

Identification of evolutionary and kinetic drivers of NAD-dependent signalling

Mathias Bockwoldt¹, Dorothée Houry², Marc Niere³, Toni I. Gossmann^{4,5}, Ines Reinartz^{6,7}, Alexander Schug⁸, Mathias Ziegler³, and Ines Heiland^{1,*}

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

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

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

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

⁵ Department of Animal Behaviour, Bielefeld University, 33501 Bielefeld, Germany

⁶ Department of Physics, Karlsruhe Institute of Technology, Wolfgang-Gaede-Str. 1, 76131 Karlsruhe, Germany

⁷ Steinbuch Centre for Computing, Karlsruhe Institute of Technology, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany

⁸ John von Neumann Institute for Computing, Jülich Supercomputing Centre, Forschungszentrum Jülich, 52425 Jülich, Germany

* Corresponding author: ines.heiland@uit.no

Significance

NAD is best known as essential cofactor of biochemical reactions. In addition, it is involved in the regulation of virtually all major cellular events. These NAD-dependent regulatory functions are mediated by enzymes (e.g. sirtuins, PARPs, ADP-ribosylcyclases) that cleave the molecule to liberate nicotinamide (Nam). We show that diversification of NAD-dependent signaling in deuterostomes was accompanied by an optimization of NAD biosynthesis to ensure efficient high affinity recycling of Nam into NAD through Nam-phosphoribosyltransferase (NamPT). In addition, a Nam-methyltransferase (NNMT) emerged which ensures high NAD-dependent signaling turnover by preventing accumulation of inhibitory Nam. This unexpected kinetic interplay between NamPT and NNMT needs to be considered in therapeutic strategies targeting these enzymes.

Abstract

NAD provides an important link between metabolism and signal transduction and has emerged as central hub between bioenergetics and all major cellular events. NAD-dependent signalling, e.g. by sirtuins and PARPs, consumes considerable amounts of NAD. To maintain physiological functions, NAD consumption and biosynthesis need to be carefully balanced. Using extensive phylogenetic analyses, mathematical modelling of NAD metabolism and experimental verification, we show that the diversification of NAD-dependent signalling in vertebrates depended on three critical evolutionary events: i) the transition of NAD biosynthesis to exclusive usage of nicotinamide phosphoribosyltransferase (NamPT); ii) the occurrence of nicotinamide N-methyltransferase (NNMT), which diverts nicotinamide (Nam) from recycling into NAD, preventing Nam accumulation and inhibition of NAD-dependent signalling reactions and iii) structural adaptation of NamPT, providing an unusually high affinity towards Nam, necessary to maintain NAD levels. Our results reveal an unexpected co-evolution and kinetic interplay between NNMT and NamPT that enables extensive NAD signalling. This has implications for therapeutic strategies of NAD supplementation and the use of NNMT or NamPT inhibitors in disease treatment.

Keywords: NAD-dependent signalling; NAD biosynthesis, nicotinamide N-methyltransferase (NNMT); nicotinamide phosphoribosyltransferase (NamPT); vitamin supplementation; pathway evolution; NAD pathway dynamics; mathematical modelling of NAD metabolism; phylogenetic pathway analysis;

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47 Introduction

48 NAD metabolism has received increasing attention, as a number of pathological states including
49 neurodegeneration (1), diabetes (2, 3), obesity (4-7), heart diseases (8, 9), muscle dystrophy (10), renal
50 dysfunction (11) and different types of cancer (12-14) have been associated with changes in this complex
51 network. It has been established that a gradual decline in NAD during ageing is one of the major driving
52 forces of these age-related pathologies (15-18). In addition, NAD metabolism has been identified to be a
53 key regulator for axonal integrity (19-21). It is therefore not surprising that NAD metabolism has emerged
54 as promising pharmacological target for disease treatment (22-25). However, to fully exploit the therapeutic
55 potential of NAD metabolism, the dynamic and functional interplay between the individual NAD pathway
56 components need to be established.

57 NAD represents one of the most critical links between cellular signal transduction and energy metabolism.
58 Even though it is best known as cofactor for a multitude of redox-reactions, NAD is involved in a number
59 of signalling processes that consume NAD by cleaving the molecule to nicotinamide (Nam) and ADP-
60 ribose (14). These NAD-dependent signalling reactions include poly- and mono-ADP-ribosylation (26, 27),
61 sirtuin-mediated protein deacetylation (28), and the synthesis of calcium-mobilizing molecules such as cyclic
62 ADP-ribose (29), and participate in the regulation of virtually all cellular activities. The enzymes involved
63 in these processes are sensitive to the available NAD concentration. Therefore, NAD-dependent signalling
64 can act as a transmitter of changes in the cellular metabolism, for example, to regulate gene expression or
65 metabolic activity (30).

66 The significance of NAD-dependent signalling for NAD homeostasis has long been underestimated. It has
67 now become clear that inhibition of NAD biosynthesis leads to a rapid decline of the cellular NAD
68 concentration (13, 31). This observation documents that NAD-dependent signalling reactions consume
69 substantial amounts of NAD. The resulting NAD turnover differs in a cell-type-specific manner.
70 Measurements of cellular NAD half-life have revealed that it can be as short as 15 minutes (32). To maintain
71 the NAD concentration at physiological levels, NAD biosynthesis needs to act at an equally rapid rate.
72 Imbalances in NAD homeostasis have been associated with a number of different diseases. In this context,
73 it is conceivable that several recent studies have demonstrated impressive health benefits of dietary
74 supplementation with intermediates of NAD biosynthesis including Nam mononucleotide (NMN) (16) and
75 Nam riboside (NR) (2, 6, 17). Apparently, the exploitation of physiologically less active NAD biosynthetic
76 routes, in addition to the use of Nam as precursor (Figure 1), results in increased NAD concentrations that
77 stimulate beneficial NAD-dependent signalling processes, in particular, protein deacetylation by sirtuins
78 (3, 33).

