### Title

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# Cross-talk in NAD+ metabolism – insights from *Saccharomyces cerevisiae*

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## Abstract

$NAD^+$ (nicotinamide adenine dinucleotide) is an essential metabolite involved in a myriad of cellular processes. The $NAD^+$ pool is maintained by three biosynthesis pathways, which are largely conserved from bacteria to human with some species-specific differences. Studying the regulation of $NAD^+$ metabolism has been difficult due to the dynamic flexibility of $NAD^+$ intermediates, the redundancy of biosynthesis pathways, and the complex interconnections among them. The budding yeast *Saccharomyces cerevisiae* provides an efficient genetic model for the isolation and study of factors that regulate specific $NAD^+$ biosynthesis pathways. A recent study has uncovered a putative cross-regulation between the *de novo* $NAD^+$ biosynthesis and copper homeostasis mediated by a copper-sensing transcription factor Mac1. Mac1 appears to work with the Hst1-Sum1-Rfm1 complex to repress the expression of *de novo* $NAD^+$ biosynthesis genes. Here we extend the discussions to include additional nutrient- and stress-sensing pathways that have been associated with the regulation of $NAD^+$ homeostasis. $NAD^+$ metabolism is an emerging therapeutic target for several human diseases. $NAD^+$ preservation also helps ameliorate age-associated metabolic disorders. Recent findings in yeast contribute to the understanding of the molecular basis underlying the cross-regulation of $NAD^+$ metabolism and other signaling pathways.

### Keywords

$NAD^+$ metabolism; Sir2 family; nutrient signaling; stress signaling; transcription; gene silencing

## Introduction

$NAD^+$ and its derivatives NADH, $NADP^+$ and NADPH (pyridine nucleotides) are essential cofactors involved in various redox reactions. $NAD^+$ also contributes to the regulation of chromatin structure, DNA repair, circadian rhythm and lifespan (Chini et al. 2016; Imai and Guarente 2014; Kato and Lin 2014a; Nikiforov et al. 2015; Yoshino et al. 2018). Several human diseases have been associated with aberrant $NAD^+$ metabolism, including diabetes,

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cancer, and neuron degeneration (Canto et al. 2015; Chini et al. 2016; Garten et al. 2015; Liu et al. 2018; Nikiforov et al. 2015; Poyan Mehr et al. 2018; Schwarcz et al. 2012; Verdin 2015; Yang and Sauve 2016; Yoshino et al. 2018). Administration of $NAD^+$ precursors such as nicotinamide mononucleotide (NMN), nicotinamide (NAM), nicotinic acid riboside (NaR) and nicotinamide riboside (NR) has been shown to increase $NAD^+$ levels and ameliorate associated deficiencies in various model systems including human cells (Belenky et al. 2007; Brown et al. 2014; Canto et al. 2015; Chini et al. 2016; Garten et al. 2015; Lin et al. 2016; Liu et al. 2018; Poyan Mehr et al. 2018; Verdin 2015; Williams et al. 2017; Yang and Sauve 2016; Yoshino et al. 2018). However, the molecular mechanisms underlying the beneficial effects of $NAD^+$ precursor treatments are not completely understood. For example, it remains unclear how eukaryote cells transport and utilize $NAD^+$ precursors. Also, signaling pathways and cellular processes that contribute to the regulation of $NAD^+$ homeostasis remain largely unknown. Studying $NAD^+$ homeostasis is complicated by the dynamic flexibility of precursors cells use to generate $NAD^+$. For example, NAM can replenish $NAD^+$ pools either by entering the salvage pathway or by de-repressing the *de novo* pathway (Fig. 1). NAM also inhibits the activity of $NAD^+$ consuming Sir2 family proteins (Bitterman et al. 2002; Jackson et al. 2003). Recent studies in budding yeast *S. cerevisiae* have helped shed some light on the regulation of $NAD^+$ homeostasis.

