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REVIEW ARTICLE

The human NAD metabolome: Functions, metabolism and compartmentalization

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Abstract

The metabolism of NAD has emerged as a key regulator of cellular and organismal homeostasis. Being a major component of both bioenergetic and signaling pathways, the molecule is ideally suited to regulate metabolism and major cellular events. In humans, NAD is synthesized from vitamin B3 precursors, most prominently from nicotinamide, which is the degradation product of all NAD-dependent signaling reactions. The scope of NAD-mediated regulatory processes is wide including enzyme regulation, control of gene expression and health span, DNA repair, cell cycle regulation and calcium signaling. In these processes, nicotinamide is cleaved from NAD^+ and the remaining ADP-ribosyl moiety used to modify proteins (deacetylation by sirtuins or ADP-ribosylation) or to generate calcium-mobilizing agents such as cyclic ADP-ribose. This review will also emphasize the role of the intermediates in the NAD metabolome, their intra- and extra-cellular conversions and potential contributions to subcellular compartmentalization of NAD pools.

Keywords

ADP-ribosylation, calcium signaling, extracellular NAD degradation, NAD biosynthesis, protein deacetylation, subcellular NAD pools

History

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Introduction

NAD is an essential redox carrier, whereas its degradation is a key element of a wide range of signaling pathways (Belenky *et al*., 2007a; Berger *et al*., 2004; Houtkooper *et al*., 2010; Magni *et al*., 2008). The molecule is used for protein modifications (deacetylation, ADP-ribosylation) and as precursor of messenger molecules. It is becoming increasingly clear that NAD-mediated signaling processes are ubiquitously present throughout all the organisms and that they are involved not just in the metabolic adjustment to environmental changes, but rather participate in the control of fundamental cellular events. In addition, there are also cell- and tissue-specific processes which are subject to NAD-mediated regulation. For example, insulin secretion is regulated by four different mechanisms of NAD-dependent signaling (Antonelli & Ferrannini, 2004; Haigis *et al*., 2006; Takasawa & Okamoto, 2002). Over the last years, NAD-dependent processes have emerged as highly promising targets for therapeutic applications to treat diseases such as cancer, diabetes, obesity and others. It is also noteworthy that supplementation of NAD precursors has a variety of beneficial effects, in particular, to improve cellular bioenergetics and metabolism.

The realization that, unlike redox reactions, signaling causes permanent NAD degradation prompted in-depth analyses of the biosynthetic pathways. They, too, constitute a complex array of conversions with emerging regulatory roles and relationships to the signaling processes, besides feeding energetic needs. There is no doubt that the studies of NAD-mediated signaling processes and the molecular understanding of NAD biosynthesis have moved far beyond the stage of being an ''emerging'' field. However, while the importance of NAD-dependent signaling pathways has been impressively demonstrated, even at the organismal level, some key issues have still remained elusive. To address these open questions, it appears important to understand the interconversions of intermediates of NAD biosynthesis and signaling, how these intermediates are provided to different subcellular compartments, and how they contribute to the maintenance of individual NAD pools. Rather surprisingly, it has also remained largely unclear how extracellular NAD and its derivatives can be utilized to serve as intracellular NAD precursors. This article is intended to provide an overview of the current knowledge regarding NAD biosynthesis and NAD-dependent signaling pathways,

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topics that have been covered in much detail in several recent reviews (see below). Here, we have particularly focused on the intra- and extra-cellular conversions and the subcellular distribution of NAD and its precursors. As will be discussed, these aspects are important to understand the physiology of NAD metabolism and signaling and therefore need to be explored in more detail.

NAD biosynthesis in human cells

Maintenance of intracellular NAD pools is not only important to fuel redox metabolism, but also to support NAD-dependent signaling pathways (Figure 1). Since NAD-dependent signaling is accompanied by the degradation of NAD^+, cells need to permanently replenish these pools. Indeed, blocking NAD biosynthesis, for example, by inhibiting the transformation of precursors, leads to a marked decrease of the intracellular NAD content within a few hours (Pittelli *et al*., 2010).

NAD is synthesized from extracellular precursors that are provided by the diet and need to enter cells (Figure 1). These precursors encompass the bases (nicotinamide [Nam] and nicotinic acid [NA]) and nucleosides (nicotinamide riboside [NR] and nicotinic acid riboside [NAR]) now collectively referred to as vitamin B3 (Bogan & Brenner, 2008; Magni *et al*., 2008). Moreover, tryptophan degradation via the kynurenine pathway leads to the generation of quinolinic acid (QA), which is converted to NA mononucleotide (NAMN), an intermediate of NAD metabolism. The uptake of NAD precursors into cells is so far poorly understood. Transporters for tryptophan (SLC7A5 and SLC36A4) and NA (SLC5A8 and SLC22A13) have been identified (Bahn *et al*., 2008; Gopal *et al*., 2007; Kanai *et al*., 1998; Pillai & Meredith, 2011), whereas the uptake of NR and NAR through equilibrative nucleoside transporters (ENTs) has been suggested based on pharmacological sensitivity (Nikiforov *et al*., 2011) (Figure 1).

In general, when considering different pathways of NAD biosynthesis, the major aspect is the generation of the mononucleotide (NMN or NAMN), because formation of the dinucleotide is common to all known routes (Figure 1). Moreover, dinucleotide formation consists of the adenylation of the pyridine mononucleotide. The adenylyl group originates from ATP, a molecule that is abundantly available. It has now been established that formation of pyridine mononucleotides follows the same principal scheme as found for others such as purine and pyrimidine mononucleotides. That is, bases can be converted directly to the mononucleotides using phosphoribosyltransferases (PRTs). These enzymes catalyze the reaction between the base and phosphoribosylpyrophosphate (PRPP), resulting in the formation of the 5′-mononucleotide and the release of pyrophosphate (PPi; Figure 1).

Thus, the reaction using NA as base is catalyzed by NAPRT yielding NAMN. This pathway including the subsequent dinucleotide formation and amidation of the nicotinic acid moiety (Figure 1) is called the Preiss–Handler pathway in honor of its discoverers (Preiss & Handler, 1958a,b). NAMN is also the product of the reaction mediated by QAPRT. A specific aspect of this reaction is the simultaneous decarboxylation at the 2′ position of QA (pyridine-2,3-dicarboxylic acid). QAPRT activity could be important, especially in the brain, to remove QA, which is highly neurotoxic (Guillemin, 2012). NAD biosynthesis from tryptophan via QA has been termed *de novo* pathway, because it includes the formation of the pyridine ring.

