Assimilation of Endogenous Nicotinamide Riboside Is Essential for Calorie Restriction-mediated Life Span Extension in Saccharomyces cerevisiae*S

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The pyridine nucleotide NAD⁺ (nicotinamide adenine dinucleotide) is an essential cofactor involved in various biological processes including caloric restriction-mediated life span extension. Administration of nicotinamide riboside (NmR) has been shown to ameliorate deficiencies related to aberrant NAD⁺ metabolism in both yeast and mammalian cells. However, the biological role of endogenous NmR remains unclear. Here we demonstrate that salvaging endogenous NmR is an integral part of NAD⁺ metabolism. A balanced NmR salvage cycle is essential for caloric restriction-induced life span extension and stress resistance in yeast. Our results also suggest that partitioning of the pyridine nucleotide flux between the classical salvage cycle and the NmR salvage branch might be modulated by the NAD⁺-dependent Sir2 deacetylase. Furthermore, two novel deamidation steps leading to nicotinic acid mononucleotide and nicotinic acid riboside production are also uncovered that further underscore the complexity and flexibility of NAD⁺ metabolism. In addition, utilization of extracellular nicotinamide mononucleotide requires prior conversion to NmR mediated by a periplasmic phosphotyrosine Pho5. Conversion to NmR may thus represent a strategy for the transport and assimilation of large nonpermeable NAD⁺ precursors. Together, our studies provide a molecular basis for how NAD⁺ homeostasis factors confer metabolic flexibility.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1–S3.

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NAD⁺ levels in mammalian cells (24). Finally, we discussed the role of Sir2 in modulating the flux of pyridine nucleotides between alternate routes.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—Yeast strain BY4742 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 was acquired from Open Biosystems (25). Rich mediaYPD and synthetic media SD were made as described (26). The growth medium used for replicative life span analysis was YPD (2% bacto peptone, 1% yeast extract, 1.5% agar) supplemented with filter-sterilized glucose at a final concentration of 2 or 0.5%. Growth medium used for chronologic life span analysis was either YPD or SD (supplemented with 4× auxotrophic amino acids leucine, histidine, lysine, and uracil). All gene deletions were made by replacing wild type genes with a reusable loxP-Kan’-loxP marker as described (27) and verified by PCR using oligonucleotides flanking genes of interest. Multiple deletions were carried out by popping out the Kan’ marker using a galactoside inducible Cre-recombinase. The Nrk1 overexpression construct was made as follows: a pair of oligonucleotides adding a NotI site to the 5’ end and a NheI site to the 3’ end of the NRK1 gene was designed to amplify the coding region of NRK1 via PCR. After PCR amplification, DNA was digested with NotI and NheI and then cloned into ppp81 (7), resulting in pADH1-NRK1, which was verified by DNA sequencing. Anaerobic growth conditions were achieved by using the BBL GasPak anaerobic chamber system.

**NAD⁺, NmR, and NaR Measurements**—Total intracellular levels of NAD⁺ were determined using enzymatic cycling reaction as described (7). NmR and NaR were determined by LC-MS as described (28) at the metabolomics core laboratory at University of California, Davis. The cell extracts were prepared from 10¹⁰ cells grown to late log phase by beads beating in ice-cold 50 mM ammonium acetate solution (29). Culture supernatants (50 ml) were collected and lyophilized along with cell extracts at −80 °C. Lyophilized products were resuspended in 100 μl (cell lysate) or 2 ml (culture supernatant) of 13 mM ammonium acetate/acetonitrile (1:1, v/v). 10 μl was used for LC-MS analysis. Chemically synthesized NmR and NaR, which were kindly provided by Dr. A. Sauve (30), were used to establish standard curves. Detection and quantification of NmR and NaR were performed using the MS-multiple reaction mode methods (NmR, retention time 6 min; NaR, retention time 8 min).

