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NAD+ Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus

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NAD+ has emerged as a vital cofactor that can rewire metabolism, activate sirtuins, and maintain mitochondrial fitness through mechanisms such as the mitochondrial unfolded protein response. This improved understanding of NAD+ metabolism revived interest in NAD+-boosting strategies to manage a wide spectrum of diseases, ranging from diabetes to cancer. In this review, we summarize how NAD+ metabolism links energy status with adaptive cellular and organismal responses and how this knowledge can be therapeutically exploited.

Introduction

The importance of nicotinamide adenine dinucleotide (NAD+) metabolism became apparent subsequent to the study of pellagra, a disease characterized by a darkly pigmented skin rash, dermatitis, diarrhea, and dementia, later resulting in death (Sydenstricker, 1958). A century ago, pellagra was common in rural areas of Europe and became an epidemic in the southern United States (Sydenstricker, 1958). However, in 1914, Joseph Goldberger tested whether pellagra was caused by a dietary deficiency and discovered that substituting corn-based diets with milk, eggs, and meat prevented and cured the condition (reprinted essay, Goldberger, 2006). Later, Conrad Elvehjem found that a nicotinamide (NAM)-enriched fraction from deproteinized liver and a sample of crystalline nicotinic acid (NA) cured pellagra (Elvehjem, 1940). NA and NAM, collectively termed niacin or vitamin B3, are now known as precursors for NAD+, an essential element for all cells (Bogan and Brenner, 2008; Chi and Sauve, 2013; Houtkooper et al., 2010a). Whereas pellagra remains endemic in underdeveloped countries (Seal et al., 2007), it is rare in developed countries and mostly occurs in association with tuberculosis, malabsorption, alcoholism, and eating disorders (Hegyi et al., 2004). Less severe niacin deficiencies are more difficult to detect and are linked with low metabolism, cold intolerance, and delayed brain development (Forbes and Duncan, 1961; Williams and Dunbar, 2014).

So, what is NAD+, and why is it important? NAD+ was originally described more than 100 years ago by Sir Arthur Harden and colleagues as a cofactor in fermentation (Harden and Young, 1906). Years later, another Nobel prize laureate, Hans von Euler-Chelpin, identified this factor as a nucleoside sugar phosphate (1940 Nobel lecture; Euler-Chelpin, 1929). Yet it took a third Nobel laureate, Otto Warburg, to isolate NAD(P)+ and discover its key role for hydrogen transfer in biochemical reactions (Warburg et al., 1935). NAD+ and NADP+ perform similar redox functions within the cell, but the latter is more confined to biosynthetic pathways and redox protective roles (reviewed in Ying, 2008). Playing a vital role in energy metabolism within

eukaryotic cells, NAD+ accepts hydride equivalents to form reduced NADH, which furnishes reducing equivalents to the mitochondrial electron transport chain (ETC) to fuel oxidative phosphorylation. The roles of NAD+, however, have expanded beyond its role as a coenzyme, as NAD+ and its metabolites also act as degradation substrates for a wide range of enzymes, such as sirtuins (Blander and Guarente, 2004; Haigis and Sinclair, 2010; Hall et al., 2013; Houtkooper et al., 2010a). Through these activities, NAD+ links cellular metabolism to changes in signaling and transcriptional events. Here, we give an overview of the current knowledge on NAD+ metabolism, including its biosynthesis, compartmentalization, degradation, and actions as a signaling molecule.

NAD+: Metabolic and Therapeutic Interests

Food Sources and Bioavailability of NAD+

The daily requirements for NAD+ biosynthesis can be met with the consumption of less than 20 mg of niacin (Bogan and Brenner, 2008). Four major molecules have been described as the root substrates for different NAD+ biosynthetic pathways: the amino acid tryptophan (Trp), NA, NAM, and nicotinamide riboside (NR) (Figures 1A, 1B, and 1D). However, intermediate compounds of these NAD+ biosynthetic pathways, such as nicotinamide mononucleotide (NMN), can also directly stimulate NAD+ synthesis. Vitamin B3 deficiency occurs on low-protein diets or diets relying mostly on untreated maize. Interestingly, niacin is found in maize but is not bioavailable unless given an alkali treatment, a process used in Aztec and Mesoamerican times termed nixtamalization (Gwirtz and Garcia-Casal, 2014). In animal products, and probably in all uncooked foods, the NAD+ and NADP+ cellular content accounts for much of their dietary niacin content (Gross and Henderson, 1983), yet, as exemplified above with corn nixtamalization, their bioavailability might be affected by food processing or cooking.

Bioavailability studies indicated that ingested NAD+ was primarily hydrolyzed in the small intestine by brush border cells (Baum et al., 1982; Gross and Henderson, 1983). As a first step,
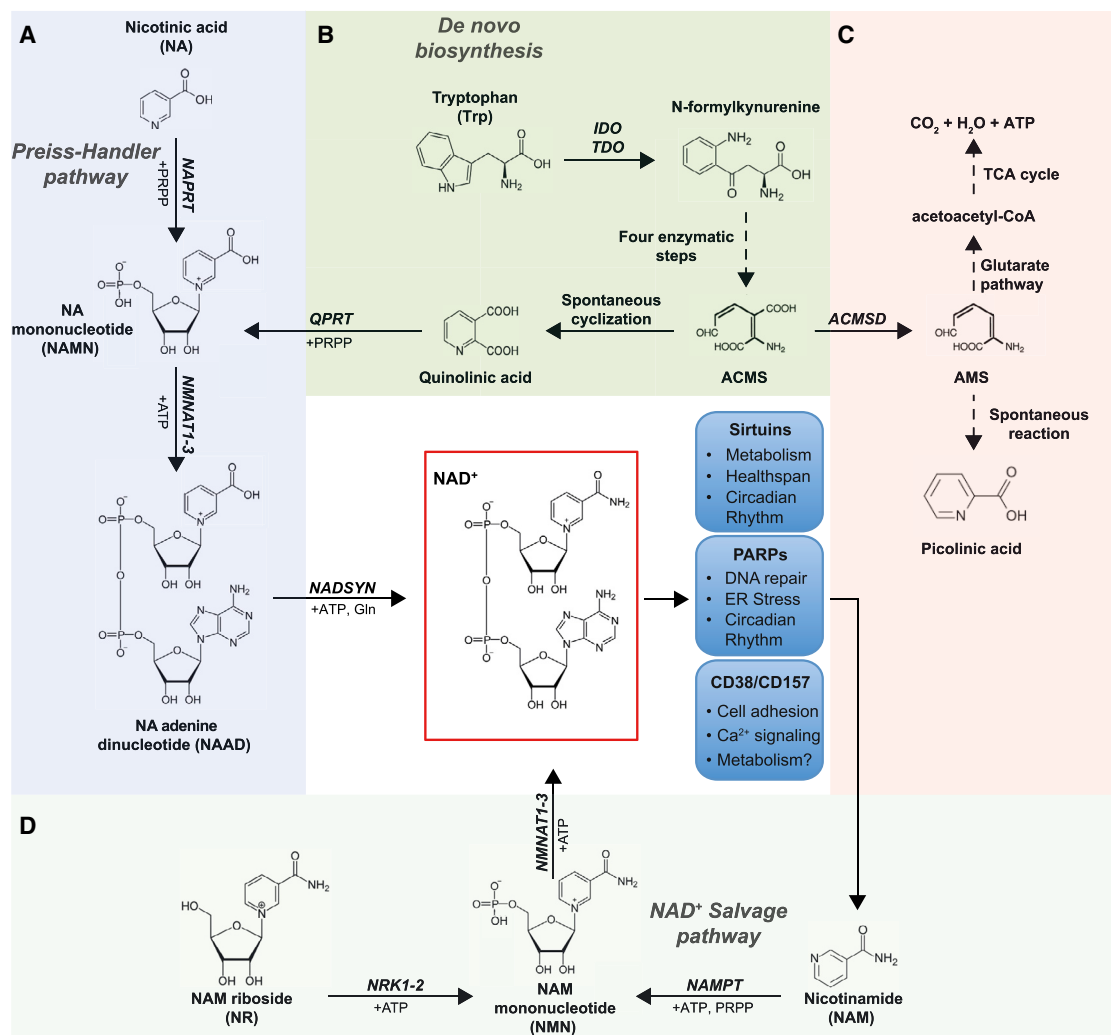
Figure 1. NAD+ Precursor Metabolism and NAD+-Consuming Enzymes
Trp, NA, NAM, and NR are utilized through distinct metabolic pathways to form NAD+.
(A) NAD+ synthesis from NA, also known as the Preiss-Handler pathway, is initiated by the NAPRT, which uses PRPP to form NAMN. Together with ATP, NAMN is then converted into NAAD by the NMNAT1–3 enzymes. Finally, NAAD is transformed to NAD+ through an amidation reaction catalyzed by the NADSYN enzyme.
(B) The de novo biosynthesis of NAD+ from Trp starts with the conversion of Trp to N-formylkynurenine by either IDO or TDO. After four reaction steps, N-formylkynurenine can be subsequently converted to the unstable ACMS, which can undergo nonenzymatic cyclization to quinolinic acid. The last step of the de novo biosynthesis component is comprised of the QPRT-catalyzed formation of NAMN, using PRPP as a co-substrate, which is converted to NAD+ via the remaining pathway described in (A).
(C) ACMS can also be diverted away from NAD+ synthesis, by ACMSD, to form AMS and can then be oxidized via the glutarate pathway and TCA cycle to CO_2 and water, or nonenzymatically converted to picolinic acid.
(D) The synthesis of NAD+ from NAM or NR is more direct and relies on only two steps each. NAM is converted by the rate-limiting NAMPT to form NMN, using PRPP as cosubstrate. NMN is also the product of phosphorylation of NR by the NR kinases (NRK1 and NRK2). The subsequent conversion of NMN to NAD+ is catalyzed by the NMNAT enzymes. The blue boxes depict the three families of NAD+-consuming enzymes and some of the key processes to which they have been linked. ACMS, α-amino-β-carboxymuconate-ε-semialdehyde; ACMSD, ACMS decarboxylase; AMS, α-amino-β-muconate-ε-semialdehyde; IDO, indoleamine 2,3-dioxygenase; NA, nicotinic acid; NAAD, NA adenine dinucleotide; NADSYN, NAD+ synthetase; NAMN, NA mononucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NAPRT, NA phosphoribosyltransferase; NMN, NAM mononucleotide; NMNAT, NMN adenylyltransferase; NR, nicotinamide riboside; NRK, NR kinase; PRPP, phosphoribosyl pyrophosphate; QPRT, quinolinate phosphoribosyltransferase; TDO, tryptophan 2,3-dioxygenase; Trp, Tryptophan.

NAD+ is cleaved to NMN and 5′-AMP by a pyrophosphatase found either in intestinal secretions (Gross and Henderson, 1983) or in the brush border (Baum et al., 1982). Next, NMN is rapidly hydrolyzed to NR, which in turn is more slowly converted into NAM (Gross and Henderson, 1983). NAM can also be formed directly by the cleavage of NAD+, obtaining ADP-ribose derivates as a side product (Gross and Henderson, 1983). The intestinal production of NAM from NAD+ or NR required the presence of intestinal cells, indicating that the enzymes for this process are membrane-bound or intracellular (Baum et al., 1982; Gross and Henderson, 1983). The direct perfusion with NAM, however, did not give rise to any of these species, indicating that NAM is the final degradation product and directly absorbed (Collins and Chaykin, 1972; Gross and Henderson, 1983; Henderson and Gross, 1979). In contrast, perfusion of the intestine with NA revealed a substantial cellular accumulation of labeled

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intermediates of the NAD+ biosynthetic pathway, including NAM, which suggest the presence of active NA metabolism in intestinal cells (Collins and Chaykin, 1972; Henderson and Gross, 1979). In line with this, blood concentrations of NA are relatively low (~100 nM) yet, when pharmacologically primed (Jacobson et al., 1995; Tunaru et al., 2003), can increase and be rapidly converted to NAM by the liver (Collins and Chaykin, 1972). Strikingly, NAM levels in fasted human plasma are also too low to support NAD+ biosynthesis in cells (between 0.3 and 4 μM) (Hara et al., 2011; Jacobson et al., 1995). All of these results suggest that these NAD+ precursors are metabolized very quickly in mammalian blood and tissues.