Nam is converted to NMN by NamPRT. This enzyme is also widely known as NAMPT, however, we will use NamPRT in this article, in keeping with the general denomination of phosphoribosyltransferases as PRTs. NamPRT has been intensely studied over the last years, because it appears to be crucial for human NAD metabolism, but also, because the enzyme may have roles not only within cells, but may also be secreted by some cell types such as adipocytes (Fukuhara *et al*., 2005), refer the ''Extracellular NAD metabolites and their degradation'' section. The critical importance of NamPRT to maintain mammalian NAD homeostasis has been established by the finding that treatment of mice with a pharmacological inhibitor of the enzyme is lethal (Watson *et al*., 2009). Most likely, the essentiality of Nam as NAD precursor is related to the fact that all NAD-dependent signaling pathways include NAD cleavage accompanied by the liberation of the Nam moiety (Figure 1). Consequently, NamPRT is critical to reutilize Nam for NAD biosynthesis. Therefore, the NAD biosynthetic route using Nam as precursor is also referred to as salvage pathway. In fact, considering the estimated daily NAD turnover, at least several grams of NAD precursors per day would be required to maintain organismal NAD levels (Chiarugi *et al*., 2012). However, the daily recommended allowance (RDA) for vitamin B3 is only about 15 mg [Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, 1998]. These considerations indicate that Nam, liberated during NAD-dependent signaling, is nearly completely reutilized for the regeneration of NAD pools. This supposition is also in line with the unusually high affinity of NamPRT towards Nam ($K_M \sim 5$ nM, in the presence of ATP; Burgos & Schramm, 2008) which indicates that even minute amounts of Nam will be detected and converted to NMN.

Similar to the synthesis of other nucleotides, pyridine mononucleotides can also be generated by phosphorylation of the corresponding nucleosides (or ribosides) NR and NAR (Figure 1). While NR had long been known as a metabolic intermediate, only a decade ago its integral role in NAD metabolism was established by the identification of nicotinamide riboside kinases (NRKs) in yeast and humans (Bieganowski & Brenner, 2004). When cells are unable to metabolize NA or Nam, their viability and NAD pools can be fully maintained by supplementation with NR (Grozio *et al*., 2013; Nikiforov *et al*., 2011). In humans, NR is converted to NMN by NRK1 and 2 (Bieganowski & Brenner, 2004; Tempel *et al*., 2007). In recent studies, a variety of health benefits has been reported that were the result of NR treatment. For example, in a mouse model of Alzheimer's disease, NR treatment significantly increased the NAD level in the cerebral cortex and improved cognitive function (Gong *et al*., 2013). Moreover, NR protected from noise-induced hearing loss and spiral ganglia neurite degeneration in mice (Brown *et al*., 2014). The nucleoside also prevented weight gain in mice challenged with a high-fat diet (Canto *et al*., 2012). Similarly, dietary NR supplementation effectively

