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Studying the Interconnection of NAD⁺ Homeostasis, Mitochondrial Function, and Autophagy in *Saccharomyces cerevisiae*

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Studying the Interconnection of NAD⁺ Homeostasis, Mitochondrial Function, and Autophagy in
Saccharomyces cerevisiae

By

LAN-HSUAN (ANNIKA) LEE
THESIS

Submitted in partial satisfaction of the requirements for the degree of

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ABSTRACT

Nicotinamide adenine dinucleotide (NAD⁺) and its derivatives are essential metabolites participating in redox reactions and cellular signaling, regulating metabolism. NAD⁺ homeostasis is closely tied to mitochondria activity and autophagy, though the underlying mechanisms are not yet fully understood. We previously showed that NatB complex mediated N-terminal acetylation of two major NAD⁺ biosynthetic enzymes, Nma1 and Nma2, is important for the proper maturation of these proteins. Consequently, the NatB mutant *nat3Δ* exhibits elevated NAM release and reduced NAD⁺ levels. However, how the elevated NAM release and reduced NAD⁺ levels in *nat3Δ* cells connect to mitochondrial function and autophagy remained unclear. To address this, we further study the *nat3Δ* mutant with another NatB substrate, the tropomyosin Tpm1. We show that overexpression of *TPM1* and *NMA1* in the *nat3Δ* background partially restores respiratory growth and autophagy defects. In *hst1Δ* cells, used to distinguish the contribution of *de novo* and NAM accumulation, QA-induced NAM release is suppressed by *NMA1* but not *TPM1* overexpression. We also show that NAD⁺ depletion may enhance autophagy and mitophagy response because the low NAD⁺ mutant *npt1Δ* shows moderate increase in both autophagy and mitophagy. We further characterize whether Urh1, a nucleosidase that produces NAM, is a potential NatB substrate. Two translation start sites were predicted for Urh1 and one of them would make Urh1 a NatB substrate. Our studies suggest that Urh1 is not a direct target of NatB, suggesting that the accumulation of NAM in *nat3Δ* cells is likely due to defective Nma1 and Nma2. Our findings help advance our understanding of how NatB, NAD⁺ salvage, and autophagy are coordinated.

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LIST OF STRAINS

Strain Name (#)	Genotype	Reference
BY4742 (WT)	<i>Wild-type MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Open Biosystem
LSC 89-1	<i>BY4742 pPP81</i>	Chapter 2
LSC 1980	<i>BY4742 nat3Δ::kanMX</i>	(1); Chapter 2
LSC 3485	<i>BY4742 pPP81-TPM1 nat3Δ::kanMX</i>	(1); Chapter 2, 3
LSC 3486	<i>BY4742 pPP81-NMA1 nat3Δ::kanMX</i>	Chapter 2
LSC 2841	<i>BY4742 pho8Δ60pho13Δ</i>	Chapter 2
LSC 3158	<i>BY4742 pho8Δ60pho13Δnat3Δ::kanMX</i>	Chapter 2
LSC 3194	<i>BY4742 pho8Δ60pho13Δnat3Δ pPP81-TPM1</i>	Chapter 2
LSC 3195	<i>BY4742 pho8Δ60pho13Δnat3Δ pPP81-NMA1</i>	Chapter 2
LSC 404-1	<i>BY4742 npt1Δ::kanMX</i>	(1); Chapter 2
LSC 403-3	<i>BY4742 pnc1Δ::kanMX</i>	(1); Chapter 2
LSC 1982	<i>BY4742 tpm1Δ::kanMX</i>	(1); Chapter 2
LSC 3205	<i>BY4742 pho8Δ60pho13Δnpt1Δ::kanMX</i>	Chapter 2
LSC 3202	<i>BY4742 pho8Δ60pho13Δpnc1Δ::kanMX</i>	Chapter 2
LSC 2706	<i>BY4742 pho8Δpho13Δ+ PRS313-PCC4</i>	Chapter 2
LSC 3293-1	<i>BY4742 pho13Δmpho8Δ60</i>	Chapter 2
LSC 3212-1	<i>BY4742 pho8Δpho13Δpnc1Δ::kanMX +PRS313-PCC4</i>	Chapter 2
LSC 3213-1	<i>BY4742 pho8Δpho13Δnpt1Δ::kanMX +PRS313-PCC4</i>	Chapter 2
LSC 434-1	<i>BY4742 hst1Δ::kanMX</i>	(1); Chapter 2
LSC 2079	<i>BY4742 hst1Δnat3Δ::kanMX</i>	(1); Chapter 2
LSC 2073	<i>BY4742 pPP81-TPM1 hst1Δ::kanMX</i>	(1); Chapter 2
LSC 2854	<i>BY4742 pPP81-NMA1 hst1Δ::kanMX</i>	Chapter 2
LSC 2751	<i>BY4742 bna6Δnrk1Δnrt1Δpnc1Δ +PRG205</i>	Chapter 2, 3
LSC 2677-1	<i>BY4742 bna6Δnrk1Δnrt1Δ +PRG205</i>	Chapter 2, 3
LSC 2750-1	<i>BY4742 bna1Δnpt1Δnrk1Δ +PRG205</i>	Chapter 2, 3
LSC 2752-1	<i>BY4742 bna4Δnpt1Δnrk1Δ +PRG205</i>	Chapter 2, 3
LSC 2016	<i>BY4742 urh1Δlpnp1Δnat3Δ::kanMX</i>	(1); Chapter 3
LSC 3481-1	<i>BY4742 URH1-HA::His3MX6 (C-terminal)</i>	Chapter 3
LSC 3483-1	<i>gal80Δ::kanMX ura3Δ0 leu2Δ0 URH1::His3MX6-PGAL1-HA (short, N-terminal)</i>	Chapter 3
LSC 3484-1	<i>gal80Δ::kanMX ura3Δ0 leu2Δ0 URH1::His3MX6-PGAL1-HA (long, N-terminal)</i>	Chapter 3

LIST OF PLASMIDS

LAB ARCHIVE #	PLASMID NAME	Reference
SJLP 1	pPP81	(2) ; Chapter 2,
SJLP 132	pUG6	(3) ; Chapter 2, 3
SJLP 392	pPP81-NMA1	(1); Chapter 2
SJLP 384	pPP81-TPM1	(1); Chapter 2
SJLP 409	pRG205	(4); Chapter 2, 3
SJLP 9	pFA6a-3HA-His3MX6	(5); Chapter 3
SJLP 309	pFA6a-His3MX6-PGAL1-3HA	(5); Chapter 3

LIST OF PRIMERS

LAB ARCHIVE #	NAME	SEQUENCE	Purpose	Chapter
SJLO 86	86	GGATGTATGGGCTAAATG	Pair w/ <i>kanMX</i> confirmation primers	2,3
SJLO 229	NPT1-Kan 5'	ATTTAGCCTTTTGTACCTATTTTCG CCAAGCAAGATAACCGCATAGGC CACTAGTGGATC	<i>npt1Δ::kanMX</i>	2
SJLO 230	NPT1-Kan 3'	TATGTCATATTTATAAAATCGCAG AGATGTCAGTACCCATAACTGAA TAACAGCTGAAGCTTCGTACGC	<i>npt1Δ::kanMX</i>	2
SJLO 234	NPT1-Conf 3'	CGTACTCGCAACTTCATA	<i>npt1Δ::kanMX</i> confirmation w/ 86	2
SJLO 3214	PNC1-Kan 5'	ATCTATATCTTTGTTAGAAAGAAT AAAATACAGTACAAAAGCATAGG CCACTAGTGGATC	<i>pnc1Δ::kanMX</i>	2
SJLO 3215	PNC1-Kan 3'	CCACCCTAGTTCATCAGGTTGAAG AAGTATTATTCAGCTCCAGCTGAA GCTTCGTACGC	<i>pnc1Δ::kanMX</i>	2
SJLO 3216	PNC1-Conf 3'	GAGGCCCAGCCAAATCTTTT	<i>pnc1Δ::kanMX</i> confirmation w/ 86	2
SJLO 2459	NAT3-Kan 5'	ATGACAACAATTCAGCCTTTTGAA CCTGTCGATTTATTCAGCATAGGC CACTAGTGGATC	<i>nat3Δ::kanMX</i>	2
SJLO 2460	NAT3-Kan 3'	TTAAAATCTTACATCATGTGGATA GCATTTATGGCTTCTTCAGCTGAA GCTTCGTACGC	<i>nat3Δ::kanMX</i>	2
SJLO 2461	NAT3-Conf 3'	GCCAACCAGTTTTTCGCAACC	<i>nat3Δ::kanMX</i> confirmation w/ 86	2
SJLO 2462	TPM1-Kan 5'	ATGGACAAAATCAGAGAAAAGCT AAGCAACTTGAAGTTGGGCATAG GCCACTAGTGGATC	<i>tpm1Δ::kanMX</i>	2
SJLO 2464	TPM1-Kan 3'	TCACAAGTTTTCCAGAGATGCAGC AATTCGTCCAGTTCCCAGCTGAA GCTTCGTACGC	<i>tpm1Δ::kanMX</i>	2

SJLO 2463	TPM1-Conf 3'	TTGTCCATGTGATAAACCGTGC	<i>tpm1</i> Δ:: <i>kanMX</i> confirmation w/ 86	2
SJLO 3459	URH1-C-tag-HA-F2	TGAAGCATTAAATAGAGCAGACA AAATGTCAACGATTGGACGGATC CCCGGGTTAATTAA	URH1-HA tag C-terminal	3
SJLO 3460	URH1-C-tag-HA-R1	AAAGGTCAGTAGAAAAAAAAAAAA GCCTTTTATAGAAGTTCGAATTCG AGCTCGTTTAAAC	URH1-HA tag C-terminal	3
SJLO 3469	URH1-N-tag-HA-F4	GATTACGTTTTCCCTCTGTTTACC ACCACGTAACGCGATAGAATTCG AGCTCGTTTAAAC	URH1-HA tag N-terminal	3
SJLO 3470	URH1-N-tag-HA- R3 (short)	GATCACAATCTAGCCATATGGGT ATTTTACTAACAGTCATGCACTGA GCAGCGTAATCTG	URH1-HA tag N-terminal for short sequence	3
SJLO 3471	URH1-N-tag-HA- R3 (long)	TTAATAAGTTTCGTGAGGTAAAA AAATCAGCAGATTCCATGCACTG AGCAGCGTAATCTG	URH1-HA tag C-terminal for long sequence	3
SJLO 3472	URH1-N-tag-HA- Conf 3'	AGAAGTGCCGTCTAAACCTG	Use with SJLO 2957 to confirm	3
SJLO 3483	URH1-C-tag-HA- Conf 5'	AGAGAATGGACATATCCTGC	Use with SJLO 2503 to confirm	
SJLO 2503	tADH1 reverse	AAAGCAACCTGACCTACAGG	Confirm HA tag pair for C-tag	3
SJLO 2957	Gall promoter Conf 5'	AATATACCTCTATACTTTAACGTC	Confirm HA tag pair for N-tag	3

LIST OF ABBREVIATIONS

- AMP (nucleotide): Adenosine Monophosphate
- ATP: Adenosine Triphosphate
- NA: Nicotinic acid
- NAAD: Nicotinic acid adenine dinucleotide
- NAD⁺: Nicotinamide adenine dinucleotide (oxidized)
- NADH: Nicotinamide adenine dinucleotide (reduced)
- NADP⁺: Nicotinamide adenine dinucleotide phosphate (oxidized)
- NADPH: Nicotinamide adenine dinucleotide phosphate (reduced)
- NAD(P)⁺: NAD⁺ and NADP⁺
- NAD(P)H: NADH and NADPH
- NAM: Nicotinamide
- NR: Nicotinamide riboside
- QA: Quinolinic acid
- NAMN: Nicotinic acid mononucleotide
- NMN: Nicotinamide mononucleotide
- NMA1: Nicotinamide mononucleotide adenylyltransferase 1
- NMA2: Nicotinamide mononucleotide adenylyltransferase 2
- Nmnat: Nicotinamide mononucleotide adenylyltransferase
- Nt-Ac: N-terminal acetylation
- TPM1: Tropomyosin 1
- PNC1: Pyrazinamidase/Nicotinamidase 1
- NPT1: Nicotinate Phosphoribosyltransferase 1
- PNP1: Purine Nucleoside Phosphorylase 1

Chapter 1: Introduction to NAD⁺ Metabolism in Budding Yeast

1.1 Introduction to NAD⁺

NAD⁺, short for nicotinamide adenine dinucleotide, is a metabolic cofactor that plays an essential role in cellular reduction-oxidation (redox) reactions in cellular metabolism (6). NAD⁺ is a dinucleotide molecule that consists of two nucleotides joined through their phosphate groups, with one nucleotide containing an adenine nucleobase and the other containing nicotinamide. As an oxidizing agent, NAD⁺ accepts electrons from other molecules, becoming reduced in the process. Specifically, NAD⁺ accepts a hydride ion (H⁻) and electrons from substrate molecules during the reduction process during metabolism, transforming from NAD⁺ into NADH. This reversible interconversion between NAD⁺ and NADH is what allows it to play its role in cellular redox chemistry and energy metabolism. Paired with the reduced form NADH, and in conjunction with another coenzyme flavin adenine dinucleotide (FAD⁺), NAD⁺ acts as the acceptor for high-energy electrons in the electron transport chain (ETC) (7). Conversely, NADH acts as an electron donor. During oxidative phosphorylation, NADH generated from glycolysis, the citric acid cycle, and fatty acid oxidation donates electrons to Complex I of the ETC, where it is oxidized back to NAD⁺, regenerating the oxidized form. This cyclical process of NAD⁺ reduction to NADH during the catabolic reactions, followed by NADH oxidation back to NAD⁺ in the ETC, is fundamental to synthesizing adenosine triphosphate (ATP), the primary energy currency of the cell, thereby contributing critically to overall energy metabolism (8). Therefore, the balance for NAD⁺/NADH ratio is important for maintaining normal cell function and viability (9). In addition to its reduction to NADH, NAD⁺ serves as the precursor for NADP⁺ (nicotinamide adenine dinucleotide phosphate) through phosphorylation catalyzed by NAD⁺ kinase. NAD⁺ is phosphorylated to NADP⁺ and subsequently reduced to NADPH via hydride transfer reactions. While NADH is primarily involved in catabolic processes and ATP generation, NADPH serves as the principal reducing agent during anabolic metabolic pathways such as fatty acid synthesis, cholesterol

biosynthesis, and nucleotide synthesis (10). NADPH also plays crucial roles in maintaining cellular antioxidant defenses by reducing oxidized glutathione and thioredoxin, highlighting the multifaceted importance of NAD⁺ metabolism beyond energy production alone (11). Besides playing a role in energy metabolism, NAD⁺ is also an essential substrate and signaling molecule for NAD⁺-dependent enzymes that regulate cellular functions (12). These enzymes include sirtuins, poly (ADP-ribose) polymerases (PARPs), and cyclic ADP-ribose synthases (CD38/CD157) (13). Sirtuins are a family of NAD⁺-dependent protein deacetylases that regulate cellular processes including DNA repair, chromatin structure remodeling, histone modification, and gene expression (14). These factors place NAD⁺ at a critical position for maintaining the body's metabolic homeostasis.