**Chronological Life Span**—Four single colonies from each strain were analyzed in each experiment as described (31) with a few modifications. The cells were grown in YPD or SD supplemented with 2 or 0.5% glucose at starting A₆₀₀ of 0.1. The cells were grown in 50-ml tubes on a roller drum set at maximum speed to ensure proper aeration. After 2 days, the cells were collected by centrifugation and washed three times with sterile water. The cells were then resuspended in 10 ml of sterile water to A₆₀₀ of 1 and then were incubated at 30 °C. Cell viability was monitored every 1–2 days by plating a fraction of culture onto fresh growth medium to determine the colony-forming units. The cell survival rate was calculated by normalizing the colony-forming units to the cell number obtained at day 2 (maximum A₆₀₀).

**Heat Shock Resistance**—The cells were grown in SD at a starting A₆₀₀ of 0.1. After 2 days, the cells were spotted onto YPD plates in 5-fold serial dilutions (started at A₆₀₀ of 1) and then were incubated at 55 or 25 °C for 45 or 60 min. After heat shock, the plates were transferred to 30 °C for another 2–3 days.

**Replicative Life Span**—All RLS analyses were carried out on YPD plates supplemented with glucose at a final concentration of 2 or 0.5% with 50 cells/strain/experiment (7) using a micro-manipulator. Statistical analysis was carried out using the JMP statistics software (SAS), and the Wilcoxon rank sum test p values were calculated for each pair of life spans.

**Deamidase Activity Assay**—300 A₆₀₀ unit cells grown overnight in YPD were harvested, and cell lysate was obtained by beads beating in breaking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, Roche protease inhibitors). Cell lysate containing 80 μg of total cellular proteins was added to 300 μl of deamidase reaction mix (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂) (32, 33) using 8 μM of NAD⁺, NMN, NmR, or Nam as substrates followed by incubation at 30 °C for 45 min. 100 μl of the deamidase reaction mix was then added to 1 ml of ammonia assay mix (3.4 mM α-ketoglutarate, 0.23 mM NADPH, 50 mM phosphate buffer, pH 7.4, 10 units of glutamate dehydrogenase) followed by a reaction at room temperature for 15 min (32, 33). The amount of ammonia was calculated by the decrease in absorbance at 340 nm using standard curve derived from the ammonia standard solutions (Sigma).

**RESULTS**

**NmR Is a Major NAD⁺ Precursor Released by NAD⁺ Prototrophic Cells**—Yeast cells lacking both the NPT1 and QPT1 genes or the QNS1 gene are inviable in regular growth media because a functional salvage or de novo NAD⁺ biosynthesis pathway is essential for growth (Fig. 1A) (3, 14). Our previous studies (3) led to a fortuitous discovery that the lethality of the npt1Δqpt1Δ and qns1Δ mutants could be rescued by growing these cells adjacent to wild type (WT) cells (Fig. 1B, left panel). These data suggested that NAD⁺ prototrophic yeast cells (feeders) constantly released certain NAD⁺ precursors, which rendered the growth of NAD⁺ auxotrophic cells (recipients) via cross-feeding (Fig. 1B, right panel). Because NmR supplementation could rescue the lethality of the qns1Δ mutant (14), it was possible that WT cells cross-fed the npt1Δqpt1Δ and the qns1Δ mutants with NmR. We first examined whether cross-feeding would be prevented by deleting NRK1 (Nrk1 kinase) in recipient cells. Anaerobic growth conditions (−O₂) were utilized to block de novo NAD⁺ biosynthesis (11) in the npt1Δ mutants. As shown in Fig. 1C, npt1Δ cells grown anaerobically became auxotrophic for NAD⁺, and as expected, utilization of exogenous NmR required functional Nrk1 (Fig. 1C, left panel). Nrk1 was also required for the npt1Δ mutant to utilize cross-feeding molecules released by WT cells (Fig. 1D). Furthermore, deleting NmR assimilation enzymes Nrk1, Urh1, and Pnp1 in WT cells dramatically enhanced cross-feeding activity (Fig. 1E, left panel). Conversely, overexpressing Nrk1 reduced the cross-feeding ability of WT cells (Fig. 1E, right panel). Interestingly, preventing NmR import by deleting NmR transporters NRT1 and FLIN26 also conferred strong cross-feeding (Fig. 1E, middle panel), indicating that yeast cells constantly released NmR.
Endogenous Nicotinamide Riboside in NAD\(^+\) Metabolism

