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# NAD+ metabolism, a therapeutic target for age-related metabolic disease

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## Abstract

Nicotinamide adenine dinucleotide (NAD) is a central metabolic cofactor by virtue of its redox capacity, and as such regulates a wealth of metabolic transformations. However, the identification of the longevity protein Sir2, the founding member of the sirtuin protein family, as being $NAD^+$-dependent reignited interest in this metabolite. The sirtuins (SIRT1-7 in mammals) utilize $NAD^+$ to deacetylate proteins in different subcellular compartments with a variety of functions, but with a strong convergence on optimizing mitochondrial function. Since cellular $NAD^+$ levels are limiting for sirtuin activity, boosting its levels is a powerful means to activate sirtuins as a potential therapy for mitochondrial, often age-related, diseases. Indeed, supplying excess precursors, or blocking its utilization by PARP enzymes or CD38/CD157, boosts $NAD^+$ levels, activates sirtuins and promotes healthy aging. Here, we discuss the current state of knowledge of $NAD^+$ metabolism, primarily in relation to sirtuin function. We highlight how $NAD^+$ levels change in diverse physiological conditions, and how this can be employed as a pharmacological strategy.

### Keywords

Aging; Metabolism; Mitochondria; PARPs; Sirtuins

## 1. Introduction

Nicotinamide adenine dinucleotide (NAD) is a metabolic cofactor that is present in cells either in its oxidized ($NAD^+$) or reduced (NADH) form. Its function as a cofactor for a multitude of enzymatic reactions has been appreciated since the early 1900's, when $NAD^+$ was described as a "cozymase" in fermentation and its characteristics were elucidated, not in the last place by several Nobel prize winners (Berger et al., 2004). In its function as an oxidoreductase cofactor, $NAD^+$ is critical for a wide range of enzymatic reactions, including for instance GAPDH in glycolysis. NAD redox balance is tightly regulated (we refer the reader for more information of this aspect to (Houtkooper et al., 2010a)). After a period of relative anonymity, $NAD^+$ became again in the spotlight because it was identified as a substrate for a major class of deacetylase proteins, the sirtuins, named after the founding member of the family yeast Sir2p (Ivy et al., 1986; Rine and Herskowitz, 1987). Sirtuins have pleiotropic metabolic effects, and since $NAD^+$ levels reflect the energy state of the cell,

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it was hypothesized that sirtuins could function as metabolic sensors that use $NAD^+$ as a messenger and cosubstrate, translating this signal to a cellular adaptation (Canto and Auwerx, 2011). Due to this development, it has become apparent that pathways involved in synthesis or consumption of $NAD^+$ are attractive targets for the management of conditions with dysfunctional metabolism, including not only obesity and diabetes, but also cancer and neurodegenerative diseases (Houtkooper and Auwerx, 2012).

In this review, we will describe the pathways contributing to $NAD^+$ homeostasis, and will discuss their potential benefits in the management of metabolic disease.

## 2. $NAD^+$ metabolism

$NAD^+$ metabolism is a careful balance between biosynthesis on one hand and its breakdown on the other. Importantly, both sides of the balance are composed of several pathways.

### $NAD^+$ biosynthesis and salvage

$NAD^+$ can be synthesized from various precursors (figure 1). De novo biosynthesis, which starts from the amino acid tryptophan, occurs primarily in the liver and kidney but is considered a minor contributor to the total pool of $NAD^+$ (reviewed in detail in (Houtkooper et al., 2010a)). On the other hand, biosynthesis from nicotinic acid (NA) or nicotinamide (NAM)—both present in our diet as vitamin B3—is the primary source of $NAD^+$. These pathways, also known as the salvage or Preiss-Handler pathway, are important for $NAD^+$ homeostasis. This is illustrated by the human disease pellagra, which is caused by $NAD^+$ deficiency subsequent to poor dietary intake of precursors. Pellagra is clinically characterized by the 4 “D’s”, i.e. diarrhea, dermatitis, dementia, and if untreated ultimately death. Pellagra, in dogs prevalent as black tongue disease, is caused by deficiency of $NAD^+$ (precursors) and can be easily treated by providing the vitamin in the diet (Elvehjem et al., 1937). Synthesis of $NAD^+$ from NA or NAM involves phosphoribosyl transfer followed by adenylyl transfer. In the case of NA, the resulting product requires a final ATP-dependent amidation step by NAD synthase to complete the synthesis of $NAD^+$ (for more detail on $NAD^+$ enzymology we refer the reader to (Houtkooper et al., 2010a; Magni et al., 2004).