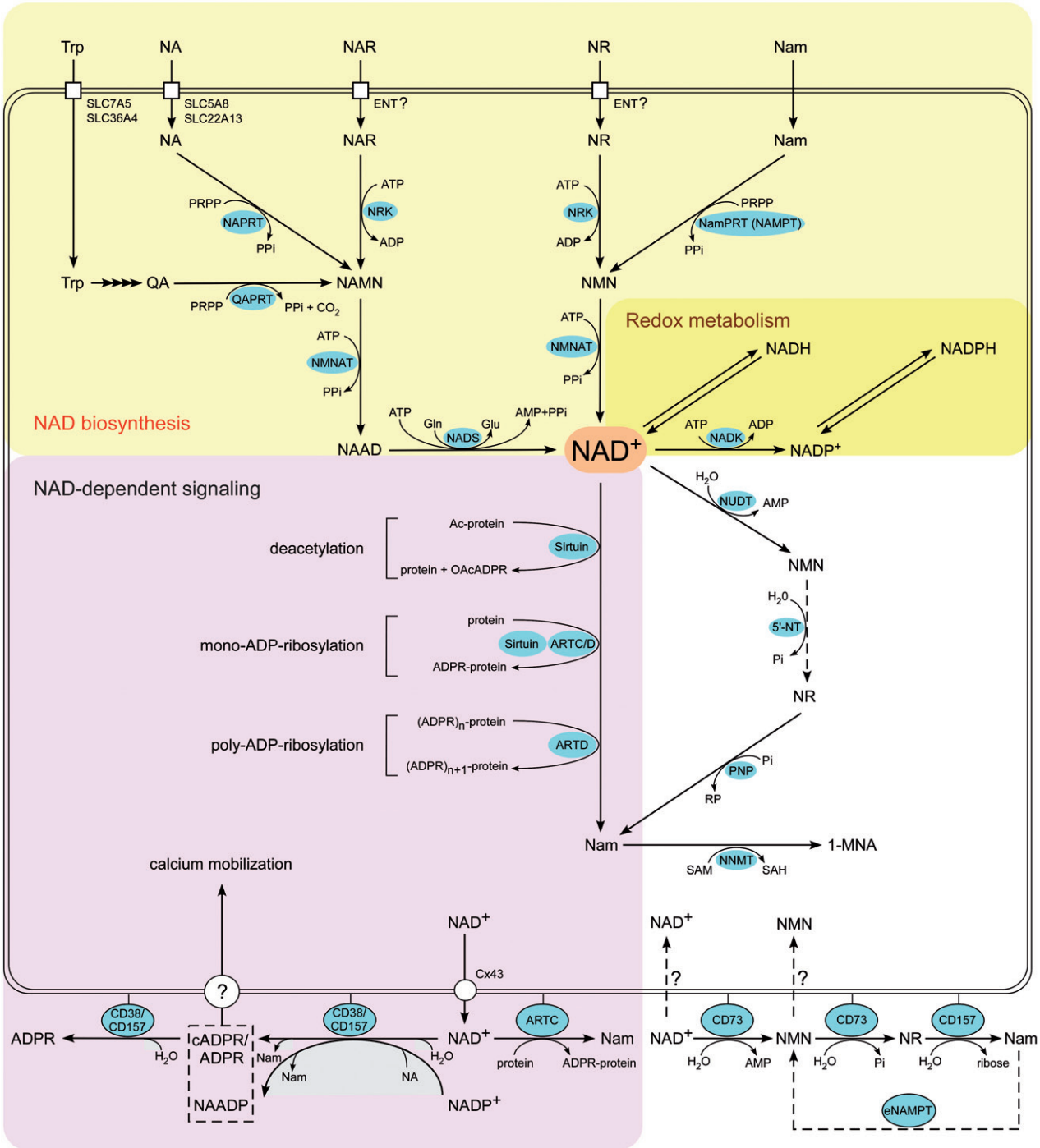

Figure 1. Overview of the NAD metabolome in humans. NAD can be synthesized from five precursors: tryptophan (Trp), the pyridine bases nicotinamide (Nam) and nicotinic acid (NA) or the nucleosides Nam riboside (NR) and NA riboside (NAR), which enter cells by different transport mechanisms. Quinolinic acid (QA), a Trp degradation product, is transformed to NA mononucleotide (NAMN) by quinolinic acid phosphoribosyltransferase (QAPRT). Nam and NA are converted to the corresponding mononucleotides (NMN and NAMN) by nicotinamide phosphoribosyltransferase (NamPRT, also known as NAMPT) and nicotinic acid phosphoribosyltransferase (NAPRT), respectively. NMN might also be synthesized by an extracellular NamPRT form (eNAMPT). NMN and NAMN are also generated through phosphorylation of NR and NAR, respectively, by nicotinamide riboside kinases (NRK). NAMN and NMN are converted to the corresponding dinucleotide (NAAD or NAD^+) by NMN adenylyltransferases (NMNAT). NAD synthetase (NADS) amidates NAAD to NAD^+. Phosphorylation by NAD kinase (NADK) converts NAD^+ to $NADP^+$. The oxidized and reduced forms of the dinucleotides, $NAD(P)^+$ and NAD(P)H, serve as reversible hydrogen carriers in redox reactions. Members of the Sirtuin family of protein deacetylases catalyze the transfer of the protein-bound acetyl group onto the ADP-ribose moiety, thereby forming O-acetyl-ADP ribose (OAcADPR). The transfer of a single (mono-ADP-ribosylation) or several (poly-ADP-ribosylation) ADP-ribose units from NAD^+ to acceptor protein is catalyzed by diphtheria toxin-like ADP-ribosyltransferases (ARTD). Mono-ADP-ribosylation is also catalyzed by clostridial toxin-like ADP-ribosyltransferases (ARTC) and some Sirtuin proteins. NAD^+ and $NADP^+$ are also used for the synthesis of second messengers, nicotinic acid adenine dinucleotide phosphate (NAADP), cyclic ADP-ribose (cADPR) and ADPR, which mediate intracellular calcium mobilization. All the three molecules are synthesized by ecto-NAD glycohydrolases CD38 and CD157. The mechanism of how messengers reach their cytosolic targets is still debated. Signaling-independent interconversions of NAD and its intermediates include NAD hydrolysis to NMN and AMP by Nudix pyrophosphatases (NUDT); NMN dephosphorylation to NR by cytosolic 5′-nucleotidases (5′-NT); phosphorolytic cleavage of NR to Nam by purine nucleoside phosphorylase (PNP); and conversion of Nam to N-methylnicotinamide (1-MNA) by nicotinamide-*N*-methyltransferase (NNMT). NAD^+ can possibly be released from cells through connexin 43 hemichannels (Cx43), and can be degraded to NR by ecto-nucleotidase CD73. NR is hydrolyzed to Nam by CD157. Whether cells can take up NAD or NMN is debated. (see colour version of this figure at www.informahealthcare.com/bmg).

delayed the progression of early and late stage mitochondrial myopathy, caused increased mitochondrial biogenesis and improved insulin sensitivity (Khan *et al*., 2014). The beneficial action of NR on mitochondrial biology was further highlighted in a mouse model of mitochondrial disease characterized by impaired cytochrome c oxidase biogenesis. Supplementation with NR led to marked improvement of the respiratory chain defect and exercise intolerance (Cerutti *et al*., 2014).

NAD biosynthesis from all known precursors merges at the step of dinucleotide formation (Figure 1). The reaction between the pyridine mononucleotide and ATP is carried out by NMN adenylyltransferases (NMNATs) resulting in the generation of NAD from NMN or NAAD (NA adenine dinucleotide) from NAMN. Interestingly, analyses of the subcellular location of enzymes involved in human NAD biosynthesis revealed that they all reside in the cytosol or nucleus, except for one of the three NMNAT isoforms, namely, NMNAT3 (Nikiforov *et al*., 2011). NMNAT3 has been localized to the mitochondrial matrix and thereby represents the only NAD biosynthetic enzyme that is separated from the nucleo-cytosolic pool of intermediates. This finding suggested that mammalian mitochondria may have an autonomous route of NAD generation which, by deduction, would depend on the import of NMN from the cytosol. However, a mitochondrial carrier that mediates NMN import into the organelles has not been identified (Figure 2). Interestingly, besides being associated with the Golgi complex, NMNAT2 is associated with axonal vesicles and appears to have a critical role in the maintenance of axonal integrity (Conforti *et al*., 2014).

To form NAD, amidation of the nicotinic acid moiety of NAAD is carried out by NAD synthetase, which uses glutamine as donor of the NH_2 group (Figure 1). NAD synthetase has also been localized to the cytosol suggesting that mitochondria can only use NMN as precursor to generate NAD. Indeed, it has been found that the mitochondrial NAD pool is rather sensitive to alterations of NamPRT activity (Nikiforov *et al*., 2011; Yang *et al*., 2007), the enzyme that directly generates NMN from Nam.

Research over the past 10–15 years has thus established that there are several routes which enable the use of alternative precursors to maintain intracellular NAD pools. Whether this redundancy is vital or whether it has, for example, tissue-specific significance still needs to be explored. Most of the enzymes involved have been scrutinized and important structural as well as regulatory properties have emerged from these studies (for more detailed analyses and comparisons, see, for example, these recent reviews (Dölle *et al*., 2013b; Houtkooper *et al*., 2010; Magni *et al*., 2008, 2009). However, as indicated above, there are still open questions that need further research to be answered.