Lipid-Lowering Effect of Niacin

NA attracted clinical attention for its cholesterol lowering actions (Altschul et al., 1955), and became the first drug used to treat dyslipidemia. Gram dosages of NA reduce plasma triglyceride and low-density lipoprotein (LDL) levels, while concomitantly increasing high-densitiy lipoproteins (HDL). However, the clinical use of NA has been limited by the fact that it induces cutaneous flushing, which compromises compliance (Birjmohun et al., 2005). This flushing does not derive from the ability of NA to drive NAD+ synthesis, but rather from the activation of a G protein-coupled receptor, GPR109A (Benyó et al., 2005). Given the low presence of NA in blood, the activation of this receptor is unlikely to be a native function of NA, but rather an effect from pharmacological dosing. It was also assumed that the beneficial effects of NA on plasma lipids are mediated via a receptor rather than a vitamin mechanism because of the high dose required (100-fold higher than that required to prevent pellagra) and the failure of NAM to provide similar benefits (Tunaru et al., 2003). Indeed, some evidence supports that GPR109A is necessary for NA to raise HDL cholesterol (Li et al., 2010; Tunaru et al., 2003). However, the absence of GRP109A expression in the liver (Soga et al., 2003; Tunaru et al., 2003; Wise et al., 2003), a central hub for HDL and LDL metabolism, also questions whether the effects of NA on blood lipids derive from GPR109A activation. Alternatively, strong evidence for the ability of NAD+ to enhance the activity of sirtuins provides a mechanism of action that also drives benefits on lipid homeostasis (Cantó and Auwerx, 2012). In addition, sirtuin activity is inhibited by NAM (Anderson et al., 2003), which could explain why NAM failed to provide the benefits of NA; however, in some situations, NAM treatments can have beneficial effects as discussed in New Perspectives in NAD+ Therapeutics (I): Metabolic Disease. The intricate relationship between NAD+ and sirtuins will be discussed further in NAD+-Consuming Enzymes (I): Sirtuins.

Introducing NAD+ as a Metabolic Regulator

The role of NAD+ as a coenzyme in most metabolic pathways suggests that NAD+ limitations could affect metabolic efficiency. Decreasing NAD+ levels could therefore prompt the development of many of the ailments associated with aging. Indeed, NAD+ levels can change during a number of physiological processes. Diverse lines of research on worms, rodents, and human cellular models indicate that declining NAD+ levels are a hallmark for senescence (Braidy et al., 2011; Gomes et al., 2013; Khan et al., 2014; Massudi et al., 2012; Mouchiroud et al., 2013; Ramsey et al., 2008; Yoshino et al., 2011). Along a similar line, a reduction in muscle progenitor cell NAD+ content leads to a SIRT1-mediated metabolic switch that induces premature differ-

entiation and a loss of regenerative capacity, reflecting a phenotype typical of aging muscle (Ryall et al., 2015). The link between metabolism and NAD+ is further solidified by observations that tissue NAD+ levels decrease with high-fat diets (HFDs) (Bai et al., 2011b; Cantó et al., 2012; Kraus et al., 2014; Pirinen et al., 2014; Yang et al., 2014; Yoshino et al., 2011). In contrast, NAD+ increases in mammalian cells and tissues in response to exercise (Cantó et al., 2009, 2010; Costford et al., 2010) or calorie restriction (CR) (Chen et al., 2008), both of which are interventions associated with metabolic and age-related health benefits. In line with this, supplementation with NAD+ precursors has proven to enhance lifespan in budding yeast (Belenky et al., 2007) and worms (Mouchiroud et al., 2013). Also, in mammals, the enhancement of NAD+ levels has been linked with improved mitochondrial function under stress (Cerutti et al., 2014; Khan et al., 2014; Mouchiroud et al., 2013; Pirinen et al., 2014), leading to protection against dietary (Bai et al., 2011b; Cantó et al., 2012) and age-related (Gomes et al., 2013; Yoshino et al., 2011) metabolic complications. Finally, hepatic NAD+ levels dynamically change in a circadian fashion (Asher et al., 2010; Nakahata et al., 2009; Ramsey et al., 2009), weaving an intricate relationship with nutritional states. Therefore, despite the classical misconception that intracellular NAD+ levels rarely change (Kaelin and McKnight, 2013), the evidence above unequivocally demonstrates the ability of NAD+ to respond dynamically to physiological stimuli. So, how do changes in NAD+ levels take place innately?

NAD+ Synthesis and Salvage: New Ways to Boost NAD+
NAD+ Biosynthesis and the Discovery of New NAD+ Precursors

NAD+ availability is determined by the relative rates of NAD+ biosynthesis and degradation. Ergo, the enhancement of NAD+ biosynthesis could provide a way to elevate NAD+ content. There are several known NAD+ precursors. First, dietary Trp can serve as an NAD+ precursor through an eight-step de novo pathway (Bender, 1983), which has been described in detail elsewhere (Houtkooper et al., 2010a); so, we will only focus on some of its most interesting features (Figures 1A–1D). The first and rate-limiting step in this path includes the conversion of Trp to N-formylkynurenine by either indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO) (Figure 1B). These enzymes are strongly overexpressed in diverse cancers, and the subsequent synthesis of kynurenines may act as potential second messengers in cancer immune tolerance (Stone and Darlington, 2002), possibly through binding to the aryl hydrocarbon receptor (AHR) (Bessede et al., 2014). An interesting branch point in the Trp catabolic pathway is the formation of the unstable α-amino-β-carboxymuconate-ϵ-semialdehyde (ACMS) (Bender, 1983). ACMS can be enzymatically converted to α-amino-β-muconate-ϵ-semialdehyde (AMS) by ACMS decarboxylase (ACMSD), leading to complete oxidation via the glutarate pathway and the tricarboxylic acid (TCA) cycle or to the production of picolinic acid via a spontaneous reaction (Figures 1B and 1C) (Houtkooper et al., 2010a). Alternatively, ACMS can undergo spontaneous cyclization forming quinolinic acid, which subsequently serves as an NAD+ precursor (Bender, 1983). This latter nonenzymatic possibility seems to be only relevant when the metabolism of ACMS is limited in the cell. This

might explain why, in general, Trp is considered a rather poor NAD+ precursor in vivo, as it will only be diverted to NAD+ synthesis when its supply exceeds the enzymatic capacity of ACMSD (Ikeda et al., 1965). In humans, diets ranging from 34 mg to 86 mg of Trp provide the equivalent of 1 mg of niacin (reviewed in Horwitt et al., 1981). Interestingly, the formation of NAD+ following Trp injections is further reduced in diabetic rats (Ikeda et al., 1965). When ACMSD capacity is surpassed, Trp-derived quinolinic acid is produced and used by quinolinate phosphoribosyltransferase (QPRT) to form NA mononucleotide (NAMN). NAMN is then converted to NA adenine dinucleotide (NAAD), using ATP, by the enzyme NMN adenylyltransferase (NMNAT) (Figure 1A) (Houtkooper et al., 2010a). This is a key enzyme for NAD+ synthesis in mammals, irrespective of the precursor used, since it is also needed for NAD+ salvage. Three NMNAT isoforms (NMNAT1–3) with different tissue and subcellular distributions have been described in mammals (Lau et al., 2009). NMNAT1 is a nuclear enzyme that is ubiquitously expressed, with its highest levels in skeletal muscle, heart, kidney, liver, and pancreas, yet it is almost undetectable in the brain (Emanuelli et al., 2001). In contrast, NMNAT2 is mostly located in the cytosol and Golgi apparatus (Berger et al., 2005; Yalowitz et al., 2004). Finally, NMNAT3 is highly expressed in erythrocytes with a moderate expression in skeletal muscle and heart and has been identified in both cytosolic and mitochondrial compartments, with cell-/tissue-specific subcellular localization patterns (Berger et al., 2005; Felici et al., 2013; Hikosaka et al., 2014; Zhang et al., 2003). The possible implications of the subcellular localization of NMNAT enzymes will be discussed in Cell Compartmentalization of NAD+. The last step in the primary biosynthesis of NAD+ includes the ATP-dependent amidation of NAAD by NAD+ synthase (NADSYN) using glutamine as a donor. NADSYN is mainly expressed in the small intestine, liver, kidney, and testis, where this pathway may be more relevant to NAD+ synthesis (Hara et al., 2003; Houtkooper et al., 2010a).

NAD+ can also be synthesized from metabolite recycling or the dietary uptake of other NAD+ precursors (Houtkooper et al., 2010a). NA can lead to NAD+ through the shorter, three-step Preiss-Handler pathway (Figure 1A). Here, NA is initially metabolized by the NA phosphoribosyltransferase (NAPRT) into NAMN, converging with the de novo pathway.

In mammals, NAM can also be an NAD+ precursor through its metabolism into NAM mononucleotide (NMN) by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT) (Figure 1D) (Revollo et al., 2004; Rongvaux et al., 2002). NMN can be then converted into NAD+ through a single additional reaction catalyzed by the NMNAT enzymes. NAM is also the product of NAD+ degradation by several enzyme families (see The Enzymatic Use of NAD+). Consequently, NAMPT is key to not only metabolizing circulating NAM, but also to recycling intracellularly produced NAM via the NAD+ salvage pathway. As a key enzyme, SNPs found in non-coding regions of human NAMPT are correlated with glucose and lipid metabolism alterations and type 2 diabetes, among other disease associations (Zhang et al., 2011).

Lastly, NR metabolism constitutes an additional path for NAD+ biosynthesis (Bieganowski and Brenner, 2004) (Figure 1D). NR is transported into cells by nucleoside transporters (Nikiforov et al., 2011) and is then phosphorylated by the NR kinases 1 and 2 (NRKs) (Bieganowski and Brenner, 2004), generating NMN. This phosphorylation step is a conserved feature in all eukaryotes (Bieganowski and Brenner, 2004), underscoring its evolutionary relevance. After the generation of NMN, NMNAT enzymes can then catalyze the formation of NAD+. While additional ways for NR metabolism have been described in yeast (Belenky et al., 2007), the phosphorylation by NRKs is still the only pathway described in mammalian cells for the transformation of NR into NAD+.

Whole-Body NAD+ Transport

Despite Trp being the canonical NAD+ precursor, its action may be up to 60 times less efficient than NA (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline, 1998), as Trp is also used for protein translation and other biosynthetic purposes. Indeed, the use of Trp as an NAD+ precursor would not be solely sufficient to support the physiological NAD+ requirements in mammals (Henderson, 1997). NA, in contrast, can act as a potent NAD+ precursor, primarily in liver and kidney where NAPRT demonstrates the highest activity levels (Hara et al., 2007). However, mammalian tissues are rarely exposed to NA, as its levels in blood are generally very low (Jacobson et al., 1995; Tunaru et al., 2003). Also, as discussed in Food Sources and Bioavailability of NAD+, most evidence suggests that NA might be quickly metabolized to NAM in the gut and the liver (Collins and Chaykin, 1972). However, the low plasma concentration of NAM (Hara et al., 2011) is ~1,000-fold less than that required to increase NAD+ levels in cultured cells (Hara et al., 2007; Revollo et al., 2004).

