The histone deacetylases Rpd3 and Hst1 antagonistically regulate \textit{de novo} NAD\textsuperscript{+} metabolism in the budding yeast \textit{Saccharomyces cerevisiae}

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NAD\textsuperscript{+} is a cellular redox cofactor involved in many essential processes. The regulation of NAD\textsuperscript{+} metabolism and the signaling networks reciprocally interacting with NAD\textsuperscript{+}-producing metabolic pathways are not yet fully understood. The NAD\textsuperscript{+}-dependent histone deacetylase (HDAC) Hst1 has been shown to inhibit \textit{de novo} NAD\textsuperscript{+} synthesis by repressing biosynthesis of nicotinic acid (BNA) gene expression. Here, we alternatively identify HDAC Rpd3 as a positive regulator of \textit{de novo} NAD\textsuperscript{+} metabolism in the budding yeast \textit{Saccharomyces cerevisiae}. We reveal that deletion of \textit{RPD3} causes marked decreases in the production of \textit{de novo} pathway metabolites, in direct contrast to deletion of \textit{HST1}. We determined the BNA expression profiles of \textit{rpd3Δ} and \textit{hst1Δ} cells to be similarly opposed, suggesting the two HDACs may regulate the \textit{BNA} genes in an antagonistic fashion. Our chromatin immunoprecipitation analysis revealed that Rpd3 and Hst1 mutually influence each other’s binding distribution at the \textit{BNA2} promoter. We demonstrate Hst1 to be the main deacetylase active at the \textit{BNA2} promoter, with \textit{hst1Δ} cells displaying increased acetylation of the N-terminal tail lysine residues of histone H4, H4K5, and H4K12. Conversely, we show that deletion of \textit{RPD3} reduces the acetylation of these residues in an Hst1-dependent manner. This suggests that Rpd3 may function to oppose spreading of Hst1-dependent heterochromatin and represents a unique form of antagonism between HDACs in regulating gene expression. Moreover, we found that Rpd3 and Hst1 also coregulate additional targets involved in other branches of NAD\textsuperscript{+} metabolism. These findings help elucidate the complex interconnections involved in effecting the regulation of NAD\textsuperscript{+} metabolism.

NAD\textsuperscript{+} is a met abolite with crucial roles in a variety of cellular processes. It is involved in the oxidative steps of glycolysis and in mitochondrial energy production as a redox cofactor, in epigenetic regulation as a cosubstrate for class III histone deacetylases (HDACs), also known as sirtuins (1–3), and in DNA repair as a substrate for poly-ADP-ribose polymerases (4). As such, defects of NAD\textsuperscript{+} metabolism are associated with a broad range of disorders, such as diabetes, Alzheimer’s disease, and various cancers (5–23).

NAD\textsuperscript{+} may be produced by three major pathways: (1) \textit{de novo} biosynthesis from L-tryptophan (TRP), (2) salvage of nicotinic acid (NA) and nicotinamide (NAM), and (3) salvage of nicotinamide riboside (NR) (Fig. 1A). These pathways are largely conserved, with a few species-specific differences, and consume cellular pools of ATP, phosphoribosyl pyrophosphate, and glutamine (24, 25). The \textit{de novo} pathway is also known as the kynurenine (KYN) pathway of TRP degradation. In yeast, this pathway is characterized by the synthesis of quinolinic acid (QA) from TRP by five enzymatic reactions mediated by the Bna (biosynthesis of nicotinic acid) proteins (Bna2, Bna7, Bna4, Bna5, and Bna1) and a spontaneous cyclization (26). Bna6 then converts QA into nicotinic acid mononucleotide (NaMN), which is also produced by the NA–NAM salvage branch (Fig. 1A). Under standard NA-abundant growth conditions, NA–NAM salvage is the preferred NAD\textsuperscript{+} biosynthesis route (27). NAM can come from NAD\textsuperscript{+}-consuming reactions including sirtuin-mediated protein deacetylation (1–3). NAM can be converted to NA by a nicotinamide phosphoribosyltransferase (Pnc1) (28), leading to NaMN production by Npt1 (Fig. 1A). Although human cells do not have Pnc1-like nicotinamidases, recent studies suggest that NAM salvage to NA may also take place in humans with the aid of gut bacterial nicotinamidases (29). In the NR salvage branch, NR can enter the NA–NAM salvage branch when converted to NAM by nucleotidases Urh1 and Pnp1 (30, 31). NR can also be converted to nicotinamide mononucleotide (NMN) by the NR kinase, Nrk1 (32). NMN adenylyltransferases (Nma1, Nma2, and Pof1 in yeast) are responsible for the conversion of NMN to NAD\textsuperscript{+} (33–35). Nma1 and Nma2 also convert NaMN to nicotinic acid adenine dinucleotide (33, 34), which is converted to NAD\textsuperscript{+} by the glutamine-dependent NAD\textsuperscript{+} synthetase Qns1 (36). Under NAD\textsuperscript{+}-repleted conditions, the \textit{de novo} pathway BNA genes are repressed by the NAD\textsuperscript{+}-dependent sirtuin Hst1 (37, 38). Conversely, NAD\textsuperscript{+} depletion results in decreased Hst1 activity leading to transcription activation of the BNA genes. Yeast cells also release and reuptake small NAD\textsuperscript{+} precursors, such as NA, NAM, QA, and NR (Fig. 1A) (35, 38–41). The
Figure 1. Cells lacking RPD3 are deficient for de novo QA production. A, model of the NAD⁺ biosynthetic pathways in *Saccharomyces cerevisiae*. De novo NAD⁺ metabolism begins with TRP, which is converted into NaMN by the Bna enzymes (Bna2, Bna7, Bna4, Bna5, Bna1, and Bna6) (left). NaMN is also produced by salvage of NA and NAM, which is further connected with salvage of NR (right). NR is metabolized to NMN by Nrk1, which is then converted to NAD⁺ by Nma1, Nma2, and Pof1. Abbreviations of NAD⁺ intermediates are shown in bold and italicized. 3-HA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; ACMS, 2-amino-3-carboximuconate-6-semialdehyde; KA, kynurenic acid; KYN, kynurenine; NA, nicotinic acid; NaAD, deamido-NAD⁺; NAM, nicotinamide; NaMN, nicotinic acid mononucleotide; NFK, N-formylkynurenine; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; QA, quinolinic acid; TRP, L-tryptophan. Abbreviations of protein names are shown in ovals. Aro9/Aro8 and Bna3, kynurenine aminotransferase; Bna1, 3-hydroxyanthranilic acid 3,4-dioxygenase; Bna2, tryptophan 2,3-dioxygenase; Bna4, kynurenine 3-monoxygenase; Bna5, kynureninase; Bna6, quinolinic acid phosphoribosyl transferase; Bna7, kynurenine formamidase; Nma1/2, NaMN/NMN adenylyltransferase (NMNAT); Npt1, nicotinic acid phosphoribosyl transferase; Pof1, NMN adenylyltransferase (NMNAT); Pnc1, nicotinamidase; Qns1, glutamine-dependent NAD⁺ synthetase; Sir2 family, NAD⁺-dependent Epigenetic antagonism governs de novo NAD⁺ metabolism.

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mechanisms of precursor release remain unclear, and it is suggested that vesicular trafficking may play a role (24, 39, 42). Transport of NAD⁺ precursors into yeast cells is mediated by specific transporters Tna1 (for NA and QA) (40, 43) and Nrt1 (for NR) (44).

