The copper-sensing transcription factor Mac1, the histone deacetylase Hst1, and nicotinic acid regulate de novo NAD\(^+\) biosynthesis in budding yeast

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NADH (NAD\(^+\)) is an essential metabolite involved in various cellular biochemical processes. The regulation of NAD\(^+\) metabolism is incompletely understood. Here, using budding yeast (Saccharomyces cerevisiae), we established an NAD\(^+\) intermediate–specific genetic system to identify factors that regulate the de novo branch of NAD\(^+\) biosynthesis. We found that a mutant strain (mac1Δ) lacking Mac1, a copper-sensing transcription factor that activates copper transport genes during copper deprivation, exhibits increases in quinolinic acid (QA) production and NAD\(^+\) levels. Similar phenotypes were also observed in the hst1Δ strain, deficient in the NAD\(^+\)-dependent histone deacetylase Hst1, which inhibits de novo NAD\(^+\) synthesis by repressing BNA gene expression when NAD\(^+\) is abundant. Interestingly, the mac1Δ and hst1Δ mutants shared a similar NAD\(^+\) metabolism–related gene expression profile, and deleting either MAC1 or HST1 de-repressed the BNA genes. ChIP experiments with the BNA2 promoter indicated that Mac1 works with Hst1-containing repressor complexes to silence BNA expression. The connection of Mac1 and BNA expression suggested that copper stress affects de novo NAD\(^+\) synthesis, and we show that copper stress induces both BNA expression and QA production. Moreover, nicotinic acid inhibited de novo NAD\(^+\) synthesis through Hst1-mediated BNA repression, hindered the reuptake of extracellular QA, and thereby reduced de novo NAD\(^+\) synthesis. In summary, we have identified and characterized novel NAD\(^+\) homeostasis factors. These findings will expand our understanding of the molecular basis and regulation of NAD\(^+\) metabolism.

NAD\(^+\) and its reduced form NADH are primary redox carriers in cellular metabolism. NAD\(^+\) is also a cosubstrate in protein modifications, such as protein deacetylation mediated by the sirtuins (Sir2 family proteins) and ADP-ribosylation mediated by the poly(ADP-ribose) polymerases. These protein modifications contribute to the maintenance and regulation of chromatin structure, DNA repair, circadian rhythm, metabolic responses, and life span (1–4). NAD\(^+\) is also an NADP\(^+\) precursor, which, like NAD\(^+\), is carefully balanced with its reduced form NADPH to maintain a favorable redox state. Aberrant NAD\(^+\) metabolism is associated with a number of diseases, including diabetes, cancer, and neuron degeneration (2, 3, 5–11). Administration of NAD\(^+\) precursors, such as nicotinamide mononucleotide (NMN),\(^2\) nicotinamide (NAM), nicotinic acid riboside, and nicotinamide riboside (NR), has been shown to ameliorate deficiencies related to aberrant NAD\(^+\) metabolism in yeast, mouse, and human cells (3, 5–10, 12–15). However, the molecular mechanisms underlying the beneficial effects of NAD\(^+\) precursor supplementation are not yet completely understood.

The NAD\(^+\) pool is maintained by multiple NAD\(^+\) biosynthesis pathways, which are conserved from bacteria to humans. Depending on the cell types, growth conditions, and availability of specific NAD\(^+\) precursors, one pathway may dominate the others. In yeast, NAD\(^+\) can be synthesized de novo from tryptophan or salvaged from intermediates, such as NA, NAM, and NR (Fig. 1A). In the de novo pathway, tryptophan is converted to QA through a series of enzymatic reactions catalyzed by Bna2, Bna7, Bna4, Bna5, and Bna1 (Fig. 1A) (16). QA is then phosphoribosylated by Bna6, producing nicotinic acid mononucleotide (NaMN). Because several steps in the de novo pathway require molecular oxygen as a substrate (Bna2, Bna4, and Bna1), cells grown under anaerobic conditions rely on the salvage pathways for NAD\(^+\) synthesis (16). In the NA/NaMN salvage pathway, yeast cells retrieve NADM from NAD\(^+\) consumption reactions or uptake NA from the environment via NA transporter Tna1, leading to NaMN production. NaMN is also the converging point of the de novo pathway and NA/NAM salvage, which is converted to NAD\(^+\) by NaMN adenylytransferases (Nma1/2) (17) and glutamine-dependent NAD\(^+\) syn-

\(^2\) The abbreviations used are: NMN, nicotinamide mononucleotide; NAM, nicotinamide; NA, nicotinic acid; NR, nicotinamide riboside; 3-HK, 3-hydroxykynurenine; 3-HA, 3-hydroxyanthranilic acid; QA, quinolinic acid; NaMN, nicotinic acid mononucleotide; KP, kynurenine pathway; KA, kynurenic acid; QA, quinolinic acid; 3-HK, 3-hydroxykynurenine; KYN, kynurenine; SD, synthetic minimal; SC, synthetic complete; 3-HA, 3-hydroxyanthranilic acid; GO, gene ontology; QPRT, quinolinate phosphoribosyl transferase; YPD, yeast extract-peptone-dextrose; Ab, antibody.
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NR salvage also contributes to NAD\(^+\) synthesis. In yeast, NR is assimilated into NMN or NAM, catalyzed by the NR kinase Nrk1 (19) and nucleosidases Ure1/Pnp1/Meu1 (15), respectively. NMN is directly converted to NAD\(^+\) via NMN adenylyltransferases (Nma1/2) (17), whereas NAM merges into the NA/NAM salvage pathway. It has also been shown that small NAD\(^+\) precursors, such as NR, NA, NAM, and QA, constantly exit and re-enter yeast cells (20–22), which constitutes an extended NAD\(^+\) precursor pool.

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, metabolites of the de novo pathway appear to have additional function. The de novo pathway is also known as the kynurenine pathway (KP) or tryptophan degradation pathway, and alterations of the KP metabolites have been linked to several brain disorders (11, 23). Interestingly, KP metabolites have been shown to exhibit both neuroprotective (kynurenic acid (KA)) and neurotoxic (QA and 3-hydroxykynurenine (3-HK)) effects (11, 23, 24) (Fig. 1A). KA is produced from kynurenine (KYN) by the KYN aminotransferase. Yeast cells also produce KA from KYN by the Aro8/9 aminotransferases (25); however, the function of KA in yeast remains unclear.

