NamPRT and NNMT – key drivers of NAD-dependent signalling

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1 Summary

NAD is best known as cofactor in redox reactions, but it is also substrate of NAD-dependent signalling reactions that consume NAD and release nicotinamide (Nam). In eukaryotes, two different Nam salvage pathways exist. While in lower organisms the initial deamidation of Nam is prevalent, in animals the direct conversion of Nam to the mononucleotide by Nam phosphoribosyltransferase (NamPRT) dominates and eventually remains as the single Nam recycling route in vertebrates.

Strikingly, loss of the deamidation pathway in early vertebrates is accompanied by the occurrence of a new enzyme that marks Nam for excretion by methylation – nicotinamide N-methyltransferase (NNMT). The physiological relevance of NNMT is still enigmatic. Why is there an enzyme that removes Nam from recycling, seemingly not having any other physiological role? And why is the occurrence of NNMT accompanied by a diversification of NAD-consuming enzymes?

We have used mathematical modelling approaches to resolve these counterintuitive observations. Our results indicate that NNMT is required to enable high NAD consumption fluxes necessitated by the increasing diversification of NAD-dependent signaling pathways. This kinetic regulation requires a high substrate affinity of the key enzyme for Nam salvage, NamPRT. Indeed, the affinity of NamPRT to Nam has previously been measured to be in the nanomolar range. Mathematical modelling supports the hypothesis that NNMT exerted an evolutionary pressure on NamPRT enforcing the development of its unusually high substrate affinity. Using multiple sequence alignments, we identified a sequence insertion, first occurring in vertebrates, that parallels an – experimentally verified – increase in the substrate affinity of the enzyme. Further simulations show that the deamidation pathway became obsolete owing to the high substrate affinity of NamPRT. Collectively, our results illustrate a close evolutionary relationship between NAD biosynthesis and the diversification NAD-dependent signaling pathways, potentially driven by the concomitant occurrence of a regulator of Nam salvage, NNMT.

2 Keywords

3 Introduction

NAD metabolism represents one of the most critical links that connect cellular signal transduction and energy metabolism. Even though best known as cofactor for various redox-reactions , NAD is involved in a number of signalling processes that consume NAD+ by cleaving the molecule to nicotinamide (Nam) and ADP-ribose [19]. These NAD+-dependent signalling reactions include but are not limited to poly- and mono-ADP-ribosylation, NAD+-dependent protein deacylation by sirtuins as well as the synthesis of calcium mobilizing molecules such as cyclic ADP-ribose [15]. These NAD+-dependent signalling processes participate in the regulation of virtually all cellular activities. The enzymes involved in these processes are sensitive to the available NAD+ concentration [16], which in turn is dependent on the NAD+/NADH redox ratio. Therefore, NAD+-dependent signalling can act as a transmitter of changes in the cellular energy homeostasis, for example, to regulate gene expression or metabolic activity [11].

The significance of NAD+-dependent signalling for NAD homeostasis has long been underestimated. However, it has now been established that substances affecting NAD biosynthesis lead to a rapid decline of the NAD concentration [4] suggesting that NAD+-dependent signalling consumes substantial amounts of NAD, which is why we later refer to them also as NAD-consuming reactions. The resulting NAD turnover differs in a cell type specific manner and can lead to an NAD-halflife as short as 2 hours [13]. To maintain the NAD concentration at physiological levels, NAD biosynthesis needs to act at an equally rapid rate. Imbalances in NAD-homeostasis have been linked to various, in particular, ageing-related diseases such as diabetes, neurodegenerative disorders and cancer [6, 19]. Several recent studies have demonstrated impressive health benefits of dietary supplementation with intermediates of NAD biosynthesis including NMN and nicotinamide riboside, NR [22]. Apparently, the exploitation of NAD biosynthetic routes, in addition to the use of nicotinamide as precursor (Fig. 1), results in increased NAD concentrations that stimulate NAD+-dependent signalling processes, in particular, protein deacetylation by sirtuins [14].