## Overview of $NAD^+$ biosynthesis

Cellular $NAD^+$ pool is maintained by three major $NAD^+$ biosynthesis pathways (Fig.1), which are largely conserved from bacteria to humans. One pathway may dominate the others depending on the availability of specific $NAD^+$ precursors and the expression levels of specific $NAD^+$ biosynthesis enzymes. For example, nicotinic acid (NA) is the primary $NAD^+$ precursor present in standard yeast growth media, and NA/NAM salvage (Fig. 1, *center panel*) is the major $NAD^+$ synthesis route until NA is depleted (Bedalov et al. 2003; Sporty et al. 2009). During NA/NAM salvage, yeast cells recycle NAM from $NAD^+$ consusmption reactions (catalyzed by Sir2 family proteins) or uptake NA from the environment via NA transporter Tna1 (Llorente and Dujon 2000), leading to nicotinic acid mononucleotide (NaMN) production. NaMN is converted to deamido-$NAD^+$ (NaAD) by NaMN adenylyltransferases (Nma1/2) (Emanuelli et al. 1999), which is turned into $NAD^+$ by the glutamine-dependent $NAD^+$ synthetase (Qns1) (Bieganowski et al. 2003) (Fig. 1, *center panel*). Unlike higher eukaryotes including mammals, yeasts do not possess NAM phosphorybosyltransferase (NAMPT) (Revollo et al. 2004), the enzyme that converts NAM to NMN. Instead, NAM is deamidated to NA by Pnc1, and shunted into NA/NAM salvaging (Ghislain et al. 2002).

In the NR salvage cycle, NR is assimilated into NMN by the NR kinase Nrk1 (Bieganowski and Brenner 2004) or turned into NAM by nucleosidases Urh1/Pnp1/Meu1 (Belenky et al. 2007). NMN is converted to $NAD^+$ via NMN adenylyltransferases (Nma1/2 and Pof1) (Emanuelli et al. 1999; Kato and Lin 2014b), whereas NAM merges into the NA/NAM salvage route (Fig. 1, *right panel*). Nrk1 can also convert nicotinic acid riboside (NaR), a deamidated form of NR, to NaMN (Belenky et al. 2007). The nucleotidases, Isn1 and Sdt1 (Bogan et al. 2009), and phosphatases, Pho5 and Pho8 (Lu et al. 2009; Lu and Lin 2011), also contribute to NR metabolism by converting NMN to NR.

In the *de novo* branch, tryptophan is converted to quinolinic acid (QA) through five steps of enzymatic reactions catalyzed sequentially by Bna2, Bna7, Bna4, Bna5 and Bna1 (Fig. 1*, left panel*) (Panozzo et al. 2002). QA is then phosphoribosylated by Bna6 producing NaMN, which is also the converging point of the *de novo* pathway and NA/NAM salvage. Because Bna2, Bna4 and Bna5 require molecular oxygen as a substrate (Panozzo et al. 2002), yeast cells grown under anaerobic conditions rely on the salvage pathways for $NAD^+$ synthesis (Panozzo et al. 2002). The *de novo* pathway is also known as the kynurenine (KYN) pathway or tryptophan degradation pathway in mammals. In addition to becoming $NAD^+$, KYN can be converted to kynurenic acid (KA), which in yeast is mediated by the Aro8/9 aminotransferases (Ohashi et al. 2017).

It has also been shown that small $NAD^+$ precursors such as NR, NA, NAM and QA are released and re-uptake by yeast cells (Croft et al. 2018; Lu et al. 2009; Ohashi et al. 2013). This extended $NAD^+$ precursor pool may confer metabolic flexibility in response to environmental changes. Uptake of NA, QA and NR is mediated by specific transporters Tna1 (NA and QA) (Llorente and Dujon 2000; Ohashi et al. 2013) and Nrt1 (NR) (Belenky et al. 2008) (Fig. 1, *bottom*). Transport of large $NAD^+$ precursors, for example NMN, may require prior conversion to smaller precursors such as NR and NAM (Lu et al. 2009; Nikiforov et al. 2011). Release and re-uptake of $NAD^+$ precursors have also been reported in human cells (Kulikova et al. 2015). Although transporters for specific $NAD^+$ precursors in higher eukaryotes remain largely unclear, it was suggested that NR enters cells via the equilibrative nucleoside transporters (ENTs) (Ratajczak et al. 2016; Trammell et al. 2016), and that NA transport is likely mediated by specific carriers (Said et al. 2007). In a recently study, a $Na^+$-dependent NMN transporter has been identified in mice (Grozio et al. 2019).