**FIGURE 1.** Nicotinamide riboside (NmR) is an endogenous metabolite in yeast. A, the current model of the NAD\(^+\) biosynthesis pathways. Extracellular NmR enters the salvage cycle through Nrk1, Urh1, Pnp1, and Meu1. B, NAD\(^+\) prototrophic cells release metabolites into growth medium to cross-feed NAD\(^+\) auxotrophic cells (the \(\text{npt}1\Delta\) and \(\text{qns}1\Delta\) mutants). Micro-colonies of the NAD\(^+\) auxotrophic mutants become visible after 2-day incubation at 30 \(^\circ\)C, which show “gradient” growth patterns descending from the side adjacent to WT. C, Nrk1 is required for NAD\(^+\) auxotrophic cells to utilize NmR. Anaerobic growth conditions (–\(\text{O}_2\)) are utilized to block de novo NAD\(^+\) biosynthesis in the \(\text{npt}1\Delta\) and \(\text{nkr}1\Delta\) mutants. D, Nrk1 is required to utilize cross-feeding metabolites. E, cross-feeding activity is modulated by factors in NmR metabolism. Cells defective in NmR utilization (left panel) or transport (middle panel) show increased cross-feeding in spot assays. Overexpressing Nrk1 decreases cross-feeding activity (right panel). The results show growth of the \(\text{npt}1\Delta\) mutant (plated on YPD at a density of \(\sim 9000\) cells/cm\(^2\)) supported by feeder cells (\(\sim 2 \times 10^6\) cells spotted directly onto the recipient lawn). oe, overexpression.

Together, these data showed that cross-feeding activity of the feeder cells appeared to be inversely correlated with NmR salvage and import activities, supporting NmR as the major released NAD\(^+\) precursor that rescued the lethality of recipients.

To understand the significance of the NmR pool, we first exploited our cross-feeding reporter system to determine the amount of NmR released by WT and NmR assimilation mutants. Fractions of cell-free culture supernatants of the feeders were collected to supplement the recipients. Fig. 2A showed that the magnitude of cross-feeding conferred by the supernatants of WT and \(\text{nrk}1\Delta\text{urh}1\Delta\text{pnp}1\Delta\) and \(\text{nrt}1\Delta\text{fun}26\Delta\) mutants in liquid culture-based assays was correlated with that determined by agar plate-based assays (Fig. 1E). Using a standard curve derived from defined concentrations of NmR and their corresponding abilities to support growth of the recipients (supplemental Fig. S1), the cumulative concentration of NmR released by the \(\text{nrk}1\Delta\text{urh}1\Delta\text{pnp}1\Delta\) mutant was estimated to be \(\sim 6.74 \mu\text{M}\). The amount of NmR released by each \(\text{nrk}1\Delta\text{urh}1\Delta\text{pnp}1\Delta\) mutant cell was about \(250 \times 10^{-10}\) nmol (\(0.3\) mm). For comparison, the level of total NAD\(^+\) in a WT cell was \(\sim 840 \times 10^{-10}\) nmol (1.2 mm) (see Fig. 4B). These data highlighted the significance of the NmR pool in NAD\(^+\) metabolism.

