# Chapter 2
# NAD$^+$ as a Pharmacological Tool to Boost Sirtuin Activity

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## 2.1 Introduction

The sirtuins constitute a family of proteins with homology to the yeast protein *silent information regulator 2* (Sir2p), a protein involved in yeast mating type regulation (Ivy et al. 1986; Rine and Herskowitz 1987). The sirtuin field was boosted by the discovery of the anti-aging effect of the yeast Sir2p. Overexpression of *sir2* in yeast extends lifespan while deletion of the gene results in short lifespan (Kaeberlein et al. 1999). These results were soon extended to other species, demonstrating involvement of the Sir2p ortologues in *C. elegans* (*sir-2.1*) and *D. melanogaster* (Sir2) in the aging process (Rogina and Helfand 2004; Tissenbaum and Guarente 2001). Even though these studies are now contested (Burnett et al. 2011), it is well accepted that sirtuins play a major role in metabolic control and are a central target for the treatment of metabolic diseases (reviewed in (Houtkooper et al. 2012)).

A second major discovery that catapulted the field forward is the clarification of the enzymological characteristics of sirtuin enzymes. Yeast Sir2 was shown to possess deacetylase activity, for which it is dependent on nicotinamide adenine dinucleotide (NAD), in particular its oxidized form NAD$^+$ (Imai et al. 2000). Considering that NAD is a classical cofactor in enzymatic redox reactions (Houtkooper et al. 2010), it was tempting to speculate that sirtuins could act as metabolic sensors. Indeed, when yeast or flies were calorie restricted (CR), lifespan increased in a Sir2-dependent fashion (Lin et al. 2000; Rogina and Helfand 2004). These results led to the hypothesis that regulation of NAD$^+$ levels could modulate sirtuin activity and thereby mimic the beneficial effects of CR.

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In this chapter, I will discuss the pathways that are involved in $NAD^+$ homeostasis and how this balance can be tipped into a favorable direction to prevent metabolic decline.

## 2.2 NAD Biosynthesis

Initial work on the biosynthesis of NAD stems from research into the disease pellagra. Pellagra is clinically characterized by the 4 D's—diarrhea, dermatitis, dementia, and ultimately death—and is the result of poor nutritional supply of NAD precursors. Supplementation studies, particularly in a dog model of pellagra, led to the discovery of several NAD precursors that were able to treat pellagra (Elvehjem et al. 1937), while follow-up studies exposed the underlying enzymology (for more information on this topic, I refer the reader to (Houtkooper et al. 2010; Magni et al. 2004)).

### *2.2.1 Primary Biosynthesis from Tryptophan*

The amino acid tryptophan serves as a precursor for primary biosynthesis of NAD (Fig. 2.1). Synthesis through this route is only limited and tryptophan also serves as a precursor for other biologically active molecules such as serotonin (Houtkooper et al. 2010). Nevertheless, in patients suffering from pellagra, supplementation of tryptophan is sufficient as a treatment, suggesting that enough NAD is produced (Elvehjem et al. 1937). In five enzymatic reactions, tryptophan is converted to α-amino-β-carboxymuconate-ε-semialdehyde (ACMS; Fig. 2.1), the first branch point in the pathway. For NAD biosynthesis, ACMS undergoes spontaneous cyclization, after which its product quinolinic acid enters the Preiss-Handler pathway, and is enzymatically converted to NAD through two additional enzymatic reactions (Fig. 2.1). In parallel, ACMS can also be metabolized to glutaryl-CoA, which can be converted to acetyl-CoA and subsequently enter the TCA cycle (Houtkooper et al. 2010).

Although the localization of NAD biosynthesis, and cycling of its intermediates or products is still subject of intense investigation, tryptophan metabolism to NAD is likely confined to the liver and kidney, as the rate-limiting enzyme QPRT is strictly localized to these tissues (Houtkooper et al. 2010). The more downstream enzymes, which converge with the salvage and Preiss-Handler pathways (see below), are more ubiquitously expressed, indicating that intermediates of these pathways may serve as cycling precursors of NAD in other tissues.

It is unclear whether switches to the alternative tryptophan metabolism pathways, i.e. serotonin or the branch towards glutaryl-CoA, play a role in the modulation of NAD levels. Since the primary NAD biosynthesis pathway contributes only a limited supply to cellular NAD levels, however, this seems unlikely, and the control of NAD levels rather involves other parts of NAD metabolic pathways.

