# Identifying evolutionary and kinetic drivers of NAD-dependent signalling

Mathias Bockwoldt1, Dorothée Houry2, Marc Niere3, Toni I. Gossmann4, Ines Reinartz5,6, Alexander Schug7, Mathias Ziegler3, and Ines Heiland1,§

1Department of Arctic and Marine Biology, UiT The Arctic University of Norway, Biologibygget, Framstredet 39, 9017 Tromsø, Norway

2Department of Biological Sciences, University of Bergen, Thormøhlensgata 53 A/B, 5020 Bergen, Norway

3Department of Biomedicine, University of Bergen, Jonas Lies Vei 91, 5020 Bergen, Norway

4Department of Animal and Plant Sciences, Western Bank, University of Sheffield, Sheffield, S10 2TN, United Kingdom

5Department of Physics, Karlsruhe Institute of Technology, Wolfgang-Gaede-Str. 1, 76131 Karlsruhe, Germany

6Steinbuch Centre for Computing, Karlsruhe Institute of Technology, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany

7John von Neumann Institute for Computing, Jülich Supercomputing Centre, Forschungszentrum Jülich, 52425 Jülich, Germany

§ Corresponding author: ines.heiland@uit.no

## 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, 13, 14. It has been established that the gradual decrease in NAD during ageing is one of the major driving forces of these age-related pathologies15, 16, 17. In addition, NAD metabolism has been identified to be a key regulator for axonal degradation18, 19, 20. It is therefore not surprising that NAD metabolism has emerged as promising pharmacological target for disease treatment21, 22, 23. But still, a much better understanding of the dynamic interplay of NAD pathway components is required to develop efficient new therapeutic strategies with minimal side effects.

NAD represents one of the most critical links between 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-ribose14. These NAD-dependent signalling reactions include poly- and mono-ADP-ribosylation24, 25, sirtuin-mediated protein deacylation26, and the synthesis of calcium-mobilizing molecules such as cyclic ADP-ribose27, and participate in the regulation of virtually all cellular activities. The enzymes involved in these processes are sensitive to the available NAD concentration28, 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 activity29.

The significance of NAD-dependent signalling for NAD homeostasis has long been underestimated. But, it has now been established that compounds affecting NAD biosynthesis lead to a rapid decline of the NAD concentration30, 31. This suggests that NAD-dependent signalling reactions consume substantial amounts of NAD. The resulting NAD turnover differs in a cell-type-specific manner and can lead to an NAD half-life as short as 15 minutes32. To maintain the NAD concentration at physiological levels, NAD biosynthesis needs to act at an equally rapid rate, and imbalances in NAD homeostasis have been associated with a large number of different diseases. It is therefore not surprising that several recent studies have demonstrated impressive health benefits of dietary supplementation with intermediates of NAD biosynthesis including Nam mononucleotide (NMN)17 and Nam riboside (NR)2, 33. 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 sirtuins34.

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 catalysed by the Nam phosphoribosyltransferase (NamPT) using phosphoribosyl pyrophosphate (PRPP) as cosubstrate. 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 range35. This appears to be mediated by an ATP-dependent phosphorylation of a histidine residue in the catalytic core36. Despite the importance of its salvage, Nam can also be marked for excretion by methylation. The presence of Nam N-methyltransferase (NNMT) in vertebrates37 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 acts as a metabolic methylation sink 38.

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. In this pathway 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 organisms39. The recycling pathway via NA finally requires reamidation 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 coexist in some eukaryotes37, 40, as well as in some bacterial species41. Why these pathways coexist 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 very limited number of species 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 41 and some marine invertebrates 37.

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 content42 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 enzymes37, 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 PARPs43.

