A Functional Link Between NAD⁺ Homeostasis and N-terminal Protein Acetylation in
Saccharomyces cerevisiae

Trevor Croft*, Christol James Theoga Raj*, Michelle Salemi#, Brett S. Phinney# and Su-Ju Lin*†

From *Department of Microbiology and Molecular Genetics, College of Biological Sciences, and
#Proteomic Core Facility, University of California, One Shields Ave., Davis, CA 95616, USA

Running title: NatB is a NAD⁺ homeostasis factor

†To whom correspondence should be addressed: Su-Ju Lin, Department of Microbiology and
Molecular Genetics, University of California, One Shields Ave., Davis, CA 95616, Tel. 530-754-6081;
Fax. 530-752-9014; E-Mail: slin@ucdavis.edu

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ABSTRACT
Nicotinamide adenine dinucleotide (NAD⁺) is an essential metabolite participating in cellular redox chemistry and signaling, and the complex regulation of NAD⁺ metabolism is not yet fully understood. To investigate this, we established a NAD⁺-intermediate specific reporter system to identify factors required for salvage of metabolically-linked nicotinamide (NAM) and nicotinic acid (NA). Mutants lacking components of the NatB complex, NAT3 and MDM20, appeared as hits in this screen. NatB is an Nα-terminal-acetyltransferase responsible for acetylation of the amino terminus of specific Met-retained peptides. In NatB mutants, increased NA/NAM levels were concomitant with decreased NAD⁺. We identified the vacuolar pool of nicotinamide riboside (NR) as the source of this increased NA/NAM. This NR pool is increased by nitrogen starvation, suggesting NAD⁺ and related metabolites may be trafficked to vacuole for recycling. Supporting this, increased NA/NAM release in NatB mutants was abolished by deleting the autophagy protein ATG14. We next examined Tpm1 (tropomyosin) whose function is regulated by NatB mediated acetylation, and Tpm1 overexpression (TPM1-oe) was shown to restore some NatB mutant defects. Interestingly, although TPM1-oe largely suppressed NA/NAM release in NatB mutants, it did not restore NAD⁺ levels. We showed that decreased nicotinamide mononucleotide adenyllytransferases (Nma1/Nma2) levels likely caused the NAD⁺ defects, and NMA1-oe was sufficient to restore NAD⁺. NatB mediated N-terminal acetylation of Nma1 and Nma2 appears essential for maintaining
NAD$^+$ levels. In summary, our results support a connection between NatB mediated protein acetylation and NAD$^+$ homeostasis. Our findings may contribute to understanding the molecular basis and regulation of NAD$^+$ metabolism.

NAD$^+$ and its reduced form NADH are essential metabolites mediating redox reactions in cellular metabolism. NAD$^+$ is a substrate in protein modifications, such as sirtuin (Sir2 family proteins) mediated protein deacetylation, and ADP-ribosylation by poly ADP-ribose polymerases. These protein modifications contribute to regulation of chromatin structure, DNA repair, circadian rhythm, metabolic responses and life span (1-4). Aberrant NAD$^+$ metabolism is associated with a number of diseases including diabetes, cancer, and neuron degeneration (2,3,5-8). Administration of NAD$^+$ precursors such as nicotinamide mononucleotide (NMN), nicotinamide (NAM), and nicotinamide riboside (NR) has been shown to ameliorate deficiencies related to aberrant NAD$^+$ metabolism in yeast, mouse, and human cells (3,5-12). However, molecular mechanisms that underlie these beneficial effects remain unclear.

NAD$^+$ biosynthesis is highly conserved between yeast and vertebrates. In yeast, NAD$^+$ is synthesized de novo from tryptophan or salvaged from intermediates such as NA, NAM, and NR (Fig. 1A). The NAD$^+$ levels maintained by these pathways converge at several different points, and share cellular pools of ATP, PRPP (phosphoribosyl pyrophosphate), and glutamine, while adding to total pools of ribose, AMP, phosphate, formate, alanine, glutamate, and others. NAD$^+$ is a NADP$^+$ precursor, which like NAD$^+$, is carefully balanced with its reduced form to maintain a favorable redox state. Some of these molecules contribute to other biosynthesis pathways, or have signaling functions. Therefore the cell must maintain these metabolites and their flux in a controlled manner. We do not fully understand all the mechanisms by which the cell can sense and tune these metabolites, but some known NAD$^+$ homeostasis regulatory mechanisms include transcriptional control, feedback inhibition, and enzyme or metabolite compartmentalization (1,13-18). The complex and dynamic flexibility of NAD$^+$ precursors makes studying NAD$^+$ metabolism complicated. For example, NAM can both replenish NAD$^+$ pools and inhibit the activity of NAD$^+$ consuming enzymes. In addition, little is known about the signaling pathways that regulate NAD$^+$ precursor homeostasis in part due to the lack of sensitive and specific genetic screening systems to identify these NAD$^+$ homeostasis factors.

We have previously shown that NR-mediated NAD$^+$ synthesis plays important roles in the maintenance of NAD$^+$ pools and calorie restriction-induced lifespan (19). To further understand the regulation of NAD$^+$ homeostasis, we developed a NR-specific reporter-based bioassay to screen for mutants with altered NR release. This screen system was based on our observations that yeast cells release and transport NR back into the cell (19), a phenomenon that is also conserved in human cells (20). Our studies have identified novel NAD$^+$ homeostasis factors including the phosphate responsive signaling (PHO) pathway (17), the SPS amino acid sensing pathway (21),
a conserved vacuolar NR transporter Fun26 (1,17) and several NAD⁺ metabolic enzymes (22).

In this study, we developed a reporter system targeting the NA/NAM branch of NAD⁺ metabolism. The hypothesis is that cells defective in NA/NAM salvage (Fig. 1A) would show altered NA/NAM release. The mdm20Δ and nat3Δ mutants were among the top hits that showed increased NA/NAM release. Nat3 and Mdm20 are the catalytic and auxiliary subunits of NatB, respectively (23,24). NatB belongs to a family of N-terminal acetyltransferases (NATs) that add an acetyl group from acetyl-CoA to the alpha-amino group on the first amino acid of a protein. In yeast, there are four functional NATs, and each has distinct substrate specificity (23,25,26). About 20% of proteins are potential substrates of NatB in yeast and humans (27). N-terminal acetylation plays important roles in regulating protein stability, complex formation and subcellular localization, and has been implicated in various cellular processes and human diseases (27). Our studies are the first to link NatB to NAD⁺ homeostasis. Here we characterized the NatB mutants as well as the NatB downstream targets to provide a molecular basis underlying NatB mediated regulation of NAD⁺ homeostasis.