79 Owing to the continuous release of Nam through NAD-consuming signalling reactions, the NAD salvage
80 pathway, using Nam as precursor, is the most important NAD synthesis pathway. There are two principal
81 pathways that recycle Nam. Vertebrates use a direct two-step pathway starting with the conversion of Nam
82 into the mononucleotide NMN catalysed by Nam phosphoribosyltransferase (NamPT) using
83 phosphoribosyl pyrophosphate (PRPP) as co-substrate. At least in mammals, a nearly complete recycling
84 of Nam by NamPT is achieved by an extraordinarily high substrate affinity to Nam, the K_M being in the
85 low nanomolar range (34). This appears to be mediated by an ATP-dependent phosphorylation of a histidine
86 residue in the catalytic core (35). Despite the importance of its salvage, Nam can also be marked for
87 excretion by methylation. This reaction is catalysed by Nam N-methyltransferase (NNMT). The presence
88 of this enzyme in vertebrates (36) is among the most enigmatic and counterintuitive features of NAD
89 metabolism. While NamPT is seemingly optimised to recycle even the faintest amounts of Nam back into
90 NAD synthesis, NNMT seems to have no metabolic function other than to remove Nam from NAD
91 metabolism. However, since NNMT uses the general methylation source S-adenosylmethionine, it has been
92 suggested that Nam methylation may act as a metabolic methylation sink (37).

93 In most prokaryotes as well as in plants and fungi, another pathway consisting of four reactions starting

94 with the deamidation of Nam to nicotinic acid (NA) by the Nam deamidase (NADA) is used. (Figure 1).
95 The three enzymes that act downstream of NADA belong to the Preiss-Handler pathway that also exists in
96 vertebrates. In this pathway NA is converted into the corresponding mononucleotide (NAMN), in a reaction
97 performed by the NA-specific phosphoribosyltransferase NAPRT. The conversion of both
98 mononucleotides, NMN and NAMN, into their corresponding dinucleotides, NAD and NAAD, is catalysed
99 by the Nam/NA adenylyltransferases (NMNATs) that are essential in all organisms (38). The recycling
100 pathway via NA finally requires reamidation of NAAD by NAD synthase. This final reaction includes an
101 enzyme adenylation step that consumes ATP. Therefore, the Nam recycling by NADA appears to be
102 energetically less efficient than the recycling pathway starting with NamPT.

103 We and others have shown earlier that the two NAD biosynthesis pathways starting from Nam (Figure 1)
104 coexist in some eukaryotes (36, 39), as well as in some bacterial species (40). Why these pathways coexist
105 in some organisms and over a very long evolutionary time frame and why NADA nevertheless disappeared
106 in vertebrates, is not known. Whether the occurrence of NNMT may have contributed to these evolutionary
107 processes has also remained unexplored.

108 In the present study, we performed a comprehensive phylogenetic analysis of the NAD pathways using 793
109 eukaryotic and 7892 prokaryotic genomes. This large scale analysis revealed that there has been an
110 evolutionary transition resulting in the coexistence of NamPT and NNMT in deuterostomes, while the
111 deamidation pathway, which is dominant in bacteria, became superfluous. Importantly, this selection for
112 NamPT and NNMT was accompanied by a marked increase in the number of NAD-consuming signalling
113 enzymes. Mathematical modelling of the pathway revealed an unexpected positive kinetic role of NNMT
114 for NAD-consuming signalling fluxes, through prevention of accumulation of Nam, the product of NAD-
115 dependent signalling reactions. In addition, our model predicts that NNMT likely exerted an evolutionary
116 pressure on NamPT to develop a high affinity towards its substrate Nam. Indeed, we identified a short
117 sequence insertion in NamPT, which first occurs in deuterostomes and appears to modulate the affinity of
118 NamPT. Simulating resource competition, we furthermore show that the presence of high affinity NamPT
119 together with NNMT makes the NADA-dependent pathway obsolete, providing a rationale for the
120 evolutionary transition of the pathway in metazoans.

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

124 Results

125 Paradoxical evolutionary correlation between NAD-dependent signalling and 126 precursor metabolism

127 To understand the functional roles and potential interplay between the three known enzymes that use Nam
128 as substrate (NamPT, NADA and NNMT, Figure 1), we conducted a comprehensive analysis of the
129 phylogenetic distribution of these three enzymes. As shown in Figure 2a, bacteria, fungi, and plants
130 predominantly possess NADA and only a very limited number of species harbour NamPT. In contrast, most
131 metazoa lost NADA, and rather possess NamPT together with NNMT. NADA and NamPT, the two
132 enzymes that initiate the two different NAD salvage pathways, show a scattered distribution in bacteria.
133 Co-occurrence of these enzymes is rather rare, and has occasionally been found in bacteria (40) and some
134 marine invertebrates (36).