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 had very few cross species kinetic data, we did furthermore mainly rely on kinetic constants found for human or yeast enzymes. Wherever possible, we included substrate affinities and known product inhibitions as well as 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, 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 enzyme35 as well as for some bacterial enzymes 44, 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. Pointing to a role of NNMT for NAD homeostasis at changing cell division and consumption 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 enzyme44 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, reaching a plateau when the *KM* of NNMT is concomitantly elevated to the submicromolar range. This 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 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 an insert of ten amino acids in most deuterostomes that possess only NamPT and NNMT (indicated by the blue circle, fig. 6A). This insert corresponds to positions 42 to 51 in the human enzyme and overlaps with a predicted weak nuclear localisation signal (NLS). The NLS prediction is lost when the insert is removed. The ten amino acid insert is furthermore part of a loop structure at the surface of the enzyme that is unresolved in all available crystal structures of human NamPT (e.g. structure visualisation in fig. 6B from45). Intriguingly, this presumed loop, depicted in red in figure 6B, is connected to one of the β-sheets involved in substrate binding36.

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 led 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 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 FK86646 for 30 minutes, neither wildtype nor mutant NamPT did synthesize NMN, suggesting that binding of FK866 is not affected by the mutation (suppl. fig. S4). Using 100 µM Nam and PRPP the wildtype showed a turnover rate of 6.5 1 s-1 at 30 °C, while the mutant did not have any detectable activity. With 1mM of both substrates the turnover rate of the wildtype enzyme increased to 11.5 0.5 s-1, whereas the turnover rate of the mutant enzyme under the same condition was significantly reduced to 9.3 0.8 (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 site (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. These elevated mobilities were dominated by rare events during the simulation time of 1µs. They therefore do not appear as a robust change of structural dynamics upon loop insertion. The observed alterations could nevertheless provide a hint 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 model details see suppl. table. 2 and suppl. file 2). Without NNMT, the compartment containing NADA shows a higher NAD consumption rate (fig. 7A), and 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, but NAD-consumption rates are still slightly higher in the NADA containing compartment. As bacteria often have relatively high growth rates and a low number of NAD consuming enzymes, this might explain why in bacteria 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). This is, however, dependent on the affinity of NamPT for Nam. If the substrate affinity of NamPT is too low (high *KM*), the NADA compartment is able to maintain higher NAD concentrations and 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

NNMT has been identified as potential marker for some types of cancer47 and as potential drug target for the treatment of metabolic disorders7. Its physiological role is, however, still enigmatic. Our phylogenetic analysis showed that the appearance of NNMT in Protostomia is followed by a diversification of NAD-consuming reactions. To understand a possible interplay of NNMT and other enzymes of the pathway, we built a mathematical model of the pathway and simulated NAD pathway dynamics with and without NNMT, and show that the presence of NNMT enables higher NAD-consumption fluxes and contributes to NAD-pathway homeostasis. The effect on NAD-consumption can be explained by the inhibition of NAD-consuming enzymes by their product Nam. It furthermore appears that NNMT might have driven the development of the high affinity NAD biosynthesis through NamPT. This finally made the alternative pathway using NADA obsolete in vertebrates. Our simulations also suggest that the diversification of NAD-consuming enzymes and thus the potentially increased NAD-consumption flux in mammals might have been enabled by the presence of NNMT. The positive effect of NNMT on NAD-consumption flux especially on sirtuins, is in line with a lifespan extension observed in worms overexpressing NNMT48.

The effect of NNMT overexpression or silencing has been controversially discussed and is presumably tissue and context specific38. And although NNMT expression is expected to lower Nam availability and thus reduce cellular NAD concentrations, this has not been supported by in vivo or in vitro experiments so far5, 49. It has been suggested that this might be due to adjustment of cellular Nam levels under normal conditions through fast equilibrium of Nam38. It has, however, repeatedly been shown that NNMT removes excess Nam, as MNam excretion is mostly proportional to Nam uptake50, supporting our findings that NNMT contributes to NAD-pathway homeostasis. As shown in several recent studies this homeostatic control by NNMT can be circumvented by supplying NR2, 51, 52, 53 which is not a substrate of NNMT. At the cellular and subcellular level NNMT is presumably mainly advantageous if high NAD-consumption rates are required for tissue function, or even more likely, might be important to prevent spatio-temporal accumulation of Nam within cells due to temporally increased NAD-consumption, e.g. PARP activation through DNA-damage.

