A Study in NAD+ Metabolism: Yeast Provides a Model System

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Abstract

NAD+ (Nicotinamide adenine dinucleotide) is a coenzyme found in all living cells and a central molecule in biological functions, being involved in redox reactions, cellular respiration, and protein modification. Abnormal levels of NAD+ have proven to play important roles in the emphasis of age-related diseases, which is why NAD+ precursor treatments are a potential therapeutic target to slow cell aging or to alleviate aging-associated disorders. However, the complicated connections between NAD+'s biosynthetic pathways make such mechanisms currently unclear. Budding yeast, *Saccharomyces cerevisiae*, was used as a model system to discover what factors may contribute to the regulation of NAD+ homeostasis. In order to do so, the lab has attempted to identify elements that affect the three pathways of NAD+ metabolism, which include the *de novo*, NA/NAM and NR salvage pathways. This study also investigated how the pathways are interconnected and how the overexpression of certain genes can influence the NAD+ pathway yields by performing a cross-feeding spot assay. Specifically, the overexpression of the *BNA1* gene has shown a small increase of QA from the *de novo* pathway, and an unexpected large increase of NA/NAM from the NA/NAM salvage pathway, which could mean that this gene may be connected to secondary mechanisms or other intermediates indirectly. These possibilities may lead to other experiments uncovering the ways NAD+ pathways are connected and to further research in NAD+ precursor treatments.

Introduction

NAD+ is used in a myriad of redox reactions that are central to eukaryotic cells, such as cellular respiration, being an electron transporter in glycolysis and oxidative phosphorylation. It is a substrate for enzymes that are involved in longevity, making it an important molecule in a cell's lifespan (Lautrup, Sofie et al., 2020). NAD+ also plays a role in assisting proteins at sites of DNA damage and is consumed

by enzymes involved in hydrolyzing ribose-ribose bonds (Imai et al., 2014; Groth et al., 2021). In particular, poly (ADP-ribose) polymerases (PARPS) and the deacetylases sirtuins heavily rely on consuming NAD+ for retaining genomic stability and immune response, which consequently contributes to the noted age-related decline of NAD+ (Chini et al., 2016).

Because of its importance in all living cells, abnormal levels of NAD+ have shown to significantly contribute to diseases, such as diabetes, cancer, mitochondrial dysfunction, and neurological decline. Thus, administration of NAD+ precursors has become an emerging therapeutic treatment as in recent studies, it has been shown to prevent type 2 diabetes in mice, as well as increase NAD+ levels in other systems including humans (Imai et al., 2016).

NAD+ metabolism contains three main pathways (**Fig. 1**) that release three different NAD+ precursors which include the *de novo* pathway and the nicotinamide riboside (NR) and niacin (NA)/nicotinamide (NAM) salvage pathways. The *de novo* pathway produces quinolinic acid (QA), the NA/NAM salvage pathway produces niacin/nicotinamide (NA/NAM), and the NR salvage pathway produces nicotinamide riboside (NR). All of the pathways have three possible uses of these NAD+ precursors: release them into the extracellular area, transport them for other functions, or keep them in the intracellular area for use. Particularly, in the *de novo* pathway, NAD+ is metabolized "*de novo"*, or anew, from L-tryptophan, while in the salvage pathways, NAD+ is recycled from its intermediates. Many of the factors in NAD+ metabolism are retained from yeast to eukaryotic cells. In yeast, the *de novo* pathway creates QA by five enzymatic reactions and spontaneous cyclization (Canto et al., 2015). The Bna proteins mediate these reactions and many of these reactions require oxygen. Specifically, the *BNA*1 protein mediates 3-hydroxyanthranilic acid (3-HA) being converted to QA by spontaneous cyclization. QA then either gets released in the extracellular environment or eventually converted to NAD+ (Raj et al. 2019). Additionally, this particular pathway plays a role in more than just NAD+ synthesis, which makes it a highly important object of study. The intermediates involved in the *de novo* pathway showed reduced

activity in mice with chronic kidney disease, even while retaining levels of NAD+ (Faivre et al., 2021). They are also either neurotoxic or neuroprotective, so dysregulation of this pathway can lead to oxidative stress and neurological damage (Lautrup, Sofie et al., 2019).