RESULTS

A cell-based reporter assay to study NA/NAM homeostasis factors—We first determined if cells indeed release more NA and/or NAM when NA/NAM salvage is blocked. A cross-feeding assay is established using bna6Δnrt1ΔΔnrt1Δ mutants as “recipient cells” (depend on NA or NAM for growth) and mutants of interest as “feeder cells”. In this system, recipient cells cannot grow on niacin-free SD (NA/NAM-free), but when feeder cells are placed in proximity, feeder cell-released NA or NAM supports recipient cell growth by “cross-feeding”. As a result this assay determines relative levels of total NA and NAM released by feeder cells. As shown in Fig. 1B (top panel), recipient cells were spread onto niacin-free SD plates as a lawn. Next, npt1Δ (blocks NA salvage) and pnc1Δ (blocks NAM salvage) feeder cells were spotted on top of the lawn and allowed to grow. Recipient cells near the npt1Δ and pnc1Δ feeder spots appeared as satellite colonies after 3 days, and the number and size of the satellite colonies positively correlate with the amount of NA/NAM released from the feeders. To separate NAM release from NA/NAM release, PNC1 was deleted in the recipient cell strain to block their ability to utilize NAM. Based on the biochemical function of Npt1 and Pnc1 (Fig. 1A), we expect that npt1Δ cells release more NA (and perhaps NAM) and pnc1Δ cells release more NAM. Indeed, as shown in Fig. 1B (bottom panel), pnc1Δ feeder cells failed to support the growth of the new recipient strain (bna6Δnrt1ΔΔnrt1ΔΔpnc1Δ, which cannot utilize NAM). In contrast, npt1Δ cells still supported the growth of bna6Δnrt1ΔΔnrt1ΔΔpnc1Δ. These results confirmed that mutants with altered NA/NAM homeostasis could be identified using this system.

Components of the NatB complex are novel NA/NAM homeostasis factors—Next, we used the haploid yeast deletion collection as feeder cells to identify mutants with increased NA/NAM release (Fig. 1C). Mutants were
assigned a score of 2 through 5 (WT=1). A higher score represents more NA/NAM release. To eliminate false-positives, mutants with a score of ≥ 3 (81 mutants) were re-examined. A total of ~70 mutants passed the secondary screen (Supplemental Table 1). Known NAD⁺ homeostasis factors were identified, including npt1Δ and pnc1Δ. The mdm20Δ and nat3Δ mutants were among the top hits. Nat3 and Mdm20 are subunits of NatB, a member of the N-terminal acetyltransferases (NATs) that add an acetyl group from acetyl-CoA to the alpha-amino group on the first amino acid of a protein (23,24). Deletion of either NatB subunit results in stress sensitivity growth defects (23,24,28,29). To verify that observed phenotypes are due to the featured mutations and not to secondary cryptic mutations in the deletion collection, we re-constructed all deletion mutants used in this study. We confirmed both nat3Δ and mdm20Δ indeed released more NA/NAM (Fig. 1D, upper panel). Similar to pnc1Δ mutant (Fig. 1B), both nat3Δ and mdm20Δ released mostly NAM not NA (Fig.1D, lower panel). Fig. 1E showed that increased NA/NAM release in nat3Δ and mdm20Δ cells correlates with increased intracellular NA/NAM levels determined by a quantitative liquid assay.

NR metabolism contributes to increased NAM release in NatB mutants—To understand how NatB complex affects NAM/NAD⁺ homeostasis, we first tested whether Pnc1 level or activity was reduced in NatB mutants since Pnc1 is a major NAM metabolic factor (nicotinamidase), and the pnc1Δ mutant showed strong NAM release (Fig. 1B). Although Pnc1 is not a predicted NatB substrate, Pnc1 may be indirectly regulated by NatB. Our results showed that Pnc1 activity (Fig. 2B) and protein expression (Fig. 2A) were only slightly decreased in nat3Δ cells. Interestingly, although pnc1Δ cells show WT level of NAD⁺ under normal (NA rich) growth conditions (19), the levels of NAD⁺ were significantly reduced in NatB mutants (Fig. 2C). These studies suggest additional factors likely contribute to the altered NAM/NAD⁺ homeostasis observed in NatB mutants. Increased production of NR has been associated with increased activity of the Pi-sensing PHO signaling pathway (17). We therefore determined whether NatB mutants showed altered PHO activity by measuring the activity of rAPase (Pho5, a periplasmic phosphatase activated by PHO signaling). As shown in Fig. 2D (left panel), PHO activity was moderately increased in the nat3Δ mutant. As controls, deleting PHO5 largely blocked the increase of rAPase activity. The residual rATPase activity observed in nat3Δpho5Δ cells likely came from Pho11 and Pho12. Interestingly, observed PHO activation in nat3Δ cells appeared independent of the conventional PHO transcription factors Pho4/Pho2 complex, because deleting PHO4 did not significantly reduce the PHO activation in nat3Δ cells (Fig. 2D, left panel). Consistent with this result, deleting PHO4 did not prevent NA/NAM release in nat3Δ cells (Fig. 2D, right panel). Next, we determined whether and how NR contributes to increased NAM in NatB mutants. Intracellular NAM may come from NR via the reactions of cytoplasmic nucleosidases Urh1 and Pnp1 (Fig. 2E, top panel) (30). As shown in Fig. 2E, (bottom panel), deleting URH1 and PNP1 largely diminished NA/NAM
release in NatB mutants. Moreover, deleting the vacuolar phosphatase PHO8 (Fig. 2E, top panel) (17) diminished NA/NAM release (Fig. 2E, bottom panel), whereas deleting the cytoplasmic nucleotidases ISN1 and SDT1 (Fig. 2E, top panel) had a lesser effect (Fig. 2E, bottom panel). These results suggest increased NAM in NatB mutants likely results from vacuolar NR, which is converted to NAM by cytoplasmic nucleosidases Urh1 and Pnp1. Vacuolar NR may exit the vacuole through Fun26 transporter (human lysosomal hENT homolog) into the cytoplasm (17) where it is converted to NAM by Urh1 and Pnp1 (Fig. 2E, top panel). Supporting this, deleting FUN26 indeed blocked NAM release in the NatB mutant (Fig. 2E, bottom panel). Moreover, nat3Δ cells carrying deletions of URH1 and PNP1 (convert NR to NAM) showed increased intracellular NR levels (Fig. 2F, middle left panel) and reduced NA/NAM levels (Fig. 2F, middle right panel). Deleting PHO8 (produces NR in the vacuole) blocked the increase of both NR and NA/NAM in nat3Δ cells (Fig. 2F, bottom panels). All these results support vacuolar NR is the main source of increased NA/NAM in nat3Δ cells.

