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## Nicotinamide and *PNC1* govern lifespan extension by calorie restriction in *Saccharomyces cerevisiae*

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### Abstract

Calorie restriction extends lifespan in a broad range of organisms, from yeasts to mammals. Numerous hypotheses have been proposed to explain this phenomenon, including decreased oxidative damage and altered energy metabolism. In *Saccharomyces cerevisiae*, lifespan extension by calorie restriction requires the NAD<sup>+</sup>-dependent histone deacetylase, Sir2 (ref. 1). We have recently shown that Sir2 and its closest human homologue SIRT1, a p53 deacetylase, are strongly inhibited by the vitamin B<sub>3</sub> precursor nicotinamide<sup>2</sup>. Here we show that increased expression of *PNC1* (pyrazinamidase/nicotinamidase 1), which encodes an enzyme that deaminates nicotinamide, is both necessary and sufficient for lifespan extension by calorie restriction and low-intensity stress. We also identify *PNC1* as a longevity gene that is responsive to all stimuli that extend lifespan. We provide evidence that nicotinamide depletion is sufficient to activate Sir2 and that this is the mechanism by which *PNC1* regulates longevity. We conclude that yeast lifespan extension by calorie restriction is the consequence of an active cellular response to a low-intensity stress and speculate that nicotinamide might regulate critical cellular processes in higher organisms.

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Lifespan in the budding yeast *S. cerevisiae* is extended by a variety of stimuli such as heat stress, osmotic stress and the restriction of amino acids or glucose<sup>1,3–5</sup>. The latter two regimens are considered to be mimics of calorie restriction in higher organisms. In *S. cerevisiae*, replicative age is defined as the number of divisions that a cell undergoes before dying. The yeast *SIR2* gene, which encodes the founding member of a conserved family of NAD<sup>+</sup>-dependent deacetylases<sup>6–9</sup>, is required for lifespan extension by glucose restriction<sup>1</sup>. Cells with an additional copy of *SIR2* live 30% longer than the wild type, whereas *sir2* strains age prematurely<sup>10</sup> owing to increased recombination at the ribosomal DNA (rDNA) locus<sup>10,11</sup>. The importance of elucidating the yeast *SIR2* pathway is underscored by increasing evidence that Sir2 proteins in higher organisms promote longevity and cell viability<sup>12–15</sup>.

Because Sir2 protein levels do not increase in response to calorie restriction<sup>16</sup>, lifespan extension must involve an increase in enzymatic activity of Sir2. One hypothesis is that Sir2

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is activated by an increased availability of NAD<sup>+</sup> (ref. 1). Nicotinamide, a product of the Sir2 reaction<sup>17</sup>, is a strong non-competitive inhibitor of Sir2-like enzymes *in vitro*<sup>2,17</sup> and can accelerate yeast ageing by inhibiting Sir2 *in vivo*<sup>2</sup>. Thus an alternative explanation is that Sir2 is regulated by changes in nicotinamide levels.

To explore the latter hypothesis, we focused on *PNC1*, a gene whose product converts nicotinamide to nicotinic acid in the NAD<sup>+</sup> salvage pathway (Fig. 1a, b). Most wild-type yeast strains have an average lifespan of 21–23 divisions, with a maximum lifespan of about 40 divisions. A wild-type strain that was calorie restricted (0.5% glucose) or heat stressed (37 °C) exhibited a longer lifespan than an untreated control (2.0% glucose or 30 °C, respectively; Fig. 1c, d). The *sir2* strain had a short lifespan, consistent with previous reports<sup>1,10</sup>, and neither calorie restriction (0.5% or 0.1% glucose) nor heat stress extended lifespan in this strain (Fig. 1c, d, and data not shown). The *pnc1* strain did not exhibit a lifespan extension under either of these conditions, demonstrating that *PNC1* is necessary for lifespan extension by calorie restriction and low-intensity stress.