## Regulation of $NAD^+$ homeostasis

Given the complex interconnections among $NAD^+$ biosynthetic pathways, studying the regulation of $NAD^+$ homeostasis has been challenging. Based on the observations that yeast cells constantly release and re-uptake small $NAD^+$ precursors (Lu et al. 2009; Ohashi et al. 2013), genetic screens targeting specific $NAD^+$ biosynthesis pathways have been developed. Yeast mutants with altered release activities of specific $NAD^+$ precursor(s) are likely defective in corresponding $NAD^+$ biosynthetic pathway(s). These $NAD^+$ precursor-specific screens have resulted in the identification of novel $NAD^+$ homeostasis factors (Croft et al. 2018; James Theoga Raj et al. 2019; Kato and Lin 2014a; Lu and Lin 2011). For example, a QA release-specific reporter system targeting the *de novo* branch was employed in a recent study, which has identified Mac1 as a novel $NAD^+$ homeostasis factor (James Theoga Raj et al. 2019). Mac1 is a copper-sensing transcription factor that activates copper transport genes during copper deprivation (Graden and Winge 1997; Gross et al. 2000; Jungmann et al. 1993). Interestingly, cells lacking *MAC1* shared similar $NAD^+$ phenotypes with the *hst1Δ* mutant and that deleting either *MAC1* or *HST1* was sufficient to abolish *BNA* gene repression (James Theoga Raj et al. 2019). Hst1 is a $NAD^+$-dependent histone deacetylase previously reported to inhibit *de novo* $NAD^+$ synthesis by repressing *BNA* gene expression when $NAD^+$ is abundant (Bedalov et al. 2003) (Fig. 2). Mac1 proteins likely work with the Hst1-containing repressor complexes (Hst1-Rfm1-Sum1) formed at the *BNA* promoters to

repress gene expression. Detailed mechanisms of how Mac1 proteins contribute to Hst1-mediated *BNA* gene repression remain to be determined.

The mechanisms of Mac1 regulation by copper help explain how may Mac1 function both as a transcription activator (for copper transport genes) and a co-repressor (for *BNA* genes). During copper deprivation, Mac1 proteins are stable and activate expression of copper transport genes. Upon copper repletion (nutritional level), copper binding to Mac1 results in a transcriptionally inactive state due to an intra-molecular interaction. When cells are exposed to high-copper conditions, Mac1 is quickly degraded to prevent excess copper import and toxicity (Jensen et al. 1998; Serpe et al. 1999; Zhu et al. 1998). Therefore, it is likely that under normal nutritional copper levels, the transcriptionally inactive (copper-bound) form of Mac1 works with the Hst1 complex to represses *BNA* genes. These studies also suggest copper stresses may impact *de novo* $NAD^+$ biosynthesis via Mac1. Supporting this, high-copper stress conditions, which cause Mac1 degradation, also induce *BNA* gene expression and QA production. Interestingly, copper deprivation also increases *BNA* expression and QA production, suggesting copper may regulate *de novo* activity via additional stress-signaling mechanisms (Fig. 2).

Several stress- and nutrient-sensing pathways have also been associated with $NAD^+$ metabolism (Fig. 2). Although these signaling pathways can increase the levels of specific $NAD^+$ precursors, cellular $NAD^+$ level does not always increase accordingly (Anderson et al. 2002; James Theoga Raj et al. 2019). It is possible that $NAD^+$ is converted to other pyridine nucleotides, which may be critical for maintaining proper redox state and cellular function under stress. Also, since specific $NAD^+$ intermediates as well as $NAD^+$ biosynthesis enzymes have additional function in other cellular processes (Kato and Lin 2014a; Tsang and Lin 2015), it is anticipated that $NAD^+$ homeostasis is under the control of multiple signaling pathways. Activation of the low phosphate (Pi)-sensing *PHO* pathway has been associated with increased NR metabolism (Lu and Lin 2011). Interestingly, *PHO* activation was also observed in the low-$NAD^+$ *npt1Δ* mutant, suggesting a cross-regulation between low-$NAD^+$ and low-Pi signaling (Lu and Lin 2011). Although the mechanisms remain unclear, coupling these pathways may be metabolically advantageous in certain conditions. For example, the Pi moiety of $NAD^+$ derivatives is potential target for Pi scavenging during Pi limitation. *PHO* activation was also observed in the *hst1Δ* mutant (James Theoga Raj et al. 2019) and cells with reduced amino acid sensing activity (Tsang et al. 2015). Other signaling pathways that may play a role in $NAD^+$ metabolism include PKA (cyclic-AMP activated protein kinase A), Sch9 (yeast Akt), and TOR (target of rapamycin) (Tsang and Lin 2015). Interestingly, these pathways appear to converge on Rim15 to regulate stress response transcription factors, Msn2 and Msn4 (Carroll et al. 2001; Swinnen et al. 2006; Wei et al. 2008). Msn2 and Msn4 have been shown to increase the expression of Pnc1, a NA/NAM salvaging enzyme (Medvedik et al. 2007), in response to various mild stresses including glucose and amino acid restriction (Anderson et al. 2003). Cells lacking Pnc1 accumulate excess NAM (Croft et al. 2018), which may result in inhibition of Sir2 and Hst1 activities (Bitterman et al. 2002; Jackson et al. 2003). It remains unclear whether these and yet-to-be-identified signaling pathways also regulate additional $NAD^+$ homeostasis factors.