Blocking NA/NAM production and release is not sufficient to restore NAD⁺ levels in the NatB mutants—So far our results suggest that in NatB mutants, increased NA/NAM release is likely due to increased NAM production, which is originated from vacuolar NR. It remains unclear how NAD⁺ and its intermediates are transported into the vacuole for turnover and storage. Since most cellular NAD⁺ and NADH are bound to proteins, NAD⁺ intermediates may enter vacuole via vesicular trafficking and/or autophagy. Since autophagy is a critical regulator of organelle homeostasis and recycling of macromolecules (31,32), we therefore asked whether inducing autophagy in yeast cells would indeed increase intracellular NR and NA/NAM levels. As shown in Fig. 3A, both NR (left panel) and NA/NAM (right panel) levels were increased by nitrogen starvation, a condition known to induce autophagy (31,33,34). These increases were largely dependent on Atg14, a key regulator of autophagic membrane tethering and fusion (31). These data suggest autophagy may contribute to increased NA/NAM in NatB mutants. Supporting this, we showed that deleting ATG14 largely blocked NA/NAM release in the nat3Δ mutant (Fig. 3B). Next, we tested whether deleting ATG14 was sufficient to restore NAD⁺ levels in the NatB mutants. As shown in Fig. 3C (left panel), deleting ATG14 did not rescue the low NAD⁺ levels in nat3Δ cells. Similarly, deleting other factors contributing to NR and NAM production (Fig. 1A and Fig. 2E) in nat3Δ cells also failed to restore the NAD⁺ level back to wild type (Fig. 3C, right panel). Most reported NatB mutant phenotypes were attributed to defective N-terminal acetylation of tropomyosins (Tpm1 and Tpm2 in yeast) (23,28,29). Tropomyosins are actin filament-binding proteins that stabilize actin cables (29,35-38). Overexpression of TPM1 (TPM1-oe) and expression of a gain-of-function allele of TPM1 (TPM1-5) were shown to restore actin cable formation in NatB mutants (23,24,28,29). Interestingly, both TPM1-oe and TPM1-5 largely blocked NA/NAM release in the nat3Δ mutant (Fig. 3D), suggesting a role for actin cytoskeleton in NAM homeostasis regulation. However, TPM1-oe and TPM1-5 still failed to restore the NAD⁺ levels in
nat3Δ cells (Fig. 3E). These studies indicate that the low NAD⁺ phenotype of nat3Δ cells cannot be rescued simply by decreasing NAM production and/or release and that additional factors are likely involved.

Nma1 and Nma2 are responsible for the low NAD⁺ levels in NatB mutants—To gain further insight, we tested whether supplementing particular NAD⁺ precursors to nat3Δ cells can restore the NAD⁺ levels. Interestingly, all NAD⁺ precursors tested (NA, NAM, NR and QA) failed to restore NAD⁺ levels (Fig. 4A). This suggests that nat3Δ cells may be defective in a step of NAD⁺ biosynthesis that is common to the utilization of QA, NA and NR. Nma1 and Nma2 are dual functional NMMATs (nicotinamide mononucleotide adenylyltransferases), which utilize both NMN and NaMN (nicotinic acid mononucleotide) as substrates and are required for all three NAD⁺ biosynthesis pathways (Fig. 1A). N-terminal acetyltransferases have specific targets that are largely determined by the sequence of the first two amino acids. NatB acetylates proteins with a MET-retained residue, followed by ASP, GLU, GLN, or ASN as the second residue (25,26,39,40). Nma1 and Nma2 have a MET-ASP N-terminus, but neither protein has been identified as a NatB target. Similar to nat3Δ mutant, nma1Δ cells showed decreased NAD⁺ levels, which could not be rescued by supplementing NR (Fig. 4B). As for the controls, NR efficiently restored the NAD⁺ level in the npt1Δ mutant, which has functional NR salvage (Fig. 4B). The similarities between nma1Δ and nat3Δ mutants suggested that Nma1 and Nma2 activities are likely reduced in nat3Δ cells. Since N-terminal acetylation may affect the turnover of target proteins, we first determined whether reduced Nma1/Nma2 activities were due to decreased Nma1/Nma2 protein levels. As shown in Fig. 4C, Nma1 and Nma2 protein levels were indeed decreased in nat3Δ cells (Fig. 4C). In addition to Nma1 and Nma2, NAD⁺ homeostasis factors Bna2, Bna5 and Hst1 are also potential NatB targets. Bna2 and Bna5 are biosynthesis enzymes in the de novo pathway (Fig. 1A), and Hst1 represses the expression of BNA genes (41). Since blocking the de novo pathway does not affect NAD⁺ levels under standard growth conditions (NA rich media), the low NAD⁺ phenotype of nat3Δ mutant is unlikely due to reduced de novo activities. To determine de novo activities in nat3Δ cells, we developed a cell-based readout for QA production. In this assay, more QA release indicates increased de novo activities and vice versa. As expected, hst1Δ released more QA (Fig. 4D, right panel) due to de-repression of BNA genes, and deletions of BNA genes completely blocked QA release (data not shown). Similar to hst1Δ mutant, nat3Δ mutant also released more QA, and double deletions of NAT3 and HST1 did not further increase QA release (Fig. 4D). In comparison, hst1Δ cells did not release more NA/NAM (Fig. 4D, left panel) indicating that increased NA/NAM in nat3Δ mutant is not simply due to increased de novo activities. This is in line with our findings that in nat3Δ cells, increased NA/NAM arises from NR (Fig. 2E). Overall, these results demonstrate that the low NAD⁺ phenotype of nat3Δ cells is mainly due to reduced protein levels of Nma1 and Nma2. To confirm this, we examined whether over-expression of NMA1 is sufficient to reduce NA/NAM release. As shown in Fig. 4E (left panel), NMA1-oe completely blocked...
NA/NAM release in nat3Δ cells. Moreover, NMA1-oe also reduced QA release in nat3Δ cells (Fig. 4E, right panel). The reduction in QA and NA/NAM release is likely due to more efficient NA/NAM and QA assimilation by NMA1-oe. Supporting this, NMA1-oe was sufficient to restore the NAD⁺ levels in nat3Δ cells back to WT levels (Fig. 4F). Interestingly, NMA1-oe also increased NAD⁺ levels in WT cells. Next we examined whether Nma1 and Nma2 are N-terminal acetylated by NatB. Immunoprecipitated Nma1-HA protein complexes obtained from WT and nat3Δ cells were treated with chymotrypsin and then analyzed by tandem mass spectrometry. Both Nma1 and Nma2 N-terminal peptides were identified in WT and nat3Δ cells. As shown in Fig. 4G, among identified specific Nma1 and Nma2 N-terminal peptides from WT cells, all of them (66 for Nma1, 25 for Nma2) were acetylated on the first methionine. Conversely, identified Nma1 and Nma2 N-terminal peptides from nat3Δ cells (20 for Nma1, 10 for Nma2), only 1 Nma1 peptide showed acetylation (Fig. 4G). These results indicate that Nma1 and Nma2 are indeed N-terminal acetylated in a NatB dependent manner.

