

Special Issue: The evolving role of mitochondria in metabolism

The dynamic regulation of NAD metabolism in mitochondria

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Mitochondria are intracellular powerhouses that produce ATP and carry out diverse functions for cellular energy metabolism. Although the maintenance of an optimal NAD/NADH ratio is essential for mitochondrial function, it has recently become apparent that the maintenance of the mitochondrial NAD pool is also of crucial importance. The biosynthesis, transport, and catabolism of NAD and its key intermediates play an important role in the regulation of NAD-consuming mediators, such as sirtuins, poly-ADP-ribose polymerases, and CD38/157 ectoenzymes, in intra- and extracellular compartments. Mitochondrial NAD biosynthesis is also modulated in response to nutritional and environmental stimuli. In this article, we discuss this dynamic regulation of NAD metabolism in mitochondria to shed light on the intimate connection between NAD and mitochondrial function.

Mitochondria; tiny cellular powerhouses

Mitochondrial dysfunction has now been associated with over 40 major diseases and health problems including type 2 diabetes, cancer, Alzheimer's disease, and other neurodegenerative diseases [1–4]. With the electron transport chain (ETC) and FOF1-ATP synthase located within their invaginated inner membrane, and enzymes of the tricarboxylic acid (TCA) cycle and fatty acid oxidation in their matrix, mitochondria carry out crucial functions for cellular energy metabolism, generating the majority of cellular ATP in eukaryotes. Mitochondria also regulate amino acid catabolism, ketone body formation, heme biosynthesis, the urea cycle, and calcium storage. With such important functions in energy metabolism, and with the major consequences of mitochondrial dysfunction, it has become critically important to investigate the various mechanisms that preserve and promote optimal mitochondrial function.

Mitochondrial ATP production and membrane potential require the universal cofactor nicotinamide adenine dinucleotide (NAD; see [Glossary](#)). As an essential coenzyme, NAD gains two electrons and a proton from substrates at multiple TCA cycle steps, being reduced to NADH. Mitochondrial NADH is oxidized upon donating its electrons to Complex I (NADH:ubiquinone oxidoreductase) of the ETC. These electrons are sequentially relayed from Complex I to ubiquinone (Coenzyme Q10), Complex III (coenzyme Q-cytochrome *c* oxidoreductase), cytochrome *c*, and Complex IV (cytochrome *c* oxidase), resulting in the reduction of

oxygen to water. The flow of electrons is coupled to the pumping of protons by Complexes I, III, and IV across the impermeable inner membrane and into the inner membrane space, generating a proton gradient [5]. Protons can reenter the matrix through FOF1-ATP synthase, a key flow that drives ATP synthesis. Because the TCA cycle and the ETC require NAD and NADH, respectively, an optimal NAD/NADH ratio is needed for efficient mitochondrial metabolism. To date, numerous studies have investigated the factors that influence the mitochondrial NAD/NADH ratio [6–8].

Recent studies have demonstrated that NAD levels are limiting, making the availability of NAD pivotal for mitochondrial function [9–11]. It has also been shown that the biosynthesis, subcellular localization, and systemic transport of NAD and its intermediates, play an important role

Glossary

CD38 and CD157: cADP-ribose synthases; ectoenzymes that use NAD to generate cADP-ribose (cADPR) and NAADP, generating NAM in the process.

Indoleamine-2,3-dioxygenase (IDO): an enzyme that converts L-tryptophan to *N*-formylkynurenin. IDO functions redundantly with TDO but is primarily expressed in immune cells.

Nicotinamide (NAM): a water-soluble vitamin and the amide form of the essential nutrient nicotinic acid (also known as vitamin B₃ or niacin).

Nicotinamide adenine dinucleotide (NAD): an essential cofactor for hydride transfer enzymes and a substrate for NAD-consuming enzymes. NAD consists of a nucleotide containing an adenine base and NAM.

Nicotinamide mononucleotide adenyl transferase (NMNAT): an enzyme that belongs to the family of nucleotidyltransferases, enzymes that transfer phosphorus-containing nucleotide groups. NMNAT isoforms, of which there are three, convert NaMN to NaAD as well as NMN to NAD.

Nicotinamide phosphoribosyltransferase (NAMPT): also termed visfatin or PBEF, is an enzyme that belongs to the family of type II phosphoribosyltransferases. NAMPT converts NAM and 5-phosphoribosyl-pyrophosphate to NMN.

Nicotinamide riboside kinase (NRK): an enzyme that phosphorylates NR to NMN using ATP. Of the two mammalian isoforms, NRK1 is ubiquitously expressed, whereas NRK2 is specifically expressed in the heart, brain, and muscle.

Nicotinic acid adenine dinucleotide phosphate (NAADP): a dinucleotide and the most potent Ca²⁺-mobilizing second messenger synthesized in response to extracellular stimuli.

Nicotinic acid phosphoribosyltransferase (NPT): an enzyme that belongs to the family of phosphoribosyltransferases. NPT converts NA and 5-phosphoribosyl-pyrophosphate to NaMN.

