Phosphate-responsive Signaling Pathway Is a Novel Component of NAD\(^+\) Metabolism in *Saccharomyces cerevisiae*¹

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Nicotinamide adenine dinucleotide (NAD\(^+\)) is an essential cofactor involved in various cellular biochemical reactions. To date, the signaling pathways that regulate NAD\(^+\) metabolism remain unclear due to the dynamic nature and complexity of the NAD\(^+\) metabolic pathways and the difficulty of determining the levels of the interconvertible pyridine nucleotides. Nicotinamide riboside (NmR) is a key pyridine metabolite that is excreted and re- assimilated by yeast and plays important roles in the maintenance of NAD\(^+\) pool. In this study we establish a NmR-specific reporter system and use it to identify yeast mutants with altered NmR/NAD\(^+\) metabolism. We show that the phosphate-responsive signaling (PHO) pathway contributes to control NAD\(^+\) metabolism. Yeast strains with activated PHO pathway show increases in both the release rate and internal concentration of NmR. We further identify Pho8, a PHO-regulated vacuolar phosphatase, as a potential NmR production factor. We also demonstrate that Fun26, a homolog of human ENT (equilibrative nucleoside transporter), localizes to the vacuolar membrane and establishes the size of the vacuolar and cytosolic NmR pools. In addition, the PHO pathway responds to depletion of cellular nicotinic acid mononucleotide (NaMN) and mediates nicotinamide mononucleotide (NMN) catabolism, thereby contributing to both NmR salvage and phosphate acquisition. Therefore, NaMN is a putative molecular link connecting the PHO signaling and NAD\(^+\) metabolic pathways. Our findings may contribute to the understanding of the molecular basis and regulation of NAD\(^+\) metabolism in higher eukaryotes.

NAD\(^+\) and its reduced form NADH are essential pyridine nucleotides mediating redox reactions in cellular metabolism. In addition, NAD\(^+\) is an essential substrate in several protein modification reactions, in particular sirtuin-mediated protein deacetylation and the addition of ADP-ribose moieties. These modifications are essential for the proteins functioning in Ca\(^{2+}\) signaling, chromatin structure, DNA repair, and lifespan (1–5). The cellular pool of NAD\(^+\) is maintained by biosynthesis from nicotinic acid mononucleotide (NaMN)² or nicotinamide mononucleotide (NMN) (Fig. 1A). The production of NaMN involves the transfer of phosphorylase fromophosphoribose-pyrophosphate to nicotinic acid (NA) catalyzed by Npt1 or to quinolinic acid (QA) by Qpt1 (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\(^+\) utilizing reactions. In yeast, synthesis of NMN is achieved by Nrk1-mediated phosphorylation of nicotinamide riboside (NmR) at the expense of ATP (9, 10). NmR is a recently identified endogenous precursor for NAD\(^+\) synthesis and the salvage of endogenous NmR is important in maintaining NAD\(^+\) homeostasis and lifespan (11, 12). Because yeast cells constantly release and re- assimilate NmR, it has been suggested that this NmR pool might confer metabolic flexibility for prompt adjustment of cellular NAD\(^+\) levels (11, 12). Although the NA/nicotinamide (Nam) salvage pathway appears to be the dominant route of NAD\(^+\) synthesis when exogenous pyridine is available (13), the NmR salvage pathway also contributes significantly to the NAD\(^+\) pool and supports NAD\(^+\)-dependent reactions (9, 10). It remains unclear how NmR is produced and how its metabolism is regulated in response to different growth conditions.