Strikingly, under non-stressing conditions (2% glucose, 30 °C), a strain with additional copies of *PNC1* (*5×PNC1*) lived 70% longer than the wild type and some cells lived for more than 70 divisions, which is the longest reported lifespan extension in this organism (Fig. 1e). Neither calorie restriction nor heat stress further increased the lifespan of the *5×PNC1* strain (not shown). Deletion of *SIR2* in the *5×PNC1* background shortened lifespan to that of the *sir2* mutant (Fig. 1e). Furthermore, the *pnc1 sir2* double mutant had a lifespan similar to that of the *sir2* mutant (Fig. 1e) and its lifespan was unaffected by glucose restriction (not shown). These findings indicate that *PNC1* and *SIR2* function in the same pathway and that *PNC1* increases lifespan through *SIR2*. Together, these results show that *PNC1* is necessary for lifespan extension by both calorie restriction and heat stress, and that additional *PNC1* is sufficient to mimic these stimuli.

Given that additional *PNC1* is sufficient to extend lifespan, we examined whether *PNC1* expression is upregulated in response to stimuli that extend lifespan. We found that Pnc1 levels were greatly induced in a dose-dependent manner by glucose restriction (Fig. 2a) and in cells carrying a *cdc25-10* allele, which mimics calorie restriction<sup>1</sup> (Fig. 2b). *MSN2* and *MSN4*, which encode transcription factors that coordinate the response to carbon source starvation and intense stress, were not required for Pnc1 induction (not shown). This is consistent with the previous observation that these two genes are not required for lifespan extension by glucose restriction<sup>1</sup>.

Pnc1 levels were elevated under every other condition known to extend yeast lifespan, including amino acid restriction, salt stress and heat stress (Fig. 2c), in agreement with whole-genome mRNA analyses of stressed yeast cells<sup>18</sup>. Pnc1 activity in extracts from treated cells was correlated with Pnc1 concentrations in western blots (Fig. 2d), showing that these cells have increased rates of nicotinamide hydrolysis.

We and others have previously shown that two other enzymes in the NAD<sup>+</sup> salvage pathway, Npt1 (nicotinic acid phosphoribosyltransferase) and Nma2 (nicotinic acid mononucleotide adenylyltransferase), are concentrated in the nucleus<sup>16,19</sup>. Surprisingly, a

fusion protein of Pnc1 with green fluorescent protein (Pnc1–GFP) was not only localized in the nucleus and the cytoplasm but was also concentrated in three to six discrete cytoplasmic foci per cell (Fig. 3a–d). Calorie-restricted (Fig. 3a) or stressed (Fig. 3b) cells showed a marked increase in the intensity of fluorescence, consistent with the western data. Interestingly, under conditions of amino acid restriction or salt stress, the fluorescence was predominantly localized to the foci (Fig. 3b), suggesting that Pnc1 localization is regulated.

To determine the identity of the foci, we searched for cellular markers that co-localized with Pnc1–GFP and observed significant overlap with a peroxisomally targeted red fluorescent protein (RFP) (Fig. 3c). Pnc1–GFP foci were no longer observed in a peroxisome-deficient *pex6* mutant, confirming that Pnc1–GFP was peroxisomal (Fig. 3d). Because our studies indicated that the localization of Pnc1 to peroxisomes might be regulated, we sought to identify the transporter responsible for its import into this organelle. Although Pex5 imports the vast majority of peroxisomal proteins, the localization of Pnc1 to peroxisomes required the less-used transporter Pex7 (Fig. 3d). The localization of Pnc1 to sites outside the nucleus implies that this enzyme could regulate proteins other than Sir2 (such as the homologues of Sir2, Hst1–Hst4). The peroxisomal localization of Pnc1 is of particular interest because these organelles are a major source of reactive oxygen species and have been implicated in mammalian ageing<sup>20</sup>.

