# Identifying evolutionary and kinetic drivers of NAD-dependent signalling

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## Summary

## Introduction

NAD metabolism has received increasing interest as changes therein are associated with a large number of diseases including but not limited to neurodegeneration1, diabetes2, 3, obesity 4, 5, 6, 7, heart diseases 8, 9, muscle dystrophy10 , renal dysfunction 11 and different types of cancer 12, and it has been established that the gradual decrease in NAD during ageing is one of the major driving forces of age-related pathologies 13, 14, 15. In addition, NAD metabolism has been identified to be a key regulator for axonal degradation16, 17, 18. It is therefore not surprising that NAD metabolism has emerged as promising pharmacological target for disease treatment19, 20, 21. However, to develop efficient new therapeutic strategies a better understanding of the pathway dynamics and the pathway components determining it is urgently required.

NAD represents one of the most critical links connecting cellular signal transduction and energy metabolism. Even though it is 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-ribose22. These NAD-dependent signalling reactions include poly- and mono-ADP-ribosylation23, 24, NAD-dependent protein deacylation by sirtuins25, and the synthesis of calcium-mobilizing molecules such as cyclic ADP-ribose26. 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 concentration27, 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 activity28.

The significance of NAD-dependent signalling for NAD homeostasis has long been underestimated. But, it has now been established that substances affecting NAD biosynthesis lead to a rapid decline of the NAD concentration29. This suggests that NAD-dependent signalling reactions consume substantial amounts of NAD. Therefore, we hereafter 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 half-life as short as 15 minutes 30. 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 mainly ageing related diseases, such as neurodegeneration1, diabetes2, 3, obesity 4, 5, 6, 7, heart diseases 8, 9 and cancer12, 22, 31. Several recent studies have demonstrated impressive health benefits of dietary supplementation with intermediates of NAD biosynthesis including Nam mononucleotide (NMN)15 and Nam riboside (NR)2, 32. Apparently, the exploitation of NAD biosynthetic routes, in addition to the use of Nam as precursor (fig. 1), results in increased NAD concentrations that stimulate NAD-dependent signalling processes, in particular, protein deacetylation by sirtuins33.

Due to the constant release of Nam through NAD-consuming signalling reactions, the NAD salvage pathway, using Nam as precursor, is the most important NAD synthesis pathway. In general, two principal pathways exist that recycle Nam. Vertebrates use a direct two-step pathway starting with the conversion of Nam into the mononucleotide NMN by the Nam phosphoribosyltransferase (NamPT) using phosphoribosyl pyrophosphate (PRPP) as co-substrate. The nearly complete recycling of Nam by NamPT is achieved by an extraordinary high substrate affinity to Nam, the *KM* being in the low nanomolar range34. This appears to be mediated by an ATP-dependent phosphorylation of a histidine residue in the catalytic core35. Despite the importance of its salvage, Nam can also be marked for excretion by methylation. The presence of Nam N-methyltransferase (NNMT) in vertebrates36 is among the most enigmatic and counterintuitive features of NAD metabolism. While NamPT is seemingly optimised to recycle even the faintest amounts of Nam back into NAD synthesis, NNMT seems to have no function other than to remove Nam from NAD metabolism. It has, however, been suggested that the process potentially acts as a metabolic methylation sink 37.

In most prokaryotes as well as in plants and fungi, another pathway consisting of four reactions starting with the deamidation of Nam to nicotinic acid (NA) by the Nam deamidase (NADA) is used. (fig. 1). The three enzymes that act downstream of NADA belong to the Preiss-Handler pathway that also exists in vertebrates. NA is converted into the corresponding mononucleotide (NAMN), in a reaction performed by the NA-specific phosphoribosyltransferase NAPRT. The conversion of both mononucleotides, NMN and NAMN, into their corresponding dinucleotides, NAD and NAAD, is catalysed by the Nam/NA adenylyltransferases (NMNATs) that are essential in all organisms38. The recycling pathway via NA finally requires re-amidation of NAAD by NAD synthase. This final reaction includes an enzyme adenylation step that consumes ATP. Therefore, the Nam recycling by NADA appears to be energetically less efficient than the recycling pathway starting with NamPT.

We and others have earlier shown that the two NAD biosynthesis pathways starting from Nam coexists in some eukaryotes 36, 39, as well as in some bacterial species40. Why these pathways coexists in some organisms and over a very long evolutionary time frame and why NADA nevertheless disappeared in vertebrates, is not known. We furthermore have little understanding of the physiological role of NNMT and its impact on NAD-metabolism so far.