Studying the regulation of specific NAD\(^+\) biosynthetic pathways has been challenging in part due to the redundancy and interconnections among them. Employing the properties of yeast cells that constantly release and retrieve small NAD\(^+\) precursors (20–22), we have previously carried out precursor-specific genetic screens to identify novel NAD\(^+\) homeostasis factors in yeast (1, 21, 26). In this study, we developed a QA release–based reporter system targeting the de novo branch of NAD\(^+\) metabolism. The hypothesis was that cells with abnormal de novo NAD\(^+\) synthesis activities would show altered QA release. The mac1\(\Delta\) mutant was among the top hits that showed increased QA release. MAC1 encodes a copper-sensing transcription factor (27–29), and our studies are the first to link Mac1 to NAD\(^+\) homeostasis. Here, we characterized the mac1\(\Delta\) mutants as well as additional factors that regulate the de novo pathway. Our studies help provide a molecular basis underlying the interconnection and cross-regulation of NAD\(^+\) biosynthesis pathways.

Results

A cell-based reporter assay to identify factors that modulate the de novo pathway

Saccharomyces cerevisiae cells have been reported to secrete QA (22). We exploited this phenomenon and established a cross-feeding assay using the bna4\(\Delta\)npt1\(\Delta\)nrk1\(\Delta\) mutant as “recipient cells” (which depend on QA for growth) and mutants of interest as “feeder cells.” In this system, recipient cells cannot grow on standard growth media (which lack QA). When feeder cells are placed in proximity, feeder cell–released QA supports recipient cell growth by “cross-feeding.” This assay determines relative levels of total QA released by feeder cells and can be considered as readout for the de novo pathway activity. As shown in Fig. 1B, recipient cells were spread onto synthetic minimal (SD) medium plates as a lawn. Next, WT, bna4\(\Delta\), bna4\(\Delta\), and bna6\(\Delta\) feeder cells were spotted on top of the lawn and allowed to grow. Recipient cells near the feeder spots appeared as satellite colonies after 3 days, and the number and size of the satellite colonies positively correlate with the amount of QA released from the feeders. According to the function of Bna proteins in the de novo pathway (Fig. 1A), we expected bna4\(\Delta\) and bna4\(\Delta\) cells to release no QA and bna6\(\Delta\) cells to release more QA. Indeed, as shown in Fig. 1B, both bna4\(\Delta\) and bna4\(\Delta\) feeder cells failed to support the growth of the recipient cells. In contrast, bna6\(\Delta\) cells supported the growth of recipient cells more robustly than WT cells. We also tested a few mutants that have been associated with regulation of the de novo pathway. The NAD\(^+\)-dependent histone deacetylase Hst1 represses BNA gene expression. During NAD\(^+\) deprivation, decreased Hst1 activity results in de-repression of BNA genes (30). Cells lacking NPT1 and TNA1 are defective in NA/NAM salvage and NA transport, respectively, and thus have decreased NAD\(^+\) levels (31, 32). In addition, Tna1 was reported to also function as a QA transporter (22). Fig. 1C showed that hst1\(\Delta\), npt1\(\Delta\), and tna1\(\Delta\) mutants all released more QA. These results demonstrated that mutants with altered QA levels and de novo activities could be identified using this system.

Transcription factor Mac1 is a novel NAD\(^+\) homeostasis factor

Next, we used the haploid yeast deletion collection as feeder cells to identify mutants with altered QA release (Fig. 1D). After incubation at 30 °C for 3 days, we scored the cross-feeding activity (which indicates the level of QA release) of each mutant by comparing the diameter of the cross-feeding zones with that of the WT. A relative QA release score was assigned to each mutant with the WT baseline score set at 0. Scores of −4, −3, and −2 indicate decreased QA release; scores of +2, +3, and +4 indicate increased QA release. To eliminate false-positives, mutants with scores of −4, −3, 3, and 4 (169 mutants) were re-examined. A total of 81 mutants passed the secondary screens on both SD and synthetic complete (SC) growth media (Table S1). Among the 12 mutants that show strongest QA release score (±4), most are known NAD\(^+\) homeostasis factors, including hst1\(\Delta\), npt1\(\Delta\), pnc1\(\Delta\), nat3\(\Delta\), and their interacting partners (Table S1). MAC1 encodes a copper-sensing transcription factor (27–29), which has not been associated with NAD\(^+\) homeostasis. To verify that observed phenotypes are due to the featured mutations and not to secondary cryptic mutations in the deletion collection, we reconstructed all deletion mutants used in this study. Fig. 1E showed that mac1\(\Delta\) released more QA, and deleting HST1 did not further increase QA release in the mac1\(\Delta\) mutant. Increased QA release correlates with increased levels of extracellular QA determined by quantitative liquid assays (Fig. 2A). Similar results were obtained using either bna4\(\Delta\)npt1\(\Delta\)nrk1\(\Delta\) (Fig. 2A, left) or bna1\(\Delta\)npt1\(\Delta\)nrk1\(\Delta\) (Fig. 2A, right) mutants as recipient cells. This suggests that QA is a major de novo pathway intermediate produced in feeder cells because bna1\(\Delta\)npt1\(\Delta\)nrk1\(\Delta\) cells can only utilize QA, whereas bna4\(\Delta\)npt1\(\Delta\)nrk1\(\Delta\) can utilize QA and additional intermediates, such as 3-hydroxykynurenine (3-HK)
and 3-hydroxyanthranilic acid (3-HA) (Fig. 1A). Interestingly, cells did not appear to retain excess QA intracellularly (Fig. 2A). Thus, increased QA may be converted to NAD⁺ or released extracellularly. To examine whether Mac1 and Hst1 function in the same pathway to regulate NAD⁺ homeostasis, we determined the levels of NR, NA/NAM, and NAD⁺/NADH in WT, mac1Δ, hst1Δ, and hst1Δmac1Δ cells. Similar to the hst1Δ mutants, mac1Δ cells showed moderate increases in intracellular...
lar levels of NR (Fig. 2B) and NA/NAM (Fig. 2C). Increased expression of NA and NR transporters was reported in hst1Δ mutants (30, 33), which could contribute to observed increases of intracellular NR and NA/NAM. The hst1Δ mutant has also been shown to maintain higher steady-state NAD$^+$ levels (30). Consistent with this study, mac1Δ, hst1Δ, and hst1Δmac1Δ mutants showed WT levels of NAD$^+$/NADH during early log-phase growth (6 h) (Fig. 2D); however, as cells entered late log phase (16 h), these mutants showed higher NAD$^+$/NADH levels when compared with WT cells (Fig. 2E). This result suggested that mac1Δ- and hst1Δ-induced QA production was indeed coupled to increased de novo NAD$^+$ synthesis. However, these mutants might also uptake more NA from the medium due to increased TNA1 expression (Fig. 3B), which could contribute to observed increases in NAD$^+$/NADH levels. We therefore determined NAD$^+$/NADH levels in cells grown in NA-free medium to eliminate the contribution of NA. As shown in Fig. 2F, an increase in NAD$^+$/NADH level was still observed in mac1Δ and hst1Δ cells. Interestingly, we were able to observe this NAD$^+$/NADH increase in early log-phase cells grown in NA-free medium. These results were in line with the observations that the presence of NA (and/or NA salvage to NAD$^+$) inhibits de novo activity (30, 34). Overall, these studies showed a connection between increased QA production and increased de novo NAD$^+$ synthesis activity in mac1Δ and hst1Δ cells.
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Mac1 is a copper-regulated transcription factor, which activates the expression of genes involved in high-affinity copper transport in response to copper deprivation (27–29). Our studies showed that deleting MAC1 increases de novo NAD\(^+\) synthesis activity and that the mac1\(^\Delta\) and hst1\(^\Delta\) mutants shared a similar effect on NAD\(^+\) homeostasis (Fig. 2). To understand how Mac1 regulates NAD\(^+\) metabolism, we carried out gene expression profile and differential gene expression studies in mac1\(^\Delta\), hst1\(^\Delta\), and WT cells (three biological triplicates of each strain were analyzed). A total of 370 genes were differentially