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In mammals, NAD biosynthesis predominantly relies on nicotinamide , a form of vitamin B3, as precursor. In the first step, Nam is converted to the mononucleotide, NMN, by Nam phosphoribosyltransferase (NamPRT) using phosphoribosylpyrophosphate (PRPP) as co-substrate. The key role of this enzyme is related to the nature of NAD+-dependent signalling reactions, namely, that in all these reactions Nam is cleaved off and potentially lost. If the released Nam were not recycled into NAD biosynthesis, humans would require a much higher daily vitamin B3 intake than the 16 mg that are the current daily recommendation [7]. The nearly complete recycling of Nam is achieved by an extraordinary high affinity of NamPRT to Nam, the Km being in the low nanomolar range [5]. Despite the importance of its salvage, Nam can also be marked for excretion by methylation. Indeed, the presence of nicotinamide N-methyltransferase (NNMT) in vertebrates [9] is among the most enigmatic and counterintuitive features of NAD metabolism. Why is there one enzyme (NamPRT) seemingly optimized to recycle even the faintest amounts of Nam back into NAD synthesis, while at the same time there is another enzyme (NNMT) that seems to have no metabolic function other than to remove Nam from NAD metabolism, This puzzle becomes even more intriguing when considering that

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the majority of lower organisms and plants deaminated Nam to nicotinic acid (NA) using the nicotinamide deamidase (NADA) before it can enter NAD biosynthesis via the Preiss-Handler pathway (Fig. 1).

We here present a phylogenetic analys of NAD-pathway in eukaryotes that in contrast to our previous analysis that has been lmited by the low number of eularyotic genomes available at the time, now comprehensively analysis the evolution of the pathway. In the present study, we furthermore combine the phylogenetic analysis with a mathematical modelling approach. This has proven to be a powerful tool to analyse the relationship between NAD biosynthetic and signalling fluxes when different subsets of Nam-converting enzymes are present. that enable us to explain the counterintuitive pathway evolution, such as that in Deuterostomes, there has been a strong tendency towards the co-existence of NamPRT and NNMT, while the pathway dominant in bacteria is lost in vertebrates. Moreover, this selection for the co-existence of NamPRT and NNMT was accompanied by a marked increase in the number of genes encoding NAD+-consuming signalling reactions. The model demonstrated that NNMT has a critical role to maintain high NAD+-consuming signalling fluxes by preventing accumulation of inhibitory Nam. We show that the presence of high affinity NamPRT together with NNMT makes the NADA dependent pathway obsolete. We furthermore identify a sequence insertion only found in Deuterostomes that encode NNMT but not NADA and experimentally verify that this insertion is indeed changing the affinity of NamPRT.

Taken together, our analyses suggest that the co-existence of NamPRT and NNMT has been a prerequisite to enable the evolutionary development of versatile NAD+-dependent signalling mechanisms present in vertebrates, and that NNMT likely exerted an evolutionary pressure on the NamPRT affinity development.

4 Results

4.1 Phylogenetic analysis of NAD biosynthesis and consumption

As shown in Fig. 1, NAD can be synthesized using several routes from three main precursors: tryptophan, nicotinamide (Nam) and nicotinic acid (NA). Nam and NA are together known as vitamin B3 or niacin. Alternatively, nicotinamide ribose (NR) can be used omitting the energetically unfavourable reaction of Nam phosphoribosyltransferase (NamPRT), requiring nicotinamide ribose kinase (NRK) instead [22]. Du to the hight turnover of NAD observed and the fact that only 1% of the tryptophan taken up with our diet is converted into NAD, vitamin B3 (Nam and NA) and to a lower extend NR are the major precursor of NAD-biosynthesis.