The connection of NAD⁺ levels, health, and aging has been established in numerous studies (15). Abnormal levels of NAD⁺ and defects in its biosynthesis pathways have been associated with various age-related chronic diseases and metabolic disorders, including diabetes, cardiovascular disease, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, and cancer (16). In type 2 diabetes, reduced NAD⁺ levels impair insulin secretion and glucose homeostasis (17). The NAD⁺/NADH ratio directly influences mitochondrial ATP production, which is essential for the signaling of glucose-stimulated insulin secretion. When NAD⁺ levels decline, this metabolic signaling is disrupted, leading to reduced insulin release (17). The perturbation in NAD⁺ levels has been shown to have be a risk factor of cardiovascular diseases due to NAD⁺ depletion being accompanied with normal aging, obesity and hypertension (18). The relationship between NAD⁺ depletion and neurodegeneration diseases involves impaired mitochondrial quality control, compromised DNA repair, increased oxidative stress, and neuroinflammation, with further research needed to understand the role of NAD⁺ in brain aging (19). Age-associated decline of NAD⁺ levels may also cause defects in DNA repair, which leads to an increase in cancer

development (20,21). On the other hand, the notable treatment of nicotinamide mononucleotide (NMN) supplementation for interventions in cancer patients remains to be further studied (22). Together, the relationship of NAD^+ to diseases and possible treatments marks the significance of understanding the regulatory mechanisms governing NAD^+ homeostasis for a better understanding of aging and age-related diseases. However, in addition to the knowledge of NAD^+ metabolism and its effect on diseases, there remain gaps in our understanding of NAD^+ metabolism that must be further studied. For example, it is unclear how cells sense NAD^+ depletion within specific subcellular compartments, and whether this depletion is sufficient to directly trigger autophagy or mitophagy. Furthermore, the mechanisms governing the transport of NAD^+ and its intermediates across membranes remain largely uncharacterized.

1.2 Overview of NAD^+ Metabolism

In eukaryotes like yeast and humans, NAD^+ biosynthesis is maintained by three pathways: (i) the de novo biosynthesis, (ii) the NA-NAM salvage pathway that recycles the NAD^+ intermediates nicotinamide (NAM) and nicotinic acid (NA), and (iii) the NR salvage pathway that utilizes nicotinamide riboside (NR) (Fig. 1A).

The de novo pathway is characterized by the conversion of the amino acid tryptophan (Trp) to the intermediate quinolinic acid (QA) via a series of enzymatic activities catalyzed by members of the Bna (Biosynthesis of Nicotinic Acid) protein family, such as Bna1, 2, 4, 5 and Bna7. This pathway is also known as the kynurenine pathway, because intermediates include formylkynurenine, kynurenine, and 3-hydroxyanthranilic acid. The final step of the de novo pathway leads to the conversion of QA to NaMN (nicotinic acid mononucleotide) mediated by Bna6 (QA phosphoribosyltransferase), where the de novo pathway converges with the NA-NAM salvage pathway. While this pathway is energetically costly, it provides cells with the capacity to

synthesize NAD^+ independent of dietary precursors by utilizing its own sources, which is particularly important for cells during nutrient limitation. However, Bna2, Bna4, and Bna1 require oxygen for their enzymatic activity, so cells grown under anaerobic conditions must rely on the NA-NAM and NR salvage pathways (23).

The NA-NAM salvage pathway is the predominant pathway for NAD^+ biosynthesis for most cells due to the energy efficiency to recycle NAM and NA. NA is very abundant in standard growth media, and it can be taken up by the yeast cell from the extracellular environment via the transporter Tna1 (24). In this salvage pathway, the product of NAD^+ degradation NAM is converted to NA through Pnc1 (nicotinamidase) (25). NA is then converted to NaMN by Npt1 (phosphoribosyltransferase) (26), producing NaMN (nicotinic acid mononucleotide). NaMN is adenylylated to NaAD (nicotinic acid adenine dinucleotide) by nicotinamide mononucleotide-specific adenylyltransferases (NMNATs) Nma1 and Nma2 (27), before finally converting to NAD^+ by Qns1 (NAD^+ synthetase) (28). The NA-NAM salvage pathway maintains the NAD^+ pool by recycling NAM produced during NAD^+ consumption by the sirtuin family enzymes. This recycling prevents potentially toxic accumulation of NAM, an inhibitor of sirtuin activity (29).

The NR salvage pathway synthesizes NAD^+ through phosphorylation of NR to NMN (nicotinamide mononucleotide) via the kinase Nrk1 (30). NMN is then adenylylated by the NMNATs Nma1/2 and Pof1 to form NAD^+ (31). In yeast, this pathway can bypass the Pnc1-dependent conversion of NAM to NA in the NA-NAM salvage pathway when cells have access to extracellular NR (30). Additionally, the NR salvage pathway can intersect with the NA-NAM pathway by Urh1 and Pnp1-mediated conversion of NR to NAM (32), providing cells with pathway flexibility based on precursor availability.

1.3 Regulation of NAD⁺ Metabolism

Beyond the three biosynthesis routes, NAD⁺ pools are further compartmentalized within membrane-bound organelles including mitochondria, peroxisomes, the nucleus and vacuole (33). The compartmentalization allows for NAD⁺ pools to serve specialized metabolic and signaling function, and allows for the mitochondrial and cytosolic NAD⁺(H) pools to be regulated independently, where distinct redox ratios are maintained (34). Notably, the mitochondrial NAD⁺ pool is specifically maintained robustly for the organelle to perform critical cellular functions such as oxidative phosphorylation and TCA cycle function (35). This maintenance of mitochondrial NAD⁺ pool suggests potential for cells to regulate NAD⁺ levels under cellular stress, such as NAD⁺ depletion; however, the mechanisms remain to be further elucidated.

The yeast silencing information regulator (SIR) family, also known as the sirtuins, include the five proteins Sir2 and Hst1-4. Sir2 encodes a NAD⁺-dependent deacetylase, which is conserved from bacteria to humans (36). In yeast, Sir2 localizes primarily to the nucleus, where it regulates chromatin silencing through transcriptional silencing of heterochromatin domains and is associated with longevity in yeast replicative lifespan (37). Sir2 and Hst1 consume NAD⁺ as a co-substrate during the deacetylation reaction, releasing nicotinamide (NAM) as a product, which can re-enter the NA-NAM salvage pathway. In yeast, NAM also plays an important regulatory role on the *de novo* pathway by inhibiting the sirtuins, and because sirtuins inhibit *BNA* gene expression, NAM accumulation derepresses the *de novo* pathway. Thus, the regulation of NAD⁺ levels and its precursors act as a bridge between sirtuin modulation and influence transcription, DNA repair, metabolic enzyme activity, stress responses, and aging (21).

The mechanisms that govern the transport of NAD⁺ and its intermediates across membranes remain to be fully elucidated. Yeast cells continuously release and reuptake the NAD⁺ intermediates, also known as NAD⁺ precursors, including quinolinic acid (QA), nicotinic acid

(NA), nicotinamide (NAM), and nicotinamide riboside (NR). Our lab has been utilizing this phenomenon of intracellular and extracellular NAD⁺ precursor exchange to study the regulation of NAD⁺ metabolism, using budding yeast as a model organism. We have developed and employed specific NAD⁺ intermediate reporter systems (Fig. 1B) to identify numerous regulators of NAD⁺ homeostasis through gene deletion and overexpression strains (1,38). Mutants that release abnormal levels of specific NAD⁺ precursors are likely defective in related steps of NAD⁺ metabolism and help reveal which genes play essential roles in the NAD⁺ biosynthetic pathway. Overexpression screens identify genes that function in alternative NAD⁺ pathways and can reveal rate-limiting factors of the pathway. Using this system, we have identified several novel NAD⁺ homeostasis factors including transcription factor Mac1 and the histone deacetylases Hst1 and Rpd3, which regulate the expression of *de novo* BNA genes (39,40).

1.4 NAD⁺ Metabolism and Autophagy

While NAD⁺ biosynthesis has been better understood, questions remain regarding how cells regulate and salvage compartmentalized NAD⁺ pools and their derivatives. In particular, it is unclear how cells respond when mitochondrial function is compromised or when cellular NAD⁺ levels become depleted, and whether they preferentially degrade certain organelles to recover NAD⁺ and its precursors (41).

Recent research has shown that autophagy, and selective forms of autophagy such as mitophagy, play important roles in NAD⁺ turnover and cell survival during metabolic stress conditions (42). Autophagy is a critical cellular response for cellular degradation and recycling pathway in which cytoplasmic components and organelles are sequestered within double-membrane vesicles called autophagosomes and delivered to the vacuole (in yeast) or lysosome (in mammals) for breakdown (43,44). This highly regulated recycling process allows for

mitochondrial turnover in a selective autophagy process called mitophagy where the mitochondria are the organelles being degraded (45). Despite the emerging link between autophagy and NAD⁺, the mechanistic interconnections between NAD⁺ homeostasis regulation and mitochondrial turnover remain incompletely understood. It is unclear how cells sense NAD⁺ depletion in different subcellular compartments, and whether this depletion is sufficient to directly trigger autophagy or mitophagy. It also remains unknown how specific NAD⁺ metabolic enzymes are regulated during these processes.

1.5 NAD⁺ Metabolism and Mitochondrial Turnover

Through the reporter systems described above, previous studies in our laboratory have identified mutants displaying increased nicotinamide (NAM) release for strains with defective mitochondrial function (1), suggesting a functional link between mitochondrial function and NAD⁺ homeostasis. This pointed to potential association between mitochondrial turnover and NAD⁺ metabolism.

One example is the NatB N-terminal acetyltransferase complex mutant *nat3Δ*, which exhibits substantial NAM release and an approximately 50% reduction in total cellular NAD⁺ levels (46). The NatB complex mediates N-terminal acetylation (Nt-Ac) of specific substrate proteins, including the tropomyosin isoforms Tpm1 and Tpm2. Previous studies attributed NatB mutant phenotypes primarily to defective Nt-Ac of Tpm1/2, which disrupts actin cable organization and impairs proper mitochondrial segregation during cell division (47). Based on these established roles, we hypothesized that mitochondrial turnover would be elevated in NatB mutants as a compensatory response to mitochondrial dysfunction. Supporting this hypothesis, our studies demonstrated that autophagy contributes to the increased NAM release observed in the *nat3Δ* mutant, consistent with enhanced mitochondrial degradation. However, this interpretation

is complicated by other studies that show NatB deficiency can also block autophagosome formation (48). These conflicting observations highlight the complexity of NatB's role in autophagy and indicate that further mechanistic studies are required to fully elucidate the role of mechanisms of NatB in autophagy processes.

Figure 1-1

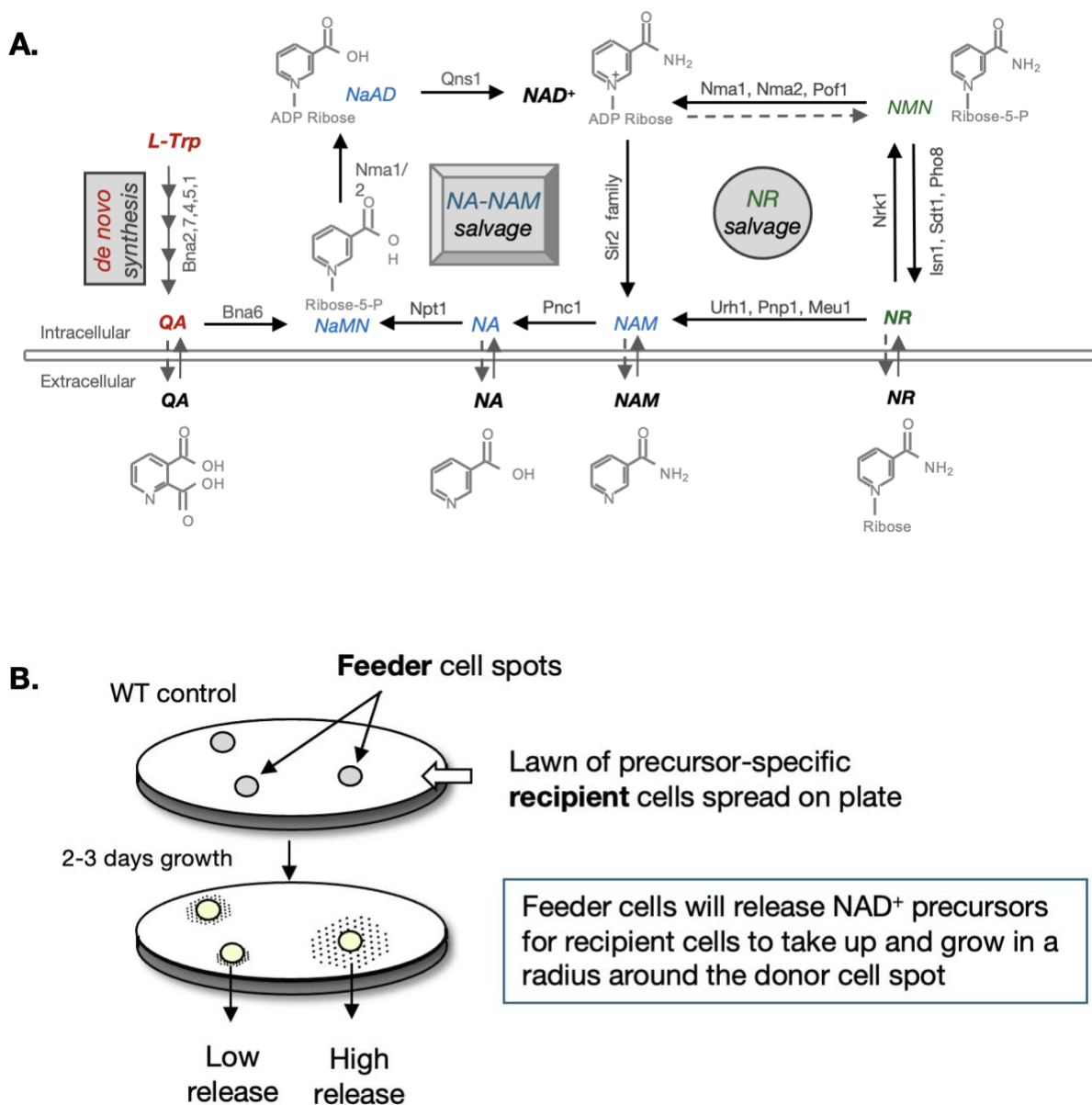


Figure 1-1. Studying the NAD^+ Biosynthesis Pathway.

A, NAD^+ biosynthesis pathways in yeast. In the simplified *de novo* synthesis pathway (left panel), L-tryptophan (L-Trp) is converted to quinolinic acid (QA) via Bna2,7,4,5,1. QA is then converted to nicotinic acid mononucleotide (NaMN) via Bna6. The NA-NAM salvage pathway (middle panel) converts nicotinamide (NAM) to nicotinic acid (NA) via Pnc1. NA is converted to NaMN via Npt1, which is then converted to nicotinic acid adenine dinucleotide (NaAD) by the NMNATs, Nma1 and Nma2. NaAD is converted to NAD^+ via Qns1. NAD^+ is converted to NAM by the

sirtuin family enzymes. In the NR salvage pathway (right panel), nicotinamide riboside (NR) is converted to nicotinamide mononucleotide (NMN) by Nrk1. NMN is then converted to NAD⁺ by Pof1, Nma1, and Nma2. NMN can be converted to NR via nucleotidases/phosphatases Isn1, Sdt1, and Pho8. NR is also converted to NAM via nucleosidases Urh1 and Pnp1, which then enters the NA-NAM salvage.