**Figure 3.** Hst1 and Mac1 regulate gene expression of the de novo NAD\(^+\) biosynthesis pathway. A, gene expression profile analyses of WT, hst1\(^\Delta\) and mac1\(^\Delta\) cells. The Venn diagrams show genes that are up-regulated (left) or down-regulated (right) in the hst1\(^\Delta\) and mac1\(^\Delta\) mutants relative to WT, de novo NAD\(^+\) biosynthesis–associated BNA genes are among the 172 genes co-up-regulated in hst1\(^\Delta\) and mac1\(^\Delta\) cells. B, validating BNA gene expression in WT, hst1\(^\Delta\), and mac1\(^\Delta\) cells by qPCR. Results showed relative expression levels of BNA, MAC1, HST1, SUM1, RFM1, (SUM1 and RFM1 encode components of the Hst1 protein complex), TNA1, and CTR1 genes. Relative gene expression (normalized to TAF10) is shown. C, relative repressible acid phosphatase (rAPase, Pho5) activities in WT, hst1\(^\Delta\), and mac1\(^\Delta\) cells. Increased rAPase activity in hst1\(^\Delta\) cells indicates increased PHO signaling activity. D, relative BNA gene expression in log-phase cells grown in copper-free (0 \(\mu\)M), nutritional copper level (10 \(\mu\)M, normal), and high-copper (250 \(\mu\)M) SD media. E, QA production is altered by high copper stress and copper deprivation. Results show relative levels of QA released by WT cells grown in copper-free SD supplemented with different concentrations of copper for 16 h. F, NAD\(^+\) and NADH levels are slightly decreased by high copper stress. Results show WT cells grown in copper-free SD supplemented with different concentrations of copper for 16 h. Error bars, S.D. The \(p\) values are calculated using Student's t test (*, \(p\) < 0.05).
expressed in mac1Δ and WT cells (255 up-regulated, 115 down-regulated), and 498 genes were differentially expressed in hst1Δ and WT cells (342 up-regulated, 156 down-regulated) (Files S1 and S2). Gene ontology (GO) term enrichment analysis showed that de novo NAD⁺ metabolism–associated GO terms were significantly enriched for the 498 differentially expressed genes in hst1Δ and WT cells. For the 370 differentially expressed genes in mac1Δ and WT cells, “copper ion import” and “iron ion homeostasis” GO terms were significantly enriched, which were in line with reported Mac1 function. In addition, “de novo NAD⁺ biosynthetic process from tryptophan” and “quinolinate biosynthetic process” GO terms were also enriched, supporting a role for Mac1 in de novo NAD⁺ metabolism. KEGG pathway mapping analysis also identified “tryptophan metabolism” as the most significantly enriched pathway (first, ranked by p values) in genes co-up-regulated by mac1Δ and hst1Δ. Consistent with these studies, expression of the de novo NAD⁺ metabolism–associated BNA genes (BNA1, -2, -4, -5, and -6) was up-regulated in hst1Δ and mac1Δ cells (Fig. 3A, left). On the other hand, high-affinity copper transporter CTR1 was specifically down-regulated in mac1Δ cells (Fig. 3A, right). These studies showed that in addition to being a transcription activator, Mac1 appeared to repress BNA gene expression.

Differential expression of additional NAD⁺ homeostasis genes was also observed in mac1Δ and hst1Δ cells (Fig. 3A), including NAD⁺ intermediate transporters TNA1 and NRT1, and components of the phosphate-sensing PHO signaling pathway (26). Co-up-regulation of TNA1 (NA and QA transporter) and NRT1 (NR transporter) in hst1Δ and mac1Δ cells (Fig. 3A, left) was in line with the function of Hst1 as a NAD⁺-regulated transcriptional repressor of NAD⁺ homeostasis genes, and with the fact that hst1Δ and mac1Δ cells shared similar NAD⁺ phenotypes. Expression of phosphatases (PHOS, -11, -12) and high-affinity phosphate transporter (PHO84) was increased in hst1Δ cells (Fig. 3A, left); however, it was either unchanged or decreased in mac1Δ cells (Fig. 3A, right). To further understand the roles of these factors in NAD⁺ metabolism and to validate the expression profile results, we first carried out qPCR analysis in WT, mac1Δ, and hst1Δ cells. Fig. 3B showed that mac1Δ mutation increased BNA gene expression to a similar extent as seen in the hst1Δ mutant. The expression patterns of BNA, PHO, and CTR1 genes were in agreement with expression profile analysis (Fig. 3, A and B). Next, we examined whether altered PHO gene expression indeed correlated with PHO signaling activities and whether it contributed to increased de novo NAD⁺ synthesis. Previous studies associated PHO activation with increased NR and NA/NAM production (21, 26). We determined PHO activity by measuring the activity of rAPase (repressible acid phosphatase) Pho5, a periplasmic phosphatase activated by PHO signaling (35). As shown in Fig. 3C, PHO activity was moderately increased in the hst1Δ mutants but not in the mac1Δ mutant, which was consistent with observed PHO gene expression patterns (Fig. 3, A and B). Interestingly, observed PHO activation in hst1Δ cells appeared partially independent of the conventional PHO transcription factors Pho4-Pho2 complex, because deleting PHO4 did not completely reduce PHO activation in hst1Δ cells. This study suggested that PHO activation is not essential for enhanced de novo NAD⁺ synthesis in mac1Δ cells, and that Hst1 can activate specific PHO downstream targets independent of Pho4-Pho2.