We next directly quantitated the levels of intracellular and released NmR by LC-MS. Cell extracts (intracellular fractions) and culture supernatants (extracellular fractions) of WT and the \(\text{nrk}1\Delta\text{urh}1\Delta\text{pnp}1\Delta\) mutant were prepared and analyzed as described (28, 29). As shown in Fig. 2C, NmR was detected in the intracellular fractions of WT and the \(\text{nrk}1\Delta\text{urh}1\Delta\text{pnp}1\Delta\) mutant, which confirmed that NmR was an endogenous metabolite. As expected, the concentrations of both intracellular and released NmR were higher in the \(\text{nrk}1\Delta\text{urh}1\Delta\text{pnp}1\Delta\) mutant compared with WT (Fig. 2C, left panel). In both strains, extracellular concentrations of NmR were maintained at much lower levels compared with the intracellular fractions, indicating that NmR transporters Nrt1 and Fun26 efficiently retrieved NmR back to the cell. Fig. 2C (right panel) showed that the amount of NmR released by each \(\text{nrk}1\Delta\text{urh}1\Delta\text{pnp}1\Delta\) mutant was \(\sim 40\)-fold higher than that of WT cell. Overall, results obtained by LC-MS analysis correlated well with results acquired by cross-feeding assays (Figs. 1E and 2A).

It has been reported that exogenous NaR could function as a NAD\(^+\) precursor, which also relied on Nrk1 and Urh1/Pnp1/Meu1 for assimilation (16). We therefore examined whether NaR was also present in the intracellular and the extracellular fractions. Interestingly, intracellular concentrations of NaR were higher (\(\sim 6\)-fold) than NmR in both WT and the \(\text{nrk}1\Delta\text{urh}1\Delta\text{pnp}1\Delta\) mutant (Fig. 2D, left panel). However, extracellular NaR concentrations were extremely low in both strains. Consistent with a recent study (21), we found that NaR was a much less efficient (\(\sim 100\)-fold less) NAD\(^+\) precursor supplement compared with NmR (supplemental Fig. S2). This was likely due to inefficient transport of NaR across the cell membrane (21). Collectively, our in vivo cross-feeding data and LC-MS quantitative results provide evidence that both NmR and NaR are endogenous metabolites. Because most NaR is retained intracellularly, NmR is likely the key NAD\(^+\) precursor that rescues the growth of recipients. The constant release and re-uptake cycle of NmR may represent a novel extended pool of NAD\(^+\).
Perturbations of NmR Salvage Shorten Life Span and Increase Sensitivity to Heat Stress—We next determined whether deficiencies in salvaging endogenous NmR would cause any growth defects. Because NmR was mainly produced during late log phase (Fig. 2B), NmR salvage might be central for cell survival in the stationary phase. Yeast chronological life span (CLS) is defined as the length of time cells remain viable in a nondividing state (stationary phase or post-diauxic shift) and is suggested to be a model for studying life span regulation of post-mitotic cells in metazoans (31). As shown in Fig. 3A, the \( \text{nrt}1\Delta \text{fun}26\Delta \) mutant displayed short CLS. CR-induced CLS extension was largely abolished in the \( \text{nrt}1\Delta \text{fun}26\Delta \) mutant. The \( \text{nrt}1\Delta \) mutant showed moderate decrease in CLS. However, CR-induced CLS extension was not affected in this mutant (Fig. 3B). NmR supplement has been shown to restore the NAD\(^+\) level and the life span of cells grown in media lacking NA (15). However, NA supplement failed to rescue the short CLS of the \( \text{nrk}1\Delta \text{urh}1\Delta \text{pnp}1\Delta \) mutant (Fig. 3B), indicating that NmR salvage played a more important role than the classical NA/Nam salvage in CLS. Yeast CLS extension has been attributed to enhanced stress resistance (31, 34–36). In line with these studies, the \( \text{nrk}1\Delta \text{urh}1\Delta \text{pnp}1\Delta \) and the \( \text{nrt}1\Delta \text{fun}26\Delta \) mutants were sensitive to heat shock stress (Fig. 3, C and D). Also consistent with CLS shown in Fig. 3A, CR-induced heat resistance was abolished in the \( \text{nrk}1\Delta \text{urh}1\Delta \text{pnp}1\Delta \) mutant (Fig. 3C) but was unaffected in the \( \text{nrt}1\Delta \text{fun}26\Delta \) mutant (Fig. 3D). We next examined whether NmR salvage was also required for the CR-induced RLS (division potential of individual cells) extension. As shown in Fig. 3E, CR-induced RLS extension was completely abolished in the \( \text{nrk}1\Delta \text{urh}1\Delta \text{pnp}1\Delta \) mutant and was only partially prevented in the \( \text{nrt}1\Delta \text{fun}26\Delta \) mutant. Collectively, our data demonstrated that the severity of growth defects observed in the \( \text{nrk}1\Delta \text{urh}1\Delta \text{pnp}1\Delta \) mutants correlated with the amount of NmR released (Fig. 2A) and that NmR assimilation was essential for CR-induced benefits.