The regulation of release and re-uptake of $NAD^+$ metabolites also contributes to $NAD^+$ homeostasis. It appears that yeast cells release and uptake small $NAD^+$ precursors via different mechanisms. Yeast cells uptake low μM levels of NR, NA and QA via specific transporters Nrt1 (NR) and Tna1 (NA and QA) (Belenky et al. 2008; Llorente and Dujon 2000; Ohashi et al. 2013). When supplemented at high levels, these $NAD^+$ precursors may enter cells by additional mechanisms. Interestingly, high μM levels of NA can hinder the re-uptake of released QA (James Theoga Raj et al. 2019) because QA and NA share the same transporter Tna1. Although it remains unclear how $NAD^+$ metabolites are made and released from yeast cells, it was suggested that both vesicular trafficking and vacuolar function play a role (Croft et al. 2018; Kato and Lin 2014a; Lu and Lin 2010). Vacuolar degradation of $NAD^+$ intermediates appears to coincide with $NAD^+$ salvage. $NAD^+$ metabolites may enter vacuole through vesicular transport and autophagy, which are then broken down into smaller precursors for storage or reuse. $NAD^+$ precursors produced in the cytoplasm can also enter vacuole for storage. The equilibrative nucleoside transporter Fun26 (human lysosomal hENT homolog) controls the balance of NR and likely other nucleosides between the vacuole and the cytoplasm (Lu and Lin 2011). For example, excess cytoplasmic NR is released extracellularly or transported into the vacuole via Fun26 if not converted to $NAD^+$. Likewise, stored vacuolar NR may re-enter cytoplasm to support $NAD^+$ synthesis when needed. Interestingly, although a vacuolar storage pool for $NAD^+$ precursors generated from the NA/NAM and NR salvage pathways has been observed, most excess QA (if not converted to $NAD^+$) is released extracellularly (James Theoga Raj et al. 2019; Ohashi et al. 2013). In addition, wild type yeast cells appear to release very low level of NA (Croft et al. 2018). It is possible intracellular NA is efficiently converted into NaMN by Npt1 (Fig. 1). Supporting this, in a recent screen for mutants that release more NA and/or NAM, the *npt1Δ* mutant shows the most significant NA release phenotype (Croft et al. 2018). Together these studies demonstrate that compartmentalization of $NAD^+$ metabolites as well as cellular processes also play important roles in the regulation of $NAD^+$ homeostasis.

## Conclusions and Perspectives

$NAD^+$ metabolism is dynamic and flexible, which may help cells adapt to environmental changes. Due to this complex nature, factors regulating $NAD^+$ metabolism and homeostasis are not completely understood. Although recent studies have identified novel regulators of $NAD^+$ homeostasis in yeast, many questions remain unanswered. For example, it is unclear whether NA and/or $NAD^+$ also repress the *de novo* pathway activity in other systems. Repression of *de novo* activity by $NAD^+$ has been observed in bacteria (Chandler and Gholson 1972; Grose et al. 2005). *In Salmonella*, this repression is mediated by a tri-functional protein NadR, which can function as a NMNAT (central domain), a $NAD^+$-sensing transcription repressor (N-terminal domain) of $NAD^+$ biosynthesis genes, and a NR kinase (C-terminal domain) (Grose et al. 2005). In addition, NAM does not appear to de-repress *de novo* $NAD^+$ synthesis activity in *E. coli* (Chandler and Gholson 1972), and instead it inhibits QA production. This is probably because NAM is efficiently converted to $NAD^+$ in *E coli*. In yeast, NAM is deamidated into NA and later becomes $NAD^+$ due to the lack of NAMPT. However, NAM is also a potent competitive inhibitor of the Sir2-family deacetylases (IC(50) < 50 μM) (Bitterman et al. 2002; Jackson et al. 2003), suggesting that

we are most likely to observe NAM-mediated inhibition on Hst1 activity under standard growth conditions in yeast. The Since Sir2 family proteins (sirtuins) are highly conserved across species (Brachmann et al. 1995; Frye 2000), it will be informative to determine whether *de novo* $NAD^+$ biosynthesis activity is repressed by $NAD^+$ (and specific $NAD^+$ precursors) and de-repressed by NAM in a sirtuin-dependent manner in higher eukaryotes. Future studies to understand the multiple roles of $NAD^+$ intermediates, as well as novel factors regulating $NAD^+$ homeostasis are also highly anticipated