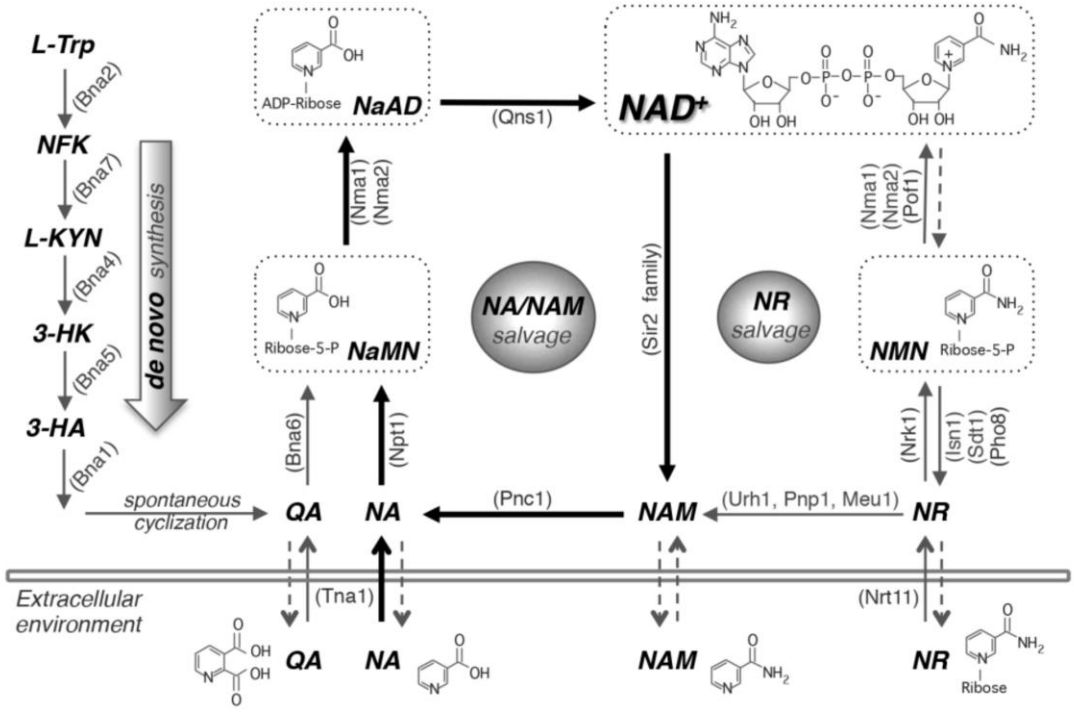

Fig. 1 Diagram of NAD+ metabolism pathways (Croft et al., 2020). Cells generate NAD+ intermediates (QA, NA/NAM, NR) and recycle them in the metabolic pathways or release them into the extracellular environment. The *de novo* pathway produces QA from new materials (L-trp), while the NA/NAM and NR salvage pathways use existing intermediates to produce their own and eventually NAD+.

The Lin Lab is employing budding yeast *Saccharomyces cerevisiae* as a model system to identify novel factors that regulate NAD+ homeostasis because many of the factors in NAD+ metabolism are conserved from budding yeast to eukaryotic cells. The lab has designed genetic screens to identify factors that affect each of the three pathways of NAD+ metabolism. Although research in NAD+'s supplement possibilities is very difficult due to the complicated connections between NAD+ metabolism pathways and their

intermediates, information about the factors regulating NAD+ homeostasis may provide another step towards understanding its exact role in molecular diseases and may help future studies develop new targeted therapies (Groth et al., 2021).

Methods

Previous Genetic Screens using the yeast overexpression collection

Previously, the Molecular Barcoded Yeast ORF 2.0 library (**Fig. 2**) created by Dr. Charlie Boone's lab at the University of Toronto (Horizon Inc.) was used to screen for genes when overexpressed that can affect NAD+ metabolism.