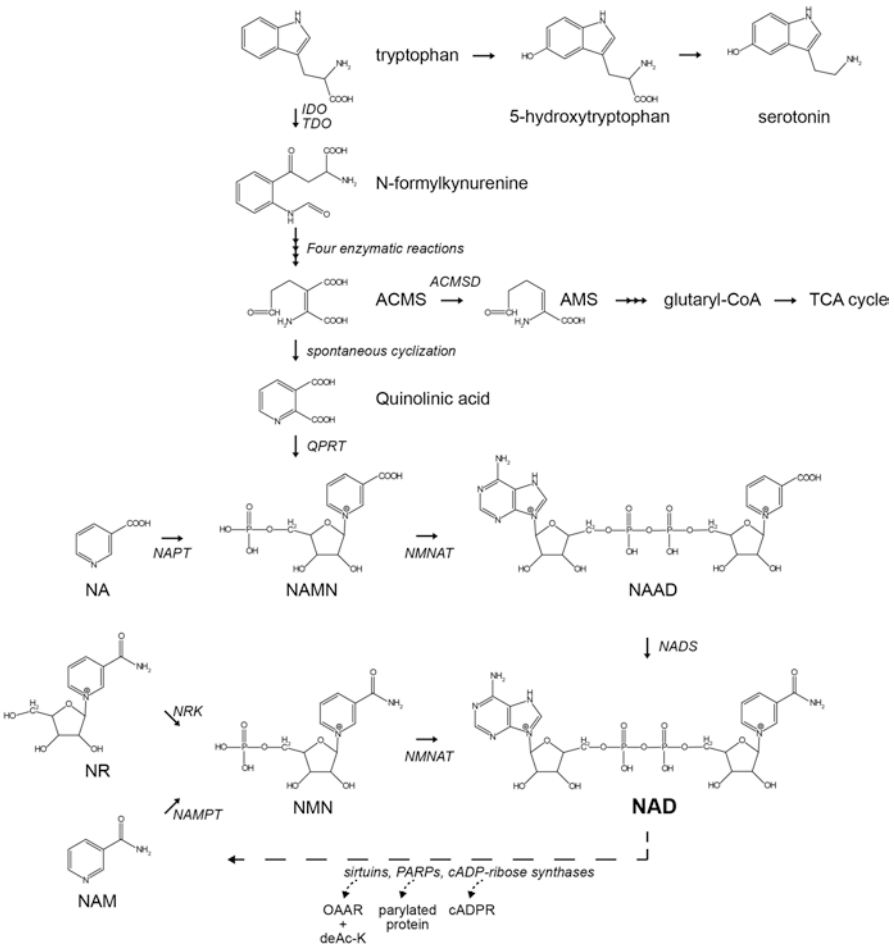


**Fig. 2.1** Biochemistry of NAD metabolism. NAD can be synthesized *de novo*, through the Preiss-Handler pathway, or from the salvage pathway. *De novo* biosynthesis starts from the precursor tryptophan, while salvage is initiated from the precursors NA, NAM, or NR

### *2.2.2 Preiss-Handler Pathway and NAD Salvage*

The main pathways to synthesize NAD involve the precursors nicotinic acid (NA) and nicotinamide (NAM) (Fig. 2.1). Both precursors have a rapid effect when supplemented in dogs suffering from pellagra (Elvehjem et al. 1937), suggesting that these can be efficiently taken up and converted. NA enters the Preiss-Handler pathway for NAD biosynthesis. Following the initial phosphoribosyltransferase step, this pathway converges with the biosynthetic pathway from tryptophan at the level of NA mononucleotide for further synthesis of NAD+ (Fig. 2.1). The adenylyltransferase activity is catalyzed by one of three NMNAT enzymes, which are localized to

different subcellular compartments (Houtkooper et al. 2010). The final enzymatic step in the Preiss-Handler pathway, however, is confined to the cytosol (Hara et al. 2003), suggesting that the conversion of NA to NAD is in fact purely cytosolic.