DISCUSSION

In this study we characterized NatB complex as a novel NAD⁺ homeostasis factor. Mutants lacking components of the NatB complex, NAT3 and MDM20, produce and release excess amount of NA and NAM. Our studies showed two pathways downstream of NatB contribute to NAD⁺ homeostasis (Fig. 5). In one, NatB is important for proper NAD⁺ biosynthesis by regulating Nma1/Nma2. NatB mutants have low NAD⁺ levels (Fig. 2C), and all NAD⁺ precursors examined failed to restore the NAD⁺ levels (Fig. 4A and 4B). This suggests that a NAD⁺ biosynthesis factor(s) required for utilization of all NAD⁺ precursors is defective in NatB mutants. Nma1 and Nma2 are such targets because they are the only factors required for all three NAD⁺ biosynthesis pathways (de novo, NA/NAM salvage, and NR salvage) (Fig. 1A), and the N-terminal amino acid sequences are a match for NatB acetylation. In addition, nma1Δ and nat3Δ mutants showed similar NAD⁺ utilization defects (Fig. 4B). Although Nma2 is present in both mutants, Nma2 is known to play a minor role in NAD⁺ metabolism. Supporting this model, decreased Nma1 protein level was observed in the nat3Δ mutant (Fig. 4C), and over-expressing NMA1 was sufficient to restore the NAD⁺ levels (Fig. 4F). Our studies showed that Nma1 and Nma2 are indeed acetylated at the N-terminus in a NatB dependent manner (Fig. 4G). Further studies are required to understand the mechanisms of NatB mediated regulation of Nma1/Nma2, but the decreased level of both proteins in nat3Δ suggests the nonacetylated counterparts may be degraded at a higher rate by the N-end rule pathway. The N-end rule pathway recognizes N-terminal degradation signals and marks proteins for degradation by specific E3 ligases. This pathway can be broken up into three branches called the Arg/N-rule pathway, Pro/N-rule pathway, and the Ac/N-rule pathway (42,43). The Ac/N-rule branch recognizes N-acetylated proteins. However, in the absence of acetylation, the proteins may be stabilized or targeted by other N-end rule branches, which can lead to different rates of degradation (42,44,45). On the other hand, N-acetylated
proteins may also bury their acetylated N-termini within physically associated proteins (46-48). This provides two important functions. First it shields the N-terminus from Ac/N-rule E3 ligases.Secondly, it stabilizes the protein complex. The absence of a normally present acetylated N-terminus can drastically decrease the affinity of a protein to complex with its partners (24,47), leaving it more dissociated and prone for targeted degradation (42). Therefore, it is possible that in addition to protein stabilization, N-terminal acetylation may also be important for Nma1 and Nma2 protein-protein interactions. Current research suggests Nma1 and Nma2 may function as tetramers (49-51).

In the other pathway, NatB and Tpm1 mediated proper vesicular trafficking appears to specifically affect NA/NAM homeostasis (Fig. 5). TPM1 encodes one of two tropomyosin proteins in yeast, which bind to actin filaments and stabilize actin cables. Acetylation of TPM1 by NatB greatly improves its affinity for actin filaments (24). During cell division and growth, actin cables provide tracks for organelle segregation and endocytic trafficking (35,38,52,53). Interestingly, although over-expression of TPM1 and expression of a dominant TPM1-5 completely blocked NA/NAM release (Fig. 3D), they failed to restore the NAD$^+$ levels in the NatB mutants. This is in line with the model that Nma1 and Nma2 abnormalities are responsible for observed NAD$^+$ defects in NatB mutants. NMA1 over-expression, however, was sufficient to block NA/NAM release (Fig. 4E) and restore NAD$^+$ level in NatB mutants (Fig. 4F). These results show that the two NatB controlled NAD$^+$ homeostasis pathways are interconnected. How may TPM1 and actin cytoskeleton affect NAD$^+$ homeostasis? It is possible NAD$^+$ and related precursors (free and protein-bound) are trafficked by means of vesicles, which would require Tpm1 and actin cables to mediate their directed movement (35,37). Deleting TPM1 alone abolishes the appearance of actin cables, whereas deleting TPM2 does not (36). Neither tpm1Δ nor tpm2Δ single mutant (tpm1Δtpm2Δ double mutant is lethal) appeared in our screen for high NA/NAM release mutants. It is likely because Tpm1 and Tpm2 have some functional redundancy (36,54), and they can substitute for each other when one is absent. During asymmetrical cell division, targeted and increased vesicular transport to daughter may concomitantly release NA/NAM and other NAD$^+$ intermediates. It is possible that unacetylated Tpm1 fails to efficiently assist the formation and delivery of endocytic vesicles, leading to increased NA/NAM release observed in the NatB mutants.