Inorganic phosphate (P\(_i\)) is essential for biomolecule synthesis, energy metabolism, and protein modification. The sensing, acquisition, and storage of P\(_i\) are mainly mediated by the phosphate-responsive signaling (PHO) pathway that controls P\(_i\) transporters, regulatory factors, and effectors (Fig. 1B) (14–16). When P\(_i\) availability is high, the downstream components of the PHO pathway are repressed mainly by the cyclin-dependent kinase complex (Pho80-Pho85), which phosphorylates and inactivates the major transcription factor Pho4. Upon P\(_i\) limitation, the synthesis of inositol heptakisphosphate (IP\(_7\)) was found to be increased (17, 18). It has been shown that inositol heptakisphosphate allosterically binds to the tertiary complex of Pho81 (a cyclin-dependent kinase inhibitor)-Pho80-Pho85 and induces a conformational change of Pho81 that prevents the phosphorylation of Pho4 by Pho85 (19). Unphosphorylated Pho4 accumulates in the nucleus and cooperates with another transcription factor Pho2 to activate PHO-responsive genes whose expression products include high affinity P\(_i\) transporters (Pho84 and Pho89), repressible phosphatases (Pho5 and Pho8), and factors for phosphate mobilization from vacuolar polyphosphate storage. It is not yet fully understood how cells
sence the level of phosphate availability to elicit proper metabolic responses. Interestingly, the pho84Δ mutant shows constitutive expression of PHO5 (20, 21). It was shown that the defect in phosphate transport caused by PHO84 mutations resulted in a low level of intracellular phosphate that led to constitutive de-repression of the PHO pathway and its downstream genes (22, 23). In addition to the changes of \( P_i \) availability, recent studies have shown that the PHO signaling pathway also responds to the alterations of intermediate metabolites in purine synthesis pathway in an inositol heptakisphosphate-independent manner (24, 25).

Due to the dynamic nature and redundancy of \( \text{NAD}^+ \) synthesis pathways, to date the signaling pathways that regulate \( \text{NAD}^+ \) metabolism remain unclear. One major barrier has been the lack of a specific and sensitive genetic screen system. Because yeast cells constantly release and retrieve NmR (11, 12), we reasoned that the changes in NmR release activity might reflect the alterations of cellular \( \text{NAD}^+ \) metabolism. In this study we employed a unique NmR reporter-based genetic screen to identify novel components and regulators of \( \text{NAD}^+ \) biosynthesis to elucidate the regulation of this pathway. We showed that components of the PHO pathway played important roles in \( \text{NAD}^+ \) metabolism. We also further characterized the molecular basis underlying the interconnection between these two pathways.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Growth Media, and Plasmids**—Yeast strain BY4742 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 acquired from Open Biosystems was used for this study (26). Yeast extract-peptone-dextrose (YPD) medium was made as described (27). Low phosphate (Low-Pi) medium was prepared by phosphate precipitation from YPD as previously described (28). In brief, for each liter of Low-Pi YPD medium, 10 g of yeast extract, 20 g of peptone, and 2.46 g of \( \text{MgSO}_4 \) were first dissolved in deionized water. 8 ml of concentrated ammonium was then slowly added with gentle stirring to precipitate inorganic phosphate as \( \text{MgNH}_4\text{PO}_4 \). After filtration, the clear solution was adjusted to indicated concentrations. All of peptone, and 2.46 g of \( \text{MgSO}_4 \) were first dissolved in deionized water. 8 ml of concentrated ammonium was then slowly added with gentle stirring to precipitate inorganic phosphate as \( \text{MgNH}_4\text{PO}_4 \). After filtration, the clear solution was adjusted to pH 7 with HCl and subjected to autoclave. To prepare media with various concentrations of phosphate, Low-Pi YPD was supplemented with \( \text{KH}_2\text{PO}_4 \) to indicated concentrations. All gene deletions were generated by replacing wild type genes with a reusuable loxP-Kan-loxP cassette as described (27). The Qpt1 overexpression construct pADH1-Qpt1 was made in the integrative pPP81 (LEU2) vector as described (27). The resulting construct was verified by DNA sequencing.

**Genetic Screen Using the Yeast Deletion Collection**—The haploid yeast deletion collection (~4500 strains) established in the BY4742 background was acquired from Open Biosystems (29). To screen for mutants with altered NmR release, 2 \( \mu \)l of each strain was directly taken from the frozen stock and then spotted onto YPD plates spread with \( \text{NAD}^+ \) auxotrophic recipient cells (the npt1\( \Delta \)gpt1Δ mutant) at a density of ~9000 cells/cm². The growth of the recipient cells relies on the NmR released from the interested \( \text{NAD}^+ \) prototrophic strains, which also indicates the levels of NmR release. After incubation at 30 °C for 3 days, we scored the cross-feeding activity of each strain from the mutant collection by comparing the diameter of the cross-feed-
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Intracellular NmR Levels

