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

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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 Ca^{2+} 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. 1*A*). NaMN is produced by transferring the phosphoribose moiety of phosphoribosyl pyrophosphate to nicotinic acid (NA) or to tryptophan-derived quinolinic acid (QA), catalyzed by phosphoribosyltransferases Npt1 and Bna6, respectively (6–8) (see Fig. 1*A*). 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 reassimilate 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 *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* acquired

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[2] The abbreviations used are: NaMN, nicotinic acid mononucleotide; NR, nicotinamide riboside; NMNAT, nicotinamide mononucleotide adenylyltransferase; NMN, nicotinamide mononucleotide; NA, nicotinic acid; QA, quinolinic acid; Nam, nicotinamide; RLS, replicative life span; rPof1, recombinant Pof1; 5-FOA, 5-fluoroorotic acid.

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 reusable loxP-*Kan*^{r}-loxP cassette as described (16). Multiple deletions were carried out by removing the *Kan*^{r} marker using a galactose-inducible Cre-recombinase. The Nrk1 overexpression construct p*ADH1*-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 XhoI, 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, 3×10^4 cells of each strain were spotted onto YPD plates spread with NAD^+ auxotrophic recipient cells (the *npt1*Δ*bna6*Δ*pho5*Δ mutant or the *qns1*Δ*pho5*Δ mutant) (14) at a density of ~9,000 cells/cm^2. 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, $\sim 1.5 \times 10^9$ (~150 A_{600} unit cells) of cells (donors of interest) grown to late log-phase (~12 h growth from an A_{600} 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 *npt1*Δ*bna6*Δ*pho5*Δ mutant) (12, 14) with starting A_{600} of 0.05 in YPD. To determine NR release levels, supernatant of donor cell culture was collected and filter-sterilized, and then 1 ml was added to 7 ml of recipient cell culture with starting A_{600} of 0.05 in YPD. A control culture of recipient cells in YPD without supplementation was included in all experiments. After incubation at 30 °C for 24 h, growth of the recipient cells (A_{600}) was measured and normalized to the cell number of each donor strain. A_{600} readings were then converted to NR concentrations using the NR standard curve as previously described (12).

Fluorescence Microscopy—Strain expressing Pof1-GFP fusion protein was made by integrating GFP gene directly into the yeast genome at a site immediately upstream of WT *POF1* using pFA6a-kanMX6-PGAL1-GFP as described (18). The resultant strain carries only the modified *GFP-POF1* gene on its chromosome because haploid strains are used for our studies. *GAL80* gene was deleted to allow Pof1-GFP expression in glucose-containing medium. Proper integration was verified by PCR, and the function of *POF1* fused to the GFP tag was confirmed by a cell-based cross-feeding assay (see Fig. 3*A*, *bottom panel*). Localization analysis of Pof1-GFP was performed by fluorescence microscopy with a Nikon Eclipse 80i microscope at ×1,000 magnification.

Cloning, Expression, Purification, and Kinetic Analysis of Recombinant Pof1 (rPof1)—The *POF1* ORF was amplified from yeast genomic DNA using primers 5′-ATTAGGATCCTATGAAGAAGACGTTCGAGCAGTTTCGAAA-3′ and 5′-AGGCCTCGAGATCAAATATTGTCTTATTATTGATCAAATAAT-3′. The amplified DNA was cloned into BamHI and XhoI sites of plasmid pET28a (Novagen). The resulting plasmid pET28-*POF1* was transformed into *Escherichia coli* Rosetta (DE3) cells (Novagen), and cells were grown in M9 medium supplemented with 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 lysozyme, 1 mg/ml DNase I, 0.1% Triton X-100), and lysed by multiple freeze and thaw cycles. Purification of Pof1 with N-terminal His_6 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 $MgCl_2$ 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 A_{600} of 0.1). Total RNA was isolated using RNeasy 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-