As earlier evolutionary analyses have been limited by few eukaryotic genomes available at the time, we here performed a comprehensive phylogenetic analysis of the NAD pathways using 793 eukaryotic and 7892 prokaryotic genomes. Our results suggest that there has been an evolutionary transition resulting in the coexistence of NamPT and NNMT in deuterostomes, while the deamidation pathway, which is dominant in bacteria, became superfluous. This was accompanied by a marked increase in the number of NAD-consuming signalling enzymes. Mathematical modelling of the pathway revealed an unexpected positive kinetic role of NNMT for NAD-consuming signalling fluxes, through prevention of accumulation of Nam. In addition, our model predicts that NNMT likely exerted an evolutionary pressure on NamPT to develop a high affinity towards its substrate Nam. Indeed, we identified a short sequence insertion in NamPT, which first occurs in Deuterostomes and that appears to modulate the affinity of NamPT. Simulating resource competition, we furthermore show that the presence of high affinity NamPT together with NNMT makes the NADA-dependent pathway obsolete, providing an explanation for the evolutionary transition of the pathway in Metazoa.

Taken together, our analyses suggest that the coexistence of NamPT and NNMT has been a prerequisite to enable the evolutionary development of versatile NAD-dependent signalling mechanisms present in vertebrates.

## Results

### Paradoxical evolutionary correlation between NAD-dependent signalling and precursor metabolism

To understand the functional roles and potential interplay between the three known enzymes that use Nam as substrate (NamPT, NADA and NNMT), we conducted a comprehensive analysis of the phylogenetic distribution of these three enzymes. As shown in figure 2A, bacteria, fungi, and plants predominantly possess NADA and only a few of them harbour NamPT. In contrast, Metazoa predominantly lost NADA and encode for NamPT together with NNMT. NADA and NamPT, the two enzymes that initiate the two different NAD salvage pathways, show a scattered distribution in bacteria. Cooccurrence of these enzymes is rather rare, and has mainly been found in bacteria 40 and some marine invertebrates 36.

NNMT seems to have arisen *de novo* or diverged rapidly in the most recent common ancestor of Ecdysozoa and Lophotrochozoa (fig. 2B). We were unable to find any indication for the presence of NNMT in fungi or plants (Blastp e-value cutoff 0.1).

Nematodes are the only organisms, where we observed a concomitant presence of NADA and NNMT. In deuterostomes, the only large clade that possesses only NamPT and seems to have lost NNMT are Sauropsida, and among them especially birds. The reason why about half of the sequenced bird genomes do not seem to encode for *NNMT* remains unclear. The distribution of *NNMT* in birds is quite scattered (suppl. fig. S2). It is possible that detection of *NNMT* in some bird genomes failed because of their high GC content 41 or because of difficulties in assembling very small chromosomes common in birds. The lack of NNMT might alternatively be related to the differences in the excretion system, as the product of NNMT, methyl-Nam, is in mammals excreted with the urine. There are few metazoan species for which we could not find NamPT or NADA, while NNMT was detected. We assume that this is due to incomplete genomes in the database, as these species are scarce and their distribution is widely scattered.

In addition to the phylogenetic distribution of the two Nam salvage enzymes NADA and NamPT, we analysed the phylogenetic diversity of enzymes catalysing NAD-dependent signalling reactions. To do so, we used the previously established classification into ten different families of NAD-consuming signalling enzymes 36, including PARP1-3,PARP4, PARP6/8, PARP7/9-15, PARP16, sirtuins, tankyrases, ADPR-cyclases, mono-ADP-ribosyltransferases and t-RNA-phosphotransferases. The detailed list of templates used for the phylogenetic analyses can be found in supplementary table S1. The numbers shown in figure 2B denote the average number of NAD-dependent signalling enzyme families found in each clade. With the exception of Cnidaria and Lophotrochozoa, we find an average of three to four families in protostomes, whereas most deuterostome species have, on average, more than eight families with an increasing diversification of enzymes within some of these families, especially PARPs42.

Taken together, we found that NADA is lost in vertebrates, but strongly preserved in most other organisms, despite the higher energetic requirement of this pathway. Moreover, the transition for having both NamPT and NNMT coincides with an increased diversification of NAD-dependent signalling. This observation seems counter-intuitive, as one would expect that increased NAD-dependent signalling should be accompanied by an increase of substrate availability for NAD biosynthesis.