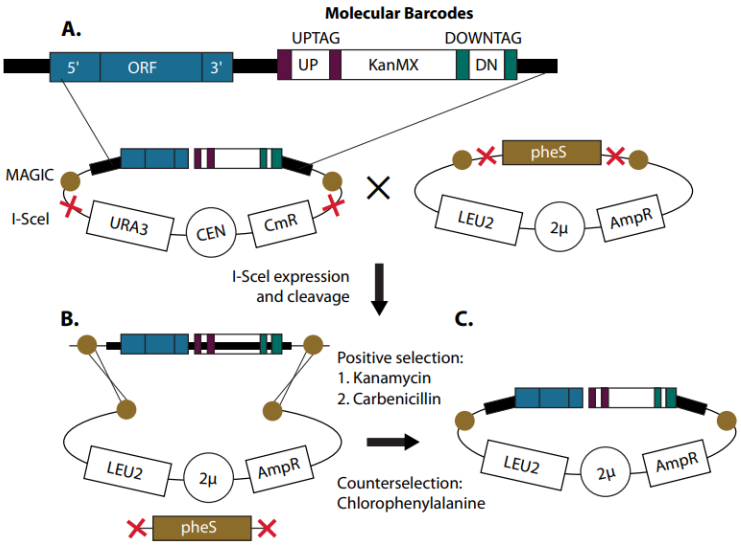

Fig. 2 A model of how the plasmids in the overexpression library are built so viable clones are able to be produced. The blue strip represents the gene intended to be overexpressed. The *LEU2* marker allows only the yeast or bacteria with this plasmid to grow in a plate without Leucine, which is why -leu plates are used in the cross-feeding spot assay. The *2μ* vector allows the plasmid to be replicated multiple times, thus overexpressing the intended gene. The AmpR marker allows only the bacteria with this plasmid to grow in plates with antibiotics. The construction of these plasmids helps only the intended yeast or

bacteria with these plasmids to grow when used in experiments so that no other organisms can interfere. It also helps us to selectively overexpress the genes being tested.

About 100 genes were identified in the screen, which either alter the level of QA, NA/NAM and/or NR release. We are currently studying the first eight that were hypothesized to show the most direct effect on the pathways, which include, *BNA1*, *NMA1*, *NMA2, CIT2, ATF1*, *PHO84, PHM8*, and *POF1*. This screen helped to fine-tune and narrow down the conditions for the secondary screening.

Current secondary cross-feeding spot assays

Yeast strain BY4742 *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* was used for the control/wild type as well as for making gene deletion strains. Yeast strain BY4741*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* was the parental strain carrying the gene overexpression library.

To validate whether the genes observed from the previous genetic screening indeed induced phenotypes consistently and to confirm that no mutations emerged, a secondary cross-feed plate assay was performed. For example, the *npt1*Δ*nrk1*Δ*bna1*Δ mutants, which are QA-dependent (requires QA supplementation for growth), were used as "recipient cells". Yeast strains being tested, which included a wild type (WT) control, positive and negative controls, and yeast strains with overexpression of *BNA*1, were used as "donor cells". To create solutions with the same concentration of cells, a spectrophotometer was used to calculate the optical density with OD_{600} of each sample to create proper dilutions.

The recipient cells ($\sim 10^4$ cells/cm^2) were first spread on three different kinds of agar plates: yeast extract-peptone-dextrose or YPD (NR-free), SC -leu (QA-free), and SC -NA -leu (NA/NAM-free). 2 µl (with sterile water at OD_{600}) of donor cells were then spotted onto the lawn of recipient cells (**Fig 3)**. The cells

were incubated for 2-3 days at 30° C. After incubation, data collected from these plates were a measurement of the diameter of growth.

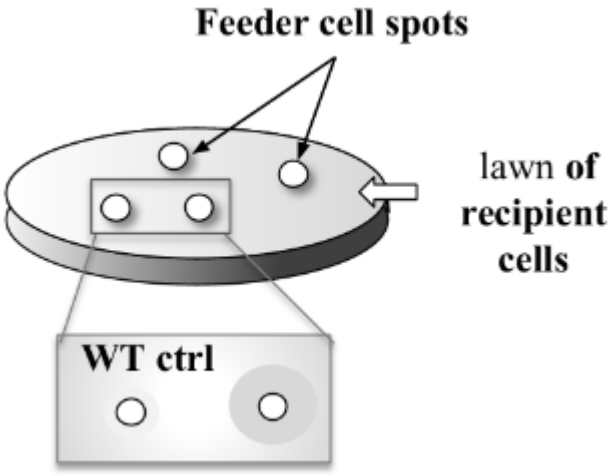

Fig. 3 Model of the cross-feeding spot assay. The recipient cells are spread onto the agar plate, while a WT and 3 other donor cell samples are spotted on top of the recipient cells (Raj et al., 2019).

Plasmid Extraction

We extracted the plasmid DNA from the yeast cells with the overexpression genes using beads beating techniques. We then introduced the plasmid into bacterial *E. Coli* cells (XL1-blue) by electroporation to allow the *E. Coli* cells to reproduce the plasmid to obtain sufficient amounts of DNA. After we extracted plasmid DNA from bacterial cells, we ran them through an electrophoresis gel to check their mass (**Fig. 4**). If there was not enough DNA extracted, as shown in the gel, additional bacterial colonies will be selected for DNA extraction. If sufficient concentration of DNA was generated, these samples were sent for sequencing. The sequencing was then reviewed and compared to standard DNA sequences with certain gene overexpression to confirm their identity and to reveal whether any mutations had emerged. The bacterial clones carrying correct plasmids were stored at -80˚C for future use. Confirmed plasmids were then transformed into yeast through heat shock. Finally, a cross-feeding assay was performed once again to confirm results from the first one.

