved in salvage synthesis of NAD<sup>+</sup> from NR, an inhibitor of the parasite nucleoside hydrolase might inactivate two pathways enzymologically distinct

from those of mammalian hosts. Pnp1 and Meu1 play lesser roles in synthesis of NAD<sup>+</sup> from NR in yeast. However, because human Pnp is highly expressed in lymphoid tissues (Markert, 1991) and *NRK1* and *NRK2* gene expression may be variable, it is possible that phosphorylases play significant roles in human NAD<sup>+</sup> metabolism in some tissues.

Because phosphorylation of NR to a Nam product would compete with phosphorylation of NR to an NMN product, phosphorylation also has the potential to be destructive to NAD<sup>+</sup> signaling. This is the case because Nam produced by phosphorylation can be either converted to NMN by action of Nam PRTase or cleared by methylation to *N*-methylnicotinamide; it can also accumulate and potentially inhibit Sirtuins and PARPs. One would expect that production of Nam and then conversion to NMN (Revollo et al., 2004) would be constructive to NAD<sup>+</sup> signaling, whereas conversion to *N*-methylnicotinamide or accumulation of Nam would inhibit NAD<sup>+</sup> signaling. Thus, there may be conditions in which application of a Pnp inhibitor (Lewandowicz et al., 2003) with NR may improve the metabolic efficacy of NR by shunting the precursor into the Nrk pathway.

Vitamins are small molecules that are required in diets to prevent malnourishment. Metabolites are compounds that are naturally produced in particular biosynthetic pathways. Data presented in this study and in a previous study (Bieganski and Brenner, 2004) establish the vitamin functions of NR in yeast. New data establish the role of NR salvage enzymes in maintaining NAD<sup>+</sup> homeostasis in nonsupplemented cells. NR appears to be unique among NAD<sup>+</sup> precursors in stably elevating NAD<sup>+</sup> levels in yeast and may have advantages in human systems in which activation of Sirtuins is desired. Indeed, providing supplements to boost NAD<sup>+</sup> synthesis may be a therapeutically important treatment modality for neurodegenerative conditions stemming from chemotherapy (Araki et al., 2004), physical nerve damage (Wang et al., 2005), Alzheimer's disease (Qin et al., 2006), and multiple sclerosis (Kaneko et al., 2006). In *ex vivo* experiments to protect dorsal-root-ganglion neurons from transection-induced axonopathy, the combination of Nam plus overexpression of Nam PRTase and the combination of nicotinic acid plus overexpression of nicotinic acid PRTase had neuroprotective activity. However, NR was the only nonphosphorylated, drug-like NAD<sup>+</sup> precursor to protect DRG neurons from axonopathy without concurrent gene therapy (Sasaki et al., 2006). This appears to be because Nrk2 transcription is induced by nerve damage (Sasaki et al., 2006).

In addition to the potential value of increased NAD<sup>+</sup> synthesis in neuroprotection, genetic and pharmacological data validate increasing Sirt1 activity in the effective metabolism of a high-fat diet (Lagauge et al., 2006). Because nicotinic acid causes flushing through a receptor mechanism unrelated to NAD<sup>+</sup> synthesis (Benyo et al., 2005) and Nam inhibits Sirtuins (Bitterman et al., 2002), it will be important to test whether increasing net NAD<sup>+</sup> synthesis with

NR is as effective in promoting health of vertebrates as it is in extending lifespan of *S. cerevisiae*.

## EXPERIMENTAL PROCEDURES

### *S. cerevisiae* Strains, Plasmids, and Media

Yeast strains used for measuring NAD<sup>+</sup> levels and longevity were derivatives of BY4742 prepared by ourselves or by the deletion consortium (Winzeler et al., 1999) and verified herein. Yeast strains used for measuring rDNA recombination and silencing were derivatives of JS306 (Smith et al., 1999) and YSB348 (Buck et al., 2002), respectively. Yeast strains used for measuring telomeric silencing were derivatives of YCB647 (Smith et al., 2000). Genetic deletions were introduced by direct transformation of PCR products (Brachmann et al., 1998) or crosses and tetrad dissection as described in the Supplemental Data. Plasmids encoding *URH1* and *PNP1* were made as described in the Supplemental Data. Yeast media and transformations were prepared as described (Burke et al., 2000) except that, when indicated, yeast nitrogen base (Wickerham, 1946) was prepared without nicotinic acid and/or with 10 μM NR, which was prepared as described (Bieganski and Brenner, 2004). For elimination of NMN and Nam impurities, NR was HPLC purified on a strong anion exchange column with a 10 mM Na phosphate monobasic running buffer.

### Calculation of Intracellular NAD<sup>+</sup> Content

Yeast cultures were grown with agitation in 0.5 l cultures. During growth, the OD<sub>600 nm</sub> of 1:10 diluted cells were recorded and 20 ml cultural volumes were pelleted, washed with water, repelleted, and frozen at -80°C. Cell pellets were extracted in 250 μl of ice-cold 1 M formic acid saturated with butanol. After 30 min, 62.5 μl of 100% (w/v) trichloroacetic acid was added to each extract, and the samples were allowed to precipitate on ice for 15 min. Samples were microcentrifuged for 5 min, and the acid soluble supernatants were recovered. Pellets were washed with 125 μl of 20% TCA and repelleted. First and second supernatants were pooled and measured volumetrically. In three 1 ml cuvettes, reactions were assembled containing 10 μl 5 mg/ml alcohol dehydrogenase (two samples) or 10 μl water (control sample), and this was followed by addition of 840 μl 360 mM Tris (pH 9.7), 240 mM lysine, 0.24% (v/v) EtOH, and finally, 150 μl extract. After a 5 min incubation at room temperature, the spectrophotometer was zeroed against the control sample for determining the alcohol dehydrogenase-dependent increase in absorbance at 340 nm of the duplicate reactions. Mean net absorbances were converted to molar NAD<sup>+</sup> with the extinction coefficient of NADH (6220 M<sup>-1</sup> · cm<sup>-1</sup>). Molar NAD<sup>+</sup> in the cuvette was converted to molar NAD<sup>+</sup> in the extract by a factor of 6.67. Moles of NAD<sup>+</sup> in the extract were determined from the fraction of the extract assayed. To determine the intracellular volumes corresponding to the extracts and the corresponding intracellular NAD<sup>+</sup> concentrations, we used a nonlinear conversion between the 1:10 diluted OD<sub>600 nm</sub> values and the cell number (Burke et al., 2000) and took the volume of a haploid cell to be 7 × 10<sup>-14</sup> l (Sherman, 1991). For cells grown in media containing nicotinic acid, NAD<sup>+</sup> concentrations were determined, in duplicate, 6 to 18 times during the growth of a liquid culture. For cells grown in media without nicotinic acid, the cells were taken with 1:10 diluted OD<sub>600 nm</sub> values of 0.095–0.105, and the NAD<sup>+</sup> concentrations were determined, in duplicate, from three to eight independent cultures.

### Recombination, Silencing, and Aging

Assays of rDNA recombination and rDNA silencing were performed as described (Smith and Boeke, 1997). Assays of telomeric silencing (Smith et al., 2000) and replicative lifespan were performed as described (Kennedy et al., 1994), with at least 50 virgin mothers per strain, with careful control of plate osmolarity.

### Supplemental Data

Supplemental Data include additional Experimental Procedures, one figure, and two tables and can be found with this article online at <http://www.cell.com/cgi/content/full/129/3/473/DC1/>.

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