Wild type yeast cells constantly release NmR and re-assimilate it. The release of NmR was first detected by cell-based cross-feeding assays in which the growth of NAD$^+$ auxotrophic cells reflects the level of NmR released from neighboring NAD$^+$ prototrophic cells (11). To identify factors that regulate NmR/NAD$^+$ metabolism, we screened a collection of yeast deletion mutants (29) for defects in cross-feeding ability. After two rounds of screening, 140 mutants were identified and classified based on cross-feeding abilities and other nutritional phenotypes (“Experimental Procedures”) (supplemental Table 1). Notably, the NmR assimilation mutant (nrk1Δ) (9) and NmR transport mutants (urt1Δ and fun26Δ) (36) showed the strongest cross-feeding ability, validating the specificity of our screen (Group I, supplemental Table 1). In addition, three mutants with deletions of NAD$^+$ salvage enzymes (Npt1, Nma1, Hst1) (37–39), and eight mutants with deletions of purine synthesis enzymes also displayed significantly reduced cross-feeding activity (Group III and IV, supplemental Table 1). The identification of these NAD$^+$ synthesis enzymes suggested that our screen might have also uncovered additional novel components of NAD$^+$ metabolism.

Interestingly, defects in the phosphate signaling (PHO) pathway alter NmR release behavior (Group II and IV, supplemental Table 1). To verify that the observed phenotypes are due to the featured mutations and not to secondary cryptic mutations in the strain collection, we constructed new mutants with defects in the phosphate transporters or the PHO regulatory factors (Fig. 1B) for further analysis. Among the phosphate transport mutants, pho84Δ (major high affinity phosphate transporter) and pho90Δ (major low affinity phosphate transporter) showed modest increases in NmR release that were consistent with the screen results (Fig. 1C, upper two panels). Moreover, the pho4Δ and pho2Δ (PHO-regulated transcription factors) mutants displayed reduced NmR release, whereas the pho85Δ (inhibitor of Pho4) mutant showed increased NmR release (Fig. 1C, lower two panels). Because pho84Δ and pho85Δ both lead to de-repression of the PHO pathway (20, 21), our results showed a positive correlation between the level of NmR release and the PHO pathway activity.

To understand whether the PHO pathway activity also correlates with NmR production, we determined intracellular NmR levels of the pho84Δ (increased PHO activity) and pho4Δ (decreased PHO activity) mutants by a liquid-based cross-feeding bioassay (“Experimental Procedures”). In this assay the growth of the reporter cells (NAD$^+$ auxotroph) is dependent on NmR supplement in culture medium. We first established a dose-response standard curve to show the correlation between the growth of the reporter cells and the concentrations of supplemented NmR (Fig. 1D). Next, we determined intracellular NmR concentrations of the PHO mutants by supplementing reporter cells with the cell extracts prepared from these PHO mutants. As shown in Fig. 1E, intracellular NmR levels were significantly increased in the pho84Δ mutant and were attenuated by a PHO4 deletion. Together, our studies demonstrate a positive correlation between NmR level and the activity of PHO pathway.