### Functional properties of NamPT and NNMT have evolved to maximise NAD-dependent signalling

To resolve this apparent contradiction, we wished to scrutinize the NAD metabolic network. Given the complexity of this network, we turned to modelling approaches and built an ordinary equation based dynamic model of NAD metabolism using previously reported kinetic data (for details, see Experimental Procedures and suppl. tab. S2).

To be able to compare metabolic features of quite different organisms in our simulations and as we had limited information about species-specific expression levels of enzymes, we initially assumed equal expression rates for all enzymes. As we have very few cross species kinetic data, we were furthermore mainly relying on kinetic constants found for human or yeast enzymes. Wherever possible, we included both substrate affinities and known product inhibitions or inhibition by downstream metabolites. As we in addition assumed 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 unexpected correlation between the transition to the cooccurrence of NamPT and NNMT and the increase in the number of NAD-consuming enzymes. We calculated steady state NAD concentrations and NAD consumption fluxes by simulating NAD biosynthesis via NamPT in the presence or absence of NNMT. To achieve free NAD concentrations in the range reported in the literature and due to the very low turnover of NamPT, we used tenfold higher NamPT levels compared to other enzymes. We also adjusted the amount of NMNAT accordingly to avoid that NAD synthesis rates are limited by this enzyme. Surprisingly, as shown in figure 3, the presence of NNMT enables higher rather than lower NAD consumption fluxes (fig. 3A), although it diminishes the steady state concentration of NAD (fig. 3B). The decline in NAD concentration can be compensated by a higher expression of NamPT, further increasing NAD consumption flux (dashed lines in fig. 3A and B).

These results can be explained by the kinetic parameters of NamPT and NAD-consuming enzymes such as Sirtuin 1 (Sirt1). Most NAD-consuming enzymes are inhibited by their product Nam. Thus, the presence of NNMT enables higher NAD consumption fluxes, by removing excess Nam from the cells. At the same time, high substrate affinity of NamPT maintains a sufficiently high NAD concentration, although the concentration is, as expected, lower than in the system without NNMT.

Kinetic parameters of NamPT were previously measured for the human enzyme 34 as well as for some bacterial enzymes 43, the latter having a much lower substrate affinity for Nam. We thus analysed the potential effect of NamPT affinity (*KM*) on NAD steady state concentration and NAD consumption flux. In the absence of NNMT, a variation of the substrate affinity of NamPT for Nam has very little effect on steady state NAD concentration and NAD consumption flux (fig. 4A and B). In the presence of NNMT, however, NAD consumption flux and NAD concentration increases with decreasing *KM* values of NamPT (fig. 4C and D).

Remarkably, NAD concentration and consumption flux are both considerably affected by cell division rates in a system without NNMT. Our simulations furthermore predict a trade-off between maintainable NAD concentration and consumption flux, in the absence of NNMT. In the presence of NNMT, however, NAD consumption rates and concentrations are almost independent of cell division rates. Again we assumed constant expression of all enzymes to make the systems comparable, although, one would assume organisms to regulate enzyme expression under physiological conditions.

Given that a lower affinity of NamPT has been described for the bacterial enzyme43 where NNMT is not present, we were wondering if the advantage provided by NNMT is bound to a high affinity of NamPT. In figures 4E and F we show the direct comparison of simulations assuming different affinities of NamPT, in the presence or absence of NNMT. Interestingly, at an affinity of *KM* = 1 µM, which is in the range of the *KM* of NADA for Nam and below the measured affinity of bacterial NamPT, NAD consumption flux is only higher in the presence of NNMT if cell division rates are low (fig. 4E). However, if the affinity of NamPT is high enough, consumption rates are always higher with NNMT than without. The NAD concentration is, as would be assumed, always lower with NNMT (fig. 4F).

To understand the interplay and competition for Nam between NamPT and NNMT, we furthermore analysed a wide range of possible *KM* values for both enzymes. As shown in figure 5, the simulations indicate that both NAD consumption flux and NAD concentration would be minimal in case of a high *KM* for NamPT and a low *KM* for NNMT. Conversely, lowering the *KM* of NamPT to the nanomolar range substantially increases NAD consumption and concentration, which reach a plateau when the *KM* of NNMT is concomitantly elevated to the submicromolar range. The asterisks in figure 5 denote the *KM* values found for the human enzymes. It shows that the naturally occurring *KM* values are in an optimal range, where further adjustment would lead to little or no increase of NAD consumption flux or NAD concentration.