One of the products of NAD utilization is NAM. This NAM enters the so-called salvage pathway for the resynthesis of NAD. In line with the various subcellular compartments where NAD is being consumed, the salvage of NAD is similarly distributed around the cell. While lower organisms have an enzymatic pathway to convert NAM to NA, mammalian cells initialize the salvage through the rate-limiting enzyme NAMPT, which has been found in the cytosol, nucleus and mitochondria and produces NMN (Houtkooper et al. 2010). The central role of NAMPT in the synthesis of NAD as well as the regulation of NAM levels suggest that NAMPT could dictate the activity of NAD-dependent enzymes. The second step in the resynthesis of NAD from NAM, catalyzed by NMNAT enzymes, also takes place in various subcellular compartments (Houtkooper et al. 2010). This conversion is even more relevant since NMN can be transported into the cell and between organelles. Using a reporter system based on the NAD-dependent poly (ADP ribose) polymerase PARP1, the Ziegler group demonstrated that NMN enters mitochondria and is converted to $NAD^+$ (Nikiforov et al. 2011). How $NAD^+$ levels are sustained or increased in other organelles is subject of future investigation. Similarly, which transporters are involved in the channeling of NAD precursors is an open question.

### *2.2.3 Nicotinamide Riboside*

In 2004, nicotinamide riboside (NR) was discovered as a bona-fide $NAD^+$ precursor in humans (Bieganowski and Brenner 2004). NR was long known as a bacterial substrate for NAD biosynthesis, but the Brenner group identified the human genes coding for the first enzymatic step in NR conversion to $NAD^+$, the NR kinases *NRK1* and *NRK2* (Bieganowski and Brenner 2004) (Fig. 2.1). The resulting product, NMN, converges with the salvage pathway for a one-step resynthesis of $NAD^+$.

## 2.3 NAD Utilization

Besides the enzymes that use $NAD^+$ for redox-coupled reactions there are three key classes of enzymes that utilize $NAD^+$ as a substrate for enzyme activity, (1) sirtuins, (2) poly(ADP-ribose)polymerases (PARPs), and (3) cyclic ADP-ribose synthases. Because of their $NAD^+$-dependence these enzymes can be regulated by the levels of $NAD^+$. Along a different line, as these enzymes compete for the cellular pool of $NAD^+$, inhibiting one of them could increase the NAD availability for the other $NAD^+$ consumers.

### 2.3.1 Sirtuins

The mammalian sirtuin family of proteins consists of seven proteins with varying subcellular localization (Guarente 2013; Houtkooper et al. 2012). The sirtuins are named after the yeast *silent regulator 2* (Sir2) (Ivy et al. 1986; Rine and Herskowitz 1987). The sirtuins were originally classified as histone deacetylases (Imai et al. 2000) but over the years many non-histone targets have been described. In addition, sirtuins are not only deacetylase enzymes, but also perform other posttranslational modifications, such as ADP-ribosylation by SIRT4 (Haigis et al. 2006), demalonylation, desuccinylation, and deglutarylation by SIRT5 (Du et al. 2011; Peng et al. 2011; Tan et al. 2014), and long-chain deaceylation by SIRT6 (Jiang et al. 2013). In addition to their specific enzymatic reactions, sirtuin activity is also regulated by their subcellular distribution. SIRT1, SIRT6 and SIRT7 are considered nuclear, SIRT2 is cytosolic, and SIRT3, SIRT4 and SIRT5 are in mitochondria (Pirinen et al. 2012). For its enzymatic activity, sirtuins require $NAD^+$ and typically produce NAM (Fig. 2.1) (Imai et al. 2000). The dependence on a central metabolite optimally places sirtuins as metabolic sensors, adapting protein activity and gene expression according to the metabolic needs of the cell. Indeed, yeast Sir2 is a critical protein for the lifespan extension that is observed in response to caloric restriction (Howitz et al. 2003; Lin et al. 2002).

For more information on specific sirtuins and their role in mammalian physiology I refer the reader to the other chapters in this book, or to extensive reviews on the topic (Blander and Guarente 2004; Chalkiadaki and Guarente 2012; Haigis and Sinclair 2010; Houtkooper et al. 2012; Menzies et al. 2016).

### 2.3.2 Poly(ADP-Ribose)Polymerases

The poly(ADP-ribose)polymerase enzymes (PARPs) are best known for their role in DNA damage. A family of 17 proteins in humans, the PARPs utilize $NAD^+$ as a substrate for PARylation, a post-translational modification that typically changes the conformation of target proteins and thereby modulates their activity (Bai et al. 2015). For instance, in response to DNA breaks, PARylation leads to the recruitment and activation of DNA repair proteins. Considering that tumors often accumulate DNA mutations, PARP inhibitors have been developed as treatment for various types of cancer (Curtin and Szabo 2013). Interestingly, similar to sirtuins, PARP enzymes require $NAD^+$ as a substrate (Figs. 2.1 and 2.2), but the Km for $NAD^+$ is much lower—in the low micromolar range—suggesting that the regulation of PARP activity is not so much at the level of $NAD^+$ but rather by DNA binding (D'Amours et al. 1999). Upon DNA damage PARPs become activated, in particular PARP1 and PARP2, and deplete a large portion of the cellular $NAD^+$ pool (Houtkooper et al. 2010). Considering that PARPs are such major $NAD^+$ consumers, blocking its activity could mobilize a pool of $NAD^+$ that can serve as a substrate for other enzymes