Because *PNC1* converts nicotinamide to nicotinic acid as part of the NAD<sup>+</sup> salvage pathway, it could theoretically activate Sir2 either by increasing the availability of its co-substrate, NAD<sup>+</sup>, or by depleting levels of the inhibitor nicotinamide. Although these mechanisms are not mutually exclusive, and mutations that alter NAD<sup>+</sup> levels can affect silencing<sup>1,3,19,21</sup>, current evidence favours the nicotinamide model. We and others have been unable to detect increases in NAD<sup>+</sup> levels or the NAD<sup>+</sup>/NADH ratio in calorie-restricted cells<sup>22</sup> or in genetic mimics of calorie restriction<sup>16</sup>, even when unbound NAD<sup>+</sup> was measured (R.M.A., A. R. Neves, H. Santos and D.A.S., unpublished observations). In addition, we have previously shown that Sir2 is inhibited *in vitro* by physiological concentrations of nicotinamide and that exogenous nicotinamide can abolish silencing *in vivo*<sup>2</sup>. Perhaps most persuasive is the observation that cells lacking *PNC1* have a silencing defect, yet they show no change in NAD<sup>+</sup> levels<sup>19</sup>. Although these observations are supportive of the nicotinamide model, we sought more conclusive evidence.

First, we reasoned that if Pnc1 activates Sir2 by stimulating the NAD<sup>+</sup> salvage pathway (by converting nicotinamide to nicotinic acid), then an increase in the intracellular nicotinic acid pool should have the same effect as increasing Pnc1 levels (see Fig. 1b). Exogenous nicotinic acid is readily taken up by yeast cells and can significantly increase the intracellular pool (R.M.A., A. R. Neves, H. Santos and D.A.S., unpublished observations)<sup>23</sup>. A common indicator of Sir2 activity is the extent to which a reporter gene inserted at the rDNA locus (*RDNI*) is silenced. As shown in Fig. 4a, exogenous nicotinic acid did not increase rDNA silencing, indicating that nicotinic acid is not limiting for the salvage pathway. Furthermore, genetic analysis demonstrates that the contribution of *PNC1* to NAD<sup>+</sup> synthesis is minimal, even in the absence of NAD<sup>+</sup> synthesis *de novo* (Fig. 4b). Taken together, these data argue against a model in which Pnc1 stimulates Sir2 by providing additional nicotinic acid for NAD<sup>+</sup> synthesis.

Second, we tested whether the manipulation of *PNC1* could increase silencing even when its contribution to NAD<sup>+</sup> synthesis was blocked. In *S. cerevisiae*, the only other NAD<sup>+</sup> salvage pathway gene that can be deleted without a loss of viability is *NPT1* (see Fig. 1b). We have previously shown that additional copies of *PNC1* increase rDNA silencing in wild-type cells<sup>16</sup>. Additional copies of *PNC1* led to a partial rescue of the silencing defect in the *npt1* strain (Fig. 4c). Because cells lacking *NPT1* have NAD<sup>+</sup> levels one-half of those in wild-type strains<sup>6</sup>, we included an NAD<sup>+</sup> precursor, quinolinic acid, in the medium, which in mammals has been shown to compensate for a low NAD<sup>+</sup> concentration<sup>24</sup>. In the presence of this compound, additional *PNC1* restored rDNA silencing in the *npt1* strain to near wild-type levels (Fig. 4c), showing that Pnc1 can increase Sir2 activity even in the absence of the NAD<sup>+</sup> salvage pathway.