### Sequence variance acquired in metazoans enhances substrate affinity

Given the kinetic interdependence of NNMT and NamPT shown in figure figures 4 and 5, it seems possible that NNMT has exerted an evolutionary pressure on the development of NamPT. In this case, one would expect to observe adaptations that are reflected in the NamPT protein sequence arising shortly after the occurrence of NNMT. To explore this, we created a multiple sequence alignment of NamPT protein sequences from Metazoa. An alignment of selected sequences is shown in figure 6A and a more comprehensive multiple sequence alignment containing a larger number of species can be found in supplementary figure S1. We found that most deuterostomes that possess only NamPT and NNMT (indicated by the blue circle, fig. 6A) have an insert of ten amino acids corresponding to positions 42 to 51 in the human enzyme. This insert overlaps with a predicted weak nuclear localisation signal (NLS). The NLS prediction is lost when the insert is removed. These ten amino acids furthermore correspond to a stretch at the protein surface that is unresolved in all available crystal structures of human NamPT (e.g. structure visualisation in fig. 6B from44). Intriguingly, this presumed loop, depicted in red in figure 6B, is connected to one of the β-sheets involved in substrate binding35 and in the functional homodimer, the two loops are placed side-by-side.

From these observations, we derived two possible hypotheses regarding the role of the loop in NamPT function. The first hypothesis is that the presence of the loop could affect the subcellular localisation of NamPT, as it is overlapping with a predicted NLS. To test this hypothesis, we created a mutant NamPT lacking the loop and recombinantly expressed FLAG-tagged wildtype and mutant NamPT in HeLa S3 cells. Immunofluorescence imaging showed a mixed cytosolic nuclear localisation for both the wildtype and the mutant NamPT (fig. 6C). Thus, deletion of the loop did not compromise nuclear localisation.

The second hypothesis is based on our model simulations that predict that the presence of NNMT might have lead to a shift in the evolutionary pressure on NamPT kinetics and that therefore the sequence insertion might have an effect on substrate binding of NamPT. To analyse this possibility, we expressed and purified both the wildtype and the mutant enzyme in *E. coli*., N-terminally fused to a 6xHis-tag. The size exclusion chromatography profile showed that both wildtype and mutant protein were expressed as dimers (see suppl. fig. S3), indicating that the mutant protein is likely to be folded correctly. The enzymatic activity was measured by NMR spectroscopy using the detection of NMN. Upon incubation with the NamPT inhibitor FK866 45 for 30 minutes, both wildtype and mutant NamPT did not produce any NMN, suggesting that binding of FK866 is not affected by the mutation (suppl. fig. S4). The mutant enzyme showed furthermore no detectable activity with 100 µM substrate after 10 min of incubation at 30 °C, while with 1mM substrate enzymatic activity could be detected with the mutant enzyme. The measured activity was, however, reduced by 20% compared to the wildtype enzyme under the same condition (fig. 6D). These observations suggest that the mutant enzyme is catalytically active, retains its dimeric state and sensitivity to FK866, but, has a lower activity and affinity to Nam, supporting the predictions derived from the metabolic modelling approach.

To see whether we can find a molecular explanation for the reduced affinity of the mutant enzyme, we analysed different available protein structures of NamPT and tested whether the loop insertion could potentially lead to dynamic structural rearrangements. To this end we applied homology modelling (fig. 6C) and molecular dynamics simulations for structures with and without the loop insertion (fig. 6F) Taken together we did not observe substantial structural rearrangements and the molecular dynamics simulations showed only limited structural changes upon loop insertion and we observed a mostly structurally stable catalytic core. This might be based on the fact that all available protein structures of NamPT differ very little even at the catalytic side (between 0.33Å and 0.95Å see supplementary table S3). Some residues close to the catalytic, nevertheless, showed slightly elevated mobilities in the wildtype structure, although these elevated mobilities were dominated by rare events during the simulation time of 1 µs. They thus do not appear as a robust change of structural dynamics upon loop insertion. The observed alterations could nevertheless provide a hind towards reduced mobility of the mutant enzyme that explain the observed changes in affinity and activity upon loop deletion.