Recent studies have demonstrated $NAD^+$ metabolism is a therapeutic target for several human diseases (Poyan Mehr et al. 2018; Williams et al. 2017). $NAD^+$ preservation may also help ameliorate age-associated metabolic disorders (Katsyuba et al. 2018; Verdin 2015; Yoshino et al. 2018; Zhang et al. 2016). Understanding the molecular basis and inter-connection of multiple $NAD^+$ metabolic pathways is important for the development of disease-specific therapeutic strategies. These strategies are more effective if associated defects in specific $NAD^+$ biosynthesis pathways/steps are characterized in the disease models or in patients (Liu et al. 2018; Poyan Mehr et al. 2018). In addition, supplementation of specific $NAD^+$ precursors is often combined with the use of genetic modifications and inhibitors of specific $NAD^+$ biosynthesis steps to help channel the precursors flow through a more efficient $NAD^+$ synthesis route (Braidy and Grant 2017; Katsyuba et al. 2018; Liu et al. 2018). It is therefore important to understand all possible interconnections and cross-regulation of $NAD^+$ precursors and associated pathways in specific cell and tissue types.

Specific $NAD^+$ metabolites and $NAD^+$ biosynthesis enzymes have also been reported to have additional function (Kato and Lin 2014a; Tsang and Lin 2015). Moreover, metabolites of the *de novo* pathway (KYN pathway) have been linked to several brain disorders (Amaral et al. 2013; Schwarcz et al. 2012). For example, QA (QUIN) is considered neurotoxic because excess QA can induce toxicity in the central nervous system (CNS) via the glutamatergic NMDA (N-methyl-d-aspartate) receptors. It was also suggested that QA inhibits the uptake of extracellular glutamate by astrocytes, which is an important mechanism to maintain extracellular glutamate at non-toxic levels (Tavares et al. 2002). On the other hand, kynurenic acid (KA) is considered neuroprotective (Amaral et al. 2013; Chang et al. 2018; Schwarcz et al. 2012). KA is produced from KYN by the KYN aminotransferase (KAT). Yeast cells also produce KA from KYN by the Aro8/9 aminotransferases (Ohashi et al. 2017), however the function of KA in yeast remains unclear. Lower KA and KA/KYN ratio as well as higher QA and QA/KA ratio were observed in Parkinson's diseases patients (Chang et al. 2018). Recent studies have shown that inhibiting the activities of *de novo* pathway enzymes such as tryptophan-2,3-dioxygenase (TDO; Bna2 in yeast) (Breda et al. 2016) and kynurenine-3-monooxygen (KMO; Bna4 in yeast) (Mole et al. 2016) via chemical inhibitors and/or genetic modifications may help alleviate specific neurological disorders. These strategies mostly center on increasing the ratio of neuroprotective KA over neurotoxic QA or 3-HK (Breda et al. 2016; Chang et al. 2018). Recent studies in yeast have expanded our understanding of the molecular basis and regulation of $NAD^+$ metabolism. Future studies to understand the multiple roles of $NAD^+$ intermediates, as well as novel factors regulating $NAD^+$ homeostasis are highly anticipated.