Supplementation of precursors in each pathway has been shown to produce positive outcomes in some disease models (6, 20). However, given the complexity of NAD⁺ metabolism and its interaction with other signaling networks, it is often necessary to understand the pathways affected in each particular disease state in order to derive the greatest advantage from treatment and determine the most useful precursor for each case. This represents one major benefit that may be won by the detailed study of the molecular mechanisms governing the regulation of each level of NAD⁺ metabolism. In addition to being crucial for the maintenance of cellular NAD⁺ pools, the intermediates of these pathways often have multifarious other roles (12, 42, 45). For instance, intermediates of de novo metabolism have been associated with both beneficial and adverse influences on neurological health (16, 46, 47) and have also been shown to interact with immune signaling (16, 46–49). Indeed, de novo metabolism, despite being a relatively minor contributor to the NAD⁺ pool compared with salvage of NA, NAM, and NR, is increasingly recognized as an important element of NAD⁺ metabolism with a variety of far-ranging influences on cellular health (47, 50).

The regulation of the de novo pathway of NAD⁺ biosynthesis is still incompletely understood. Previous studies in yeast have shown the sirtuin Hst1 (37) and the copper-sensing transcription factor Mac1 (38) to be negative regulators of de novo metabolism, whereas a complex of Bas1 and Pho2 transcriptionally activates de novo metabolism under conditions of adenine depletion (25). In addition, a fragment of the Huntington protein was shown to activate production of several de novo intermediates, an effect that is ameliorated by the inhibition of class I HDAC Rpd3 (51). Deletion of RPD3 has also been shown to reduce expression of BNA1 (52). In this study, we identified Rpd3 as a positive regulator of the majority of the BNA genes of de novo NAD⁺ biosynthesis, the deletion of which results in markedly diminished production of several de novo pathway metabolites. In addition, we characterized the interaction of Rpd3 with the NAD⁺-dependent Hst1 in the regulation of de novo NAD⁺ metabolism. This work helps to elaborate the mechanism by which BNA expression is regulated and to clarify the connection of de novo NAD⁺ metabolism to other branches of metabolism and signaling in the cell.

### Results

**The HDAC Rpd3 is a positive regulator of QA production**

The signaling pathways that regulate NAD⁺ metabolism remain unclear in part because of the dynamic nature and complexity of NAD⁺ synthesis pathways. Making use of the tendency of yeast cells to constantly release and retrieve small NAD⁺ precursors (35, 39–41), we developed genetic screens to identify and study novel NAD⁺ homeostasis factors. The rpd3Δ mutant was identified in a screen for mutants that showed altered QA release using a “cross-feeding” assay as described previously (38). As illustrated in Figure 1, B, a lawn of QA-dependent “recipient cells” (bna4Δnpt1Δnurk1Δ) is first spread onto an agar plate. These cells cannot grow because they are dependent on exogenous QA for NAD⁺ synthesis, whereas standard growth medium does not contain QA. Next, “feeder cells” (strains of interest) are spotted onto the lawn of “recipient cells.” In this manner, the “feeder cells” release QA to support the growth of “recipient cells” by crossfeeding. This assay determines relative levels of total QA produced and released by “feeder cells” and is considered as a readout for de novo pathway activity (Fig. 1B). The rpd3Δ mutant caught our attention because it did not appear to release QA when compared with WT cells (Fig. 1C). This is to our surprise, because we previously reported that cells lacking another HDAC, Hst1, exhibited the opposite phenotype (38) (Fig. 1C).

Interestingly, the hst1Δrpd3Δ double mutant showed increased QA release, closely matching those seen for the hst1Δ single mutant (Fig. 1C), suggesting Hst1 may function downstream of Rpd3. Next, we examined whether the QA cross-feeding deficiency observed in the rpd3Δ mutant (Fig. 1C) was due to decreased QA production or altered QA transport. To answer this question, we determined the QA content in the cell lysate and growth medium using quantitative liquid assays. Our results showed that the rpd3Δ mutant is likely defective in QA production, because it has a significant reduction in both extracellular (Fig. 1D) and intracellular QA pools (Fig. 1E). Interestingly, despite releasing a significantly higher amount of QA (Fig. 1, C and D), the hst1Δ and hst1Δrpd3Δ mutants appeared to have slightly lower intracellular QA levels (Fig. 1E). This is in line with our previous report that yeast cells seem to maintain low intracellular QA levels and that excess QA is either released extracellularly or converted to NAD⁺ (38). QA accumulation may be detrimental to cells; for example, it has been linked with the production of reactive oxygen species (16). However, the factors leading hst1Δ and hst1Δrpd3Δ cells to store less QA remain unclear.
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Cells lacking Rpd3 also show altered NA–NAM salvage, NR salvage, and de novo activities

Next, we examined whether Rpd3 also plays a role in regulating the other two branches of NAD⁺ metabolism, and whether there is an interaction between Rpd3 and Hst1 for this regulation as observed in the regulation of de novo QA production. As shown in Figure 2, A, the hst1Δ and hst1Δrpd3Δ mutants showed increased NA–NAM release, whereas the rpd3Δ single mutant did not. This suggests that Rpd3 plays a role in NA–NAM salvage and that Hst1 may function downstream of Rpd3. Note that WT cells did not show visible NA–NAM release (Fig. 2A), and therefore, it was unclear whether the rpd3Δ mutant has reduced or unchanged NA–NAM production. This observation is consistent with a previous report that the majority of NA–NAM is stored intracellularly (39). To address this question, we determined intracellular NA–NAM levels and were able to reduce a selection in NA–NAM levels in rpd3Δ cells (Fig. 2B). Conversely, release of NR is increased in rpd3Δ cells in comparison with WT, whereas little difference is evident in both hst1Δ and hst1Δrpd3Δ cells (Fig. 2C). Interestingly, intracellular NR levels were raised not only in rpd3Δ but also in hst1Δ cells and, to a remarkably high degree, hst1Δrpd3Δ cells (Fig. 2D). Although both the hst1Δ and rpd3Δ mutants showed increased NR production (Fig. 2D), increased NR release was only observed in the rpd3Δ but not in the hst1Δ mutants (Fig. 2C). This is likely because of an increase in NR import in the hst1Δ mutants since the expression of NR transporter, Nrt1, is increased in these cells (38). These results also suggest some degree of synergy between Rpd3 and Hst1 in the regulation of the NR salvage pathway.

We next investigated the influence of rpd3Δ and hst1Δ on cellular NAD⁺ pools. As shown in Figure 2, E, a moderate, yet significant, reduction in NAD⁺ levels was observed in both rpd3Δ and hst1Δrpd3Δ mutants (Fig. 2E) when cells were grown in standard synthetic complete (SC) media. It has been suggested that NA–NAM salvage is the favored route for NAD⁺ synthesis when NA is present (27) (which is the case for all standard growth media). Therefore, observed NAD⁺ defects in rpd3Δ cells may be due to deficient NA–NAM salvage (37, 38). To directly examine whether the rpd3Δ mutant is defective in de novo NAD⁺ synthesis, cells were cultivated in medium lacking NA (NA-free SC), removing exogenous precursors for the salvage pathways and isolating de novo metabolism for analysis. As shown in Figure 2, F, decreased NAD⁺ levels were observed in rpd3Δ cells (Fig. 2F), which was in line with the defects in QA production exhibited by these cells (Fig. 1, C and D). Conversely, NAD⁺ levels were raised to a similar degree in both hst1Δ and hst1Δrpd3Δ cells, in alignment with the increased QA production observed in both strains (Fig. 1D). This also suggests that the reduced levels of intracellular QA observed in these two strains (Fig. 1E) may be due to more efficient NAD⁺ synthesis.