Phosphate Limitation Alters NmR Metabolism—We next tested whether cellular NmR level would respond to changes of P$_i$ availability. As shown in Fig. 2A, wild type cells grown in Low-P$_i$ medium showed a significant elevation in the level of intracellular NmR, whereas the level of released NmR appeared to be decreased. Cells grown in Low-P$_i$ media re-supplemented with P$_i$ also displayed an inverse correlation between P$_i$ availability and intracellular NmR level (Fig. 2B). In addition, the
intracellular NmR level of the pho84Δ (constitutively PHO active) mutant was not further increased by P limitation, suggesting the alterations in NmR levels induced by low P were signaled through the PHO pathway (Fig. 2C). Interestingly, a mutant that is completely defective for assimilation of NmR (nrk1Δurh1Δpnp1Δ) exhibited an increase in NmR level similar to that seen for the wild type during P limitation (Fig. 2D). Therefore, the elevation of intracellular NmR level induced by low P appears to be caused by increased NmR production rather than by attenuated NmR utilization. Collectively, these data suggest that cellular NmR level could be modulated by P availability via the PHO pathway.
The PHO-regulated Pho8 Alkaline Phosphatase Is an NmR-producing Enzyme—The positive correlation between NmR levels and PHO pathway activities suggested that NmR might be generated by a PHO-regulated factor(s), in particular the PHO-regulated phosphatase(s). We have previously reported that the periplasmic acid phosphatase Pho5 could mediate the conversion of exogenous NMN to NmR for subsequent uptake and assimilation (11). However, Pho5 appeared to be dispensable for endogenous NmR production because the deletion of Pho5 did not affect intracellular NmR level (data not shown). The PHO-regulated alkaline phosphatase Pho8 functions to scavenge Pi from phosphate-containing molecules intracellularly (40–42). We found that the level of NmR was significantly reduced in the pho8Δ mutant (Fig. 3A), suggesting Pho8 might play an important role in NmR production. Two 5′-nucleotidases Isn1 and Sdt1 have been reported to generate NmR and NaR from NMN and NaMN, respectively (12). The enzyme Isn1 was first identified as an inosine 5′-monophosphate (IMP)-specific nucleotidase mediating the production of inosine in purine salvage pathway (43). The function of Sdt1 protein was first related to the suppression of 6-azauracil sensitivity of yeast (44) and later characterized as a specific pyrimidine 5′-nucleotidase (45). To gain a better understanding of NmR production, we examined the role of Isn1 and Sdt1 in the determination of intracellular NmR level as a comparison. Under our assay conditions, deletions of Isn1 and Sdt1 only slightly affected intracellular NmR levels (Fig. 3A). This result indicates that out methods may not be sensitive enough to reflect intracellular NmR levels in vivo. Our LC-mass spectrometry analysis further confirmed that the level of NmR was substantially reduced in the pho8Δ mutant (Fig. 3B, left panel). Moreover, this reduction of NmR was accompanied by concomitant increases in NMN and NAD+ in the pho8Δ mutant (Fig. 3B, right panel).

We next determined whether Pho8 could mediate NmR production from NMN in vitro. Because Pho8 is a vacuolar membrane-located enzyme whose activation requires vacuolar peptidase (46), we employed a cell extract-based alkaline phosphatase activity assay (35) in conjunction with our reporter bioassay (“Experimental Procedures”) to determine Pho8-dependent NmR production activity. As shown in Fig. 3C, WT cell extract indeed exhibited NMN phosphatase activity that resulted in NmR production in the alkaline phosphatase activity reaction (Fig. 3C). Deleting Pho8 completely reduced this NMN-dependent NmR-producing activity, whereas deleting the cytosolic alkaline phosphatase Pho13 had no effect (Fig. 3C). This Pho8-dependent NMN phosphatase activity was further confirmed by LC-mass spectrometry analysis of the alkaline phosphatase activity reaction products (Fig. 3D). Because the pho8Δ mutant also showed decreased NaR and increased NaMN levels (Fig. 3B, right panel), it is possible that Pho8 could also contribute to the production of NaR from NaMN. As shown in Fig. 3E, a Pho8-dependent NaMN phosphatase activity was observed in WT cell extract. These results suggested that Pho8 might function as a NMN and NaMN phosphatase.