Our studies showed that increased NA/NAM in NatB mutants came from the NR pool (Fig. 2E). Increased NA/NAM release in NatB mutants was abolished by deleting cytosolic nucleosidases (URH1/PNP1), which convert NR to NAM. Increased NR mostly originates from the vacuolar pool since deleting vacuolar NR-producing phosphatase (PHO8) abolished NA/NAM increase whereas deleting cytosolic NR-producing nucleotidases (ISN1/SDT1) had a lesser effect (Fig. 2E, bottom panel). Vacuolar NR may exit the vacuole through Fun26 transporter (human lysosomal hENT homolog) and enters cytoplasm (17) where it is converted to NAM by nucleosidases. Indeed, deleting FUN26 blocked NAM release
in the NatB mutant (Fig. 2E, bottom panel). It remains unclear how NAD⁺ and its intermediates are transported into the vacuole. Our studies suggest autophagy may also play a role. Autophagy is a critical regulator of organelle homeostasis and recycling of macromolecules (55). It has been reported that NatB mutants failed to properly segregate mitochondria during cell division (mitochondrial inheritance defects) (23,24,28), which may lead to increased mitochondrial turnover by autophagy. Supporting this, many mutants we identified in our screen affect mitochondrial function (Supplemental Table 1). In addition, we showed that intracellular levels of both NA/NAM and NR were increased by starvation conditions known to induce autophagy (Fig. 3A). Finally, deleting ATG14, a key regulator of autophagic membrane tethering and fusion (56), blocked NAM release in nat3Δ mutant (Fig. 3B). Although TPM1-oe shared similar phenotypes with ATG14 deletion, it is unclear whether Tpm1 mediated vesicular trafficking and autophagy function in the same pathway to regulate NA/NAM homeostasis. Further evidence is required to detail the functional link between these factors.

It remains unclear why NatB mutants release more NA/NAM and not NR. Our studies show the NR pools in NatB mutants are quickly converted to NAM by cytoplasmic Urh1 and Pnp1 (Fig. 2E). In line with this, the NatB mutants were previously identified in a different screen as “low NR” mutants (17). Although Urh1 and Pnp1 are not predicted direct targets of NatB (23,25,26), their activities and localization could be indirectly affected by NatB mutations. Some NatB mutant phenotypes may also be due to aberrant energy homeostasis (57). Supporting this, the Glc7 phosphatase, which inhibits Snf1 (yeast AMPK) activity, is acetylated by NatB and that Snf1 activation was observed in nat3Δ cells (57). In addition, Snf1 activation was associated with Pnp1 activity in starvation induced ribonucleotide salvage (58). Another NAM metabolic factor that may contribute to increased NA/NAM release is Pnc1. Although Pnc1 levels and activities are only slightly decreased in NatB mutants (Fig. 2A and 2B), its localization may be altered in NatB mutants. Pnc1 has been shown to localize in peroxisomes in normal conditions. In response to nutrient starvation and stress, Pnc1 expression is induced and becomes ubiquitous (15,59). In addition, Pnc1 has been shown to be released extracellularly (60), and it is most likely retained in the periplasmic space (61). It is possible that during asymmetrical cell division, increased vesicular transport to daughter may concomitantly increase NAM release. Pnc1 may be released to convert NAM to NA to support NAD⁺ biosynthesis and/or eliminate NAM toxicity (62-64). In mammals, eNAMPT is also released extracellularly to help metabolize NAM (65), however yeast does not have NAMPT.

In summary, our studies have uncovered novel NAD⁺ homeostasis factors. It would be informative to further investigate the regulation of NAD⁺ homeostasis by NatB in future studies. These studies may also provide insights into the regulation of NAD⁺ metabolism and the molecular basis of disorders associated with aberrant NAD⁺ metabolism.

**EXPERIMENTAL PROCEDURES**
Yeast Strains, Growth Media and Plasmids—Yeast strain BY4742 \( \text{MATa his3A1 leu2A0 lys2A0 ura3A0} \) acquired from Open Biosystems (66) was used for this study. Standard media such as yeast extract-peptone-dextrose (YPD), synthetic minimal (SD), and synthetic complete (SC) media were made as described (67). Niacin-free SD and SC were made by using niacin-free yeast nitrogen base (Sunrise Science Products). Niacin (vitamin B3) comprises both NA and NAM. All gene deletions were generated by replacing wild type genes with reusable loxP-Kanr-loxP cassettes as described (68). Multiple deletions were carried out by removing the Kan\(^r\) marker using a galactose-inducible Cre recombinase (68). \( PNC1, NMA1, \) and \( NMA2 \) were tagged by the HA or EGFP epitope tag in the genome using the pFA6a-3HA-HIS3MX or pFA6a-link-yoEGFP-CaURA3 plasmid as previously described (69,70).

The \( NMA1 \) and \( TPM1 \) over-expression constructs, pADH1-NMA1 and pADH1-TPM1, were made in the integrative pPP81 (LEU2) vector as described (71). The resulting constructs were verified by DNA sequencing. \( TPM1-5 \) in pRS316 was a gift from Dr. B. Polevoda. \( TPM1-5 \) encodes an extended 7 amino acids at the N-terminus of \( TPM1 \) (23).

**NA/NAM cross-feeding spot assays**—The bna6\( \Delta \)nrk1\( \Delta \)nrt1\( \Delta \) mutant (utilizes both NA and NAM) and bna6\( \Delta \)nrk1\( \Delta \)nrt1\( \Delta \)pnc1\( \Delta \) mutant (only utilizes NA) were used as “recipient cells”. First, recipient cells were plated onto niacin-free SD (NA/NAM free) as a lawn (~10\(^4\) cells/cm\(^2\)). Next, 2 \( \mu l \) of each feeder cell strain (~10\(^4\) cells, cell suspension was made in sterile water at \( A_{600} \) of 2) was spotted onto the lawn of recipient cells. Plates were then incubated at 30°C for 3 days. Extent of the recipient cell growth indicates the levels of NA/NAM released by feeder cells.

**Genetic Screen Using the Yeast Deletion Collection**—The haploid yeast deletion collection (~4500 strains) established in the BY4742 background was acquired from Open Biosystems (72). To screen for mutants with increased NA/NAM release, 2 \( \mu l \) of each strain was directly taken from the frozen stock and then spotted onto niacin-free SD plates spread with the bna6\( \Delta \)nrk1\( \Delta \)nrt1\( \Delta \) recipient cells at a density of ~10\(^4\) cells/cm\(^2\). After incubation at 30°C for 3 days, we scored the cross-feeding activity (which indicates the level of NA/NAM release) of each mutant by comparing the diameter of the cross-feeding zones to that of the wild type. Mutants were assigned a score of 2 through 5 (WT=1). A higher score represents more NA/NAM release. To eliminate false-positives, mutants with a score of \( \geq 3 \) (81 mutants) were re-examined. Cells from the frozen stock were first recovered in fresh media for 2 days. 10\(^4\) cells of each strain were then spotted and scored as described above. Both the bna6\( \Delta \)nrk1\( \Delta \)nrt1\( \Delta \) mutant (utilizes both NA and NAM) and bna6\( \Delta \)nrk1\( \Delta \)nrt1\( \Delta \)pnc1\( \Delta \) mutant (only utilizes NA) were used as “recipient cells”. A total of 63 mutants passed the secondary screen and interestingly, most mutants release more NAM. Known NAD\(^+\) homeostasis factors were identified including npt1\( \Delta \) and pnc1\( \Delta \) (Supplemental Table 1).