It has been shown that during copper deprivation, Mac1 protein is stabilized and turns on copper homeostasis genes, including CTR1, whereas high copper concentrations facilitate the degradation of Mac1 (36, 37). Copper deprivation was also reported to induce BNA gene expression in strains defective in copper homeostasis (29, 38, 39). We therefore examined whether copper alterations would affect BNA gene expression in WT cells. As controls, expression of CTR1 (Fig. 3D, top right) was induced by copper deprivation (0 μM), which was repressed by nutritional level (10 μM, normal) and a high level (250 μM) of copper. Interestingly, both low- and high-copper conditions appeared to induce the expression of a few BNA genes (Fig. 3D). Consistent with this, we showed that cells grown in media containing a nutritional copper level (10 μM) produced the lowest amount of QA and that both copper deprivation (0 μM) and high copper stress (≥250 μM) increased QA production (Fig. 3E). High copper stress facilitates Mac1 degradation, which could explain the de-repression of BNA genes and QA production. However, copper might affect BNA gene expression independent of Mac1 because copper deprivation also moderately increased BNA gene expression and QA production. Interestingly, NAD⁺ and NADH levels did not correlate with increased QA production under copper stress conditions (Fig. 3F). In fact, both NAD⁺ and NADH levels were slightly reduced by high copper stress (Fig. 3F). It is possible that NAD⁺ and its derivatives were consumed to offset the damages and redox changes caused by copper stresses. Together, these studies demonstrated a link between copper stress, de novo QA production, and NAD⁺ metabolite homeostasis.

We next asked how might Mac1 and Hst1 co-repress BNA genes. Because mac1Δ and hst1Δ did not show a synergetic effect on NAD⁺ homeostasis (Figs. 1E and 2 (A–E)), they likely function in a linear pathway to regulate BNA genes. As a transcription activator, Mac1 may activate HST1 expression, which then represses BNA gene expression. This scenario was unlikely because expression of HST1 was not altered in mac1Δ cells and vice versa (Fig. 3B). Mac1 has been shown to bind to the copper-response element (CuRE), TTTGC(TG)C(A/G) sequences, which are present in the promoters of copper homeostasis genes (40, 41). However, this consensus Mac1-binding motif is absent in the promoters of BNA genes. Hst1 and associated proteins have been reported to bind to the promoter of BNA2 (42). We therefore examined whether Mac1 required Hst1 and vice versa to bind to the BNA2 promoter. To address this question, ChIP studies of various BNA2 promoter fragments using HA-tagged Hst1 (Hst1-HA) and Mac1 (Mac1-HA) were carried out. Fig. 4A (top) showed Mac1 and Hst1 protein levels in strains used for this study. Deleting HST1 did not significantly alter Mac1 protein levels; likewise, deleting MAC1 also did not alter Hst1 protein levels. Note that Mac1 was overexpressed for the ChIP studies because the Mac1 protein level was low and unstable in cells grown under nutritional levels of copper (standard conditions). Because all mac1Δ-associated NAD⁺ homeostasis phenotypes were observed under standard conditions, we overexpressed Mac1 to enhance the sensitivity and to allow
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Figure 4. Mac1 binds to the promoter of BNA2 gene in an Hst1-dependent manner. A, design of the ChIP studies. HA-tagged Hst1 was generated in both WT and mac1Δ cells. HA-tagged and overexpressed Mac1 was generated in WT and hst1Δ cells. Expression was confirmed via Western blot analysis (top). BNA2 promoter regions for ChIP analysis are shown as BNA2-a, BNA2-b, BNA2-c, BNA2-d, and BNA2-e (bottom). B, Hst1 bound to the promoter regions of BNA2 in both WT and mac1Δ cells with the highest binding activity near BNA2-e. Relative IP levels were normalized to TAF10. TNA1 is a previously reported positive control. C, Mac1 showed the most significant binding to the BNA2-e region of the BNA2 promoter in WT but not in cells lacking HST1. D, cells overexpressing HA-tagged MAC1 show WT-level QA release (left). Mutants of the Hst1-Sum1-Rfm1 complex components show increased QA release (right). Error bars, S.D. The p values are calculated using Student’s t test (*, p < 0.05).

further studies to be carried out under normal copper conditions. Fig. 4A (bottom) illustrates BNA2 promoter fragments (BNA2-a, -b, -c, -d, and -e) examined in ChIP studies. As shown in Fig. 4B, Hst1 was bound to all five BNA2 promoter fragments, with the most significant binding activity near the BNA2-e region. Deleting MAC1 did not significantly alter Hst1 binding to BNA2 promoter fragments, indicating that Hst1 did not require Mac1 to bind to BNA2 promoter (Fig. 4B). On the other hand, Mac1 only showed significant binding near the BNA2-e region (Fig. 4C). Interestingly, Mac1 binding to the BNA2-e region was abolished in hst1Δ cells, indicating that Hst1 is essential for Mac1 binding. It remained possible that Mac1 also bound to other regions of the BNA2 promoter; however, our assay conditions were not sensitive enough to detect the binding. It was also likely that the Mac1 overexpression might hinder its binding activity. This was less likely because cells overexpressing Mac1 still showed normal QA release (Fig. 4D, left), indicating that overexpression did not significantly affect Mac1 function at BNA promoters. Among Hst1-associated protein complexes, the Hst1-Sum1-Rfm1 complex appeared to play a key role in BNA repression because both sum1Δ and rfm1Δ mutants were also identified in our screen and showed increased QA release (Fig. 4D, right). Overall, these studies suggested that Mac1 works with the Hst1-associated repressor complex to regulate BNA genes, and deleting MAC1 or HST1 alone is sufficient to abolish the repression.