**FIGURE 2.** Cellular NmR is modulated by phosphate availability via the PHO pathway. A, the cell releases less NmR and retains more NmR in response to Pi limitation. For A–D, the levels of NmR are determined by supplementing reporter cells with cell extracts (for intracellular NmR) or culture supernatants (for released NmR) prepared from the strains of interest. B, intracellular NmR levels inversely correlate with Pi availability in growth medium. Low-Pi medium is supplemented with various amount of KH₂PO₄ to establish different phosphate availabilities. Regular YPD is used as high-Pi control, which has been reported to contain 2–5 mM orthophosphate (59, 60). –, no KH₂PO₄ supplement. C, the pho8Δ mutant grown in YPD shows similar elevated intracellular NmR level as the WT grown in Low-Pi medium. Pi limitation does not further increase the NmR level of the pho8Δ mutant. D, the nrk1ΔΔurh1ΔΔpnp1Δ mutant also responds to low Pi availability and shows increased intracellular NmR. For A, C, and D, relative NmR levels are shown; the growth (Aₙ₀₀) of reporter cells is normalized to the set supplemented with cell extracts (or culture supernatant) of WT cells grown in YPD. Results of one representative set of three independent experiments are shown. The p values are calculated using Student’s t test (*, p < 0.05; ***, p < 0.005).
Fun26 Is a Vacuolar NmR Transporter—Because Pho8 is localized to the vacuole, we examined how yeast cells may deliver NmR produced in the vacuole to the cytosol for assimilation. The Fun26 protein was reported to have a minor role in NmR transport relative to Nrt1 (36). However, we found that the fun26/H9004 mutant displayed a comparable level of NmR release to that seen for the nrt1/H9004 mutant (Fig. 4A) (Group I, supplemental Table 1). In addition, a significant increase in intracellular NmR level was also observed in the fun26/H9004 mutant but not in the nrt1/H9004 mutant (Fig. 4B). Fun26 is the only yeast homolog of the human equilibrative nucleoside transporter (hENT) protein family, which mediates bi-directional transport of specific nucleosides across plasma membrane and intracellular membranes (47–49). Unlike the plasma membrane NmR transporter Nrt1 (36), Fun26 has been suggested to localize to intracellular membranes including vacuolar membranes (50, 51). This may explain why the fun26Δ and nrt1Δ mutants have distinct phenotypes. To further understand the role of Fun26 in NmR transport, we first determined the cellular localization of Fun26. Adding a GFP tag to Fun26 did not compromise its function, as determined by cross-feeding assays (Fig. 4C, lower panel). In line with previous reports, Fun26-GFP was clearly localized to the vacuolar membrane (Fig. 4C, upper panels). Interestingly, deleting the vacuolar resident Pho8 strongly reduced the accumulation of intracellular NmR in the fun26Δ mutant (Fig. 4D), further supporting the idea that Fun26 is a vacuolar NmR transporter that functions to balance NmR levels between the cytosol and vacuole (Fig. 4E).

The Level of NaMN Modulates the PHO Signaling Activity—We have previously shown that assimilation of extracellular NMN requires prior conversion to NmR by the phosphatase Pho5 in the periplasmic space (11). Therefore, in addition to modulating intracellular NmR production, the PHO signaling pathway might also modulate extracellular NmR production. Indeed, growth of the NAD+ auxotrophic qns1Δ mutant on exogenous NMN was dependent on the PHO-regulated transcription factor Pho4 (Fig. 5A). In addition, genetically activating PHO signaling by deleting Pho84 resulted in significant...
cell growth on NMN 20 h after inoculation when the parental qns1Δ mutant still exhibited no cell growth (Fig. 5B). These results suggest that the activities of the PHO pathway correlate with the time period required for the initiation of growth under this condition. Hence, the initiation time needed for PHO-dependent growth could be used to determine the activation status of the PHO pathway in strains of interest. However, because the PHO signaling cascade is mainly de-repressed by Pi starvation, it is unclear how the PHO pathway could be activated in Pi-rich medium, which was used for the growth assay of the qns1Δ mutant (Fig. 5C).

To test this possibility, we deleted NA/Nam salvage enzymes in the qns1Δ mutant to see whether putatively altered levels of specific NAD+ intermediates would affect the PHO-dependent growth of the qns1Δ mutant. We found that deleting NPT1 significantly shortened the lag time preceding growth initiation of the qns1Δ mutant from ~24 to ~14 h (Fig. 5C).