135 NNMT seems to have arisen de novo or diverged rapidly in the most recent common ancestor of Ecdysozoa
136 and Lophotrochozoa (Figure 2b). We were unable to find any indication for the presence of NNMT in fungi
137 or plants (Blastp e-value cutoff 0.1). Interestingly, NA can be methylated to trigonelline in plants and
138 bacteria (41), but the required enzyme has no homology to NNMT or any other enzyme in metazoan.
139 Nematodes are the only organisms, where we observed a concomitant presence of NADA and NNMT. In
140 deuterostomes, the only large clade that possesses only NamPT and seems to have lost NNMT are
141 Sauropsida, and among them especially birds. The reason why about half of the sequenced bird genomes
142 do not seem to harbour NNMT remains unclear. The distribution of NNMT in birds is quite scattered
143 (Figure S2). It is possible that detection of NNMT in some bird genomes failed because of their high GC

144 content (42) or because of difficulties in assembling very small chromosomes commonly found in birds.
145 The absence of NNMT might, alternatively, be related to the differences in the excretion systems. In
146 mammals, the product of NNMT, methyl-Nam, is excreted with the urine. There are few metazoan species
147 for which we could not find NamPT or NADA, while NNMT was detected. We assume that this is due to
148 incomplete genomes in the database, as these species are scarce and their distribution appears to be
149 randomly scattered.

150 In addition to the phylogenetic distribution of the two Nam salvage enzymes NADA and NamPT, we
151 analysed the phylogenetic diversity of enzymes catalysing NAD-dependent signalling reactions. To do so,
152 we used the previously established classification into ten different families of NAD-consuming signalling
153 enzymes (36), including PARP1-3, PARP4, PARP6/8, PARP7/9-15, PARP16, sirtuins, tankyrases, ADPR-
154 cyclases, mono-ADP-ribosyltransferases and t-RNA-phosphotransferases. The detailed list of templates
155 used for the phylogenetic analyses can be found in Table S1. The numbers shown in Figure 2b denote the
156 average number of NAD-dependent signalling enzyme families found in each clade (for a detailed
157 distribution see Table S2). With the exception of Cnidaria and Lophotrochozoa, we find an average of three
158 to four families in protostomes, whereas most deuterostome species have, on average, more than eight
159 families with an increasing diversification of enzymes within some of these families, especially PARPs
160 (43).

161 Taken together, we found that NADA is lost in vertebrates, but strongly preserved in most other organisms,
162 despite the higher energetic requirement of this pathway. Moreover, the transition to having both NamPT
163 and NNMT coincides with a considerable diversification of NAD-dependent signalling. This observation
164 seems counter-intuitive, as one would expect that increased NAD-dependent signalling should be
165 compensated by an increased efficiency of substrate (Nam) utilization for NAD biosynthesis. Since NNMT
166 removes Nam from recycling into NAD, it is not obvious how this enzyme could contribute to higher NAD
167 turnover.

168 Functional properties of NamPT and NNMT have evolved to maximise NAD- 169 dependent signalling

170 To resolve this apparent contradiction, we turned to modelling approaches permitting to simulate the
171 behaviour of the complex NAD metabolic network under different conditions. We built a dynamic model
172 of NAD metabolism based on ordinary differential equations using previously reported kinetic data (for
173 details, see Methods and Materials and Table S3).

174 Given the rather limited information about species-specific expression levels of enzymes, we first assumed
175 equal expression of all enzymes, thereby enabling an initial comparison of metabolic features in rather
176 different organisms. Moreover, due to the lack of specific kinetic data from most organisms, we mainly
177 relied on kinetic constants found for human or yeast enzymes. Wherever possible, we included substrate
178 affinities and known product inhibitions as well as inhibition by downstream metabolites, such as e.g. the
179 inhibition of NamPT by NAD (34). Finally, the models assumed that cell growth and consecutive cell
180 division is, besides NAD-consuming reactions, a major driving force for NAD biosynthesis.

181 First, we addressed the unexpected correlation between the transition to the co-occurrence of NamPT and
182 NNMT and the increase in the number of NAD-consuming enzymes. We calculated steady state NAD
183 concentrations and NAD consumption fluxes by simulating NAD biosynthesis via NamPT in the presence
184 or absence of NNMT (Figure 3 A and B). Due to the very low turnover number of NamPT ($\sim 0.01/s$), we
185 used 40fold higher NamPT levels compared to the other enzymes, to achieve free NAD concentrations in
186 the range reported in the literature (44). NAD concentrations can be further increased with higher NamPT
187 levels (see Figure S3 A and B). Due to the flux limiting effect of NamPT, NMNAT levels have no effect
188 under the conditions tested (Figure S3 A and B).

189 Surprisingly, our simulations predict that the presence of NNMT enables higher rather than lower NAD
190 consumption fluxes (Figure 3A), although it diminishes the steady state concentration of NAD (Figure 3B).
191 The decline in NAD concentration can be compensated by a higher expression of NamPT, which also
192 further increases NAD consumption flux (dashed lines in Figure 3A and B). These results indicate a

stimulatory role for NNMT solely on the basis of the enzyme kinetics, without having to invoke any regulatory mechanism (such as signalling events). It turns out that these results can be explained on the basis of 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 system (Figure S3D). At the same time, a high substrate affinity of NamPT ensures the maintenance of sufficiently high NAD concentration, although the concentration is, as expected, lower than in the system without NNMT. To show that indeed the relaxation of the Nam inhibition is responsible for the increase in NAD consumption flux, we varied the K_i for the NAD consuming reaction. As can be seen from Figure S3E and F increasing the K_i for Nam in a system without NNMT, mimics the effect caused by NNMT addition. In contrast, changing the K_i for NAD of NamPT has no effect. Thus, the flux increase with NNMT does not stem from an increase of the NAD consumption flux, due to lowered NAD concentrations. But if the NAD concentration is reduced too much due to high expression of NNMT the NAD consumption declines again (Figure S3C and D).