These findings underscore the importance of naturally occurring NR as a possible alternative substrate for NAD+ biosynthesis (Bieganowski and Brenner, 2004). Supporting this idea, NR treatment enhances NAD+ levels in all mammalian cells tested (Cantó et al., 2012; Yang et al., 2007b). Interestingly, different lines of evidence suggest that NR is the primary metabolite transported into the cell and metabolized into NAD+, even when cells are cultured in the presence NAD+ or NMN (Lu et al., 2009) (Figure 2). Thus, by using specific inhibitors, it was suggested that free NAD+ or NMN in the medium are metabolized extracellularly to NR, which may be the final metabolite transported to cells for NAD+ biosynthesis (Nikiforov et al., 2011). Interestingly, milk extracts (whey vitamin fraction) rescue the survival of yeast cells defective for QNS1, a necessary enzyme for NA and NAM-triggered NAD+ biosynthesis in yeast (Bieganowski and Brenner, 2004). This extract, however, failed to rescue survival in NRK1-deficient yeast (Bieganowski and Brenner, 2004). The presence of significant NR levels in blood after oral intake is, however, not apparent, as classic reports generally indicate that radiolabelled NR is transformed into NAM at the brush border (Gross and Henderson, 1983). In this sense, it is intriguing that mammalian cultured cells commonly require almost millimolar NR concentrations in order to enhance NAD+ biosynthesis (Cantó et al., 2012; Yang et al., 2007b), which is unlikely to be met in vivo. However, in some microorganisms, such as Haemophilus influenza, only the transport of NR across the membrane allows them to synthesize NAD+ and survive in the host bloodstream, as they are unable to use NA, NAM, or the de novo pathway for this purpose (Cynamon et al., 1988; Herbert

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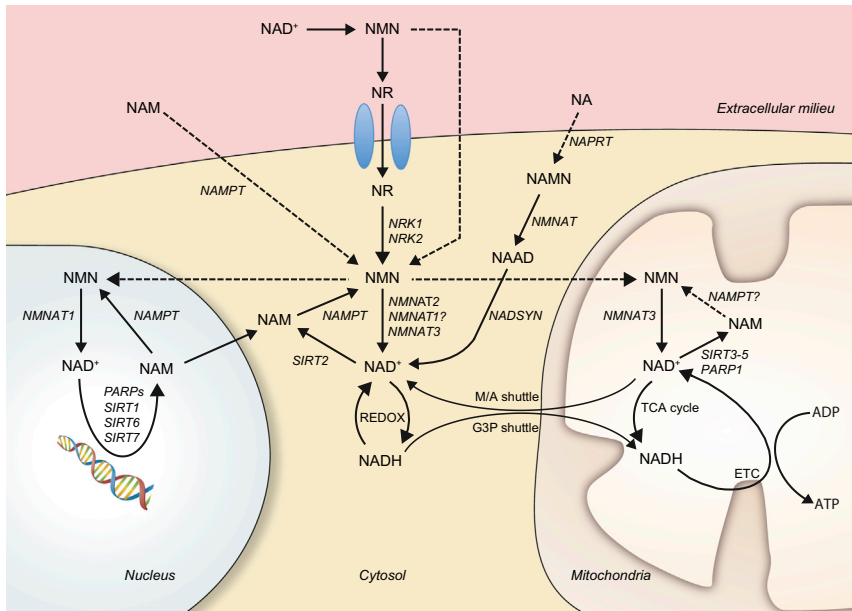
Figure 2. Central Nodes for Cellular NAD+ Metabolism

In normal circumstances, most NAD+ or NMN in blood is converted to NR, which enters the cell through specific transporters and is metabolized into NMN through NRK activity. Similarly, circulating NAM can be metabolized to NMN extracellulary by the extracellular NAMPT or enter the cell and be metabolized into NMN by the intracellular NAMPT. Extracellular NA can also enter the cell and be converted to NAD+ via a three-step reaction that is reliant on NAPRT, NMNAT, and NADSYN. NMN and, possibly, NAM are potentially transported into the mitochondrial and nuclear compartments. In those compartments, NMN can lead to NAD+ synthesis via NMNAT activity. In each subcellular compartment, NAD+ and NADH equilibriums will be determined by their unique redox states. In the mitochondria, the ETC is a major contributor to NADH oxidation into NAD+, coupling this action to ATP synthesis. In addition, the mitochondria and the cytosol can exchange redox equivalents through the malate/aspartate (M/A) and glyceraldehyde 3-phosphate (G3P) shuttles. In all compartments, the activity of NAD+-consuming enzymes, such as sirtuins or PARPs, lead to NAM production, which can be salvaged for NAD+ synthesis via NAMPT activity. Dashed arrows indicate pathways that need further validation.

et al., 2003). This suggests that either NR or NMN is, in fact, available in the blood. This is partially corroborated by a report indicating that NMN might be present in the bloodstream at concentrations around 50 μM (Revollo et al., 2007). The presence of a circulating extracellular form of the NAMPT enzyme (eNAMPT), to convert NAM to NMN, supports this possibility (Revollo et al., 2007). Recent evidence indicates that eNAMPT activity in the plasma is required to safeguard hypothalamic NAD+ levels (Yoon et al., 2015). However, other labs have failed to detect NMN in plasma (Hara et al., 2011). In addition, the marginal presence of ATP and 5-phosphoribosyl 1-pyrophosphate (PRPP) in blood (Hara et al., 2011), both substrates for the reaction catalyzed by NAMPT, would impede the generation of NMN in the circulation. Furthermore, since NAM plasma levels are also low, it is difficult to substantiate significant NMN synthesis in the bloodstream (Hara et al., 2011).

All the above suggest that plasma levels of most NAD+ precursors are probably unable to systematically sustain high NAD+ production rates. Consequently, it seems that mammalian organisms largely rely on NAD+ salvage from intracellular NAM in order to maintain NAD+ pools. In fact, NAM is an end product of NAD+-consuming activities in the cell (i.e., sirtuins, poly(ADP-ribose) polymerases and cyclic ADP-ribose hydrolases) (Houtkooper et al., 2010a). Accordingly, mice lacking NAMPT are not viable (Revollo et al., 2007). This, however, does not rule out a limited contribution of circulating NR, NMN, NAM, or Trp to NAD+ biosynthesis under basal conditions. However, further technical improvements will be needed, especially for NR, NMN, and NAM determination, to precisely evaluate the contribution of circulating precursors to NAD+ homeostasis.

Cell Compartmentalization of NAD+

In general, intracellular NAD+ levels are maintained between 0.2 and 0.5 mM, depending on the cell type or tissue. However, NAD+ levels can change up to ~2-fold in response to diverse physiological stimuli. For example, NAD+ levels increase in response to energy stresses, such as glucose deprivation (Fulco et al., 2008), fasting (Cantó et al., 2010; Rodgers et al., 2005), CR (Chen et al., 2008), and exercise (Cantó et al., 2010; Costford et al., 2010), and fluctuate in a circadian fashion (Nakahata et al., 2009; Ramsey et al., 2009). So, where and how do these changes take place in the cell?

The presence of NMNATs in the nucleus, cytosol, and mitochondria suggests that these compartments are fully capable of salvaging NAD+ from NAM (Figure 2). NAD+-degrading enzymes, such as sirtuins, are also present in these compartments. In addition, the presence of different forms of NMNATs in each cellular compartment (e.g., NMNAT1 in the nucleus or NMNAT3 in the mitochondria/cytosol) suggests that NAD+ salvage is tailored according to compartment-specific metabolic needs. However, despite some evidence that NAMPT is localized to the mitochondria (Yang et al., 2007a), there is still some debate as to whether this is really the case (Pittelli et al., 2010). Therefore, further experimental evidence is needed to confirm mitochondrial NAD+ salvage. Nonetheless, it is important to note that NAD+ is not evenly distributed in the cell. Most reports indicate that mitochondrial NAD+ content is ≥250 μM (Nakagawa et al., 2009; Yang et al., 2007a), while according to indirect estimations, nuclear NAD+ levels seem to be much lower, ~70 μM (Fjeld et al., 2003). To this effect, two-photon microscopy approaches have also been used to indirectly estimate NAD+ levels, confirming that NAD+ content in the nucleus is much lower than in the cytosol (Zhang et al., 2009). In addition, the different NAD+ pools can behave independently. As such, cells treated with methylmethane sulfonate, a genotoxic agent, can survive as long as mitochondrial NAD+ levels are maintained, irrespective of NAD+ depletion in other compartments (Yang et al., 2007a). Given that NAD+ or NADH cannot diffuse through membranes (van Roermund et al., 1995), the maintenance of NAD+ levels in each compartment is reliant on salvaging the

NAM produced by NAD+-consuming enzymes (Figure 2). Alternatively, it can be derived from the intermediates NMN or NAMN, generated from NR metabolism or the Preiss-Handler pathway, respectively. It was recently shown that exogenous NAD+ can elevate mitochondrial NAD+ levels more than cytoplasmic levels, indicating that NAD+ precursors or intermediates traverse the mitochondrial membrane (Pittelli et al., 2011). Further, NR treatment was shown to enhance mitochondrial NAD+ levels in cultured cells and in mouse liver (Cantó et al., 2012). However, the mitochondrial compartment lacks NRK activity to initiate NR conversion into NAD+ (Nikiforov et al., 2011). Hence, NR is likely converted to NMN in the cytosol, and NMN may traverse the mitochondrial membrane to produce NAD+ via NMNATs (Figure 2) (Berger et al., 2005; Yang et al., 2007a). This way, both NMN and NAM might act as the main intracellular forms for regulating NAD+ levels between compartments.

The compartmentalization of NAD+ synthesis may have even more layers of complexity than once imagined. For example, NMNAT1 is recruited to target gene promoters by either the NAD+-consuming enzyme SIRT1 (Zhang et al., 2009) or PARP1 (Zhang et al., 2012), which suggests that NAD+ production is regulated at a sub-compartmental level during transcriptional regulation or DNA repair. These observations suggest that SIRT1 and PARP1 may compete for limiting amounts of localized NMNAT1-produced NAD+. Thus, despite estimations indicating that nuclear NAD+ levels are low, NAD+ maintenance is key for survival, as testified by the fact that *Nmnat1*-KO is embryonically lethal in mice (Conforti et al., 2011).

The Enzymatic Use of NAD+
NAD+ and Redox Reactions in Metabolism
While distinct, the cytosolic/nuclear and mitochondrial pools of NAD+ are interconnected by an intricate set of cellular redox processes. These NAD+ pools can modulate the activity of compartment-specific metabolic pathways such as glycolysis in the cytoplasm and the TCA cycle/oxidative phosphorylation in the mitochondria.

In the cytoplasm, the conversion of glucose to pyruvate by glycolysis requires two NAD+ molecules per molecule of glucose. Following the conversion of glucose to two molecules of glyceraldehyde-3-phosphate (G3P), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reduces NAD+ to NADH to transform G3P into 1-3-biphosphoglycerate. Glycolysis will therefore net two NADH and two pyruvate molecules that can then be transported into to the mitochondrial matrix. Since the outer mitochondrial membrane is very porous, NADH is free to enter the intermembrane space. However, it is the reducing equivalent of NADH that is transported into the mitochondria, via either the malate-aspartate shuttle or the glycerol-3-phosphate shuttle of the inner mitochondrial membrane, rather than NADH itself (Figure 2) (McKenna et al., 2006). As discussed in Cell Compartmentalization of NAD+, cytoplasmic NAD+ levels cannot alter mitochondrial NAD+/NADH ratios directly since NAD+ is not permeable to the mitochondrial membrane (Barile et al., 1996). Therefore, changes in the cytoplasmic NAD+ pool do not acutely alter mitochondrial NAD+ levels (Pittelli et al., 2010; Yang et al., 2007a).

In the mitochondrion, the TCA cycle reduces NAD+ molecules to produce multiple NADH molecules. Mitochondrial NADH, gained from glycolysis or the TCA cycle, are oxidized by Complex I (NADH:ubiquinone oxidoreductase) of the ETC. The subsequent two electrons gained by Complex I are relayed along ubiquinone (Coenzyme Q10), complex III (coenzyme Q-cytochrome c oxidoreductase), cytochrome c, and Complex IV (cytochrome c oxidase). In parallel to the oxidation of NADH to NAD+ by the ETC, the substrate succinate from the mitochondrial TCA cycle provides additional electrons to ubiquinone in parallel with Complex I. Ultimately, the flow of electrons, generated from NADH and succinate, along the ETC is coupled to the pumping of protons from the mitochondrial matrix to the intermembrane space via Complex I, III, and IV, creating a proton gradient. The proton gradient then provides the chemiosmotic gradient to couple the flux of protons back into the matrix via F0F1-ATP synthase with oxidative phosphorylation of ADP to ATP. Overall, the ETC reduces O_2 to water and NADH to NAD+ for the purpose of generating ATP. As a result, mitochondrial NAD+ levels are 2-fold greater than the rest of the cell, as measured in mouse skeletal muscle (Pirinen et al., 2014), and 4-fold greater in mouse cardiac myocytes (Alano et al., 2007).