For the recycling of Nam, two different pathways exist. The pathway found in yeast and plants is using a four-step pathway starting with the deamination of Nam to nicotinic acid by NADA. The other three enzymes comprise the Preiss-Handler pathway that also exists in vertebrates. The recycling pathway found in mammals directly converts Nam into the corresponding mononucleotide (NMN) a reaction catalysed by NamPRT and driven by a non-stoichiometric ATP-hydrolysis. A similar reaction catalysed by an evolutionary related enzyme NAPRT, converts NA into the NA mononucleotide in the Preiss-Handler pathway. NMN and NAMN are converted into dinucleotides by the Nam/NA adenylytransferases (NMNATs). The recycling pathway via NA finally requires an amination step catalysed by NADsynthase, driven by the conversion of ATP to AMP producing pyrophosphate. Even though the latter pathway seems to be very inefficient, it is the pathway prefer-

entially used by most bacteria, fungi and plants (see Figure 2A), whereas most metazoans recycle Nam using NamPRT.

Analysing the phylogeny of the NAD recycling enzymes in Metazoa in more detail reveals that not only does NamPRT replace NADA, but in most organisms, especially in Deuterostomia, NamPRT is found together with the Nam methyltransferase NNMT (Figure 2B). NNMT methylates Nam to methyl-Nam that is in mammals excreted with the urine, thus removing Nam from recycling. NNMT seems to have arisen de novo in the common ancestor of Ecdysozoa and Lophotrochozo. We were not be able to find any gene with considerable similarity in fungi or plants, even though in fungi a gene named NNMT can be found in databases. These genes do, however, show very limited sequence similarities to the NNMT of nematodes or deuterostomes and in the yeast protein has later on be found to be a lysin-protein-methyltransferase [21]. Nematodes are the only organisms where we find NNMT together with NADA without NamPRT being present. In Deuterostomia the only large class that does only have NamPRT and seems to have lost NNMT again are Sauropsida and here especially birds. The reason why a lot of birds do not encode NNMT remains unclear, as the appearance is quite scattered (not shown). It might be related to the excretion system, as the product of NNMT methyl-nicotinamide is in mammals excreted with the urine. There are some species where we could not find NamPRT or NADA but NNMT, we assume that this is due to incomplete genomes in the database.

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In addition to the phylogenetic distribution of the two Nam salvage enzymes NADA and NamPRT, we looked at phylogenetic diversity of enzymes catalysing NAD-dependent signalling reactions. To do so we used the previously established classification into 10 different families [9] (For details see materials and methods and supplementary information). The numbers in Figure 2B denote the average number of NAD-dependent signalling enzyme families found in each taxa. With the exception of Cnidaria and Lophotrochozoa, we find average 3 to 4 families in Protostomia, whereas most Deuterostomia have on average more than 8 families with an increasing diversification of enzymes within some of these families [8].

Taken together, we found that the presence of NamPRT and NNMT coincides with an increased diversification of NAD-dependent signalling, whilst NADA is lost in vertebrates. That is counterintuitive, as one would expect that a decrease in precursor concentration caused by the precursor removal through NNMT, should cause a decrease of NAD availability and consequently less active NAD dependent signalling.

4.2 Dynamics of NAD biosynthesis and consumption

So why does the diversity of NAD-dependent signalling increase? And why does NADA disappear in Deuterostomia although it is the predominant pathway in bacteria, plants and fungi? Given the complexity of the NAD metabolic network, this question is difficult to be comprehensively addressed experimentally. Thus, to answer these questions we built a dynamic model of NAD metabolism using existing kinetic data from the literature (for details see materials and methods and supplementary material).

To be able to compare metabolic features of evolutionary quite different systems in our simulations, and as we have limited information about expression levels of enzymes or changes of kinetic constants during evolution, we initially used the kinetic constants found for yeast or human enzymes for all systems analysed and used equal

amounts of enzymes for all reactions. Wherever possible we did not only include substrate affinities but also known product inhibition or inhibition by downstream metabolites. As we assume that cell growth is, besides NAD-consuming reactions, a major driving force for NAD biosynthesis, we analysed different growth rates (cell division rates) by simulating different dilution rates for all metabolites.