Next, we examined whether deleting these NA/Nam salvage enzymes in wild-type background would be sufficient to activate the PHO pathway. As shown in Fig. 5, D and E, the npt1Δ mutant exhibited higher Pho5 activity as determined by both colorimetric acid phosphatase plate assay and liquid assay (32). Deleting TNA1 or PNC1 also slightly increased Pho5 activity. Although Npt1, Tna1, and Pnc1 contribute to the production of NaMN and NAD+ (Fig. 6A), observed PHO activation in these

**FIGURE 4. Fun26 is a vacuolar NmR transporter.** A, both fun26Δ and nrt1Δ mutants show increased NmR release determined by the cross-feeding plate assay as described in Fig. 1. B, the fun26Δ mutant, but not nrt1Δ mutant, has high levels of intracellular NmR. C, cellular localization of Fun26-GFP protein is shown. The fluorescence signal of Fun26-GFP along with the differential interference contrast (DIC) image indicates that Fun26 localizes to vacuolar membrane. Adding a GFP tag to Fun26 does not interfere with its function determined by cross-feeding plate assay (lower panel). D, deletion of vacuolar resident protein Pho8 significantly lowers intracellular accumulation of NmR in the fun26Δ mutant. E, the proposed model of the role of Fun26 in NmR metabolism is shown. Fun26 may function to balance NmR between the cytosolic and vacuolar pools (left). In Fun26-defective cells, NmR generated in the vacuole cannot be salvaged by NmR assimilation enzymes localized in the cytosol and/or nucleus (right).
mutants (Fig. 5, C, D, and E) was unlikely due to NAD$^+$ depletion because Pnc1 deletion does not decrease NAD$^+$ level (11, 52). Therefore, we tested whether enhanced PHO activation might be due to NaMN depletion by LC-mass spectrometry analysis. Fig. 6B showed that the level of NaMN was decreased by deleting Npt1 in the qns1Δ mutant. In addition, the level of NmR was concomitantly increased (Fig. 6B). This result was expected as deleting Npt1 significantly shortens the lag time preceding growth initiation of the qns1Δ mutant from −24 to −14 h. For D and E, the repressible acid phosphatase (rAPase) activity is significantly increased in the npt1Δ mutant and modestly elevated in the tna1Δ and pnc1Δ mutants as determined by colorimetric plate assay (D) and liquid assay (E). For E, cells grown to late log phase are harvested for repressible acid phosphatase activity assay. Repressible acid phosphatase activities are expressed in Miller units ([A$$_{420}$$ × 1000]/[A$$_{600}$$ × volume of cells harvested]) × 15 min). The $p$ values are calculated using Student’s t test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

**DISCUSSION**

In this study we characterized the PHO pathway as a novel component in NAD$^+$/NmR metabolism. Mutants with activated PHO signaling showed increased NmR levels, whereas mutants with decreased PHO signaling displayed reduced NmR levels. We have also identified alkaline phosphatase Pho8 as a
potential important enzyme of NmR production, likely to work by removing Pi from NMN. We demonstrated that the increase in intracellular NmR level is an integral part of the PHO response upon Pi limitation, during which cells retain more NmR instead of releasing it extracellularly. By genetically manipulating the enzymes of NA/Nam salvage, we showed that accumulations of NaMN might inhibit PHO activation, which also suggested that NaMN might function as an internal signal for the PHO pathway (Fig. 6D).

The repression and activation of the PHO pathway is an important mechanism of cellular adaptation to endure the variation of P_i availability and to maintain metabolic homeostasis. The signals of external P_i level and internal reservoir (including orthophosphate and polyphosphate) are sensed and conveyed by the PHO pathway that leads to responses at the gene expression level to adjust cellular activities in Pi transport and scavenging. Although P_i availability appears to be the major signal, recent studies have revealed novel components in the regulatory circuit of the PHO responses. It has been shown that, after prolonged P_i starvation, the repletion of P_i to cells leads to degradation of the high affinity phosphate transporter Pho84 (53, 54). This response requires the activation of the protein kinase A (53, 54), demonstrating a connection between the glucose and phosphate signaling pathways. The level of other nutrient intermediates also influences the activation of the PHO pathway. Recently, it was reported that the synthesis of purine nucleotide is co-regulated with the PHO pathway by a mutual transcription factor Pho2 (24, 25). The accumulation of the purine biosynthetic intermediate, in particular 5’-phosphoribosyl-5-amino-4-imidazole carboxamide (AICAR), activates the transcription of purine regulon genes and PHO regulon genes by Pho2 in a P_i availability-independent manner (24, 25). Moreover, deficiency in Adk1 (adenylate kinase 1), an important enzyme in energy metabolism, results in a transcriptional profile similar to the cellular response upon P_i starvation (24, 25).