Since NAD+ levels within the cell can be limiting (Bai et al., 2011b; Pirinen et al., 2014; Pittelli et al., 2011), both glycolysis in the cytoplasm and the TCA cycle in the mitochondria can influence metabolic homeostasis by altering cytosolic/nuclear NAD+ and NADH levels. In addition, following DNA damage, NAD+ levels can drop low enough that glycolysis and substrate flux to the mitochondria is blocked, leading to cell death, despite having an excess of available glucose (Alano et al., 2010; Benavente et al., 2009; Ying et al., 2005; Zhang et al., 2014). This finding highlights the need to understand the mechanisms interconnecting subcellular NAD+ pools, as their homeostasis and interactions are essential for the preservation of cell viability and ATP levels.

NAD+-Consuming Enzymes (I): Sirtuins
In mammals, there are seven sirtuin enzymes (SIRT1–SIRT7) based on the presence of a characteristic and evolutionarily conserved catalytic site, comprised of 275 amino acids (Haigis and Sinclair, 2010; Hall et al., 2013; Houtkooper et al., 2010a). Three sirtuins are located in the mitochondria (SIRT3–SIRT5), while SIRT1, SIRT6 and SIRT7 are predominantly located in the nucleus, and SIRT2 is found in the cytoplasm (Michishita et al., 2005; Verdin et al., 2010). However, some sirtuins, such as SIRT1, have been shown to shuttle in and out of the nucleus (Tanno et al., 2007).

Sirtuins use NAD+ as a cosubstrate to remove acetyl moieties from lysines on histones and proteins, releasing NAM and O-acetyl-ADP-ribose (Houtkooper et al., 2010a). The consumption of NAD+ during deacetylation is what separates sirtuins, as type III lysine deacetylases (KDACs), from type I, II, and IV KDACs. SIRT1, SIRT2, and SIRT3 have strong deacetylase activity (Imai et al., 2000; North et al., 2003; Schwer et al., 2002; Vaziri et al., 2001), while SIRT4, SIRT5, and SIRT6 are weak in comparison. However, deacetylation is not the singular function of all sirtuins, as SIRT4 can act as a lipoamidase (Mathias et al., 2014) and, along with SIRT6, as an NAD+-dependent mono-ADP-ribosyltransferase (Haigis et al., 2006; Liszt et al., 2005). SIRT6 can also efficiently remove long-chain fatty acyl groups from lysine residues (Jiang et al., 2013), while SIRT5 has strong desuccinylase, demalonylase, and deglutarylase enzymatic activities

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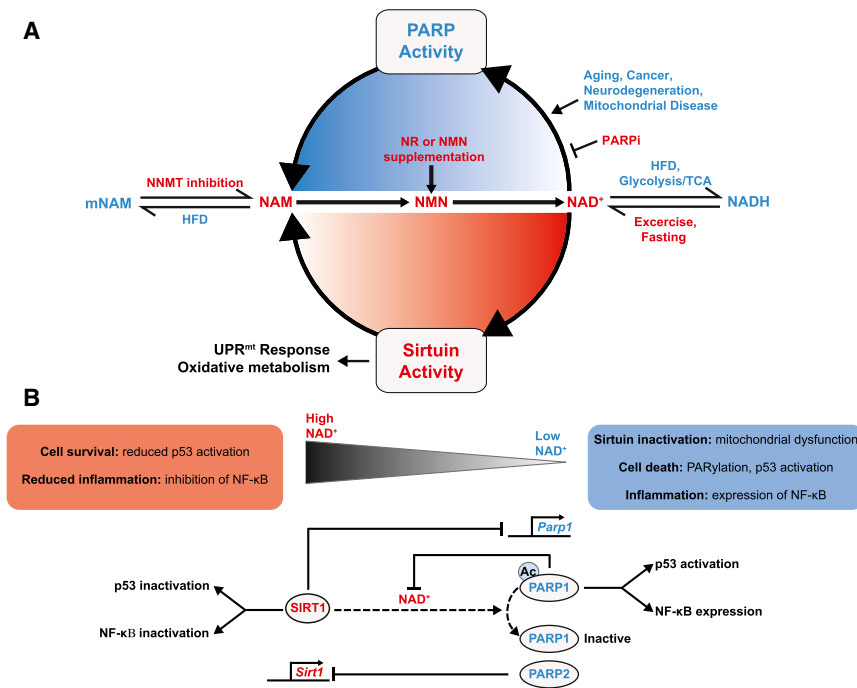
Figure 3. The Reciprocal Relationship between SIRT1 and PARPs during NAD+ Homeostasis and Metabolic Signaling in the Cell

(A) NAD+ is an essential coenzyme for sirtuin, PARP, and CD38 activity, all of which metabolize NAD+ into NAM. Glycolysis and the TCA cycle also consume available NAD+ for the production of NADH, providing reducing equivalents for either lactate dehydrogenase (LDH) or the ETC. Red font indicates environmental or physiological stimuli that activate sirtuins by increasing NAD+, while blue font indicates a reduction in NAD+, thereby diminishing sirtuin activity. NAM can be shunted away from NAD+ production following methylation by NNMT, a pathway activated by a HFD or with long-term or high doses of NAM, which can favor the development of a fatty liver, due to reductions in available methyl groups. In contrast, NNMT depletion by NNMT-antisense oligonucleotides in animals or mNAM supplementation in cells reduces NAM methylation. With a HFD, NAD+ can be reduced by elevating energy availability and NADH production, while exercise, fasting, and CR reverses this process providing more NAD+ for sirtuin activation and protein deacetylation. NR supplementation or intraperitoneal NMN increases NAD+ availability via the NAD+ salvage pathway in mice. Ultimately, SIRT1 induces mitochondrial biogenesis, energy expenditure, antioxidant defenses, and lifespan extension by a mechanism that involves the mitochondrial unfolded protein response (UPRmt). PARPs consume NAD+, reducing SIRT1 activity, by increasing PARylation of DNA and proteins during aging, cancer, neurodegeneration, and mitochondrial diseases.

(B) SIRT1 negatively regulates PARP1 through the inhibition of transcription and possibly through deacetylation. Reciprocally, PARP1 inhibits SIRT1 by limiting NAD+ levels, while PARP2 directly inhibits SIRT1 transcription. Interestingly, PARP1 is required for the transcriptional co-activation of NF-κB, while SIRT1 inhibits NF-κB activity through the deacetylation of RelA/p65. In addition, PARP1 and SIRT1 oppositely regulate p53 nuclear accumulation and activation following cytotoxic stress. Since the K_m of PARP1 for NAD+ is lower than that of SIRT1, as NAD+ levels drop following cell stress or senescence, SIRT1 becomes less effective at regulating PARP1, and inhibiting inflammation or cell death through the inactivation of NF-κB and p53. Dashed arrows indicate pathways that need further validation.

(Du et al., 2011; Tan et al., 2014). Finally, SIRT7 is an NAD+-dependent deacetylase with few known substrates, including p53 in vitro (Vakhrusheva et al., 2008), PAF53 in HeLa cells (Chen et al., 2013), and GABPβ1 in vivo (Ryu et al., 2014). Recent evidence indicates that long-chain deacylation is a general feature of all mammalian sirtuins (Feldman et al., 2013). For example, SIRT1, SIRT2, and SIRT3 can also act as effective decrotonylases (Bao et al., 2014; Feldman et al., 2013). The general deacylase activity of sirtuins, however, can differ in their preferential activity toward certain acyl chain lengths (Feldman et al., 2013).

Physiological Roles of Sirtuins. Generally, most sirtuins are activated during times of energy deficit and reduced carbohydrate energy sources, triggering cellular adaptations that improve metabolic efficiency (Figure 3A). For example, SIRT1 activity increases during exercise (Cantó et al., 2009), CR (Chen et al., 2008), fasting (Cantó et al., 2010; Rodgers et al., 2005), or low glucose availability (Fulco et al., 2008), all of which correlate with higher NAD+ levels. Due to space limitations, we will only briefly summarize the roles of sirtuins. For further information, we refer the reader to recent specialized reviews (Boutant and Canto, 2014; Chang and Guarente, 2013; Houtkooper et al., 2012).

Different nuclear sirtuin orthologs have been shown to influence lifespan in yeast, worms, flies, and mice (Bauer et al., 2009; Boily et al., 2008; Kaeberlein et al., 1999; Kanfi et al., 2012; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). Accordingly, the deacetylation of transcription factors, cofactors, and histones by SIRT1 was shown to be important to enhance mitochondrial metabolism (Boily et al., 2008; Cantó et al., 2009, 2010; Feige et al., 2008; Menzies et al., 2013; Price et al., 2012; Rodgers et al., 2005). Further evidence indicates that SIRT1 is key to linking nutrients to circadian rhythm (Asher et al., 2008; Chang and Guarente, 2013; Nakahata et al., 2008). The tight link between sirtuins and metabolism was reinforced by findings indicating that a moderate SIRT1 overexpression in mice could prevent metabolic and age-related complications, including insulin resistance, obesity, and hepatic steatosis (Banks et al., 2008; Herranz et al., 2010; Pfluger et al., 2008). In addition, pharmacological SIRT1 activation protects against lifespan reductions prompted by HFDs (Baur et al., 2006; Minor et al., 2011). Similarly, SIRT6 overexpression has been shown to increase mouse lifespan (Kanfi et al., 2012). Oppositely, loss-of-function models for SIRT1, SIRT3, and SIRT7 have been linked to a higher susceptibility to metabolic and age-related disease or reduced maximal lifespan (Boutant and Canto, 2014; Hirschey et al., 2011; Ryu et al., 2014; Vakhrusheva et al., 2008), while the absence of SIRT6 causes severe hypoglycemia, leading to mortality within the first month of life (Mostoslavsky et al., 2006; Zhong et al., 2010). *Sirt2*- and *Sirt5*-deficient mice, however, do not display an overt metabolic phenotype in the basal state (Beirowski et al., 2011; Bobrowska et al., 2012;

Table 1. NAD$^+$-Consuming Enzymes

Enzyme	K$_m$ Value (μM)	References
SIRT1	94–96	Gerhart-Hines et al., 2011; Pacholec et al., 2010
SIRT2	83	Borra et al., 2004
SIRT3	880	Hirschey et al., 2011
SIRT4	35	Laurent et al., 2013
SIRT5	980	Fischer et al., 2012
SIRT6	26	Pan et al., 2011
SIRT7	Unknown	
PARP1	50–97	Amé et al., 1999; Jiang et al., 2010; Mendoza-Alvarez and Alvarez-Gonzalez, 1993
PARP2; PARP4 (VPARP)	130; unknown	Amé et al., 1999
Tankyrase 1 (PARP5a); Tankyrase 2 (PARP5b)	1,125–1,500; unknown	Jiang et al., 2010; Rippmann et al., 2002
CD38	15–25	Cakir-Kiefer et al., 2001; Fulco et al., 2008; Sauve et al., 1998

Only poly-ADP-ribosylating PARPs were included in this table.

Yu et al., 2013), while *Sirt4* deficiency, in contrast to most sirtuins, enhances oxidative metabolism (Laurent et al., 2013).

Sirtuins as NAD$^+$ Sensors. Their ability to use NAD$^+$ as a substrate led to speculation that sirtuins could act as metabolic sensors. The activity of sirtuins for a given intracellular NAD$^+$ level is defined by the Michaelis constant, K$_m$, for the reaction. This constant describes the NAD$^+$ concentration when the reaction rate is half of the maximum during NAD$^+$ excess. The estimated total intracellular content of NAD$^+$ in mammals ranges from ∼200 to ∼500 μM (Bai et al., 2011b; Hong et al., 2014; Houtkooper et al., 2010a; Schmidt et al., 2004). The K$_m$ of SIRT1 for NAD$^+$ has been reported to be in the range of 94–96 μM (Table 1) (Gerhart-Hines et al., 2011; Pacholec et al., 2010). The K$_m$ for NAD$^+$, however, can differ very significantly between sirtuins. For example, the K$_m$ for NAD$^+$ of SIRT2, SIRT3, SIRT4, SIRT5, and SIRT6 are reported as 83 μM (Borra et al., 2004), 880 μM (Hirschey et al., 2011), 35 μM (Laurent et al., 2013), 980 μM (Fischer et al., 2012), and 26 μM (Pan et al., 2011), respectively. The affinity of SIRT7 for NAD$^+$ has not been reported to our knowledge. The above numbers help to classify sirtuins into two different categories. First, there are sirtuins, such as SIRT2, SIRT4, and SIRT6, whose activity is unlikely to be rate-limited by NAD$^+$, as NAD$^+$ availability is considerably higher than their K$_m$ values. In contrast, there are other sirtuins, such as SIRT1, SIRT3, and SIRT5, whose K$_m$ for NAD$^+$ falls within the range for physiological changes in NAD$^+$. In this respect, it is important to note that SIRT1 is a nuclear enzyme, and NAD$^+$ concentrations in the nucleus are below 100 μM, while NAD$^+$ levels in the mitochondria can reach millimolar values, suggesting that NAD$^+$ could limit SIRT3 and SIRT5 based on their K$_m$ values.