B, The NAD⁺ intermediate reporter system and the cross-feeding assay. An overview of the cell-based reporter assay to study mutants or gene overexpression with abnormal phenotype of NAD⁺ intermediate (precursor) release (QA, NA-NAM, NR). Feeder cells, also called donor cells (cells of interest), are spotted on the plates and release the precursors. Recipient cells are spread over the plate as a lawn and will take up the precursors released by the donor cells to grow in a radius around the donor cell spots (cross-feeding). The extent of the radius growth corresponds to the amount of precursor released by the donor cell. Cross-feeding plates are grown at 30°C for 2-3 days before the images are taken.

Chapter 2: The Role of NAM Metabolism and NAD⁺ Depletion in Autophagy

2.1 Introduction

2.1.1 NatB-Mediated N-Terminal Acetylation Links and Autophagy and NAD⁺ metabolism

Recent studies have shown that N-terminal acetylation (Nt-Ac), catalyzed by N-acetyltransferase complexes such as NatB, plays crucial roles in autophagy regulation (47). NatB-mediated Nt-Ac is essential for proper autophagosome formation, with NatB-deficient cells showing defects in autophagy induction. This is because NatB acetylates specific proteins critical for autophagosome biogenesis and maturation, including Act1, involved in actin filament formation necessary for vesicle transport, and Vps1, important for autophagosome-vacuole fusion. Nat3, the catalytic subunit of the NatB complex in yeast, was identified as an essential component of the autophagy core machinery that through its acetylation of these NatB substrates (49).

Beyond autophagy, NatB also regulates NAD⁺ metabolism. This connection is mediated through NatB-mediated Nt-Ac of the NAD⁺ biosynthetic enzymes Nma1 and Nma2 (NMNATs), which catalyze essential steps of NAD⁺ biosynthesis (46). Loss of NatB-mediated acetylation reduces Nma1 and Nma2 protein stability, resulting in approximately a 50% decrease of protein levels (1). Our studies also show that the NatB catalytic subunit deletion mutant *nat3Δ* releases significant amount of NAM accompanied by an approximately 50% reduction in NAD⁺ levels (1). NatB also acetylates the tropomyosin Tpm1, and previous studies attributed reported NatB mutant defects to defective Nt-Ac of Tpm1/2 (tropomyosin), which causes actin cables dysfunction and improper mitochondrial segregation during cell division (50–52). Therefore, we expected mitochondrial turnover to increase in NatB mutants. Supporting this hypothesis, our previous study showed that autophagy contributes to increased NAM in *nat3Δ* mutant (1). Overexpression of *NMA1* restores both NAM release and NAD⁺ levels, while overexpression of *TPM1* rescues the NAM release in *nat3Δ* cells, with NAD⁺ levels remaining low (1). These findings suggest that

elevated NAM release may serve as a marker of mitochondrial dysfunction and/or defective mitochondrial turnover, drawing a link between NatB to autophagy and NAD⁺ metabolism. However, the precise mechanisms connecting Nt-Ac, NAD⁺ biosynthesis, and mitochondrial dynamics remain to be fully elucidated. Whether this regulation is conserved between yeast and humans also remains to be fully established. While yeast Nma1 and Nma2 are NatB substrates, it remains unclear whether the human NMNAT isoforms (particularly NMNAT1) are similarly regulated by mammalian NatB complexes (53), highlighting the importance of future investigation for these NAD⁺ biosynthetic enzymes and NatB complexes.

2.1.2 NAM Homeostasis, Mitochondrial Turnover, and Autophagy

The association of increased NAM levels, mitochondrial defects, and autophagy provides an opportunity to study the mechanisms regulating these processes and identify which factors play a role in the regulation. Earlier studies in our lab demonstrated that disruption of NatB function results in increased NAM release and reduced NAD⁺ levels, establishing a functional link between Nt-Ac and NAD⁺ metabolism (1). The phenomenon that overexpression of *TPM1* and *NMA1* rescues the high NAM release phenotype in mitochondrially dysfunctional *nat3Δ* cells (as seen in Figure 2A) further suggests a connection between mitochondrial function and NAD⁺ homeostasis.

These findings led to a central question: does NAM release caused by mitochondrial defects and/or NAD⁺ depletion induce autophagy or mitophagy to facilitate NAD⁺ turnover? It has been shown in mammalian cells that the mitochondria contain a substantial fraction of cellular NAD⁺, the proportion is cell-type dependent, and can be up to 70% in cardiac myocytes (35). Since NAD⁺ is mainly synthesized in the cytoplasm and some in the nucleus, it must be imported into mitochondrial through dedicated inner membrane transporters. In yeast this is mediated by Ndt1 and Ndt2 (NAD⁺ transporter 1/2) (54). It is suggested that the turnover of NAD⁺ through

autophagy or mitophagy could serve as an important NAD⁺ recycling mechanism during metabolic stress (55). If increased autophagy and mitophagy are observed in NAD⁺-depleted cells, this would support the hypothesis that NAD⁺ turnover is regulated by NAD⁺ depletion or mitochondrial dysfunction as a homeostatic response.

2.2 Results

2.2.1 Loss of NatB Causes Mitochondrial Dysfunction, Impairs Respiratory Growth, and is Rescued by TPM1 and NMA1 Overexpression

Because NA and NAM are central intermediates in the NAD⁺ salvage pathway, and NAD⁺ is essential for mitochondrial function (12), having an abnormal NA-NAM (NAM) release phenotype may be indicative of compromises to the cell's mitochondrial and respiratory metabolism. Earlier studies in our lab identified the rescue effect of *TPM1* and *NMA1* overexpression on the NA-NAM release phenotype of *nat3Δ* (1). Although the cross-feeding assay measures combined NA and NAM release, the release from *nat3Δ* cells, and most mutants is predominantly NAM. (1), data not shown. For clarity, the NA-NAM release shown in figures throughout the text will be referred to as NAM release. As shown in Figure 2-1A, the *nat3Δ* strain showed an increase in NAM release, while the two strains of interest, *nat3Δ+TPM1-oe* and *nat3Δ+NMA1-oe*, showed no release of NAM, restoring that of the WT phenotype. This led to the question of whether the overexpression of these two NatB substrates could also restore mitochondrial function.

To assess mitochondrial function in the absence of NatB activity, respiratory growth of *nat3Δ* cells was evaluated on YPEG medium containing ethanol and glycerol as non-fermentable carbon sources (56) (Figure 2-1B). Yeast cells grown in YPD primarily utilize glucose as a fermentable carbon source, allowing ATP production through glycolysis and fermentation (57).

Growth on YPEG requires mitochondria function, because cells must rely on oxidative phosphorylation to metabolize ethanol and glycerol for ATP production (56,57). Therefore, impaired growth on YPEG serves as an indicator of decreased respiratory growth that is often indicative of mitochondrial dysfunction. Compared to WT cells, *nat3Δ* exhibited a severe respiratory growth defect. Overexpression of *TPM1* in the *nat3Δ* background restored respiratory growth to near WT levels. Similarly, overexpression of *NMA1* significantly improved respiratory growth on YPEG. Together, these results demonstrate that loss of NatB impairs respiratory growth and that overexpression of either *TPM1* or *NMA1* partially rescues this defect.

2.2.2 Loss of NatB Alters Autophagy Activity, and is Partially Rescued by *TPM1* and *NMA1* Overexpression

Given that the *nat3Δ* mutant is a model of mitochondrial dysfunction, respiratory growth defects, and elevated NAM release, we next investigated whether loss of NatB-mediated N-terminal acetylation affects autophagy activity. Because autophagy functions as a conserved stress response pathway that maintains cellular homeostasis through degradation and recycling of cytoplasmic organelles (58), we hypothesized that the mitochondrial defects and metabolic imbalance in the NatB mutants will influence autophagic activity. Building on our preliminary findings that *TPM1-oe* and *NMA1-oe* restore the respiratory growth and possible mitochondrial dysfunction of NatB mutant (Fig. 2-1B), we also hypothesized that these proteins may also influence autophagy activity.

Bulk autophagy was measured using the Pho8Δ60 alkaline phosphatase assay (see Section 2.4). Autophagy activity was quantified using the pNPP (p-Nitrophenyl Phosphate) assay and expressed as relative autophagy activity pNP (p-Nitrophenyl) produced per total OD cell. After four hours of nitrogen starvation (-N, 4hr), WT cells showed a strong ~14 fold of autophagy

induction compared to T₀, increasing from 2.28 to 32.40 (pNP/total OD cell), while autophagy induction in *nat3Δ* cells only reached a reduced 26% level of WT starvation-induced activity, ~3.2-fold autophagy increasing from 2.58 to 8.32 (pNP/total OD cell) (Fig. 2-1C). Overexpression of *TPM1* increased autophagy in the *nat3Δ* background from 8.32 to 15.00 (pNP/total OD cell), representing an ~1.8-fold increase relative to *nat3Δ* and reaching ~46% of WT activity. Similarly, *NMA1* overexpression elevated autophagy by ~1.6-fold relative to *nat3Δ*, from 8.32 to 13.01 (pNP/total OD cell), corresponding to ~40% of WT activity. These data indicate that loss of NatB impairs proper nitrogen starvation-induced autophagy, and that overexpression of either *TPM1* or *NMA1* partially alleviates these defects.

2.2.3 Crosstalk between NAD⁺ depletion and autophagy in NAD⁺ salvage pathway mutants

To further investigate the relationship between NAD⁺ homeostasis and precursor release, we examined NA and NAM release in the salvage pathway mutants, *npt1Δ* and *pnc1Δ*, using the cross-feeding assay (see Fig. 1 for the NAD⁺ salvage pathway and the roles of Npt1 and Pnc1). As shown in Figure 2-2A (left panel), *npt1Δ* cells exhibited increased NA release compared to wild-type, consistent with impaired conversion of NA to NaMN by Npt1. In the combined NAM release assay (Fig. 2-2A, right panel), both *pnc1Δ* and *npt1Δ* strains showed elevated NAM release, reflecting the distinct blockage and release of intermediates in the salvage pathway.

Although both *NPT1* and *PNC1* function in the NAD⁺ salvage pathway, previous studies have demonstrated that their deletions affect intracellular NAD⁺ levels differently (1). *npt1Δ* cells exhibit NAD⁺ depletion, whereas *pnc1Δ* cells do not show a substantial reduction in total NAD⁺ levels under standard growth conditions. However, it is possible that the high NAM accumulated in *pnc1Δ* cells derepressed the *de novo* pathway *BNA* genes and therefore helps maintain the NAD⁺

level. This distinction in NAD⁺ levels pose the question of whether NAD⁺ depletion specifically, or the accumulation of NAD⁺ intermediates such as NAM, drives changes in autophagy activity.

Autophagy activity of the salvage pathway mutants *npt1Δ* and *pnc1Δ* were measured using the Pho8Δ60 alkaline phosphatase assay (Fig. 2-2B). In cells grown under moderate, ideal density conditions (59), (Final optical density OD₆₀₀: WT, 1.42; *pnc1Δ*, 2.29; and *npt1Δ*, 2.24), WT cells showed a robust induction of autophagy after four hours of nitrogen starvation, increasing from 2.48 to 27.56 (pNP/total OD cell). The *pnc1Δ* strain displayed a similar response to WT, increasing from 2.46 at T0 to 29.62 (pNP/total OD cell). In contrast, *npt1Δ* cells exhibited a greater increase from 1.91 to 35.35 (pNP/total OD cell) (Fig. 2-2B, left panel). This increase was statistically significant compared to WT (P<0.05). Interestingly, as shown in the right panel, when cells were grown to a higher optical density, in which the overnight cultures of WT and *pnc1Δ* cells reached substantially greater densities than *npt1Δ* cells (Final optical density OD₆₀₀: WT, 7.98; *pnc1Δ*, 8.04; and *npt1Δ*, 2.0) the difference in nitrogen starvation-induced autophagy between *npt1Δ* and WT became more pronounced. Taking note that the *npt1Δ* mutant has a slower growth rate, *npt1Δ* cells displayed a greater increase in pNP production per cell (p<0.001). These results suggest NAD⁺ depletion may facilitate autophagy as seen in the *npt1Δ* cells, and the magnitude of activation appears to be further enhanced in cells grown to higher density and under further nutrient starvation.

To investigate whether the enhanced autophagy in *npt1Δ* cells also extends to selective mitochondrial turnover, we measured mitophagy activity using a mitochondrially targeted Pho8Δ60 reporter. After five hours of nitrogen starvation, WT and *pnc1Δ* cells showed minimal mitophagy induction, with no significant difference between them (Fig. 2-2C). *npt1Δ* cells exhibited a substantial increase in mitophagy activity, increasing from 3.52 to 29.44 (pNP/total OD cell). This increase was statistically significant compared to WT (p < 0.001), whereas *pnc1Δ*

did not significantly differ from WT ($p > 0.05$). Like the autophagy studies, further studies will be needed to determine whether the elevated mitophagy in *npt1Δ* cells contributes directly to NAD⁺ recycling or reflects a broader stress response to NAD⁺ depletion.

2.2.4 Increased NAM release in the *nat3Δ* NatB mutant is not solely due to elevated *de novo* NAD⁺ synthesis

As discussed above, low NAD⁺ also leads to the de-repression of the *de novo* pathway (39,60). Hst1 is an NAD⁺-dependent histone deacetylase that represses transcription of the *BNA* genes, which encode enzymes of the *de novo* NAD⁺ biosynthetic pathway (Fig. 2-3A) (60). Deletion of *HST1* results in de-repression of *BNA* gene expression and activation of *de novo* NAD⁺ synthesis. Therefore, *hst1Δ* cells mimic a transcriptional response of *BNA* genes typically associated with NAD⁺ limitation even under NAD⁺ abundant conditions. On the other hand, the *npt1Δ* mutant serves as a true low NAD⁺ model and show an accumulation of NAD⁺-intermediates in the NA-NAM salvage and *de novo* pathways. Notably, a positive correlation between quinolinic acid (QA) production (a *de novo* pathway intermediate) and NAM release has been observed in our recent studies (data not shown), suggesting a potential link between elevated *de novo* pathway activity and salvage precursor accumulation. Because *hst1Δ* serves as a model of de-repressed *de novo* activity with elevated *BNA* gene expression but without a substantial reduction in intracellular NAD⁺ levels, it allowed us to test whether elevated *de novo* pathway flux alone is sufficient to drive increased NAM release. We previously demonstrated that *nat3Δ* cells display elevated NAM release, however, why *nat3Δ* cells release more NAM remains unclear; we therefore asked whether this increased NAM release is due to de-repression of the *de novo* pathway.

To determine whether the elevated NAM release observed in *nat3Δ* cells results from increased *de novo* NAD⁺ biosynthesis, we measured quinolinic acid (QA) release using a QA-

specific cross-feeding reporter assay. In this assay, QA release serves as an indicator for *de novo* pathway activity. As shown in Figure 2-3B, both *nat3Δ* and *hst1Δ* cells exhibited increased QA release compared to WT, indicating enhanced *de novo* intermediates synthesis in both mutants. However, only *nat3Δ* cells displayed elevated NAM release, whereas *hst1Δ* cells did not show significant increased NAM release. These findings demonstrate that although *de novo* pathway activity is elevated in *nat3Δ* cells, increased NAM release cannot be explained solely by enhanced *de novo* NAD⁺ synthesis.