First we addressed the question of the predominant coexitence of NamPRT and NNMT in vertebrates that coincides with an increase in the number of NAD-consuming enzyme families. We thus used our dynamic model of the NAD biosynthesis and consmption pathway to simulate the effect of the presence or absence of NNMT. We see that the presence of NNMT enables higher NAD consumption rates (Figure3A) while, as exptected, reducing the steady state concentration of NAD (Figure 3B). The decline in NAD-concnetrtion can be compensated by a higher expression of NamPRT (not shown) further increasing NAD consumption flux.

These findings can be explained when looking in more detail into the kinetic parameters of NamPRT and NAD-consuming enzymes such as Sirt1p. The increase of NAD consumption flux is caused by the fact that most NAD-consuming enzymes are inhibited by their product Nam, explaing why the presence of NNMT enables higher NAD consumption fluxes. At the same time, the high substrate affinity of NamPRT maintains a sufficiently high NAD-concetration.

As kinetic parameters of NamPRT are only available for the human enzyme [5] , we went on to theoretically analysed the potential effect of the NamPRT affinity (Km) on NAD steady state concentration and NAD consumption flux, leaving all other kinetic parameters constant. In the absence of NNMT (Figure 3C-D) a change in Km has very little effect on steady state NAD concentration and NAD consumption flux. In the presence of NNMT, however, NAD consumption flux and NAD concentration increases with decreasing Km values (Figure 5E-F).

It is intersting to note that, without NNMT, NAD concentration and consumption flux are both considerably affected by cell division rates, at least if the enzyme expression is kept constant. Even though, this is of course an artificial scenario, as one would assume organisms to regulate enzyme expression to achieve similar levels of metabolite concentrations instead. However, there seems to be an apparent trade off between maintainable NAD concentration and consumption flux.

When we compare NAD consumption and NAD concentration with and without NNMT with two different substrate affinities of NamPRT, we see that at a low affinity (Km of 1 mikroM, which is in the range of the Km of NADA for Nam, NAD consumption flux is only higher with NNMT at low cell division rates. This effect becomes stronger if we further lower the affinity (not shown) (Figure 3G-H).

The pathway dynamics are of course not solely dependent on one enzyme. Thus, what is the impact of the substrate affinity of NNMT that is competing with NamPRT for the same substrate? In Figure 4A we see that the substrate affinity values found in the human enzymes (indicated by black asterisks) are actually optimal with respect to both achievable steady state NAD concentration and consumption fluxes. Thus, a further increase of the affinity of NamPRT for Nam would not provide any advantage.

4.3 Sequence variance acquired in metazoans enhances substrate affinity

As our simulations suggest that NNMT might have excerted an evolitionary pressure on the development of NamPRT. We performed multiple sequence analysis to see if

we can find sequence variations in the protein sequence of NamPRT that indicate evolutionary changes of NamPRT upon the appearance of NNMT. The multiple sequence alignment of selected sequences is shown in Figure 5A and Supplementary Figure ?We recognized that Deuterostomia that have only NamPRT and NNMT (indicated by the blue circle) have an insert of ten amino acids corresponding to positions 43 to 52 of the human enzyme, that overlaps with a predicted weak nuclear localisation signal, that is lost when removing this sequence stretch. Furthermore, the inserted sequence corresponce to an structurallly unresolved loop structure in all avaibable crystal structure of human NamPR (e.g. [20] structure visualisation Figure 5B). The loop is connected to one of the beta-sheets involved in substrate binding.

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Full MSA to be prepared as suppl figure.

From these observations we derived two hypotheses regarding the potential function of the loop for NamPRT function. Firstly, we analysed whether the deletion of the amino acids 43 to 52 has an effect on the localisation of the human enzyme. But both wildtype and mutant protein show a mixed localisation in the cytosol and nucleus (Figure 5C). We therefore conclude that the partial nuclear localisation of NamPRT is not affected by the sequence deletion. The second hypothesis was that the sequence insertion influences NamPRT affinity and activity, which is also what our mathematical simulations would predict. We thus recombinantly expressed both wildttype and mutant NamPRT in E. coli. Upon purifictaion we meassured the activity using NMR detecttion of NMN produced in the presence and absence of ATP. It appears that the enzymatic activity of the mutant enzyme is much lower than that of the wildtype enzyme both with and without ATP. We furthermore see that the activity of the mutant enzyme can be increase with higher concentration of Nam and PRPP whereas that is not the case for the wildtype enzyme. Pointing to a decrease substrate affinity of the mutant enzyme. Supporting the second hypothesis and thus supporting the predictions derived from our mathematical modelling approach.