The above observations indicate that few sirtuins (i.e., SIRT1, SIRT3, and SIRT5) fulfill the key criteria to be considered NAD$^+$ sensors. However, sirtuins are not exclusively regulated by NAD$^+$. For example, NAM, the end product of the sirtuin reaction,

acts as a potent and general sirtuin deacetylase inhibitor. NAM was in fact shown to inhibit Sir2p, the yeast SIRT1 ortholog in a non-competitive manner with NAD$^+$, with an IC$_{50}$ < 50 μM (Anderson et al., 2003; Bitterman et al., 2002; Borra et al., 2004). Thus, sirtuin activity can potentially be differentially regulated by the cellular concentrations of both NAD$^+$ and NAM.

NADH has also been proposed to act as an inhibitor of SIRT1 through competitive binding of the NAD$^+$ pocket (Lin et al., 2004). Yet, the inhibition by NADH only occurs in the millimolar range, considerably above physiological NADH levels (Schmidt et al., 2004; Smith et al., 2009; Zhang et al., 2002). For example, intracellular concentrations of NADH in muscle cells range from 50 to 100 μM (Cantó et al., 2012; Hong et al., 2014). Thus, based on the above findings, the intracellular NAD$^+$/NAM ratio may be a better predictor of sirtuin activity compared to the popularly used NAD$^+$/NADH ratio.

NAD$^+$-Consuming Enzymes (II): Poly(ADP-ribose) Polymerases

Poly(ADP-ribose) polymerases (PARPs) have been the center of intense focus due to their active role in DNA repair, inflammation, and cell death, but they have now also been shown to influence circadian rhythm, neuronal function, endoplasmic reticulum stress, and metabolism, among other cellular pathways (reviewed in Cantó et al., 2013; Gibson and Kraus, 2012; Kraus and Hottiger, 2013). There are 17 different genes encoding PARP-related proteins (Gibson and Kraus, 2012), but most research has so far focused on PARP1 and PARP2, which account for the vast majority of PARP activity in the cell (Cantó et al., 2013). In general, PARP1 and PARP2 can be activated by DNA strand breaks endowing them with a role in the response to DNA damage (Amé et al., 1999; Benjamin and Gill, 1980; Gradwohl et al., 1990). However, PARPs can also be activated by interactions with the phosphorylated form of extra signal-regulated kinases (ERKs) to amplify ERK-mediated histone acetylation events (Cohen-Armon et al., 2007). Furthermore, PARPs are also activated by HSP70 during heat shock stress to alter nucleosome structure and by Trp tRNA synthetase (TrpRS) (Petesch and Lis, 2012; Sajish and Schimmel, 2015). Active PARP catalyzes the transfer of ADP-ribose subunits from NAD$^+$ to protein acceptors, including different nuclear protein substrates, and even itself (a process called auto-poly-ADP-ribosylation), thus forming PAR chains (Kameshita et al., 1984). Classically, PARPs have been shown to play dual roles in the cell that can either result in the induction of cell death or DNA repair. PARP1, for instance, was shown to modify the effectiveness of the p53-mediated DNA damage response for different types of cytotoxic stress (Valenzuela et al., 2002). As a result, PARP inhibition can be an effective treatment for cancer (Bryant et al., 2005; Farmer et al., 2005; Fong et al., 2009), leading to the development of several potent PARP inhibitors as chemotherapeutic agents. From a purely metabolic angle, PARP1 activation has also been linked to a rapid reduction in the glycolytic rate. While this phenomenon has been classically linked to a reduction in NAD$^+$ availability, recent evidence indicates that PARP1 might also directly PARylate hexokinase, leading to a reduction in hexokinase activity and the cellular glycolytic rate (Andrabi et al., 2014; Fouquerel et al., 2014). Indeed, the possible direct impact of PARP activities on metabolic enzymes will be a fascinating area of research for years to come.

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The Competition between PARPs and Sirtuins for NAD+ as a Metabolic Determinant. Upon DNA damage, PARP enzymes utilize NAD+ to generate PAR polymers, yielding NAM as a reaction product. Excessive DNA damage dramatically reduces NAD+ levels (Berger, 1985), even down to 20%–30% of their normal levels (Houtkooper et al., 2010a). In fact, the enzymatic properties of PARP1 indicate that it is an avid NAD+ consumer, with NAD+ increasing up to 2-fold in *Parp1*-KO mouse tissues (Bai and Cantó, 2012). This, in turn, limits NAD+ availability for other nuclear enzymes such as SIRT1 (Figure 3A) (Bai et al., 2011b; Pillai et al., 2005; Qin et al., 2006; Rajamohan et al., 2009). In fact, the K_m of PARP1 is in the ~50–59 μM range, unlike like that of PARP2 (K_m 130 μM), dictating that NAD+ is rarely rate-limiting for PARP1 activity (Table 1) (Amé et al., 1999; Mendoza-Alvarez and Alvarez-Gonzalez, 1993). The lower affinity for, and consumption rate of, NAD+ by PARP2 is in agreement with the lack of change in NAD+ levels when PARP2 is knocked down in cultured cells (Bai et al., 2011a). Interestingly, however, *Parp2* deficiency increased SIRT1 expression as a consequence of a direct negative regulatory effect on the SIRT1 promoter (Figure 3B) (Bai et al., 2011a). This further illustrates how PARP activity leads to SIRT1 inactivation, either by limiting NAD+ levels, in the case of PARP1 (Bai et al., 2011b), or by acting as a transcriptional repressor, in the case of PARP2 (Bai et al., 2011a).

The complexity of this pathway was heightened when SIRT1 was shown to directly inhibit PARP1 via its deacetylation (Figure 3B). Specifically, increased PAR activity was observed in *Sirt1*-KO cells treated with H_2O_2 (Kolthur-Seetharam et al., 2006), while the ability of SIRT1 to deacetylate PARP1 was confirmed by immunoprecipitation experiments (Rajamohan et al., 2009). Furthermore, SIRT1 also negatively regulates PARP1 transcription (Rajamohan et al., 2009). Illustrating the opposing roles of both enzymes, PARP1 is required for the transcriptional co-activation of NF-κB (Hassa et al., 2003), while SIRT1 inhibits NF-κB activity through the deacetylation of RelA/p65 (Yeung et al., 2004). Furthermore, PARP1 and SIRT1 have opposing effects on p53 nuclear accumulation and activation following cytotoxic stress (Figure 3B) (Langley et al., 2002; Luo et al., 2001; Valenzuela et al., 2002; Vaziri et al., 2001). Since the K_m of SIRT1 for NAD+ is higher than that of PARP1, NAD+ levels can become so low following cell stress or senescence that SIRT1 no longer has the activity to keep PARP1 in check. This is supported by the fact that NAD+-repletion by expression of NAMPT can protect against PARP1 overexpression in a SIRT1-mediated manner (Pillai et al., 2005). Thus, it is likely that diverse cellular fates and metabolic decisions are closely regulated by the balance of the reciprocal regulation of SIRT1 and PARP1 activities, under the guidance of NAD+ levels (Figure 3B).

Recent work has further strengthened the hypothesis that PARP1 and SIRT1 have counterbalancing roles in metabolism and aging. For instance, PARP1 activity is enhanced with aging (Braidy et al., 2011; Mouchiroud et al., 2013) and high caloric intake (Bai et al., 2011b) yet reduced upon nutrient scarcity (Bai et al., 2011b). *Parp1* deletion in C57Bl/6 mice confers protection against diet-induced obesity (Bai et al., 2011b). Strikingly, *Parp1*-deficiency on a 129/SvImJ background has been reported to exacerbate HFD-induced obesity (Devalaraja-Narashimha and Padanilam, 2010). In addition, some studies have

shown that *Parp1* deficiency can limit adipocyte function and size, leading to higher hepatic lipid accumulation (Erener et al., 2012). Despite these discrepancies, pharmacological PARP inhibition has consistently rendered protection against diet-induced obesity (Lehmann et al., 2015; Pirinen et al., 2014), possibly through an upregulation of SIRT1-dependent mitochondrial biogenesis and energy expenditure via the mitochondrial unfolded protein response (UPR^mt; see New Perspectives in NAD+ Therapeutics (IV): Aging) (Pirinen et al., 2014). In addition, when the PARP1 worm homolog, *pme-1*, was knocked down in *C. elegans*, worms lived longer and maintained a more youthful phenotype at late adult stages (Mouchiroud et al., 2013). This was correlated to a marked increase in NAD+ availability, Sir2.1 activity, and mitochondrial function that were linked to the activation of the UPR^mt (Mouchiroud et al., 2013). Altogether, most studies certify that a reduction in PARP activity is beneficial against some aspects of metabolic disease.

Importantly, PARP inhibition might lead to higher NAD+ availability in a compartment-specific fashion. In line with the predominant localization of PARP1 to the nucleus, reductions in PARP1 activity/expression markedly increases nucleo/cytoplasmic NAD+ levels and SIRT1 activity, yet it does not alter mitochondrial NAD+ or SIRT3 activity (Bai et al., 2011b; Pirinen et al., 2014). However, this notion will need to be consolidated when further technical developments allow us to better directly measure NAD+ levels in a compartment-specific fashion, most notably in the nucleus.

To strengthen the hypothesis that PARPs can consume NAD+ to the point of impeding metabolism, the AHR target gene, TiPARP (TCDD-inducible poly(ADP-ribose) polymerase or PARP7), was shown to increase PARylation of proteins, reducing NAD+ levels and SIRT1-mediated PGC-1α deacetylation in liver tissue (Diani-Moore et al., 2010). Furthermore, *tankyrase 2 (PARP5b)*-KO mice also have reduced fat pad and body weights, although no connection has yet been made to improvements in tissue NAD+ levels (Chiang et al., 2006). Altogether, these results suggest that ADP-ribosylation by several PARP family members can lead to metabolic dysfunction, suggesting that PARP inhibitors may have beneficial effects in this context.

NAD+-Consuming Enzymes (III): Cyclic ADP-Ribose Synthases

Cyclic ADP-ribose (cADPR), a secondary messenger implicated in Ca^{2+} signaling, cell cycle control and insulin signaling (Malavasi et al., 2008), is produced from NAD+ by cADPR synthases. The family of cADP-ribose synthases, including CD38 and its homolog CD157, were initially described as plasma membrane antigens on thymocytes and T lymphocytes. However, these ectoenzymes have also been found in non-lymphoid tissues, including muscle, liver, and brain (Aksoy et al., 2006b; Quarona et al., 2013). In addition, recent topological studies have described the enzymatic activity of this transmembrane protein as both extra- and intra-cellular (Jackson and Bell, 1990; Lee, 2012; Zhao et al., 2012).