To further distinguish the metabolic regulation of *hst1Δ* and *nat3Δ* cells, NAM and QA release were examined in *hst1Δ* strains with or without overexpression of *TPM1* or *NMA1* (Fig. 2-3C). *hst1Δ* cells showed elevated QA release relative to WT, consistent with de-repression of the *de novo* pathway. Overexpression of *TPM1* or *NMA1* in the *hst1Δ* background showed similar QA release to *hst1Δ* cells under these conditions. Upon exogenous QA treatment, used to boost the effect of NAM release, *hst1Δ* cells displayed increased NAM release, indicating that enhanced *de novo* flux can promote downstream precursor release in this background. Notably, overexpression of *NMA1* suppressed QA-induced NAM release, whereas *TPM1* overexpression did not restore NAM levels in *hst1Δ* cells. These results suggest in *hst1Δ* cells, increased NAM likely comes from de-repressed *de novo* NAD⁺ followed by NAD⁺ turnover into NAM. *NMA1-oe* was able to decrease both QA and NAM production likely because *NMA1-oe* increases NAD⁺ level (1), which repressed *de novo* activity. It remains unclear why *TPM1-oe* was able to rescue NAM release in *nat3Δ* cells (Fig. 2-1A) but not in *hst1Δ* cells.

2.3 Discussion

In this study we characterized the interconnections between the NatB N-terminal acetyltransferase complex and NAD⁺ homeostasis, mitochondrial function, and autophagy. We

further showed the effect of NAD⁺ depletion on nitrogen starvation-induced autophagy, using the NAD⁺ salvage pathway mutants *npt1Δ* and *pnc1Δ*. Together, these findings help establish the coordination of NatB, NAD⁺ metabolic homeostasis, and autophagic regulation in *Saccharomyces cerevisiae*.

Nat3 functions as the catalytic subunit of the NatB complex, which acetylates proteins at their N-terminus to regulate their stability, localization, and activity. In our earlier studies, mutants lacking this catalytic subunit of the NatB complex (*nat3Δ*), exhibited an elevated NAM release phenotype (Fig. 2-1A) (1). We have also identified two downstream pathways through which NatB contributes to NAD⁺ homeostasis and mitochondrial function, one is through the Nt-Ac of NatB substrates NAD⁺ biosynthetic enzymes Nma1 and Nma2, and the other is through Nt-Ac of Tpm1, a tropomyosin protein required for actin cable formation (1). The NAM release phenotype is restored when *TPM1* and *NMA1* are overexpressed in the *nat3Δ* background (Fig. 2-1A). In the absence of Nat3, Tpm1 is not properly acetylated, leading to defects in actin cable and improper mitochondrial segregation during cell division (50–52). Given the absence of proper mitochondrial function in the NatB mutants, we hypothesized that the impaired mitochondrial function would affect the respiratory growth of NatB mutants and that it may be rescued when Tpm1 function is restored. As expected, we saw a decrease in cellular respiratory growth in *nat3Δ* cells, which was restored by *TPM1* overexpression (Fig. 2-1B). This likely indicates that the overexpression of *TPM1* compensates for the reduced Tpm1 functionality caused by the absence of N-terminal acetylation in *nat3Δ* cells, thereby restoring actin organization and mitochondrial integrity, and allowing for proper utilization of the mitochondria for respiratory growth. Additionally, NatB acetylates the NAD⁺ biosynthetic enzymes Nma1 and Nma2, which catalyze essential steps in NAD⁺ synthesis. In our results, overexpression of *NMA1* in the *nat3Δ* background also improved respiratory growth. Restoration of NAD⁺ biosynthesis likely enhances mitochondrial redox

balance and ATP production, thereby alleviating the metabolic stress that contributed to mitochondrial dysfunction of the mutant. In *nat3Δ* cells, reduced Nt-Ac of Nma1 and Nma2 leads to decreased protein maturation and significantly lower intracellular NAD⁺ levels, approximately a 50% reduction compared to wild-type (1). Because *NMA1-oe* increases NAD⁺ levels, the rescued growth also indicates that the respiratory growth defect may be linked to NAD⁺ depletion. Both *TPM1-oe* and *NMA1-oe* appear to partially restore mitochondrial function, but the relationship between the mitochondrial turnover and NAD⁺ balance remains to be further studied. Overall, the *TPM1* and *NMA1* overexpression studies highlight the role of NatB in coordinating cytoskeletal organization and metabolic regulation to maintain mitochondrial function.

The *nat3Δ* mutant cells exhibit various phenotypes associated with NatB dysfunction including increased NAM production (Fig. 2-1A) and defective respiratory growth (Fig. 2-1B). Moreover, we have previously shown that autophagy contributes to the increased NAM production in the NatB mutant (1). To further understand the interconnection of autophagy, mitochondrial defects, and NAD⁺ depletion, we studied whether *TPM1-oe* and *NMA1-oe* could rescue autophagy activity in *nat3Δ* cells (Fig. 2-1C). In this experiment, we demonstrated that the loss of NatB impairs autophagy, which is in agreement with a recent study (47). The reduction of autophagy in *nat3Δ* cells in comparison to WT cells indicates that disruption of NatB-mediated N-terminal acetylation of substrates such as Tpm1 and Nma1/2 may compromise the cell's ability to properly regulate and induce autophagic responses. Our data showed that overexpression of *TPM1*, which restores actin cable stability and mitochondrial inheritance, partially rescued the reduction in autophagy activity observed in *nat3Δ* cells. Interestingly, overexpression of *NMA1*, which restores NAD⁺ biosynthesis, also partially raised autophagy levels, suggesting the autophagy defect observed in *nat3Δ* cells is in part caused by NAD⁺ deficiency, and therefore could be rescued by overexpressing *NMA1*.

It is possible that the loss of Nt-Ac results in mitochondrial and metabolic stress, which could affect mitochondrial function and, consequently, autophagy regulation. Specifically, Tpm1-mediated proper actin cytoskeleton function may be important for proper vesicular trafficking and autophagy. This implies that mitochondrial defect is a downstream event of Tpm1 dysfunction, that can also impact autophagy possibly due to NAD⁺ deficiency. It is also possible that by raising NAD⁺ biosynthesis through *NMAI* overexpression, such defects are not as severe in the reduction of autophagy levels (Fig. 2-1D). Additionally, although *TPM1*-oe and *NMAI*-oe alleviated the elevated NAM phenotype, the current autophagy data do not directly implicate the role of NAM in the effect of autophagy, but as a marker of NAD⁺ salvage defect. Future studies could capture the effect of NAM directly by adding exogenous NAM to cell cultures to the autophagy assays, and by co-overexpression of *NMAI* and *TPM1*. This will lead to better understanding of the connections between Tpm1 actin cable formation/cytoskeletal organization and autophagic control.

In addition to studying the effects of the *nat3Δ* mutant in autophagy regulation, we sought to more directly determine whether NAD⁺ depletion or NAM accumulation is the primary driver of autophagic induction through the examination of mutants in the NA-NAM salvage pathway. As shown in Figure 2-2A, *pnc1Δ* and *npt1Δ* cells both exhibit elevated NAM release, reflecting their respective roles in the NA-NAM salvage pathway. Furthermore, it has been shown that *pnc1Δ* shows similar high NAM release as *npt1Δ* but maintains normal NAD⁺ levels while *npt1Δ* has reduced NAD⁺ levels (1). This difference provides a genetic opportunity to distinguish the effects of NAM accumulation from those of NAD⁺ depletion on autophagy. In Figure 2-2B, we show that the autophagic response to nitrogen starvation is enhanced in the model of *npt1Δ* (Fig. 2-2B). Deletion of *NPT1* blocks NA utilization within the salvage pathway, limiting NAD⁺ biosynthetic flux and producing measurable NAD⁺ depletion. This metabolic deficit appears to sensitize cells

to nutrient stress, resulting in elevated autophagy induction. The autophagy levels of *pnc1Δ* cells are comparable to that of wildtype, suggesting that autophagy response may be triggered by low NAD⁺ levels and not NAM accumulation. It was also interestingly noted that we had observations for experiments with higher cellular density, due to the faster growth of these strains, and where WT and *pnc1Δ* were grown to higher cell density than *npt1Δ* (~2-3 times higher). When working with these growth conditions, we initially hypothesized that additional nutrient limitation at higher cell density might further enhance autophagy activity. WT and *pnc1Δ* exhibited similar autophagy activity despite their higher density, and both remained lower than *npt1Δ*. The autophagy induction of *npt1Δ* was more significantly compared to the WT. These findings suggest that at cells with higher OD, nutrient stress may be associated with increased autophagy under these conditions. In Figure 2-2C, we showed results similar to Figure 2-2B (right panel) of autophagy. This indicates that mitophagy and autophagy activity may be enhanced in both mutant strains and brings into our attention of relationship between autophagy and mitochondrial function. Mitophagy is the selective autophagic degradation of mitochondria and mitophagy may also serve as a mechanism for recycling mitochondrial NAD⁺ and its precursors during metabolic stress. The elevated mitophagy observed in *npt1Δ* cells therefore suggests cells may turn over mitochondria as a compensatory response to NAD⁺ depletion, potentially to recover NAD⁺ or its intermediates from the mitochondrial pool. For future studies, to study whether autophagy/mitophagy responses are induced by low NAD⁺ conditions or other NAD⁺ intermediates, we can employ low NAD⁺ media with or without supplementation of various NAD intermediates including NAM in future studies. To validate whether NAD⁺ depletion alone is sufficient to promote autophagy and mitophagy independent of genetic perturbations to NAM metabolism, we anticipate that autophagy and mitophagy may be induced in wild-type cells cultured in low-NA media. This would be a study

that complements the knowledge of low NAD⁺ and NAM accumulation, which may help establish a direct mechanistic link between NAD⁺ homeostasis and autophagic regulation.

Our results further suggest that activation of the *de novo* NAD⁺ pathway alone may be insufficient to cause NAM release under normal conditions, as evidenced by the *hst1Δ* phenotype (Fig 2-3A). Hst1 is a NAD⁺-dependent sirtuin deacetylase that represses the expression of *BNA* genes encoding enzymes in the *de novo* biosynthesis pathway. In its absence, *BNA* gene de-repression leads to elevated QA production. *hst1Δ* cells exhibit increased QA production due to de-repression of the *de novo* pathway. However, the *NMA1-oe* strain in the *hst1Δ* background does not display elevated NAM release when the pathway flux is further increased by exogenous QA treatment (Fig 2-3B). This suppression of QA-induced NAM release in *NMA1* overexpression suggests that increasing NMNAT activity through *NMA1* overexpression may enhance the conversion of intermediates into NAD⁺, thereby preventing NAM accumulation and release. Alternatively, but not mutually exclusive, *NMA1-oe* increases NAD⁺, which activates NAD⁺-dependent Hst1 to repress *BNA* genes, resulting in reduced QA and NAM production. Further studies utilizing the *NMA1-oe* strain defective in NAD⁺ synthesis may provide more insight into whether the NMNAT may be a limiting factor to convert intermediates into NAD⁺ to increase or decreased the high release of NAM. Other studies in our lab are also focusing on the interconnection between the *de novo* QA synthesis and NAM production, which will help address some of these questions.

2.4 Experimental Procedures

2.4.1 Yeast Strains, Growth Media, and Plasmids - Yeast strain BY4742 *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* acquired from Open Biosystems (61) was used as the WT strain for this study. Yeast strains and plasmids used in this chapter are described in **List of Strains**. Standard growth

media such as yeast extract-peptone-dextrose (YPD), synthetic complete (SC) media, yeast extract-peptone-ethanol-glycerol (YPEG), and yeast-nitrogen base (YNB) were made as described (62). Niacin-free yeast nitrogen base acquired from Formedium was used to make NA-free SC. Gene deletions were performed using the standard PCR-based strategy to amplify gene-specific PCR products using either the *pAG32-hphMX4* (63) or the reusable *loxP-Kanr-loxP* (pUG6) (3) cassettes as templates. Double gene deletions were carried out by employing different drug-resistant markers for each round of gene deletion and by using the galactose-inducible Cre recombinase to remove the *loxP-kanMX-loxP* (*kan^r*) marker from the yeast genome (3). The *NMA1* and *TPM1* overexpression constructs (*pADHI-NMA1* and *pADHI-TPM1*) (1) were made in the integrative pPP81 (*LEU2*) vector and integrated at the *LEU2* locus. In brief, DNA fragments of the coding region were PCR amplified with NotI site added to the 5' end and NheI site added to the 3' end. The DNA fragments were then cloned into pPP81 digested with NotI and NheI. After PacI digestion, the linearized plasmids were integrated into the yeast genome, resulting in the *pADHI-NMA1* and *pADHI-TPM1* strains (1,64). The reason we chose to use the stable integrative *ADHI* promoter construct ppp81 for gene overexpression was for conditions that were previously used under our CR condition (0.5% glucose), where the *ADHI* promoter was not significantly down-regulated as described (2,65). All plasmid constructs were verified by DNA sequencing. The HA tag was added to the C-terminal of target genes in the genome using the pFA6a-3HA-HIS3MX plasmid as the template for PCR-mediated tagging (5). For N-terminal tags, the pFA6a-His3MX6-PGAL1-3HA plasmid was used as template, where the 3HA tag is expressed under the control of the *GAL1* promoter. Because *GAL1* is subject to glucose repression via Gal80-mediated inhibition of Gal4, *GAL80* was deleted in the relevant strains to relieve this repression and allow constitutive *GAL1* promoter activity (5).

2.4.2 NA-NAM and QA Cross-Feeding Spot Assays – NA-NAM and QA release were measured using NAD⁺ intermediate-specific cross-feeding reporter assays. This assay is designed based on the fact that yeast cells constantly release and re-uptake small NAD⁺ precursors such as QA, NR, NA, and NAM (38) and that standard growth media do not contain most of these intermediates. For measuring NA-NAM release, NA-NAM-dependent recipient cells (*bnaf6Δnrk1Δnrt1Δ*, utilizing both NA and NAM; or *bnaf6Δnrk1Δnrt1Δpnc1Δ*, utilizing only NA) (Fig. 1A) were spread as a lawn (~10⁴ cells/cm²) on niacin-free SC (NA-NAM-free) plates. Next, 2 μL of feeder/donor cells (strains of interest) (~10⁴ cells, cell suspension was made in sterile water at OD₆₀₀ of 1) were spotted onto the lawn of recipient cells. In this system, donor cells release NA-NAM to support the growth of the recipient cells, and the extent of recipient cell growth surrounding each donor spot reflects the relative level of the amount of NA-NAM released from the donor cells (a cell growth-based reporter system). Plates were incubated at 30°C for 2-3 days and then images were captured. QA is added onto the SC plates to a final concentration of 10 μM in some NA-NAM release assays to enhance the phenotypes (Fig. 1B). QA release was measured using the same cross-feeding approach with *npt1Δnrk1Δbna4Δ* cells or *npt1Δnrk1Δbna1Δ* cells (Fig 1A) as QA-dependent recipient cells on regular SC plates.