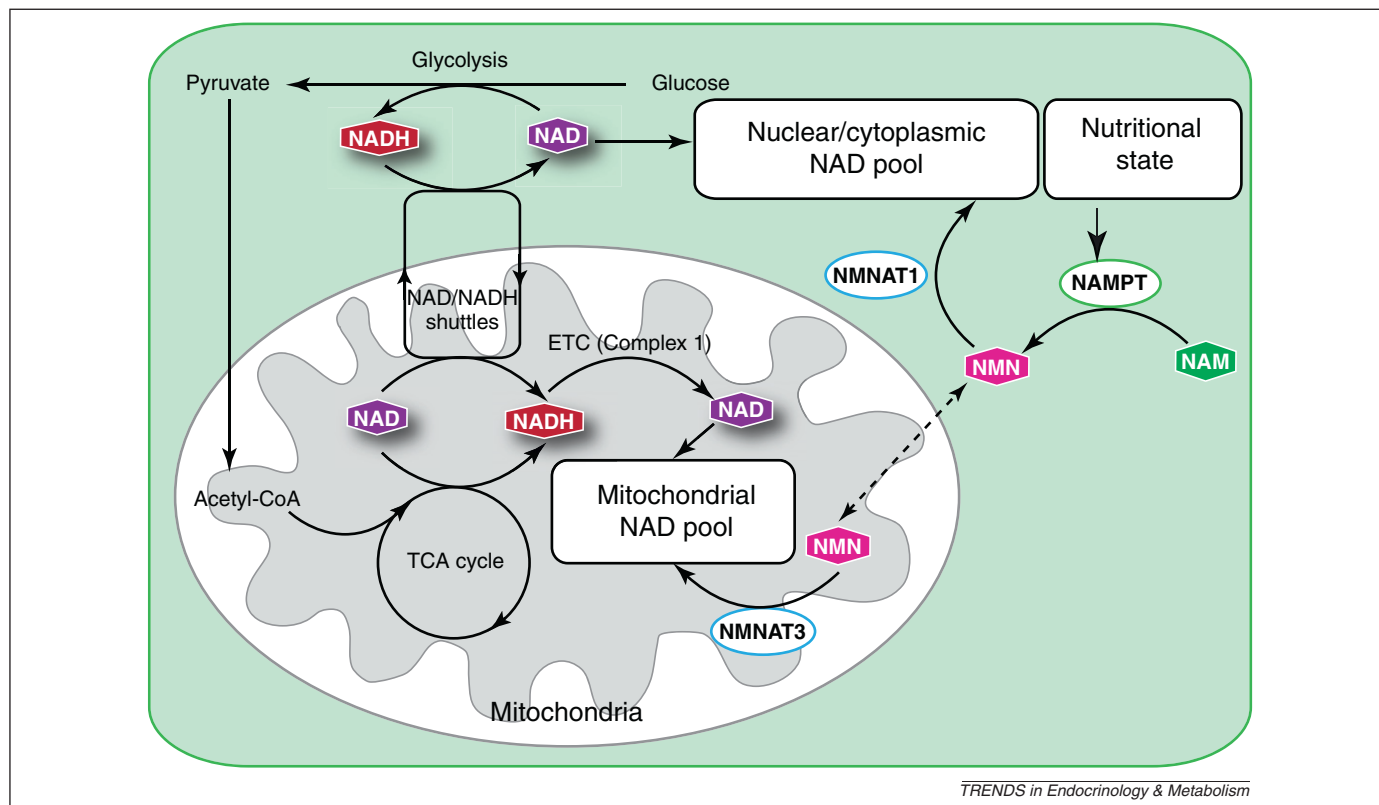
Poly(ADP-ribose) polymerases (PARPs): nuclear enzymes that use NAD to transfer long chains and branches of ADP-ribosyl polymers to their protein substrates.

Purine nucleoside phosphorylase (PNP): an enzyme that degrades NR to NAM.

Sirtuins: the silent information regulator 2 (Sir2) family of proteins. The seven mammalian sirtuin family members, SIRT1–7, use NAD to deacetylate, ADP-ribosylate, and/or demalonylate/desuccinylate their target proteins.

Tryptophan dioxygenase (TDO): an enzyme that converts L-tryptophan to *N*-formylkynurenin. TDO functions redundantly with IDO but is primarily expressed in the liver.

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Figure 1. Maintenance of the mitochondrial NAD pool. Although separate, the mitochondrial and nuclear/cytoplasmic NAD pools are intricately connected through the NAD/NADH-redox shuttles (most commonly the malate/aspartate and the glycerol-3-phosphate shuttles) and NAD biosynthetic pathways in each subcellular compartment. Multiple cellular processes play an important role in maintaining an optimal NAD/NADH ratio between mitochondria and the cytoplasm, including glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation by the electron transport chain (ETC). Mitochondrial and nuclear/cytoplasmic NAD biosynthetic pathways are balanced in response to nutritional and environmental stimuli. Abbreviations: NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; NMN, nicotinamide mononucleotide.

in the regulation of various biological processes, with significant impact upon mitochondrial functionality [8,12–14]. These studies have uncovered an intricate layer of tissue/organ-specific effects and differential roles for various NAD intermediates. In this review, we will focus on how these factors and different NAD intermediates affect the mitochondrial NAD pool in metabolic tissues/organs. We will further discuss the regulation of NAD biosynthesis by diet and aging. Finally, we will touch upon the therapeutic potential of NAD biology for mitochondrial function.