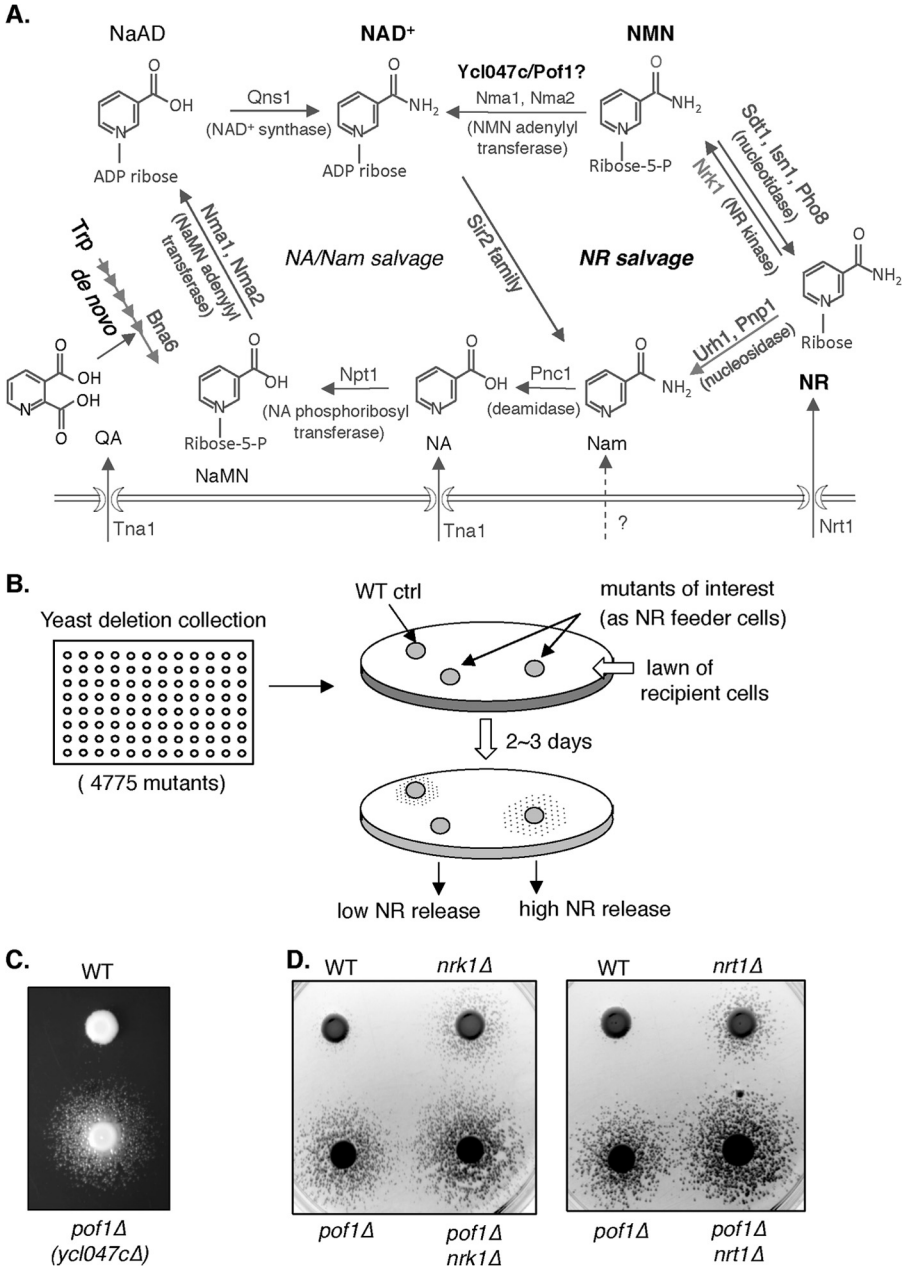


FIGURE 1. **Deletion of *YCL047C/POF1* causes altered NR metabolism.** *A*, a simplified model of *Saccharomyces cerevisiae* NAD^+ synthesis pathway. In yeast, NAD^+ is synthesized *de novo* from tryptophan (*Trp*) and by salvaging NA, Nam, QA, and NR. Cells can also salvage nicotinic acid riboside by 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 *npt1Δbna6Δpho5Δ* (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 unitization 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 are spotted onto a lawn of pyridine auxotrophic *npt1Δbna6Δpho5Δ* recipient cells. The number and size of the satellite colonies surrounding the feeder spots 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.