### NamPT and NNMT made NADA obsolete in vertebrates

Finally, we wanted to understand why NADA was lost in vertebrates. As shifts in evolutionary selection pressure may result from competition for resources, we built a two-compartment model, based on the pathway model described above. One compartment contains NADA, while the other one contains either NamPT alone or together with NNMT. Both compartments share a limited Nam source (for details se suppl. Table. 2 and suppl. file 2). Without NNMT, the compartment containing NADA shows slightly lower NAD consumption rates (fig. 7A), but is able to maintain much higher NAD concentrations especially at low cell division rates (fig. 7B). At high cell division rates, steady state concentrations in both compartments are similar. This might explain why in bacteria that often have relatively high growth rates, both systems coexist.

In the presence of NNMT, the NamPT compartment has both higher NAD consumption rates and higher steady state NAD concentrations than the compartment containing NADA (fig. 7C and D). The higher NAD concentrations in the compartment containing NamPT and NNMT can, however, only be maintained if the affinity of NamPT for Nam is high enough. If the substrate affinity of NamPT is too low (high *KM*), the NADA compartment is able to maintain higher NAD concentrations, but still has a lower NAD consumption flux. Taken together, the results suggest that the NADA pathway might have become obsolete upon emergence of a high affinity NamPT. This in turn might have been induced by the appearance of NNMT.

## Discussion

Although NNMT has been identified a as potential marker for some types of cancer46 and potential drug target for the treatment of metabolic disorders7, its presence in most vertebrates and physiological role is still enigmatic. Using phylogenetic analysis we showed that the appearance of NNMT in Protostomia is followed by a diversification of NAD-consuming reactions. To explain these results, we built a mathematical model of the pathway to simulate NAD pathway dynamics with and without NNMT. It appears that the presence of NNMT enables higher NAD-consumption fluxes, as many NAD-consuming enzymes are inhibited by their product Nam. This suggests that the diversification of NAD-consuming enzymes and thus the potentially increased NAD-consumption flux observed in mammals might have been enabled by the presence of NNMT.

As NAD concentrations are lowered by the removal of NAD precursor through NNMT, a high affinity of NamPT is required for high NAD consumption fluxes and to maintain a sufficiently high NAD concentration. It therefore seems plausible that NNMT might have driven NamPT evolution. Looking at the enzyme affinities of the human enzymes, it furthermore appears that both NNMT and NamPT reached an almost optimal state with respect to their substrate affinity, as further changes in the affinity of either NamPT or NNMT would not result in much higher steady state NAD concentrations or NAD consumption fluxes.

The detailed analysis of the sequence evolution of NamPT revealed that organisms that encode NNMT and have lost NADA have a conserved insertion in the N-terminal sequence of NamPT that corresponds to a structurally unresolved loop on the surface of the enzyme. Experimental analysis showed that this loop insertion indeed affects the substrate affinity of NamPT supporting the hypothesis derived from our phylogenetic analysis.

The main healthy tissue expressing NNMT are the liver and adipose tissues, while no or only little expression of NNMT is observed in most other organs47. Increased NNMT expression is observed in some types of cancer46, and might serve to remove Nam derived by increased NAD-dependent signalling. To maintain high NAD concentrations, a simultaneous higher expression of NamPT is required, which is what has been found in some types of cancer48, 49. It is worth noticing that NNMT is only advantageous as long as NamPT affinity is sufficiently high. This suggests that certain types of cancer expressing NNMT at a high level, would potentially be more susceptible to competitive inhibitors of NamPT. Several of such inhibitors are currently tested in clinical studies19, 50. Based on our analysis, we would suggest that it might be reasonable to test patients before treatment whether or not NNMT expression can be detected in the tumour tissue, as non-NNMT expressing tumours might respond less to competitive NamPT inhibitors and missing Nam degradation in those cancer cells would potentially lead to an accumulation of Nam that could outcompete the inhibitor.

Neither the scattered distribution of NamPT and NADA that is especially pronounced in bacteria40, nor the loss of NADA in the ancestor of vertebrates has been understood earlier. Our combined phylogenetic-modelling analysis now provides a potential explanation for both observations. Using simulated competition between two compartments that share the same limited source of Nam, we show that the compartment that contains NamPT and NNMT can maintain a higher steady state NAD concentration and NAD consumption rate than the compartment containing NADA. This is, however, only the case if NamPT substrate affinity is sufficiently high. The dominant enzyme combination found in vertebrates, a high-affinity NamPT with NNMT, thus seems to provide a competitive advantage.