2.4.3 Respiratory Growth Assay - To assess mitochondrial respiratory function, yeast strains were grown to log phase in YPD medium, serially diluted (10-fold) in sterile H₂O, and then spotted (2 μL) onto YPD and YPEG plates. Plates were incubated at 30°C for 2-3 days, and images captured after incubation period.

2.4.4 Pho8Δ60 Alkaline Phosphatase (rALPase) Assay - Bulk autophagy activity was measured using the Pho8Δ60 reporter made in the *pho13Δ* background as previously described (48,59). *PHO13* was deleted because its presence would increase non-Pho8-dependent background. Normally,

Pho8 is anchored to the vacuolar membrane by its N-terminal transmembrane domain, where it is activated by vacuolar proteases. In the Pho8 Δ 60 allele, the first 60 amino acids including this transmembrane anchor are deleted, so the truncated protein is instead expressed in the cytoplasm. Since it can no longer insert into the vacuolar membrane on its own, it can only reach the vacuole and gain phosphatase activity by being engulfed during autophagy and delivered to the vacuolar lumen. This makes its phosphatase activity a surrogate measure of how much autophagy is occurring in the cell. We made the strain by replacing the first 60 aa of Pho8 with GPD (Tdh3) promoter and a new start codon using pYM-N14 as the PCR template to amplify this GPD-Pho8 Δ 60 chimera fragment. We did not use the pYM-N14 plasmid directly to make the Pho8 Δ 60 strain for autophagy assay as described (59) because we do not want the G418 marker on the plasmid. We then employed CRISPR (66) to target this chimera fragment to the yeast genome, which replaces the native promoter along with the first 60 aa of Pho8 with GPD-*Pho8 Δ 60*. No additional marker was introduced after removing the CRISPR plasmid (Lin Lab data not shown), which allows us to introduce additional gene deletions using the KanR marker.

Mitophagy was initially assessed using a parental strain expressing mitochondrially targeted Pho8 Δ 60 from the pCC4 episomal plasmid, which requires constant selection for the *HIS3*-plasmid (67). pCC4 carries a mitochondrial targeted Pho8 Δ 60; a Pho8 Δ 60 with COXIV leader seq under the control of the *ADHI* promoter in pRS313 (*CEN-HIS3*) plasmid. To avoid reliance on plasmid-based expression, we PCR amplified the *ADHI-COXIV-PHO8 Δ 60* expression cassette using the pCC4 plasmid as template, and then employed CRISPR (66) to target this cassette to yeast genome (Lin Lab data not shown). For autophagy activity, cells were pre-grown on YPD plate overnight, then shifted to YPD liquid medium for 16 hours at 30°C to mid-log phase (OD₆₀₀ ~1–2). For mitophagy activity, cells were pre-grown in YPEG plate overnight and then shifted to YPEG liquid medium at 30°C for 16 hours to OD₆₀₀ ~1–2. Growing on a non-fermentable

carbon source for the mitophagy process forces cells to rely on mitochondrial respiration, making mitophagy's degradation of the mitochondria detectable upon subsequent nitrogen starvation.

To induce autophagy/mitophagy, cells were washed twice in sterile water and then shifted to nitrogen starvation medium (YNB + 2% glucose, referred to as –N) for 4 hours, or five hours for mitophagy. T0 samples were collected before the starvation shift as a basal activity control. After starvation, cells were washed twice with 0.85% NaCl containing 1 mM PMSF and cell lysates were prepared by bead-beating in lysis buffer (20 mM PIPES, 0.5% Triton X-100, 50 mM KCl, 100 mM potassium acetate, 10 mM MgSO₄, 10 μM ZnSO₄, 1 mM PMSF). After lysis, 200 μL of supernatant was incubated with reaction buffer containing 1.66 mM pNP at 37°C for 20 minutes. Alkaline phosphatase activity was measured using *p*-nitrophenyl (pNP) as a substrate. Activity was expressed as nmol pNP produced per cell and used as a quantitative measurement of autophagy or mitophagy activity. The alkaline phosphatase activity is analyzed and graphed using GraphPad Prism 10 software.

Figure 2-1

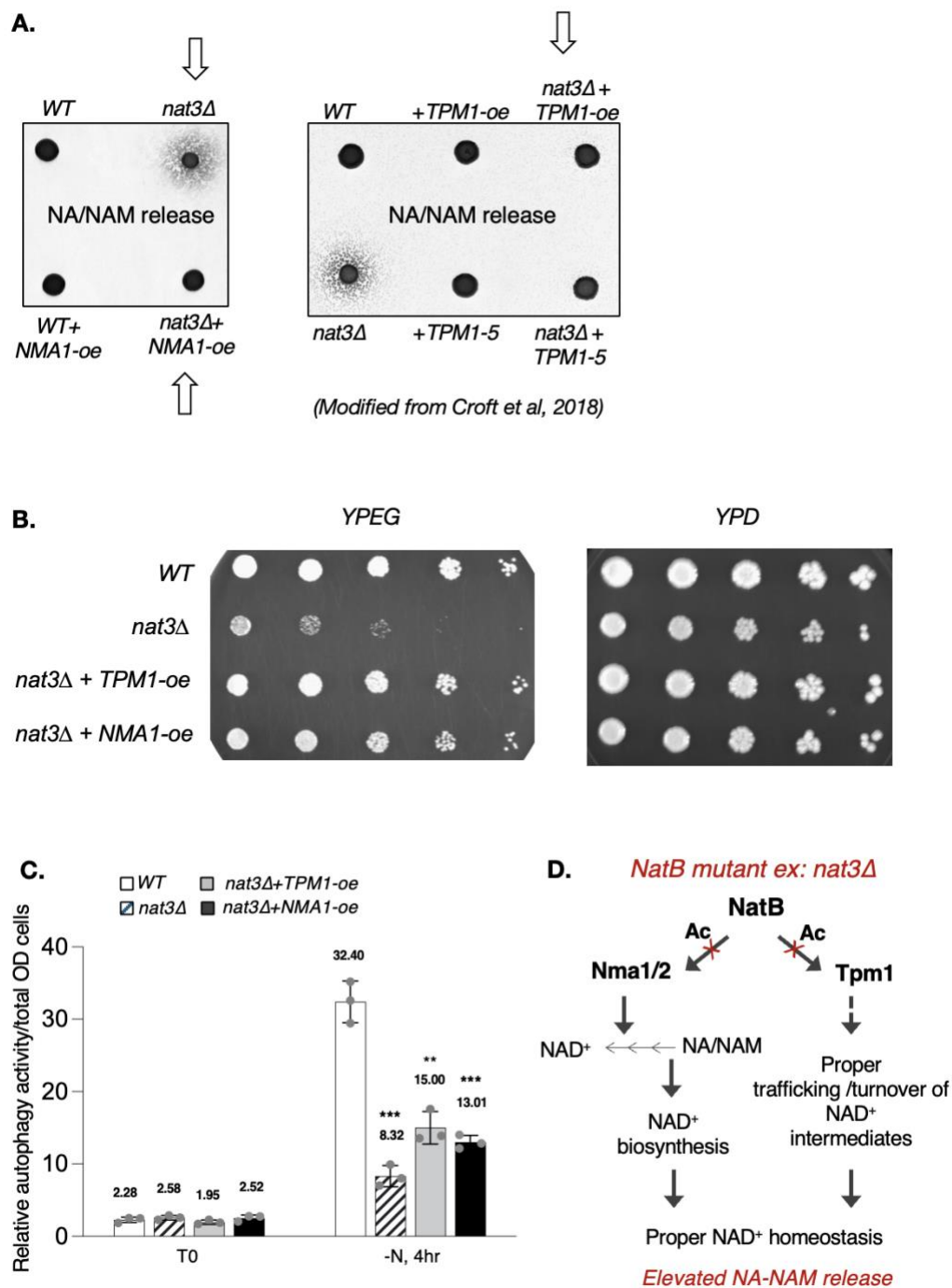


Figure 2-1. NatB substrates *TPM1* and *NMA1* affect the regulation of NAD⁺ metabolism, mitochondrial function, and autophagy.

A, defective NatB causes abnormal NA-NAM release, which is rescued by *TPM1* or *NMA1*-oe. NA-NAM release was assessed using a cross-feeding assay in which donor strains were spotted onto a lawn of NA-NAM-specific recipient cells. Release of NAD⁺ intermediates by the donor

cells is represented in a radial growth of recipient cells surrounding the donor spot. In the left panel, compared to WT, *nat3Δ* cells showed increased NA-NAM release. Overexpression of either *TPM1* (right panel) or *NMA1* (left panel) in the *nat3Δ* background restored NA-NAM release, restoring the phenotype to WT. Feeder cells were grown on YPD plates, and recipient cells were grown on SC plates at 30 °C overnight before use in the cross-feeding assay on SC without nicotinic acid (SC(-NA)) plates. Cross-feeding plates were grown at 30 °C for 2–3 days. Images shown are captured on day 2 of growth. (1)

B, loss of NatB impairs respiratory growth and is rescued by *TPM1* or *NMA1* overexpression. Respiratory growth was assessed by spotting WT, *nat3Δ*, *nat3Δ+TPM1-oe*, and *nat3Δ+NMA1-oe* strains (in 5-fold serial dilutions) onto YPEG medium containing ethanol and glycerol as non-fermentable carbon sources (left panel). Growth on YPEG requires functional mitochondria and oxidative phosphorylation. Compared to WT cells, *nat3Δ* exhibited a severe growth defect on YPEG, indicating impaired mitochondrial function. Overexpression of *TPM1* in the *nat3Δ* background restored respiratory growth to near WT levels. Similarly, *NMA1* overexpression partially restored respiratory growth on YPEG. Growth on YPD is shown as control (right panel). Cells show similar growth to WT. Cells were grown at 30 °C for 2–3 days. Images shown are captured on day 3 of growth.

C, loss of NatB impairs starvation-induced autophagy and is partially rescued by *TPM1* or *NMA1* overexpression. Autophagy activity was quantified using the Pho8Δ60/pNP assay and expressed as relative autophagy activity (pNP) produced per total OD cell. T0 represents cells collected immediately prior to nitrogen starvation (-N). After nitrogen starvation for four hours (-N, 4 hr), WT cells showed an increase of autophagy activity from 2.28 to 32.40 (pNP/total OD cell). *nat3Δ* cells showed an increase from 2.58 to 8.32. *nat3Δ+TPM1-oe* cells increased autophagy from 2.55 to 15.00 (pNP/total OD cell) relative to *nat3Δ*. *nat3Δ+NMA1-oe* cells increased autophagy from 2.52 to 13.01 (pNP/total OD cell). Error bars denote standard deviations of three biological replicates. Significance was determined using Student's unpaired *t*-test comparing each strain to WT under nitrogen starvation (-N, 4hr). Significance is indicated as (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001). *nat3Δ* exhibited a highly significant reduction in autophagy activity compared to WT (*p* ~0.00029, ***). *nat3Δ+TPM1-oe* also showed a significant reduction relative to WT (*p* ~ 0.0017, **). *nat3Δ+NMA1-oe* similarly displayed a highly significant difference compared to WT (*p* ~ 0.00046, ***). Final optical density (OD₆₀₀) measurements: WT, 3.96; *nat3Δ*, 2.82; *nat3Δ+TPM1-oe*, 4.34; *nat3Δ+NMA1-oe*, 2.66. The experiment was repeated three times, and a representative experiment is shown.

D, a proposed model depicting factors contributing to altered NAD⁺ homeostasis in NatB mutants. Nma1/2 and Tpm1 are acetylation targets of NatB. NatB regulates NAD⁺ biosynthesis and NAD⁺ intermediate trafficking via Nma1/Nma2 and Tpm1, respectively. Dashed line, the mechanism of this pathway remains largely unclear. In the NatB mutant, the acetylation of Tpm1 and Nma1/2 is disrupted and lead to a phenotype of elevated NA-NAM release.

Figure 2-2

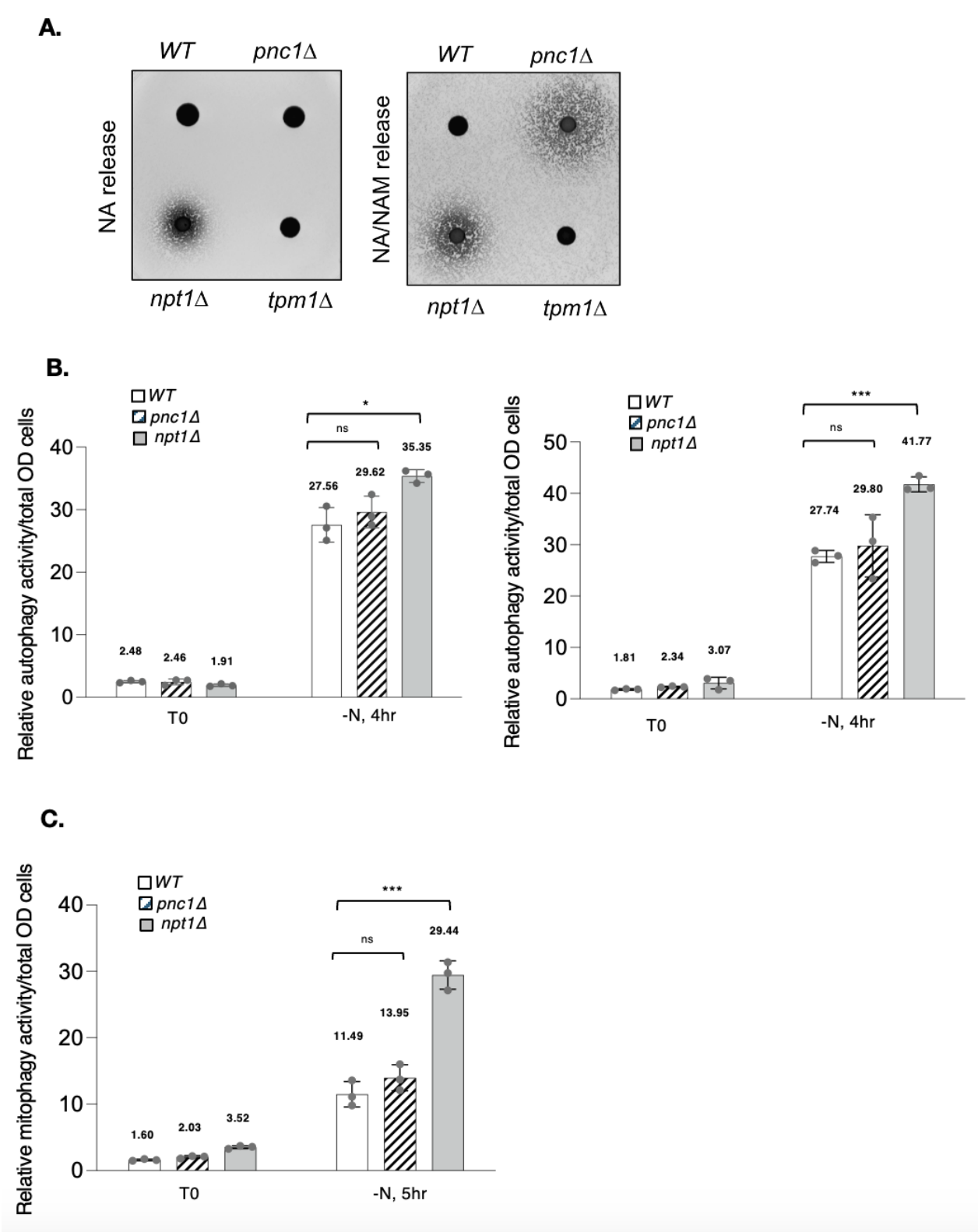


Figure 2-2. NAD⁺ salvage pathway mutant *npt1Δ* increases autophagy and mitophagy activity.