NmR Production Requires Functional NA/Nam-mediated NAD\(^+\) Salvage—We next investigated the endogenous sources of NmR. Because the nicotinamide moiety of NmR is likely to originate from de novo synthesis or NA/Nam-mediated salvage (Fig. 1A), we compared the cross-feeding activities of the qpt1\Delta and npt1\Delta mutants. As shown in Fig. 4A, the qpt1\Delta mutant was...
unable to cross-feed the recipients, whereas the qpt1Δ mutant exhibited similar cross-feeding ability as the WT cells, suggesting that NA/Nam-mediated salvage was required for NmR production. However, it was also possible that in the npt1Δ mutant, NmR assimilation was activated, thereby resulting in decreased NmR levels. Deleting NmR assimilation enzymes in the npt1Δ mutant only slightly restored NmR release (Fig. 4C). Therefore, NmR production was indeed compromised in the npt1Δ mutant. Because the level of NAD+/H11001 in the npt1Δ mutant was significantly reduced compared with WT and the qpt1Δ mutant cells (Fig. 4B) (37), it appeared that the cross-feeding activities were determined by intracellular NAD+ levels. We therefore determined the contribution of each step of the NA/Nam salvage pathway to cross-feeding activities and NAD+ levels. Deleting TNA1 (the major NA transporter) (Fig. 1A) significantly decreased total cellular NAD+ levels (Fig. 4B); however, the cross-feeding activity of the tna1Δ mutant remained similar to that of WT cells (Fig. 4B). Deleting other components of the NA/Nam salvage pathway, such as PNC1, NMA1, and NMA2, only slightly reduced cross-feeding (Fig. 4B). These results demonstrated that the amount of NmR released did not simply reflect the levels of intracellular NAD+. Interestingly, unlike other components in the NA/Nam salvage pathway, deleting SIR2 increased cross-feeding (Fig. 4, B and C).

Sir2 Modulates the Flux of the NmR Salvage Cycle—The NAD+-dependent Sir2 deacetylase family has been proposed to support the NA/Nam salvage pathway via producing Nam in deacetylation reactions (38) (Fig. 1A). However, the precise role of Sir2 in NAD+ metabolism remained unclear because deleting SIR2 did not significantly affect total NAD+ levels in WT cells (Fig. 4B). Increased NmR release observed in the sir2Δ mutant suggested a specific role of Sir2 in NmR