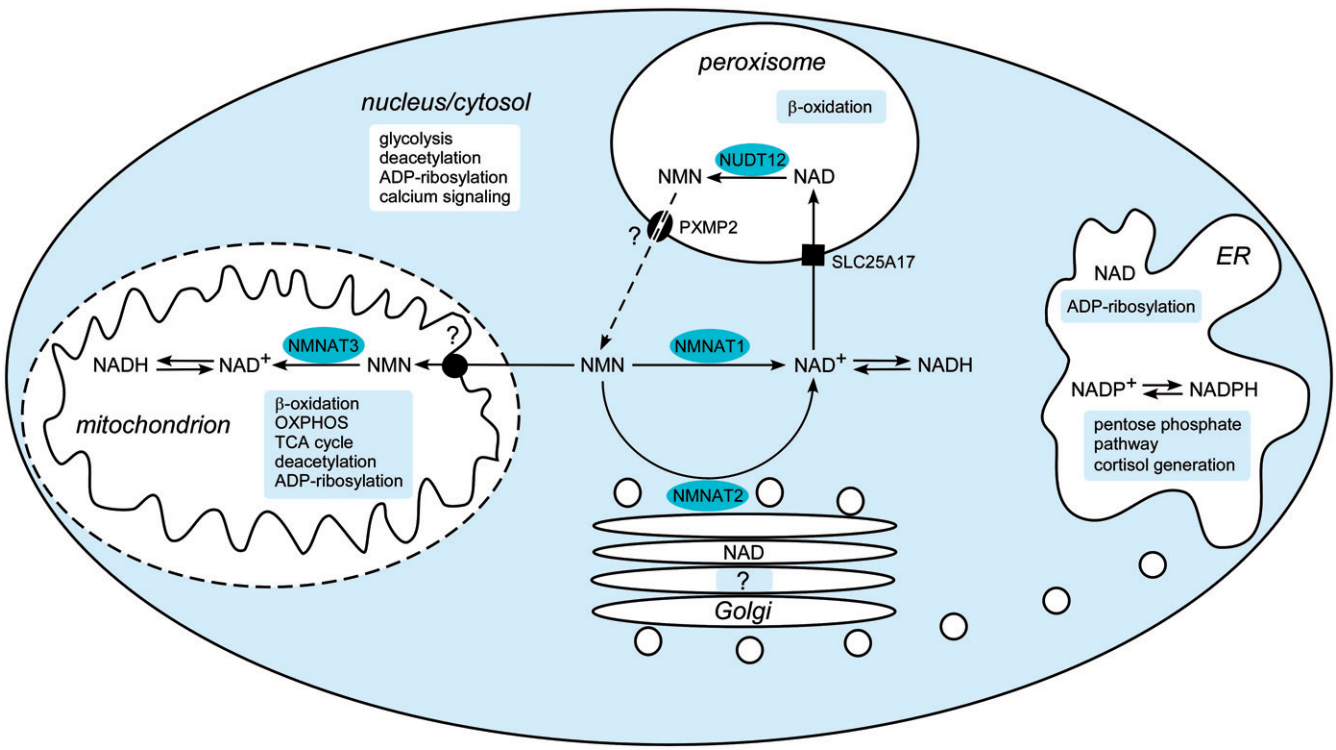

Figure 2. Subcellular compartmentalization of NAD pools. An outline of four subcellular NAD pools (nuclear/cytosolic, mitochondrial, peroxisomal and ER/Golgi) is shown. NAD(P)-dependent processes in each compartment are indicated in frames. Cytosolic and nuclear pools of NAD are combined based on the assumption that NAD can freely exchange between these two compartments. Nuclear/cytosolic and mitochondrial pools are maintained by NAD biosynthesis in these compartments. The three NMNAT isoforms catalyzing the generation of NAD from NMN are localized within the nucleus (NMNAT1), at the surface of the Golgi apparatus (NMNAT2) and within the mitochondria (NMNAT3). It is suggested that NMN is imported from the cytosol into the mitochondrial matrix, but the mechanism of this transport is unclear. The peroxisomal NAD pool can be maintained by import of the dinucleotide from the cytosol through the carrier protein SLC25A17. Peroxisomal NAD can be cleaved to AMP and NMN by Nudix hydrolase NUDT12. It is assumed that the generated NMN is released from the organelle to the cytosol through the PXMP2 channel. Although NAD(P)-dependent processes in ER have been identified, the mechanism of NAD supply to this organelle remains unknown. Moreover, a possible role of NAD in the Golgi lumen is obscure. (see colour version of this figure at www.informahealthcare.com/bmg).

NAD-dependent signaling

During the past two decades, the understanding of the biological roles of NAD has considerably changed. It is now beyond doubt that this dinucleotide is important not only for the cellular energy metabolism, but also represents a key molecule of cellular signaling being substrate for various regulatory proteins (Berger *et al*., 2004; Houtkooper *et al*., 2010). NAD-dependent post-translational protein modifications have decisive impact on vital processes including gene expression, cell cycle progression, insulin secretion, DNA repair, apoptosis, aging and many others (Haigis & Sinclair, 2010; Hassa & Hottiger, 2008; Koch-Nolte *et al*., 2008). Moreover, NAD is the biosynthetic precursor of metabolites that mediate intracellular calcium mobilization (Fliegert *et al*., 2007). As a result of all signaling conversions of NAD, the molecule is cleaved into nicotinamide and an ADP-ribose derivative (Figure 1). Therefore, NAD-dependent signaling processes are also referred to as ADP-ribosyl transfer reactions. Eventually, all ADP-ribosyl derivatives are converted to ADP-ribose, which, in turn, can be further degraded, for example, to AMP and ribose-5-phosphate (Formentini *et al*., 2009a).

NAD-dependent deacetylation

NAD-dependent deacetylation of various protein substrates is performed by members of the highly conserved family of silent information regulator-2 (Sir2) like proteins (Sirtuins). Sirtuins have been found in all living organisms from bacteria to man (Brachmann *et al*., 1995).

In the course of the deacetylation reaction, nicotinamide is cleaved off and the protein-bound acetyl group is transferred onto the ADP-ribose moiety, thereby forming O-acetyl-ADP ribose (OAADPR) (Jackson & Denu, 2002; Tanner *et al*., 2000).

In humans, seven members of the Sirtuin family have been characterized (SIRT1-7). SIRT1, 6 and 7 are located in the nucleus, SIRT2 has been found both in the nucleus and cytosol, while SIRT3, 4 and 5 are mitochondrial proteins (Michishita *et al*., 2005), even though for SIRT3 nuclear functions have also been proposed (Iwahara *et al*., 2012; Scher *et al*., 2007). Besides a deacetylase activity, some Sirtuins also display other catalytic activities. It was shown that SIRT6 exhibits both deacetylase and ADP-ribosyltransferase activities (Mao *et al*., 2011; Michishita *et al*., 2008), SIRT4 was found to have only ADP-ribosyltransferase activity (Haigis *et al*., 2006), whereas SIRT5 may act as a lysine demalonylase and desuccinylase (Du *et al*., 2011).

Originally, Sirtuins were described as NAD-dependent histone deacetylases which mediate transcriptional silencing and longevity (Imai *et al*., 2000; Landry *et al*., 2000; Smith *et al*., 2000). Later, it was demonstrated that, besides histones, Sirtuins deacetylate a broad spectrum of protein substrates including regulatory, structural and catalytically active proteins (Martinez-Redondo & Vaquero, 2013). NAD-dependent deacetylation results in the modulation of target protein activity, stability or localization thereby regulating aging, transcription, cell cycle progression, genome stability, apoptosis, mitochondrial biogenesis and many other vital processes (Haigis & Sinclair, 2010). It is important to emphasize the critical role of Sirtuins in the regulation of metabolism. Since these enzymes use NAD^+ as a co-substrate, their activity is dependent on the bioenergetic state of the cell (which is, in part, reflected by the NAD^+/NADH ratio and the overall NAD concentration). That is, Sirtuins directly link the metabolic state with signaling processes and thereby also regulate the cellular response to environmental signals (Chang & Guarente, 2014; Houtkooper *et al*., 2012). The biological role of OAADPR, a product of the Sirtuin reactions, is still unclear. However, evidence has been presented suggesting that this molecule could be a ligand of calcium channels in the plasma membrane, such as TRPM2 channels (Grubisha *et al*., 2006). This possibility would establish a mechanistic link between protein acetylation state and calcium signaling. OAADPR has been shown to be cleaved to ADPR by ADP-ribosyl-acceptor hydrolase 3 (ARH3) (Kasamatsu *et al*., 2011; Ono *et al*., 2006), an enzyme that is also involved in poly-ADP-ribose metabolism.

ADP-ribosylation

Another mechanism of NAD-dependent protein modification is ADP-ribosylation. This reaction mediates the transfer of a single (mono-ADP-ribosylation) or several (poly-ADP-ribosylation) ADP-ribose units from NAD^+ to specific amino acids of acceptor proteins. As a result of poly-ADP-ribosylation, polymers of ADP-ribose are attached to acceptor proteins. These polymers can be branched and consist of up to 200 ADP-ribose units (Bürkle, 2005). ADP-ribosylation is catalyzed by two families of enzymes: clostridial toxin-like ADP-ribosyltransferases (ARTC) and diphtheria toxin-like ADP-ribosyltransferases (ARTD) (Hottiger *et al*., 2010) (Figure 1). So far, five mammalian members of the ARTC family have been described. They are ectoenzymes which catalyze mono-ADP-ribosylation of secreted and membrane proteins, as well as cell surface receptors, and thus can regulate innate immunity and cell–cell and cell–matrix interactions (Koch-Nolte *et al*., 2008; Seman *et al*., 2004). Members of the ARTD family (currently including 18 mammalian proteins) catalyze mono- or poly-ADP-ribosylation inside the cell (Hottiger *et al*., 2010). In addition to the ARTDs and the ARTCs, Sirtuins SIRT4 and SIRT6 are able to mono-ADP-ribosylate proteins (Haigis *et al*., 2006; Mao *et al*., 2011).