Fig. 4 Example of an electrophoresis gel run to check the mass of plasmids from plasmid extraction. From left to right, the items run on the gel are the O'GeneRuler 1kb plus DNA Ladder, *NMA1* overexpression samples #3 and #4, *NMA2* overexpression samples #1 and #2, and *CIT2* overexpression samples #2 and #4.

Results

In this study particularly, analysis of the cross-feeding will be focusing on the results gathered of the *BNA1* overexpression gene. The levels of intermediate release of the recipient yeast cells due to the donor cells with *BNA1* overexpression were compared across the three different types of media that we plated the cells on, measured as the diameter of *BNA1* overexpression cell growth around each of the 4 donor cells spots. The size of cell growth represents the release of NAD+ intermediates, corresponding to each of the three types of media, into the extracellular area. The greater the release of intermediates, the larger the diameter of growth. QA (SC -leu) plates with donor cells carrying *BNA1* overexpression exhibited a larger circle of cell growth, compared to the WT (**Fig. 5A**). NA/NAM (SC -NA, -leu) plates exhibited a larger release of intermediates compared to the WT (**Fig. 5B**). For NR (YPD) plates, there was no observable change from the WT (**Fig. 5C**).

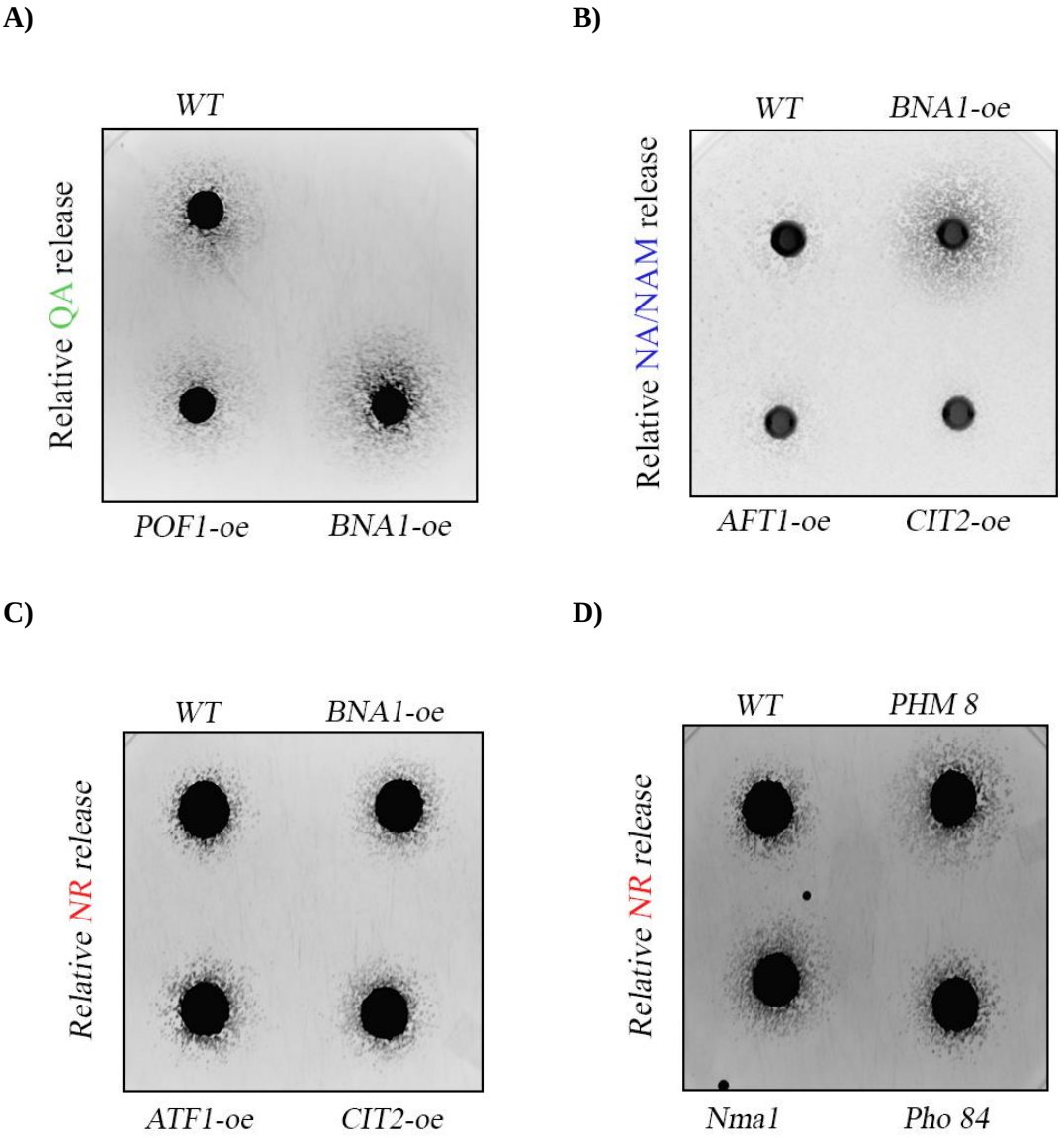

Fig. 5 Images of the results of cross-feeding spot assays after being grown at 30° Celsius for 2-3 days. The diameter of growth around each of the four "donor cell" spots indicate how much of the corresponding intermediate was released. **A**, SC (-leu) plate (QA-dependent), showed overexpression of *POF1* decreased QA release, and *BNA1* slightly increased QA release compared to the WT. **B**, SC -NA, -leu plate (NA/NAM-dependent), showed overexpression of *BNA1* increased NA-NAM release, while *AFT1* and *CIT2* overexpression abolished NA-NAM release compared to the WT. **C**, YPD (NR-dependent) plate, showed overexpression of *BNA1*, *ATF1*, and *CIT2* had relatively the same amount of

NR release as the WT. **D**, YPD (NR-dependent) plate, showed overexpression of *PHM 8* and *Nma1* had an increase of NR release compared to the WT and overexpression of *Pho 84*.

Discussion

The results of the cross-feeding showed the overexpression of *BNA*1 slightly increased growth in QA but largely increased growth in the NA/NAM salvage pathways in comparison to the WT. The increase in QA is expected, as *BNA1* overexpression was hypothesized to increase QA because it facilitates the intermediate 3-HA, which is involved in the step right before the production of QA (**Fig. 1**). However, a large increase in NA/NAM was unexpected, as *BNA*1 does not seem directly correlated to the NA/NAM salvage pathway.