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. 1*B*). We found that cells lacking the ORF *YCL047C/POF1* displayed strong cross-feeding activity (NR release), indicating altered NR metabolism in this mutant (Fig. 1*C*). 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*

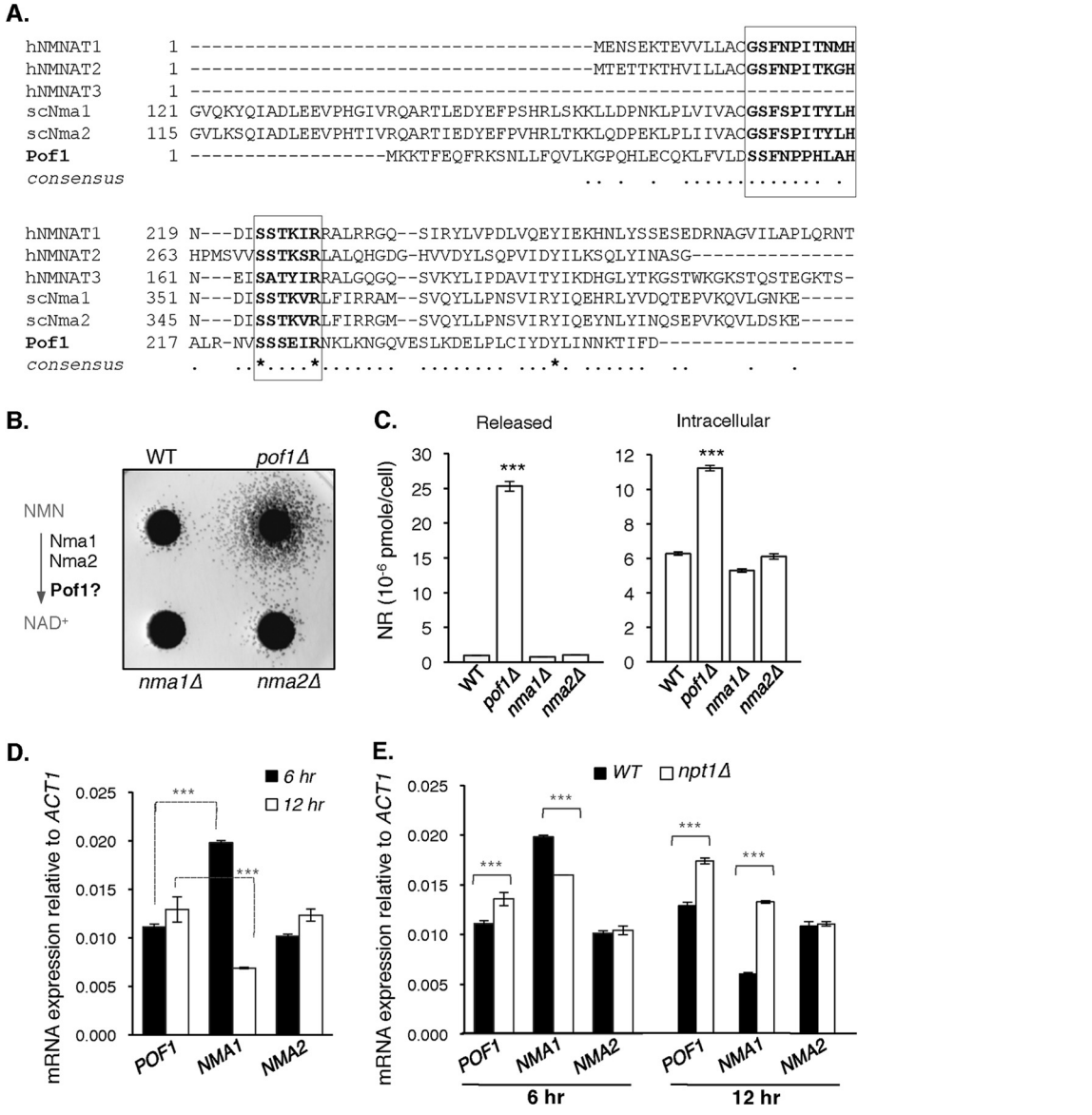


FIGURE 2. ***POF1* encodes a putative NMNAT.** *A*, amino acid sequence comparison between Pof1 and human NMNATs (hNMNAT1–3) and yeast NMNATs (scNma1 and scNma2). Pof1 possesses characterized ATP recognition motifs G*XXX*P*X*(T/H)*XX*H and S*XTXX*R. *B*, comparison of NR release in the *pof1*Δ mutant and other NMNAT deletion mutants, *nma1*Δ and *nma2*Δ. *C*, measurements of released (*left panel*) and intracellular (*right panel*) levels of NR in *nma1*Δ, *nma2*Δ, and *pof1*Δ mutants. NR is determined in both growth media (released) and cell extracts (intracellular). The *pof1*Δ mutant shows significant increases in both released and intracellular NR levels. *D*, comparison of gene expression of *POF1* and other NMNATs (*NMA1* and *NMA2*). *E*, comparison of NMNAT gene expression in wild type and the low NAD^+ *npt1*Δ mutant. For *C–E*, 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. ***, $p < 0.005$.

appeared to further enhance NR release in these mutants (Fig. 1*D*).