Last, if *PNC1* regulates Sir2 activity by modulating nicotinamide levels, we reasoned that manipulation of nicotinamide using a gene outside the NAD<sup>+</sup> salvage pathway should have the same effect. In humans, nicotinamide is converted to N-methylnicotinamide by nicotinamide N-methyltransferase (NNMT)<sup>25</sup> and then excreted. As predicted by the nicotinamide model, overexpression of human *NNMT* in yeast increased rDNA silencing (Fig. 4d). By homology we also identified a putative *S. cerevisiae* *NNMT* gene, YLR285W. The predicted protein contains the four signature motifs of S-adenosylmethionine-dependent methyltransferases<sup>26</sup> and its core domain is 23% identical to that of human NNMT<sup>25</sup>. Additional copies of YLR285W increased silencing, whereas deletion of this gene led to a loss of silencing, similar to the effect of manipulating *PNC1* (ref. 16) (Fig. 4d). Additional copies of YLR285W increased yeast lifespan and this effect was not enhanced by glucose restriction (Fig. 4e). Unlike *PNC1*, YLR285W is not a true longevity regulator because its expression is not apparently modulated by stimuli that extend lifespan<sup>18</sup>, and its deletion does not abolish lifespan extension by glucose restriction (Fig. 4e).

Our results show that lifespan extension by either calorie restriction or mild stress is the result of an active cellular response that requires the upregulation of a specific longevity gene, *PNC1* (Fig. 4f). This system of longevity regulation explains how multiple, disparate stimuli can lead to the same longevity response and how species can rapidly evolve strategies to suit a changing environment. We also provide multiple lines of evidence that *PNC1* regulates Sir2 by modulating intracellular nicotinamide. It has been proposed that Sir2 is regulated by passive means, by changes in either NAD<sup>+</sup> availability<sup>1,3,6,21</sup> or the NAD<sup>+</sup>/NADH ratio<sup>1,3,21</sup>. We do not exclude the possibility that these mechanisms can function in tandem with nicotinamide depletion. However, an attractive feature of nicotinamide-based regulation is that it does not require the modulation of NAD<sup>+</sup>, an essential cofactor involved in cellular homeostasis.

Nicotinamide has been shown to promote apoptosis in mammalian cells by inhibiting the Sir2 homologue SIRT1 (refs 2, 15), a regulator of p53 (refs 14, 15). Moreover, the poly(ADP-ribose) polymerase family of proteins, which are involved in many processes including DNA repair, telomere-length regulation and the opening of chromatin associated with stress-activated genes, are also inhibited by nicotinamide<sup>27</sup>. Interestingly, an increased expression of *NNMT* is correlated with tumorigenesis<sup>28</sup> and a decreased expression is

correlated with radiosensitivity<sup>29</sup>. These findings raise the possibility that nicotinamide regulates critical cellular processes in higher organisms.

## Methods

### Media and strains

All strains were grown at 30 °C in complete 2.0% (w/v) glucose (YPD) medium except where stated otherwise. Glucose restriction medium contained 0.5% or 0.1% glucose. Mild stress conditions were one of the following: defined medium (SD); amino acid restriction (SD lacking non-essential amino acids); salt stress (NaCl, 300 mM); heat stress (37 °C); sorbitol (1 M). In all experiments, auxotrophic markers were matched between strains by integrating empty vector. The copy number of integrated genes was determined by Southern blotting. Deletions were generated with a kan-MX6 PCR-based technique<sup>16</sup> and confirmed by PCR. Additional copies of *PNC1* were integrated as described previously<sup>16</sup>. The entire open reading frame and 700 bases of the upstream sequence of YLR285W were amplified from genomic DNA and cloned into pSP400, then sequenced and integrated as described previously<sup>16</sup>. A GFP cassette was integrated in frame at the 3' end of the native *PNC1* gene as described previously<sup>16</sup>. The RFP-PTS1 (for peroxisomal targeting signal 1) plasmid (pSG421) was a gift from S. J. Gould (Johns Hopkins University). The coding region of human *NNMT* was subcloned from p91023(B), a gift from R. Weinshilboum (Mayo Clinic), into pSP400 downstream of the *ADHI* promoter. Strains derived from PSY316AT<sup>16</sup> were used for lifespan analysis. Strains derived from W303AR<sup>11</sup> were used for western blots, microscopy and silencing assays. W303AR cdc25-10 was a gift from L. Guarente (MIT).