Maintenance of the mitochondrial NAD pool

The mitochondrial NAD pool is relatively distinct from that of the rest of the cell [15,16]. Whereas cytoplasmic NAD/NADH ratios range between 60 and 700 in a typical eukaryotic cell, mitochondrial NAD/NADH ratios are maintained at 7 to 8 [17,18]. Mitochondrial NAD levels can also be higher than cytoplasmic levels, but the relative difference is cell type-specific [14]. For instance, the NAD pool is 70% mitochondrial in cardiac myocytes (10.0 ± 1.8 nmol/mg protein) [19,20], 50% mitochondrial in neurons (4.7 ± 0.4 nmol/mg protein) [19], and 30–40% mitochondrial in hepatocytes [14] and astrocytes (3.2 ± 1.0 nmol/mg protein) [19]. These differences are presumably due to differential requirements for maximal oxidative phosphorylation [20]. Another important aspect of the mitochondrial NAD pool is that it is sufficiently robust and isolated to preserve oxidative phosphorylation. Even upon massive depletion of cytoplasmic NAD, mitochondrial NAD levels

can be maintained for at least 24 h, and possibly up to three days [8,15,16,19–22]. These findings indicate that a pool of NAD is sequestered within mitochondria, preserving cell viability and ATP levels until the mitochondrial membrane is breached.

Although separate, the cytoplasmic and mitochondrial NAD pools are intricately connected by two processes: glycolysis and NAD biosynthesis (Figure 1). In the cytoplasm, glycolysis converts glucose to pyruvate. In this process, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) requires two NAD per glucose to oxidize glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. The NADH and pyruvate produced by glycolysis are transported from the cytoplasm into the mitochondrial matrix to provide reducing equivalents for the TCA cycle and ETC [5,6,8]. Mitochondrial NADH transport is conducted by NAD/NADH-redox shuttles, most commonly the malate/aspartate and the glycerol-3-phosphate shuttles [5,6,8]. With its requirement for GAPDH catalysis and other key regulators in glycolysis, cytoplasmic NAD availability is an important determinant of the rate of glycolysis and thus NADH and pyruvate flux to mitochondria. Indeed, in particular circumstances where glucose is the predominant exogenous substrate, depletion of cytoplasmic NAD by 50% or more can block glycolysis and mitochondrial substrate flux, and eventually lead to loss of cellular viability [6,9,21,22].

A growing body of evidence also suggests that mitochondria have their own NAD biosynthetic machinery which

Box 1. NAD biosynthetic pathways

In mammals, NAD biosynthesis can proceed via four different routes: *de novo* synthesis from tryptophan (TRP), synthesis from either form of vitamin B₃, nicotinamide (NAM), or nicotinic acid (NA), or conversion of nicotinamide riboside (NR) (Figure 1) [59]. Together, these pathways generate a cellular NAD concentration of 300 to 800 μ M, depending upon the tissue/organ [8,16,84,85]. In mammals, NAM has a better capability to stimulate NAD biosynthesis than NA in multiple organs [86,87]. Nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the NAD biosynthetic pathway from NAM, converts NAM and 5-phosphoribosyl-pyrophosphate to a key NAD intermediate, nicotinamide mononucleotide (NMN). NMN is adenylated to NAD by nicotinamide mononucleotide adenylyl transferase (NMNAT) [8,14,88]. Accordingly, increased gene dosage of NAMPT, but not NMNAT, increases NAD levels [16,28–90].

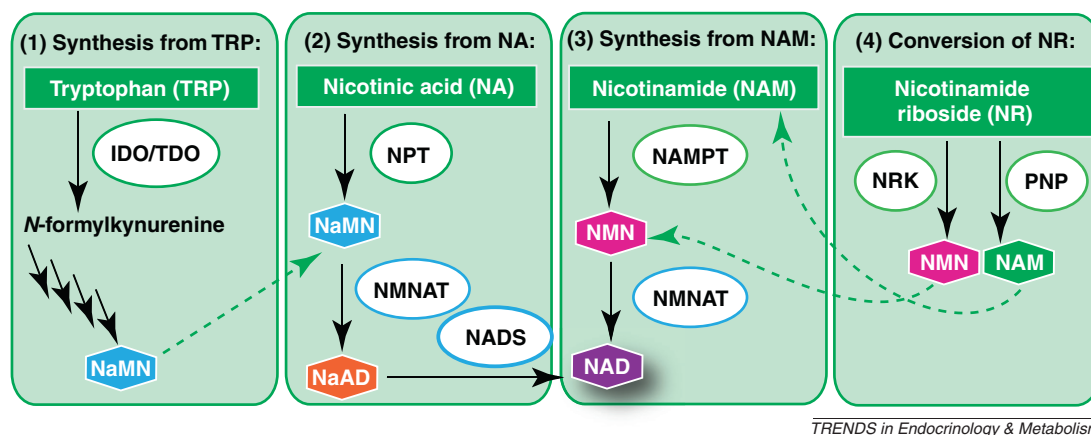
Conversion of NA to NAD takes place via the Preiss–Handler pathway [91]. Nicotinic acid phosphoribosyltransferase (NPT) converts NA to nicotinic acid mononucleotide (NaMN), which is then adenylated to nicotinic acid adenine dinucleotide (NaAD) by NMNAT [85,91]. NaAD is amidated to NAD by NAD synthetase (NADSYN1 or NADS) [91].