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. 2*A*, comparison of the amino acid sequence of Pof1 to known human NMNATs (hNMNAT1–3) and yeast NMNAT (Nma1 and Nma2) revealed that Pof1 possesses two ATP recognition motifs G*XXX*PX(T/H)*XX*H and S*XTXX*R, signature of all NMNATs characterized to date (22–24). To further understand the role of Pof1 in NR metabolism, we first examined whether *pof1*Δ and other yeast NMNAT mutants displayed similar phenotypes. Interestingly, only the *pof1*Δ mutant showed increased NR release determined by cross-feeding plate assays (Fig. 2*B*). This result was further confirmed using a quantitative liquid-based assay (12). As shown in Fig. 2*C*, *pof1*Δ mutant cells showed a ~10-fold increase in NR release (Fig. 2*C*, *left panel*), which was accompanied by increased intracellular NR level (Fig. 2*C*, *right panel*). This unique phenotype of *pof1*Δ 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. 2*D*). On the contrary, the expression of *NMA2* and *POF1* was slightly induced in the late log phase (Fig. 2*D*). Next, we

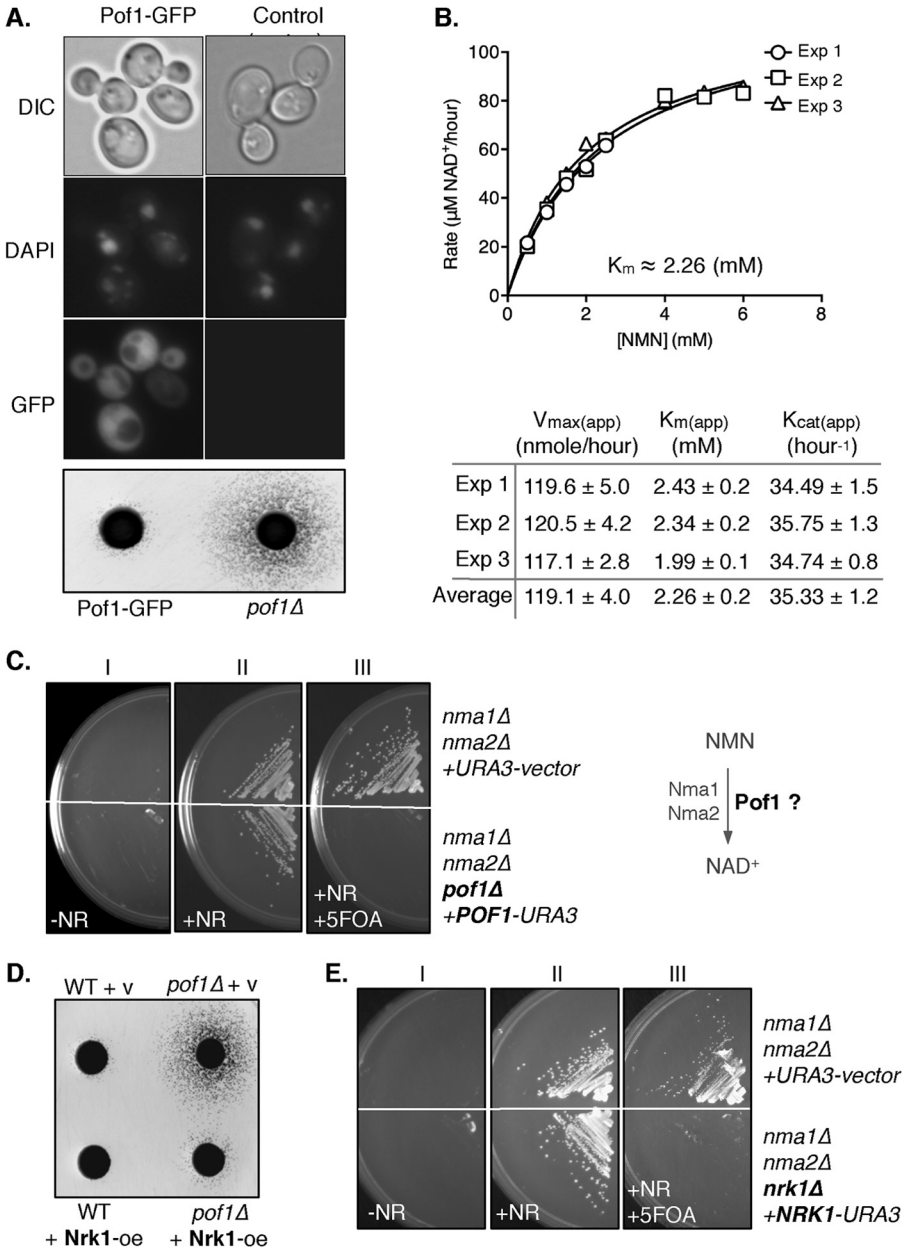
determined whether their expression was affected by low intracellular NAD^+. As shown in Fig. 2*E*, the expression of *POF1* was significantly induced in a low NAD^+ *npt1*Δ mutant (both 6 and 12 h), whereas *NMA2* expression remained constant. Although *NMA1* expression was not increased in the *npt1*Δ 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 *npt1*Δ mutant, the NA/Nam salvage route is blocked; therefore cells rely more on the NR salvage pathway for NAD^+ synthesis (Fig. 1*A*). Dual function of *NMA1* in both NA/Nam and NR salvage pathways may contribute to its complex expression patterns. Induction of *POF1* expression in the *npt1*Δ mutant suggested that Pof1 might be a dominant NMNAT in the NR salvage pathway in conditions where NR is the main NAD^+ precursor.