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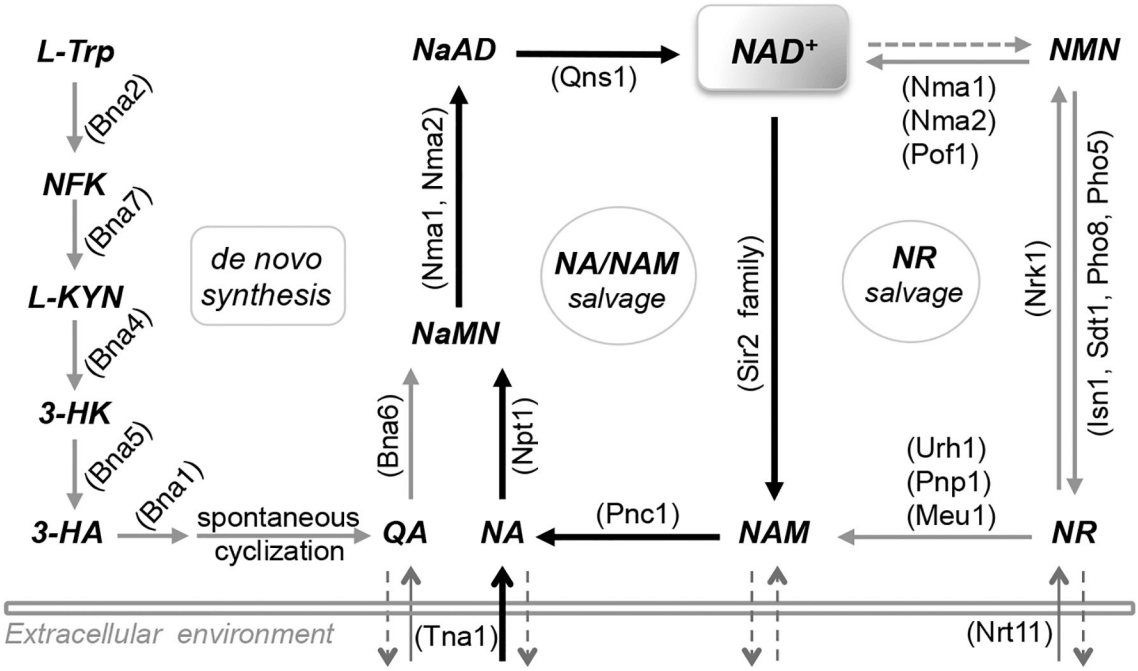


**Fig. 1. NAD$^+$ biosynthesis pathways in budding yeast *Saccharomyces cerevisiae*.**

NAD$^+$ can be synthesized *de novo* or salvaged from intermediates and small precursors such as NA, NAM and NR. Yeast cells also release and re-uptake these precursors. The *de novo* NAD$^+$ synthesis (*left panel*) is mediated by Bna2,7,4,5,1,6 proteins leading to the production of NaMN. The *NA/NAM* salvage pathway (*center panel*) also produces NaMN, which is then converted to NaAD and NAD$^+$ by Nma1/2 and Qns1, respectively. NR salvage (*right panel*) connects to the NA/NAM salvage pathway by Urh1, Pnp1 and Meu1. NR turns into NMN by Nrk1, which is then converted to NAD$^+$ by Nma1, Nma2 and Pof1. For clarity, this model centers on NA/NAM salvage (highlighted with bold black arrows) because standard growth media contain abundant NA. Arrows with dashed lines indicate the mechanisms of these pathways remain largely unclear. NA, nicotinic acid. NAM, nicotinamide. NR, nicotinamide riboside. QA, quinolinic acid. L-TRP, L-tryptophan. NFK, N-formylkynurenine. L-KYN, L-kynurenine. 3-HK, 3-hydroxykynurenine. 3-HA, 3-hydroxyanthranilic acid. NaMN, nicotinic acid mononucleotide. NaAD, deamido-NAD$^+$. NMN, nicotinamide mononucleotide. Abbreviations of protein names are shown in parentheses. Bna2, tryptophan 2,3-dioxygenase. Bna7, kynurenine formamidase. Bna4, kynurenine 3-monooxygenase. Bna5, kynureninase. Bna1, 3-hydroxyanthranilate 3,4-dioxygenase. Bna6, quinolinic acid phosphoribosyl transferase. Nma1/2, NaMN/NMN adenylyltransferase. Qns1, glutamine-dependent NAD$^+$ synthetase. Npt1, nicotinic acid phosphoribosyl transferase. Pnc1, nicotinamide deamidase. Sir2 family, NAD$^+$- dependent protein deacetylases. Urh1, Pnp1 and Meu1, nucleosidases. Nrk1, NR kinase. Isn1 and Sdt1, nucelotidases. Pho8 and Pho5, phosphatases. Pof1, NMN adenylyltransferase. Tna1, NA and QA transporter. Nrt1, NR transporter.