Mice deficient in *Cd38* show significantly elevated levels of NAD+ (10- to 30-fold) in tissues such as liver, muscle, brain, and heart, with corresponding SIRT1 activation, confirming the role of CD38 as a major NAD+ consumer (Figure 3A) (Aksoy et al., 2006b; Barbosa et al., 2007). Conversely, cells

overexpressing CD38 showed reductions in NAD$^+$ levels and in the expression of proteins related to energy metabolism and antioxidant defense, as measured by quantitative proteomic analysis (Hu et al., 2014). Similar to *Parp1*-deficient mice, *Cd38*-KO animals were protected from diet-induced obesity, liver steatosis, and glucose intolerance due to enhanced energy expenditure (Barbosa et al., 2007). In fact, the influence of *Cd38* defiency on metabolism is so dramatic that, despite having lower physical activity compared to WT animals, they still expend more total energy. One potential issue is that CD38-independent cADPR synthase and NAD$^+$-glycohydrolase activity remained present in the developing brain of *Cd38*-KO mice (Ceni et al., 2003). Similarly, studies in heart (Kannt et al., 2012; Xie et al., 2005), skeletal muscle (Bacher et al., 2004), and kidney (Nam et al., 2006) also demonstrated that cADPR synthesis occurs independently of CD38 and CD157, suggesting the existence of other cADPR synthase family member(s). In further support of the existence of additional cADPR synthases, a small-molecule compound screen discovered two potent inhibitors, SAN2589 and SAN4825, that do not inhibit CD38 yet blunt cardiac cADPR synthase activity (Kannt et al., 2012). Despite observations that CD38 inhibition appears to enhance NAD$^+$ levels, further work should clarify its cellular location and specific roles in various tissues to make it a viable therapeutic target.

## Recent Advances in NAD$^+$-Related Therapeutics

Although NA is effective to treat dyslipidemia (Altschul et al., 1955), due to its undesirable effects, niacin derivatives including acipimox and prolonged release forms, such as niaspan and enduracin, have largely replaced NA use in the clinical management of hyperlipidemia. The core of the hypothesis explaining the effectiveness of niacin rested in part on the activation of GPR109A in adipocytes, which apparently mediated the transient reduction of plasma free fatty acid (FFA) levels (Tunaru et al., 2003; Zhang et al., 2005). Yet, more recently, using both a mouse line deficient in *Gpr109* and clinical trials with two GPR109 agonists, it became clear that GPR109 did not mediate niacin's lipid efficacy, thus questioning the GPR109-mediated FFA hypothesis (Lauring et al., 2012). This, in turn, gave strength to the possibility that the effects of niacin relied on the ability of NA or NAM to elevate NAD$^+$ levels and activate sirtuins (Cantó and Auwerx, 2012). Beyond niacin, other NAD$^+$ precursors, such as NMN and NR, are being considered as alternatives to niacin, since they do not activate GPR109A receptors, yet still activate SIRT1 in mice (Cantó et al., 2012). Similarly, the inhibition of PARP or CD38 activities has also proven to enhance NAD$^+$ levels and sirtuin action (Figure 3A). Further disqualifying a GPR109-mediated effect and in support an NAD$^+$-mediated metabolic response, in human type 2 diabetes patients, acipimox increases muscle mitochondrial function, which is accompanied by a mitonuclear protein imbalance and the induction of the UPR^{mt} (see New Perspectives in NAD$^+$ Therapeutics (I): Metabolic Disease and New Perspectives in NAD$^+$ Therapeutics (IV): Aging), hallmarks of SIRT1, in lieu of GPR109, activation (van de Weijer et al., 2014). In the next section, we will hence discuss the therapeutic targets, the prospective clinical indications, and the potential limitations for NAD$^+$-boosting compounds that activate sirtuins.

New Perspectives in NAD$^+$ Therapeutics (I): Metabolic Disease

Introducing New NAD$^+$ Precursors: NR and NMN. NR was recently demonstrated to have a surprisingly robust effect on systemic metabolism. First, dietary supplementation with NR protected against diet-induced obesity (Cantó et al., 2012). NR treatment increased both intracellular and mitochondrial liver NAD$^+$ levels, concomitant to an enhancement of SIRT1 as well as SIRT3 activities (Cantó et al., 2012). As a result, there was a SIRT1-dependent increase in FOXO1 deacetylation, along with elevations in SOD2 expression, a FOXO1 target gene. Furthermore, in the mitochondrial compartment, NR led to the deacetylation of the well-established SIRT3 targets SOD2 and NDUFA9. In line with the activation of SIRT1 and SIRT3 targets, mitochondrial content was higher in skeletal muscle and brown adipose tissue of NR-treated high-fat-fed animals, which increased the use of lipids as energy substrates, boosted energy expenditure, and improved insulin sensitivity (Cantó et al., 2012). In alignment, impaired glucose tolerance and glucose-stimulated insulin secretion, induced by NAD$^+$ shortages in NAMPT-deficient heterozygous animals, could be corrected by the administration of NMN (Revollo et al., 2007). Similarly, intraperitoneally administered NMN ameliorates glucose homeostasis in age- and diet-related insulin-resistant states (Ramsey et al., 2008; Yoshino et al., 2011). Importantly, NMN reversed the loss of NAD$^+$ levels observed in both circumstances. As with NR, NMN also safeguarded mitochondrial function in mice and improved age-related mitochondrial dysfunction (Gomes et al., 2013). Knockdown of the nuclear-localized NMNAT1 attenuated the effect of NMN, consistent with the effect of NMN being driven by increases in NAD$^+$ levels (Gomes et al., 2013). Furthermore, as NMNAT1 is located in the nucleus, the nuclear NAD$^+$ pool may play a more dominant role for the induction of mitochondrial-encoded OXPHOS transcripts, potentially through alterations in SIRT1-directed HIF1α destabilization, leading to c-Myc activation of the nuclear-encoded mitochondrial factor TFAM (Gomes et al., 2013). Although these findings support the use of NR or NMN as a strategy for healthy aging, their efficacy in humans still needs testing. In fact, the dosages used for NR and NMN in mice, 400–500 mg/(kg·day), are high and potentially suboptimal for human application. Unlike NR, the use of NMN in mice has relied on intraperitoneal delivery, which could further complicate clinical use. Thus, the dosages, routes of administration, and efficacy of NAD$^+$ boosters need to be optimized for human use.

Rejuvenating Old NAD$^+$ Precursors: The Complexities around NAM. NAM, was first associated with diabetes when it was shown to protect against streptozotocin (STZ)-induced diabetes (Schein et al., 1967), which is accompanied by a robust reduction of NAD$^+$ levels in pancreatic islet cells. NAM, but not NA, can recover this drop in NAD$^+$ levels (Ho and Hashim, 1972). Later, it was demonstrated that the NAD$^+$ reduction induced by STZ was due to increased DNA damage, stimulating PARP1 activity (Yamamoto et al., 1981).

Unlike other NAD$^+$ precursors, NAM has the capacity to exert end product inhibition on SIRT1 deacetylase activity. However, long-term NAM treatment increases NAD$^+$ levels via the NAD$^+$ salvage pathway, which likely tips the balance of the NAD$^+$/NAM ratio such that SIRT1 is activated. Despite NAM being
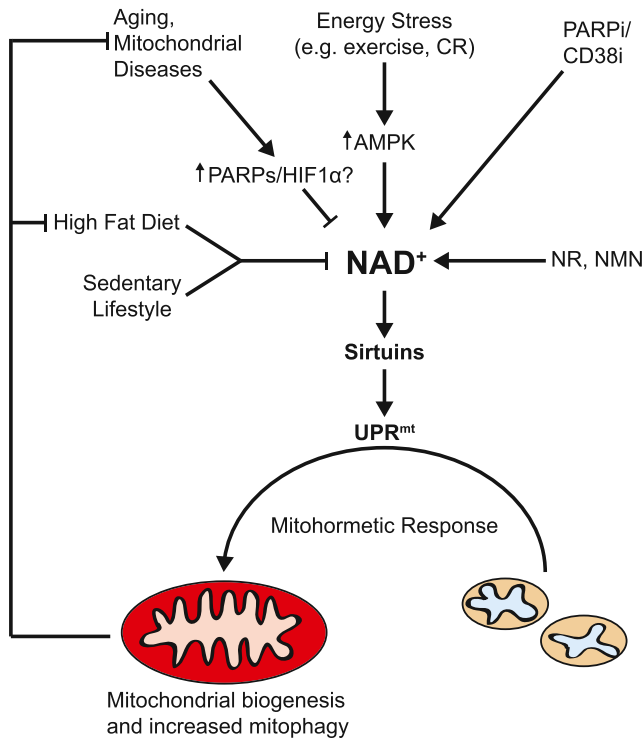
Figure 4. Energy Stress, NAD+-Dependent UPR[mt] Signaling, and Mitochondrial Health
The aging process and associated metabolic diseases, including obesity and mitochondrial diseases, can be improved in mice and *C. elegans* using NAD+ boosters or PARP/CD38 inhibitors (PARPi/CD38i) in much the same way as has been demonstrated by CR. Part of the metabolic decline during aging is due to a PARP-directed reduction in NAD+ levels, attenuating SIRT1 and FOXO3A activities and leading to the activation of HIF1α and an increased reliance on glycolysis. Recently, a mechanism has been proposed for these NAD+-mediated improvements that include the induction of the UPR[mt], which is triggered by SIRT1- and SIRT3-induced mitochondrial biogenesis, creating an imbalance in mitochondrial- versus nuclear-encoded mitochondrial proteins. This mitonuclear imbalance activates the UPR[mt], a retrograde signal that induces a mitohormetic and adaptive nuclear response, ultimately repairing and improving mitochondrial function. These mitohormetic signals can attenuate the impact of aging, mitochondrial diseases, or a HFD on metabolism.

suggested as a treatment for type 1 diabetes (Olmos et al., 2006), clinical trials failed to confirm this hypothesis (Cabrera-Rode et al., 2006; Gale et al., 2004). More recently, OLETF rats, a rodent model of obesity and type 2 diabetes, exhibited profound metabolic improvements following NAM treatment (100 mg/kg for 4 weeks). This treatment induced liver NAD+ levels, which were complimented by enhanced glucose control (Yang et al., 2014). However, some reports indicate that long-term or high doses of NAM are detrimental because they favor the development of a fatty liver, due to reductions in available methyl groups (Kang-Lee et al., 1983). For instance, NAM administration for 8 weeks (1–4 g/kg) resulted in methyl group deficiency, which is likely due to the conversion of NAM into 1-methyl-NAM (mNAM) by nicotinamide n-methyltransferase (NNMT) (Figure 3A). NNMT shunts NAM away from NAD+ using S-adenosylmethionine (SAM) as a methyl donor (Aksoy et al., 1994; Riederer et al., 2009). In line with this hypothesis, supplementation of methionine, a methyl group donor, prevented the

formation of steatohepatosis caused by high doses of NAM (Kang-Lee et al., 1983).

Recently, NNMT expression was found to be negatively correlated with GLUT4, the insulin-responsive glucose transporter, in adipose tissue (Kraus et al., 2014). In adipose-specific *Glut4*-KO mice, *Nnmt* transcripts are increased, while they are reduced in adipose-specific *Glut4*-overexpressing mice *Nnmt* (Kraus et al., 2014). Similarly, *Nnmt* transcripts were increased in the WAT of *ob/ob*, *db/db*, and high-fat-fed mice compared to lean insulin-sensitive controls (Kraus et al., 2014). In addition, tissue specific knockdown of *Nnmt* in WAT and liver, using antisense oligonucleotides, protected against diet-induced obesity by increasing the expression of *Sirt1* target genes and energy expenditure. Accordingly, treating adipocytes with mNAM, which acts as an end product inhibitor of NNMT (Aksoy et al., 1994), increased O2 consumption (Kraus et al., 2014). Coming from a totally different angle, a germline mouse model deficient in *Maf1*, a repressor of RNA polymerase III transcription of highly abundant cellular RNAs (Upadhya et al., 2002), also underscored the importance of NNMT in NAD+ homeostasis. *Maf1*−/− mice are resistant to obesity due to metabolic inefficiency as a consequence of futile tRNA production, which led to extreme reductions of NNMT levels, boosting NAM salvage to regenerate NAD+ (Bonhoure et al., 2015). In combination, these independent studies in widely different mouse models support that NNMT inhibition enhances NAD+-dependent SIRT1 activity and protects mice against obesity and type 2 diabetes. In a seemingly contradictory fashion, work in *C. elegans* has shown that NNMT and the methylation of NAM might actually be an integral part of the mechanism by which sirtuins provide healthspan and lifespan benefits (Schmeisser et al., 2013). For this, NNMT-produced mNAM would act as a substrate to the ortholog of the mammalian aldehyde oxidase (AOx1), GAD-3, to generate hydrogen peroxide, which acts as a mitohormetic reactive oxygen species signal (Schmeisser et al., 2013). Taken together, however, NNMT activity seems strongly regulated in diverse metabolic contexts and has a major impact on NAD+ homeostasis. The discrepancies in the current findings might arise from the distinct models used (i.e., worms vs. mice) and the amplitude of the mitohormetic response in different metabolic scenarios.

