**YCL047C/POF1 Is a Novel Nicotinamide Mononucleotide Adenylyltransferase (NMNAT) in *Saccharomyces cerevisiae***

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Michiko Kato and Su-Ju Lin

From the Department of Microbiology and Molecular Genetics, College of Biological Sciences, University of California, Davis, California 95616

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**Background:** Factors regulating NAD⁺ metabolism and homeostasis remain unclear because of the dynamic nature of NAD⁺ synthesis pathways.

**Results:** Pof1 is a novel NMN-specific NMNAT that mediates NAD⁺ production.

**Conclusion:** Enzymes with redundant function may provide flexibility to maintain NAD⁺ homeostasis.

**Significance:** Novel NAD⁺ metabolic factors are identified, aiding our understanding of the complex NAD⁺ pathways.

NAD⁺ is an essential metabolic cofactor involved in various cellular biochemical processes. Nicotinamide riboside (NR) is an endogenously produced key pyridine metabolite that plays important roles in the maintenance of NAD⁺ pool. Using a NR-specific cell-based screen, we identified mutants that exhibit altered NR release phenotype. Yeast cells lacking the ORF YCL047C/POF1 release considerably more NR compared with wild type, suggesting that *POF1* plays an important role in NR/NAD⁺ metabolism. The amino acid sequence of Pof1 indicates that it is a putative nicotinamide mononucleotide adenylyltransferase (NMNAT). Unlike other yeast NMNATs, Pof1 exhibits NMN-specific adenylyltransferase activity. Deletion of *POF1* significantly lowers NAD⁺ levels and decreases the efficiency of NR utilization, resistance to oxidative stress, and NR-induced life span extension. We also show that NR is constantly produced by multiple nucleotidases and that the intracellular NR pools are likely to be compartmentalized, which contributes to the regulation of NAD⁺ homeostasis. Our findings may contribute to the understanding of the molecular basis and regulation of NAD⁺ metabolism in higher eukaryotes.

Pyridine nucleotides NAD⁺ (H) and NADP⁺ (H) are essential coenzymes participating in many cellular redox reactions in all living systems. NAD⁺ and its derivatives also function as substrates and signaling molecules in key cellular processes such as regulation of Ca²⁺ signaling, chromatin structure, DNA repair, and life span (1–5). Many of these processes consume NAD⁺; therefore, cells have developed complex interconnecting biosynthetic and signaling pathways to monitor and replenish intracellular NAD⁺ levels.

NAD⁺ is synthesized from multiple precursors. In yeast, the cellular pool of NAD⁺ is maintained by biosynthesis from nicotinic acid mononucleotide (NaMN)² or nicotinamide mononucleotide (NMN) (see Fig. 1A). NaMN is produced by transferring the phosphoribose moiety of phosphoribosyl pyrophosphate to nicotinic acid (NA) or to tryptophan-derived quinolonic acid (QA), catalyzed by phosphoribosyltransferases Npt1 and Bna6, respectively (6–8) (Fig. 1A). QA and NA are intermediate metabolites generated by *de novo* synthesis or by salvaging reactions that utilize exogenous pyridines or internal pyridines derived from NAD⁺ recycling. NMN is generated by phosphorylating nicotinamide riboside (NR), which is catalyzed by the kinase Nrk1 at the expense of ATP (9, 10). It has been shown that yeast cells produce NAD⁺ predominantly via the NA/Nam salvage pathway during exponential growth (11). More recently, NR has been shown to be an efficient NAD⁺ precursor that contributes to the NAD⁺ pool and supports NAD⁺-dependent reactions (9, 10). Intact NR salvaging pathway is essential for maintaining NAD⁺ homeostasis and life span (12, 13). Because yeast cells constantly release and resimulate NR, it has been suggested that this NR pool might confer metabolic flexibility for prompt adjustment of cellular NAD⁺ levels (12, 13). How NAD⁺ synthesis routes are regulated in response to different growth conditions remains to be elucidated.

To date, factors regulating NAD⁺ metabolism and homeostasis remain unclear because of the dynamic nature and redundancy of NAD⁺ synthesis pathways. One major challenge has been the lack of a specific and sensitive genetic screen system. Employing the property of yeast cells that constantly release and retrieve NR, we developed a unique NR reporter-based assay (14). A genetic screen utilizing this reporter system led to the identification of a novel NAD⁺ biosynthesis pathway component. In this study, we describe the role of *POF1/YCL047C* in NR and NAD⁺ homeostasis. We show evidence supporting the function of Pof1 as the third nicotinamide adenylyltransferase (NMNAT) in yeast.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Growth Media, and Plasmids**—Haploid yeast strain BY4742 *MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* acquired...
from Open Biosystems was used for this study (15). Yeast extract-peptone-dextrose (YPD) medium (2% Bacto peptone, 1% yeast extract, 1.5% agar supplemented with sterilized glucose at a final concentration of 2%) was made as described (16).