Regulation of de novo NAD⁺ synthesis by NA salvage

An unexpected phenomenon was observed while developing the QA cross-feeding reporter system, which indicated that de novo NAD⁺ synthesis is also regulated by an Hst1/Mac1-independent mechanism. In the reporter system, QA released by the feeder cells (mutants of interest) supported the growth of the QA-dependent recipient cells (readout for QA release). When grown on standard rich medium YPD, all strains tested, including the hst1Δ and mac1Δ mutants, appeared to be defective in QA production and failed to support the recipient cell growth (Fig. 5A, panel 1). On the other hand, cells grown on minimal synthetic medium SD were able to produce QA in an Hst1- and Mac1-regulated manner (Fig. 5A, panel 2). NA was shown to repress de novo NAD⁺ synthesis, and the amount of NA differed significantly in standard SD (0.4 mg/liter, ~3.3 μM) and YPD (~13–61 mg/liter, ~100–500 μM). However, NA-mediated repression was suggested to require NA salvage into NAD⁺ and NAD⁺-dependent activation of Hst1 (30). These observations prompted us to examine whether NA played a role in this seemingly Hst1/Mac1-independent repression. As shown in Fig. 5A (panels 2–5), QA levels released by WT, mac1Δ, and hst1Δ cells indeed inversely correlated with NA levels (0 mg/liter, NA-free SD; 0.4 mg/liter, SD/normal; ~61 mg/liter, high NA) in growth media. In contrast, NAM enhanced QA release, and it did not further increase QA release
in hstΔ and mac1Δ cells (Fig. 5A, panel 6). Although NAM can be deamidated into NA, it also inhibits the activity of the Sir2 family deacetylases (43, 44). Therefore, NAM was anticipated to induce hstΔ-like phenotypes. These results indicated that high NA–mediated inhibition of QA production did not require Hst1 and Mac1 (Fig. 5A, panels 2–4). However, elevated QA release observed in WT cells grown in NA-free media could be rescued by restoring NAD⁺ levels via supplementing NR (Fig. 5B), and that NR-mediated rescue required functional HST1 (Fig. 5B). Thus, it appeared that NA could inhibit de novo pathway activity in both an NAD⁺/Hst1-dependent (Fig. 5B) and an NAD⁺/Hst1-independent manner (Fig. 5A), depending on the NA levels in growth media.

Interestingly, NA-induced inhibitions were not completely due to repression of BNA genes. In WT cells, the expression of most BNA genes inversely correlated with the levels of NA in growth media (Fig. 5C). However, in hstΔ and mac1Δ cells, elevated BNA expression was not altered by NA treatments.

**Figure 5. NA inhibits the activity of the de novo pathway.** A, levels of QA production inversely correlate with the concentrations of NA present in the growth media. Results show released QA levels in WT, hstΔ, and mac1Δ cells grown in the following media: YPD, NA-free SD, SD (normal), and SD supplemented with NA (+NA) or NAM (+NAM). NAM is a competitive inhibitor of the NAD⁺–dependent deacetylases. B, NA inhibits QA production in an NAD⁺/Hst1-dependent manner. Elevated QA release in WT cells grown in NA-free media was rescued by restoring NAD⁺ levels via supplementing NR (at 20 μM, a NAD⁺ precursor). Hst1 was required for the rescue. C, most BNA gene expression in WT cells shows an inverse correlation with NA levels in growth media. Cells were grown in NA-free, SD (normal) and + NA (at 123 mg/liter) media. Relative gene expression determined by qPCR (normalized to TAF10) is shown. D, BNA gene expression in hstΔ and mac1Δ cells is not altered by NA levels. Cells were grown in NA-free, SD (normal), and + NA (at 123 mg/liter) media. Relative gene expression determined by qPCR (normalized to TAF10) is shown. E, QA uptake is inhibited by NA in a dose-dependent manner, which is alleviated by increasing QA concentrations. The QA-dependent npt1Δ/Δnrk1Δ/bna4Δ mutant was grown in SD containing different levels of QA and NA. The extent of growth (A₆₀₀) of this mutant correlates with its QA uptake efficiency. F, NA-mediated inhibition of QA production can be rescued by supplementing specific intermediates of the de novo pathway. Results show relative QA production in WT cells grown in SD supplemented with NA (40 mg/liter) and L-Kyn (1 mM) or 3-HK (0.25 mM). G, BNA2 overexpression (Bna2-oe) restores QA release in WT cells grown in SD supplemented with NA (20 mg/liter). Error bars, S.D. The p values are calculated using Student’s t test (*, p < 0.05).
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(Fig. 5D), indicating that mac\(\Delta\) and \(hst1\Delta\) cells still produced elevated levels of QA regardless of NA levels. This suggested that high NA might actually hinder growth of the QA-dependent recipient cells, masking the QA cross-feeding readout (Fig. 5A, panels 1, 3, and 4). The NA transporter Tna1 has been reported to also transport QA (22). It was shown that the \(tna1\Delta\) mutant failed to transport QA (1–3.3 \(\mu\)M) and thus exhibited growth defects in NA-free media under anaerobic conditions in which the de novo NAD\(^+\) pathway is completely blocked and the cells depend on QA transport for growth (22). Because Tna1 can transport both NA and QA, it is possible that high NA competes with QA for Tna1-mediated transport. To address this, we examined whether high NA could inhibit QA uptake by exploiting the QA-dependent \(npt1\Delta nrk1\Delta bna4\Delta\) recipient cell mutant. This mutant cannot utilize NA (due to \(npt1\Delta\) mutation); therefore, its de novo pathway gene expression is not altered by NA treatments. As a result, the extent of cell growth (\(A_{600}\)) of this mutant in media containing different levels of QA and NA correlates with its QA uptake efficiency. As shown in Fig. 5E, NA indeed inhibited QA uptake in a dose-dependent manner. At 5–10 \(\mu\)M NA (approximate NA levels in SD), no inhibition of QA uptake was observed. High NA (\(\geq 100 \mu\)M) showed the most significant inhibition of QA uptake when QA was supplemented at 1–5 \(\mu\)M, which was similar to reported QA levels released by WT cells in SD (22). Observed NA inhibitions of QA uptake could be alleviated by increasing QA concentrations (at 10–50 \(\mu\)M) (Fig. 5E), suggesting that NA competes with QA for Tna1-mediated transport. It remains possible that NA may also inhibit intracellular QA utilization. One possible target is the enzymatic activity of Bna6, a quinolinate phosphoribosyltransferase (QPRT) that converts QA into NaMN (Fig. 1A). However, it has been shown that NA does not inhibit QPRT activity (45, 46). Together, these results suggested that high NA could also inhibit the de novo pathway activity by competing with QA transport, which is independent of NA salvage into NAD\(^+\) and Hst1-mediated BNA gene repression.

Nevertheless, Hst1-mediated BNA gene repression remains the major mechanism of NA/NAD\(^+\)-induced inhibition of de novo QA/NAD\(^+\) synthesis. To further understand this regulation, we first determined which BNA gene was most sensitive to NA inhibition using QA production as readout. A simplified de novo QA production pathway was illustrated in the bottom panel of Fig. 5F. The QA-dependent \(npt1\Delta nrk1\Delta bna1\Delta\) mutant was used as the recipient cells whose growth correlates with the levels of QA released by the WT donor cells. As shown in Fig. 5F (top), levels of QA released by WT donor cells were inhibited by NA (at \(\leq 40\) mg/liter NA to minimize its inhibition of QA uptake). This inhibition could be rescued by supplementing 1-KYN or 3-HK (Fig. 5F, top) suggesting that the expression of BNA2 or BNA7 in the WT donor cells was most sensitive to NA-induced Hst1-mediated repression (Fig. 5F, bottom). Because BNA7 expression appeared less sensitive to NA alterations (Fig. 5C) and \(hst1\Delta\) mutation (Fig. 5D), we directly examined BNA2. As shown in Fig. 5G, overexpressing BNA2 was sufficient to increase QA release (left) and rescue NA inhibition (right) in WT cells. As controls, overexpressing BNA4 did not show significant effects on QA release. These results indicate that BNA2 expression is most sensitive to Hst1- and NA-mediated repression, and therefore it is likely a rate-limiting step in de novo QA production.