The main healthy tissues expressing NNMT are the liver and adipose tissues, while no or only little expression of NNMT is observed in most other organs54. Increased NNMT expression is observed in some types of cancer47, 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 has been found in some types of cancer55, 56. 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 inhibitors of NamPT. Several of such inhibitors are currently tested in clinical studies21, 57. Based on our analysis, 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 deficient 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 bacteria41, 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 along 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, or phosphoribosyl pyrophosphate (PRPP) and ATP 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 NNMT58 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 (schematic overview see figure 8). This transition appears to be accompanied by the loss of NADA in vertebrates and the first gene duplication of NMNATs. 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). This might point to a n important role of NNMT in NAD pathway compartmentalisation. Just recently the role of the interaction between subcellular compartments for adipogenic gene regulations has been demonstrated59. Which role NNMT plays in the spatio-temporal regulation of the pathway still maintains to be investigated.

We here used an approach that combines detailed phylogenetic analysis with dynamic metabolic modelling and have been able to provide an explanation for the 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 toolkit60 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 used61. 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 4.2562 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 onto microscope slides using ProLong Gold (ThermoFisher, Invitrogen). Confocal laser scan imaging of cells was performed at the Molecular Imaging Center at the Department of Biomedicine (University of Bergen), using a Leica TCS SP8 STED 3x microscope equipped with a 100x oil immersion objective (numerical aperture 1.4).

### NamPT expression

BL21- codonPlus (DE3) RIL were transformed with pQE-30 NamPT WT/pREP4 or pQE-30 NamPT Δ42-51/pREP4. Bacterial cells were grown at 37°C in 1 L of Luria-Bertani broth containing 100 µg/mL ampicillin, 50 µg/mL kanamycin and 32 µg/mL chloramphenicol. Protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactoside at 0.4∼0.6 OD600. Induction was done at 18°C overnight.

### 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 at 13000 g for 30 min, 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, 1 mM DTT, 300 mM imidazole).

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

### Enzymatic Assay

In a final volume of 1.2 ml reaction buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 6 mM MgCl*2*, 0.03% (w/v) BSA), 2 µM of enzyme were incubated with 5-phospho-D-ribose 1-diphosphate and nicotinamide (100 µM or 1 mM both). The reaction was incubated at 30 °C for 10 min and stopped by adding 100 µM of FK866. Subsequently, 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% (v/v) deuterated H2O 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. Data were acquired with 2000 scans, 1 s relaxation delay, 1.6 s acquisition time, and contained 65 000 data points with 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

All-atom molecular dynamics simulations were performed with explicit solvent for NamPT (PDB Code: 2H3D 45) with and without loop insertion. AMBER99SB-ILDN force field 63 was used with the TIP3P water model 64 in GROMACS 5.1.2 65. 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.

## Acknowledgements

We thank the Norwegian Research Council for funding (grant no. 250395/F20 and grant no. 226244/F50). We furthermore thank for the computation time provided through UNINETT Sigma2 – the National Infrastructure for High Performance Computing and Data Storage in Norway. IR and AS received support by the Helmholtz Association Initiative and Networking Fund under project number ZT-I-0003. TIG is supported by a Leverhulme Early Career Fellowship (Grant ECF-2015-453) and a NERC grant (NE/N013832/1).

## 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 the corresponding ribosides NR and NAR. 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-ribose polymerases). 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). Further biosynthesis via the Preiss-Handler pathway, which also exists in vertebrates, requires three subsequent enzymatic steps catalysed by Nicotinic acid phosphoribosyltransferase (NAPRT), Nicotinic acid/Nicotinamide mononucleotide adenylyltransferase (NMNAT) and NAD synthase (NADS). In vertebrates, Nam is directly converted to nicotinamidemononucleotide (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. The colour marking of three different enzymes utilizing Nam will be used in successive figures to denote the presence of these enzymes in different organism.