All gene deletions were generated by replacing wild type genes with a reusuable loxP-Kan’-loxP cassette as described (16). Multiple deletions were carried out by removing the Kan’ marker using a galactose-inducible Cre-recombinase. The Nrk1 over-expression construct pADH1-Nrk1 was made in the integrative pPP81 (LEU2) vector as described (16). The resulting construct was verified by DNA sequencing. The POF1-URA3 episomal plasmid (pRS316-POF1) was derived from pADH1-POF1, which was constructed in the integrative pPP81 (LEU2) vector as described (16). pADH1-POF1 was digested with SacI and Xhol, and the excised fragment was ligated into pRS316 (URA3). The NRK1-URA3 episomal plasmid (pPS1527-NRK1) was constructed by PCR amplification of the NRK1 coding region with 1 kb upstream and downstream sequence. The PCR fragment was digested with NotI and XhoI and then ligated into pPS1527 (URA3).

NR Cross-feeding Spot Assays—To study mutants with altered NR release, 3 × 10⁴ cells of each strain were spotted onto YPD plates spread with NAD⁺ auxotrophic recipient cells (the nptIΔbna6Δpho5Δ mutant or the qns1Δpho5Δ mutant) (14) at a density of ~9,000 cells/cm². Growth of the recipient cells relies on NR released from the spotted NAD⁺ prototrophic strains, and the extent of the recipient cell growth indicates the levels of NR release. After incubation at 30 °C for 3 days, we scored the cross-feeding activity of each strain by comparing the diameter of the cross-feeding zones to that of the wild type. Mutants with increased cross-feeding activity showed larger cross-feeding zones, whereas mutants with decreased cross-feeding activity showed smaller cross-feeding zones.

Determinations of NR and NAD⁺—Total intracellular levels of NAD⁺ were determined using an enzymatic cycling reaction as described (16, 17). Relative NR levels were determined by a liquid-based cross-feeding bioassay (12). To prepare cell extracts for intracellular NR determination, ~1.5 × 10⁶ (~150 A₆₀₀ unit cells) of cells (donors of interest) grown to late log-phase (~12 h growth from an A₆₀₀ of 0.1) were lysed by bead beating in 800 μl of ice-cold 50 mM ammonium acetate solution. After filter sterilization, 16 μl of cleared extract was used to supplement 8 ml of culture of recipient cells (the nptIΔbna6Δpho5Δ mutant) (12, 14) with starting A₆₀₀ cell concentrations of 250 mM imidazole, 10% glycerol, 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml N-acetyl-l-lysylzyme, 1 mg/ml DNaseI, 0.1% Triton X-100, and lysed by multiple freeze and thaw cycles. Purification of Pof1 with N-terminal His₆ tag was carried out using nickel-nitrilotriacetic acid-agarose resin (MCLab). The column was washed with buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole) containing 250 μM imidazole, and the recombinant protein was eluted with buffer containing 50 μg/ml of kanamycin and 25 μg/ml of chloramphenicol. Overexpression of Pof1 was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside at 37 °C for 16–18 h. Harvested cells were frozen, resuspended in 10 volumes of lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, 10% glycerol, 1 mM PMSF, 1 μg/ml leupeptin, 1 mg/ml N-acetyl-l-lysylzyme, 1 mg/ml DNaseI, 0.1% Triton X-100), and lysed by multiple freeze and thaw cycles. Purification of Pof1 with N-terminal His₆ tag was carried out using nickel-nitrilotriacetic acid-agarose resin (MCLab). The column was washed with buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl) containing 30 mM imidazole, and the recombinant protein was eluted with buffer containing 250 mM imidazole.

Recombinant Pof1 activity was measured based on the NMNAT discontinuous assay described (19) with modifications. Briefly, 100 μg of rPof1 was added to a reaction mix containing 20 mM AMP-HCL, pH 10, 1 mM ATP, 5 mM MgCl₂ and various concentrations of NMN and incubated for 1 h at 30 °C. NAD⁺ produced in this reaction was subsequently amplified by enzymatic cycling and measured in the same manner described for NAD⁺ measurement. The kinetic parameters for NMN were estimated from the results of three independent measurements (each carried out in triplicate) using GraphPad Prism 6. Fresh rPof1 was purified for each experiment shown in this study.

Replicative Life Span—All replicative life span (RLS) analyses were carried out on YPD plates supplemented with glucose at a final concentration of 2% with 50 cells/strain for each experiment (17) using a micromanipulator. 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.