In our analyses, we did not consider the potential effects of cosubstrates of the investigated pathway. Such cosubstrates include targets of the NAD-consuming enzymes, such as acylated proteins for sirtuins, for example, or phosphoribosyl pyrophosphate (PRPP) and ATP that are required for NMN synthesis by NamPT. Furthermore, the presence of the methyl donor *S*-adenosyl methionine (SAM) and its precursor methionine that have been shown to potentially limit the effect of NNMT51 was not considered here. As cosubstrate availability might alter the behaviour of the system, these should thus be included in future analyses. Unfortunately, information about the *in vivo* concentrations of these cosubstrates is currently very limited.

Taken together, we have been able to comprehensively analyse the functional coevolution of several enzymes of the NAD pathway. The appearance of NNMT seemingly initiated and drove complex alterations of the pathway such as an increase and diversification of NAD-dependent signalling, followed by an increase in NamPT substrate affinity. A schematic overview is given in figure 8. This transition appears to be accompanied by the loss of NADA in vertebrates and the first gene duplication of NMNATs52. We also noted that the second gene duplication of NMNATs and thus the further compartmentalisation of NAD metabolism is cooccurring with a site-specific positive selection event in NNMT (see suppl. fig. S6).

We here used an approach that combines detailed phylogenetic analysis with dynamic metabolic modelling and have been able to explain observed evolutionary changes in the NAD biosynthesis and consumption pathway. Based on the simulated pathway dynamics, we have furthermore derived predictions for physiological interdependencies between several enzymes of the pathway that are potentially relevant for new disease treatments. Our results, including the experimental verification of our predictions, demonstrate the potential of those approaches for the analysis of dynamic networks and how it can be used to unravel functional interdependencies within pathways of interest.

## Experimental Procedures

### Phylogenetic Analysis

Functionally verified sequences of NNMT, NADA, NamPT, and NAD-consuming enzymes were used as sequence templates for a Blastp analysis against the NCBI non-redundant protein sequence database. For a list of template sequences see supplementary table S1. 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. To prevent cross-hits, a matrix was created in which the lowest e-values were given at which Blast yielded the same result for each query protein pair. With help of the matrix, the e-value cut-off was set to 1e-30 for all enzymes. To further prevent false positives, a minimal length limit was set based on a histogram of the hit lengths found for each query protein, excluding peaks much lower than the total protein length. Length limits are given in supplementary table S1. In addition, 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 3.5, using the ETE3 toolkit53 and are available at …..

### 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 mammalian species were used if available. For enzymes not present in mammals, values from yeast were integrated. The full list of kinetic parameters including reference to original literature can be found in supplementary table S2. For NMNAT, the previously developed rate law for substrate competition was used54. Otherwise, Henri-Michaelis-Menten kinetics were applied for all reactions except the import and efflux of Nam, which were simulated using constant flux and mass action kinetics, respectively. Steady state calculation and parameter scan tasks provided by COPASI 4.2455 were used for all simulations. The model files are provided in SBML as supplementary file 1 and 2 and will be made available at the Biomodels database upon publication. Related figures were generated using Gnuplot 5.0.

### Generation of expression vectors encoding wild-type and mutant human NamPT

For eukaryotic expression with a C-terminal FLAG-epitope, the open reading frame (ORF) encoding human NamPT was inserted into pFLAG-CMV-5a (Merck - Sigma Aldrich) via EcoRI/BamHI sites. Using a PCR approach, this vector provided the basis for the generation of a plasmid encoding a NamPT deletion mutant lacking amino acid residues 42-51 (Δ42-51 NamPT). For prokaryotic expression with an N-terminal 6xHis-tag, the wild-type and mutant ORFs were inserted into pQE-30 (Qiagen) via BamHI and PstI-sites. All cloned sequences were verified by DNA sequence analysis.

### Transient transfection, immunocytochemistry, 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).

### Purification of NamPT

The cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 4 mM dithiothreitol (DTT), 1 mg/mL lysozyme, 1X Complete EDTA-free protease inhibitor cocktail (Roche)). After sonification, the lysate was centrifuged for 30 min at 13000 g, and the clear lysate was incubated with 2 mL of Nickel-NTA resin (Qiagen). Non-specific protein binding was removed with washing buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM DTT, 20 mM imidazole). The protein was eluted with 2.5 mL of elution buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 300 mM imidazole).

The eluted protein was immediately subjected to size exclusion chromatography (SEC) on an ÄKTA pure system (GE Healthcare) and loaded onto a HiLoad 16/60 Superdex 200 pg column (GE Healthcare), run at a flow rate of 1 mL/min with SEC buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl). Fractions corresponding to the size of recombinant protein were pooled and used for enzymatic assay. The purity and size of the protein were assessed by SDS-PAGE.