Pof1 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. 3*A*, *bottom panel*). Similar to Nma1, Nma2, and other salvage enzymes (25), Pof1 is localized both in the cytoplasm and the nucleus (Fig. 3*A*, *top three panels*). Next we examined whether Pof1 possessed NMNAT activity using His_6-tagged rPof1. For comparison and as a validation of our assay system, we tested the activity of recombinant Nma1, which exhibited robust activity toward NMN (23, 26) (data not shown). A steady-state kinetic study was conducted on the conversion of NMN to NAD^+ by rPof1. Using 100 μg of rPof1, $K_m^{(app)}$ 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. 3*B*). 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 adenylylation of NaMN or NMN is a required step in NAD^+ synthesis (Fig. 1*A*), deleting both *NMA1* and *NMA2* is expected to result in synthetic lethality. However, we found that *nma1*Δ*nma2*Δ cells were viable in media supplemented with NR (Fig. 3*C*, *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 *nma1*Δ*nma2*Δ mutant could grow when *POF1* is lost. We constructed a triple deletion mutant *nma1*Δ*nma2*Δ*pof1*Δ carrying wild type *POF1* 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, *nma1*Δ*nma2*Δ*pof1*Δ mutant was unable to grow, even in the presence of NR, which indicated that Pof1 functions as the third NMNAT (Fig. 3*C*, *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 *pof1*Δ mutant suggests that Pof1 may also possess NR kinase activity. To test this, we first examined whether *pof1*Δ-induced NR release could be rescued by overexpressing Nrk1. Nrk1 overexpression complemented *pof1*Δ and lowered NR release back to the wild type level, suggesting that Pof1 and Nrk1 might have overlapping function (Fig. 3*D*). We next asked whether Pof1 could function as a NR kinase by testing whether Nrk1 is the only NR kinase in yeast. In the *nma1*Δ*nma2*Δ mutant, Pof1 is essential for growth on NR (Fig. 3*C*). If Pof1 indeed has NR kinase activity, the *nma1*Δ*nma2*Δ mutant should remain viable upon loss of *NRK1* because they still have *POF1*. To test this, we constructed the *nma1*Δ*nma2*Δ*nrk1*Δ mutant carrying the wild type *NRK1* on an episomal *URA3* plasmid. Fig. 3*E* 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. 3*E*). These results indicated that Nrk1 is the only NR kinase for the utilization of NR and that Pof1 does not possess NR kinase activity.