Many possible explanations of these results emerge. Firstly, while the NA/NAM pathway as a whole was increased in activity, it is unknown whether it was both NA/NAM or just NA precursors that were produced more. Secondly, an explanation for the large release of NA/NAM could be that overexpression of *BNA1* affects other genes, such as *NPT*1 or *PNC*1, which facilitate more transformations to NA/NAM. Finally, there could be secondary pathways/other mechanisms contributing to the production of QA that we are not aware of, or there could be other requirements, along with the overexpression of *BNA*1, to increase QA yields. We are continuing to test the *de novo* pathway with the overexpression of *BNA1*, but without the blockage of the two enzymes 3-HK (3-Hydroxykynurenine) and 3-HA, to see if this will increase even more QA yields.

The Lin Lab is continuing to carry out more trials for the genes observed during initial screening so that results are consistent. Inconsistent results stemmed from not enough DNA being acquired when isolating yeast plasmid, uneven spreading of recipient cell lawns, some phenotypes were not as strong as observed in initial screening, and contamination easily occurred. A mutation in cells carrying overexpression of *NMA2* has been observed by sequencing, so it requires further trials to be done. Furthermore, the

identification of the role of other genes requires further investigation, as well as more in-depth experiments regarding unknown secondary mechanisms that *BNA1* overexpression may affect.

Conclusion

The present study is a novel attempt to understand the interconnectedness of NAD+ metabolism pathways. This research conducted on the *BNA*1 gene has led us to understand that the gene has not only affected the de novo pathway, but also the NA/NAM salvage pathway as well. Our original hypothesis positively correlated with the QA data gathered from our experiments, but not with the NA/NAM data, so future research is required to investigate the *BNA1* gene further to understand how it facilitates reactions in relation to NA/NAM and NR salvage pathways. Furthermore, the lab will continue to perform cross-feeding assays to test such questions as described earlier in the discussion section and others in the lab will be performing NAD+ assays, which will identify how genes in the metabolism cycle directly affect overall NAD+ production. This study largely contributes to existing knowledge about NAD+, and we have partially confirmed the role certain genes play in NAD+ metabolism pathways. If any genes are found to increase NAD+ levels, possible clinical trials for new therapies may be created.

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