### Enzymatic Assay

2 µM of enzyme were incubated with 5-phospho-D-ribose 1-diphosphate (PRPP, 0.1 mM or 1 mM) and nicotinamide (Nam, 0.1 mM or 1 mM) in reaction buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 6 mM MgCl*2*, and 0.03% BSA), in absence or presence of 1 mM of adenosine triphosphate (ATP). The 1.2 mL reaction was incubated for 10 minutes at 30 °C and the enzymatic activity stopped with 0.1 mM of FK866, the samples were frozen in liquid nitrogen.

### Sample preparation and NMR spectroscopy

The samples were dried with an Eppendorf Vacufuge Concentrator, and then resuspended with 200 µl of NMR buffer containing 5% deuterated H2O (D2O) and 1 mM 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

1D 1H NMR spectra were acquired on a 850 MHz Ascend Bruker spectrometer equipped with 5 mm TCI triple-resonance CryoProbe and a pulse field gradients along the z-axis. The experiments were acquired with the zgesgppe pulse sequence, allowing water suppression using excitation sculpting with gradients and perfect echo. The temperature was kept constant at 300 K and the acquisition was started with 2000 scans, 1 s relaxation delay, 1.6 s acquisition time, 65 000 data points, and a spectral width of 14 ppm.

The spectra phase and baseline were automatically and manually corrected using TopSpin 3.5 software (Bruker Biospin). Quantification of nicotinamide mononucleotide (NMN) was done by the integration of the peak at 9.52 ppm and DSS used as an internal standard.

### Molecular dynamics simulations

We performed all-atom molecular dynamics simulations with explicit solvent for NamPT (PDB Code: 2H3D 44) with and without loop insertion. We used the AMBER99SB-ILDN force field 56 with the TIP3P water model 57 in GROMACS 5.1.2 58. The structures were simulated each in a box of water with distance between the solute and the box set to 0.2 nm at a temperature of 300 K for a total time of 1 µs. A time step of 2 fs and the stochastic dynamics integrator were used. For the evaluation of the root mean square fluctuations (RMSF) the first 100 ns of the simulations were omitted.

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## Author contribution

MB and TG performed the phylogenetic analysis, IH performed the mathematical modelling, DH and MN performed the experiments, IR performed the MD analyses guided by AS, MZ and IH were supervising and guiding the investigations. All authors contributed to the manuscript preparation.

## Figure Legends

### Figure 1

**Schematic overview of NAD biosynthesis pathways.** NAD can be synthesised from tryptophan (Trp), nicotinamide (Nam), nicotinic acid (NA), and to a lesser extend nicotinamide ribose (NR). Nam is the main precursor in human and also the product of NAD-consuming signalling reactions by enzymes such as sirtuins (NAD-dependent deacylases) or PARPs (poly-ADP-ribosylases). For the recycling of Nam, two different pathways exist. The pathway found in yeast, plants, and many bacteria starts with the deamidation of Nam by Nam deamidase (NADA). The other three enzymes comprise the Preiss-Handler pathway that also exists in vertebrates. The pathway found in vertebrates directly converts Nam into the corresponding mononucleotide (NMN) by the Nam phosphoribosyltransferase (NamPT). The Nam N-methyltransferase (NNMT) degrades Nam to methyl-Nam (MNam), which is in mammals excreted with the urine.

### Figure 2

**Phylogenetic distribution of NADA, NNMT, and NamPT and their relation to the number of NAD consumers.** A) Distribution of NADA, NNMT, and NamPT in selected clades. NADA is dominant in bacteria, fungi, and plants (Viridiplantae), whereas NamPT together with NNMT is dominant in Metazoa. Numbers at the pie charts show, how many species of the clades possess the respective enzyme combination indicated by the colour explained in the lower right of the figure. The number of species in a clade is given below its name. B) Common tree of selected clades within the Metazoa, including 334 species. The pie charts indicate the distribution of species within the respective clade that encode the enzyme combination indicated by the different colours. The size of the pie charts is proportional to the logarithm of the number of species analysed in the particular clade. The numbers below the clade names indicate the average number of NAD-consuming enzyme families found in all species of that clade. The branch length is arbitrary.