### Yeast assays

Lifespan measurements were performed as described previously<sup>16</sup> except for the heat-stress experiments, in which strains were incubated after each dissection at 37 °C. Silencing was assayed as described previously<sup>16</sup>.

### Protein expression analysis

Strains were pretreated under the indicated conditions and grown to mid-exponential phase. Western blots were performed as described<sup>16</sup> with whole-cell extracts (75 mg). Proteins were detected with anti-GFP antibodies (Santa Cruz) or anti-actin antibodies (Chemicon). Fluorescent microscopy images were captured at the same exposure (1 s) at  $\times 100$  magnification with a Hamamatsu Orca100 CCD camera and processed with Openlab software. Cultures were grown in complete medium containing 0.5% glucose to enhance the detection of fluorescence.

### Nicotinamidase activity assay

The activity of Pnc1 in extracts obtained from pretreated mid-exponential-phase cultures was determined as described previously<sup>30</sup>. In brief, 0.16 mg of protein was incubated with either 0 or 8 mM nicotinamide for 45 min at 30 °C in a final volume of 400  $\mu$ l of 10 mM Tris-HCl pH 7.5, 150 mM NaCl and 1 mM MgCl<sub>2</sub>. Pnc1 activity was determined by measuring the final concentration of the reaction product, ammonia, with an ammonia diagnostic kit (Sigma). Baseline ammonia was accounted for by subtracting a no-

nicotinamide control. Nicotinamidase activity was expressed as nmol ammonia min<sup>-1</sup> per mg total protein. Pnc1 activity was obtained by subtracting the background value for the *pnc1* strain ( $0.06 \pm 0.004$  nmol min<sup>-1</sup> per mg).

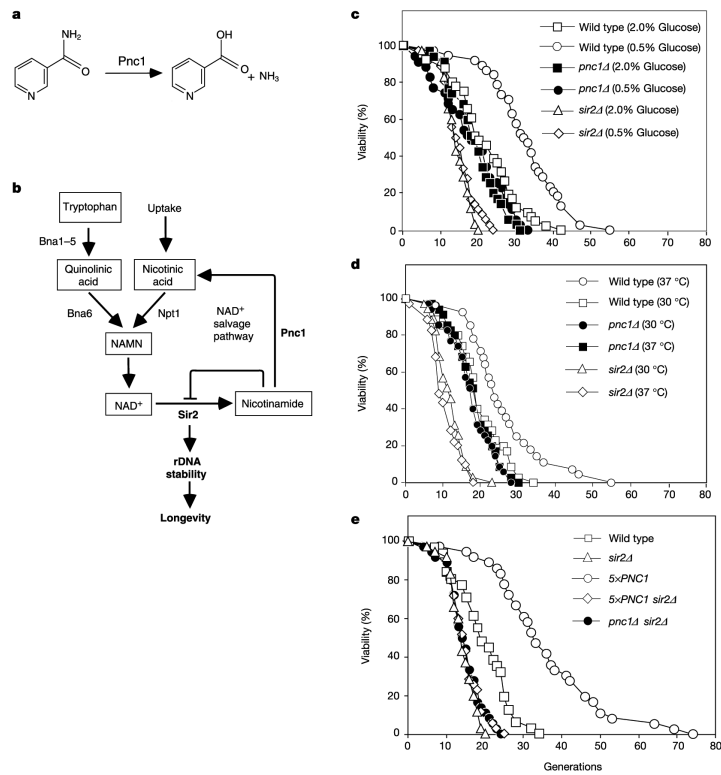
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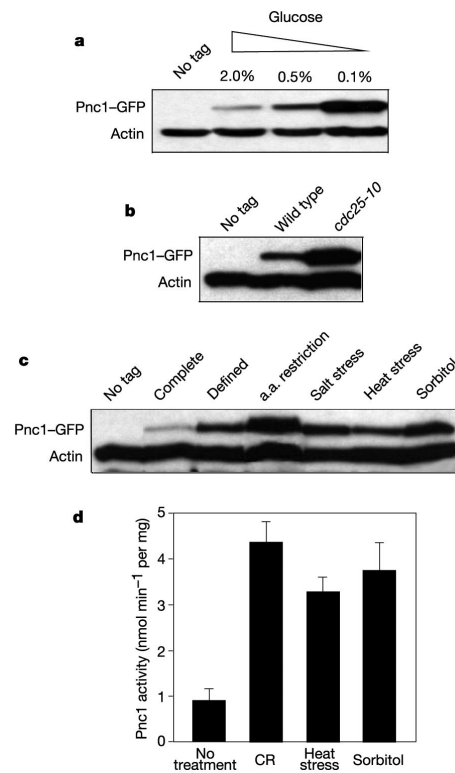
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**Figure 1.**