In order to determine whether the defects seen in QA and NAD⁺ production were reflective of a general dysfunction in de novo NAD⁺ metabolism (Fig. 1A), we analyzed several intermediates of the pathway present in the cell lysate by mass spectrometry (MS). In accordance with expectations, rpd3Δ cells displayed increased TRP levels, whereas hst1Δ and hst1Δrpd3Δ cells showed reduced accumulation of TRP (Fig. 3A). This suggests that rpd3Δ cells are deficient for TRP utilization via the de novo NAD⁺ biosynthesis pathway, whereas hst1Δ and hst1Δrpd3Δ cells consume more TRP because of increased de novo pathway activity. Indeed, rpd3Δ cells showed reduced levels of KYN (Fig. 3B), 3-hydroxykynurenine (3-HK) (Fig. 3C), and 3-hydroxyanthranilic acid (3-HA) (Fig. 3D). Although hst1Δ cells showed reduced levels of KYN, an early intermediate of de novo NAD⁺ metabolism, both hst1Δ and hst1Δrpd3Δ cells exhibited increased levels of the downstream intermediates 3-HK and 3-HA, likely because of the rapid flux of de novo metabolism expected for cells lacking Hst1 (38). Because of the extremely low concentration of QA stored in the cell (Fig. 1E) (38), we were unable to detect QA in the cell lysate by MS using same standard conditions. Since most excess QA is released extracellularly, the QA cross-feeding plate assay appears to be a convenient readout for released QA. Moreover, the plate assay measures QA accumulation in the growth media during the entire time course of recipient cell growth, and therefore, the readout signal may be amplified. MS analysis also confirmed increased intracellular QA accumulation in hst1Δ and hst1Δrpd3Δ cells (Fig. 3E), suggested by the cross-feeding studies of NA–NAM (Fig. 2, A and B).

Rpd3 is important for optimal activation of the BNA genes in de novo metabolism

To further study the role of Rpd3 in NAD⁺ metabolism, we first asked whether the defects in de novo NAD⁺ metabolism shown in rpd3Δ cells were due to dysregulation of the BNA gene expression (Fig. 1A). As expected, rpd3Δ cells showed strongly reduced expression of most of the BNA genes involved in de novo metabolism (Fig. 4A). BNA7 appears to be insensitive to the factors affecting the other BNA genes (38), whereas BNA3 is not strictly a part of the course of the de novo pathway (Fig. 1A) (53); hence, the two were not investigated. Consistent with previous studies (37, 38), hst1Δ cells exhibited markedly increased BNA expression, whereas hst1Δrpd3Δ cells showed an expression phenotype most closely resembling hst1Δ, yet in most cases slightly but significantly below the levels seen in hst1Δ alone (Fig. 4A). The ability of hst1Δrpd3Δ cells to override the expression deficits observed in rpd3Δ3A cells suggests that Hst1 functions downstream of Rpd3 and that Rpd3 promotes transcription possibly by opposing the repressive activity of Hst1 on the BNA gene promoters. However, additional factors are likely involved, as BNA gene expression in hst1Δrpd3Δ cells was not fully restored to level observed in hst1Δ cells (Fig. 4A). We further confirmed that observed BNA expression changes were reflective of Bna protein expression. As shown in Figure 4, B, significant decreases of the three Bna proteins examined (Bna1, Bna2, and Bna5) were observed in rpd3Δ cells, whereas significant increases of these proteins were observed in hst1Δ and hst1Δrpd3Δ cells.
Having shown Rpd3 to be a positive regulator of BNA expression, we then sought to investigate whether decreased BNA expression is ultimately responsible for the low QA and NAD⁺ levels in rpd3Δ cells. We began with BNA2, the rate-limiting enzyme of the de novo pathway (38), and inquired whether restoration of this step could replenish the low QA
Figure 3. Rpd3 and Hst1 regulate homeostasis of de novo intermediates. A, mass spectrometry analysis of TRP levels in rpd3Δ, hst1Δ, and hst1Δrpd3Δ cells. Deletion of RPD3 leads to accumulation of TRP, whereas hst1Δ and hst1Δrpd3Δ cells show reduced TRP levels. B, rpd3Δ cells exhibit defective KYN production. C, rpd3Δ cells show reduced 3-HK levels, whereas hst1Δ and hst1Δrpd3Δ cells show increased 3-HK levels. D, rpd3Δ cells produce reduced levels of 3-HA, whereas hst1Δ and hst1Δrpd3Δ cells produce greater levels of 3-HA. E, deletion of HST1 and especially deletions of RPD3 and HST1 together increase NA levels. All values for each metabolite are normalized to levels in WT cells. Error bars represent data from three technical replicates. The p values are calculated using Student’s t test (p < 0.05; ns, not significant), 3-HA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; KYN, kynurenine; NA, nicotinic acid; TRP, l-tryptophan.

levels seen in rpd3Δ cells. We found that overexpression of BNA2 (from the ADH1 promoter, so as to decouple it from regulation by Rpd3) was only sufficient to induce a small but significant increase in QA levels, which nevertheless remained far below those seen in WT cells (Fig. 4, C and D). Pairing overexpression of BNA2 with BNA6, which increases QA assimilation into NAD⁺ (Fig. 1A), we found that BNA6-oe effectively cleared the small amount of QA accumulated in rpd3Δ BNA2-oe cells (Fig. 4D). We then examined whether this modest increase of QA induced by BNA2-oe could restore the NAD⁺ levels of rpd3Δ cells. As shown in Figure 4, E, neither BNA2-oe alone nor BNA2-oe + BNA6-oe was sufficient to stimulate any visible increase in the NAD⁺ pool in rpd3Δ cells. This indicated that each step of de novo metabolism would need to be accounted for in order to achieve full restoration of NAD⁺ levels in rpd3Δ cells. To test whether this was the case, we attempted to reinstate de novo activity by bypassing the earlier steps of the pathway. To achieve this, we cultured cells in NA-free SC supplemented with QA and overexpressed BNA6 (Fig. 4F, right), feeding directly into the main pathway of NAD⁺ biosynthesis via NaMN (Fig. 1A). As controls, we also included cells grown in standard SC (Fig. 4F, left) and NA-free SC (Fig. 4F, middle) without QA supplementation. As shown in Figure 4, F (right), neither QA supplementation nor BNA6-oe alone was sufficient to raise NAD⁺ levels in rpd3Δ cells to any significant degree. When paired however, the two adjustments were able to restore NAD⁺ levels in rpd3Δ back to those observed in WT cells. Interestingly, the NAD⁺ levels under these conditions were still slightly below that of WT cells with BNA6-oe and QA supplementation, suggesting that other factors downstream of BNA6 may also be impacted because of rpd3Δ.