FIGURE 3. Balanced NmR salvage is required for CR-induced life span extension and heat resistance. A, CLS analyses of the NmR salvage mutants grown under normal and CR conditions. CR fails to extend the CLS of the nrk1Δ urh1Δ pnp1Δ mutant. B, NA does not rescue the short CLS of the nrk1Δ urh1Δ pnp1Δ mutant. One representative set of three independent experiments, each conducted in quadruplicate, is shown. The error bars denote standard deviations. C, deleting Nrk1, Urh1 and Pnp1 abolishes CR-induced heat resistance. D, deleting Nrt1 and Fun26 has no effect on CR-induced heat resistance. E, RLS analyses of NmR salvage mutants grown under normal and CR conditions. CR requires NmR assimilation to confer RLS extension. Statistical analysis of RLS was carried out using the JMP statistics software, and the Wilcoxon rank-sum test p values are calculated for each pair of life spans. The p values for WT versus CR and nrk1Δ urh1Δ pnp1Δ versus nrk1Δ urh1Δ pnp1Δ, CR are both <0.05. One set of representative data is shown. For A–E, WT, wild type control; CR, 0.5% glucose.
The extent of NmR release compared with WT. One set of representative experiments conducted in triplicate is shown. The release in WT (\(npt1\Delta\)) was significantly lower than in the WT (left panel). This contrast between NMN and NmR salvage that further underscored the complexity of NAD\(^+\) metabolism. Utilization of Extracellular NMN Requires Prior Conversion to NmR—NMN supplement has also been shown to replenish the intracellular NAD\(^+\) pool (19, 40, 41). Because the structure of NMN is unfavorable for direct diffusion across the cell membrane, either specific NMN transporters or extracellular NMN catabolizing enzymes would be required to utilize NMN. It has been reported that some bacteria utilize exogenous NMN via conversion to NmR, which is then imported into cell (40, 41).

To date, it remained unclear how eukaryotic cells utilized extracellular NMN. To address this question, we first demonstrated that yeast cells were able to utilize exogenous NMN (Fig. 6A). Interestingly, growth of yeast cells on NMN-supplemented medium required functional Qns1 (Fig. 6B), indicating that conversion of NMN to NmR was necessary prior to entering cells. However, NMN supported cell growth less efficiently compared with NmR (Fig. 6B, right panels). This contrast between NMN and NmR in supporting cell growth was correlated with their abilities in replenishing the NAD\(^+\) pool (Fig. 6C).

We next determined how exogenous NMN was converted to NmR. In bacteria, NMN assimilation is facilitated by an acid phosphatase, which cleaves NMN to NmR within the periplasmic space (40). In yeast, there are four identified periplasmic acid phosphatases localized to the cell wall or the periplasmic space (42). Among those, Pho5 accounts for more than 90\% of acid phosphatase activity in this compartment (43). In addition, Npp1 and Npp2 are two ecto-nucleotide pyrophosphatases that exhibit overlapping function with Pho5 in salvaging extracellular phosphates from nucleotides (44). Here we showed that deleting PHOS totally abolished growth of the \(qns1\Delta\) mutant on NMN containing medium, whereas deleting \(NPP1\) and \(NPP2\) had no effect (Fig. 6D). These results indicated Pho5 played a major role in the utilization of exogenous NMN.
These results also raised the possibility that released NAD\(^+\) precursors might include NMN. However, deleting PHO5 did not significantly reduce the ability of recipients to utilize released metabolites (Fig. 6E), indicating that NMN was not a major cross-feeding molecule. We concluded that prior conversion of NMN to NmR by Pho5 and NmR salvage enzymes were central for assimilating exogenous NMN. Other organisms including mammals might utilize extracellular NMN by a similar mechanism.