Discussion

In this study, we identified Mac1 as a novel NAD\(^+\) homeostasis factor in yeast. Mac1 was previously characterized as a copper-sensing transcription activator (27–29). Cells lacking \(MAC1\) shared similar NAD\(^+\) phenotypes with the \(hst1\Delta\) mutant. The NAD\(^+\)-dependent histone deacetylase Hst1 has been suggested to be an NAD\(^+\) sensor, which inhibits de novo NAD\(^+\) synthesis by repressing BNA gene expression when NAD\(^+\) is abundant (Fig. 6) (30). We showed that deleting either \(MAC1\) or \(HST1\) was sufficient to abolish BNA gene repression (Fig. 3, A and B). Although BNA gene promoters lack Mac1-binding sequences CuRE, Mac1 proteins likely associate with BNA promoters with help from the Hst1 complex (Fig. 4C). On the other hand, Hst1 binding to the BNA2 promoter does not require functional Mac1 (Fig. 4B), indicating that binding of the Hst1 complex precedes Mac1 at the BNA2 promoter. Together, these results suggest that Mac1 proteins work with the Hst1-containing repressor complexes (Hst1, Rfm1, and Sum1) formed at the BNA promoters to repress gene expression. It remains unclear how Mac1 proteins work with the Hst1 complexes to mediate BNA gene repression. Although Mac1 and Hst1 complex components did not appear to directly interact in systematic pulldown studies (47, 48), they may interact under...
specific conditions. Mac1 and Sum1 were reported to share a common interacting partner, histone H4 (Hhf1) (47, 48), suggesting a possible interaction model. Interestingly, both Mac1 and Hst1 proteins showed higher binding activities at the BNA2 promoter regions near the start codon (Fig. 4, B and C), suggesting that Mac1 and Hst1 binding to these regions is more critical for BNA2 gene repression. Further studies are required to understand how Mac1 contributes to Hst1-mediated BNA gene repression.

Our studies also uncovered additional NA-mediated regulation of the de novo pathway. The NAD⁺ pool is maintained by NA and NR salvage pathways and de novo NAD⁺ synthesis from tryptophan (Fig. 6). Under NA-abundant conditions, NA salvage is the preferred NAD⁺ synthesis route (30, 34), which leads to Hst1 activation and BNA gene repression. As cells consume NA, eventually NA/NAD⁺ deprivation decreases Hst1 activity, leading to de-repression of BNA genes, QA production, and de novo NAD⁺ synthesis. Supporting this, cells grown in NA-free media showed increased BNA gene expression (Fig. 5C) and QA production (Fig. 5A). Supplemented NR to NA/NAD⁺-deprived cells was sufficient to restore their NAD⁺ levels and repression of QA production (Fig. 5B). It appears that QA production occurs before complete depletion of NA/NAD⁺ because QA release is detectable in log-phase WT cell culture, which is further increased in stationary phase culture (22). Most excess QA (if not converted to NAD⁺) is released extracellularly (Fig. 2A) and can re-enter cells via Tna1 (also a high-affinity NA transporter) (22) as the NA levels decline. Supporting this, the high QA-producing hst1Δ and mac1Δ mutants showed more significant increases in NAD⁺ levels when NA was depleted in the growth media (Fig. 2, E and F). Thus, NA inhibits de novo NAD⁺ synthesis both by repressing QA production (Fig. 5, B and C) and by competing with QA transport (Fig. 5E); the latter is particularly significant when cells are grown in rich media, which contain high levels of NA (100–500 μM) (Fig. 5A, panel I). We have also shown BNA2 expression is most sensitive to NA-mediated repression (Fig. 5F), and BNA2 is a rate-limiting step in QA production (Fig. 5G). Overall, these studies provide further understanding of the dynamic interactions between NA salvage and de novo NAD⁺ synthesis.

Additional nutritional stresses also play a role in regulating de novo NAD⁺ synthesis. Copper has been shown to negatively regulate Mac1-dependent transcription activation. Under copper deprivation, Mac1 remains stable and activates expression of genes in the high-affinity copper transport system (36, 37, 49). Upon copper repletion, copper binding to Mac1 causes an intramolecular interaction between its N-terminal DNA binding domain and C-terminal trans-activating domain, resulting in a transcriptionally inactive state. Under high-copper conditions, Mac1 is quickly degraded to avoid copper toxicity (36, 37, 49). Thus, the copper-bound form of Mac1, although transcriptionally inactive, is likely to bind to and repress BNA gene promoters because our studies were carried out under nutritional copper levels. High copper stress caused Mac1 degradation (36, 37, 49), which was in line with increased BNA gene expression (Fig. 3D) and QA production (Fig. 3E). Interestingly, copper deprivation also increased BNA expression (Fig. 3D) and QA production (Fig. 3E). It is possible that the transcriptionally active form of Mac1 binds to CuRE promoters more efficiently than to the BNA promoters during copper deprivation. Alternatively, copper may regulate BNA gene expression independent of Mac1. Together, these studies demonstrate that dynamic Mac1 pool is shared between the Hst1-containing repressor complexes at BNA promoters and the transcription-activating complexes at CuRE-containing promoters. However, NAD⁺ levels did not appear to correlate with increased QA production under copper stress conditions (Fig. 3F). It is likely that NAD⁺ is consumed or converted to NAD⁺ derivatives, such as NADH, NADP⁺, and NADPH, to maintain proper redox state and to prevent and counteract copper stress-induced oxidative damages. In addition, copper may inhibit the activities of NAD⁺ metabolic enzymes. For example, copper was shown to inhibit the activity of QPRT (50, 51). It is also possible that under stress conditions, it is beneficial for cells not to increase NAD⁺ levels to maintain an optimal metabolic state.