**Measurement(s) of NAD\(^+\), NADH, NR, NA and NAM**—Total intracellular levels of NAD\(^+\) and NADH were determined using enzymatic
cycling reactions as described (71). NR levels were determined by a liquid-based cross-feeding bioassay (17,19). Relative NA/NAM levels were determined similarly as NR with some modifications. To prepare cell extracts for intracellular NA/NAM determination, approximately 2.5x10⁹ (~250 A₆₀₀ unit) of cells (donors of interest) grown to late log-phase (~16 hr growth from an A₆₀₀ of 0.1) were lysed by bead-beating (Biospec Products) in 800 μl ice-cold 50 mM ammonium acetate solution. After filter sterilization, 50 to 200 μl of clear extract was used to supplement 8 ml cultures of recipient cells (the NA/NAM dependent bna6Δnrk1Δnrt1Δ mutant) with starting A₆₀₀ of 0.05 in niacin-free SC. To determine extracellular NA/NAM levels, supernatant of donor cell culture was collected, filter-sterilized, and then 4 ml was added to 4 ml of recipient cell culture with total starting A₆₀₀ of 0.05 in 2X niacin-free SC. A control culture of recipient cells in niacin-free SC without supplementation was included in all experiments. After incubation at 30°C for 24 hours, growth of the recipient cells (A₆₀₀) was measured and normalized to the cell number of each donor strain. A₆₀₀ readings were then converted to NA/NAM concentrations using the standard curve established as previously described (19).

Deamidase Activity Assay–About 300 A₆₀₀ unit cells grown overnight in YPD were harvested and cell lysate was obtained by beads beating in breaking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, Roche protease inhibitors). Cell lysate containing 70 μg of total cellular proteins was added to 300 μL deamidase reaction mix (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM MgCl₂) (15,73) using 8 mM of NAD⁺, NMN, NmR or Nam as substrates followed by incubation at 30°C for 45 min. 100 μL of the deamidase reaction mix was then added to 1 mL of ammonia assay mix (3.4 mM α-ketoglutarate, 0.23 mM NADPH, 50 mM phosphate buffer, pH 7.4, 10 units of glutamate dehydrogenase) followed by reaction at room temperature for 15 min (15,73). The amount of ammonia was calculated by the decrease in absorbance at 340 nm using standard curve derived from the ammonia standard solutions (Sigma).

Repressible Acid Phosphatase (rAPase) Activity Assay–The rAPase liquid assay was performed as described (74) on cells grown to late log phase in SDC. In brief, ~ 2.5 X 10⁷ cells were harvested, washed and resuspended in 150 μL water. 600 μL of substrate solution (5.6 mg/ml p-nitrophenylphosphate in 0.1 M sodium acetate, pH 4) was added to cell suspension, and the mixture was incubated at 30°C for 15 min. The reaction was stopped by adding 600 μL ice-cold 10% trichloroacetic acid. 600 μL of this final mixture was then added to 600 μL saturated Na₂CO₃ to allow color (neon yellow) development. Cells were pelleted to acquire the supernatant for A₄₂₀ determination. The rAPase activities were determined by normalizing A₄₂₀ readings to total cell number (A₆₀₀).

Nitrogen starvation–We followed previous nitrogen starvation regimens that were shown to induce autophagy in yeast cells with some modifications (31,33,34). 8 mL of cells grown in SC overnight were harvested by centrifugation, washed twice before inoculating into 250 mL SC –niacin at A₆₀₀ of 0.2. SC –niacin was used to reduce the interference of extracellular niacin (NA/NAM) while determining intracellular
NA/NAM. After 6 hours at 30˚C, 2x100 mL cells were collected, and washed twice in water. One tube of washed cells were subject to lysate collection (Control, 0 hr) for NA/NAM and NR measurements. The other tube of washed cells were inoculated into 250 mL SC –niacin –nitrogen (without ammonium sulfate and amino acids) and allowed to grow for 4 hours (N-starvation, 4 hrs) before lysate collection for NA/NAM and NR measurements. SC –niacin and SC –niacin –nitrogen were made with special yeast nitrogen base from Sunrise Science Products.

Protein Extraction and Western Blot Analysis—A total of 100 mL cells were grown in SC or SD to mid-logarithmic phase (A_600 of 1-2), concentrated by centrifugation. Total protein extract was from cell lysate was obtained by beads beating in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% NP-40, and Roche cOmplete Mini protease inhibitor cocktail). 30 or 70 µg of total protein was loaded in each lane. After electrophoresis, the protein was transferred to nitrocellulose membrane (Whatman). The membranes were then washed, blotted with either anti-HA antibody (Pierce) or anti-actin antibody (Abcam). Protein was visualized using anti-mouse (GE) or rabbit (Pierce) antibody conjugate to the horseradish peroxidase and the ECL-reagents (GE). The chemiluminescent image was analyzed using the Alpha Innotech imaging system and software provided by the manufacturer.