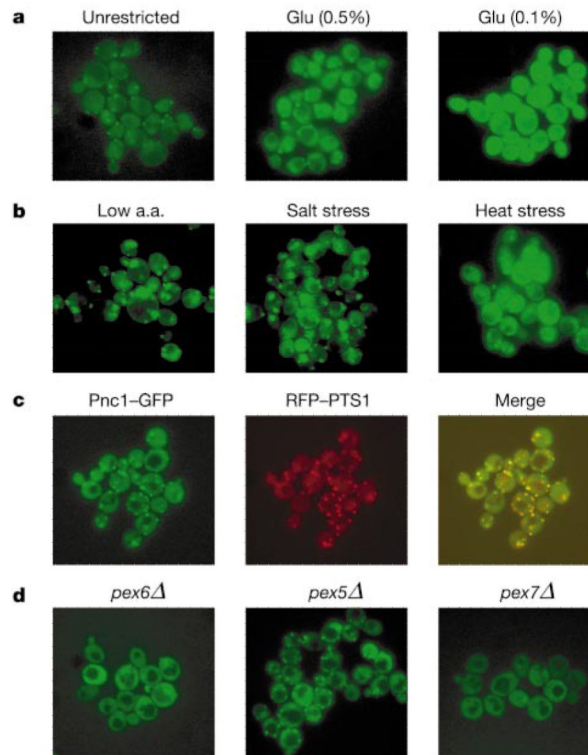
Calorie restriction and heat stress extend lifespan in a *PNC1*-dependent manner. **a**, Pnc1 converts nicotinamide to nicotinic acid. **b**, In *S. cerevisiae*,  $\text{NAD}^+$  is synthesized *de novo* from tryptophan via Bna1–6 or recycled from nicotinamide. **c**, Average lifespan on 2.0% (w/v) glucose: wild type, 21.6 generations; *pnc1*, 19.1; *sir2*, 14.2. Average lifespan on 0.5% glucose: wild type, 32.7 generations; *pnc1D*, 18.1; *sir2*, 14.7. **d**, At 30 °C, average lifespans: wild type, 19.4 generations; *pnc1*, 18.5; *sir2*, 12.0. At 37 °C, average lifespans: wild type, 23.4 generations; *pnc1*, 17.5; *sir2*, 10.6. **e**, Average lifespans on 2.0% glucose at 30 °C: wild type, 19.7 generations; *5×PNC1*, 36.1; *sir2*, 14.2; *5×PNC1 sir2*, 15.1; *pnc1 sir2*, 14.4.