### 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, the percentage of species per clade, which possess the respective enzyme combination indicated by the colour code explained in the lower right of the figure (n = number of species per clade included in the analysis). 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.** A dynamic model of NAD biosynthesis and consumption (for details, see Experimental Procedures) was used 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 curves), steady state NAD consumption rates are higher despite reduced NAD concentrations. Increasing the amount of NamPT in the simulation fourfold (dotted blue curves) partially compensates for the decreased NAD concentration caused by Nam degradation through NNMT.

### Figure 4

**NNMT is a potential driver of NamPT affinity transition** Using our dynamic model, the effect of different Michaelis-Menten constants (*KM*) of NamPT for Nam on the steady state NAD consumption flux and NAD concentration was simulated at different cell division rates. All other 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 curves) and high cell division rates NNMT no longer enables higher NAD consumption rates compared to NamPT alone (green curves and dashed grey curves).

### Figure 5

**The substrate affinities of human NNMT and NamPT are close to optimum.** Simulating the impact of changes in the *KM* for both NamPT and NNMT on NAD consumption rates (A) and NAD concentration (B) showed that both increase 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.** (A) A multiple sequence alignment of NamPT revealed a sequence insertion in the N-terminal region of this enzyme in most deuterostomes that possess NamPT and NNMT. 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. (B) The structure visualisation of human NamPT is based on a structure prediction by SWISS-MODEL66, 67 using the model 2H3D of the human NamPT as template45. The inserted region (shown in red) 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. (C) Confocal laser scan micrographs of HeLaS3 cells expressing C-terminally FLAG-tagged wild-type (wt) and mutant (Δ42-51) NamPT lacking the unresolved loop. Both proteins showed a heterogeneous nuclear-cytosolic localisation. Nuclei were stained with DAPI. The C-terminally FLAG-tagged human poly-ADP-ribose glycohydrolase isoform PARG60 was used as a control for exclusive cytosolic localization. (D) The purified recombinant deletion mutant Δ42-51 NamPT had no detectable activity with 100 µM Nam. At a tenfold higher substrate concentration, the activity of the deletion mutant was significantly lower than the activity of wild-type (wt) NamPT (p ≤ 0.05; non-parametric one-tailed Mann-Whitney test). (E) 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 NamPT without loop are aligned to accord with the wildtype structure 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, a two-compartment model was created. In this model 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 a higher NAD consumption rate (A), and a 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* for Nam.

### Figure 8

**Schematic representation of evolutionary events in the NAD pathway.** Based on the phylogenetic analysis presented here (roman font) and earlier work 68 (italic font) we summarised and indicated important events in the evolution of NAD metabolism in metazoa.

References

1. Ljungberg MC*, et al.* CREB-activity and nmnat2 transcription are down-regulated prior to neurodegeneration, while NMNAT2 over-expression is neuroprotective, in a mouse model of human tauopathy. *Hum Mol Genet* **21**, 251-267 (2012).

2. Trammell SA*, et al.* Nicotinamide Riboside Opposes Type 2 Diabetes and Neuropathy in Mice. *Sci Rep* **6**, 26933 (2016).

3. Yoshino J, Mills KF, Yoon MJ, Imai S. Nicotinamide mononucleotide, a key NAD(+) intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice. *Cell Metab* **14**, 528-536 (2011).

4. Mitchell SJ*, et al.* Nicotinamide Improves Aspects of Healthspan, but Not Lifespan, in Mice. *Cell Metab* **27**, 667-676 e664 (2018).

5. Kraus D*, et al.* Nicotinamide N-methyltransferase knockdown protects against diet-induced obesity. *Nature* **508**, 258-262 (2014).

6. Canto C*, et al.* The NAD(+) precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity. *Cell Metab* **15**, 838-847 (2012).

7. Kannt A*, et al.* A small molecule inhibitor of Nicotinamide N-methyltransferase for the treatment of metabolic disorders. *Sci Rep* **8**, 3660 (2018).