De novo NAD biosynthesis from the amino acid TRP proceeds by the kynurenine pathway. Tryptophan dioxygenase (TDO) and indoleamine-2,3-dioxygenase (IDO) share the first and rate-limiting step of

this pathway to produce *N*-formylkynurenine [92]. Through a series of enzymatic steps, *N*-formylkynurenine is converted to NaMN. NaMN is subsequently converted to NAD by the sequential reactions of NMNAT and NADS [92].

Two pathways use NR to synthesize NAD [91]. First, ATP can be used to phosphorylate NR, generating NMN, in a reaction catalyzed by two isoforms of nicotinamide riboside kinase in mammals (NRK1,2) (Figure 1) [34]. Second, the glycosidic linkage of NR can be broken by purine nucleoside phosphorylase (PNP), and NAM liberated from this reaction is reused [42,93].

All of these NAD biosynthetic pathways merge at the last step of the dinucleotide formation, catalyzed by NMNATs (Figure 1). In mammals, there are three NMNAT isoforms, NMNAT1–3, located in the nucleus, the Golgi complex, and the mitochondria, respectively [94–96]. All three NMNAT isoforms can use both the oxidized or reduced (NMNH) forms of NMN for NAD or NADH synthesis [96]. The catalytic efficiency of NMNAT1 ($K_m = 20.1 \mu$ M [29]) is much higher than the other isoforms, with mitochondrial NMNAT3 being the least efficient [97]. Interestingly, NMNATs can also catalyze the reverse reaction, producing NMN and ATP from NAD and pyrophosphate [96]. However, because their K_m values for pyrophosphate are relatively high, NAD synthesis is probably the predominant reaction *in vivo* [33].



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Figure 1. Pathways of NAD biosynthesis. Mammalian NAD biosynthesis can take place via *de novo* synthesis from tryptophan (TRP) as well as by conversion of the vitamin B₃ precursors nicotinamide (NAM), nicotinic acid (NA) or nicotinamide riboside (NR). These four pathways generate common downstream intermediates, as denoted by the broken green arrows. Usage of each pathway varies in different tissues/organs.

appears to play an important role in maintaining the mitochondrial NAD pool in response to environmental and nutritional stresses [16,23] (Figure 1). Although the details still remain unclear, how mitochondria maintain their NAD pool by their own NAD biosynthetic machinery, and/or through the communication with nuclear/cytoplasmic NAD biosynthetic pathways, has recently become a focus of intensive investigation. Box 1 presents a summary of how mammalian NAD is synthesized.