Immunoprecipitation and Analysis of N-terminal acetylation of Nma1 and Nma2—About 500 mL of WT and nat3Δ cells carrying HA-tagged Nma1 grown to A_600 of 1-2 were collected by centrifugation and washed twice in PBS. Cell lysate was obtained by beads beating in IP lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 3 mM EDTA, 5% glycerol, and protease inhibitor cocktail). Lysate was cleared at 100,000 g for 1.5 hours. IP was carried out using 1.8 mL of lysate at 2.8 mg/mL with 75 µL of PierceTM anti-HA Magnetic Beads. The magnetic beads were washed 5 times with end-over-end rotation: Wash 1 (20 minutes) in lysis buffer, wash 2 to 4 (15 minutes) in TBS with 0.5% Tween-20, and wash 5 with PBS. The magnetic beads were then washed 3 more times in 300 µL 50 mM ammonium bicarbonate with end-over-end rotation for 20 minutes. The magnetic beads were then brought up in 300 µL 50 mM ammonium bicarbonate for on-bead digestion, and 2.5 µL of 250 ng/µL PierceTM Chymotrypsin Protease (90056) was added. Digest was left at room temperature overnight with end-over-end rotation. Next day the supernatant was separated from the magnetic beads and collected using a magnetic stand. The magnetic beads were washed with 100 µL 50 mM ammonium bicarbonate for 20 minutes, and the supernatant was pooled. Samples were immediately sent to the UC Davis proteomics core for tandem mass spectrometry analysis (LC-MS/MS). Digested peptides were analyzed by LC-MS/MS on a Thermo Scientific Q Exactive Plus Orbitrap Mass spectrometer in conjunction with Proxeon Easy-nLC II HPLC (Thermo Scientific) and Proxeon nanospray source. The digested peptides were loaded a 100 micron x 25 mm Magic C18 100Å 5U reverse phase trap where they were desalted online before being separated using a 75 micron x 150 mm Magic C18 200Å 3U reverse phase column. Peptides were eluted using a 60 minute
gradient with a flow rate of 300 nL/min. An MS survey scan was obtained for the m/z range 350-1600, MS/MS spectra were acquired using a top 15 method, where the top 15 ions in the MS spectra were subjected to HCD (High Energy Collisional Dissociation). An isolation mass window of 1.6 m/z was for the precursor ion selection, and normalized collision energy of 27% was used for fragmentation. A fifteen second duration was used for the dynamic exclusion. The total number of identified Nma1 and Nma1 specific peptide sequences (peptide spectrum matches, PSM) are determined using the Byonic software (75) with 1% max spectra decoy FDR (false discovery rate). The Skyline software (76) was used to calculate the peak area intensity of the acetylated N-terminal peptides identified by Byonic. Percentage of N-terminal acetylated Nma1 and Nma2 peptides shown in Figure 4G were calculated from two independent experiments using the Scaffold software with peptide threshold set at 1% FDR.

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Conflict of interest statement—The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions—TC designed and performed the experiments. CJTR performed experiments shown in Figures 2A, 2B, 4D and 4E. MS and BSP performed Mass Spectrometry studies and data analysis shown in Figure 4G. SJL helped conceive the experiments, analyzed the data, and co-wrote the manuscript with TC. All authors approved the final version of the manuscript.
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FIGURE LEGENDS

FIGURE 1. Identification of yeast mutants with altered NA and NAM homeostasis. A, a simplified model of *Saccharomyces cerevisiae* NAD\(^+\) synthesis pathway. NAD\(^+\) can be synthesized *de novo* from tryptophan (Trp), and by salvaging NAD\(^+\) intermediates through the NA/NAM and NR cycles. NA, nicotinic acid. NAM, nicotinamide. QA, quinolinic acid. NR, nicotinamide riboside. NaMN, nicotinic acid mononucleotide. NaAD, deamido NAD\(^+\). NMN, nicotinamide mononucleotide. Abbreviations of protein names are shown in parentheses. B, deletions of *NPT1* and *PNC1* confer increased NA/NAM and NAM release, respectively. NA/NAM dependent recipient cells were plated onto niacin-free SD plates as a lawn. Next, WT, *npt1*Δ and *pnc1*Δ feeder cells were spotted on top of the lawn and allowed to grow for 3-4 days at 30˚C. Recipient cells on the top panel (*bna6*Δ*nrk1*Δ*nrt1*Δ) can utilize both NA and NAM whereas recipient cells on the bottom panel (*bna6*Δ*nrk1*Δ*nrt1*Δ*pnc1*Δ) can only utilize NA. The number and size of the satellite colonies surrounding the feeder spots positively correlate with the amount of NA/NAM released from the feeders. C, overview of the genetic screen to identify mutants with increased NA/NAM release. Haploid single deletion mutants (feeder cells) were spotted onto a lawn of NA/NAM dependent *bna6*Δ*nrk1*Δ*nrt1*Δ recipient cells, whose growth is dependent on NA/NAM released from the feeder cells in a dose-dependent manner. D, deletions of *NAT3* and *MDM20* confer increased NAM release. Various feeder strains were spotted onto a lawn of *bna6*Δ*nrk1*Δ*nrt1*Δ (top panel) and *bna6*Δ*nrk1*Δ*nrt1*Δ*pnc1*Δ (bottom panel) recipient cells. For clarity, inverse images are shown. E, The *nat3*Δ and *mdm20*Δ mutants show significant increases in both released and intracellular NA/NAM levels. NA/NAM levels were determined in both growth media (released) and cell extracts (intracellular). Data shown are representative of three independent experiments. Feeding of collected lysate to recipient cells was conducted in triplicate. The error bars denote standard deviations. The *p* values are calculated using Student’s *t* test (*, p < 0.05).

FIGURE 2. Characterizations of NAD\(^+\) homeostasis factors in NatB mutants. A, Pnc1 protein levels in WT and *nat3*Δ cells. Pnc1 level is slightly decreased in *nat3*Δ mutant during late log phase (13 hr). B, relative Pnc1 deamidase activities determined in WT and *nat3*Δ cell lysates. Pnc1 activity is slightly decreased in *nat3*Δ cells during late log phase (13 hr). C, measurements of intracellular NAD\(^+\) levels. Deleting *NAT3* significantly decreases NAD\(^+\) levels. D, relative repressible acid phosphatase (*rAPase*) activities in WT and *nat3*Δ cells. Increased *rAPase* activity in *nat3*Δ cells indicates increased PHO signaling activity (left panel). Deleting *PHO4* does not abolish *rAPase* activity whereas deleting *PHO5* largely diminishes the *rAPase* activity (left panel). Deleting *PHO4* does not block NA/NAM release in *nat3*Δ cells (right panel). Data shown are representative of three independent experiments. E, NR salvage contributes to increased NA/NAM production in NatB mutants. Intracellular compartmentalization of NR salvage factors (top panel). Deleting cytosolic nucleosidases *URH1* and *PNP1* (convert NR to NAM), diminishes NA/NAM release in *nat3*Δ and
mdm20Δ cells (bottom panel). Deleting vacuolar NR producing factor Pho8 also diminishes NA/NAM release, whereas deleting the cytosolic NR producing factors Isn1 and Sdt1 has a lesser effect (bottom panel). Deleting vacuolar transporter FUN26 also abolishes NA/NAM release in nat3Δ cells. This suggests increased NAM in NatB mutants comes from vacuolar NR, which is converted to NAM by Uhr1 and Pnp1 (top panel). F, intracellular NR (top left panel) and NA/NAM (top right panel) levels are increased in nat3Δ mutant. Deleting URH1 and PNP1 diminishes NA/NAM production (middle right panel) with a commitment increase of intracellular NR level (middle left panel). Deleting PHO8 diminishes both NR and NA/NAM levels (bottom panels). For B, C, D and F, the error bars denote standard deviations of triplicated samples. The p values are calculated using Student’s t test (*, p < 0.05).