Intracellular mono- and poly-ADP-ribosylation has been shown to be involved in many essential cellular processes including: DNA repair, apoptosis, transcription, cell cycle progression, energy metabolism and many others. For many of these functions, the molecular mechanisms have been explored. These functions have been reviewed in detail in several excellent recent articles (Bürkle & Virag, 2013; Feijs *et al*., 2013; Grimaldi *et al*., 2015; Hassa & Hottiger, 2008).

Since ADP-ribosylation participates in the regulation of important cellular processes, its extent needs to be tightly controlled. Indeed, as other post-translational modifications, both mono- and poly-ADP-ribosylation are reversible. It was shown that ADP-ribosyl-acceptor hydrolase 1 (ARH1) hydrolyses the *N*-glycosidic bond linking ADP-ribose to an arginine residue of the modified protein (Moss *et al*., 1992). On the other hand, poly-ADP-ribose glycohydrolase (PARG) and

ADP-ribosyl-acceptor hydrolase 3 (ARH3) cleave the O-glycosidic bond of poly-ADP-ribose chains (Lin *et al*., 1997; Mashimo *et al*., 2013; Mueller-Dieckmann *et al*., 2006; Niere *et al*., 2012; Oka *et al*., 2006; Slade *et al*., 2011), while the remaining ADP-ribose, linked to glutamate residues, is removed by specialized enzymes – MacroD1, MacroD2 and terminal ADP-ribose protein glycohydrolase 1 (TARG1) (Jankevicius *et al*., 2013; Rosenthal *et al*., 2013; Sharifi *et al*., 2013). These processes lead to release of ADP-ribose and the regeneration of the unmodified protein.

Calcium mobilization

$NAD(P)^+$-derived molecules are now also well established as key elements of intracellular calcium signaling. Several of these derivatives, namely, nicotinic acid adenine dinucleotide phosphate (NAADP), cyclic ADP-ribose (cADPR) and ADPR have been characterized as potent calcium-mobilizing agents (Gasser *et al*., 2006; Lee *et al*., 1989; Lee & Aarhus, 1995) (Figure 1). The calcium mobilization systems activated by NAD(P) derivatives have been broadly addressed. Both cADPR and NAADP stimulate calcium release from endogenous stores. While cADPR activates ryanodine receptors, thereby stimulating calcium release from the endoplasmic reticulum, NAADP activates two-pore channels which have been found in endolysosomes (Galione, 2014; Guse, 2014; Lee, 2012; Patel *et al*., 2010). On the other hand, ADPR can trigger calcium entry from the extracellular space via activation of plasma membrane cation channel TRPM2 (Guse, 2014; Sumoza-Toledo & Penner, 2011). All three molecules, ADPR, cADPR and NAADP are synthesized by multifunctional NAD glycohydrolases, in humans represented by the ecto-ADP-ribosyl cyclase CD38 and its structural and functional homolog, CD157 (Malavasi *et al*., 2008) (Figure 1). Besides the synthesis, these proteins also catalyze the hydrolysis of cADPR to ADPR. NAADP is generated by exchanging nicotinamide for nicotinic acid in $NADP^+$. Since the catalytic activity of CD157 is by far less compared to that of CD38 (Malavasi *et al*., 2008), the biological role of CD157 is not well understood.

No other (intracellular) mammalian NAD glycohydrolases have been identified so far. Moreover, many of the intracellular calcium signaling events mediated by NAD derivatives have been ascribed to CD38 activity. Given that CD38 is an ectoenzyme, it has remained difficult to explain these intracellular effects. This has been referred to as the topological paradox of CD38. That is, CD38 being a type II transmembrane protein with its active site facing the extracellular space, seems to produce messengers that are used for intracellular calcium signaling. It is also not entirely clear, how the presence of its substrates, NAD^+ and $NADP^+$, in the extracellular space is mediated and regulated. A variety of studies have been conducted to address these issues. De Flora and co-workers showed that NAD can be released from various types of cells through connexin 43 (Cx43) hemi-channels (Bruzzone *et al*., 2001; Fruscione *et al*., 2011; Verderio *et al*., 2001). It was also observed that one of the second messengers, cADPR, can enter the cell via nucleoside transporters (Podesta *et al*., 2005) or by channeling across oligomeric CD38 (Franco *et al*., 1998). Further observations led to the suggestion that Cx43 has a dual function exporting NAD and importing cADPR into the cell (Song *et al*., 2011).

Despite these efforts, a conclusive mechanism explaining the topological paradox of CD38 is still lacking. It was proposed that intracellular vesicles containing CD38 could arise from endocytosis. If NAD or NADP were present or transported into these vesicles, the calcium messengers could be released upon stimulation (Zocchi *et al*., 1999). Such a mechanism was demonstrated for cADPR-dependent calcium mobilization in sea urchin eggs (Davis *et al*., 2008). Recently, an interesting alternative explanation was presented. It was shown that CD38 could be expressed as type III transmembrane protein. The resultant protein would be fully active and could therefore produce cADPR in the cytosol (Zhao *et al*., 2012, 2014). Nevertheless, the mechanism how these metabolites are produced and accessible within the cytoplasm still remains to be established.

Recycling of NAD degradation products

Nam and ADPR are the common products generated as a result of NAD-dependent signaling conversions. In human cells, ADPR can be cleaved by several members of the Nudix hydrolase family (McLennan, 2006). Among these enzymes, NUDT9 appears to have the highest specificity towards ADPR. It was therefore suggested that NUDT9 may be responsible for ADP-ribose elimination by its splitting to AMP and ribose 5-phosphate (Lin *et al*., 2002; Perraud *et al*., 2001, 2003). The reutilization of the degradation products arising from NAD-dependent signaling would require three molecules of ATP to regenerate NAD. Therefore, NAD-dependent signaling events, especially poly-ADP-ribosylation, are energetically rather expensive adding further weight to their functional roles.

Taken together, NAD-dependent signaling pathways comprise a complex network of regulatory mechanisms. Different pathways ''compete'' for the same substrate, NAD^+, whose intracellular concentration is tightly regulated. Therefore, even though NAD availability is an important factor in the coordination of NAD-dependent signaling, the interrelationships between different pathways are likely governed by additional mechanisms.

Intracellular degradation and interconversion of NAD and its metabolites

Degradation of NAD to NMN

In prokaryotes and lower eukaryotes, the intracellular NAD concentration is regulated through both biosynthesis and enzymatic cleavage to NMN and AMP by pyrophosphatases of the Nudix family. Nudix (nucleoside diphosphate linked to moiety X) hydrolases or pyrophosphatases are found in all the types of organisms, from archaea to mammals. The substrates of these enzymes (NDP-X) comprise molecules consisting of a nucleoside diphosphate (NDP) linked to various other moieties (X). Well-characterized substrates include (d)NTPs, dinucleoside polyphosphates (Np_nN), NAD and capped RNAs. When the pyrophosphate bond is cleaved, the

corresponding mononucleotide (NMP) and the phosphorylated compound X (P-X) are generated. Nudix pyrophosphatases control the levels of metabolites and signaling molecules and cleave potentially mutagenic, oxidized nucleotides (McLennan, 2006).