Rpd3 binding to the BNA2 promoter is altered by deleting Hst1 and vice versa

Next, we sought to investigate the interaction between Rpd3 and Hst1 at the promoter of the BNA genes. Hst1 has been shown to bind to the promoter of BNA2 (38), which mediates the first and rate-limiting step of de novo QA synthesis. Therefore, we determined whether Rpd3 affects the binding activity of Hst1 at the BNA2 promoter and vice versa. To achieve this, we carried out chromatin immunoprecipitation (ChIP) studies of various BNA2 promoter fragment using HA-tagged Rpd3 (Rpd3-HA) and Hst1 (Hst1-HA) strains. Figure 5, A (top) shows Rpd3 and Hst1 protein levels used for this study. It appeared that deletion of HST1 resulted in slightly increased expression of Rpd3-HA, whereas deletion of RPD3 resulted in somewhat decreased levels of Hst1-HA (Fig. 5A, top). However, we did not expect this to have a significant influence on Rpd3 binding to the BNA2 promoter. As shown in Figure 5, A (top), the Rpd3-HA WT strain showed a normal level of QA release and was still sensitive to the dosage of Hst1, as deleting HST1 in this strain was able to significantly increase QA release (Fig. 5B, top). Similarly, deleting RPD3 in Hst1-HA WT cells also drastically reduced QA release (Fig. 5B, bottom), suggesting that Hst1-HA remained an efficient repressor and was recruited to the BNA2 promoter in both WT and rpd3Δ backgrounds. Figure 5, A (bottom) illustrates BNA2 promoter fragments (BNA2 #1, #2, #3, #4, and #5) used in ChIP studies. As shown in Figure 5, C, the binding activity of Rpd3 was increased near the middle of the BNA2 promoter (BNA2 #2 and #3) and tapered off toward the -1000 and 0 sites. Interestingly, when Hst1 is absent, Rpd3 appears to move away from the middle of the promoter (BNA2 #3) and instead occupying the ends (BNA2 #1, #2, #4, and #5) (Fig. 5C). In
Figure 4. Rpd3 positively regulates de novo NAD⁺ metabolism. A, gene expression quantitative PCR (qPCR) analysis of BNA mRNA in WT, rpd3Δ, hst1Δ, and hst1Δrpd3Δ cells. Values shown are relative expression levels normalized to TAF10 as a control. Deletion of RPD3 decreases expression of all BNA genes shown. BNA expression in hst1Δrpd3Δ cells is generally increased relative to WT cells and slightly less than levels in hst1Δ cells. B, comparisons of Bna protein expression in HDAC mutants. HA-tagged Bna1, Bna2, and Bna5 proteins were generated in WT, rpd3Δ, hst1Δ, and hst1Δrpd3Δ cells. Protein expression was determined by Western blot analysis. Arrows make the positions of molecular weight markers. C, overexpression of BNA2 (BNA2-oe) slightly increases the levels of QA release. D, BNA2-oe increases QA release in rpd3Δ, whereas overexpression of both BNA2 and BNA6 in rpd3Δ clears accumulated QA. E, BNA2-oe alone or BNA2-oe and BNA6-oe together is insufficient to raise NAD⁺ levels in rpd3Δ cells grown in SC. F, restoration of de novo pathway activity is necessary to rescue NAD⁺ levels in rpd3Δ cells. NAD⁺ levels in rpd3Δ cells are increased to WT levels when supplemented with QA (at 10 μM) and with BNA6-oe. For A, C, E, and F, the graphs are representative of the trend observed across three independent experiments. For A and C, error bars represent data from three technical replicates for each strain in an experiment. For E and F, error bars represent data from two biological replicates each with two technical replicates for each strain in an experiment. The p values are calculated using Student’s t test (*p < 0.05; ns, not significant). BNA, biosynthesis of nicotinic acid; HDAC, histone deacetylase; QA, quinolinic acid.
particular, we noted the highest increase of Rpd3 occupancy near site #5 (BNA2 #5), directly proximal to the transcription start site. This is in accordance with expectation, as Hst1 was previously shown to exhibit the highest binding activity in this region of the BNA2 promoter (38). Ultimately, when Hst1 is absent, Rpd3-binding distribution on the BNA2 promoter is altered, with the most significant increase near the transcription start site. This result suggests that Hst1 may oppose Rpd3 binding at specific regions of the BNA2 promoter. Next, we examined whether the absence of Rpd3 would affect Hst1 binding to the BNA2 promoter. In agreement with previous studies (38), Hst1-binding activity was the highest near the transcription start site (BNA2 #5) in an ascending pattern. Hst1-binding activity is decreased when Rpd3 is absent. For C and D, the graphs are representative of the trend observed across three independent experiments. Error bars represent data from three technical replicates for each strain in an experiment. The p values are calculated using Student’s t test (*p < 0.05; ns, not significant). E, model of Rpd3 and Hst1 binding to the BNA2 promoter. BNA, biosynthesis of nicotinic acid; QA, quinolinic acid.
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promoter but also raises the possibility that Rpd3 may be important for Hst1 binding. However, the latter contradicts the expectation that Rpd3, as a positive regulator of BNA expression, would most likely oppose Hst1 binding. It is clear that Hst1 in the rpd3Δ background is still highly proficient in silencing BNA expression and limiting de novo activity (Figs. 4A and 5B) and remains bound at levels significantly above those at the TAF10 control site (Fig. 5D). Otherwise, the rpd3Δ/hst1Δ cells would have shown a similar high QA release phenotype as seen in hst1Δ cells, which was not the case here (Fig. 5B). Therefore, it is more likely that the ascending binding pattern of Hst1 is due to higher Rpd3-binding activity toward the middle of the BNA2 promoter and that in the absence of Rpd3, Hst1 is able to redistribute more evenly on the BNA2 promoter. This conclusion supports a model in which Rpd3 may oppose Hst1 binding at specific regions of the BNA2 promoter, possibly by preventing the overpressing of silenced chromatin, which is important for proper BNA gene expression. The cause of this generally reduced Hst1 binding may also be the reduced NAD+ levels exhibited by the rpd3Δ mutant (Fig. 2E). As an NAD+-dependent HDAC, the activity of Hst1 is expected to decrease under conditions of NAD+ limitation, which is reflected in the observed reduction in Hst1-binding activity. Supporting this, it has been reported that of all the sirtuins, Hst1 is most sensitive to NAD+ levels, responding quickly to perturbations to the NAD+ pool within the range of physiological concentration (37). It is also possible that without the opposing effect of Rpd3, Hst1 does not need to access the BNA2 promoter frequently in order to maintain silent chromatin.

**Rpd3 and Hst1 deacetylate the N-terminal lysine residues of the core histone protein H4**

Having determined how Rpd3 and Hst1 interact in the binding of the BNA2 promoter, we then sought to determine how each HDAC affects the histone acetylation status of the BNA2 promoter. In investigating this question, we examined histone acetylation at two of the promoter sites indicated in Figure 5, A in order to account for any possible local effects of each HDAC. Rpd3 binding peaks at the middle of the promoter near site #3 (Fig. 5C), whereas Hst1 binding peaks immediately upstream of the transcription start site at site #5 (Fig. 5D). Rpd3 has previously been shown to deacetylate a wide variety of lysine residues on the N-terminal tail of core histone proteins, among these being H4K5, H4K8, H4K12, H3K9, H3K14, H3K18, H3K23, H3K27, and H2AK7 (54–56). Hst1, on the other hand, has been identified as a deacetylase of H3K4 (57), H4K5, and potentially H4K12 (58). Owing to Rpd3 and Hst1 having H4K5 and H4K12 as shared targets, we chose to focus on histone H4 as a potential site of competition between Rpd3 and Hst1. Our ChIP results showed that Hst1 is the primary deacetylase for H4K5-Ac and H4K12-Ac at the BNA2 promoter, as the levels of H4K5-Ac and H4K12-Ac were increased in hst1Δ cells (at both site #3 and site #5). Deleting RPD3 slightly yet significantly decreases the level of H4K5-Ac and H4K12-Ac (at site #5, near the transcription start site) (Fig. 6, A and B). This is interesting in light of the fact that deletion of RPD3 was previously associated with increased acetylation of both these residues (56). In this case, however, Rpd3 seems to interfere with the deacetylation of both by an unknown mechanism, whereas Hst1 appears to be the primary deacetylase for these residues at the BNA2 promoter. These results suggest that in the absence of Rpd3, another HDAC, likely Hst1, deacetylates H4K5-Ac and H4K12-Ac more efficiently at the BNA2 promoter. Supporting this, deleting HST1 in rpd3Δ cells restored acetylation to similar levels observed in the hst1Δ cells (Fig. 6, A and B).