It appears that once QA is produced from de novo synthesis, it is either released to the extracellular environment or converted to NAD⁺ (Fig. 6). In yeast, released QA can re-enter cells to support NAD⁺ synthesis via the NA transporter (22). Yeast cells also release and re-uptake other small NAD⁺ precursors that arise from NA/NAM salvage and NR salvage, such as NA, NAM, and NR (1, 21, 52). It has been suggested that vesicular trafficking and vacuolar function play a role in the production and release of these NAD⁺ precursors (1, 21, 52). However, the detailed mechanisms are not completely understood. There appears to be a storage pool for NAD⁺ precursors generated from the salvage pathways. For example, cytoplasmic NR is released to the environment or transported into the vacuole for storage if not converted to NAD⁺. Likewise, vacuolar NR may exit the vacuole to support NAD⁺ synthesis in the cytoplasm. The equilibrative nucleoside transporter Fun26 (human lysosomal hENT homolog) controls the balance of NR and possibly other nucleosides between the vacuole and the cytoplasm (26). It is possible that vacuolar degradation of NAD⁺ intermediates coincides with NAD⁺ salvage. NAD⁺ intermediates may enter the vacuole through vesicular transport and autophagy and are then broken down into smaller precursors for storage or reuse. On the other hand, when excess QA is made in the cytoplasm, it is mostly released and not stored in the vacuole. Interestingly, the fun26Δ mutant showed increased QA production/release (Table S1). This is likely due to the blockage of NR salvage-mediated NAD⁺ synthesis because NR is trapped inside the vacuole in the fun26Δ mutant. We have shown that NAD⁺ depletion causes BNA gene de-repression and that NR supplementation restores the repression in an Hst1-dependent manner (Fig. 5B). In line with this, cells lacking the NAM deamidase PNC1 also showed increased QA production (Table S1). The pnc1Δ mutation causes accumulation of NAM (21), which is anticipated to inhibit Hst1 activity. Therefore, increased QA production observed in the fun26Δ and pnc1Δ mutants was likely due to loss of Hst1-mediated repression of BNA genes. Together, these studies demonstrate a complex interconnection between the de novo and salvage pathways.
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In summary, our studies have uncovered novel NAD$^+$ homestasis factors. Hst1 and Mac1 may play important roles in the cross-regulation of de novo NAD$^+$ synthesis and other nutrient-sensing pathways. Copper levels determine the conformation and stability of the Mac1 proteins, resulting in corresponding changes in de novo QA production and copper homestasis. In addition, increased phosphate-sensing PHO activity was also observed in the hst1Δ mutant. Activation of PHO has been associated with increased NR (26) and NA/NAM (21) production, suggesting that Hst1 may also affect NR and NA/NAM salvage activities via regulating specific components in the PHO pathway. Recent studies have demonstrated that NAD$^+$ metabolism is a therapeutic target for several human diseases. This strategy was more effective when specific defects in NAD$^+$ biosynthesis were identified and associated with the progression of 2,3-dioxygenase (TDO; Bna2 in yeast) (55) and kynurenine (24). Recent studies have also shown that inhibiting the activities of de novo pathway enzymes, such as tryptophan 2,3-dioxygenase (TDO; Bna2 in yeast) (55) and kynurenine 3-monooxygenase (KMO; Bna4 in yeast) (56), as well as increasing the ratio of neuroprotective KA over neurotoxic QA or 3-HK, may help to alleviate a few neurological disorders (24, 55). Understanding the molecular basis and interconnection of multiple NAD$^+$ metabolic pathways is important for the development of disease-specific therapeutic strategies. Overall, our studies contribute to the understanding of the regulation of NAD$^+$ metabolic pathways and may provide insights into the underlying mechanisms of disorders associated with aberrant NAD$^+$ metabolism.

Experimental procedures

**Yeasts, growth media, and plasmids**

Yeast strain BY4742 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 was acquired from Open Biosystems (57). Standard media, including yeast extract-peptone-dextrose (YPD), SD, and SC media were made as described (58). NA-free and NC-free SC were made by using niacin-free yeast nitrogen base (Sunrise Science Products). Single gene deletion mutants were generated by replacing the WT genes with a reusable loxp-kanMX-loxp cassette as described (59). Making mutants with multiple gene deletions employed a galactose-inducible Cre recombination to remove the reusable loxp-kanMX-loxp cassette, followed by another round of gene deletion (59). In strains carrying nonreusable kan markers, gene deletion was introduced by using a hygromycin resistance marker (pAG32-hphMX4) (60). The HA epitope tag was added to target genes directly in the genome using the pFA6a-3HA-kanMX6 (HST1) or pFA6a-kanMX6-PGA1–3HA (MAC1) plasmids as template for PCR-mediated tagging (61). The BNA2 and BNA4 overexpression plasmids, pADH1-BNA2 and pADH1-BNA4, were made in the integrative pPP81 (LEU2) vector and were introduced to yeast cells as described (62).

**QA cross-feeding spot assays**

The npt1Δntrk1Δbna1Δ and npt1Δntrk1Δbna4Δ mutants (which depend on QA for growth) were used as “recipient cells,” and yeast strains of interest were used as “feeder cells.” First, recipient cells were plated onto SD as a lawn (~$10^6$ cells/cm$^2$). Next, ~$2 \times 10^4$ cells of each feeder cell strain (2 μl of cell suspension made in sterile water at $A_{600}$ of 1) were spotted onto the lawn of recipient cells. Plates were then incubated at 30 °C for 3 days. The extent of the recipient cell growth indicates the levels of QA released by feeder cells.

**Genetic screen using the yeast deletion collection**

The haploid yeast deletion collection (~4,500 strains) established in the BY4742 strain was acquired from Open Biosystems (63). To screen for mutants with altered QA release, 2 μl of each strain was directly taken from the frozen stock and then spotted onto SD plates spread with the npt1Δntrk1Δbna4Δ recipient cells at a density of ~$10^6$ cells/cm$^2$. After incubation at 30 °C for 3 days, we scored the cross-feeding activity (which indicates the level of QA release) of each mutant by comparing the diameter of the cross-feeding zones with that of the WT. Mutants were assigned a score of −4 through 4 (WT = 0). Scores of −4, −3, and −2 indicate decreased QA release; scores of +2, +3, and +4 indicate increased QA release. To eliminate false-positives, mutants with scores of −4, −3, 3, and 4 (169 mutants) were re-examined. A total of 81 mutants passed the secondary screen on both SD and SC growth media (Table S1).