Kinetic parameters of NamPT were previously reported for the human enzyme (34) as well as for some bacterial enzymes (45), the latter having a much lower substrate affinity for Nam. We thus simulated the potential effect of NamPT affinity (K_M) on NAD steady state concentration and NAD consumption flux. In the absence of NNMT, a variation of the substrate affinity of NamPT for Nam is predicted to have very little effect on steady state NAD concentration and NAD consumption flux (Figure 3C and D). In the presence of NNMT, however, NAD consumption flux and NAD concentration would increase with increasing affinity of NamPT (Figure 3E and F).

Remarkably, NAD concentration and consumption flux are both considerably affected by cell division rates in a system without NNMT. Our simulations predict a trade-off between sustainable 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. These observations point to a role of NNMT for NAD homeostasis at varying cell division and consumption rates.

Given that a lower affinity of NamPT has been described for the bacterial enzyme (45) where NNMT is not present, we were wondering if the advantage provided by NNMT is dependent on a high affinity of NamPT. In Figures 3G and H we show the direct comparison of simulations assuming different affinities of NamPT, in the presence or absence of NNMT. Interestingly, a low affinity that is in the range of the affinity of NADA for Nam and far above those measured for bacterial NamPT, leads to higher NAD consumption flux in the presence of NNMT only if cell division rates are low (Figure 3G). However, if the affinity of NamPT is high enough ($K_m \ll 1 \text{ ! M}$), consumption rates are always higher with NNMT than without. The NAD concentration is, as would be assumed, always lower with NNMT (Figure 3H).

To understand the interplay and competition for Nam between NamPT and NNMT, we conducted simulations in which we scanned a wide range of possible substrate affinities for both enzymes. As shown in Figure 4, these simulations indicate that both NAD consumption flux and NAD concentration would be minimal in case of a low substrate affinity of NamPT and high affinity of NNMT. Conversely, increasing the affinity of NamPT, increases NAD consumption, NAD concentration and the flux ratio between NamPT and NNMT reaching a plateau when the substrate affinity of NNMT is sufficiently low. Remarkably, as indicated by the asterisks in Figure 4A, B and C the substrate affinities for human NamPT and NNMT (K_m of 5nM and 400 ! M, respectively) are within the predicted optimal range, where further adjustment would lead to little or no increase of NAD consumption flux, NAD concentration or NamPT to NNMT flux ratio.

Sequence variance acquired in metazoan NamPT enhances substrate affinity

Given the kinetic interdependence of NNMT and NamPT revealed above, 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 in conjunction with 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 5A, more comprehensive multiple sequence alignment containing a larger number of species can be found in Figure S2. We found

an insert of ten amino acids in most deuterostomes that possess only NamPT and NNMT (indicated by the blue circle, Figure 5A). 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 has so far not been resolved in any of the available crystal structures obtained for NamPT. When modelling this stretch into the known homodimeric structure, the predicted loop, depicted in red in Figure 5B, is connected to one of the α -sheets involved in substrate binding (35). Intriguingly, the loops of the two subunits are in close proximity.

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 (Figure 5C). 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 exerted 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 lacking the stretch of amino acids 42-51 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 Figure S4C), 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 (31) for 30 minutes, neither wildtype nor mutant NamPT did synthesize NMN, suggesting that binding of FK866 is not affected by the mutation (Figure S4D). Using 100 μ M Nam and PRPP the wildtype showed a turnover rate of $0.0065 \pm 0.0010 \text{ s}^{-1}$ and $0.0077 \pm 0.0006 \text{ s}^{-1}$ without and with ATP, respectively, while the mutant did not have any detectable activity (Figure 5D). With 1mM of both substrates, the turnover rate of the wildtype enzyme increased to $0.0115 \pm 0.0005 \text{ s}^{-1}$ and $0.0098 \pm 0.0010 \text{ s}^{-1}$ without and with ATP, respectively. Under these conditions, the activity of the mutant enzyme was $0.0093 \pm 0.0008 \text{ s}^{-1}$ and $0.0077 \pm 0.0006 \text{ s}^{-1}$ without and with ATP, respectively. The decrease in turnover with ATP at high concentration of substrates has been observed earlier (34) and has been attributed to the competitive binding of ATP and PRPP (35). Overall our observations suggest that human NamPT lacking the amino acid stretch 42-51 is catalytically active, retains its dimeric state and sensitivity to FK866. However, it has a lower activity and affinity to Nam. These observations lend support to the conclusion that the acquisition of this loop in the NamPT of higher vertebrates has led to an increased affinity to Nam, as predicted by our 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 (Figure 5B) and molecular dynamics simulations for structures with and without the loop insertion (Figure 5E) 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 Table S4). Some residues close to the catalytic site, showed slightly elevated mobilities in the wildtype structure. However, these elevated mobilities were dominated by rare events during the simulation time of 1! s. They therefore do not appear as a robust change of structural dynamics upon loop insertion.

NNMT made NADA obsolete in vertebrates

Finally, we wanted to understand whether NADA may have been lost in vertebrates due to kinetic constraints. 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 Table S3). Without NNMT, the compartment containing NADA shows a higher NAD consumption rate (Figure 6A), and is able to maintain much higher NAD concentrations especially at low cell division rates (Figure 6B). At high cell division rates, steady state concentrations in both compartments are similar, as are NAD-consumption rates. As bacteria often have

296 relatively high growth rates and a low number of NAD consuming enzymes, this might explain why in
297 bacteria both systems co-exist.

298 In the presence of NNMT, the NamPT compartment has both higher NAD consumption rates and higher
299 steady state NAD concentrations than the compartment containing NADA (Figure 6C and 6D). This is,
300 however, dependent on the affinity of NamPT for Nam. If the substrate affinity of NamPT is too low, the
301 NADA compartment is able to maintain higher NAD concentrations and consumption flux. Taken together,
302 the results suggest that the NADA pathway might have become obsolete upon emergence of a high affinity
303 NamPT. This in turn might have been induced by the appearance of NNMT.