4.4 Loss of NADA in vertebrates

But why is NADA lost in vertebrates? Our simulations show that in direct competition NADA is outcompeted by the combined presence of NamPRT and NNMT but not by NamPRT alone. This effect deisappears of NamPRT affinity is lowered. ....

5 Discussion

5.1 Evolutionary and physiological role of NNMT

The dynamic interactions among metabolism, signal transduction, and gene regulation is still not very well understood. New approaches are required to disentangle the underlying network, helping us to understand how alterations affect human physiology. We here combine phylogenetic analysis and mathematical modelling of a central metabolic pathway supported by experimental analysis to reveal the likely evolutionary development of the NAD biosynthesis and consumption pathway and the physiological role of the methyltransferase NNMT.

We show that the vitamin B3-degrading enzyme NNMT plays a vital role for the diversification of NAD-dependent signalling reactions and potentially also for NAD homeostasis. Due to the fact that NamPRT has a very high affinity for Nam whereas NNMT has a rather low affinity for it, NNMT is able to remove excess Nam that

would lead to inhibition of NAD-dependent signalling reactions while maintaining NAD concentrations and even increasing NAD consumption fluxes.

The affinities measured for the human enzymes seem to be optimal and further increase in NAD consumption rates might only enabled by compartmentalisation of NAD biosynthesis.

In contrast to our results, it was proposed that NNMT is present in yeast (*Saccharomyces cerevisiae* [1]. The enzyme was later further characterised as methyltransferase of the transcription elongation factors TEF1 and TEF2 but not Nam [21].

5.2 General applicability

It is of course impossible to extend our conclusions to other metabolic processes without analysing them in more detail, but we have noted that the metabolism of other vitamins, such as pyridoxal (vitamin B6) metabolism, also contains vitamin degrading enzymes , that might thus have similar roles. Degrading enzymes do in general not receive the same attention as biosynthetic enzymes, reflected by the observation that NNMT has only recently been analysed in more detail, besides the much earlier recognition of NNMT as potential marker for some types of cancer (e.g. Ref?) and routine clinical measurements of urine methyl-Nam concentrations in the context of different diseases (e.g. Ref?). Our analysis show, however, that degrading enzymes do not solely modify substrates for better excretion, but can play a vital role both in human physiology as well as in the evolutionary development of biological processes.

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5.3 Limitations of our analysis and outlook

Among others, we neglected the potential effects of the methyl donor S-adenosylmethionin (SAM) and its precursor methionine in our analysis, although it is most likely contributing yet another regulatory level for Nam availability and thus an additional interaction point between gene regulation and metabolism. It has been shown earlier that in turn, NNMT expression influences protein methylation dependent on methionine availability [18]. One of the challenges in the analysis of this aspect is the availability of in vivo concentration measurements for SAM and the large amount of reactions using it as substrate. The same holds for the analysis of enzyme acetylation versus deacetylation by sirtuins that would enable prediction about enzyme activation or histone state if available.

6 Experimental Procedure

6.1 Dynamic modelling

Kinetic parameters (substrate affinity (Km) and turnover rates (kcat), substrate and product inhibitions) were retrieved from the enzyme database BRENDA and additionally evaluated by checking the original literature especially with respect to measurement conditions. Parameter values from mammals were used if available. For enzymes not present in mammals, values from yeast were used. The full list of kinetic parameters including reference to original literature can be found in Supplementary table 1. For NMNAT, the previously developed rate law for substrate

competition was used [17]. Despite these modifications, Henri-Michaelis-Menten kinetics were used for all reactions except the import and efflux of Nam, which were simulated using constant flux and mass action kinetics, respectively. All simulations were performed using the steady state calculation and parameter scan options provided by COPASI 4.22 [10]. The model will be available at the Biomodels database accession number

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. Related figures were generated using gnuplot.