**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 (62). Levels of NAD$^+$ intermediates (QA, NR, and NA/NAM) were determined by a liquid-based cross-feeding bioassay as described previously (20,21,26) with modifications. To prepare cell extracts for intracellular NAD$^+$ intermediates determination, ~150 $A_{600}$ unit (1 $A_{600}$ unit = $1 \times 10^7$ cells/ml) donor cells grown to late logarithmic phase in SC (~16 h of growth from an $A_{600}$ of 0.1) were collected by centrifugation and lysed by bead-beating (Biospec Products) in 800 μl of ice-cold 50 mM ammonium acetate solution. After filter sterilization, 100 μl of clear extract was used to supplement 8-ml cultures of recipient cells with starting $A_{600}$ of 0.05 in SC. To determine extracellular NAD$^+$ intermediates levels, supernatant of donor cell culture was collected and filter-sterilized, and then 500 μl was added to 7.5 ml of recipient cell culture with total starting $A_{600}$ of 0.05 in SC. A control culture of recipient cells in SC without supplementation was included in all experiments. For measuring relative QA levels, npt1Δntrk1Δbna4Δ and npt1Δntrk1Δbna1Δ mutants were used as recipient cells. The npt1Δbna6Δpho5Δ recipient cells were used to measure relative NR levels. To measure relative NA/NAM levels, the bna6Δntrk1Δnrt1Δ recipient cells were grown in NA-free SC. After incubation at 30 °C for 24 h, growth of the recipient cells ($A_{600}$) was measured and normalized to the cell number of each donor strain. $A_{600}$ readings were then converted to concentrations of QA, NR, and NA/NAM using the standard curves established as described previously (Fig. S1) (20, 21).
**Gene expression profile analysis**

Approximately 40 $A_{600}$ unit cells grown to early logarithmic phase in SD (6-h growth from an $A_{600}$ of 0.1) were collected by centrifugation. Total RNA was extracted using the GeneJET RNA purification Kit (Thermo Scientific). High-quality DNA-free RNA for sequencing was made using the RNA Clean and Concentrator kit (Zymo Research). For each sample, 10 μl of RNA (at 100 ng/μl) was used to generate 3’-Tag-Seq libraries (a single initial library molecule per transcript, complementary to 3’-end sequences, was generated). The libraries were then sequenced by single-end sequencing on the HiSeq 4000 (DNA Technology Core, University of California, Davis, CA). Three biological triplicates of each strain were sequenced. Differential gene expression testing was conducted using a single-factor analysis of variance model in the limma-voom Bioconductor pipeline (Bioinformatics Core, University of California, Davis, CA). Prior to analysis, genes with expression less than 1 count per million reads were filtered, leaving 6,023 of 7,126 genes. The multidimensional scaling plot shows the distance between samples based only on normalized counts. In this experiment, the WT samples clustered together and were very different than the mutants, as expected. GO enrichment analyses of differential gene expression results were conducted using Kolmogorov–Smirnov tests in the Bioconductor package topGO. For each GO term, the KS test tests whether genes annotated with that GO term are smaller than for genes not annotated with that GO term. KEGG enrichment analyses were conducted using Wilcoxon rank sum tests, in conjunction with the Bioconductor package KEGGREST. For each KEGG pathway, the Wilcoxon rank-sum test tests whether $p$ values for genes in that pathway are smaller than genes not in that pathway. Enrichment analyses of genes that are significantly up or down in both $mac1Δ$ and $hst1Δ$ mutants relative to WT were obtained by KEGG and GO enrichment analyses of gene lists from Venn diagrams. Fisher’s exact test was used to test whether the number of genes in the Venn list in a given pathway/GO term was greater than would be expected by chance.

**Quantitative PCR (qPCR) analysis of gene expression levels**

Approximately 40 $A_{600}$ unit cells grown to early logarithmic phase in SD (6 h of growth from an $A_{600}$ of 0.1) were collected by centrifugation. Total RNA was isolated using the GeneJET RNA purification Kit (Thermo Scientific), and cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer’s instructions. For each qPCR, 50 ng of cDNA and 500 nM each primer were used. The qPCR was run on a Roche LightCycler 480 using LightCycler Green I Master Mix (Roche Applied Science) as described previously (64). The average size of the amplicon for each gene was ~150 bp. The target mRNA transcript levels were normalized to TAF10 transcript levels.

**rAPase activity assay**

The rAPase liquid assay was carried out as described (35) using cells grown to late log phase in SC. In brief, 2.5 $A_{600}$ unit cells were harvested, washed, and resuspended in 150 μl of water. Next, 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 of ice-cold 10% TCA. 600 μl of this final mixture was then added to 600 μl of saturated Na₂CO₃ to allow color (neon yellow) development. Cells were pelleted to acquire the supernatant for $A_{420}$ determination. The rAPase activities were determined by normalizing $A_{420}$ readings to total cell number ($A_{600}$).

**Protein extraction and Western blot analysis**

A total of 100 ml of cells were grown in SD to early logarithmic phase ($A_{600}$ of ~1) and 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% Nonidet P-40 (Sigma), and protease inhibitor mixture (Pierce™)). The protein concentration was measured using the Bradford assay (Bio-Rad), and 30 μg of total protein was loaded in each lane. After electrophoresis, the protein was transferred to a nitrocellulose membrane (Amer sham Biosciences™ Protran™, GE Healthcare). The membranes were then washed and blotted with either anti-HA antibody (Invitrogen) or anti-β-actin antibody (Abcam). Protein was visualized using anti-mouse IgG antibody conjugate to the horseradish peroxidase (GE Healthcare) and the ECL reagents (Amer sham Biosciences™, GE Healthcare). The chemiluminescent image was analyzed using the Amer sham Biosciences Imager 600 (GE Healthcare) system and software provided by the manufacturer.

**ChiP assay**

Approximately 500 $A_{600}$ unit cells grown to early logarithmic phase in SD were cross-linked with 1% formaldehyde for 30 min at room temperature and stopped by adding glycin e to a final concentration of 125 mM. Cells were pelleted by centrifugation and washed two times with cold TBS (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 HEPES, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1 mM PMSF, 1× protease inhibitor mixture (Pierce)) (33). 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 three tubes (labeled “input,” “IP,” and “no-Ab”). The IP samples were incubated overnight at 4 °C with anti-HA mAb (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 (33). DNA was then eluted from the beads two times with 125 μl of elution buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS). The combined DNA solution and input samples were incubated at 65 °C overnight to reverse the cross-linking. The purified DNA samples were analyzed by qPCR, and results were compared with a standard curve prepared from input DNA. The amount of immunoprecipitated specific promoter DNA

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was determined relative to no-Ab DNA. S.D. values were calculated from the results of three independent biological replicates.

**Author contributions**—C. J. T. R. and S.-J. L. conceptualization; C. J. T. R., G. D., T. Cater, and S.-J. L. data curation; C. J. T. R. and T. Croft formal analysis; C. J. T. R., T. Croft, B. G., and G. D. validation; C. J. T. R., T. Croft, P. V., B. G., G. D., and T. Cater investigation; C. J. T. R., T. Croft, P. V., B. G., and S.-J. L. visualization; C. J. T. R., T. Croft, P. V., and B. G. methodology; C. J. T. R. and S.-J. L. writing-original draft; C. J. T. R., T. Croft, P. V., B. G., and S.-J. L. writing-review and editing; S.-J. L. supervision; S.-J. L. funding acquisition; S.-J. L. project administration.

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