304 **Discussion**

305 The present study has revealed fundamental new insights into the evolution and dynamic interplay of the
306 enzymes in NAD metabolism. Our results show that the occurrence of NNMT enabled the enormous
307 diversification of NAD-consuming signalling enzymes in deuterostomes. NNMT promotes the removal of
308 excessive Nam produced in the signalling reactions. This is necessary to overcome Nam inhibition of the
309 corresponding enzymes. To enable both high NAD turnover and continuous salvage of Nam into NAD, the
310 kinetic parameters of both human NamPT and NNMT have attained values that are in the optimal range
311 predicted through our simulations (Figure 4). Our analyses have identified a stretch of 10 amino acids in
312 the structure of NamPT which contributes to the unusually high substrate affinity of this enzyme in higher
313 vertebrates. While it remains unclear why lower organisms and plants use primarily the Nam salvage
314 pathway through NADA, our analyses demonstrate that the combination of NamPT and NNMT
315 outcompetes this alternative when high turnover of NAD is required for signalling processes. Consequently,
316 NADA has been lost in vertebrates.

317

318 The positive effect of NNMT on NAD-consumption flux especially on sirtuins, is in line with a lifespan
319 extension observed in worms overexpressing NNMT (46). The effect of NNMT overexpression or silencing
320 has been controversially discussed and is presumably tissue and context specific (37). In this context it
321 should be noted that although we predict an overall positive effect of NNMT on NAD consumption, too
322 high expression of NNMT can indeed lead to adverse effects (Figure S3C). And although our analyses
323 predict that the presence of NNMT generally lowers Nam concentration and reduce cellular NAD
324 concentration, expression changes in NNMT do not necessarily have strong effects on NAD concentrations
325 (Figure S3D), which might explain why experimental results reported in the literature are not consistent.
326 For example, a considerable decrease of liver NAD levels have been reported upon NNMT overexpression
327 (47), while no changes in NAD concentrations could be detected in other approaches (5, 48). In addition,
328 this might of course be attributed to either adjustment of cellular Nam levels through fast equilibrium of
329 Nam (37) or the upregulation of NamPT (49, 50). It has furthermore been shown that M_{Nam} excretion is
330 mostly proportional to Nam uptake (51), supporting our findings that NNMT contributes to NAD-pathway
331 homeostasis. As shown in several recent studies this homeostatic control by NNMT can be circumvented
332 by supplying NR (2, 52-54) which is not a substrate of NNMT. At the cellular level NNMT is presumably
333 mainly advantageous, when high NAD-consumption rates are required for tissue function, or even more
334 likely, might be important to prevent spatio-temporal accumulation of Nam within cells due to temporally
335 increased NAD-consumption, e.g. PARP activation through DNA-damage.

336 The main healthy tissues expressing NNMT are the liver and adipose tissues, while no or only little
337 expression of NNMT is observed in most other organs (55). Increased NNMT expression is observed in
338 several types of cancer (56, 57), and might serve to remove Nam derived by increased NAD-dependent
339 signalling. To maintain high NAD concentrations in the tumours, a concomitant increase of NamPT
340 expression is required, which has indeed been found for some cancers (49, 50). It is worth noticing that
341 NNMT is only advantageous as long as NamPT affinity and activity are sufficiently high. This suggests
342 that certain types of cancer expressing NNMT at a high level could potentially be more susceptible to
343 inhibitors of NamPT. Several of such inhibitors are currently tested in clinical studies (23, 58). Based on
344 our analyses, it might be reasonable to test patients for NNMT expression in the tumour tissue. Non-NNMT
345 expressing tumours might respond less to competitive NamPT inhibitors, because deficient Nam
346 degradation in those cancer cells would potentially lead to an accumulation of Nam that could outcompete
347 the inhibitor.

Neither the scattered distribution of NamPT and NADA that is especially pronounced in bacteria (40), nor the loss of NADA in the ancestor of vertebrates has been understood earlier. Our combined phylogenetic-modelling analysis 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 (Figure 6). This is, however, only the case, if NamPT's 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 when high NAD turnover rates are needed. This is not necessarily the case in organisms that use Nam recycling through NADA

In our analyses, we did not consider the potential effects of most co-substrates of the investigated pathway. These co-substrates 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. But, we did perform a limited analysis of the effect of concentration changes in the methyl donor S-adenosyl methionine (SAM). Its precursor methionine has been shown to potentially limit the effect of NNMT (57). As shown in Figure S3 G and H SAM can have positive as well as negative effects on the NAD consumption flux, depending on the SAM concentration range. The effects observed in the physiological SAM concentration range are, however, much smaller than those observed for expression changes in NNMT or NamPT (Figure S3C and A respectively). Nevertheless, changes in methionine metabolism, might under some conditions influence cellular NAD concentration and NAD consumption rates. As NNMT in turn consumes SAM, NNMT might not only provide a kinetic advantage for NAD metabolism, but is likely to have a role in regulating other cellular processes through its impact on SAM availability (37). It has for example been shown that the product MNam is inducing the expression of sirtuins (46, 48, 59), the underlying mechanisms is, however, still unknown.