**DISCUSSION**

In this study, we showed that yeast cells constitutively produce, release, and import NmR. The lethality of mutants defective in both de novo and salvage synthesis of NAD\(^+\) could be rescued by NAD\(^+\) precursors released by WT cells grown in proximity (Fig. 1B), which we defined as “cross-feeding.” We developed NmR-specific cross-feeding reporter systems to further study factors that regulate NmR/NAD\(^+\) metabolism. Through LC-MS (Fig. 2, C and D) and cross-feeding analyses (Fig. 6) of WT and the NmR utilization mutants, we demonstrated that NmR was the major NAD\(^+\) precursor released by NAD\(^+\) prototrophic feeders that rescued the lethality of the npt1\(\Delta\) and npt1\(\Delta\)nrk1\(\Delta\) recipients. We showed that functional NmR salvage was required for CR-induced life span extension and stress resistance (Fig. 3). We also provided evidence that endogenous NmR generation was largely dependent on NA/Nam-mediated salvage and was negatively regulated by Sir2 (Fig. 4). Deleting SIR2 in cells defective in NAD\(^+\) salvage further decreased intracellular NAD\(^+\) levels, resulting in reduced growth fitness and short life span (Fig. 5, A–C). We also demonstrated that utilization of exogenous NMN generation was largely dependent on NA/Nam-mediated salvage and was negatively regulated by Sir2 (Fig. 4). Deleting SIR2 in cells defective in NAD\(^+\) salvage further decreased intracellular NAD\(^+\) levels, resulting in reduced growth fitness and short life span (Fig. 5, A–C). We also demonstrated that utilization of exogenous NMN required prior conversion to NmR by a periplasmic acid phosphatase Pho5 (Fig. 6D). We proposed that similar mechanisms might take place in mammalian cells.

Our studies have uncovered novel paths for NmR-mediated NAD\(^+\) salvage (Figs. 5H and 7), in which both NmR and NMN were deamidated by Pnc1 (and perhaps other deamidases), giving rise to NaR and NaMN respectively. Conversion of NmR to

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**FIGURE 5. Analyses of the role of Sir2 in NmR salvage.** A, measurements of intracellular NAD\(^+\) levels. Deleting Sir2 decreases NAD\(^+\) levels in the npt1\(\Delta\) and npt1\(\Delta\)nrk1\(\Delta\) mutants. B, deleting Sir2 in combination with deleting Npt1 and Nrk1 shortens CLS. C, cells lacking Sir2, Npt1 and Nrk1 show severe growth defect in rich medium. D, NmR supplement supports growth of the npt1\(\Delta\)mutant, which is further enhanced by deleting Sir2. E, deleting Sir2 does not further enhance growth of the npt1\(\Delta\)mutant supported by NmR. F and G, relative deamidase activities toward various substrates. Cell extracts of WT show deamidase activities toward Nam, NmR, and NMN (F) but not NAD\(^+\) (G). Deleting Pnc1 abolishes most deamidase activities in cell extracts. H, a model showing additional routes for NmR assimilation. For A–G, one set of representative experiments conducted in triplicate is shown. The error bars denote standard deviations. The p values are calculated using Student’s t test. **, p < 0.01; ***, p < 0.005. For D and E, NmR is supplemented at a final concentration of 0.1 M.

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Endogenous Nicotinamide Riboside in NAD⁺ Metabolism

**A** + NMN, 2 days
+ NMN, 2 days
+ NMN, 4 days

**B** - O₂
- O₂, + NmR
+ O₂, + NmR

**C** No treatment
(+) NmR

**D** periplasmic space
NMN ➔ Pho5
NAD⁺

**E** 1. qns1Δ
2. qns1Δ
3. qns1Δ
4. qns1Δ
5. qns1Δ

NaR might represent a mechanism to preserve the NAD⁺ pool because most NaR remained intracellular (Fig. 2D). Supporting this model, our LC-MS analysis showed that NaR was an abundant endogenous metabolite (Fig. 2D). Multiple NmR/NMN assimilation pathways might help confer metabolic flexibility in response to changes in growth conditions. It would be interesting to investigate whether this metabolic strategy could be extrapolated to mammals. Although there is no direct Pnc1 homolog in mammals, it is possible that other aminotransferases could mediate the deamination of NmR and NMN.