Transport of NAD and its intermediates between cytoplasm and mitochondria

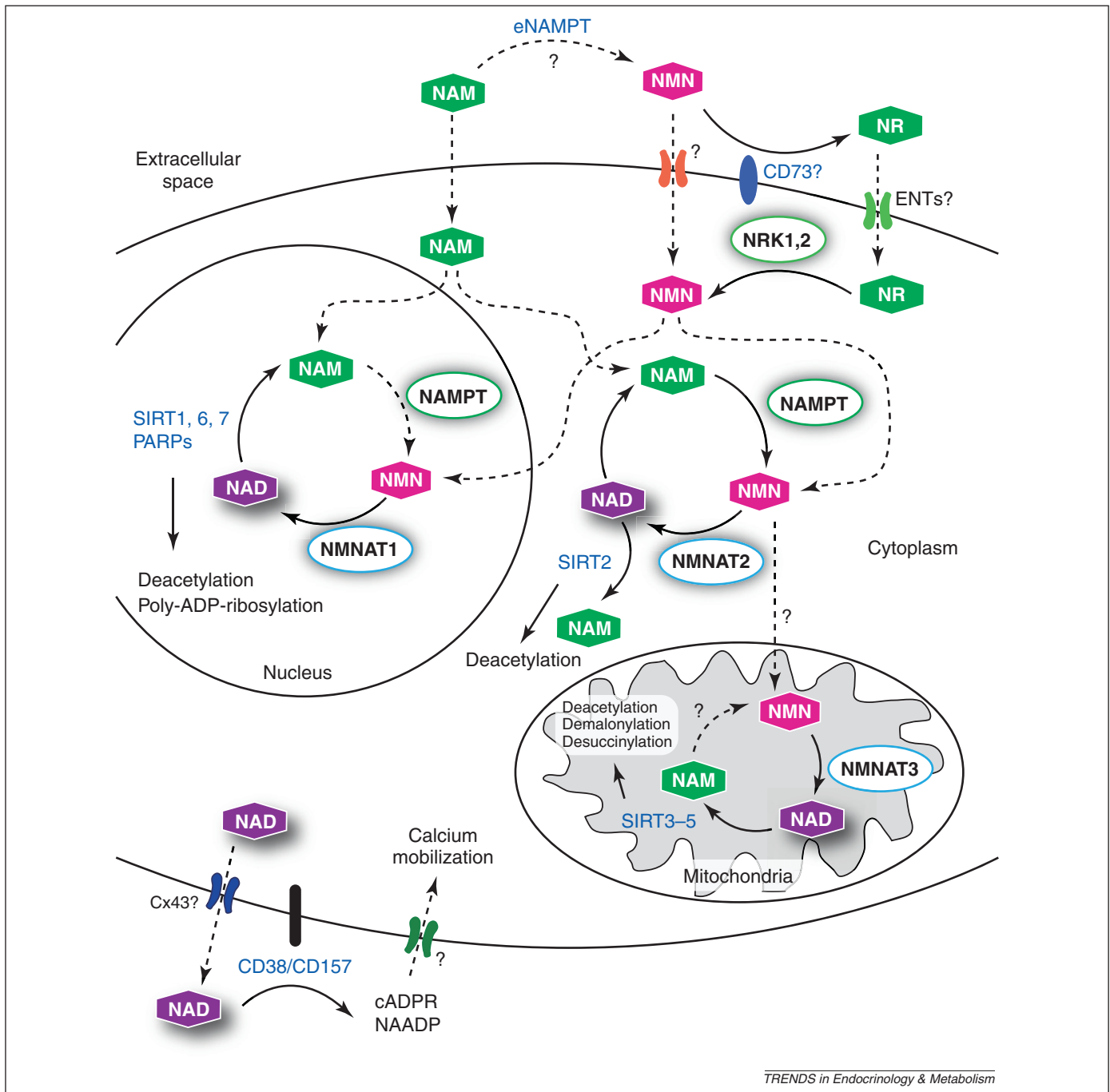
Whereas the outer mitochondrial membrane is porous and freely permeable to small molecules and ions, the permeability of the inner membrane is strictly regulated by the mitochondrial permeability transition pore (mtPTP) under normal physiological conditions [5]. The existence of mitochondrial NAD biosynthetic machinery raises the question as to how NAD precursors and/or intermediates can enter the mitochondrial matrix. In yeast, NAD is not synthesized in mitochondria [24]. Instead, the 70%

homologous nucleoside deoxyribosyltransferases Ndt1 and Ndt2 function to transport NAD across the inner mitochondrial membrane by unidirectional exchange with mitochondrial (d)AMP and (d)GMP [24]. NAD transport by Ndt1 is highly specific for NAD, with an apparent K_m of 0.38 mM [24]. Deletion of these transporters in yeast reduces mitochondrial NAD levels (>fourfold), impairs the activity of mitochondrial NAD-requiring enzymes, and delays growth on nonfermentable carbon (respiratory conditions) [24,25]. Conversely, overexpression of Ndt1 doubles NAD levels [24,25]. A mitochondrial NAD transporter, AtNDT2, has also been found in *Arabidopsis*, which transports NAD in a counter-exchange mode for ADP or AMP [26].

A mammalian mitochondrial NAD transporter has yet to be found [16,24], and it has been shown that NAD is unable to cross the mitochondrial membrane [6], such as in isolated liver mitochondria [27]. As described in Box 1, NMNAT3 is partially localized in mitochondria. However, the majority of studies have so far reported that NAMPT is localized in the cytoplasm [15,28–30], with cell

type-specific nuclear localization [31]. Because exogenous NAD increases mitochondrial NAD levels more than cytoplasmic levels (2.4- and 1.8-fold, respectively) [11], it is conceivable that mitochondrial transport mechanisms might exist for NAD precursors and/or intermediates.

Using the activity of a mitochondrion-targeted poly ADP-ribose polymerase (PARP1) as a readout of mitochondrial NAD levels, a recent study showed that NMN is the cytoplasmic precursor of mitochondrial NAD [28]. NMN is able to cross the mitochondrial membrane [27,28] and is