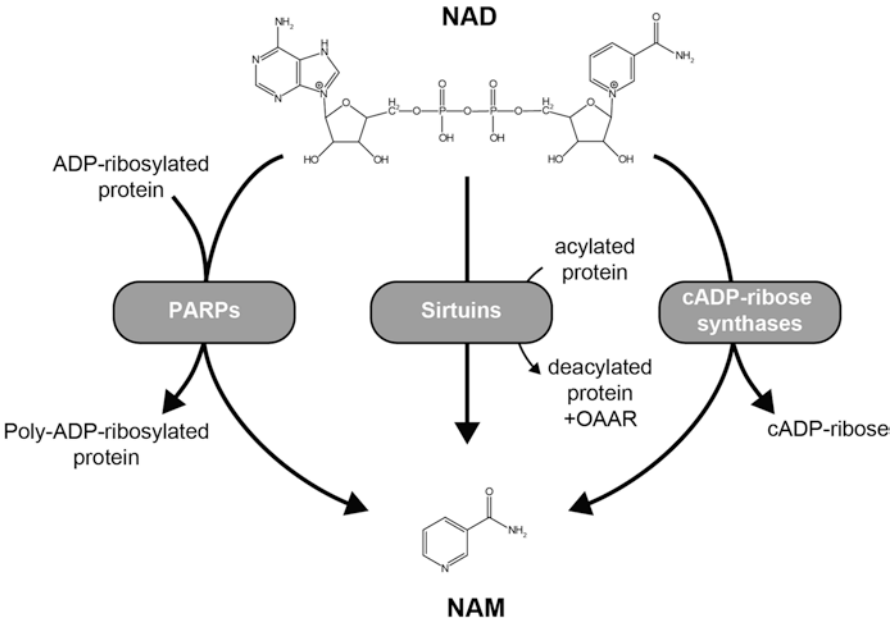


**Fig. 2.2** Enzymatic activities of NAD consuming enzymes. Sirtuins, PARPs and cADP-ribose synthases are major classes of NAD-consuming enzymes. The three enzyme classes display a different activity but all form NAM as a product

such as the sirtuins. Indeed, deletion of *Parp1*—the major PARP enzyme—in mice leads to a marked accumulation of $NAD^+$ levels in various tissues (Bai et al. 2011b). This increase in $NAD^+$ levels is accompanied by activation of nuclear SIRT1, while cytosolic SIRT2 and mitochondrial SIRT3 are not activated, suggesting that $NAD^+$ levels only increase locally. As a consequence, PGC-1α is deacetylated leading to mitochondrial biogenesis in brown adipose tissue and skeletal muscle. Phenotypically, *Parp1*$^{-/-}$ mice display reduced body weight, improved glucose homeostasis, cold tolerance, and resistance to high fat diet (Bai et al. 2011b). Deletion of Parp2 also improved metabolic parameters, but rather than increasing $NAD^+$ levels these mice exhibited markedly increased SIRT1 expression (Bai et al. 2011a). Importantly, SIRT1 was not only activated in genetic models of PARP deficiency, but also in mice that were treated with PARP inhibitors (Bai et al. 2011b). Along the same line, *C. elegans* that were treated with RNAi against the main PARP *pme-1* or with the PARP inhibitor olaparib lived longer than controls, in a *sir-2.1*-dependent manner (Mouchiroud et al. 2013). Together, these studies identify PARP enzymes as regulators for $NAD^+$ levels that are tractable for pharmacological intervention.