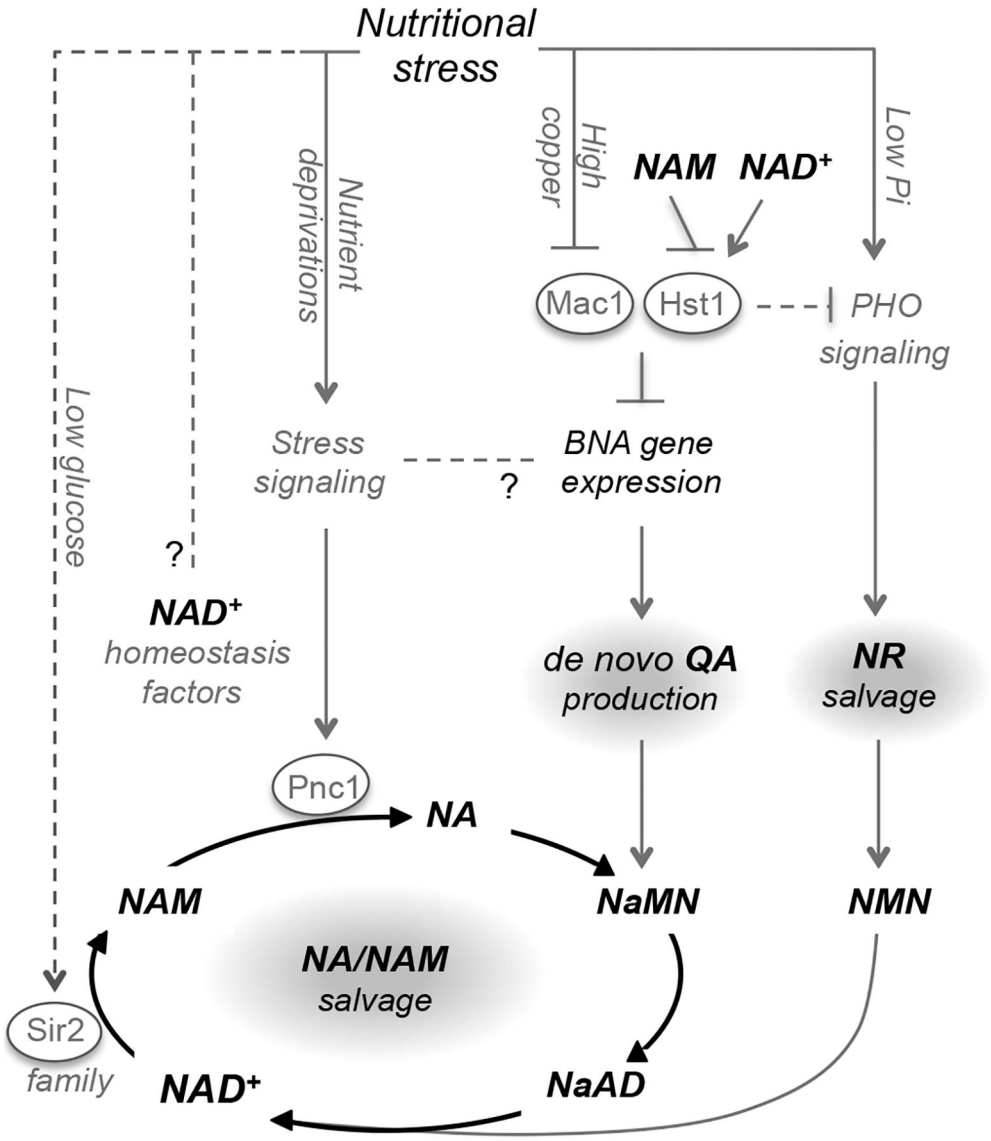


**Fig. 2. A simplified model depicting the interactions of NAD$^+$ metabolism and cellular signaling pathways.**

Under NA abundant conditions (standard growth media), NA/NAM salvage is the preferred NAD$^+$ biosynthesis route, and *BNA* genes are repressed by the NAD$^+$-dependent histone deacetylase Hst1. NAM contributes to NAD$^+$ synthesis via NA/NAM salvage. NAM also de-represses *BNA* gene expression and *de novo* QA synthesis by inhibiting Hst1 activity. The copper-sensing transcription factor Mac1 appears to work in concert with the Hst1-containing repressor complex to repress *BNA* genes. Several nutrient sensing pathways including glucose sensing, amino acid sensing and phosphate (Pi) sensing pathways have also been connected to NAD$^+$ metabolism. Dashed lines indicate the mechanisms of these pathways remain largely unclear.