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Figure 2. The biosynthesis, transport, and catabolism of NAD and its intermediates in intra- and extracellular compartments. In mammals, NAD can be synthesized from tryptophan, nicotinamide (NAM), and nicotinic acid. NAM is a major substrate for mammalian NAD biosynthesis (Box 1). Thus, NAD biosynthetic pathways from tryptophan and nicotinic acid are not shown in this scheme. In the cytoplasm, NAM is converted to nicotinamide mononucleotide (NMN), a key NAD intermediate, by nicotinamide phosphoribosyltransferase (NAMPT). This process might also be mediated by extracellular NAMPT (eNAMPT). It remains unclear whether a similar process occurs in the nucleus and mitochondria. Nicotinamide ribose (NR), another key NAD intermediate, can be produced from NMN extracellularly, likely by the ectoenzyme CD73, and transported into the cell, possibly through equilibrative nucleoside transporters (ENTs). Inside the cell, NR is re-phosphorylated to NMN by NR kinases 1 and 2 (NRK1 and 2). In each subcellular compartment, NMN is converted to NAD by NMN adenylyltransferases, NMNAT1–3. NMN also appears to be transported from the cytoplasm to mitochondria, although the mechanism underlying this NMN transport is currently unknown. NAD is consumed intracellularly by key mediators, particularly sirtuins and poly-ADP-ribose polymerases (PARPs). NAD can also be transported to the outside of the cell, probably through connexin 43 hemichannels [83]. CD38/157 ectoenzymes produce cyclic ADP-ribose (cADPR) and nicotinic acid adenosine dinucleotide phosphate (NAADP), which reenter the cell and induce calcium mobilization.

enriched in mitochondria, with 1.5- to 2.0-fold higher levels than in the cytoplasm [32]. Furthermore, NMN can be converted to NAD in isolated mitochondria [27], and mitochondrial NMN content rises when NAD intermediates are administered to cells [32]. Thus, the current model is that NAD precursors and intermediates are converted to NMN in the cytoplasm, and NMN is transported into mitochondria for NAD biosynthesis by NMNAT3 [28] (Figure 2). Whereas NMNATs can also catalyze the reverse reaction (Box 1), the equilibrium of their reaction probably leans toward NAD biosynthesis, because their affinity for pyrophosphate is relatively low [33]. So far, no NMN-specific transport system has been identified, making it a prime area for future investigation. It will also be interesting to examine whether cytoplasmic NAMPT activity, or the as-yet uncharacterized mitochondrial NMN shuttling system, is actually the limiting factor in mitochondrial NAD levels.

Systemic distribution of key NAD intermediates

In mammals, key NAD intermediates, such as NMN and NR, can exist in food as well as systemically in tissues and blood circulation [34–36]. Both intermediates can also be systemically delivered to cells in different tissues and organs. Recently, it has been shown that NMN and NR can be efficiently transported into cells and multiple tissues and utilized for NAD biosynthesis [36,37]. For instance, within 15 min after administration of a single dose of NMN to mice, NMN levels in the liver, pancreas, and white adipose tissue (WAT) increase significantly, and NMN levels in the pancreas and WAT reach 10-fold higher levels than NMN levels in the liver. These changes are accompanied by a concomitant significant increase in NAD levels (2.0-fold) in the liver, and a more modest one in the pancreas and WAT (1.4-fold and 1.2–1.5-fold, respectively) [36]. Because NMNAT activity is not thought to be limiting for cellular NAD biosynthesis, these differences probably indicate differential NMN uptake in each tissue. Interestingly, in the liver, NR also shows a mild increase (~fivefold) compared to a ~15-fold increase of NMN after NMN administration, suggesting that some NMN may be converted to NR before uptake [36]. Similarly to NMN, NR is also able to stimulate NAD biosynthesis efficiently in mammalian cells and tissues, accompanied with a significant enhancement of mitochondrial NAD levels [37].

How NMN and NR are transported into mammalian cells remains an open question. In yeast, NR is an intracellular and a secreted NAD precursor [38–41]. Thus, balancing import and export of NR contributes to the regulation of intracellular NAD metabolism. In fact, yeast cells utilize NR much more efficiently than NMN to augment NAD levels, promote cell growth, and increase the activity of NAD-consuming enzymes [39,42]. In yeast, extracellular NMN is converted to NR by the periplasmic acid phosphatase Pho5, and NR is transported into cells through the NR transporter Nrt1 and re-phosphorylated to NMN by NRK [39,40]. Although there are no direct homologs of Pho5 and Nrt1 in mammals, CD73, an ecto-5'-nucleotidase, and equilibrative nucleoside transporters (ENTs) could be functional counterparts to Pho5 and Nrt1 [39,43] (Figure 2). Indeed, in transformed HEK293 and HeLa cells, the pharmacological suppression of CD73 and ENTs by chemical inhibitors can

significantly reduce NAD biosynthesis stimulated by exogenous NMN and NR [28]. Because NMN can also be degraded by CD38/CD157 ectoenzymes (described below) to ribose-5-phosphate and NAM [44], an alternative possibility is that NAM liberated from NMN can be reused for NAD biosynthesis. Further investigation will be required to elucidate the mechanisms for the transport of NAD intermediates across plasma and mitochondrial membranes.