Although both NA/Nam salvage and Sir2 have been demonstrated to play important roles in regulating RLS (3, 15), their roles in CLS remained unclear. Because both the npt1Δ and urh1Δ mutants had low basal NAD⁺ levels (15), it was unexpected that the urh1Δ mutant showed reduced CLS (Fig. 3A), whereas the npt1Δ mutant exhibited normal CLS (Fig. 3B). Moreover, NA supplement failed to rescue the short CLS of the urh1Δ mutant (Fig. 3B). These data reinforced the distinct role of NmR salvage in CLS. Interestingly, it was reported that sir2Δ extended the maximum CLS of certain long-lived mutants (45). Because deleting Sir2 enhanced the pyridine nucleotide flow into the NmR branch (Fig. 5D), it would be interesting to investigate whether NmR metabolism plays a role in sir2Δ-induced CLS extension. Our studies suggested that Sir2, as a metabolic sensor, not only senses the NAD⁺ levels but also modulates the pyridine nucleotide flux. This unique role of Sir2 might provide a feedback control mechanism for cells to respond to metabolic changes. Because deleting other Sir2 family members, such as Hst1 and Hst2, did not show similar effects (supplemental Fig. S3), the growth benefits conferred by sir2Δ was not likely due to decreased NAD⁺ consumption. It was possible that Sir2 directly regulated yet to be identified components in the NmR pathway. Our data showed that the increase in NmR release resulted from sir2Δ was not affected by deleting any known components of the NmR assimilation pathway (Fig. 4C and data not shown). Future studies to
identify novel components in the NmR pathway will provide insights into the molecular basis underlying the roles of Sir2 and NAD\(^+\) metabolism in life span regulation.

It is intriguing that cells allow NmR to traffic between intracellular and extracellular compartments, which poses the potential risk of losing NAD\(^+\). NAD\(^+\) participates in many biological processes (1, 46). It is possible that keeping a flexible NmR/NAD\(^+\) pool facilitates prompt adjustments of the activities of NAD\(^+\)-dependent enzymes. In bacteria, it has been suggested that conversion of intracellular NMN to NmR and subsequent release of NmR relieve NMN inhibition toward the NAD\(^+\)-dependent DNA ligase during active aerobic growth (40). NmR assimilation might also protect cells against stress because NmR assimilation mutant was sensitive to heat stress (Fig. 3C), and intracellular NmR level in WT yeast was decreased upon heat treatment (data not shown). Interestingly, recent studies on the role of NAD\(^+\) in protecting neuronal degeneration also showed that expression levels of most NAD\(^+\) biosynthetic enzymes were moderately increased after injury (19). In particular, Nrk2, the isoform of Nrk1 identified in mammals (14), was dramatically up-regulated in response to neuronal stresses (19). In addition, it has been suggested that NmR also circulated in the peripheral bloodstream in mammals (47). It will be of great interest to further investigate whether intra- and extracellular cycling of NmR identified in yeast represents a primordial design for metabolic flexibility that also functions in higher eukaryotes.

In mammals, extracellular NMN circulating in peripheral bloodstream has been shown to exert systemic function by regulating NAD\(^+\) requiring enzymes in response to altered physiological demand signaled by remote tissues (24, 48). In addition, NMN administration effectively elevated NAD\(^+\) level and SIRT1 function in old BESTO mice in which SIRT1 was over-expressed specifically in the pancreatic \(\beta\) cells (24, 48). The additions of NaMN, NMN, NmR, and NAD\(^+\) were also shown to delay axonal degeneration in \textit{vivo} (18, 19). Therefore, administrating NAD\(^+\) precursors appeared to be a promising therapeutic or preventive strategy for certain age-associated diseases (17, 19, 48–50). Here we showed that yeast cells utilized exogenous NMN through conversion to NmR by the periplasmic acid phosphatase Pho5 (Fig. 6D). Although there seemed no direct homologs of Pho5 in mammals, CD73, an ecto-5′-nucleotidase, has been suggested to break down extracellular pyridine nucleotides to salvagable precursors (49). In summary, our results have uncovered novel components involved in NmR and NMN assimilation. These studies may also provide insights into the molecular basis of diseases associated with aberrant NAD\(^+\) metabolism and aging.

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