In conclusion, we have comprehensively analysed the functional co-evolution of several enzymes of the NAD pathway. The appearance of NNMT apparently initiated and drove complex alterations of the pathway such as an increase and diversification of NAD-dependent signalling, paralleled by an increase in NamPT substrate affinity. To see whether there have been other coevolutionary developments in the pathway we analysed the possible co-evolutionary of NNMT/NamPT with NMNATs and see that the loss of NADA and the loop insertion in NamPT co-occurs with the appearance of a human-like NMNAT2 (schematic overview see Figure 7, details Figure S6-S8). We furthermore noted that the occurrence of human-like NMNATs 1 and 3 and thus the further compartmentalisation of NAD metabolism (60) does coincide with a site-specific positive selection event in NNMT (see Figure S6 and S7). This might point to a role of NNMT in NAD pathway compartmentalisation as well as the spatio-temporal regulation of the pathway in general. Just recently the importance of the interaction between subcellular compartments for adipogenic gene regulations has been demonstrated (61).

Methods and Materials

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 2000 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 toolkit (62) and are accessible through the following GitHub repository <https://github.com/MolecularBioinformatics/Phylogenetic-analysis>.

400 Dynamic modelling

401 Kinetic parameters (substrate affinity (K_M) and turnover rates (k_{cat}), substrate and product inhibitions) were
402 retrieved from the enzyme database BRENDA and additionally evaluated by checking the original literature
403 especially with respect to measurement conditions. Parameter values from mammalian species were used
404 if available. For enzymes not present in mammals, values from yeast were integrated. The full list of kinetic
405 parameters including reference to original literature can be found in supplementary table S3. For NMNAT,
406 the previously developed rate law for substrate competition was used (63). Otherwise, Henri-Michaelis-
407 Menten kinetics were applied for all reactions except the import and efflux of Nam, which were simulated
408 using constant flux and mass action kinetics, respectively. Steady state calculation and parameter scan tasks
409 provided by COPASI 4.25 (64) were used for all simulations. The model files are provided in SBML format
410 are available at the Biomodels database accession no. Related figures were generated using
411 Gnuplot 5.0.

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

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

419 Transient transfection, immunocytochemistry, and confocal laser scanning 420 microscopy

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

434 NamPT expression

435 BL21- codonPlus (DE3) RIL were transformed with pQE-30 NamPT WT/pREP4 or pQE-30 NamPT Δ 42-
436 51/pREP4. Bacterial cells were grown at 37°C in 1 L of Luria-Bertani broth containing 100 μ g/mL
437 ampicillin, 50 μ g/mL kanamycin and 32 μ g/mL chloramphenicol. Protein expression was induced with 0.2
438 mM isopropyl- β -D-thiogalactoside at 0.4 $\pm$ 0.6 OD₆₀₀. Induction was done at 18°C overnight.

439 Purification of NamPT

440 The cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris-HCl pH 8.0,
441 500 mM NaCl, 4 mM dithiothreitol (DTT), 1 mg/mL lysozyme, 1X Complete EDTA-free protease inhibitor
442 cocktail (Roche)). After sonification, the lysate was centrifuged at 13000 g for 30 min, and the clear lysate
443 was incubated with 2 mL of Nickel-NTA resin (Qiagen). Non-specific protein binding was removed with
444 washing buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM DTT, 20 mM imidazole). The protein was
445 eluted with 2.5 mL of elution buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM DTT, 300 mM
446 imidazole).

447 The eluted protein was immediately subjected to size exclusion chromatography (SEC) using an ÄKTA
448 pure system (GE Healthcare) and loaded onto a HiLoad 16/60 Superdex 200 μ g column (GE Healthcare).
449 The chromatography was performed at a flow rate of 1 μ L/min with SEC buffer (20 μ M Tris-HCl pH 8.0,
450 500 μ M NaCl). Fractions containing the recombinant protein were pooled and used for enzymatic assay.
451 The purity and size of the protein were assessed by SDS-PAGE.

452 Enzymatic Assay

453 In a final volume of 1.2 ml reaction buffer (20 μ M Tris-HCl pH 8.0, 500 μ M NaCl, 6 μ M MgCl₂, 0.03%
454 (w/v) BSA), 2 μ M of enzyme were incubated with 5-phospho-D-ribose 1-diphosphate (PRPP) and Nam
455 (100 μ M or 1 μ M both). The reaction was incubated at 30 $^{\circ}$ C for 10 min and stopped by adding 100 μ M of
456 FK866. Subsequently, the samples were frozen in liquid nitrogen. The amount of NMN produced was
457 analysed using NMR spectroscopy. To do so the samples were dried with an Eppendorf Vacufuge
458 Concentrator, and then resuspended with 200 μ l of NMR solvent containing 5% (v/v) deuterated H₂O and
459 1 μ M 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). 1D ¹H NMR spectra were acquired on a 850 μ MHz
460 Ascend Bruker spectrometer equipped with 5 μ m TCI triple-resonance CryoProbe and a pulse field
461 gradients along the z-axis. The experiments were acquired with the zgpg30 pulse sequence, allowing
462 water suppression using excitation sculpting with gradients and perfect echo. The temperature was kept
463 constant at 300 μ K. Data were acquired with 2000 scans, 1 μ s relaxation delay, 1.6 μ s acquisition time, and
464 contained 65 μ 000 data points with a spectral width of 14 μ ppm.
465 The spectra phase and baseline were automatically and manually corrected using TopSpin 3.5 software
466 (Bruker Biospin). Quantification of nicotinamide mononucleotide (NMN) was done by the integration of
467 the peak at 9.52 μ ppm and DSS used as an internal standard.
468 All experiments were conducted at the Norwegian NMR Platform, NNP (grant 226244/F50).