The Potential for PARP Inhibition in Cell Metabolism. The potential of PARP inhibition as a treatment for metabolic complications was first suggested by the observation that *Parp1*-KO mice were protected from STZ-induced β cell death and dysfunction by maintaining NAD+ levels and therefore glucose tolerance (Masutani et al., 1999). *Parp1*-KO animals exhibit higher mitochondrial content, increased energy expenditure, and protection against metabolic disease brought on by a HFD (Bai et al., 2011b). Correspondingly, PARP inhibitors also prevent carbon-tetrachloride-induced liver mitochondrial dysfunction and fibrosis (Mukhopadhyay et al., 2014), and diet-induced obesity in mice (Pirinen et al., 2014). Long-term treatment of up to 18 weeks with the dual PARP1 and PARP2 inhibitor MRL-45696 was shown to enhance exercise capacity and muscle mitochondrial function in chow-diet-fed mice (Cerutti et al., 2014; Pirinen et al., 2014). Both in worm and mouse models, the effect of PARP inhibition on mitochondrial function was linked with the activation of the UPR[mt], as reflected by the

induction of HSP60 and CLPP, two UPR^{mt} biomarkers (Mouchiroud et al., 2013; Pirinen et al., 2014) (Figure 4). In fact, PARP inhibitors increased mitochondrial translation without coordinate changes in cytosolic translation rates, thus leading to a mitonuclear protein imbalance (Pirinen et al., 2014), which in turn triggers the UPR^{mt} to maintain optimal mitochondrial function (Houtkooper et al., 2013). This finding is in line with the recent discovery that mitochondrially located PARP1 activity may PARylate and disrupt the interaction between key mitochondrial-specific DNA base excision repair (BER) enzymes, namely EXOG and DNA polymerase gamma (Polγ), and the mitochondrial DNA (mtDNA), hindering mitochondrial biogenesis and reducing mtDNA copy numbers (Szczesny et al., 2014).

While the above observations set the stage for PARP inhibition to treat complex human metabolic diseases, it is important that inhibitors are selective for PARP1 and do not affect other members of the PARP family. For instance, although *Parp2*-KO mice were protected from diet-induced obesity, they were glucose intolerant due to defective pancreatic function (Bai et al., 2011a). In this sense, although diverse and highly efficient PARP inhibitors exist and are currently used in humans for anti-cancer therapy (Curtin and Szabo, 2013), none of them are selective for PARP1. Furthermore, since several of the PARPs play key roles in DNA damage repair upon genotoxic stress (Curtin and Szabo, 2013), further work must also ensure the long-term safety of selective PARP1 inhibition to treat metabolic diseases.

Inhibition of cADP-Ribose Synthases Improves Metabolism. As described above, cADP-Ribose synthases, such as CD38, are primary NADases in mammalian tissues with a strong impact on SIRT1 activity (Aksoy et al., 2006a; Escande et al., 2010). This led to the hypothesis that CD38 inhibition (and subsequent increases in NAD$^+$ levels) could be applied to treat metabolic disorders. In line with this, mice lacking CD38 are protected against diet-induced metabolic disease (Barbosa et al., 2007). Some natural flavonoids, such as quercetin, apigenin, luteolinidin, kuromanin, and luteolin, were found to inhibit CD38 in the low micromolar range (Escande et al., 2013; Kellenberger et al., 2011). Accordingly, quercetin and apigenin increased liver NAD$^+$ levels and SIRT1 activity resulting in improved glucose homeostasis and fatty acid oxidation in the liver of these mice (Escande et al., 2013). However, the recent development of potent thiazoloquin(az)olinone inhibitors for CD38, which can enhance NAD$^+$ levels in multiple tissues, may prove to be effective for the design of future therapies (Haffner et al., 2015). Yet, as discussed in NAD$^+$-Consuming Enzymes (III): Cyclic ADP-Ribose Synthases, there remain several issues that require further work before CD38 inhibitors can be recommended to treat metabolic dysfunction. First, it is not entirely clear that CD38 is the main cADP-ribose synthase enzyme, therefore potentially compromising the efficacy of CD38 inhibitors for clinical use. Second, despite evidence indicating that it might also exist in nuclear and mitochondrial fractions (Aksoy et al., 2006a), CD38 activity is highest on the extracellular side of the plasma membrane, where NAD$^+$ levels are generally very low (De Flora et al., 1997). Finally, the increase in NAD$^+$ observed in *Cd38*-deficient mice is $\sim$30-fold, while most other strategies described to date lead to a $\sim$2-fold increase in NAD$^+$ at best. The massive effect of CD38 on NAD$^+$ levels could therefore be indicative for major alterations in additional NAD$^+$-utilizing metabolic pathways.

New Perspectives in NAD$^+$ Therapeutics (II): Neurodegenerative Disease

Although the elimination of neurons by axonal degradation plays a role in normal nervous system development, aberrant neuronal cell death is typical of insults such as trauma, and chemical toxicity or of aging and neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease (AD), and amyotrophic lateral sclerosis (for review, see Wang et al., 2012).

Controversial Links between NMNAT and Neurodegenerative Phenotypes. Axon degradation had originally been assumed to be a passive process. However, this view changed with the characterization of the naturally occurring Wallerian degeneration slow (Wld^S) dominant mutation (Conforti et al., 2000). Rodent carriers of this mutation displayed a dramatic reduction in axonal degeneration in both central and peripheral neurons. The Wld^S mutant protein is a chimeric protein composed of the complete sequence of NMNAT1 fused to the ubiquitination factor E4B at the N terminus (Conforti et al., 2000; Mack et al., 2001). Efforts from diverse labs have since confirmed that it is the NMNAT enzymatic activity that is required to delay axon degeneration (Araki et al., 2004; Conforti et al., 2009; Gilley and Coleman, 2010; Llopis et al., 2000; Sasaki et al., 2009; Yahata et al., 2009; Yan et al., 2010), probably by promoting an increase in NAD$^+$-directed SIRT1 activity (Araki et al., 2004). Interestingly, Wld^S mutant mice exhibit enhanced insulin secretion from isolated islets with an improvement in glucose homeostasis, also via an NAD$^+$-directed activation of SIRT1 (Wu et al., 2011). Another study specifically demonstrated that it is the cytosolic distribution of NMNAT proteins that is crucial for slowing Wallerian degeneration (Sasaki et al., 2009). Further work should define changes in nuclear and cytosolic NAD$^+$ levels, as most studies measure NAD$^+$ in whole brain lysate, the outcome of which is confounded by the high level of NAD$^+$ found in neuronal mitochondria.

NAD$^+$ Precursors Protect against Neurodegenerative Disease. Following the injury of neurons, there is an induction of multiple transcripts for NAD$^+$ biosynthetic enzymes, including a more than 20-fold increase in NRK2, which catalyzes the synthesis of NAD$^+$ from NR, suggesting a compensatory response to elevate NAD$^+$ levels (Sasaki et al., 2006). In line with this, the pretreatment of neurons with either high levels of NAD$^+$ in cell culture, or precursors such as NMN or NR, protects against axonal degeneration following axotomy, hearing loss caused by excess manganese toxicity, or even noise-induced hearing loss in mice (Brown et al., 2014; Gerdts et al., 2015; Sasaki et al., 2006; Wang et al., 2014b). Similarly, rodent studies have demonstrated that pharmacological doses of NAM increases NAD$^+$ biosynthesis and provides protection against ischemia (Klaidman et al., 2003; Sadanaga-Akiyoshi et al., 2003), fetal-alcohol-induced neurodegeneration (Ieraci and Herrera, 2006), and fetal ischemic brain injuries (Feng et al., 2006) by preventing NAD$^+$ depletion. Further supporting the role of NAD$^+$ in neuroprotection, a high-throughput screen identified an aminopropyl carbazole chemical P7C3 (Pieper et al., 2010), which was only recently discovered to be a pharmacological activator of NAMPT (Wang et al., 2014a), but had previously been shown to possess neuroprotective activity in models of traumatic brain injury

(Yin et al., 2014), Parkinson's disease (De Jesús-Cortés et al., 2012), and amyotrophic lateral sclerosis (Tesla et al., 2012). Increasing the activity of existing NAMPT using similar pharmacological approaches may therefore improve NAD$^+$ depletion in aged animals, exhibiting reduced NAMPT and impairments in neural stem/progenitor cell self-renewal and differentiation, a treatment phenomenon already demonstrated using NMN on aging mice (Stein and Imai, 2014). In another model of neuronal degeneration, raised NAD$^+$ levels after CR attenuated increases in AD-type β-amyloid content in a rodent model of AD (Qin et al., 2006). NAM was also able to improve β-amyloid peptide (1–42)-induced oxidative damage and therefore protect against neurodegeneration (Turunc Bayrakdar et al., 2014a; Turunc Bayrakdar et al., 2014b). Similarly, exposing neuronal cells to toxic prion proteins, to model protein misfolding in Alzheimer's and Parkinson's disease, induced NAD$^+$ depletion that was improved with exogenous NAD$^+$ or NAM (Zhou et al., 2015). Additionally, NR has been shown to improve the AD phenotype via PGC-1α-mediated β-secretase (BACE1) degradation and the induction of mitochondrial biogenesis (Gong et al., 2013).

Maintaining NAD$^+$ levels seems to, hence, sustain basal metabolic function and health in neurons. Furthermore, based on the preliminary evidence above, NR might have a privileged position among different NAD$^+$ precursors in the prevention of neurodegeneration, as the effect of NR may be enhanced by the increase in NRK2 during axonal damage.

The Role of PARPs in Neurodegeneration. The depletion of NAD$^+$ in neurodegeneration has been generally attributed to the activation of PARP enzymes. Well-known neurodegenerative DNA repair disorders include ataxia-telangiectasia (AT), Cockayne syndrome (CS), and xeroderma pigmentosum group A (XPA), all of which demonstrate mitochondrial dysregulation due to SIRT1 inhibition and a reduction in mitophagy, the process of autophagic clearance of defective mitochondria (Fang et al., 2014). The reduction in SIRT1 activity and mitophagy in XPA-, CSB-, and ATM-deficient cells can be attributed to the aberrant activation of PARP1, as reflected by the ability of PARP inhibitor AZD2281 (olaparib) to rescue the mitochondrial defect in cells and to extend the lifespan of *xpa-1* mutant worms (Fang et al., 2014). In extension of these findings, using NR or a PARP inhibitor both improved the phenotype of a mouse model of Cockayne Syndrome group B (CSB), an accelerated aging disorder featuring the disinhibition of PARP activity by CSB protein, through SIRT1-mediated improvements of metabolic, mitochondrial, and transcriptional alterations (Scheibye-Knudsen et al., 2014). Similarly, augmented PARylation in the *Csa$^{-/-}$/Xpa$^{-/-}$* (CX) mouse model of cerebellar ataxia was reduced upon NR treatment, which improved NAD$^+$ levels, SIRT1 activity, and mitochondrial function (Fang et al., 2014). Both interventions using NAD$^+$ precursors or PARP inhibition could hence be helpful to improve neurodegenerative phenotypes.