### *2.3.3 Cyclic ADP-Ribose Synthases*

A third family of NAD consuming enzymes is the cyclic ADP-ribose (cADPR) synthases. The cADPR synthases—including their most prominent family members CD38 and CD157—regulate processes such as calcium homeostasis and cell cycle, through the production of the second messenger cADPR (Malavasi et al. 2008). Both are highly conserved ectoenzymes that are ubiquitously expressed (Lee 2006). In addition to its role as a receptor on many different cell types, CD38 and CD157 also have enzymatic activity that occurs both intra- and extracellularly (Jackson and Bell 1990; Lee 2012; Zhao et al. 2012). This pH dependent activity utilizes $NAD^+$ as a substrate for the cyclization reaction that leads to the formation of cADPR and the concurrent release of NAM. As such, CD38 and CD157 could regulate $NAD^+$ levels similar to PARPs. In line with this idea, tissues from CD38 knockout mice displayed 2-10x higher $NAD^+$ levels that resulted in SIRT1 activation and increased mitochondrial biogenesis (Aksoy et al. 2006a; Aksoy et al. 2006b; Barbosa et al. 2007). Phenotypically, these mice have enhanced energy expenditure and are resistant to high-fat diet induced obesity (Barbosa et al. 2007). These phenotypes are reminiscent of SIRT1 activation with resveratrol (Baur et al. 2006; Lagouge et al. 2006), and both the biochemical and clinical phenotypes of the CD38 knockout mice can be attenuated by treating the mice with the sirtuin inhibitor sirtinol (Barbosa et al. 2007). Even though these experiments suggest a marked similarity in the mode of action, and a favorable clinical outcome in the mice, more work is needed to establish CD38 and/or CD157 as pharmacological targets for the treatment of metabolic diseases. First, the biochemical routing of $NAD^+$ metabolites upon CD38/CD157 enzymatic action needs to be determined. Second, it is currently unclear whether inhibition of CD38/CD157 is strictly beneficial or may have adverse effects related to the high consumption rates of $NAD^+$.

## 2.4 Pharmacological Opportunities to Activate Sirtuins

After the identification of $NAD^+$ as an essential substrate for sirtuin enzymatic activity, the idea emerged that this could serve as a target for therapeutic intervention. Indeed, supplementation of $NAD^+$ precursors NA or NAM alleviated the clinical symptoms associated with pellagra (Elvehjem et al. 1937). Furthermore, NA is effective in the treatment of hyperlipidemia, despite causing undesired flushing as an adverse effect (Houtkooper et al. 2010). Developments over the last couple of years have established a potent role for other NAD precursors such as NMN and NR. Indeed, supplementing these compounds to the diet of mice leads to an overall improved metabolic profile. NR supplementation attenuated the body weight gain on a high-fat diet, and thereby prevents the development of insulin resistance (Canto et al. 2012). Furthermore, these mice are resistant to cold and perform better on a treadmill (Canto et al. 2012). Consistent with the molecular events that are expected

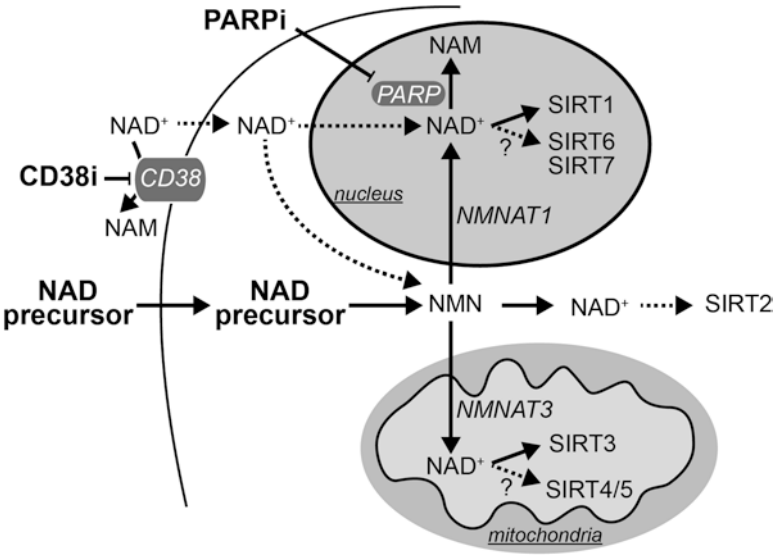


**Fig. 2.3** Subcellular regulation of NAD metabolism and sirtuin activity. Little is known about the subcellular distribution of NAD synthesis, and which are the NAD intermediates that are transported between these subcellular compartments. Considering the ubiquitous localization of NMNAT enzymes it is likely that supplementation of NAD precursors increases NAD levels and sirtuin activity in all compartments. In contrast, PARP inhibitors might only increase nuclear NAD and sirtuins considering the nuclear localization of the PARPs