Our present and earlier studies suggest that NAD^+ metabolism is also connected to the PHO pathway (Fig. 6D). The coupling of these two pathways may render a more efficient metabolic support under specific conditions. For instance, the phosphate moiety of NMN is a putative target for phosphate scavenging during Pi limitation. Indeed, activation of the PHO pathway by deleting Pho84 or by decreasing Pi availability in growth medium increases intracellular NmR levels (Figs. 1E and 2B). Moreover, the increase of intracellular NmR in response to Pi starvation is likely mediated by PHO-regulated Pho8 in the vacuole and by Pho5 in the periplasmic space (11).

Our results show that the PHO pathway also responds to NaMN depletion. The mutant with a deletion of the major NaMN generation enzyme Npt1 showed de-repression of the PHO pathway as determined by the acid phosphatase activity assay (Fig. 5, D and E). The deletion of NPT1 stimulates the
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PHO pathway-dependent growth of the qus1Δ mutant (Fig. 5C), whereas overexpression of Oqp1 (which putatively leads to increases of NaMN) inhibits the growth of the qus1Δ mutant (Fig. 6C). Since NaMN is an important intermediate for NAD⁺ biosynthesis (Fig. 1A), low levels of NaMN might reflect impaired NAD⁺ biosynthesis and lead to de-repression of an alternative NAD⁺ salvage route-NmR utilization (Fig. 1A). Because NmR-mediated NAD⁺ synthesis requires phosphate (in form of ATP), a coordinated activation of the PHO pathway is important in supporting NAD⁺ synthesis and homeostasis.

In addition to the PHO pathway, our genetic screen also revealed potential novel players in NmR/NAD⁺ metabolism. We found 12 mutants harboring defects in the regulation or the assembly of vacuolar ATP synthase complex showed strong or moderately higher NmR release (Group I and II, supplemental Table 1). The vacuolar ATP synthase complex is essential in establishing and maintaining the acidity of vacuolar matrix, and the impairment in vacuolar ATP synthase complex will alter the activity of vacuolar proteins, including peptidases and phosphatases (55). The identification of vacuolar ATP synthase assembly mutants in our genetic screen suggests that vacuole is a putative compartment important for NmR homeostasis in yeast. In addition, 19 mutants with deficiencies in the protein trafficking process or in the vesicle-mediated transport machinery were found to release more NmR (Group I and II, supplemental Table 1). One important function of vesicle-mediated transport is to deliver secreted proteins and membrane proteins to their final destinations (56, 57). It is, therefore, possible that the aberrant NmR release might result from defects in the transport of NmR-metabolizing proteins to certain locations; for example, NmR transporter Nrt1 to plasma membrane. A comprehensive characterization of the turnover of Nrt1 would help address the phenotypes of this group of mutants. The identification of phosphatase Pho8 as a NmR-producing enzyme raises another question; if vacuole is a cellular compartment for NmR production, how does cell salvage vacuolar NmR to synthesize NAD⁺ in other compartments, for example cytosol? Previous reports (50, 51) and our current studies collectively suggest that Fun26 could balance NmR between the cytosolic and vacuolar pools, which would enable the utilization of NmR originated from the vacuole. In addition, the mechanism of NmR export has not yet been addressed. Because the fun26Δ mutant also releases more NmR (Fig. 4A), it is possible that the increase of NmR release in this mutant is due to the accumulation of NmR in the vacuole. A direct investigation to determine whether NmR molecules could be exported extracellularly, for example, along with other protein cargos through vesicle-mediated transport, would help depict the dynamics of endogenous NmR.

We have previously shown that the ability to salvage both the intracellular and extracellular NmR is important for cell survival and stress resistance (11). To date, the mechanisms of NmR uptake and its transport between cellular compartments in higher eukaryotes are still unknown. Four Fun26-related human ENT proteins have been characterized (47). Given the role of Fun26 in NmR metabolism in yeast, it would be informative to determine whether the plasma membrane-localized hENT1 and hENT2 and lysosome membrane-resided hENT3 also participate in NmR homeostasis and NAD⁺ metabolism in human.

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