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6.2 Phylogenetic Analysis

NADA, NamPRT, and NNMT enzymes or enzyme candidates were identified using Blastp with known enzymes against the NCBI non-redundant protein sequence database (nr). A list of functionally verified enzymes used as templates is given in supplementary table 2. This table also includes the length cut-offs for identified enzymes. The e-value cut-off was 1e-30 for all enzymes. Blastp parameters were set to yield maximum 20,000 target sequences, using the BLOSUM62 matrix with a word size of 6 and gap opening and extension costs of 11 and 1, respectively. Low-complexity filtering was disabled. Obvious sequence contaminations were removed by manual inspection of the results. The taxonomy IDs of the species for each enzyme was derived from the accession2taxonomy database provided by NCBI. Scripts for creating, analysing, and visualising the phylogenetic tree were written in Python, using the ETE3 toolkit (Huerta-Cepas, 2010).

6.3 Generation of eukaryotic expression vectors encoding C-terminally FLAG-tagged NamPT proteins

The open reading frame (ORF) encoding human NamPRT was inserted into pFLAG-CMV-5a (Merck - Sigma Aldrich) via EcoRI/BamHI sites. Using a PCR-based approach, this vector provided the basis for the generation of a plasmid encoding a NamPRT deletion mutant lacking amino acid residues 42-51 (NamPRT-Δ42-51). The sequences of the inserted ORFs were verified by DNA sequence analysis.

6.4 Transient transfection, immunocytochemstry and confocal laser scanning microscopy

HeLa S3 cells cultivated in Ham's F12 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, and penicillin/streptomycin, were seeded on cover slips in a 24 well plate. After one day, cells were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer's recommendations. Cells were fixed with 4% paraformaldehyde in PBS 24 hours post transfection, permeabilised (0.5% (v/v) Triton X-100 in PBS) and blocked for one hour with complete culture medium. After overnight incubation with primary FLAG-antibody (mouse M2, Sigma-Aldrich) diluted 1:2500 in complete medium, cells were washed and incubated for one hour with secondary AlexaFluor 594-conjugated goat anti mouse antibody (ThermoFisher, Invitrogen) diluted 1:1000 in complete culture medium. Nuclei were stained with DAPI and the cells washed. The cover slips were mounted on microscope slides using ProLong Gold (ThermoFisher, Invitrogen). Confocal laser scan imaging of cells was performed using a Leica TCS SP8 STED 3x microscope equipped with a 100x oil immersion objective (numerical aperture 1.4).

7 Figure Legends

7.1 Figure1

Schematic overview of NAD biosynthesis and consumption. NAD can be synthesized using several routes from altogether three main precursors: tryptophan (Trp), nicotinamide (Nam), and nicotinic acid (NA). Nam and NA are together known as vitamin B3 or niacin. To a lesser extend, nicotinamide ribose (NR) can be used omitting the energetically unfavourable reaction of Nam phosphoribosyltransferase (NamPRT), requiring nicotinamide ribose kinase (NRK) instead. Only 1% of the tryptophan taken up with our diet is converted into NAD, thus, vitamin B3 and, to a lower extend, NR are essential components of our diet, with Nam being the major NAD precursor in humans. Nam is furthermore the product of NAD-consuming signalling reactions such as sirtuins (NAD-dependent histone deacetylases) or PARPs (poly-ADP-ribosylases).

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For the recycling of Nam, two different pathways exist. The pathway found in yeast and many bacteria is using a four-step pathway starting with the deamination of Nam to nicotinic acid (NA) by Nam deamidase (NADA). The other three enzymes comprise the Preiss-Handler pathway that also exists in vertebrates. The recycling pathway found in vertebrates directly converts Nam into the corresponding mononucleotide (NMN) a reaction catalysed by NamPRT and driven by a non-stoichiometric ATP-hydrolysis. A similar reaction catalysed by an evolutionary related enzyme NAPRT, converts NA into the NA mononucleotide (NAMN). NMN and NAMN are converted into dinucleotides by the Nam/NA adenylytransferases (NMNATs). The recycling pathway via NA finally requires an amination step catalysed by NADsynthase (NADS), driven by the conversion of ATP to AMP producing pyrophosphate.