469 Molecular dynamics simulations

470 All-atom molecular dynamics simulations were performed with explicit solvent for wildtype and mutant
471 (42-51) NamPT (PDB Code: 2H3D (65)). AMBER99SB-ILDN force field (66) was used with the TIP3P
472 water model (67) in GROMACS 5.1.2 (68). The structures were simulated each in a box of water with
473 distance between the solute and the box set to 0.2 μ m at a temperature of 300 μ K for a total time of 1 μ s. A
474 time step of 2 μ s and the stochastic dynamics integrator were used. For the evaluation of the root mean
475 square fluctuations (RMSF) the first 100 μ s of the simulations were omitted.

476 Identification of human-like NMNATs and test of positive selection in NNMNTs

477 For the identification of human-like NMNATs we clustered the retrieved sequences using BALi-Phy (69)
478 (Supplementary FigureS8). Human-like NMNATs 1, 2, and 3 were identified based on the isoform-specific
479 targeting and interaction domains described in (60).
480 We conducted a test of positive selection for orthologs of human NNMT from 60 vertebrate species. We
481 obtained coding sequences for all species and aligned the respective protein-translated sequences using
482 MUSCLE (70) and prepared codon-based alignments for further processing with PAL2NAL (71). We used
483 codeml from the PAML package (72) to conduct a branch-site model A test of positive selection. The
484 species names and the underlying tree topology for the codeml runs is depicted in Supplementary Figure
485 S7b. As a null model we assumed neutrality (e.g. diversifying site class with dN/dS = % = 1) which then
486 was compared to a model with positive selection (dN/dS = % > 1). Significance between the two models is
487 assessed using a likelihood ratio test assuming that twice the likelihood difference is χ^2 distributed. The
488 critical value is 3.84 at the 5 % level. Additionally, we identify codons with a site-specific signal of positive
489 selection using a Bayes Empirical Bayes (BEB) analysis with a probability > 0.9 (73).
490

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498 **Author contribution**

499 IH and MZ conceived the study. MB and TG performed the phylogenetic analysis, IH performed the
500 mathematical modelling, DH and MN performed the experiments, IR performed the MD analyses guided
501 by AS. MZ and IH supervised and guided the investigations. All authors analysed data and contributed to
502 the manuscript preparation.

503 **Declaration of interests**

504 The authors declare no competing interest.

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- 667

668 Figure Legends

669 Figure 1: Schematic overview of NAD biosynthesis pathways. NAD can be synthesised from tryptophan
670 (Trp), nicotinamide (Nam), nicotinic acid (NA) and the corresponding ribosides NR and NAR. Nam is the
671 main precursor in human and also the product of NAD-consuming signalling reactions by enzymes such as
672 sirtuins (NAD-dependent deacetylases) or PARPs (poly-ADP-ribose polymerases). For the recycling of Nam,
673 two different pathways exist. The pathway found in yeast, plants, and many bacteria starts with the
674 deamidation of Nam by Nam deamidase (NADA). Further biosynthesis via the Preiss-Handler pathway,
675 which also exists in vertebrates, requires three subsequent enzymatic steps catalysed by Nicotinic acid
676 phosphoribosyltransferase (NAPRT), Nicotinic acid/Nicotinamide mononucleotide adenylyltransferase
677 (NMNAT) and NAD synthase (NADS). In vertebrates, Nam is directly converted to
678 nicotinamidemononucleotide (NMN) by the Nam phosphoribosyltransferase (NamPT). The Nam N-
679 methyltransferase (NNMT) degrades Nam to methyl-Nam (MNam), which is in mammals excreted with
680 the urine. The colour marking of three different enzymes utilizing Nam will be used in subsequent figures
681 to denote the presence of these enzymes in different organisms.

682 Figure 2: Phylogenetic distribution of NADA, NNMT, and NamPT and their relation to the number
683 of NAD consumers. A) Distribution of NADA, NNMT, and NamPT in selected clades. NADA is dominant
684 in bacteria, fungi, and plants (Viridiplantae), whereas NamPT together with NNMT is dominant in metazoa.
685 Numbers at the pie charts show, the percentage of species per clade, which possess the respective enzyme
686 combination indicated by the colour code explained in the lower right of the figure (n = number of species
687 per clade included in the analysis). B) Common tree of selected clades within the metazoa, including 334
688 species. The pie charts indicate the distribution of species within the respective clade that encode the
689 enzyme combination indicated by the different colours. The size of the pie charts is proportional to the
690 logarithm of the number of species analysed in the particular clade. The numbers below the clade names
691 indicate the average number of NAD-consuming enzyme families found in all species of that clade. The
692 branch length is arbitrary. A detailed analysis of birds is provided in Figure S1 and the template sequences
693 used for the analysis are listed in Table S1.

694 Figure 3: NNMT enables high NAD consumption flux and is a potential driver of NamPT affinity
695 transition. A dynamic model of NAD biosynthesis and consumption (for details, see Methods and
696 Materials and Table S2) was used to simulate A) steady state NAD consumption flux and B) concentration.
697 Except for the results shown as dashed line in A and B, the enzyme amounts were kept constant for all
698 simulations shown. In the presence of NNMT (blue curves), steady state NAD consumption rates are higher
699 despite reduced NAD concentrations. Increasing the amount of NamPT in the simulation fourfold (dotted
700 blue curves) partially compensates for the decreased NAD concentration caused by Nam degradation
701 through NNMT. Using our dynamic model, the effect of different affinities of NamPT for Nam
702 (represented by Michaelis-Menten constants K_M) on the steady state NAD consumption flux and NAD
703 concentration were simulated at different cell division rates. In the absence of NNMT, the affinity of
704 NamPT has little influence on C) NAD consumption and D) NAD concentration, but both are strongly
705 influenced by cell division rates. E and F) In the presence of NNMT, increasing affinity of NamPT enables
706 increasing NAD consumption flux and NAD concentration. The presence of NNMT makes both NAD
707 consumption flux and concentration almost independent of cell division rates. G and H) calculated NAD
708 consumption fluxes and free NAD concentrations, respectively, are shown for the assumption of high
709 affinity of NamPT ($K_M=5$ nM, as found in the human enzyme) and low affinity (1 ! M, dashed lines).
710 Comparing the situation with and without NNMT reveals that at low substrate affinity of NamPT and high
711 cell division rates, NNMT, no longer enables higher NAD consumption rates compared to NamPT alone

712 (green curves and dashed grey curves).