A, salvage pathway mutants show altered NAD⁺ precursor NA-NAM release. NA-NAM release was assessed using a cross-feeding cell assay in which donor strains were spotted onto a lawn of NA-NAM-specific recipient cells. Release of NAD⁺ intermediates by the donor cells is represented in a radial growth of recipient cells surrounding the donor spot. Compared to WT, *npt1Δ* cells exhibited increased NA release (left panel). Compared to WT, *pnc1Δ* and *npt1Δ* cells exhibited increased NA-NAM release (right panel). Feeder and recipient cells were grown on YPD plates at 30 °C overnight prior to the cross-feeding assay on SC-NA. Cross-feeding plates were incubated at 30 °C for 2–3 days, and images were captured on day 2 of growth.

B, the salvage pathway *npt1Δ* mutant display increased autophagy activity. (Left Panel – Low Cell Density) After nitrogen starvation for four hours (–N, 4 hr), WT cells showed relative increased autophagy activity from 2.48 to 27.56 (pNP/total OD cell). *pnc1Δ* cells showed an increase from 2.46 to 29.62. *npt1Δ* cells showed increased autophagy from 1.91 to 35.35 (pNP/total OD cell). Under nitrogen starvation, *npt1Δ* exhibited significant increased pNP production (autophagy activity) relative to WT ($p \sim 0.0103$, *); *pnc1Δ* did not significantly differ from WT ($p \sim 0.39$, ns). Final optical density (OD₆₀₀) measurements: WT, 1.42; *pnc1Δ*, 2.29; *npt1Δ*, 2.24.

(Right Panel – High Cell Density) After nitrogen starvation for four hours (–N, 4 hr), WT cells showed increased autophagy activity from 1.81 to 27.74 (pNP/total OD cell). *pnc1Δ* cells showed an increase from 2.34 to 29.80. *npt1Δ* cells showed increased autophagy from 3.07 to 41.77 (pNP/total OD cell). *npt1Δ* exhibited a significant increase in pNP production (autophagy activity) relative to WT ($p \sim 0.0003$, ***); *pnc1Δ* did not significantly differ from WT ($p \sim 0.59$, ns). Final optical density (OD₆₀₀) measurements: WT, 7.98; *pnc1Δ*, 8.04; *npt1Δ*, 2.0. Error bars denote standard deviations of three biological replicates. Experiments were performed at least three times with similar results. A representative experiment is shown. Statistical significance was calculated using Student's two-tailed t-test comparing each mutant to WT after nitrogen starvation (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

C, the salvage pathway *npt1Δ* mutant display increased mitophagy activity. After nitrogen starvation for five hours (–N, 5 hr), WT and *pnc1Δ* cells did not show increased mitophagy activity. Relatively, the *npt1Δ* cell showed increased autophagy activity from 3.52 to 29.44 (pNP/total OD cell), reaching approximately 7.5 times of WT's activity. Error bars denote standard deviations of three biological replicates. Experiments were performed at least three times with similar results. A representative experiment is shown. Statistical significance was calculated using Student's two-tailed t-test comparing each mutant to WT after nitrogen starvation (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *npt1Δ* exhibited a significant increase in pNP production (mitophagy activity) relative to WT ($p \sim 0.0004$, ***); *pnc1Δ* did not significantly differ from WT ($p \sim 0.19$, ns). Final optical density (OD₆₀₀) measurements: WT, 1.87; *pnc1Δ*, 1.54; *npt1Δ*, 1.08.

Figure 2-3.

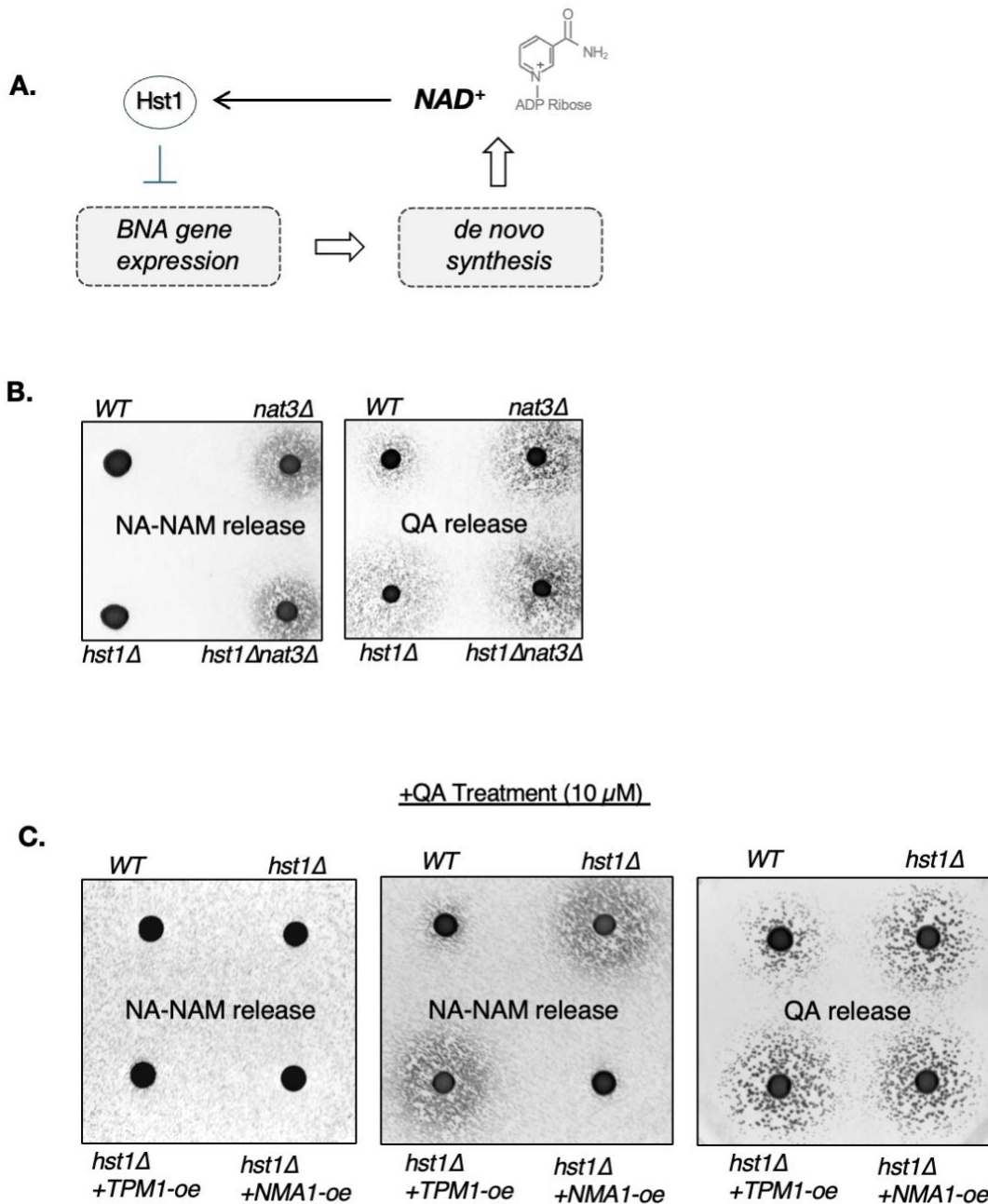


Figure 2-3. *NMA1* overexpression reduces QA-induced NA-NAM release.

A, model of relationship between Hst1 and the NAD^+ biosynthesis pathway. The NAD^+ -dependent histone deacetylase Hst1 represses *BNA* expression under NAD^+ abundant conditions. *BNA* genes encode enzymes in the *de novo* pathway of NAD^+ synthesis.

B, *nat3Δ* cells increased NA-NAM release is not solely due to increased *de novo* activity. QA release was assessed using a QA-specific cross-feeding reporter assay in which donor strains were spotted onto a lawn of QA-specific recipient cells. QA release reflects increased *de novo*

NAD⁺ synthesis. Both *nat3Δ* and *hst1Δ* cells show increased QA release, which is indicative of increased *de novo* activities (right panel). However, unlike *nat3Δ* cells, *hst1Δ* cells did not display increased NA-NAM release (left panel). These results indicate that the elevated NA-NAM release observed in *nat3Δ* cells cannot be explained solely by increased *de novo* pathway activity. Feeder and recipient cells were grown on YPD plates at 30 °C overnight prior to the cross-feeding assay on SC and SC(-NA) plates. Cross-feeding plates were incubated at 30 °C for 2–3 days, and images were captured on day 2 of growth.

C, QA enhanced NA-NAM release in the *hst1Δ* mutant is reduced by *NMA1* overexpression. NA-NAM and QA release were assessed using a precursor reporter assay in which donor strains were spotted onto a lawn of precursor-specific recipient cells. Release of NAD⁺ intermediates by the donor cells is represented in a radial growth of recipient cells surrounding the donor spot. Neither WT and *hst1Δ* cells or *TPM1-oe* and *NMA1-oe* cells in the *hst1Δ* background exhibited increased NA-NAM release (left panel). Compared to WT, *hst1Δ* cells and *TPM1-oe* and *NMA1-oe* cells in the *hst1Δ* background exhibited increased QA release (middle panel). When treated with 10 μM QA, *hst1Δ* mutants displayed increased NA-NAM release, which is reduced by *NMA1* overexpression but not *TPM1* overexpression. These results indicate *NMA1* overexpression suppresses QA-induced NA-NAM release. Feeder and recipient cells were grown on YPD plates at 30 °C overnight prior to the cross-feeding assay in SC and SC(-NA) plates. Cross-feeding plates were incubated at 30 °C for 2–3 days, and images were captured on day 2 of growth.

**Chapter 3: Investigation of the Urh1 nucleosidase as a putative NatB N-Terminal
Acetylation Target and its Regulation by NAD⁺ Metabolism**

3.1 Introduction

In *Saccharomyces cerevisiae*, NAD⁺ biosynthesis is maintained by three pathways: *de novo* biosynthesis from tryptophan, the NA-NAM salvage pathway, and the NR salvage pathway. In the NR salvage pathway, NR can be converted to the NAD⁺ through two routes: phosphorylation to NMN by Nrk1 (nicotinamide riboside kinase 1), or cleavage to NAM by cytoplasmic nucleosidases, most notably Urh1 (uridine hydrolase 1) and Pnp1 (purine nucleoside phosphorylase 1) (32). The Urh1/Pnp1 cleavage route provides a secondary means of NR utilization that operates in parallel with the Nrk1-mediated phosphorylation (68). The phenotype that we have identified and studied in Chapter 2, elevated NAM release, and its association with low NAD⁺ led us to the attention of two enzymes that govern the upstream and downstream of NAM: Urh1 and Pnc1, and to the question of whether the upregulation of Urh1 or downregulation of Pnc1 plays a role in the NAM accumulation observed in the NatB mutant (Fig 3-1A). Because NAM generated by the cleavage of Urh1 must be deamidated to NA by Pnc1 before being subsequently entering through the NA-NAM pathway, the activity of Urh1 plays a crucial role in the balance between the salvage routes.

Our previous work identified the NatB N-terminal acetyltransferase (Nt-Ac) complex as a novel NAD⁺ homeostasis factor. We identified the connection between the NatB mutant and elevated NA-NAM release phenotype, as well as its corresponding substrates such as Tpm1 and Nma1 (1). Notably, deleting the nucleosidase genes *URH1* and *PNP1* largely diminished NA-NAM release in NatB mutants, identifying Urh1 as the primary enzymatic source of elevated NAM in *nat3Δ* cells (1). While the shorter predicted sequence Urh1 did not initially indicate it to be a NatB substrate, where the defining feature of a NatB substrate is a N-terminal methionine followed by an acidic or amidic residue: aspartate (D), glutamate (E), asparagine (N), or glutamine (Q) (Fig

3-1B) (69), a recent RNA sequencing study, suggests Urh1 have additional 38 aa at the N-terminus under standard growth conditions (70). This extension places a compatible recognition motif at the new N-terminus, making Urh1 a potential candidate for Nt-Ac.

This raises two questions: (i) Whether Urh1 is a NatB substrate, and if so, (ii) whether loss of its N-terminal acetylation in *nat3Δ* cells account for its elevated activity and the resulting increase in NAM release observed.

If Urh1 itself is a direct substrate of NatB-mediated N-terminal acetylation, the functional consequences of N-terminal acetylation on Urh1 are still unknown but may include effects on protein stability, gene expression, or enzyme activity (71). In the context of NAD⁺ metabolism, changes in any of these parameters could alter the regulation of NR through the NAM-producing branch relative to the NMN-producing branch (Fig. 1A), with downstream effects on sirtuin activity via NAM inhibition of Hst1 and Sir2 and on the *de novo* pathway via *BNA* gene de-repression. Furthermore, nitrogen starvation conditions that induce autophagy were shown to increase intracellular NR and NA-NAM levels in an Atg14-dependent manner (1). These studies suggest a connection between autophagy and NAD⁺ turnover however, detailed mechanisms remain to be further studied.

This chapter focuses on investigating whether Urh1 is a target of NatB and examining how Urh1 protein expression and activity are regulated by nutritional conditions that relate to NAD⁺. These studies aim to clarify the molecular mechanism connecting Nt-Ac to the regulation of NAD⁺ precursors in the NR and NA-NAM salvage pathways.

3.2 Results

3.2.1 Study whether Urh1 is responsible for elevated NAM release in NatB mutants

Previous studies (1) established that elevated NAM release in *nat3Δ* cells is dependent on the nucleosidases Urh1 and Pnp1, which convert vacuolar NR (exiting through the Fun26 transporter) to NAM in the cytoplasm (Fig 3-1C). To determine the contributions of Urh1 and Pnp1 in NAD⁺ turnover in NatB mutants, we examined NA-NAM release in *nat3Δ* and *urh1Δpnp1Δ* double mutants using the cross-feeding spot assay described in Chapter 2. As shown in Fig 3-1C, the double deletion *urh1Δpnp1Δ* in the *nat3Δ* background reduced NAM release to near WT levels. In summary, we showed that increased NAM in NatB mutants comes from vacuolar NR, which is converted to NAM by Urh1 and Pnp1.

3.2.2 Identification of whether Urh1 is a NatB Substrate

With evidence regarding the possible Urh1 N-terminal extension (70) and our sequence analysis revealing that the 38-amino acid N-terminal extension begins with Met-Glu (M-E), as opposed to the canonical start site of Met-Thr (M-T) (Fig 3-2A), we proposed Urh1 to be a potential NatB substrate. To further evaluate whether Urh1 is a substrate of NatB-mediated N-terminal acetylation, we first examined the protein level of Urh1 by Western Blot using two different N-terminally tagged Urh1-HA strains: the predicted start site that is shorter (URH1-HA short) and the predicted start site that is longer with the 38-amino acid N-terminal extension (URH1-HA long). We tagged both constructs at the N-terminus with an HA epitope (5) to distinguish and directly compare the two strains on the same Western blot, where the expression of each specific start site were driven by a strong *GAL1* promoter. To remove glucose repression of the *GAL1* promoter, we also deleted the *GAL80* gene. The Urh1 protein level with the predicted long start

site was shown to be weaker than the predicted short start site (Fig. 3-1B), thus, indicating it may not be the naturally expressed form in normal growth conditions.