Pof1 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. 1*A*), we employed the *qns1*Δ 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. 1*A*) and therefore depend on NR supplementation for viability. We took advantage of this NR-dependent property of *qns1*Δ cells and examined whether deleting *POF1* would affect NR utilization. As shown in Fig. 4*A* (*left panel*), the *qns1*Δ*pof1*Δ mutant exhibited a growth defect in standard NR-supplemented (10 μM) YPD medium. This *pof1*Δ-induced growth defect was specific to NR utilization deficiency because higher concentration of NR (50 μM) rescued the growth defect (Fig. 4*A*, *right panel*). Next, we determined intracellular NAD^+ levels in *qns1*Δ mutant lacking *POF1*, *NMA1*, or *NMA2*. Deletion of *POF1* or *NMA1* significantly impaired NAD^+ production, suggesting that *POF1* and *NMA1* both played important roles in the conversion of NMN to NAD^+ (Fig. 4*B*). We also examined how *POF1* deletion might affect cellular fitness. In line with the observation by Costa *et al.* (21), deleting *POF1* in *qns1*Δ cells conferred sensitivity to H_2O_2, which was partially rescued by supplementing a higher concentration of NR (Fig. 4*C*). Functional NR salvage has been shown to play important roles in maintaining the replication potential



	$V_{max(app)}$ (nmole/hour)	$K_{m(app)}$ (mM)	$K_{cat(app)}$ ($hour^{-1}$)
Exp 1	119.6 ± 5.0	2.43 ± 0.2	34.49 ± 1.5
Exp 2	120.5 ± 4.2	2.34 ± 0.2	35.75 ± 1.3
Exp 3	117.1 ± 2.8	1.99 ± 0.1	34.74 ± 0.8
Average	119.1 ± 4.0	2.26 ± 0.2	35.33 ± 1.2

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 *pof1*Δ 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^+. The Michaelis-Menten plot of rPof1 is shown. The apparent kinetic parameters of rPof1 protein are shown in the table. *C*, Pof1 exhibits NMNAT activity *in vivo*. The results show that *POF1* is required for the growth of *nma1*Δ*nma2*Δ 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*-expressing plasmid (*POF1-URA3*) in the triple deletion mutant *nma1*Δ*nma2*Δ*pof1*Δ 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 *pof1*Δ mutant. *E*, Pof1 does not have NR kinase function. The results show that *NRK1* is required for the growth of *nma1*Δ*nma2*Δ on NR-supplemented medium. The loss of the *NRK1*-expressing plasmid (*NRK1-URA3*) in the triple deletion mutant *nma1*Δ*nma2*Δ*nrk1*Δ 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.

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. 5*D*, RLS of the NR-dependent *qns1*Δ mutant was extended by higher concentrations of NR, and *POF1* deletion blocked NR-induced RLS extension.

*Multiple Phosphatases and Nucleotidases Contribute to NR Production in the pof1*Δ *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