Quantitative PCR Analysis of Gene Expression Levels—Cells were grown to log phase or late log phase in YPD (~6 or 12 h growth from A₆₀₀ of 0.1). Total RNA was isolated using RNaseasy mini kit, and cDNA was synthesized using Quantitect reverse transcription kit (Qiagen) according to the manufacturer’s instructions. For each quantitative PCR, 50 ng of cDNA and 500 nm of each primer were used. Quantitative PCR was run on Roche LightCycler 480 using LightCycler 480 SYBR green I Master Mix (Roche) with the following cycle conditions: preincubation (95 °C for 5 min) and 43 cycles of amplification (dena-
turation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s) followed by melting curve generation (65–97 °C, 0.11 °C per s). Average size of the amplicon for each gene was ~150 bp. The target mRNA transcript levels were normalized to ACT1 transcript levels.

RESULTS

YCL047C/POF1 Affects NR Metabolism and Encodes a Putative NMNAT—To identify novel players in the NR/NAD^+ metabolic pathway, we exploited the NR release property of yeast cells (12) and carried out a genetic screen to identify mutants that showed altered NR release activity, using the nonessential haploid single gene deletion mutants (20) (Fig. 1A). We found that cells lacking the ORF YCL047C/POF1 displayed strong cross-feeding activity (NR release), indicating altered NR metabolism in this mutant (Fig. 1C). When compared with previously characterized NR utilization mutants, nrk1Δ (defective in NR assimilation) and nrt1Δ (defective in NR transport), the pof1Δ mutant showed the strongest NR release phenotype, supporting a role for Pof1 in NR metabolism. Interestingly, pof1Δ

FIGURE 1. Deletion of YCL047C/POF1 causes altered NR metabolism. A, a simplified model of Saccharomyces cerevisiae NAD^+ synthesis pathway. In yeast, NAD^+ is synthesized de novo from tryptophan (Trp) and by salvaging NA, Nam, QA, and NR. Cells can also salvage nicotinic acid riboside by converting it to NA or NaMN. NaAD, deamido NAD^+. B, overview of the genetic screen to identify mutants that exhibit altered NR metabolism. Haploid single deletion mutants (feeder cells) are spotted onto a lawn of pyridine auxotrophic mutant npt1Δ bna6Δ pho5Δ (recipient cells), whose growth is dependent on NR released from the feeder cells in a dose-dependent manner. We have previously reported that Pho5 can convert extracellular NMN to NR. To rule out NMN utilization as a possible confounding factor in our assay, PHO5 was deleted in the recipient cell strain. C, deletion of YCL047C/POF1 confers increased NR release. Various feeder strains positively correlate with the amount of NR released from the feeder cells. D, deleting POF1 in nrk1Δ (left panel) or nrt1Δ (right panel) mutant further enhances NR release. For clarity, inverse images are shown. ctrl, control.
appeared to further enhance NR release in these mutants (Fig. 1D).

Pof1 was previously reported to harbor ATPase activity, which might be involved in protein quality control (21). Although the amino acid sequence of Pof1 does not show significant overall homology with any characterized protein, Pof1 possesses the characteristics of NMNAT. As shown in Fig. 2A, comparison of the amino acid sequence of Pof1 to known human NMNATs (hNMNAT1–3) and yeast NMNATs (Nma1 and Nma2) revealed that Pof1 possesses two ATP recognition motifs GXXXPX(T/H)XXH and SXXTR, signature of all NMNATs characterized to date (22–24). To further understand the role of Pof1 in NR metabolism, we first examined whether pof1Δ and other yeast NMNAT mutants displayed similar phenotypes. Interestingly, only the pof1Δ mutant showed increased NR release determined by cross-feeding plate assays (Fig. 2B). This result was further confirmed using a quantitative liquid-based assay (12). As shown in Fig. 2C, pof1Δ mutant cells showed a ~10-fold increase in NR release (Fig. 2C, left panel), which was accompanied by increased intracellular NR levels (Fig. 2C, right panel). This unique phenotype of pof1Δ suggested that Pof1, Nma1, and Nma2 may play different roles in NR metabolism and therefore may be regulated differently. In line with this hypothesis, NMA1 expression is higher than NMA2 and POF1 during the early log phase (6 h), which significantly dropped as the cells entered the late log phase (12 h) (Fig. 2D). On the contrary, the expression of NMA2 and POF1 was slightly induced in the late log phase (Fig. 2D). Next, we
determined whether their expression was affected by low intracellular NAD^+ . As shown in Fig. 2E, the expression of POF1 was significantly induced in a low NAD^+ npt1Δ mutant (both 6 and 12 h), whereas NMA2 expression remained constant. Although NMA1 expression was not increased in the npt1Δ mutant during the early log phase (6 h), it remained stable and did not significantly drop (as in the WT cells) as cells entered late log phase (12 h). These results suggested that NMA1 is the dominant NMNAT during early phases of growth when NA, the most abundant NAD^+ precursor in standard growth media, is still abundant. As NA becomes depleted during the late log phase, NMA1 expression is decreased accordingly. In the npt1Δ mutant, the NA/Nam salvage route is blocked; therefore cells rely more on the NR salvage pathway for NAD^+ synthesis (Fig. 1A). Dual function of NMA1 in both NA/Nam and NR salvage pathways may contribute to its complex expression patterns. Induction of POFl expression in the npt1Δ mutant suggested that Pof1 might be a dominant NMNAT in the NR salvage pathway in conditions where NR is the main NAD^+ precursor.