Interestingly, the acetylation patterns seen for H4K8 differed from that of H4K5 and H4K12 (Fig. 6C). H4K8-Ac was comparatively difficult to detect at site #3, possibly indicating that it is less abundant than the other acetyl marks. Moreover, H4K8-Ac abundance was increased in rpd3Δ cells, in conformity with the previously established role of Rpd3 as a deacetylase of H4K8. On the other hand, any enrichment of H4K8-Ac in hst1Δ cells was minor and nonsignificant, unlike acetylation of the other two residues. However, H4K8-Ac levels were strongly increased in hst1Δ/rpd3Δ cells, pointing to the possibility of synergy between the two HDACs in the deacetylation of H4K8. These results suggest that Hst1 is the primary deacetylase of H4K5 and H4K12 at the BNA2 promoter, whereas Rpd3 works to limit deacetylation of these residues in this context, in close agreement with the antagonism seen between the two in the regulation of BNA expression and de novo NAD+ metabolism. On the other hand, the two HDACs also appear to both deacetylate H4K8 to some degree, suggesting a variety of different interactions between Rpd3 and Hst1 at the BNA2 promoter, all of which ultimately combine to regulate BNA2 expression in a specific manner. Ultimately, Hst1 seems to promote repressed chromatin status at the BNA2 promoter (Fig. 6D). H4 acetylation (Figure 6, A–C), Rpd3 binding (Fig. 5C), and BNA2 expression are all increased in hst1Δ cells (Fig. 4A). On the other hand, Rpd3 activity is likely associated with derepressed chromatin status in this context (Fig. 6D); H4K5 and H4K12 acetylation is somewhat decreased in rpd3Δ cells (Fig. 6, A and B), whereas BNA2 expression is very strongly decreased (Fig. 4A). Moreover, the reduced binding of Hst1 to the BNA2 promoter in rpd3Δ cells (Fig. 5D) may in part be caused by this putative repressed chromatin structure.

**De novo NAD+ metabolism, NA–NAM salvage, NR salvage, and transport of NAD+ precursors are integrated by Rpd3 and Hst1**

Next, we further examined how Rpd3 and Hst1 may affect other branches of NAD+ metabolism. We tested the expression of genes known to be involved in NA–NAM and NR salvage in WT, rpd3Δ, hst1Δ, and hst1Δ/rpd3Δ cells by quantitative RT–PCR (qRT–PCR) (Fig. 7A). As shown in Figure 7, A, differential expression of NA–NAM salvage genes including NPT1, TNA1, and PNC1 was observed. A significant decrease in NPT1 expression was seen in rpd3Δ and hst1Δ/rpd3Δ cells (Fig. 7A). These changes may account for the low NAD+
Figure 6. Rpd3 and Hst1 have opposing effects on histone H4 acetylation status at the BNA2 promoter. A, relative abundance of acetylated H4K5 (H4K5-Ac) at sites 3 and 5, depicted in Figure 5, A, of the BNA2 promoter (left). Deletion of RPD3 slightly decreases the amount of H4K5-Ac, whereas deletion of HST1 as well as deletions of RPD3 and HST1 together increase the level of H4K5-Ac, suggesting that Hst1 is the main deacetylase for this residue (right). B, relative abundance of H4K12-Ac at sites #3 and #5 of the BNA2 promoter (left). rpd3Δ cells show reduced acetylation of H4K12, whereas hst1Δ and hst1Δrpd3Δ cells show increased acetylation of H4K12, suggesting that Hst1 is primarily responsible for the deacetylation of this residue (right). C, relative abundance of H4K8-Ac at sites #3 and #5 of the BNA2 promoter (left). rpd3Δ and hst1rpd3Δ cells show increased acetylation of H4K8, whereas deletion of...
HST1 alone does not have a significant influence on H4K8-Ac levels, suggesting that Rpd3 is the main deacetylase for this residue. Values are relative to levels of H4 protein-bound DNA in each strain, and all values are normalized to those of WT cells. The graphs are representative of the trend observed across three independent experiments. Error bars represent data from three technical replicates for each strain in an experiment. The \( p \) values are calculated using Student’s \( t \) test (* \( p < 0.05 \); ns, not significant). NA, nicotinic acid; NAM, nicotinamide; NR, nicotinamide riboside.

**Discussion**

In this study, we have established a role for Rpd3 as a positive regulator of de novo NAD\(^+\) metabolism. In particular, it opposes the negative regulation of the BNA genes by another HDAC Hst1 (Figs. 4A and 6). The rpd3\(\Delta\) and hst1\(\Delta\) mutants show contrasting QA release phenotypes. Interestingly, hst1\(\Delta\) appears to override rpd3\(\Delta\), and the hst1\(\Delta\)rpd3\(\Delta\) double mutant behaves like the hst1\(\Delta\) single mutant (Fig. 1, C and D), with the exception of intracellular QA levels (Fig. 1E). Our studies suggest that the high BNA6 expression in hst1\(\Delta\) and hst1\(\Delta\)rpd3\(\Delta\) cells (Fig. 4A) may facilitate QA assimilation and therefore reduce intracellular QA accumulation. The same pattern of reduced de novo metabolism in rpd3\(\Delta\) cells and increased metabolism in hst1\(\Delta\) cells is seen for several intermediates upstream of QA, namely 3-HK (Fig. 3C) and 3-HA (Fig. 3D). Moreover, rpd3\(\Delta\) cells accumulate TRP (Fig. 3A), likely because of reduced Bna2 activity at the first and rate-limiting step of de novo NAD\(^+\) metabolism. Rpd3 has also been implicated in recycling of Tat2, a TRP transporter. The growth of a strain defective in Tat2-dependent transport is significantly inhibited under low TRP conditions by deletion of RPD3 (59). We saw, however, that rpd3\(\Delta\) cells remain proficient for TRP uptake, accumulating the metabolite above levels seen in WT cells (Fig. 3A). This suggests that reduced assimilation of TRP into NAD\(^+\) via the de novo pathway may contribute to the growth defects of rpd3\(\Delta\) cells in low TRP media (59). In addition to defective de novo NAD\(^+\) metabolism, cells lacking Rpd3 also show altered NA–NAM salvage pathways in WT, rpd3\(\Delta\), hst1\(\Delta\), and hst1\(\Delta\)rpd3\(\Delta\) cells by quantitative PCR (qPCR). B, relative expression analysis of the genes of the NR salvage pathway in WT, rpd3\(\Delta\), hst1\(\Delta\), and hst1\(\Delta\)rpd3\(\Delta\) cells by qPCR. All values shown are relative expression levels normalized to TAF10 as a control. The graphs are representative of the trend observed across three independent experiments. Error bars represent data from three technical replicates for each strain in an experiment. The \( p \) values are calculated using Student’s \( t \) test (* \( p < 0.05 \); ns, not significant). NA, nicotinic acid; NAM, nicotinamide; NR, nicotinamide riboside.
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and NR salvage activities. Although contrasting NA–NAM release phenotypes are also observed in rpd3Δ and hst1Δ mutants (Fig. 2, A and B), it appears that different downstream genes are dysregulated in each mutant. The lower NA–NAM levels in the rpd3Δ mutant (Fig. 2, A and B) are likely because of overall lower NAD⁺ levels (Fig. 2, E and F). It is also possible that reduced URH1 expression (Fig. 7B) results in less NR to NAM conversion (24). In hst1Δ (including hst1Δrpd3Δ) cells, increased intracellular NA–NAM levels (Fig. 2B) are likely because of increased expression of the NA transporter TNA1 as well as reduced nicotinamidase PNC1 expression (Fig. 7A). On the other hand, the rpd3Δ and hst1Δ mutants also show contrasting NR release patterns, but this time, the rpd3Δ mutant releases more NR (Fig. 2C). The hst1Δ mutant appears to release less NR because most NR is accumulated intracellularly (Fig. 2D) because of high expression of the NR transporter NRT1 (Fig. 7B). Increased NR release in the rpd3Δ mutant is likely because of decreased NRT1 expression (Fig. 7B). Notably, rpd3Δ and hst1Δ have a synergistic effect on NR production (Fig. 2D), which has also been seen for the expression of other genes involved in NR salvage including POF1 (Fig. 7B). Overall, our results show that Rpd3 and Hst1 opposingly coregulate the de novo BNA genes, yet they seem to affect different target genes in the salvage pathways. Among the genes examined, only the NAD⁺ precursor transporters NRT1 and TNA1 (Fig. 7, A and B) appear to be regulated by the two HDACs in a manner resembling the BNA genes (Fig. 4A).