3.2.3 Evaluate if Urh1 Expression is Induced by NAD⁺ Depletion and Nitrogen Starvation

To study the regulation of Urh1 protein expression by nutritional stress, we constructed a C-terminal HA-tagged Urh1 for our studies because this provides a more physiologically relevant readout of Urh1 protein levels. The C-terminal HA tag, unlike the N-terminal tag, does not require choosing between the predicted short or long start sites. The tag is added downstream of whichever start site is naturally used. Given that Urh1 activity drives NAM production and that NAM can inhibit sirtuin activity, we hypothesized that *URH1* protein levels might be altered in response to NAD⁺ status and nutritional conditions. To test this, we first examined and compared the Urh1 protein levels in cells grown in YPD (nutrient rich) and SC (synthetic media, nutrient limiting) (Fig 3-1C). Our results showed that the expression of Urh1 appeared to be slightly decreased in cells grown in YPD compared to cells grown in SC. To extract defining results, we evaluated the Urh1 protein levels in more defined media conditions including normal SC, low glucose, low NA, and high NA (Fig 3-1D). The high NA condition is to mimic YPD media conditions. However, no significant differences in protein expression were observed for the conditions measured, which was indicative that Urh1 is not induced by NAD⁺ depletion or nitrogen starvation. Overall, these studies suggest Urh1 protein levels are maintained constitutively regardless of nutritional or NAD⁺ status, or the conditions may not be induced.

3.3 Discussion

Our results provided evidence that Urh1 is one of the nucleosidases responsible for elevated NAM release observed in *nat3Δ* cells (Fig3-1C). This is consistent with the known role of Urh1 in

cleaving NR to produce NAM, feeding into the NA-NAM pathway. We hypothesized Urh1 to be a target for NatB, however, the protein expression results indicate that the predicted start site with the extended N-terminus (long) is a relative weaker translation start site compared to the short start site under normal conditions (Fig 3-2B). It has been shown that loss of NatB leads to dysfunction the acetylation of NMNATs Nma1 and Nma2 (64), impairing NAD⁺ biosynthesis. A possible explanation for the elevated NAM release in *nat3Δ* cells and its connection to Urh1 is that the disrupted pathway of NMN converted to NAD⁺ by the Nma1/2 leads to accumulation of the upstream intermediates such as NR. The excess NR then becomes available for Urh1 and Pnp1, resulting in elevated NAM production and release. Moreover, since reduced Nma1/2 activity also slows down the assimilation of NaMN, a reduced NA-NAM salvage activity is anticipated, which may also contribute to the accumulation of NAM. The comparatively weaker expression of the longer Urh1 protein under normal growth conditions (Fig. 3-2B) suggests that the upstream start site may be used infrequently or only under specific physiological contexts not captured in our current experimental conditions. The weak but visible detection of the longer form under GAL1-driven expression demonstrates that this protein can be produced and is sufficiently stable to be detected, indicating that its low abundance under native conditions is likely due to inefficient use of the upstream start site rather than rapid protein degradation. The existence of this upstream extension raises the possibility that in some induced conditions Urh1 could be subject to NatB-mediated acetylation that may favor the use of the longer start site. For example, conditions that increase cellular dependence on the salvage pathways, such as anaerobic growth, in which the oxygen-dependent *de novo* pathway is inactive, may favor expression of the longer Urh1 isoform and represent a particularly relevant context to test whether Urh1 is subject to NatB-mediated regulation. It would be interesting to determine whether strains expressing the longer Urh1 isoform

vs shorter Urh1 isoform exhibit altered metabolic outputs, such as changes in NA-NAM release or NAD⁺ levels.

Our results shown in Fig. 3-2C and Fig. 3-2D suggest that Urh1 protein levels remain comparable across the different nutritional conditions tested, including YPD, SC, low glucose, low NA, and high NA media. The absence of significant differences across conditions implies that Urh1-mediated NAM production may not be modulated through regulation of Urh1 but could be through other substrate availability. Nevertheless, since Urh1 also plays a role in overall nucleotide degradation (72), maintaining a constant level of Urh1 may be important for proper cellular function. However, as mentioned, the conditions tested so far may not fully represent the regulation of Urh1's protein expression and localization. Protein expression of Urh1 has not been directly compared between *nat3Δ* and WT backgrounds. Moreover, Urh1 may exhibit differences in protein stability, subcellular localization, or interaction with binding partners in response to the different conditions and/or in the *nat3Δ* background. Future studies examining Urh1 protein expression and localization in the *nat3Δ* mutant would help us clarify whether and how NatB loss affects Urh1 abundance, and whether the elevated NAM production reflects increased or decreased changes in its enzymatic activity.

Altogether, the data presented in this chapter support an indirect model rather than a direct N-terminal acetylation of NatB's role in the Urh1-mediated NAM release. As represented in the model (Fig. 3-2B), NR accumulation is channeled through Urh1 and Pnp1 to produce excess NAM. Our findings of the Urh1 protein levels indicates that the NAM increase we observe in the *nat3Δ* cells may not be due to the Nt-Ac of Urh1. It is possible that NatB loss impairs NatB-dependent acetylation of Nma1/2, reducing flux through the NA-NAM salvage pathway, leading to the resulting NAM release. These results have implications for understanding how N-terminal

acetylation coordinates NAD⁺ homeostasis more broadly. Whether similar indirect mechanisms contribute to NAD⁺ dysregulation in other NatB substrate mutants is also an important question for future investigation.

3.4 Experimental Procedures

3.4.1 Yeast Strains, Growth Media, and Plasmids - Yeast strain BY4742 MAT α *his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ* (Open Biosystems) was used as the wild-type strain for this study. Yeast strains and plasmids used in this chapter are described in the **List of Strains**. Standard growth media including yeast extract-peptone-dextrose (YPD), synthetic complete (SC), were prepared as described (61). Niacin-free yeast nitrogen base (YNB) (acquired from Formedium) was used to prepare NA-free SC media. Gene deletions, including *nat3 Δ* , *urh1 Δ* , and *pnp1 Δ* , were performed using a standard PCR-based strategy with gene-specific PCR products amplified from either the *pAG32-hphMX4* (63) or the reusable *loxP-Kanr-loxP* (pUG6) (3) cassettes as templates. The *urh1 Δ pnp1 Δ* double deletion strain (1) was generated using distinct drug-resistance markers for each deletion round, the *loxP-Kanr-loxP* marker was subsequently removed by galactose-inducible Cre recombinase expression (3). All plasmid constructs were verified by DNA sequencing. HA tags were introduced at either the C-terminus or N-terminus of *URH1* in the genome by PCR-mediated tagging using the pFA6a-3HA-HIS3MX plasmid as the template for PCR-mediated tagging (5).

3.4.2 NA-NAM Cross-Feeding Spot Assays - NA-NAM release was studied using NAD⁺ intermediate-specific cross-feeding reporter assays as described in **2.4.2 NA-NAM and QA Cross-Feeding Spot Assays**. In brief, recipient cells auxotrophic for NA-NAM (*bnaf6 Δ nrk1 Δ nrt1 Δ* , utilizing both NA and NAM; or *bnaf6 Δ nrk1 Δ nrt1 Δ pnc1 Δ* , utilizing NA only) (Fig.1A) were spread as a lawn ($\sim 10^4$ cells/cm²) on niacin-free SC (SC-NA) plates. Donor cells (strains of interest) were then spotted (2 μ L, $\sim 10^4$ cells, prepared in sterile water at OD₆₀₀ = 1) onto

the recipient lawn. The extent of recipient cell growth surrounding each donor spot reflects the relative level of NA-NAM released from the donor strain.

3.4.3 Western Blot Analysis - Cells were grown to early logarithmic phase in YPD, SC, or defined media conditions (SC with low glucose, low NA, or high NA) and 15 OD₆₀₀ unit cells were collected by centrifugation. Collected cells were immediately subjected to extraction. Protein extracts were obtained by bead-beating (Biospec Products) 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). Protein concentrations were estimated using the Bradford assay (Bio-Rad, Hercules, CA, USA), and 20 µg (Urh1-HA) or, 10 µg (PGK) of total protein was loaded in each lane. After SDS-PAGE, the protein was transferred to a polyvinylidene fluoride membrane (GE Healthcare, Amersham, UK). Blocking was carried out using OneBlock Western-CL Blocking buffer (Genesee, Cat#20-313) at room temperature for 1 hour. The membranes were then washed and blotted with either an anti-HA rabbit antibody (Cell Signaling, Cat# 3724S) or an anti-PGK mouse antibody (Invitrogen, Cat# 459250). Protein was visualized using anti-mouse or anti-rabbit immunoglobulin antibody conjugated to horseradish peroxidase (Invitrogen) and the ECL reagents (Amersham, GE). For each protein, multiple images were taken with multiple exposure times (10 sec increments) to avoid signal saturation. The chemiluminescent image was analyzed using the Amersham Imager 600 (GE) system and software provided by the manufacturer.

Figure 3-1

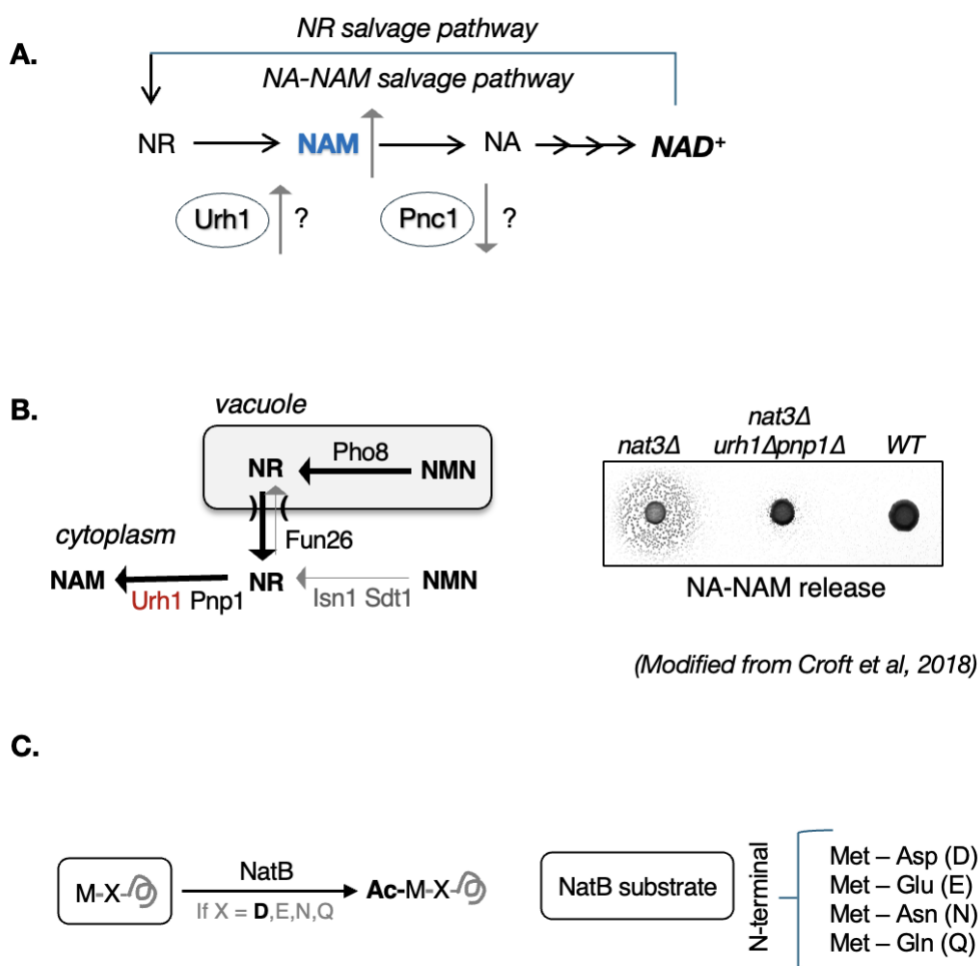


Figure 3-1. Characterizations of Urh1 as a putative NatB substrate.

A, model of the NAD^+ pathway focusing on NR salvage and NA-NAM salvage branches. The level of NAM is controlled by the activity of Urh1 and Pnc1

B, intracellular compartmentalization of NR salvage factors. NR salvage contributes to increased NA-NAM production in NatB mutants (1). Increased NAM in NatB mutants comes from vacuolar NR, which is converted to NAM by Urh1 and Pnp1 (left). Deleting cytosolic nucleosidases *URH1* and *PNP1* (which convert NR to NAM) diminishes NA-NAM release in *nat3Δ* cells (right).

C, schematic showing amino acid sequences that result in Nt-acetylation by NatB. NatB acetylates Met-retained peptides when the second amino acid is Asp (D), Asn (N), Glu (E), and Gln (Q). (69)

Figure 3-2

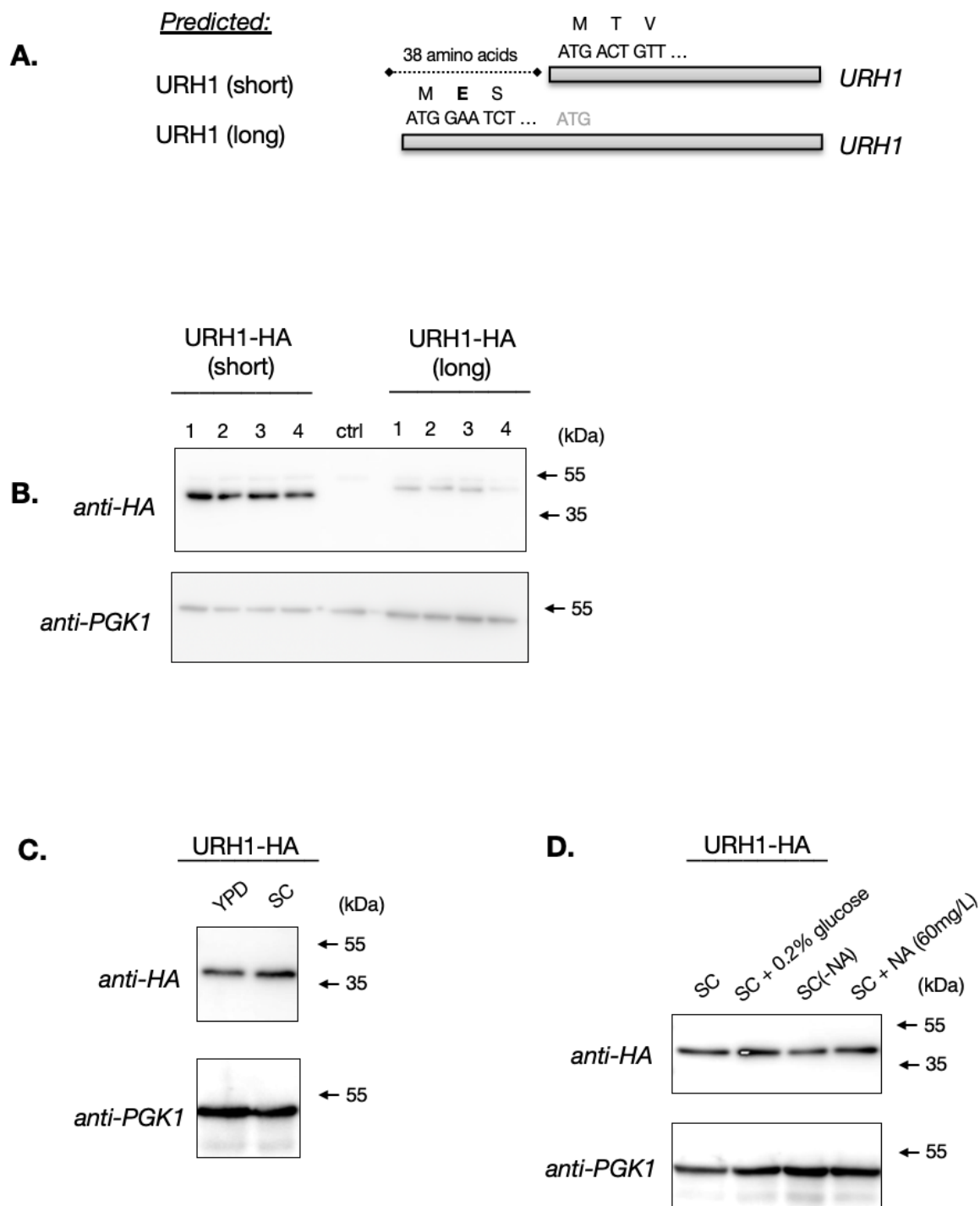


Figure 3-2. Analysis of Urh1 protein expression levels in various media conditions.