8. Hsu CP, Oka S, Shao D, Hariharan N, Sadoshima J. Nicotinamide phosphoribosyltransferase regulates cell survival through NAD+ synthesis in cardiac myocytes. *Circ Res* **105**, 481-491 (2009).

9. Diguet N*, et al.* Nicotinamide Riboside Preserves Cardiac Function in a Mouse Model of Dilated Cardiomyopathy. *Circulation* **137**, 2256-2273 (2018).

10. Ryu D*, et al.* NAD+ repletion improves muscle function in muscular dystrophy and counters global PARylation. *Sci Transl Med* **8**, 361ra139 (2016).

11. Poyan Mehr A*, et al.* De novo NAD(+) biosynthetic impairment in acute kidney injury in humans. *Nat Med* **24**, 1351-1359 (2018).

12. Buonvicino D*, et al.* Identification of the Nicotinamide Salvage Pathway as a New Toxification Route for Antimetabolites. *Cell Chem Biol* **25**, 471-482 e477 (2018).

13. Chiarugi A, Dölle C, Felici R, Ziegler M. The NAD metabolome – A key determinant of cancer cell biology. *Nature Reviews Cancer* **12**, 741--752 (2012).

14. Verdin E. NAD+ in aging, metabolism, and neurodegeneration. *Science* **350**, (2015).

15. Chini CCS, Tarrago MG, Chini EN. NAD and the aging process: Role in life, death and everything in between. *Mol Cell Endocrinol* **455**, 62-74 (2017).

16. Imai S, Yoshino J. The importance of NAMPT/NAD/SIRT1 in the systemic regulation of metabolism and ageing. *Diabetes Obes Metab* **15 Suppl 3**, 26-33 (2013).

17. Mills KF*, et al.* Long-Term Administration of Nicotinamide Mononucleotide Mitigates Age-Associated Physiological Decline in Mice. *Cell Metab* **24**, 795-806 (2016).

18. Araki T, Sasaki Y, Milbrandt J. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science* **305**, 1010-1013 (2004).

19. Beirowski B*, et al.* Non-nuclear Wld(S) determines its neuroprotective efficacy for axons and synapses in vivo. *J Neurosci* **29**, 653-668 (2009).

20. Di Stefano M*, et al.* NMN Deamidase Delays Wallerian Degeneration and Rescues Axonal Defects Caused by NMNAT2 Deficiency In Vivo. *Curr Biol* **27**, 784-794 (2017).

21. Espindola-Netto JM*, et al.* Preclinical efficacy of the novel competitive NAMPT inhibitor STF -118804 in pancreatic cancer. *Oncotarget* **8**, 85054--85067 (2017).

22. Yoshino J, Baur JA, Imai SI. NAD(+) Intermediates: The Biology and Therapeutic Potential of NMN and NR. *Cell Metab* **27**, 513-528 (2018).

23. Rajman L, Chwalek K, Sinclair DA. Therapeutic Potential of NAD-Boosting Molecules: The In Vivo Evidence. *Cell Metab* **27**, 529-547 (2018).

24. Bütepage M, Eckei L, Verheugd P, Lüscher B. Intracellular Mono- ADP -Ribosylation in Signaling and Disease. *Cells* **4**, 569--595 (2015).

25. The diverse roles and clinical relevance of PARPs in DNA damage repair: Current state of the art. *Biochemical Pharmacology* **84**, 137--146 (2012).

26. Osborne B, Bentley NL, Montgomery MK, Turner N. The role of mitochondrial sirtuins in health and disease. *Free Radical Biology and Medicine* **100**, 164--174 (2016).

27. Lee HC. Cyclic ADP -ribose and nicotinic acid adenine dinucleotide phosphate (NAADP) as messengers for calcium mobilization. *Journal of Biological Chemistry* **287**, 31633--31640 (2012).