Our studies also indicate that observed NAD⁺ deficiencies in rpd3Δ cells are likely caused by various factors discussed previously depending on specific growth conditions. In standard NA-rich growth media, de novo pathway is repressed by the NAD⁺-dependent Hst1. Therefore, observed low NAD⁺ defects in rpd3Δ (and hst1Δrpd3Δ) cells (Fig. 2E) likely result from increased NR production in conjunction with a blockage in NR flow to NA–NAM salvage and reduced NA assimilation because of decreased expression of NPT1 (Fig. 7A) and URH1 (Fig. 7B). The effects of rpd3Δ and hst1Δ on de novo pathway activity are better understood since this pathway can be studied in defined NA-free growth media (Fig. 2F). Under this condition, observed NAD⁺ deficiencies are mainly because of reduced BNA expression, and so deleting hst1Δ overrides rpd3Δ and increases NAD⁺ levels (Fig. 2F). We also show that dysregulation of multiple BNA genes contributes to NAD⁺ deficiencies in rpd3Δ cells, since overexpressing individual rate-limiting BNA2 gene and the BNA6 gene was not sufficient to restore NAD⁺ levels (Fig. 4E). However, we were able to restore the levels of NAD⁺ in rpd3Δ cells by supplementing QA and overexpressing BNA6 (Fig. 4F). These studies confirm that Rpd3 is important for optimal BNA gene expression and de novo NAD⁺ synthesis. To understand the mechanisms of Rpd3- and Hst1-mediated regulation of the BNA expression, we carried out ChIP studies to study the binding distributions of Rpd3 and Hst1 on the promoter of the BNA2 genes (Fig. 5, C and D). The results gathered point toward a model in which Hst1 serves to limit the distribution pattern of Rpd3 on the BNA2 promoter. Rpd3 also appears to affect the interaction of Hst1 with the BNA2 promoter, but the effect is less significant, as the overall Hst1-binding activity at the BNA2 promoter is reduced in rpd3Δ cells (Fig. 5, C–E). It is possible that Hst1 activity is reduced in rpd3Δ cells because of reduced NAD⁺ levels. This is interesting in light of the fact that Hst1 appears to work downstream of Rpd3, with hst1Δ sufficient to raise BNA expression and QA production to a similar extent in WT and rpd3Δ backgrounds. Therefore, we anticipate that additional factors may also play a role in the antagonistic BNA gene expression regulation exerted by Rpd3 and Hst1. One possibility is that specific transcription factors and/or chromatin remodeling factors are recruited to or excluded from the BNA promoter by specific chromatin modifications effected by the two HDACs.

Similar instances of antagonism between Rpd3 and other sirtuins have been noted in previous studies, with Rpd3 opposing repression of the silent mating-type loci by Sir2 and thereby promoting transcription (55). Rpd3 has also been shown to limit Sir2 spreading at telomere boundaries (60). While it is typical for HDACs to serve as chromatin silencers (61), Rpd3 has long been noted for seeming to promote transcription of certain genes (62). Interestingly, it was previously noted that the role played by Rpd3 in opposing Sir2 activity was indeed mediated by its catalytic HDAC activity, but that the effect did not depend on any of the known histone targets of Rpd3 (55). Indeed, there are many examples of HDACs acting on nonhistone proteins (63, 64), making the possible avenues of HDAC-dependent regulation quite extensive. Moreover, restoration of silencing by RPD3 deletion in a sir2-catalytic-dead strain was dependent on Hst3 activity (55), highlighting the complementarity and interactive roles of different HDACs. In the case of de novo pathway regulation however, our QA cross-feeding screen (Fig. 1B) did not indicate the involvement of any other HDACs aside from Rpd3 and Hst1. It appears that some or all BNA genes may be regulated in a unique fashion. For example, Sir2 has previously been identified as a negative regulator of BNA1 expression, with sir2Δ cells showing BNA1 expression increased by approximately twofold (52). The positive regulator Bas1–Pho2, for instance, appears to affect each BNA gene to significantly varying degrees (25). We have previously seen as well that BNA7 is insensitive to many of the factors that regulate other BNA genes (38). It does appear that Rpd3 and Hst1 are ubiquitous regulators of de novo NAD⁺ metabolism; however, this property may not extend to other regulators of the pathway. It will likely be necessary to study each BNA gene individually in order to fully elaborate the intricacies of de novo pathway regulation.

It has also been shown that treatment with sodium butyrate and other HDAC inhibitors is able to suppress aberrant de novo metabolism in Huntingtin-expressing cells in a similar fashion to 1μA and rxt3Δ (51), whereas treatment with the sirtuin inhibitor NAM produces effects on BNA expression similar to those observed in hst1Δ (38). These studies suggest that the catalytic activities of each HDAC are required at least to some extent and that the role each plays is not purely structural. In agreement with this notion, both HDACs seemed to have a rather significant effect on the acetylation
status of histone H4. In particular, we found that the abundance of H4K5-Ac (Fig. 6A) and H4K12-Ac (Fig. 6B) was significantly increased in hst1Δ and hst1Δrpd3Δ cells, whereas H4K8-Ac abundance was significantly increased in rpd3Δ cells and very greatly raised in hst1Δrpd3Δ cells (Fig. 6C). Altogether, these results seem to implicate Hst1 as the major deacetylase active at the BNA2 promoter and, together with increased de novo activity and BNA expression observed in hst1Δ cells, point toward a role for Hst1 in establishing a repressive chromatin architecture at the BNA promoters. Conversely, Rpd3, with the exception of H4K8-Ac, appears to generally limit deacetylation at the BNA2 promoter. In combination with decreased BNA expression seen in rpd3Δ cells, Rpd3 appears to act in the capacity of a positive regulator by limiting Hst1-dependent deacetylation.