A, representation of the two predicted Urh1 sequences. The predicted shorter Urh1 sequence has a start site that starts with Met-Thr-Val (ATGAGTGTT), while the predicted longer sequence start site with an extended 38 amino acids at the N-terminal would yield Met-Glu-Ser (ATGGAATCT). The change in the second amino acid makes Urh1 a candidate for NatB substrate.

B, the shorter Urh1 sequence displays stronger protein expression than the longer Urh1 sequence. Western blot analysis of the expression of two predicted Urh1-HA proteins. The HA tag was added to the N-terminus and immediately upstream to the predicted start sites. Expression of Urh1 is driven by the *GALI* promoter. *GLA80* was deleted in these cells so that the *GALI* promoter is active in standard glucose containing media. The result indicates that the predicted “longer” start site is not the naturally occurring start site.

C, comparison of Urh1 expression in YPD versus SC media. The shorter Urh1 was tagged with HA at the C-terminus and Urh1 is under the control of its native promoter. The higher Urh1 expression observed in SC medium compared to YPD suggests that Urh1 expression may be upregulated in conditions of nutrient limitation-induced upregulation, suggesting that Urh1 expression is induced when nutrients are scarce.

D, comparison of Urh1 expression under different nutrient conditions and NA availability. Urh1 protein expression was compared in growth medium SC (2% glucose), SC+0.2% glucose (low glucose), SC(-NA) (low NA), and SC+NA (60 mg/L). The expression was not significantly different across the media conditions, suggesting that Urh1 expression is not transcriptionally regulated by glucose availability or NA levels under the conditions tested.

Chapter 4: Conclusion, Perspective and Future Directions

The studies presented above may help advance our understanding of the interconnections between NatB-mediated N-terminal acetylation, autophagy regulation, and NAD⁺ homeostasis in *Saccharomyces cerevisiae*. While these findings answer some questions about the mechanistic and regulatory connections between precursors and genes in the biosynthesis pathway of NAD⁺, they also raise new questions. In this chapter, I will focus on the discussions of the key findings of my work as well as possible future directions on the study of NAD⁺ metabolism.

4.1 Characterizing the NatB Mutant and the NatB Substrates Tpm1 and Nma1

Previous studies in our lab have identified the phenotypic similarities and differences between the NatB substrates Tpm1 and Nma1 (1,64). A central question raised by these NatB studies is how the elevated NAM release and the reduced NAD⁺ levels observed in *nat3Δ* cells are connected. The high NAM release phenotype of the NatB mutant is rescued by both *TPM1-oe* and *NMA1-oe* (Fig. 2-1A), but only *NMA1-oe* restores the NAD⁺ levels (1). Our results here further demonstrate that overexpression of either *TPM1* or *NMA1* can partially rescue the respiratory and autophagy defects observed in *nat3Δ* cells (Fig. 2-1B & 2-1C). Because growth on YPEG requires functional oxidative phosphorylation, the impaired respiratory growth of *nat3Δ* cells indicate underlying mitochondrial dysfunction. This partial rescue on YPEG by *TPM1-oe* and *NMA1-oe* suggests that the disruption of acetylation of the two NatB substrates may play a role in the mitochondrial dysfunction in NatB mutants. We do not know if they are two distinct processes, or if the loss of Tpm1 also contributes to the defect of NAM salvage. Co-overexpression of *TPM1* and *NMA1* in the *nat3Δ* background may help answer whether these NatB substrates contribute to autophagy through separate or overlapping pathways.

Additionally, because *TPM1-oe* did not restore NAD⁺ levels but rescues the defects of NAM release and autophagy (partially), the relationship between Tpm1 and NAD⁺ remains to be

explored. In *nat3Δ* cells, both Tpm1 activity and NAD⁺ levels are reduced. It is possible that NAD⁺ depletion further compromises Tpm1-dependent actin organization, for example through affecting the organization of cytoskeletal proteins. The mechanisms remain to be studied, such as examining actin cable morphology in the *nat3Δ* cells and whether they are also affected by Nma1 overexpression. It would also be useful to determine whether the effects of NAD⁺ depletion through low-NA media in WT cells induces cytoskeletal or mitochondrial segregation defects like those observed in NatB mutants.

Similarly, even though *NMA1* overexpression rescues the three major phenotypes of the NatB mutants (NAM release, NAD⁺ levels, and autophagy defects), the mechanistic connections are not yet clear. From our results, we hypothesize that Nma1 activity could contribute to NAD⁺ turnover through the salvage pathways, reducing NAM accumulation and its inhibitory effects on sirtuin activity. Restoration of NAD⁺ biosynthesis through *NMA1-oe* likely enhances mitochondrial function and contributes to autophagy, which leads us to ask whether low NAD⁺ plays a role in autophagy regulation.

4.2 Investigation of Salvage Pathway Mutants, NAD⁺ Depletion, and Autophagy Regulation

The *npt1Δ* and *pnc1Δ* mutants allow us to more specifically ask whether autophagy activity is regulated by NAD⁺ depletion, like we suggested for Nma1, or to NAM accumulation, or even to a more general imbalance in the NA-NAM salvage pathway. Our results from Chapter 2 demonstrated that *npt1Δ* cells exhibit significantly enhanced nitrogen starvation-induced autophagy and mitophagy compared to wild-type and *pnc1Δ* cells. *npt1Δ* cells represent the low NAD⁺ genetic model because *npt1Δ* display low NAD⁺, while *pnc1Δ* has normal NAD⁺ levels. Our findings indicate that both *npt1Δ* and *pnc1Δ* cells express the high NAM release phenotype (Fig. 2-2A) but only *npt1Δ* cells show increased autophagy and mitophagy activity (Fig. 2-2B and

2-2C). This suggests that NAD⁺ depletion, rather than NAM accumulation, may be the driver of enhanced autophagy activity. However, this remains an association rather than a direct correlation. Our overall studies suggest that NAD⁺ metabolic pathways including the NA-NAM salvage pathway, can be disrupted due to NAM accumulation. To help re-establish a balanced NAD⁺ metabolism, it is likely for cells to regulate other processes such as autophagy to gain balance to the flux. Therefore, even though we do not observe strong effects of autophagy increase in the *pnc1Δ* cells, it may be that optimal conditions have not been stimulated. We used the YPD media for most of the autophagy experiments, which are rich in nutrients and may impact the sensitivity towards NAM and nitrogen starvation during the assay. Future studies should test more defined conditions in SC media.

Furthermore, it would also be valuable to directly test the effect of exogenous NAD⁺ precursors on autophagy activity in wild-type cells and in these NAD⁺ salvage mutants. Supplementation the growth culture of the WT strains with defined concentrations of QA, NAM or NR may further allow for the elucidation of the effects of NAD⁺ depletion versus individual precursor accumulation. Because NAM is an inhibitor of the sirtuins Sir2 and Hst1, exogenous NAM may affect NAD⁺-dependent deacetylase (sirtuin) activity and downstream gene regulation. These experiments would complement the genetic comparisons of *npt1Δ* and *pnc1Δ* and would help strengthen the interpretation of which conditions of NAD⁺ depletion and/or NAM accumulation affect autophagy regulation, and subsequently mitophagy.

4.3 Establishing a Connection Between Hst1 and NAD⁺ Intermediates

Besides the question of how NAD⁺ levels and NAM accumulation affect autophagy; the source of elevated NAM release is still a question that remains to be answered. The *hst1Δ* mutant is a convenient genetic tool to address this question. Hst1 normally represses the *de novo* pathway, and

its deletion leads to the production of more QA. Interestingly, *hst1Δ* cells also appear to show a modest increase of NAM in prolonged culture. This result led us to examine whether and how activating the *de novo* pathway affects NAM release/production in a controlled manner (Fig 2-3A). The cross-feeding results of the *hst1Δ* mutants shown in Figure 2-3B and 2-3C indicate that de-repressed *de novo* NAD⁺ synthesis is insufficient to drive elevated NAM release under standard conditions, but it is possible that the excess *de novo* flux can promote downstream NAM accumulation when the pathway is further enhanced by supplementing exogenous QA. Specifically, we saw that *NMA1* overexpression suppressed QA-induced NAM release in *hst1Δ* cells while *TPM1* overexpression did not. We interpret this as evidence that *NMA1-oe* upregulates the biosynthesis activity of NAD⁺ by driving the flux through the adenylation step of NaMN to NaAD or NMN to NAD⁺ catalyzed by Nma1/2, in the respective NA-NAM and NR salvage pathways (Fig. 1A). It has been shown in our recent studies that an increase in *de novo* flux may also lead to the accumulation of downstream precursors such as NAM (data not shown). In *nat3Δ* cells, NAD⁺ is depleted (~50% reduction) (1), while in *hst1Δ*, the de-repressed *BNA* genes drive excess *de novo* flux, but unlike *nat3Δ*, *BNA* gene expression in this background is no longer sensitive to changes in NAD⁺ levels, since the regulator Hst1 is absent (40). We hypothesize that the difference of NAM release restoration in *NMA1-oe* and *TPM1-oe* in the *hst1Δ* background is due to the lack of Tpm1's mechanism to directly regulate the *de novo* or salvage pathway, while *NMA1* regulates NAD⁺ directly, and in turn, reduces NA-NAM accumulation. In contrast, it suggests that the elevated NAM release in *nat3Δ* cells reflect primarily impaired Nma1/2 stability and mitochondrial dysfunction, whereas in *hst1Δ* cells it arises from de-repressed *de novo* flux and downstream NAD⁺ turnover.

Future studies should directly measure the NAD⁺ levels in *hst1Δ+NMA1-oe* cells treated with exogenous QA to confirm that Nma1-driven NAD⁺ synthesis is the mechanism of NA-NAM

suppression. In addition, NAM cross-feeding experiments of *hst1Δ* cells + *NMA1-oe* using a catalytically inactive *NMA1* could help distinguish whether the NMNAT activity is required for the response seen of the NA-NAM salvage precursors.

4.4 Studying whether Urh1 is a NatB Substrate

The work shown in Chapter 2 demonstrates that the elevated NAM release phenotype of the NatB mutants can be rescued by seemingly different but possibly interconnected mechanisms. Questions remain on the source of the NAM accumulation, where we address in Chapter 3. Pnc1 and Urh1 are the two enzymes that function downstream and upstream to NAM metabolism respectively. Pnc1 has already been examined in our previous studies; Urh1 is at the crosspoint of NR salvage and NA-NAM salvage pathway and is an enzyme of focus (Fig. 3-1A). Excess intracellular NR is a signal of disrupted NAD⁺ balance, and recent work has shown that NR production in yeast is regulated through coordination between NMNATs and nucleotidases, showing how this balance being important for NAD⁺ homeostasis under nutritional stress (73). Chapter 3 presents protein expression evidence that the predominant Urh1 start sequence suggests Urh1 is unlikely to be a direct NatB substrate. However, several important questions regarding Urh1 regulation remain unresolved.

First, a direct comparison of Urh1 protein abundance between wild-type and *nat3Δ* cells have not yet been performed. If Urh1 protein levels or activity are elevated in the NatB mutant background, this would suggest that NatB loss may indirectly upregulate Urh1, potentially through effects on NAM or NR accumulation that alter Urh1 expression or stability. Future Western blot analysis comparing C-terminally tagged Urh1 in wild-type and *nat3Δ* background would help determine whether the loss of NatB can increase Urh1 protein level.

Second, specific growth conditions may increase the expression of the longer Urh1. While the current data suggest the shorter predicted sequence of Urh1 is the isoform that predominates under normal growth conditions, the longer Urh1 isoform, which is a potential NatB substrate, could be dominant in certain nutritional conditions not tested in these studies. The conditions tested for Urh1 expression in this study included low NA, low glucose, and rich media (YPD), and future studies could include conditions that introduce different stress conditions, such as nitrogen starvation using the C-terminal HA tag of Urh1.

4.5 Conclusion

The studies presented in this thesis help advance our understanding of the mechanistic connections between NatB-mediated N-terminal acetylation, NAD⁺ homeostasis, and autophagy regulation in *Saccharomyces cerevisiae*. The balance of NAD⁺ and its precursors is essential for cellular homeostasis, as NAD⁺ serves as a critical cofactor in redox reactions, and as a co-substrate for gene expression regulation. In this study, we show how the NatB substrates Nma1 and Tpm1 contribute to the regulation of autophagy and respiratory growth. It brings to light the importance of actin cytoskeletal integrity and mitochondrial organization in parallel with NAD⁺ homeostasis. We additionally revealed that NAD⁺ depletion is likely associated with increased autophagy and mitophagy activity in normal culture conditions, as supported by the differential phenotypes of the salvage pathway mutants *npt1Δ* and *pnc1Δ*. Furthermore, the analysis of *hst1Δ* cells helped distinguish the mechanisms that govern elevated NAM release in *nat3Δ* versus *hst1Δ* cells. We showed that de-repressed *de novo* flux can contribute to downstream NAM accumulation, which is reduced by *NMA1* overexpression, indicating that Nma1 may act to facilitate the clearance of NAM. Finally, protein expression analysis of Urh1 suggests that the predominant Urh1 protein under standard growth conditions begins with a Met-Thr sequence, indicating that Urh1 is likely

not a direct NatB substrate, though its regulation under alternative stress conditions and in the NatB mutant background requires further investigation. Together with our previous findings that NatB regulates Nma1 and Nma2 proteins, this suggests that the elevated NAM release in *nat3Δ* cells more likely arises from impaired Nma1/2-mediated NAD⁺ biosynthesis rather than direct regulation of Uth1. Together, these studies contribute to the overall knowledge on how cells coordinate NatB-mediated acetylation, NAD⁺ homeostasis, and autophagy to maintain and regulate metabolic balance, and how unbalanced NAD⁺ metabolism may contribute to disease.

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