POFl Functions as an NMNAT; Which Catalyzes the Conversion of NMN to NAD^+ —To further characterize the function of Pof1, we first determined its cellular localization. Adding a GFP tag to Pof1 did not compromise its function, as determined by cross-feeding assays (Fig. 3A, bottom panel). Similar to Nma1, Nma2, and other salvage enzymes (25), Pof1 is localized both in the cytoplasm and the nucleus (Fig. 3A, top three panels). Next we examined whether Pof1 possessed NMNAT activity using His6-tagged rPof1. For comparison and as a validation of our assay system, we tested the activity of recombinant Nma1, K_m(app) and V_max for NMN were ~2.26 mM and 119 (nmol/h), respectively. K_m and V_max for ATP were not determined because a higher ATP concentration (>1 mM) was inhibitory (data not shown) and steady-state approximation was not applicable (Fig. 3B). This pattern of inhibition indicates that Pof1 may exhibit an ordered sequential mechanism, which was also observed with human NMNAT1 (27, 28). Interestingly, Pof1 displayed an alkaline optimum pH (pH 10). Some NMNATs have been reported to be more active under alkaline conditions (29, 30). It is possible that alkaline pH might affect Pof1 structure to mimic its in vivo state, thereby influencing the catalytic activity.

Next, we examined whether Pof1 could function as an NMNAT in vivo. Because the adenyllylation of NaMN or NMN is a required step in NAD^+ synthesis (Fig. 1A), deleting both NMA1 and NMA2 is expected to result in synthetic lethality. However, we found that nma1Δnma2Δ cells were viable in media supplemented with NR (Fig. 3C, panels I and II). We therefore reasoned that a third NMNAT exists, which could function in the absence of NMA1 and NMA2 to support cell growth on NR. To test whether Pof1 fulfills this function, we examined whether the nma1Δnma2Δ mutant could grow when POFl is lost. We constructed a triple deletion mutant nma1Δnma2Δpof1Δ carrying wild type POFl on an episomal URA3 plasmid for viability in NR-supplemented media. When these cells were grown in media containing 5-FOA (which is toxic to URA3^+ cells), the only way to remain viable was to lose the URA3 plasmids. Under this 5-FOA selection condition, nma1Δnma2Δpof1Δ mutant was unable to grow, even in the presence of NR, which indicated that Pof1 functions as the third NMNAT (Fig. 3C, panel III).

Furthermore, Pof1 may play other in vivo roles. Some eubacterial NMNATs are known to possess multifunctional roles (30–32). For example, NadR in Salmonella typhimurium is known as a trifunctional protein, which serves both regulatory and synthetic roles that contribute to pyridine nucleotide metabolism (32). NadR mainly functions as the transcriptional repressor of the de novo NAD^+ pathway. However, it also functions as a NR kinase and possesses weak NMNAT activity (32). The uniquely strong NR release phenotype of the pof1Δ mutant suggests that Pof1 may also possess NR kinase activity. To test this, we first examined whether pof1Δ-induced NR release could be rescued by overexpressing Nrk1. Nrk1 overexpression complemented pof1Δ and lowered NR release back to the wild type level, suggesting that Pof1 and Nrk1 might have overlapping function (Fig. 3D). We next asked whether Pof1 could function as a NR kinase by testing whether Nrk1 is the only NR kinase in yeast. In the nma1Δnma2Δ mutant, Pof1 is essential for growth on NR (Fig. 3C). If Pof1 indeed has NR kinase activity, the nma1Δnma2Δ mutant should remain viable upon loss of Nrk1 because they still have POFl. To test this, we constructed the nma1Δnma2Δnrk1Δ mutant carrying the wild type NRK1 on an episomal URA3 plasmid. Fig. 3E showed that these cells were unable to grow in NR-supplemented medium when forced to lose the NRK1-URA3 plasmid (via simultaneous selection on 5-FOA) (Fig. 3E). These results indicated that Nrk1 is the only NR kinase for the utilization of NR and that Pof1 does not possess NR kinase activity.