28. Ruggieri S, Orsomando G, Sorci L, Raffaelli N. Regulation of NAD biosynthetic enzymes modulates NAD -sensing processes to shape mammalian cell physiology under varying biological cues. *Biochimica et Biophysica Acta* **1854**, 1138--1149 (2015).

29. Koch-Nolte F, Haag F, Guse AH, Lund F, Ziegler M. Emerging roles of NAD+ and its metabolites in cell signaling. *Science Signaling* **2**, mr1 (2009).

30. Buonvicino D*, et al.* Identification of the Nicotinamide Salvage Pathway as a New Toxification Route for Antimetabolites. *Cell Chemical Biology* **25**, 471--482.e477 (2018).

31. Hasmann M, Schemainda I. FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis. *Cancer Res* **63**, 7436-7442 (2003).

32. Liu L*, et al.* Quantitative Analysis of NAD Synthesis-Breakdown Fluxes. *Cell Metabolism* **27**, 1067--1080.e1065 (2018).

33. Yoshino J, Baur JA, Imai S-i. NAD+ Intermediates: The Biology and Therapeutic Potential of NMN and NR . *Cell Metabolism* **27**, 513--528 (2018).

34. North BJ, Verdin E. Sirtuins: Sir2 -related NAD -dependent protein deacetylases. *Genome Biology* **5**, 224 (2004).

35. Burgos ES, Schramm VL. Weak coupling of ATP hydrolysis to the chemical equilibrium of human nicotinamide phosphoribosyltransferase. *Biochemistry* **47**, 11086--11096 (2008).

36. Burgos ES, Ho M-C, Almo SC, Schramm VL. A phosphoenzyme mimic, overlapping catalytic sites and reaction coordinate motion for human NAMPT . *Proceedings of the National Academy of Sciences* **106**, 13748--13753 (2009).

37. Gossmann TI, Ziegler M, Puntervoll P, de Figueiredo LF, Schuster S, Heiland I. NAD+ biosynthesis and salvage – a phylogenetic perspective. *The FEBS Journal* **279**, 3355--3363 (2012).

38. Pissios P. Nicotinamide N -Methyltransferase: More Than a Vitamin B3 Clearance Enzyme. *Trends in Endocrinology and Metabolism* **28**, 340--353 (2017).

39. de Figueiredo LF, Gossmann TI, Ziegler M, Schuster S. Pathway analysis of NAD+ metabolism. *Biochemical Journal* **439**, 341--348 (2011).

40. Carneiro J*, et al.* The Evolutionary Portrait of Metazoan NAD Salvage. *PLoS ONE* **8**, (2013).

41. Gazzaniga F, Stebbins R, Chang SZ, McPeek MA, Brenner C. Microbial NAD metabolism: lessons from comparative genomics. *Microbiology and Molecular Biology Reviews* **73**, 529--541 (2009).

42. Hron T, Pajer P, Pačes J, Bartüněk P, Elleder D. Hidden genes in birds. *Genome Biology* **16**, 4--7 (2015).

43. Gossmann TI, Ziegler M. Sequence divergence and diversity suggests ongoing functional diversification of vertebrate NAD metabolism. *DNA Repair* **23**, 39--48 (2014).

44. Sorci L, Blaby Ia. Genomics-driven reconstruction of \spec Acinetobacter NAD metabolism: Insights for antibacterial target selection. *Journal of Biological Chemistry* **285**, 39490--39499 (2010).

45. Wang T, Zhang X, Bheda P, Revollo JR, Imai S-i, Wolberger C. Structure of Nampt/PBEF/visfatin , a mammalian NAD+ biosynthetic enzyme. *Nature Structural and Molecular Biology* **13**, 661--662 (2006).

46. Hasmann M, Schemainda I. FK866 , a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis. *Cancer Research* **63**, 7436--7442 (2003).

47. Okamura A*, et al.* Increased hepatic nicotinamide N -methyltransferase activity as a marker of cancer cachexia in mice bearing colon 26 adenocarcinoma. *Japanese Journal of Cancer Research* **89**, 649--656 (1998).