713 Figure 4: Evolutionary optimality of the substrate affinities of human NNMT and NamPT. We
714 simulated the impact of changes in the substrate affinities of both NamPT and NNMT on A) NAD
715 consumption rates, B) free NAD concentration, C) NamPT/NNMT flux ratio and D) Nam concentration.
716 With increasing affinity of NamPT (decreasing K_M), but decreasing affinity of NNMT (increasing K_M)
717 NAD consumption rates and free NAD concentrations as well as the ratio between NamPT and NNMT flux
718 are increasing. The affinities reported for human enzymes (indicated by a black asterisk) appear to be in
719 the optimal range predicted through our simulations. The steady state concentration of Nam is largely
720 independent of the substrate affinity of NamPT, but strongly dependent on the affinity of NNMT.

721 Figure 5: The function of the structurally unresolved loop of NamPT. A) A multiple sequence
722 alignment of NamPT revealed a sequence insertion in the N-terminal region of this enzyme in most
723 deuterostomes that possess NamPT and NNMT. The relevant sequence section is shown for selected
724 organisms. A more comprehensive alignment can be found in Figure S2. Coloured circles indicate the
725 enzymes present in the respective species; blue: NamPT and NNMT; black: NamPT, NADA and NNMT;
726 yellow: NamPT and NADA. B) The structure visualisation of human NamPT is based on a structure
727 prediction by SWISS-MODEL (74, 75) using the model 2H3D of the human NamPT as template (65).
728 The inserted region (shown in red) is not resolved in any of currently available crystal structures of
729 NamPT and thus appears to be a flexible loop structure at the surface of the NamPT dimer. C) Confocal
730 laser scan micrographs of HeLaS3 cells expressing C-terminally FLAG-tagged wild-type (wt) and mutant
731 (\$42-51) NamPT lacking the unresolved loop. Both proteins showed a heterogeneous nuclear-cytosolic
732 localisation. Nuclei were stained with DAPI. The C-terminally FLAG-tagged human poly-ADP-ribose
733 glycohydrolase isoform PARG60 was used as a control for exclusive cytosolic localization. D)
734 Measurement of NamPT (wild-type and \$42-51 mutant) enzymatic activity in the presence of 1 mM or
735 100 μ M substrate (Nam and PRPP) with or without 1mM ATP (ND, no detection of NMN). The p-value
736 was calculated using non-parametric one-tailed Mann-Whitney test. The His-tagged proteins were
737 expressed in E. coli and purified as described in Methods and Materials (see also Figure S3). E)
738 Molecular dynamics simulations were performed for wildtype (red) and (\$42-51) NamPT (blue). Root
739 mean square fluctuations (RMSF) for every residue of chain A are shown (top). The difference RMSF for
740 every residue is shown in the lower panel (green). For better comparison the residue IDs for (\$42-
741 51) NamPT are aligned to accord with the wildtype structure and the average RMSF of residues 42 and 51
742 displayed in the blue curve between these residues. For the RMSF calculation, the first 100 ns of the
743 simulation are omitted to allow equilibration. In addition, root mean square deviation (RMSD) values
744 between different published structures of human NamPT structures were calculated and presented in
745 Table S4 and Figure S5.

746 Figure 6: NNMT provides a competitive advantage and makes NADA obsolete in vertebrates. To
747 simulate competition for common resources, a two-compartment model was created (see Methods and
748 Materials and Table S2). In this model one compartment contained NADA, but no NamPT and the other
749 compartment contained NamPT either with or without NNMT, but no NADA. NADA and NamPT were
750 simulated to be present at equal amounts. A) In the absence of NNMT the compartment containing NADA
751 has a higher NAD consumption rate, and B) a much higher steady state NAD concentration. C) In the
752 presence of NNMT, however, both NAD consumption and D) NAD concentration are lower in the NADA
753 compartment. This effect is dependent on a high affinity of NamPT for Nam.

754 Figure 7: Schematic representation of evolutionary events in the NAD biosynthesis pathway. The
755 scheme illustrates major evolutionary events in metazoans detected in our phylogenetic analyses of NAD
756 metabolism. The time of occurrence of human-like NMNAT1 and 3 has been reported previously (60), and
757 identified that human-like NMNAT2 most likely originated in the last common ancestor (LCA) of
758 vertebrates, while human-like NMNAT1/3 can be traced back to the LCA of placentalia (Supplementary
759 Figure S8). To test whether the rise of human-like NMNAT1/3s was associated with an event of rapid
760 sequence diversification in NNMT we conducted a test of positive selection specific to the branch leading
761 to the LCA of placentalia (Supplementary Figures S6 and S7) using a coding DNA substitution rate ratio
762 model. Indeed, we obtain a strong signature of positive selection for NNMT in the tested branch and can
763 pinpoint residue 171 as being significantly associated with the signature of positive selection.

764 (Supplementary Figure S7). Specific events in the evolution of NMNATs coincide with those of NamPT
765 or NNMT indicating a co-evolution of functions beyond those identified in the present study. The tree is a
766 schematic representation of selected taxa and is based on information provided by the Tree of life Web
767 Project (76).

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