POFl Contributes to NAD^+ Pool by Functioning in the NR Utilization Pathway—Next, we examined whether Pof1 plays a role in NAD^+ production. Because of the complex functional redundancies in the NAD^+ synthetic pathways (Fig. 1A), we employed the qns1Δ mutant to study the role of Pof1 specifically in the NR salvage pathway. In this NR-specific background, cells cannot synthesize NAD^+ de novo or via the NA/Nam salvage pathway (Fig. 1A) and therefore depend on NR supplementation for viability. We took advantage of this NR-dependent property of qns1Δ cells and examined whether deleting POFl would affect NR utilization. As shown in Fig. 4A (left panel), the qns1Δpof1Δ mutant exhibited a growth defect in standard NR-supplemented (10 μM) YPD medium. This pof1Δ-induced growth defect was specific to NR utilization deficiency because higher concentration of NR (50 μM) rescued the growth defect (Fig. 4A, right panel). Next, we determined intracellular NAD^+ levels in qns1Δ mutant lacking POFl, NMA1, or NMA2. Deletion of POFl or NMA1 significantly impaired NAD^+ production, suggesting that POFl and NMA1 both played important roles in the conversion of NMN to NAD^+ (Fig. 4B). We also examined how POFl deletion might affect cellular fitness. In line with the observation by Costa et al. (21), deleting POFl in qns1Δ cells conferred sensitivity to H_2O_2, which was partially rescued by supplementing a higher concentration of NR (Fig. 4C). Functional NR salvage has been shown to play important roles in maintaining the replication potential.
Yeast Pof1 is an NMNAT

Multiple Phosphatases and Nucleotidases Contribute to NR Production in the pof1Δ Mutant—Based on our results thus far, we have concluded that Pof1 is the third NMNAT in Saccharomyces cerevisiae. Cells lacking POF1 release and accumulate high levels of NR, and it is possible that blocked NMN utilization shifts/increases the flux toward NR production. Cytosolic

of yeast cells (10, 12), and NR can restore the RLS of NAD^+ biosynthetic mutants (10, 12). We therefore examined whether deleting POF1 would affect NR mediated RLS extension. As shown in Fig. 5D, RLS of the NR-dependent qns1Δ mutant was extended by higher concentrations of NR, and POF1 deletion blocked NR-induced RLS extension.

FIGURE 3. Characterization of the Pof1 protein. A, cellular localization of Pof1 in yeast cells. The fluorescence signal of Pof1-GFP along with the differential interference contrast (DIC) and DAPI (which marks the nuclear and mitochondrial DNA) images indicate that Pof1 localizes both in the cytoplasm and the nucleus. Direct genomic integration of a GFP tag (upstream of the POF1 ORF) into haploid WT yeast genome does not interfere with the normal function of Pof1, because unlike the pof1Δ mutant, the GFP-tagged strain releases WT level of NR, determined by cross-feeding plate assay (bottom panel). B, rPof1 protein catalyzes the conversion of NMN to NAD^+ (Table). C, rPof1 exhibits NMNAT activity in vivo. The results show that POF1 is required for the growth of nma1Δnma2Δ mutants on NR-supplemented medium. Cells lacking both NMA1 and NMA2 require NR for viability (panels I and II). 5-FOA is toxic to cells carrying the URA3 gene (panel III), which therefore forces cells to lose the URA3 plasmid for viability. Loss of the POF1-URA3 plasmid in the triple deletion mutant nma1Δnma2Δpof1Δ results in lethality even in the presence of NR (panel III, bottom panel), suggesting that Pof1 is indispensable in the absence of other NMNATs. D, Nrk1 overexpression rescues the high NR release phenotype of the pof1Δ mutant. E, Pof1 does not have NR kinase function. The results show that NRK1 is required for the growth of nma1Δnma2Δ on NR-supplemented medium. The loss of the NDK1-expressing plasmid (NRK1-URA3) in the triple deletion mutant nma1Δnma2Δnrk1Δ results in lethality even in the presence of NR (and POF1) (panel III, bottom panel), suggesting that Nrk1 is the only NR kinase in budding yeast. Exp, experiment.
nucleotidases Isn1 and Sdt1 and the vacuolar phosphatase Pho8 have previously been shown to hydrolyze NMN to NR (13, 14). We first tested whether these enzymes contribute to increased NR production in pof1Δ mutant. Consistent with previous reports, deletion of ISN1 and SDT1 significantly decreased NR release in pof1Δ cells, and PHO8 deletion did not further decrease the level of release (Fig. 5B). Conversely, the intracellular NR level remained high in pof1Δ isn1Δ sdt1Δ cells but significantly decreased when combined with pho8Δ (Fig. 5A). This phenomenon could be due to compartmentalization of intracellular NR pools; NR is produced both in the cytosol and the vacuole and is stored mainly in the vacuole. NR produced by cytosolic nucleotidases (Isn1 and Sdt1) is more likely to be assimilated or excreted; therefore, measured intracellular NR is predominately the vacuolar fraction (mainly affected by Pho8).