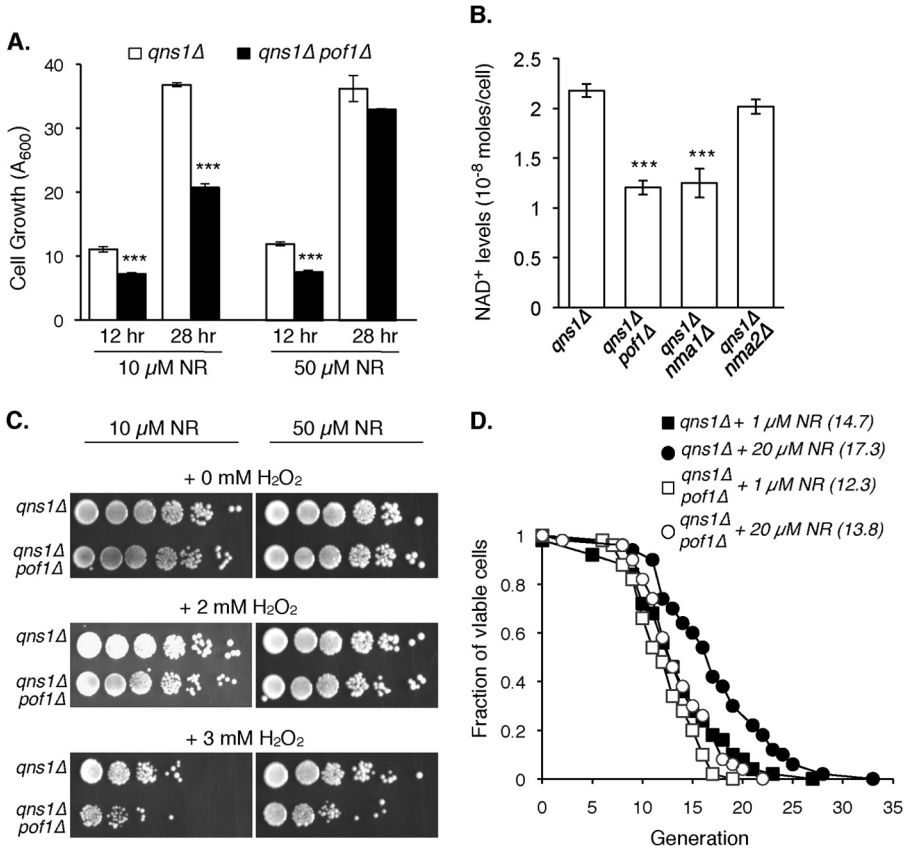


FIGURE 4. **Characterization of the *pof1*Δ mutants.** *A*, deletion of *POF1* significantly impairs the growth of NR-dependent cells. To enhance the sensitivity and specificity toward NR, we employ the pyridine auxotrophic *qns1*Δ mutant for NR-dependent growth assay. The results show the cell growth of *qns1*Δ*pof1*Δ mutant in YPD supplemented with 10 μM or 50 μM NR. The growth defect of the *qns1*Δ*pof1*Δ 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 *qns1*Δ*pof1*Δ mutant (*left panel*) is mainly due to decreased NR utilization. *B*, comparisons of intracellular NAD^+ levels in *nma1*Δ, *nma2*Δ, and *pof1*Δ deletion mutants in NR-dependent cells (*qns1*Δ). 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 H_2O_2. *D*, deletion of *POF1* abolishes the RLS extension by NR. The results show the RLS of the *qns1*Δ and *qns1*Δ*pof1*Δ cells grown in YPD containing different concentrations of NR. The average RLS of *qns1*Δ + 20 μM NR *versus qns1*Δ*pof1*Δ + 20 μM NR are significantly different ($p = 0.0013$) as determined by the Wilcoxon rank sum test.

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 *pof1*Δ mutant. Consistent with previous reports, deletion of *ISN1* and *SDT1* significantly decreased NR release in *pof1*Δ cells, and *PHO8* deletion did not further decrease the level of release (Fig. 5*B*). Conversely, the intracellular NR level remained high in *pof1*Δ*isn1*Δ*sdt1*Δ cells but significantly decreased when combined with *pho8*Δ (Fig. 5*A*). This phenomenon could be due to compartmentalization of intracellular NR pools; NR is produced both in the cytosol and the vacuole is and 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 predominantly 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 *pho4*Δ mutant, in which *PHO* signaling is blocked (35). *PHO4* deletion significantly decreased both intracellular (Fig. 5*C*) and released NR (Fig. 5*D*) in *pof1*Δ cells. Interestingly, deletion of *PHO4* also reduced intracellular NR level in the wild type background (Fig. 5*C*). 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 *pof1*Δ cells, and the NR level was further decreased when combined with *pho4*Δ (Fig. 5*D*). *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 *pof1*Δ mutant, NMN assimilation into NAD^+ is decreased; therefore, there is more NMN flow to the NR production route.