The bacterial protein YjaD was the first Nudix pyrophosphatase shown to cleave NAD (Frick & Bessman, 1995; McLennan, 2006). Recently, a fungal pyrophosphatase, NdxA, was identified. This protein controls the intracellular NAD^+ and NADH levels in *Aspergillus nidulans*, thereby regulating important NAD-dependent processes such as gene expression and glycolysis. Due to its ability to cleave NAD, NdxA negatively regulates the NAD-dependent protein deacetylase SirA, which requires NAD^+ as a co-substrate for histone H4 deacetylation. In turn, the downregulation of SirA leads to diminished expression of several genes (Shimizu *et al*., 2012). NAD degradation by NdxA also has an impact on the utilization of pyruvate and intermediates of the tricarboxylic acid cycle (Shimizu & Takaya, 2013).

In the human genome, 24 genes have been detected that encode Nudix hydrolases with different substrate specificity. The mammalian NUDT12 protein exhibits the highest similarity to the bacterial YjaD and the fungal NdxA enzymes. Moreover, NUDT12 contains the consensus sequence SQPWPFPxS, found in the conserved Nudix domain, which is present in all NAD pyrophosphatases identified until now (Abdelraheim *et al*., 2003; McLennan, 2006). However, so far, little is known about the substrate specificity and functions of NUDT12. It was shown that NUDT12 efficiently cleaves NADH, NADPH and NAD^+ *in vitro*. Moreover, the PNL sequence at the C-terminus mediates its peroxisomal localization (Figure 2), (Abdelraheim *et al*., 2003). NUDT12 could therefore regulate the levels of NAD and NADP in these organelles.

Degradation of NMN to NR

In yeast cells, the mononucleotides NMN and NAMN can be dephosphorylated to NR and NAR by the cytosolic 5′-nucleotidases Isn1 and Sdt1 and the vacuolar phosphatase Pho8 (Bogan *et al*., 2009; Lu & Lin, 2011). In general, 5′-nucleotidases dephosphorylate both purine and pyrimidine ribo- and deoxyribo-mononucleotides to their corresponding nucleosides, thereby controlling major nucleotide metabolic pathways (Hunsucker *et al*., 2005; Ipata & Balestri, 2013). These enzymes are characterized by rather broad substrate specificity. Indeed, before their identification as NMN and NAMN cleaving enzymes, Isn1 and Sdt1 were characterized as IMP- and pyrimidine mononucleotide-specific 5′-nucleotidases, respectively (Itoh *et al*., 2003; Nakanishi & Sekimizu, 2002).

In humans, three cytosolic 5′-nucleotidases have been described, CN-IA, CN-II and CN-III (Hunsucker *et al*., 2005). They exhibit different substrate specificities. It has been proposed that these enzymes might mediate the generation of NR or NAR in human cells using NMN or NAMN, respectively, as substrate (Bogan & Brenner, 2010; Magni *et al*., 2004). However, experimental evidence for such a possibility is lacking.

Degradation of NR to Nam

Besides the utilization by nicotinamide riboside kinase 1 (Nrk1) to form NMN, in yeast cells NR can also be cleaved to Nam (which then can enter an alternative NAD-biosynthetic route). Three enzymes were identified (Urh1, Pnp1 and Meu1) which catalyze this cleavage, however, using two different mechanisms. Urh1 is a nucleoside hydrolase leading to the formation of Nam and ribose, whereas both Pnp1 and Meu1 are nucleoside phosphorylases generating Nam and ribose-1-phosphate from NR (Belenky *et al*., 2007b, 2009; Ma *et al*., 2007).

In humans, the phosphorolytic cleavage of NR to Nam was shown more than half a century ago (Grossman & Kaplan, 1958; Rowen & Kornberg, 1951). However, the mammalian enzyme capable of catalyzing this conversion was only recently identified as purine nucleoside phosphorylase (PNP) (Wielgus-Kutrowska *et al*., 1997) (Figure 1). PNP has been known to catalyze the reversible phosphorolytic cleavage of purine oxy- and deoxynucleosides (Moriwaki *et al*., 1999). Even though the biochemical *in vitro* characterization of bovine PNP revealed that NR is far less preferred as substrate than, for example, inosine (Belenky *et al*., 2009; Wielgus-Kutrowska *et al*., 1997), its heterologous expression in yeast demonstrated its capacity to substitute the yeast homolog Pnp1 to mediate NAD synthesis from Nam generated by NR cleavage (Belenky *et al*., 2009).

The functional relevance of NR degradation to Nam still remains to be established. It is still unclear whether this conversion represents a mechanism necessary as alternative for NR utilization by NRKs or whether it has other, for example, regulatory functions. The reversibility of the phosphorolytic NR cleavage is another, quite intriguing aspect. Given the presence of ribose-1-phosphate in human cells, Nam could be converted to NR to enter NAD biosynthesis. However, a major contribution of such a pathway appears rather unlikely, because inhibition of NamPRT, which catalyzes NMN formation from Nam, causes death in a large variety of cell types.

Conversion of Nam to 1-MNA

The cytosolic enzyme nicotinamide-N-methyltransferase (NNMT) catalyzes the formation of N-methylnicotinamide (1-MNA) using S-adenosylmethionine (SAM) as methyl donor (Figure 1); (Aksoy *et al*., 1994; Cantoni, 1951). The involvement of SAM, the universal methyl group donor, which is also used for the methylation of proteins and nucleic acids (Jia *et al*., 2011; Lu, 2000; Luo, 2012; Ulrey *et al*., 2005) represents an intriguing link between gene regulation and epigenetics and NAD metabolism. For many years, this conversion was regarded as a catabolic pathway merely mediating the excretion of excess Nam. This notion has dramatically changed over the past few years. Elevation of NNMT expression has been observed in neurodegenerative diseases (Williams *et al*., 2005), obesity and diabetes type II (Kraus *et al*., 2014; Lee *et al*., 2005; Yaguchi *et al*., 2005). Moreover, NNMT is strongly increased in a variety of tumors and has been proposed to support tumorigenesis by promoting migration, invasion and proliferation of cancer

cells (Roessler *et al*., 2005; Sartini *et al*., 2013; Tang *et al*., 2011; Ulanovskaya *et al*., 2013; Wu *et al*., 2008).

Exploration of the mechanisms leading to these phenomena has led to several ground-breaking discoveries. It was shown that increased expression of NNMT lowers the cellular concentration of SAM (the universal methyl group donor) thereby causing a deficiency of methyl groups in other pathways. In turn, a decrease of specific histone methylation was demonstrated leading to a shift in the epigenetic state. It was proposed that the conversion of Nam to 1-MNA could thereby act as a stable sink to store methyl groups in cancer cells (Ulanovskaya *et al*., 2013).