Though the specific mechanism of this antagonism between Rpd3 and Hst1 remains to be further investigated, there are several possibilities suggested by these results. Limitation of Hst1-dependent deacetylation by Rpd3 may proceed by means of competition for the same targets, such as H4K5-Ac and H4K12-Ac, wherein limited deacetylation by Rpd3 prevents recognition and full deacetylation by Hst1. It has previously been reported that certain HDACs may be recruited by a specific acetyl mark. For instance, H4K16-Ac, a target of Sir2, has been shown to promote the binding of Sir2 to chromatin (65). It is also possible that other acetyl marks may be targeted primarily by Rpd3, potentially including H4K8-Ac, reconfiguring the chromatin so as to interfere with Hst1 activity at different marks, the net result of which might be decreased silencing by Hst1. We saw that deletion of RPD3 unexpectedly decreased Hst1 binding to the BNA2 promoter (Fig. 5D); one possible explanation for this is that the repressive heterochromatin structure formed by Hst1 HDAC activity in the absence of Rpd3 might then limit the continued binding of Hst1 itself, with only enough Hst1 binding to maintain the heterochromatin structure. Finally, histone modifications made by Rpd3 may also be involved in the recruitment of additional chromatin remodeling factors or transcription factors to the BNA2 promoter, which may then interact with Hst1. It has previously been established that the copper-sensing transcription factor Mac1 (38) and the Bas1–Pho2 complex (25) regulate BNA expression. How these factors, and possibly others, might interact with Rpd3 and Hst1 is a matter for future study.

It appears ultimately that Hst1 is involved in the formation of heterochromatin at the BNA2 promoter via deacetylation of the N-terminal lysines of H4, whereas Rpd3 opposes Hst1 HDAC activity and maintains a less repressed chromatin structure (Fig. 6D). Whether Rpd3 and Hst1 target additional residues on other histones remains a topic for further study. Rpd3 has been shown to target a very wide group of acetyllysine residues across several different histone proteins, including H3K9, H3K14, H3K18, H3K23, H3K27, and H2A-K7 (54), whereas Hst1 is known to deacetylate H3K4 (57) and may also deacetylate H4K16 in the absence of Sir2 and Pde2 (66). In addition, it remains to be investigated what effect each acetyllysine mark has on BNA expression and chromatin structure, and whether Rpd3 and Hst1 do indeed regulate heterochromatin formation in the manner proposed (38, 55).

In summary, Rpd3 and Hst1 appear to exert opposing influence on de novo NAD⁺ metabolism and also integrate the regulation of several disparate branches of NAD⁺ metabolism. This set of regulators helps coordinate a variety of inter-related metabolic signals in budding yeast. Further work will be required to explore the detailed mechanistic interactions among these regulators and to establish the means by which they compete and cooperate to influence the cellular pools of NAD⁺ and its precursors. Rpd3 and Hst1, having homologs in human HDAC1 and SIRT1, respectively, represent links between the study of the epigenetic regulation of NAD⁺ metabolism and various disease states. Aberrant NAD⁺ metabolism and associated dysregulation of sirtuin activities have been implicated in a number of human disorders (5, 6, 18–22). HDAC1 has also been associated with a variety of diseases, and HDAC inhibition generally has received attention as a therapeutic intervention in a variety of contexts (67–69). It is currently unclear whether sirtuins and HDAC1 also regulate NAD⁺ metabolism in other organisms by similar mechanisms. It would be interesting to determine what other genes are also coregulated by Hst1 and Rpd3 in future studies. Notably, many NAD⁺ intermediates also have a manifold set of relationships with cellular health. For instance, the intermediates of the de novo pathway have diverse interactions with infectious mechanisms, metabolic stress, and immune signaling (45). Altogether, this work contributes to the elaboration of the relations by which NAD⁺ metabolism is governed and helps to connect different branches of NAD⁺ metabolism among each other.

**Experimental procedures**

**Yeast strains, growth media, and plasmids**

Yeast strain BY4742 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 acquired from Open Biosystems (70) was used as the parental WT strain for this study. Standard growth media including synthetic defined (SD) minimal, SC, and yeast extract/peptone/dextrose–rich media were made as described (71). Special NA-free SD and NA-free SC were made by using niacin-free yeast nitrogen base acquired from Sunrise Science Products. Gene deletions were carried out by replacing the coding regions of WT genes with gene-specific PCR products generated using either the pAG32-hphMX4 (72) or the reusable loxP-kanMX-loxP (pUG6) (73) cassettes as templates. Multiple gene deletions employed a galactose-inducible Cre recombinase to remove the loxP-kanMX-loxP cassette, followed by another round of gene deletion (73). The HA epitope tag was added to target genes directly in the genome using the pFA6a-3HA-kanMX6 (for HST1) or pFA6a-kanMX6-PGAL1-3HA (for RPD3) plasmids as template for PCR-mediated tagging (74). The BNA6-oE and BNA2-oE plasmids pADH1-BNA6 (75) and pADH1-BNA2-URA3 were made in the integrative pPPB1 (LEU2) and pPP35 vectors, respectively. After PacI digestion, the linearized plasmids were introduced to yeast cells as described (71). The pADH1-BNA2-URA3 plasmid was made by cloning the Not1–Nhe1 DNA fragment.
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from the pADH1-BNA2 plasmid (38) into pPP35 cut with NotI and NheI.

**QA, NR, and NA–NAM cross-feeding plate assays**

These assays employed specific mutants, which depend on QA, NR, or NA–NAM for growth, as “recipient cells” and yeast strains of interest as “feeder cells.” First, recipient cells were plated as a lawn on a solid agar plate (~10⁵ cells/cm²). Next, ~2 × 10⁴ cells of each feeder cell strain (2 μl cell suspension made in sterile water at an absorbance of 1 at 600 nm) were spotted onto the lawn of recipient cells. Plates were then incubated at 30 °C for 3 days. Since the growth media do not contain the NAD⁺ intermediates needed for the growth of recipient cells, the extent of the recipient cell growth indicates the levels of specific NAD⁺ intermediates released by feeder cells. QA crossfeeding was carried out on SC or SD using the QA-dependent npt1Δnrt1Δbna6Δ mutant. NR crossfeeding was carried out on yeast extract/peptone/dextrose or SC using the NR-dependent npt1Δbna6Δapho5Δ mutant. NA–NAM crossfeeding was carried out on NA-free SC or NA-free SD using the NA–NAM-dependent bna6Δnrt1Δ mutants.