Because these mutants did not lower released NR to wild type level, we sought to identify other nucleotidases that may contribute to NR production. A link between NAD⁺ metabolism and the inorganic phosphate-sensing PHO pathway has been established in our previous studies (14). Also, Pho8 (33) and Sdt1 (34) have been either shown or suggested to be under PHO regulation. Therefore, we reasoned that additional nucleotidases/phosphatases under PHO regulation might be involved in NR production. To test this, we examined intracellular and released NR levels in the pho4Δ mutant, in which PHO signaling is blocked (35). PHO4 deletion significantly decreased both intracellular (Fig. 5C) and released NR (Fig. 5D) in pof1Δ cells. Interestingly, deletion of PHO4 also reduced intracellular NR level in the wild type background (Fig. 5C). To identify nucleotidases that are not under PHO regulation, we screened a set of genes based on previously characterized property of hydrolyzing nucleotides and pyridine nucleotides. Deletion mutants of these genes were tested for NR release (data not shown). We found that deleting NPY1 was sufficient to moderately reduce intracellular NR and NR release in pof1Δ cells, and the NR level was further decreased when combined with pho4Δ (Fig. 5D). NPY1 was characterized as a peroxisomal NAD⁺ (H) pyrophosphatase (or Nudix hydrolase) which could produce NMN(H) from NAD⁺ (H) (36). These results suggested that cells constantly convert NAD⁺ (H) to NMN by Npy1-like enzymes. NMN is subsequently converted to NR by nucleotidases/phosphatases for release, storage, or reassimilation into NAD⁺. In the pof1Δ mutant, NMN assimilation into NAD⁺ is decreased; therefore, there is more NMN flow to the NR production route.

FIGURE 4. Characterization of the pof1Δ mutants. A, deletion of POF1 significantly impairs the growth of NR-dependent cells. To enhance the sensitivity and specificity toward NR, we employ the pyridine auxotrophic qns1Δ mutant for NR-dependent growth assay. The results show the cell growth of qns1Δ pof1Δ mutant in YPD supplemented with 10 μM or 50 μM NR. The growth defect of the qns1Δ pof1Δ mutant in 10 μM NR (normal condition) (left) can be rescued with higher concentration of NR (50 μM) (right panel), suggesting that the observed growth defect in the qns1Δ pof1Δ mutant (left panel) is mainly due to decreased NR utilization. B, comparisons of intracellular NAD⁺ levels in nma1Δ, nma2Δ, and pof1Δ deletion mutants in NR-dependent cells (qns1Δ). Cells are grown in YPD supplemented with 10 μM NR. Deletion of NMA1 or POF1 significantly decreases NAD⁺ levels. For A and B, the data shown are representative of three independent experiments, each conducted in triplicate. The error bars denote standard deviations. The p values are calculated using Student’s t test. ***, p < 0.005. C, deletion of POF1 renders sensitivity toward H₂O₂. D, deletion of POF1 abolishes the RLS extension by NR. The results show the RLS of the qns1Δ and qns1Δ pof1Δ cells grown in YPD containing different concentrations of NR. The average RLS of qns1Δ + 20 μM NR versus qns1Δ pof1Δ + 20 μM NR are significantly different (p = 0.0013) as determined by the Wilcoxon rank sum test.
Overall, our studies demonstrated the dynamic nature of inter-
changeable NAD\(^+\) intermediate pools and identified several
key players.

**DISCUSSION**

In this study, we characterized Pof1 as a novel component of
yeast NAD\(^+\) synthesis pathway. Our genetic screen revealed
that deleting **POF1** significantly increased NR levels, which sug-
gested a role for Pof1 in NR and NAD\(^+\) metabolism. We
showed that Pof1 is endowed with NMNAT activity that is spe-
cific for NMN *in vitro* and *in vivo*. Although rPof1 appeared to
be a less efficient NMNAT *in vitro* when compared with rNma1
and rNma2, which exhibit low \(K_m\) toward NMN (~100 \(\mu\)M) (23), Pof1 activity may be robust in its physiological context.
Precedents exist for efficient enzymes that exhibit high
\(K_m\) for their substrates. For example, Nma1 has high \(K_m\) for NaMN (~5 mM) but is a key enzyme in the *de novo* and NA salvage
pathways for NAD\(^+\) synthesis (27), and deletion of Nma1 signifi-
cantly decreases NAD\(^+\) levels (12). Our study also suggested
that Pof1 activity is specific for NMN unlike Nma1 and Nma2,
which exhibit dual substrate specificity toward NMN and
NaMN (Fig. 1A) (23, 24, 26). Although we did not directly
determine the catalytic activity of Pof1 toward NaMN, \(K_m\) for
NMN was not affected under high concentration of NaMN (4
mM), indicating that there is no competition or inhibition by
NaMN (data not shown). In addition, if Pof1 could adenylylate
NaMN, the *nma1Δnma2Δ* mutant would not be lethal without
NR supplement (Fig. 3C). Collectively, these studies indicated
that Pof1 is the only other NMNAT, and it contributes to
NAD\(^+\) biosynthesis by converting NMN to NAD\(^+\).