7.2 Figure 2

Evolutionary distribution of NADA, NNMT and NamPRT and their relation to the number of NAD consumers. A) Distribution of NADA, NNMT and NamPRT in selected major taxa. NADA is dominant in Bacteria, Fungi, and Plants (Viridiplantae), whereas NamPRT together with NNMT is dominant in Deuterostomia. Numbers at the pie charts show, how many species of the taxon possess the respective enzyme combination indicated by the colour explained in the lower right of the figure. Below the taxon name, the number of species in that taxon is given.

B) Common tree of selected taxa within the Metazoa, including 334 species. The pie charts indicate the distribution of species within the respective taxon that have the enzyme combination indicated by the colour, explained in the lower right. The size of the pie charts is proportional to the logarithm of the number of species analysed in the particular taxon. The numbers below the taxon names indicate the average number of NAD-consuming enzyme families found in all sub-taxa. The branch length is arbitrary.

7.3 Figure 3

NAD consumption flux and NAD concentration in simulations of organisms with NADA(?) and with and without NNMT at different Nam import and cell division rates.

7.4 Figure 4

Impact of NNMT on NAD consumption and NAD concentration in simulations of organisms with NADA or NamPRT.

7.5 Figure 5

NAD consumption flux and NAD concentration in simulations of organisms with NamPRT and with and without NNMT at different KMs for NamPRT and cell division rates.

7.6 Figure 6

The substrate affinity of NNMT and NamPRT have opposite effects on NAD consumption (A) and concentration (B), as would be expected. The affinities previously measured for human enzymes (indicated by a black asterisk) appear to be close to optimal, as further improvements would have little or no effect on NAD consumption or concentration.

7.7 Figure 7

Deuterostomia that encode NNMT show a sequence insertion in the N-terminal region of NamPRT. A) Multiple sequence alignment of NamPRT of selected deuterostomes show a sequence insertion in organisms that encode NamPRT and NNMT. Coloured circles indicate the enzymes present in the species; blue: NamPRT and NNMT; black: NamPRT, NADA, and NNMT; yellow: NamPRT and NADA.

B) The inserted region is not resolved in crystal structures of human NamPRT and thus appears to be a flexible loop structure at the surface of the NamPRT dimer, coloured in red. The visualisation is based on a structure prediction of SWISS-MODEL [2, 3] of the sequence of the human NamPRT (P43490) using the model 2H3D as template [20].

8 Comments and parts of old version not included yet

Chordata: NADA disappears. These analyses also support the view that Tunicata and Branchiostoma are not part of the phylum chordata.

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Is this view in any way argued upon by anyone??

In the evolutionary context, an additional question arises: Why do only a few organisms, mostly less complex animals, possess the gene for NADA in addition to NamPRT and NNMT. When including NADA into the simulations, we see that the effect of NADA on NAD consumption is very limited in the presence of NNMT (old Figure 4F) even at high expression levels (see old Suppl. Figure S1C and D) and could be compensated by increased expression of NamPRT (not shown).

Until this point we have neglected compartmentalisation of the pathway. We do however know from previous studies that in early vertebrate development a compartmentalisation of the pathway has occurred reflected by a gene triplication of NMNAT and the occurrence of compartment-specific domains called ISTIDs (Lau, 2010). Looking at the evolutionary timepoint of appearance of NNMT and the gene triplication of NMNATs, we see that NNMT occurs prior to the gene triplication and the first occurrence of ISTIDs and we have confirmed this in our own analysis (not shown).

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This time point can only be reconstructed, but is unknown. I think a figure with the likely reconstruction would help to follow the argumentation

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