FIGURE 3. Blocking NA/NAM production or release is not sufficient to restore NAD⁺ levels in the NatB mutants. A, nitrogen starvation (N-starv) increases NR (left panel) and NA/NAM (right panel) production, which are diminished by deleting ATG14, an autophagy-specific PI 3-kinase subunit. B, increased NA/NAM levels in nat3Δ cells are diminished by deleting ATG14. C, blocking NA/NAM production by deleting ATG14, FUN26, PHO8, URH1 and PNP1 is not sufficient to restore NAD⁺ levels in nat3Δ cells. D, TPM1 over-expression (TPM1-oe) and expression of a gain-of-function allele of TPM1 (TPM1-5) diminish NA/NAM release in nat3Δ cells. E, TPM1-oe and expression of TPM1-5 are not sufficient to restore NAD⁺ levels in nat3Δ cells. Data shown are representative of three independent experiments. For A, C and E, the error bars denote standard deviations of triplicated samples. The p values are calculated using Student’s t test (*, p < 0.05).

FIGURE 4. NMNATs are responsible for the NAD⁺ decrease in NatB mutants. A, supplementing NAD⁺ precursors (NA, NAM, NR QA) does not restore NAD⁺ levels in the nat3Δ mutants. B, nat3Δ and nma1Δ mutants share similar low NAD⁺ levels, which cannot be rescued by NR supplementation. As a control, NR is able to rescue the low NAD⁺ level in npt1Δ cells. C, the levels of NMNATs proteins (Nma1 and Nma2) are decreased in nat3Δ cells. D, both nat3Δ and hst1Δ cells show increased QA release, which is indicative of increased de novo activities (right panel). Unlike nat3Δ cells, hst1Δ cells do not show increased NA/NAM release (left panel). These suggest that increased NA/NAM in nat3Δ cells is not simply due to increased de novo activity. The npt1Δnrk1Δbna4Δ mutant was used as the recipient cells for determining QA release (QA-dependent growth). E, NMA1 overexpression (Nma1-oe) diminishes both NA/NAM and QA release in nat3Δ cells. F, NMA1-oe restores the NAD⁺ levels in nat3Δ cells. Data shown are representative of three independent experiments. G, among specific Nma1 and Nma2 N-terminal peptides identified from WT cells, 100% are acetylated at the first methionine. Acetylations are mostly absent among specific Nma1 and Nma2 N-terminal peptides identified in nat3Δ mutant, indicating Nma1 and Nma2 are N-terminal acetylated in a NAT3 (NatB) dependent manner. For A, B, F and G, the error bars denote standard deviations of duplicated samples. The p values are calculated using Student’s t test (*, p < 0.05).
FIGURE 5. A proposed model depicting factors contributing to altered NAD$^+$ homeostasis in NatB mutants. We identified Nma1 and Nma2 as the acetylation targets of NatB. NatB regulates NAD$^+$ biosynthesis and NAD$^+$ intermediate trafficking via Nma1/Nma2 and Tpm1, respectively. Dashed line indicates the mechanism of this pathway remains largely unclear.
A. Trp 

\(\text{Trp} \rightarrow \text{de novo} \rightarrow \text{Npt1} \rightarrow \text{NAM} \rightarrow \text{NR} \)

\(\text{NAM} \rightarrow \text{NR Salvage} \rightarrow \text{NR} \rightarrow \text{Salvage} \rightarrow \text{NA/NAM} \rightarrow \text{NaAD} \)

B. Feeder cell spots

WT pnc1 Δ

∆ NA/NAM release

WT npt1 Δ

∆ NA release

C. Increased NA/NAM release

WT nat3 Δ mdm20 Δ

(~ 4500 yeast deletion mutants)

lawn of recipient cells

D. Intracellular

WT npt1 Δ nat3 Δ mdm20 Δ

NA/NAM release

E. Released

WT npt1 Δ nat3 Δ mdm20 Δ

NA/NAM levels (10^-6 pmole/cell)

*
A. 

[Graph showing NR levels in WT and atg14Δ with error bars and asterisks for statistical significance.]

B. 

[Image showing NA/NAM release from WT and nat3Δ.]

C. 

[Bar graph comparing NAD+ and NADH levels in WT, nat3Δ, atg14Δ, and nat3Δ atg14Δ with error bars and asterisks for statistical significance.]

D. 

[Western blot showing NA/NAM release from nat3Δ and nat3Δ+TPM1-oe.]

E. 

[Bar graph showing NAD+ and NADH levels in WT, nat3Δ, WT+TPM1-oe, nat3Δ+TPM1-oe, WT+TPM1-5, nat3Δ+TPM1-5 with error bars and asterisks for statistical significance.]
A. NAD⁺ and NADH levels (10⁻⁸ mole/cell)

B. NAD⁺ and NADH levels (10⁻⁸ mole/cell)

C. Nma1-HA Nma2-HA

anti-HA anti-Actin

D. NA/NAM release QA release

E. WT nat3Δ WT nat3Δ

WT+ NMA1-oe WT+ NMA1-oe

WT+ NMA1-oe nat3Δ+ NMA1-oe

F. NAD⁺ NADH

G. % of N-terminal acetylation

Nma1: Ac-MDPTRAPDFKPPSADEELIPPDPSKIPKKSIPY

Nma2: Ac-MDPTKAPFQQPNEELQPPPDTHTIPKSGPVPY
Proper NAD⁺ homeostasis

Proper trafficking/turnover of NAD⁺ intermediates

NAD⁺ ↔ NA/NAM

NAD⁺ biosynthesis

Nma1/2

NatB

Tpm1
A Functional Link Between NAD$^+$ Homeostasis and N-terminal Protein Acetylation in *Saccharomyces cerevisiae*

Trevor Croft, Christol James Theoga Raj, Michelle Salemi, Brett S Phinney and Su-Ju Lin

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