Although it is the third NMNAT in yeast, one striking phe-
notype associated with **POF1** deletion (but not with deletions of
the other two NMNAT) is the significantly increased NR pro-
duction. Given the NMNAT activity of Pof1 and its substrate
specificity for NMN, this mutant phenotype is likely due to
decreased utilization of NMN. One interesting question is why
*pof1Δ* mutant cells release more NR. Our study revealed that
multiple nucleotidases are responsible for increased NR pro-
duction in *pof1Δ* (Fig. 5, A and B). However, expression of these
nucleotidase genes did not appear to be significantly up-regu-
lated in *pof1Δ* mutant (data not shown). Therefore, these nucle-
otidases may constantly convert NMN to NR to maintain the
flow of NAD\(^+\) synthesis and salvage. This flow is altered in the
*pof1Δ* mutant, leading to increased NR accumulation because
NMN, a substrate for the nucleotidases, becomes more avail-
able when Pof1 is absent. Interestingly, one novel factor con-
tributing to increased NR level in *pof1Δ* mutant is Npy1, a per-

**FIGURE 5. Determining the nucleotidases that contribute to NR production in the**
*pof1Δ* **mutant.** A and B, deletions of **ISN1, SDT1, and PHO8** completely
abolish intracellular NR (A) increase but only partially reduce the NR release (B) in the
*pof1Δ* mutant. C and D, deletion of **NPY1** also significantly reduces
increased intracellular NR (C) and released NR (D) in the
*pof1Δ* mutant. Deletion of **NYP1** further decreases the NR level in the
*pho4Δ* mutant. The data shown are representative of three independent experiments, each conducted in triplicate. The error bars denote standard deviations. The \(p\) values are calculated using
Student’s *t* test. *, \(p < 0.05\); ***, \(p < 0.005\).
Yeast Pof1 Is an NMNAT

oxismal Nudix hydrolase that produces NMN (36). It remains unclear how this peroxismal enzyme contributes to NAD⁺ homeostasis. In addition, increased NR production has also been associated with activated PHO signaling (14). It would be interesting to determine the interaction between Pof1 and these NMN/NR producing factors.

Although Pof1 appears to share redundant function with NmA1 and NmA2, the significance of Pof1 in NAD⁺ synthesis in the NR salvage pathway is evident. These NMNATs may have distinct roles because their expression varies under different growth conditions (Fig. 2, D and E). It appears that POF1 expression is stimulated when NAD⁺ level decreases such as in late growth phase or in low NAD⁺ mutants. NMA1 expression is more complicated in that it can be stimulated under both high NAD⁺ and low NAD⁺ conditions. NMA1 expression may also be influenced by certain NAD⁺ precursors such as NA. Supporting this possibility, we showed that NMA1 expression remained high in the low NAD⁺ npn1Δ mutant in late growth stage (Fig. 2E). In npt1Δ mutant, NA salvaging is blocked, and depletion of NA is expected to be slower. Additional evidence supports that NA also regulates other NAD⁺ salvaging factors. Bogan et al. (13) reported that Isn1 protein expression responds positively to extracellular NA and glucose availability. This suggests that cytosolic degradation of NMN is regulated in response to available extracellular resources. It is possible that NAD⁺ synthesis is mainly mediated via the NA/Nam route when NA is abundant, and the key NMNAT involved is Nma1, whose expression is induced by NA. In saturated culture, depletion of NA might signal the cells to switch the route of NAD⁺ synthesis from NA/Nam salvage to de novo synthesis and NR salvage.