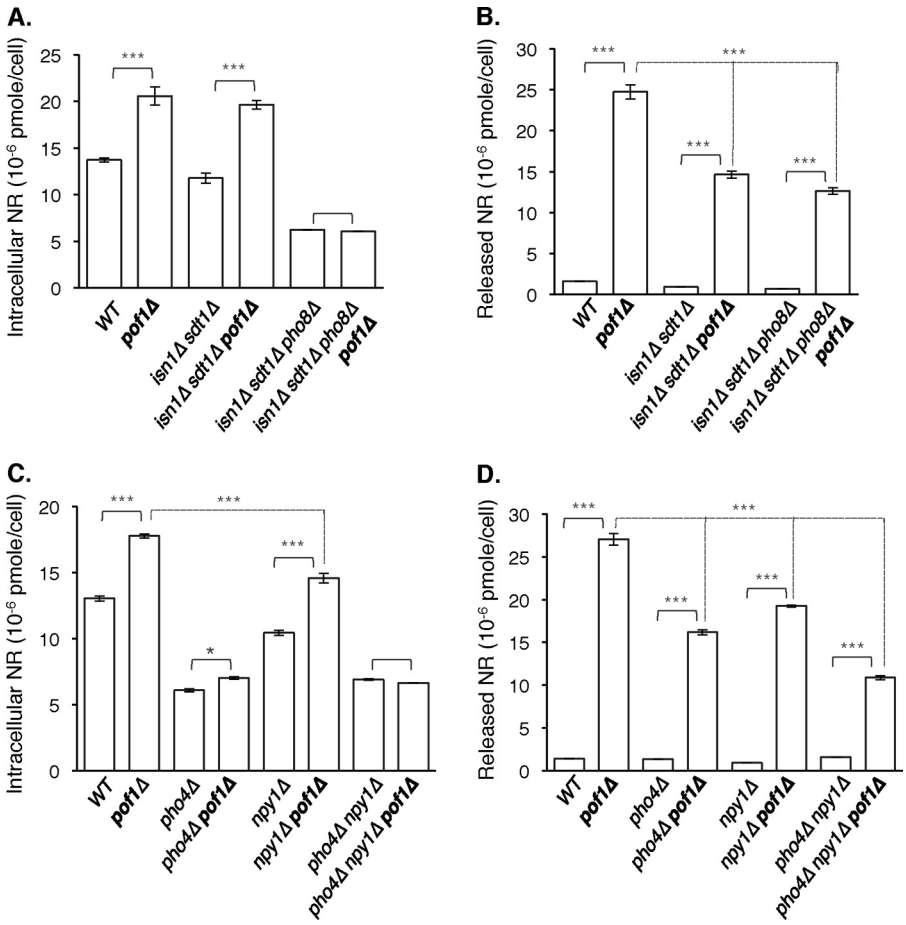


FIGURE 5. **Determining the nucleotidases that contribute to NR production in the *pof1*Δ mutant.** *A* and *B*, deletions of *ISN1*, *SDT1*, and *PHO8* completely abolish intracellular NR (*A*) increase but only partially reduce the NR release (*B*) in the *pof1*Δ mutant. *C* and *D*, deletion of *NPY1* also significantly reduces increased intracellular NR (*C*) and released NR (*D*) in the *pof1*Δ mutant. Deletion of *NPY1* further decreases the NR level in the *pho4*Δ 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. *, $p < 0.05$; ***, $p < 0.005$.

Overall, our studies demonstrated the dynamic nature of interchangeable 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 suggested a role for Pof1 in NR and NAD^+ metabolism. We showed that Pof1 is endowed with NMNAT activity that is specific 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 μM) (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 significantly 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. 1*A*) (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 *nma1*Δ*nma2*Δ mutant would not be lethal without NR supplement (Fig. 3*C*). 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 phenotype associated with *POF1* deletion (but not with deletions of the other two NMNAT) is the significantly increased NR production. 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 *pof1*Δ mutant cells release more NR. Our study revealed that multiple nucleotidases are responsible for increased NR production in *pof1*Δ (Fig. 5, *A* and *B*). However, expression of these nucleotidase genes did not appear to be significantly up-regulated in *pof1*Δ mutant (data not shown). Therefore, these nucleotidases may constantly convert NMN to NR to maintain the flow of NAD^+ synthesis and salvage. This flow is altered in the *pof1*Δ mutant, leading to increased NR accumulation because NMN, a substrate for the nucleotidases, becomes more available when Pof1 is absent. Interestingly, one novel factor contributing to increased NR level in *pof1*Δ mutant is Npy1, a per-

oxisomal Nudix hydrolase that produces NMN (36). It remains unclear how this peroxisomal 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^+ *npt1*Δ mutant in late growth stage (Fig. 2*E*). In *npt1*Δ 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 *pof1*Δ 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. 4*C*). Apparent low catalytic activity of Pof1, together with the strong NR production phenotype (Fig. 2*E*), 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 *pof1*Δ mutant may be a result of the degradation of increased free NMN. Supporting this, *pof1*Δ further enhanced the NR release phenotypes of the *nrk1*Δ and *nrt1*Δ mutants (Fig. 1*D*) (Nrk1 and Nrt1 are two major players in NR salvage pathway) (Fig. 1*A*), 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 K_m 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. 5*B*) but only slightly affected intracellular NR level (stored pool) (Fig. 5*A*) in *pof1*Δ mutant. Likewise, deleting *PHO8* largely decreased intracellular NR level (Fig. 5*A*) but only slightly affected the level of NR release (Fig. 5*B*) in *pof1*Δ 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 *pof1*Δ 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. Vacuolar 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, 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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