In another study, it was found that the NNMT reaction does not only regulate the level of SAM in adipose tissue, but also has an impact on the cellular NAD concentration. In obese mice with diabetes type II, NNMT expression was increased in both white adipose tissue and the liver. Consequently, both SAM and NAD levels were diminished, leading to hypomethylation of histones, lowered polyamine synthesis and suppression of SIRT1-dependent signaling. On the other hand, the authors demonstrated that knockdown of NNMT prevented the development of obesity in animals fed with a high-fat diet (Kraus *et al*., 2014).

These recent insights into the functional consequences of altered NNMT activity suggest that Nam potentially has a critical role not only for the synthesis of NAD, but also in the regulation of pathways seemingly unrelated to NAD metabolism.

Extracellular NAD metabolites and their degradation

Extracellular NAD

Under normal physiological conditions, the extracellular concentration of NAD (e.g. in blood plasma) has been reported to be in the submicromolar range (Kim *et al*., 1993; O'Reilly & Niven, 2003). Extracellular NAD could be the result of cell lysis. However, stressed cells can actively release NAD. For example, in a mouse model of inflammation induced by injection of polyacrylamide beads, NAD^+ was released as an early response and its extracellular concentration reached 10 μM (Adriouch *et al*., 2007). It has also been shown that NAD is released from various types of neuronal cells upon stimulation to serve as a novel neurotransmitter and neuromodulator (Breen *et al*., 2006; Mutafova-Yambolieva *et al*., 2007; Smyth *et al*., 2004). It has also been shown that extracellular NAD can regulate functions of human granulocytes and mesenchymal stem cells by activation of P2Y11 receptor (Fruscione *et al*., 2011; Moreschi *et al*., 2006). Moreover, in mice NAD activates P2X7 receptor through ART2.2-mediated mono-ADP-ribosylation and thus induces apoptosis of T cells (Adriouch *et al*., 2007, 2008; Seman *et al*., 2003).

Extracellular degradation of NAD to NR

There are several studies demonstrating that extracellular NAD, and also NMN, can be used to maintain intracellular NAD levels. However, the mechanism is still debated. The direct uptake of the nucleotides has been suggested (Billington *et al*., 2008; Durnin *et al*., 2012; Formentini *et al*., 2009b; Pittelli *et al*., 2011). Given that, except for connexin 43 hemichannels (Bruzzone *et al*., 2001), no transporter has been identified, alternative mechanisms have been studied. It has recently been found that, in order to contribute to intracellular pools, extracellular NAD needs to be cleaved to NMN, which, in turn, is cleaved to NR. NR can then be taken up by membrane transporters, which have pharmacological characteristics known for identified nucleoside transporters present in the plasma membrane (Nikiforov *et al*., 2011). This conclusion was further supported by the demonstration that the ectoenzyme CD73 successively degrades NAD to NMN and AMP and further to NR and adenosine (Garavaglia *et al*., 2012; Grozio *et al*., 2013) (Figure 1).

Extracellular degradation of NR to Nam

A recent study revealed that the conversion of NR to Nam takes place not only inside cells, but also at the cell surface. It was shown that the human CD157, known as an ecto-NAD glycohydrolase, cleaves the nucleosides NAR and NR to the corresponding bases, NA and Nam, and ribose (Preugschat *et al*., 2014). Interestingly, the efficiency of CD157 to generate ADPR or cADPR from NAD is very low (>100 times lower than that of CD38; Malavasi *et al*., 2008). On the other hand, the now discovered activity to cleave NR by far exceeds that of NAD cleavage. Moreover, the affinity of CD157 towards NR is extremely high ($K_M = 6$ nM) suggesting a highly specific mechanism. The molecular mechanism of the reaction was found to proceed via a long-lived intermediate state consisting of the ribosylated enzyme. A high level of this intermediate could also be observed in the presence of low NR concentrations. Based on these findings, it was suggested that extracellular NR could act as an activator of the receptor functions borne by CD157 (Preugschat *et al*., 2014).

Extracellular NMN and NamPRT

There have been several studies demonstrating impressive health benefits from NMN supplementation (e.g. by injection into mice; Revollo *et al*., 2007; Yamamoto *et al*., 2014; Yoshino *et al*., 2011). However, the physiological occurrence of extracellular NMN has still remained a matter of discussion. It appears unlikely that significant extracellular amounts of this nucleotide can arise as a result of cell damage, because the intracellular concentration was measured to be only around 1 μM (Trammell & Brenner, 2013). However, the possibility exists that NMN could be synthesized in plasma owing to the presence of an extracellular form of NamPRT (eNAMPT) (Revollo *et al*., 2007), previously identified as pre-B cell colony enhancing factor (PBEF) (Samal *et al*., 1994) and also as adipocytokine visfatin (Fukuhara *et al*., 2005). It has been proposed that eNAMPT possesses signaling functions in adipocytes and pancreatic β-cells (Garten *et al*., 2009).

Cellular compartmentalization of NAD

It is still widely believed that NAD is readily available in the nucleus/cytosol and mitochondria, because the overwhelming

majority of both the known bioenergetic and NAD-dependent signaling processes are found there. However, recent research has discovered an unexpected distribution of subcellular NAD pools, with readily detectable NAD in the mitochondria, peroxisomes and even in the ER and Golgi complex, that is, all cytoplasmic organelles tested by the PARAPLAY method, which is based on the formation of poly-ADP-ribose by targeted expression of the catalytic domain of PARP-1 (Dölle *et al*., 2010). At least for the Golgi, no functional role of NAD has so far been identified. Moreover, given the subcellular distribution of NAD biosynthetic enzymes in mammalian cells NAD can only be synthesized in the nucleus/cytosol or mitochondria (Nikiforov *et al*., 2011). A mammalian transporter for NAD has only been described for the peroxisomal membrane (Agrimi *et al*., 2012). Therefore, it has remained largely unclear how organellar NAD pools are generated and maintained.

A large proportion of NAD-dependent processes is located in the nucleus and the cytosol. Even though there is no conclusive experimental evidence, it is widely believed that the nuclear membrane does not constitute a diffusion barrier for NAD and consequently it is assumed that the nucleotide can freely exchange between these two compartments. Therefore, with regard to NAD compartmentalization, the nucleus and cytosol have not been separated (Figure 2). It is also interesting to note that all enzymes of human NAD biosynthesis, except for the mitochondrial NMNAT3, have been localized to the nucleus or cytosol (Nikiforov *et al*., 2011). Therefore, NAD can be readily regenerated in these compartments.

Mitochondrial NAD

In some tissues, mitochondria contain up to 70% of the total cellular NAD (Alano *et al*., 2007; Stein & Imai, 2012). Mitochondrial NAD is a vital redox coenzyme for metabolic processes such as the tricarboxylic acid cycle, fatty acid oxidation and oxidative phosphorylation.