NMNAT family proteins have been reported to affect cellular functions independent of their enzymatic function. For example, Salmonella NadR is a multifunctional protein with a role in transcriptional regulation (32). Some eukaryotic NMNATs have been reported to possess cytoprotective functions. For example, chaperone activity that contributes to axonal protection was described for Drosophila NMNAT (37). In yeast, NmA1 and NmA2 have been shown to alleviate proteotoxicity in yeast models of proteinopathies (38). Studies by Costa et al. (21) suggested that Pof1 has a role in protein quality control. Their study also showed that pof1Δ exhibited compromised resistance to oxidative stress, heat shock, and chemical-induced ER stress. In our study, cells lacking POF1 also showed increased sensitivity to oxidative stress in NR-dependent mutant background (Fig. 4C). Apparent low catalytic activity of Pof1, together with the strong NR production phenotype (Fig. 2E), suggests that Pof1 may also maintain NMN/NAD⁺ homeostasis by sequestering NMN. For example, the majority of NADH was found to be protein-bound, and only a small fraction exists as free molecules (2, 39, 40). Therefore, it is possible that a significant fraction of NMN is bound to Pof1, and increased NR accumulation in pof1Δ mutant may be a result of the degradation of increased free NMN. Supporting this, pof1Δ further enhanced the NR release phenotypes of the nrk1Δ and nrt1Δ mutants (Fig. 1D) (Nrk1 and Nrt1 are two major players in NR salvage pathway) (Fig. 1A), suggesting that Pof1 does not simply function to convert NMN to NAD⁺. In addition, low levels of free NMN can still be efficiently assimilated into NAD⁺, because Nma1 and Nma2 have low Km for NMN (23). Further studies are required to understand the interaction among these NMNATs and to elucidate whether they possess additional roles in other cellular processes.

Our results suggest that intracellular NR pools are compartmentalized into two major pools: the stored pool and the cytosolic pool. The stored NR pool is maintained mainly in the vacuole, where Pho8 is a major NR-producing enzyme. The cytosolic NR pool is more dynamic and mainly generated from NMN by nucleotidases such as Isn1 and Sdt1. NR in this pool can be excreted or transported to the vacuole if not assimilated into NAD⁺. For each individual cell, the level of released NR reflects the size of its dynamic cytosolic NR pool, and the steady-state NR level determined in total cell extract reflects the size of the stored pool. Supporting this idea, deletions of genes encoding cytosolic nucleotidases Isn1 and Sdt1 significantly decreased NR release (cytosolic pool) (Fig. 5B) but only slightly affected intracellular NR level (stored pool) (Fig. 5A) in pof1Δ mutant. Likewise, deleting PHO8 largely decreased intracellular NR level (Fig. 5A) but only slightly affected the level of NR release (Fig. 5B) in pof1Δ mutant. Deleting these genes in wild type cells caused similar effects, but at a smaller scale (Fig. 5, A and B) (14), because wild type cells have lower levels of intracellular NR and NR release compared with pof1Δ cells. This model predicts that perturbing the vacuolar NR storage will increase cytosolic NR, and therefore more NR is released. Indeed, cells lacking a putative NR transporter Fun26 showed increased NR release (14). In higher eukaryotes, stored NR pool is likely to reside in the lysosome. Human equilibrative nucleoside transporters are Fun26 homologs that facilitate the transport of variety of purine and pyrimidine nucleosides (41), which may also have a role in intracellular NR homeostasis. Vacular NR production and uptake of extracellular NR may be stimulated concurrently to replenish the cytosolic NR pool for NAD⁺ synthesis when cells are in need for alternative NAD⁺ precursors.

Intracellular concentrations of many of the NAD⁺ intermediates are maintained at low levels (42), which is characteristic of signaling molecules. NR and NMN, similar to other NAD⁺ intermediates, may function as signaling molecules to regulate NAD⁺ homeostasis or other cellular processes. We have previously discovered that low NaMN level is associated with activation of the PHO pathway (14). Moreover, nicotinic acid adenine dinucleotide phosphate has been shown to function as a signaling molecule to regulate calcium homeostasis in variety of organisms (43, 44). Supporting this possibility, our results showed that most NR is stored in the vacuole and that Pof1 may also function to sequester NMN. In addition to functioning as signaling molecules, high concentrations of intracellular NAD⁺ intermediates may be unfavorable for certain cellular processes. For example, NAD⁺-dependent DNA ligase in bacteria is inhibited by NMN, and it is suggested that NMN deamidase contributes to maintaining a small intracellular NMN pool (45). In addition, Nam is known as an inhibitor of Sir2, and clearance of Nam is critical for maintaining Sir2 activity and life span (46). Nam clearance is facilitated by a Nam deamidase Pnc1 in yeast or Nampt, a Nam phosphoribosyltransferase in
mammals (47, 48). A recent report described that Nam is also methylated, and this modification induces a hormetic response to protect cells from oxidative damage (49). In this study, we showed that yeast cells constantly convert NMN to NR to NR, which is more mobile and can be readily excreted, stored, or reassimilated. A recent report showed that QA is also produced and excreted like NR (50). Thus, it is possible that pyridine nucleotides and their metabolites are involved in a variety of cellular processes, and balancing their concentrations would be critical for the regulation of these processes. Overall, our studies have contributed to the understanding of the complex NAD⁺ homeostasis pathways and may also provide insights into the underlying mechanisms of diseases related to defects in NAD⁺ metabolism.

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