48. Schmeisser K*, et al.* Role of sirtuins in lifespan regulation is linked to methylation of nicotinamide. *Nature Chemical Biology* **9**, 693--700 (2013).

49. Hong S*, et al.* Nicotinamide N -methyltransferase regulates hepatic nutrient metabolism through Sirt1 protein stabilization. *Nature Medicine* **21**, 887--894 (2015).

50. Kang-Lee YA, McKee RW, Wright SM, Swendseid ME, Jenden DJ, Jope RS. Metabolic effects of nicotinamide administration in rats. *J Nutr* **113**, 215-221 (1983).

51. Gong B*, et al.* Nicotinamide riboside restores cognition through an upregulation of proliferator-activated receptor-gamma coactivator 1alpha regulated beta-secretase 1 degradation and mitochondrial gene expression in Alzheimer's mouse models. *Neurobiol Aging* **34**, 1581-1588 (2013).

52. Liu HW*, et al.* Pharmacological bypass of NAD(+) salvage pathway protects neurons from chemotherapy-induced degeneration. *Proc Natl Acad Sci U S A*, (2018).

53. Martens CR*, et al.* Chronic nicotinamide riboside supplementation is well-tolerated and elevates NAD(+) in healthy middle-aged and older adults. *Nat Commun* **9**, 1286 (2018).

54. Aksoy S, Szumlanski CL, Weinshilboum RM. Human liver nicotinamide N -methyltransferase. cDNA cloning, expression, and biochemical characterization. *Journal of Biological Chemistry* **269**, 14835--14840 (1994).

55. Bi TQ*, et al.* Overexpression of Nampt in gastric cancer and chemopotentiating effects of the Nampt inhibitor FK866 in combination with fluorouracil. *Oncology Reports* **26**, 1251--1257 (2011).

56. Wang B, Hasan MK, Alvarado E, Yuan H, Wu H, Chen WY. NAMPT overexpression in prostate cancer and its contribution to tumor cell survival and stress response. *Oncogene* **30**, 907--921 (2011).

57. Xu T-Y*, et al.* Discovery and characterization of novel small-molecule inhibitors targeting nicotinamide phosphoribosyltransferase. *Scientific Reports* **5**, 10043 (2015).

58. Ulanovskaya OA, Zuhl AM, Cravatt BF. NNMT promotes epigenetic remodeling in cancer by creating a metabolic methylation sink. *Nature Chemical Biology* **9**, 300--306 (2013).

59. Ryu KW, Nandu T, Kim J, Challa S, DeBerardinis RJ, Kraus WL. Metabolic regulation of transcription through compartmentalized NAD(+) biosynthesis. *Science* **360**, (2018).

60. Huerta-Cepas J, Serra F, Bork P. ETE 3: Reconstruction, Analysis, and Visualization of Phylogenomic Data. *Molecular Biology and Evolution* **33**, 1635--1638 (2016).

61. Schäuble S, Stavrum A-K, Puntervoll P, Schuster S, Heiland I. Effect of substrate competition in kinetic models of metabolic networks. *FEBS Letters* **587**, 2818--2824 (2013).

62. Hoops S*, et al.* COPASI – a COmplex PAthway SImulator. *Bioinformatics* **22**, 3067--3074 (2006).

63. Lindorff-Larsen K*, et al.* Improved side-chain torsion potentials for the Amber ff99SB protein force field. *Proteins* **78**, 1950-1958 (2010).

64. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *The Journal of Chemical Physics* **79**, 926--935 (1983).

65. Abraham MJ*, et al.* GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* **1-2**, 19--25 (2015).

66. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics* **22**, 195--201 (2006).

67. Biasini M*, et al.* SWISS-MODEL : Modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Research* **42**, 252--258 (2014).

68. Lau C, Dölle C, Gossmann TI, Agledal L, Niere M, Ziegler M. Isoform-specific targeting and interaction domains in human nicotinamide mononucleotide adenylyltransferases. *The Journal of Biological Chemistry* **285**, 18868--18876 (2010).