Mammals may even be able to coordinate NAD biosynthesis at the systemic level. NAMPT, the mammalian key NAD biosynthetic enzyme, has two different forms: intra- and extracellular NAMPT (iNAMPT and eNAMPT, respectively). eNAMPT is produced by fully differentiated white and brown adipocytes, hepatocytes, and leukocytes [35,45–47]. NAMPT does not have a cleavable signal sequence, and eNAMPT release occurs neither by the classic ER–Golgi secretory pathway, nor through the release of microvesicles, or ATP-binding cassette pathways [35,45,47]. Thus, eNAMPT secretion probably occurs by an as-yet undiscovered transport system. Although the functional significance of eNAMPT is still under investigation, it has been shown that the enzymatic activity of eNAMPT secreted from brown adipocytes is twice as high as iNAMPT, a difference likely due to a post-translational modification [35]. Intriguingly, *Nampt*-deficient heterozygous female mice exhibit reduced levels of plasma eNAMPT and NMN and defects in glucose-stimulated insulin secretion in pancreatic β cells [35]. The defects in β -cell function can be completely ameliorated by systemic administration of NMN. Based on these findings, it has been hypothesized that circulating eNAMPT plays an important role in maintaining systemic NAD biosynthesis through the extracellular biosynthesis of NMN [12,48] (Figure 2). This model has recently been challenged [49]. To address this hypothesis, it will be crucial to analyze the effect of tissue-specific *Nampt* ablation on systemic NAD biosynthesis, particularly in adipose tissue- and liver-specific *Nampt*-deficient mice.

Key mediators downstream of NAD biosynthesis

Three distinct families of enzymes consume NAD as a substrate: poly-ADP-ribose polymerases (PARPs), CD38/CD157 ectoenzymes, and sirtuins [50–52]. These enzymes generally break the glycosidic bond between NAM and ribose moieties of NAD, contributing to the short half-life of NAD (1–10 hrs) [8,14].

PARPs are nuclear enzymes that transfer long chains and branches of ADP-ribosyl polymers to their protein substrates, and mediate a wide range of biological processes, including DNA repair, transcriptional regulation, and metabolism [52]. CD38 and CD157 are ectoenzymes that generate cyclic ADP-ribose (cADPR) and NAADP, two potent second messengers for calcium signaling [50]. Because their activity constitutes the main source of cellular NAD catabolism, PARPs and CD38/CD157 significantly affect cellular NAD levels [8,10,53]. As a result, NAD availability becomes limiting for other NAD-consuming enzymes, such as sirtuins. Indeed, inhibition or deletion of PARP1 or CD38 increases NAD levels enough to increase the activity of the mammalian sirtuin SIRT1 [10,53]. The silent information regulator 2 (Sir2) family of proteins, now named sirtuins, use NAD to mediate deacetylation, ADP-ribosylation,

and demalonylation/desuccinylation of target proteins [54–56]. There are seven mammalian sirtuin family members, SIRT1–7, which share an evolutionarily conserved catalytic core domain [51,57,58], and NAD is perhaps the most important regulator of their activity [8,58,59]. Increasing lines of evidence suggest that NAMPT-mediated NAD biosynthesis plays a crucial role in the regulation of sirtuin activities in diverse subcellular compartments [58–61]. Thus, an optimal balance between production and consumption of NAD in each subcellular compartment is crucial for these key mediators to regulate diverse biological processes under various pathophysiological conditions.

In this regard, the effects of key NAD-dependent mediators on mitochondrial function have recently become a focus of intensive investigation. Of these, three mitochondrial sirtuins, SIRT3–5, have attracted much attention (Figure 2). Recent proteomics studies have demonstrated that many key metabolic enzymes are acetylated, malonylated, or succinylated in mitochondria, and that their enzymatic activities are regulated by these modifications in response to environmental stimuli [54,62,63]. Interestingly, SIRT3 and SIRT5 have NAD-dependent deacetylase and demalonylase/desuccinylase activities, respectively, in mitochondria [54,63,64]. Whereas SIRT4 has been demonstrated to act as an ADP-ribosyltransferase [55], it is suspected that SIRT4 might also function as a deacetylase, demalonylase, and/or desuccinylase, depending upon its substrate. The specific mitochondrial functions of these sirtuins are addressed in depth in other review articles in this issue. The finding that poly-ADP-ribose, cADPR, and NAADP are produced in mitochondria [65,66] may further indicate that the activities of these mitochondrial sirtuins are regulated by competition for NAD with other mitochondrial NAD-consuming pathways. Further investigation is required to elucidate how the dynamic balance between NAD production and consumption regulates various aspects of mitochondrial function.

NAD biosynthesis and NAD-consuming key mediators appear to comprise more complex feedback mechanisms throughout the body. It has been demonstrated that NAMPT-mediated NAD biosynthesis and SIRT1 comprise an interlocked transcriptional–enzymatic feedback loop that regulates the core circadian clock machinery through CLOCK/BMAL1, a key clock regulatory complex [67,68]. In peripheral tissues, such as the liver and WAT, levels of NAMPT and NAD display circadian oscillations that are regulated by CLOCK/BMAL1. In this circadian cycle, NAD functions as a ‘metabolic oscillator’ [69] and periodically activates SIRT1, which represses CLOCK/BMAL1-mediated transcription of clock target genes, including *Nampt* itself. It is very likely that this NAD oscillation also has an impact upon the activity of other NAD-consuming key mediators, such as mitochondrial sirtuins. Therefore, it will be of great interest to examine whether mitochondrial protein modifications are altered following the circadian oscillation of NAD in peripheral tissues.