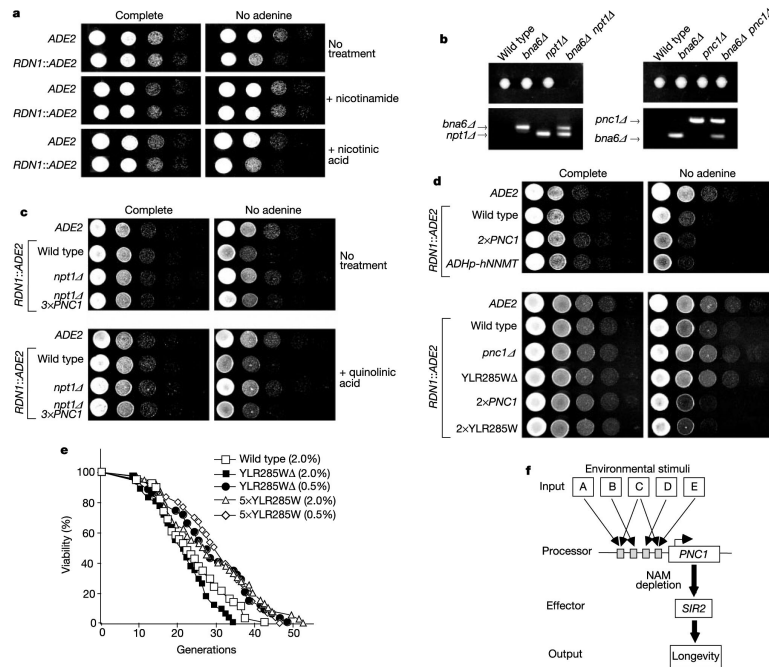
**Figure 2.**

Pnc1 levels and activity are elevated in response to calorie restriction and low-intensity stress. **a**, Western analysis of Pnc1-GFP under conditions of glucose restriction. **b**, Pnc1-GFP in wild-type or *cdc25-10* cells. **c**, Detection of Pnc1-GFP in cells subjected to mild stress as indicated (a.a., amino acid). **d**, Measurement of nicotinamide deamination by Pnc1. Activity (nmol ammonia min<sup>-1</sup> per mg protein) from three experiments (means ± s.d): no treatment (2% glucose),  $0.9 \pm 0.26$ ; calorie restriction (CR; 0.1% glucose),  $4.38 \pm 0.43$ ; heat stress (37 °C),  $3.28 \pm 0.32$ ; sorbitol (1 M),  $3.75 \pm 0.65$ .



**Figure 3.**

Pnc1-GFP is localized in the nucleus and cytoplasm, and concentrated in peroxisomes. **a**, Pnc1-GFP fluorescence in glucose-restricted cells (Glu 0.5% and 0.1%). **b**, Pnc1-GFP fluorescence under conditions of mild stress (a.a., amino acid). **c**, Co-localization of Pnc1-GFP (green) and RFP-PTS1 (red). Yellow indicates overlap. **d**, Localization of Pnc1-GFP in cells from peroxisomal mutant strains, *pex6* $\Delta$ , *pex5* $\Delta$  and *pex7* $\Delta$ .

**Figure 4.**

Manipulation of nicotinamide metabolism alters silencing and lifespan. **a**, To monitor silencing, *ADE2* was integrated at the rDNA locus. Increased growth on medium lacking adenine indicates decreased silencing. Serial dilutions of cells spotted on plates containing nicotinic acid or nicotinamide (5 mM). **b**, *PNC1* does not have a critical role in  $\text{NAD}^+$  biosynthesis, even in the absence of the *de novo*  $\text{NAD}^+$  synthesis pathway. *BNA6* encodes an enzyme in the  $\text{NAD}^+$  *de novo* synthesis pathway (see Fig. 1b). *NPT1* encodes a phosphoribosyltransferase that converts nicotinic acid to nicotinic acid mononucleotide in the  $\text{NAD}^+$  salvage pathway. Spore colonies from heterozygous *bna6 npt1* or *bna6 pnc1* diploids. Genotypes determined by PCR with a colony/ microcolony genomic template. **c**, Partial rescue of silencing by additional *PNC1* in the absence of the  $\text{NAD}^+$  salvage pathway and complete rescue in the presence of quinolinic acid (5 mM). **d**, Manipulation of genes involved in nicotinamide metabolism alters rDNA silencing. **e**, Manipulation of YLR285W affects lifespan. **f**, Model for the regulation of Sir2 activity and lifespan by PNC1 and nicotinamide (NAM).