### Figure 3

**NNMT enables high NAD consumption flux.** We used a dynamic model of NAD biosynthesis and consumption (for details, see Experimental Procedures) to simulate steady state NAD consumption flux (A) and concentration (B). The amount of NMNAT and NamPT used in the simulations where adjusted such that the free NAD concentrations were in the range reported in the literature. All other enzyme concentrations were set equal. Details are given in supplementary table S2. In the presence of NNMT (blue lines), steady state NAD consumption rates are higher despite reduced NAD concentrations. Increasing the amount of NamPT in the simulation fourfold (blue dotted lines) partially compensates for the decreased NAD concentration caused by Nam degradation through NNMT.

### Figure 4

**Role of NamPT substrate affinity.** We simulated the effect of different Michaelis-Menten constants (*KM*) of NamPT for Nam on the steady state NAD consumption flux and NAD concentration at different cell division rates. All parameters were equal to those used for the simulations in figure 3. In the absence of NNMT, the *KM* of NamPT has little influence on NAD consumption (A) and concentration (B), but both are strongly influenced by cell division rates. In the presence of NNMT, decreasing *KM* of NamPT enables increasing NAD consumption flux (C) and NAD concentration (D). NNMT furthermore makes both, NAD consumption flux and concentration, almost independent of cell division rates. Comparing the situation with and without NNMT (E and F) at two different NamPT *KM* values reveals that at high *KM* (dashed lines) and high cell division rates NNMT no longer enables higher NAD consumption rates compared to NamPT alone (green line and dashed grey line).

### Figure 5

**The substrate affinities of human NNMT and NamPT are close to optimum.** We simulated the impact of changes in the *KM* for both NamPT and NNMT on NAD consumption rates (A) and NAD concentration (B). Both are increasing with decreasing *KM* of NamPT, but increasing *KM* of NNMT. The affinities reported for human enzymes (indicated by a black asterisk) appear to be close to the theoretical optimum, as further improvements would have little effect on NAD consumption or concentration.

### Figure 6

**The function of the structurally unresolved loop of NamPT.** Most deuterostomes that possess NamPT and NNMT show a sequence insertion in the N-terminal region of NamPT that has been revealed by multiple sequence alignment of NamPT from different species (A). Coloured circles indicate the enzymes present in the respective species; blue: NamPT and NNMT; black: NamPT, NADA and NNMT; yellow: NamPT and NADA. For a more comprehensive alignment, please see supplementary figure S1. The structure visualisation of human NamPT (B) is based on a structure prediction by SWISS-MODEL 59, 60 using the model 2H3D of the human NamPT as template 44. The inserted region is not resolved in any of currently available crystal structures of NamPT and thus appears to be a flexible loop structure at the surface of the NamPT dimer, coloured in red. Immunofluorescence images (C) show that the localisation of the FLAG-tagged mutant protein lacking the unresolved loop is not changed compared to FLAG-tagged human wildtype NamPT. Both show a heterogeneous nuclear-cytosolic localisation in HeLa S3 cells. *In vitro* measurements using recombinant protein show that the mutant NamPT has a lower activity than the wildtype enzyme (D) and is not activated by ATP (E). Bars in panels D and E that have different letters indicate significant difference of measured values as estimated using a T test assuming independent samples and significance at *p < 0.05*. (F) Root mean square fluctuations (RMSF) for every residue of chain A of NamPT with loop insertion (red) and without loop insertion (blue) are shown, respectively (top). The difference RMSF for every residue is shown in the bottom (green). For better comparison the residue IDs for the structure without loop are aligned to accord with the structure with loop and the average RMSF of residues 43 and 54 displayed in the blue curve between these residues. For the RMSF calculation, the first 100 ns of the simulation are omitted to allow equilibration.

**Figure 7**

**NNMT provides a competitive advantage and makes NADA obsolete.** To simulate competition for common resources, we created a two-compartment model where one compartment contained NADA, but no NamPT and the other compartment contained NamPT either with or without NNMT, but no NADA. NADA and NamPT were simulated to be present at equal amounts. In the absence of NNMT the compartment containing NADA has slightly lower NAD consumption rates (A), but much higher steady state NAD concentrations (B). In the presence of NNMT, however, both NAD consumption (C) and NAD concentration (D) are lower in the NADA compartment. This effect is dependent on a low NamPT *KM*.

### Figure 8

**Schematic representation of evolutionary events in the NAD pathway.** Based on the phylogenetic analysis presented here (roman font) and earlier work 52 (italic font) we summarised and indicated important events in the evolution of NAD metabolism in Metazoa.

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