In recent years, it was also shown that NAD^+ has roles as a signaling molecule in the mitochondrial matrix, because it is an obligatory co-substrate for SIRT3, 4 and 5. These Sirtuins regulate the activities of key metabolic enzymes including glutamate dehydrogenase, acetyl-CoA synthetase 2, hydroxymethylglutaryl-CoA synthetase 2 and carbamoyl-phosphate synthetase 1 by deacetylation, deacylation or ADP-ribosylation (Dölle *et al*., 2013a). As a result of these NAD-dependent post-translational modifications the dinucleotide is degraded. Therefore, the mitochondrial NAD pool needs to be continuously replenished.

Even though the mitochondrial inner membrane itself is impermeable for polar molecules, a large number of carrier proteins have been identified that mediate the exchange of solutes between the cytosol and the mitochondrial matrix (Palmieri, 2013). In yeast, the mitochondrial NAD pool is maintained by means of import of the nucleotide from the cytosol through the carrier proteins Ndt1p and Ndtp2 (Todisco *et al*., 2006). A similar mechanism has been identified for mitochondria from *A. thaliana* (Palmieri *et al*., 2009). In mammalian cells, however, the situation appears to be rather different. A direct exchange of NAD between the mitochondrial matrix and the cytosol in human cells could not be detected (Yang *et al*., 2007). Moreover, experiments using isolated rat liver mitochondria also indicated that there is no exchange of NAD across the mitochondrial inner membrane (Barile *et al*., 1996). It was also found that the closest human homolog of the yeast carrier Ndt1p, the solute carrier protein SLC25A32, did not mediate the transport of NAD (Yang *et al*., 2007). These observations suggested that, in mammalian cells, the mitochondrial NAD pool is segregated from the cytosolic one and needs to be replenished by autonomous generation of NAD. Even though progress has been made in this regard over the past few years, the exact molecular mechanisms how mitochondrial NAD is generated in human mitochondria still need to be established.

In a recent study, it was shown that all four vitamin B3 precursors (Nam, NA, NR and NAR) can enter human cells and support the generation of mitochondrial NAD (Nikiforov *et al*., 2011). Moreover, among all known enzymes contributing to NAD biosynthesis, only NMNAT3 was found to be intramitochondrial, one of the three adenylyltransferases that convert NMN to NAD. The observation that increased generation of cytosolic NMN also leads to an increased mitochondrial NAD content further suggested that the mechanism of NAD generation in these organelles includes the import of NMN and its subsequent conversion to NAD by NMNAT3 (Figure 2) (Nikiforov *et al*., 2011). However, so far, no carrier protein mediating the import of NMN has been identified. This identification has therefore remained as one of the most critical tasks to fully understand how the mitochondrial NAD pool is maintained in mammalian cells.

Peroxisomal NAD

Several NAD-dependent processes have been reported to be localized within peroxisomes including redox reactions catalyzed by enzymes of the fatty acid oxidation pathway, lactate dehydrogenase (LDH) and glycerol-3-phosphate dehydrogenase (G3PDH) (Wanders & Waterham, 2006). While NAD^+ is reduced to NADH during β-oxidation, both LDH and G3PDH re-oxidize NADH to NAD^+.

It has been shown that the peroxisomal membrane is partially permeable for metabolites (Antonenkov & Hiltunen, 2012; Antonenkov *et al*., 2004). Solutes with a molecular mass up to ~300 Da can freely diffuse through the Pxmp2 channels. However, bulkier molecules such as ATP, CoA and NAD are unable to pass through the peroxisomal membrane in the absence of carrier proteins (Rokka *et al*., 2009). SLC25A17 was shown to transport CoA, FAD, FMN, AMP and to lesser extent NAD^+ and ADP by an exchange mechanism (Agrimi *et al*., 2012). As indicated above, peroxisomal NAD can be cleaved to AMP and NMN by NUDT12 (Abdelraheim *et al*., 2003). Therefore, NUDT12 could be important to regulate the NAD content in these organelles. Because mouse Pxmp2 mediated the release of AMP (Antonenkov & Hiltunen, 2012; Rokka *et al*., 2009), the generated NMN could possibly be released from the peroxisomes through this channel. NMN could then be used by NMNAT1 or 2 to regenerate NAD (Figure 2). In this scenario, the peroxisomal NAD pool could serve as a buffer to maintain a constant free NAD level in the cytosol.

NAD in the ER and the Golgi complex

The presence of NAD^+ within the ER and the Golgi complex has been demonstrated using the PARAPLAY assay (Dölle *et al*., 2010). However, the biological role of NAD in these organelles is unclear. No NAD-dependent process within the Golgi complex is known. In the ER, the luminal chaperone glucose-regulated protein 78 (GRP78), also known as immunoglobulin heavy chain-binding protein (BiP), is regulated by NAD-dependent mono-ADP-ribosylation. GRP78/BiP mediates translocation of the newly synthesized proteins from the ribosome to the ER and their correct folding in the ER lumen. Moreover, GRP78/BiP targets misfolded proteins for ER-associated protein degradation and plays a key role in the unfolded protein response (Schroder & Kaufman, 2005). When induced by various stress conditions, mono-ADP-ribosylation inactivates GRP78/BiP leading to intensification of ER stress signaling (Chambers *et al*., 2012; Fabrizio *et al*., 2015; Leno & Ledford, 1989). Recently, human ecto-ADP-ribosyltransferase ARTC1 was shown to localize to the ER lumen and to mono-ADP-ribosylate GRP78/BiP in response to ER stress (Fabrizio *et al*., 2015).

There have also been reports suggesting an independent NADP pool in the ER (Bublitz & Lawler, 1987; Piccirella *et al*., 2006). Indeed, two enzymes which require NADP have been localized to the lumen of the ER, namely, hexose-6-phosphate dehydrogenase (H6PD) and 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1). H6PD catalyzes the first two steps of the pentose phosphate pathway in which glucose-6-phosphate is oxidized to 6-phosphogluconate accompanied by the reduction of $NADP^+$ to NADPH (Senesi *et al*., 2010). NADPH, in turn, is required for the conversion of cortison to cortisol catalyzed by 11β-HSD1 (Banhegyi *et al*., 2004; Chapman *et al*., 2013).

Conclusions and future perspectives

Recent research has revealed the importance of the regulatory roles of NAD. In fact, some of these pathways have already been targeted for therapeutic treatments of cancer and other diseases. While impressive progress has been made over the last years with regard to NAD-dependent signaling mechanisms, some critical issues have remained unresolved. For example, NAD-dependent signaling uses up an unexpectedly large amount of NAD. Therefore, NAD biosynthesis has to be highly active and needs to be fine-tuned to the actual demands of the cell. However, except for the identification of the principal pathways of NAD biosynthesis and the corresponding enzymes, surprisingly little is known about the mechanisms regulating NAD synthesis and how it influences signaling pathways. Moreover, cellular NAD is compartmentalized in distinct membrane-coated organelles in human cells. Yet, almost nothing is known about whether (and how) NAD enters these organelles or is produced there. In this regard more detailed insights into the interconversions of NAD metabolites will likely facilitate understanding the maintenance of individual NAD pools. It appears as an intriguing possibility that the compartmentalization might represent a means to selectively influence NAD-dependent signaling events. The tremendous medical relevance as well as the broad scientific interest in this field will promote further research into these and other exciting questions regarding NAD biology.

Declaration of interest

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