**Measurement(s) of NAD⁺, NADH, QA, NR, and NA–NAM**

Total intracellular levels of NAD⁺ and NADH were determined using enzymatic cycling reactions as described (76). In brief, approximately one absorbance at 600 nm unit (one absorbance at 600 nm unit = 1 × 10⁷ cells/ml) cells grown to early logarithmic phase in SC (~6 h growth from absorbance of 0.1 at 600 nm) was collected in duplicate by centrifugation. Acid extraction was performed in one tube to obtain NAD⁺, and alkali extraction was performed in the other to obtain NADH for 40 min at 60 °C. Amplification of NAD⁺ or NADH in the form of maltate was carried out using 3 μl or 4 μl of neutralized acid– or alkali-extracted lysate in 100 μl of cycling reaction for 1 h at room temperature. The reaction was terminated by heating at 100 °C for 5 min. Next, malate produced from the cycling reaction was converted to oxaloacetate and then to aspartate and α-ketoglutarate by the addition of 1 ml malate indicator reagent for 20 min at room temperature. The reaction produced a corresponding amount of NADH as readout, which was measured fluorometrically with excitation at 365 nm and emission monitored at 460 nm. Standard curves for determining NAD⁺ and NADH concentrations were obtained as follows: NAD⁺ and NADH were added into the acid and alkali buffer to a final concentration of 0, 2.5, and 7.5 μM, which were then treated with the same procedure along with other samples. The fluorometer was calibrated each time before use with 0, 5, 10, 20, 30, and 40 μM NADH to ensure that the detection was within a linear range. Levels of NAD⁺ intermediates (QA, NR, and NA–NAM) were determined by a liquid-based cross-feeding bioassay as previously described (39, 41, 75) with modifications. To prepare cell extracts for intracellular NAD⁺ intermediate determination, approximately 200 absorbance at 600 nm unit (for NR and NA–NAM) or 900 absorbance at 600 nm unit (for QA) donor cells grown to late-logarithmic phase in SC (~16 h growth from an absorbance of 0.1 at 600 nm) were collected by centrifugation and lysed by bead-beating (Biospec Products) in 400 μl (per 200 absorbance at 600 nm unit cells) ice-cold 50 mM ammonium acetate solution. The supernatant was collected by centrifugation, and the pellet was extracted two more times with 600 μl ice-cold 50 mM ammonium acetate solution, which generates 1600 μl cell lysate. After filter sterilization, 100 to 200 μl of clear extract (2.5 ml for QA) was used to supplement 8 ml cultures of recipient cells with starting absorbance of 0.05 at 600 nm in SC. To determine extracellular NAD⁺ intermediate levels, 20 ml supernatant of donor cell culture was collected, filter-sterilized, and then 4 ml was added to recipient cell culture in 2× SC to a final volume of 8 ml with total starting absorbance of 0.05 at 600 nm. A control culture of recipient cells in SC without supplementation was included in all experiments. For measuring relative QA levels, npt1Δnrt1Δbna6Δ and npt1Δnrt1Δ mutants were used as recipient cells. The npt1Δbna6Δapho5Δ recipient cells were used to measure relative NR levels. To measure relative NA–NAM levels, the bna6Δnrt1Δnrt1Δ recipient cells were grown in NA-free SC. After incubation at 30 °C for 24 h, growth of the recipient cells (absorbance at 600 nm) was measured and normalized to the cell number of each donor strain. Absorbance at 600 nm readings were then converted to concentrations of QA, NR, and NA–NAM using the standard curves established as previously described (39, 41).

**MS analysis of metabolite levels**

Metabolomic data were acquired at the UC Davis West Coast Metabolomics Center. For each sample, approximately 300 absorbance at 600 nm unit (for QA) cells grown to late-logarithmic phase in SC (~16 h growth from an absorbance of 0.1 at 600 nm) were collected by centrifugation. Snap freezing was achieved by dry ice. Frozen cell pellets were kept in Eppendorf tubes and then subject to metabolite extraction and MS analysis. Cells were extracted following recommendations published before (77). GC-TOF was performed with an Agilent 6890 gas chromatography instrument with an Rtx-5Sil MS column coupled to a Leco Pegasus IV time of flight mass spectrometer (78). For data processing, ChromaTOF version 4.50.8 was used in conjunction with the BinBase algorithm as previously described (79). Metabolite identifications were performed according to the Metabolomics Standards Initiative by using chromatography-specific databases in conjunction with Mass Bank of North America (http://massbank.us) and NIST 20 mass spectral libraries (80).

**qPCR analysis of gene expression levels**

Approximately 40 absorbance at 600 nm unit cells grown to early logarithmic phase in SD (6 h growth from an absorbance of 0.1 at 600 nm) were collected by centrifugation. Total RNA was isolated using GeneJET RNA purification Kit (Thermo Fisher Scientific), and complementary DNA was synthesized using QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer’s instructions. For each qPCR, 50 ng of
complementary DNA and 500 nM of each primer were used. qPCR was run on Roche LightCycler 480 using LightCycler 480 SYBR green I Master Mix (Roche) as previously described (35). Average size of the amplicon for each gene was ~150 bp. The target mRNA transcript levels were normalized to TAF10 transcript levels.

Protein extraction and Western blot analysis

Approximately 50 absorbance at 600 nm unit cells grown in SC to early logarithmic phase (absorbance of ~1 at 600 nm) were collected by centrifugation. The cell lysate was obtained by bead beating in lysis buffer: 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 5 mM EDTA (pH = 8), 1 mM PMSF, and protease inhibitor cocktail (Pierce). The protein concentration was measured using the Bradford assay (Bio-Rad), and 20 to 25 μg (Hst1, Bna, and PGK) or 40 μg (Rpd3) of total protein was loaded in each lane. After electrophoresis, the protein was transferred to a polyvinylidene fluoride membrane (GE Healthcare). Blocking was carried out using OneBlock Western-CL Blocking buffer. The membranes were then washed, blotted with either anti-HA rabbit antibody (Cell Signaling; catalog no.: 3724S) or anti-PKG mouse antibody (Invitrogen; catalog no.: 459250). Protein was visualized using antimouse or anti-rabbit immunoglobulin antibody conjugate to horseradish peroxidase (Invitrogen) and the ECL reagents (Amersham, GE). The chemiluminescent image was analyzed using the Amersham Imager 600 (GE) system and software provided by the manufacturer.

ChIP assay

Approximately 500 absorbance at 600 nm unit cells grown to early logarithmic phase in SD were crosslinked with 1% formaldehyde for 30 min at room temperature and stopped by adding glucose to a final concentration of 125 mM. Cells were pelleted by centrifugation and washed twice with cold Tris-buffered saline (20 mM Tris–HCl, pH 7.5, 150 mM NaCl). Cells were lysed by bead beating in 1 ml of FA-140 lysis buffer (50 mM Heps, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1 mM PMSF, and 1× protease inhibitor cocktail [Pierce]) (81). The cell lysate was drawn off the beads and centrifuged at a maximum speed (13,200 rpm) for 30 min at 4 °C. The chromatin pellet was resuspended in 1 ml of FA-140 lysis buffer and sonicated on ice eight times with 20 s pulses using a Branson 450 Sonicator (output control set at 1.5 and duty cycle held at constant) to shear chromatin to an average length of ~500 bp. Sonicated chromatin solution was centrifuged twice at 10,000 rpm for 10 min at 4 °C. The supernatant was then aliquoted into two tubes (labeled “IP” [immunoprecipitated] and “no-Ab” [no antibody]). The IP samples were incubated overnight at 4 °C with anti-HA monoclonal antibody (catalog no.: ab1424; Abcam) at a dilution of 1:150. Both IP and no-Ab samples were incubated with 60 μl of ChIP-grade protein G beads (Cell Signaling Technology) for 2 h at 4 °C and then washed as described (81). DNA was then eluted from the beads two times with 125 μl of elution buffer (5× TE, 1% SDS). The combined DNA solution and input samples were incubated at 65 °C overnight to reverse the crosslinking. The purified DNA samples were analyzed by qPCR. The amount of immunoprecipitated specific promoter DNA was determined relative to no-Ab DNA. For quantitation of histone acetyl-lysine marks, each mark, along with the H4 protein itself, was precipitated as aforementioned using the following antibodies: ab10158, Abcam (H4); 71-290-6, Invitrogen (H4K5-Ac); ab15823, Abcam (H4K8-Ac); ab46983, and Abcam (H4K12-Ac). The amount of each mark was determined relative to no-Ab DNA and H4-bound DNA.

Data availability

All data are contained within this article.

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Abbreviations—The abbreviations used are: BNA, biosynthesis of nicotinic acid; ChIP, chromatin immunoprecipitation; 3-HA, 3-hydroxyanthranilic acid; HDAC, histone deacetylase; 3-HK, 3-hydroxykynurenine; KYN, kynurenine; MS, mass spectrometry; NA, nicotinic acid; NAM, nicotinamide; NaMN, nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; QA, quinolinic acid; qRT, quantitative RT; SC, synthetic complete; SD, synthetic defined; TRP, L-tryptophan.

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