New Perspectives in NAD$^+$ Therapeutics (III): Cancer and Cell Fate

Genomic stress is the root of all cancers, and so maintaining genome integrity is an essential tool for the prevention of cancer. The fact that PARPs and several sirtuin enzymes are key for genomic maintenance suggests that the regulation of NAD$^+$ could have an impact on cancer susceptibility and development (Cantó et al., 2013). As protectors of genomic stability, PARPs can potentially play a multifunctional role in various cancer-related processes, including DNA repair, recombination, cell proliferation, or cell death. In general, PARP activity protects cancer cells, especially those with high genome instability, from cellular death. Thus, PARP inhibitors are currently being clinically studied for the treatment of cancers that result from dysfunctional homologous DNA recombination repair (Curtin and Szabo, 2013). While a role for SIRT1 in cancer has been controversial, transgenic animal models demonstrate that SIRT1 protects against age-related carcinomas and sarcomas, but not lymphomas (Herranz et al., 2010). Given that PARP activity is rarely affected by physiological changes in NAD$^+$ availability, it would be intuitive to think that most effects derived from fluctuations in NAD$^+$ levels might crystalize into expected outcomes of modulating the activity of some sirtuins, such as SIRT1 (i.e., protection against cancer). In this sense, it has been demonstrated that niacin supplementation can decrease the development of skin cancer (Gensler et al., 1999), while NR can both reduce the incidence of cancer and have a therapeutic effect on fully formed tumors in a genetic mouse model for liver cancer (Tummala et al., 2014). Conversely, niacin deficiency can enhance cancer susceptibility, indicating that cellular NAD$^+$ levels are inversely related to the incidence of cancer (Benavente et al., 2012; Jacobson, 1993). In another approach, some evidence suggests that the protective effects of niacin against the development of skin cancer are due to elevation in both PARPs and SIRT1 activity (Benavente et al., 2012). First, niacin can effectively restore NAD$^+$ levels and Poly(ADP)-ribosylated proteins in keratinocytes following photodamage, indicating an increase in the activity of PARPs. Second, SIRT1, also related to DNA repair and maintaining genomic stability and nucleotide excision repair pathways (Fan and Luo, 2010; Wang et al., 2008), exhibited increased activity in this same model, as evidenced by increased protein deacetylation. However, the balance of SIRT1 and PARP activities upon genotoxic stress is further complicated by the fact that SIRT1 may reduce the expression or be a direct inhibitor of PARP1 (Kolthur-Seetharam et al., 2006; Rajamohan et al., 2009), and PARP2 is a negative regulator of SIRT1 expression (Bai et al., 2011a). This emphasizes the potential for an NAD$^+$-dependent failsafe mechanism that can decide to elicit either repair or apoptosis depending on the severity of the cellular genotoxic insult through the manipulation and balance of SIRT1 and PARP activity levels. Furthermore, SIRT6, which, as described above, most likely does not act as an NAD$^+$ sensor (see NAD$^+$-Consuming Enzymes (I): Sirtuins), can activate PARP1 to stimulate highly efficient double-strand break repair, but only in response to oxidative-stress-induced DNA damage (Mao et al., 2011). In addition, SIRT3 and SIRT5 have both been shown to play roles as either tumor suppressors or oncogenes depending on the cellular and molecular context, while SIRT4 acts as a tumor repressor due to its repression of glutamine metabolism, a process that is essential during rapid cell proliferation, as is seen in cancer (reviewed in Kumar and Lombard, 2015).

In a more speculative territory, it should also be highlighted that higher NAD$^+$ levels, either through PARP inhibition or NAD$^+$ precursor supplementation, rewire metabolism and enhance oxidative versus glycolytic metabolism. Most cancer cells rely indisputably on glycolytic metabolism. Therefore, the metabolic

remodeling by enhanced NAD+ levels could constitute a complementary mechanism to slow down cancer progression or initiate cell death. However, NAD+ depletion might also inhibit growth of several cancers. NAMPT has been found to be overexpressed in several types of tumors, and its expression is associated to tumor progression (Bi et al., 2011; Hasmann and Schemainda, 2003; Van Beijnum et al., 2002; Wang et al., 2011). Consequently, several studies showed that the NAD+ depletion triggered by the downregulation of NAMPT activity can reduce tumor cell growth and sensitize cells to chemotoxic agents (Bi et al., 2011; Hasmann and Schemainda, 2003; Wang et al., 2011; Watson et al., 2009). One must keep in mind that, in most cancer cells, PARPs are activated due to DNA damage and genome instability, leading to NAD+ depletion in cancer cells (Garten et al., 2009). As a result, the downregulation of NAMPT sensitizes cancer cells to DNA-damaging agents and apoptosis. In addition, NAD+ depletion also impairs glycolytic capacity in tumor cells (Bai and Cantó, 2012). Altogether, the above data suggest that both NAD+-boosting and depletion can impact on tumor development and progression depending on the type and the metabolic properties of the tumor.

New Perspectives in NAD+ Therapeutics (IV): Aging
We have only recently started to understand the key pathways involved in defining lifespan. Much of this insight came from studies on CR, the most consistent intervention that extends longevity (as reviewed in Cantó and Auwerx, 2009). Despite some evidence disputing the link between sirtuins and the longevity effects of CR, or longevity in general (Burnett et al., 2011; Jiang et al., 2000; Lamming et al., 2005), most data agree that sirtuin activation in mammals delays the onset of age-related degenerative processes (Herranz et al., 2010; Kanfi et al., 2012; Pearson et al., 2008; Satoh et al., 2013) and that defective sirtuin activity impairs some of the metabolic and physiological benefits triggered by CR (Boily et al., 2008; Hallows et al., 2011; Mercken et al., 2013; Someya et al., 2010). Of note, SIRT1 and SIRT3 have so far been the sirtuins most tightly linked to adaptations during CR. As discussed in NAD+-Consuming Enzymes (I): Sirtuins, the enzymatic characteristics of these sirtuins, but not of other sirtuins, may allow them to act as predominant NAD+ sensors, enabling them to monitor changes in nutrient availability.

During aging, reductions in NAD+ have been consistently observed in worms, diverse rodent tissues—including liver, pancreas, kidney, skeletal muscle, heart, and white adipose— and in human skin samples (Braidy et al., 2011; Gomes et al., 2013; Khan et al., 2014; Massudi et al., 2012; Mouchiroud et al., 2013; Yoshino et al., 2011). Several hypotheses can explain the reductions in NAD+ levels during aging. The first relies on reductions in NAMPT expression with age, which may be in part due to the dysregulation of the circadian rhythm and therefore the CLOCK/BMAL regulation of NAMPT (Nakahata et al., 2009). Another possible explanation for this lies in the higher PARP activity (due to cumulative DNA damage or alternative pathways of PARP activation, such as inflammatory or metabolic stress) observed in old worms and tissues from aged mice (Braidy et al., 2011; Mouchiroud et al., 2013). Supporting this possibility, blocking PARP activity is enough to recover NAD+ levels in aged organisms (Mouchiroud et al., 2013). The age-related reduction in NAD+, in turn, compromises mitochondrial

function, which can be recovered via PARP inhibition or NAD+ precursor supplementation (Gomes et al., 2013; Mouchiroud et al., 2013). These observations are in line with the protection from metabolic dysfunction and disease in mice with genetically or pharmacologically triggered deficiencies in PARP activity (Bai et al., 2011b; Pirinen et al., 2014) or in mice treated with NR (Cantó et al., 2012) or NMN (Yoshino et al., 2011). As found in natural aging, significant reductions in skeletal muscle NAD+ occur in Deletor mice, a mouse model containing a mutation in the mitochondrial replicative helicase Twinkle, resulting in the accumulation of damage and a progressive muscle myopathy (Khan et al., 2014). Treatment of Deletor mice with NR delayed early- and late-stage disease progression by increasing mitochondrial biogenesis in skeletal muscle and brown adipose tissue while preventing mtDNA damage (Khan et al., 2014). In line with this, NR and PARP inhibition also improve the respiratory chain defect and exercise intolerance in Sco2 knockout/knockin mice, another model for mitochondrial disease (Cerutti et al., 2014). Altogether, the above data demonstrate that NAD+ supplementation maintains mitochondrial function, not only upon age-related decline but also in genetically determined mitochondrial diseases that are known to accelerate the aging process (Figure 4).

The Induction of UPRmt by NAD+ as a Mechanism to Enhance Longevity. The ability of NAD+ to induce a mitonuclear protein imbalance could provide a key link between NAD+ and mitochondrial function. Mitonuclear protein imbalance can be defined as a stoichiometric difference between nuclear and mitochondrial-encoded respiratory subunit proteins (Houtkooper et al., 2013), an effect known to promote longevity in worms (Durieux et al., 2011; Houtkooper et al., 2013). Forced expression of genes regulating UPRmt, such as Hsp60 paralogs, in Drosophila slows age-dependent mitochondrial and muscle dysfunction due to the compensatory actions of UPRmt signaling (Owusu-Ansah et al., 2013). Likewise, the activation of the UPRmt by targeting mitochondrial ribosomal protein (Mrp) translation, using a knockdown of ribosomal proteins or antibiotics that specifically inhibit mitochondrial translation, increases longevity in C. elegans (Houtkooper et al., 2013). Strikingly, even subtle changes in expression levels of the Mrp's in the BXD mouse genetic reference population have robust effects on mouse lifespan (Houtkooper et al., 2010b; Wu et al., 2014). Similarly, both NR and PARP inhibitors increased C. elegans lifespan via an induction of the UPRmt by Sir-2.1, the worm SIRT1 ortholog (Mouchiroud et al., 2013). Part of the metabolic decline during aging is due to a PARP-directed decay in NAD+ levels, leading to reduced SIRT1, or Sir-2.1, activity and subsequent reduction in FOXO3A/Daf-16 activity and anti-oxidant defense (Mouchiroud et al., 2013) and increased HIF1α-led glycolytic reliance (Gomes et al., 2013). Furthermore, Sir-2.1-directed longevity is blunted in worms deficient in ubl-5 (Mouchiroud et al., 2013), an essential component for UPRmt-directed communication from the mitochondria to the nucleus (Durieux et al., 2011), solidifying the necessity of UPRmt induction for NAD+-induced longevity. In this sense, the decline in NAD+ observed with aging deregulates the mitonuclear protein balance and respiratory function, which accelerates the aging process. Importantly, mitochondrial dysfunction in aged mice or worms could be recovered following NMN or NR treatment (Gomes et al., 2013; Mouchiroud et al., 2013). The

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above results highlight how the UPR^{mt} triggers an adaptive mitohormetic response as long as the cell is properly furnished with NAD$^+$. However, in situations of limited NAD$^+$ availability, SIRT1 fails to drive mitohormesis and, hence, mitochondria remain dysfunctional. As a whole, the above demonstrates a tight feedback loop between the mitonuclear protein imbalance and UPR^{mt} on the one hand and NAD$^+$ metabolism and SIRT1 activity on the other. In summary, this assigns a crucial role for NAD$^+$ to synchronize the nuclear and mitochondrial genomes.

The identification of UPR^{mt} as a main mechanism by which NAD$^+$ levels modulate mitochondrial fitness constitutes a major leap forward in our understanding of the molecular mechanisms driving a healthy lifespan. Importantly, UPR^{mt} is not just the principle mechanism by which NAD$^+$ affects longevity, but is also a key mode of action for other well-studied longevity compounds, such as rapamycin and resveratrol (Houtkooper et al., 2013).

Conclusions
The association between metabolism, health, and lifespan have long been proposed based on similarities between metabolic dysfunction and disease (e.g., obesity, diabetes, neurodegeneration, and cancer) and the aging process. Only recently have these processes been linked so tightly by multiple proteins, including sirtuins and PARPs, all of which are tightly controlled by the regulation and subcellular balance of the metabolite NAD$^+$. As such, we have never been so close to solving the ancient question of how we age and what we can do to slow this process while simultaneously not compromising on our quality of life. Despite these insights, several aspects of NAD$^+$ metabolism remain obscure. On one side, the complex detection and quantification of NAD$^+$ metabolites and fluxes has not yet allowed us to obtain a clear picture of how different NAD$^+$ precursors are metabolized to feed cells and tissues. We also speculate that additional proteins controlling the supply or salvage of NAD$^+$, along with proteins that are controlled by NAD$^+$ levels, will be identified. Furthermore, the potential preventive and therapeutic use of NAD$^+$-boosting strategies requires an assessment of the bioavailability and effectiveness of various precursor doses in human therapy. In addition, new NAD$^+$ boosters are welcomed since the side effects of niacin generally lead to poor compliance, despite its known efficacy in a myriad of diseases. Therefore, the dosing and safety of these new NAD$^+$ boosters (e.g., NAD$^+$ precursors, CD38 inhibitors, and PARP inhibitors) must be thoroughly assessed to translate these exciting insights into NAD$^+$ biology toward human relevance.

AUTHOR CONTRIBUTIONS

C.C. and K.J.M. both designed the outline and wrote the text. J.A. contributed to the ideas that make up this review and provided edits to all sections.

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