to arise from NR supplementation, prominent metabolic tissues such as muscle and brown adipose tissue displayed a marked activation of both nuclear SIRT1 and mitochondrial SIRT3, which was accompanied by increased mitochondrial biogenesis (Canto et al. 2012). This is in line with the biochemical routing of these NAD precursors, which are distributed along the various subcellular compartments and lead to NAD synthesis throughout the cell (Fig. 2.3). Similar to NR, supplementation of NMN improves glucose homeostasis in diabetic mice (Yoshino et al. 2011). In addition to multifactorial, complex diseases such as diabetes, NAD precursors may also serve to treat inherited metabolic diseases in which mitochondrial function is compromised (Andreux et al. 2013). Indeed, treating the Deletor mice—a model for mitochondrial myopathy caused by Twinkle mutations—with NR rescued important aspects of their pathophysiology (Khan et al. 2014). Similarly, NR limited disease progression in the *Sco2*$^{KO/KI}$ model for mitochondrial myopathy (Cerutti et al. 2014). These mice suffer from OXPHOS deficiency, which is rescued upon NR supplementation. In addition, four weeks of supplementation fully restores the exercise capacity that is severely impaired in the *Sco2*$^{KO/KI}$ mice (Cerutti et al. 2014). Although data from NAD precursor supplementation in humans are scarce and often limited to pellagra and hyperlipidemia, a recent study demonstrated that supplementation of acipimox—a more stable version of niacin—improves glucose

homeostasis in diabetic patients (van de Weijer et al. 2015). Further human intervention studies with other NAD precursors are eagerly awaited.

Rather than increasing the supply of NAD precursors, inhibiting its consumption has also proven successful in increasing NAD levels and activating sirtuins. In cultured C2C12 muscle cells, the PARP inhibitor PJ34 dose-dependently increased NAD levels leading to SIRT1-dependent PGC-1α deacetylation, which was accompanied by increased mitochondrial gene expression and function (Bai et al. 2011b). At the organismal level, PARP inhibitors extended lifespan of *C. elegans* in a similar way to NR and other NAD precursors (Mouchiroud et al. 2013). Indeed, the PARP inhibitor olaparib increased $NAD^+$ levels, and the lifespan extension was mediated through *sir-2.1*-dependent activation of mitochondrial stress response pathways (Mouchiroud et al. 2013). Little is known about the potential applicability of PARP inhibitors in mammalian models of metabolic disease. PARP inhibitor treatment in mice led to a marked attenuation of high-fat diet induced obesity, accompanied by improved exercise capacity (Pirinen et al. 2014). Along a similar line, the flavonoid apigenin was shown to inhibit the other NAD-consuming enzyme CD38, and apigenin treatment of obese mice improved glucose tolerance, even after just a few days of treatment (Escande et al. 2013).

Even though more research is needed to validate the potential of PARP and CD38 inhibitors for the treatment of metabolic diseases it is important to mention that PARP inhibitors are being developed for the treatment of various types of cancer (Rouleau et al. 2010), and underwent extensive clinical testing, even up to phase III. As a consequence, the route to clinical development for metabolic diseases may be shorter, especially in the case of severe rare inherited metabolic diseases (Bleeker and Houtkooper 2016).

## 2.5 Conclusion

After the discovery that sirtuins are NAD dependent metabolic regulators a lot of research has refocused on the elucidation of NAD metabolism, which was long considered an old-fashioned metabolite. As the intracellular NAD levels are around the Km of sirtuins changing the NAD concentrations is a meaningful strategy to increase sirtuin activity. In this chapter, I outlined several of these strategies, including (1) enhanced NAD synthesis from various precursors, (2) reducing the activity of competitive NAD consumers, rendering this NAD pool available for sirtuins. Indeed, both strategies proved successful as treatment with NAD precursors—e.g. NR or NMN—and PARP or CD38 inhibitors led to increased NAD levels and improved cellular or organismal metabolic state. Nevertheless, more work is needed to fully understand the dynamics of this response, as the enzymology, subcellular distribution, and downstream consequences on the various sirtuins are still incompletely understood. Despite these gaps in our knowledge, it is evident that the $NAD^+$/sirtuin axis is a promising treatment avenue for rare inherited and common acquired diseases. The success stories with NR in the treatment of mice with

high-fat diet induced obesity or mitochondrial myopathy nicely illustrate this point. More recently, NR treatment also proved effective in maintaining a healthy muscle and neural stem cell pools in aged mice, extending mouse lifespan even though the treatment was only started when mice were already 700 days old (Zhang et al. 2016). Despite the immense progress showing beneficial effects of NR on simple organisms and mouse metabolism and healthspan, the translatability of these treatments needs to be further established. Such human studies are expected soon and might firmly establish these compounds as potent sirtuin boosters with human clinical relevance.

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