Modulation of NAD biosynthesis by diet and aging

In addition to its circadian oscillation, NAD biosynthesis is modulated in response to nutritional and environmental stimuli. For instance, it has been shown that calorie

restriction (CR), a dietary regimen that retards aging and extends lifespan in a wide variety of organisms, enhances NAD levels in the liver, skeletal muscle, WAT, and brain [23,70,71]. Fasting has also been shown to increase NAD levels to the same degree in the liver and skeletal muscle [16,23,72–74]. Some studies have even localized the increases in NAD levels generated by CR [23], and fasting [16], to the mitochondria. Mirroring the effect of low energy intake on NAD levels, high-fat diet (HFD) feeding significantly reduces NAD levels in the liver and WAT [36], contributing to the pathogenesis of HFD- and age-induced type 2 diabetes in mice [36]. These changes in NAD levels in response to energy intake appear to be caused by changes in NAMPT expression. Fasting and CR induce NAMPT expression two- to threefold in the liver and skeletal muscle [16,23,72,74,75]. Serum deprivation also increases NAMPT expression (1.5–2-fold), enough to increase NAD levels in cultured cells [16,75–78]. On the other hand, HFD feeding significantly reduces NAMPT protein levels in the liver and WAT [36].

Aging also has a significant impact upon NAD biosynthesis at the cellular and organismal levels, resulting in reduced NAD levels in human cells [79,80] and rodents [36,80,81]. Similarly to HFD feeding, aging significantly reduces NAMPT protein levels. Consistent with the decreases in NAD levels, aged mice have significantly decreased NAMPT levels in peripheral tissues [36]. Currently, the mechanism by which aging affects NAMPT expression remains unclear. Determining this regulatory mechanism will provide valuable insight into effective interventions to preserve appropriate NAD levels during metabolic complications and aging.

When NAMPT-mediated NAD biosynthesis is compromised, one simple way to preserve NAD levels is by supplementing key NAD intermediates, such as NMN and NR. It has been demonstrated that intraperitoneal administration of NMN (500 mg/kg/day) dramatically improves glucose tolerance and glucose-stimulated insulin secretion in HFD- and age-induced type 2 diabetic mice, and even in non-diabetic aged mice [36,82]. In addition, it has recently been shown that NR (400 mg/kg/day) enhances oxidative function in skeletal muscle and brown adipose tissue, and protects against impaired glucose metabolism in HFD-fed mice [37]. These findings suggest a preventive/therapeutic use of key NAD intermediates for diet- and age-associated complications. Because both NMN and NR can be efficiently utilized for NAD biosynthesis in multiple tissues, it is of great importance to conduct long-term administration experiments for key NAD intermediates to determine whether NAD biosynthesis can be optimized to maintain lifelong physiological functions. Such assessments will provide a firm foundation for developing effective, scientifically sound, nutraceutical interventions with key NAD intermediates for human health.

Concluding remarks

Recently, the importance of NAD biosynthesis has been revisited, generating convincing evidence that there are complex regulatory mechanisms, including NAD biosynthetic enzymes, intra- and extracellular transport of NAD precursors and intermediates, and key NAD-consuming

Box 2. Outstanding questions

- Is cytoplasmic NMN the major source of the mitochondrial NAD pool? If so, how is NMN transported into mitochondria?
- Is systemic uptake of NAD intermediates, cytoplasmic NAD biosynthesis, or the still uncharacterized mitochondrial NMN shuttling system the limiting factor in mitochondrial NAD levels?
- What fuels the mitochondrial NAD pool in tissues/organs with little to no NMNAT3 activity?
- Do NMN and NR function as a required and/or optional extracellular exchange currency in mammals? Which tissue/organ, if any, regulates the kinetics of NMN/NR production and uptake?
- What nutritional and environmental conditions regulate the usage of each NAD precursor and intermediate?
- What molecular mechanisms mediate the changes in NAD levels and NAMPT-mediated NAD biosynthesis in response to energy status and aging?
- Can long-term supplementation of NAD levels with NAD intermediates, namely NMN and NR, preserve mitochondrial function throughout life?

mediators, which function to maintain an optimal balance between NAD production and consumption in each subcellular compartment as well as systemically. With their continual requirement for NAD(H), mitochondrial NAD metabolism has become increasingly important. Despite many novel findings summarized in this article, current knowledge leaves several pressing questions unanswered (Box 2). First, further investigation is necessary to identify the source of the mitochondrial NAD pool. Namely, what transports NMN to the mitochondria and what else can fuel the mitochondrial pool of NAD in each tissue/organ? Second, we need to elucidate whether NMN and/or NR indeed function as an extracellular exchange currency in mammals. If so, what regulates the pharmacokinetics of these NAD intermediates? Third, the molecular mechanisms that alter NAMPT-mediated NAD biosynthesis in response to energy intake and aging should be determined. And finally, we need to determine if augmentation of NAD levels with key NAD intermediates, namely NMN and NR, can preserve mitochondrial function throughout life. Answers to these pressing questions will open new avenues to better understand the dynamic, multi-layered regulation of NAD metabolism in mitochondria and to develop effective interventions to preserve these tiny powerhouses over time.

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