Recently, a “new” $NAD^+$ precursor—NAM riboside (NR)—that also enhances $NAD^+$ levels through the salvage pathways was described (Bieganowski and Brenner, 2004). Even though this pathway for NAD biosynthesis was already known in bacteria, it was only recently demonstrated that NR —which is found in milk and yeast—could also be used to synthesize $NAD^+$ in eukaryotes (Bieganowski and Brenner, 2004). Indeed, supplementation of NR to cells or mice increases the levels of $NAD^+$ and results in the activation of its downstream signaling cascades (Canto et al., 2012), as will be discussed in more detail below.

### Sirtuins

Sirtuins are a class of metabolic regulators, of which seven orthologues exist in mammals (Blander and Guarente, 2004; Chalkiadaki and Guarente, 2012; Haigis and Sinclair, 2010; Houtkooper et al., 2012). The sirtuins differ in tissue expression, subcellular localization, enzymatic activity and targets. Sirtuins are named after their homology to yeast Sir2 (silent regulator 2) (Ivy et al., 1986; Rine and Herskowitz, 1987), which was originally described as a $NAD^+$-dependent class III histone deacetylases (Imai et al., 2000). Sirtuins are categorized into four different classes according to the amino acid sequence-based phylogenetic analysis (Frye, 2000): Class I includes SIRT1, SIRT2, and SIRT3, Class II and Class III SIRT4 and SIRT5, respectively, and SIRT6 and SIRT7 come under Class IV. Mammalian sirtuins show a diverse subcellular localization. SIRT1, SIRT6 and SIRT7 are mainly found in the nucleus, SIRT2 is predominantly in the cytoplasm, while SIRT3, SIRT4 and SIRT5 are localized in mitochondria (Pirinen et al., 2012). It has become clear, however, that the

sirtuins not only deacetylate histones, but also a wide range of other proteins (figure 2). Most of the targets are involved in stress response pathways, whether metabolic in nature, genotoxic or otherwise. In addition, some of the sirtuins were reported to ADP-ribosylate proteins rather than deacetylate (Haigis et al., 2006), and SIRT5 was shown to act as a demalonylase and desuccinylase (Du et al., 2011; Peng et al., 2011; Wang et al., 2011). Future research will have to determine whether other sirtuins also possess such activity, but it seems likely that multiple family members function as deacylases.

The nuclear sirtuin SIRT1 is the best-known member of the family especially after it was described as the target of the polyphenol, resveratrol (Howitz et al., 2003), which is found in low quantities in red wine (see section "Pharmacological control of $NAD^+$ levels"). SIRT1 deacetylates histones, but its key activity involves the regulation of mitochondrial biogenesis and stress response through the deacetylation of PGC-1α (Rodgers et al., 2005) and FOXO1 (Brunet et al., 2004; van der Horst et al., 2004). Its role in stress response is further confirmed by the identification of p53, HIF-1α and NF-κB as SIRT1 targets (reviewed in (Canto and Auwerx, 2011; Houtkooper et al., 2012)). Less is known about the other sirtuins, but it is clear that they also impact on metabolism in various ways. The cytosolic SIRT2 was shown to deacetylate tubulin (North et al., 2003), as well as the sterol regulatory element binding protein-2 (SREBP-2) (Luthi-Carter et al., 2010), although genetic evidence for this latter association is so far lacking. Furthermore, SIRT2 deacetylates phosphoenolpyruvate carboxykinase (PEPCK) to control gluconeogenesis (Jiang et al., 2011) and was recently shown to deacetylate the receptor-interacting protein 1 (RIP1), and thereby serve as a critical component of the TNFα-mediated programmed necrosis pathway (Narayan et al., 2012). Finally, SIRT2 controls myelin formation in vivo through the atypical-PKC regulator PAR3 (Beirowski et al., 2011). The mitochondrial sirtuins—SIRT3, SIRT4 and SIRT5—deacetylate protein targets involved in oxidative phosphorylation (Ahn et al., 2008), fatty acid oxidation (Hirschey et al., 2010), ketogenesis (Shimazu et al., 2010), oxidative stress (Someya et al., 2010), glutamate metabolism (Haigis et al., 2006), urea cycle (Nakagawa et al., 2009), as well as several other mitochondrial pathways (Hebert et al., 2013), and thereby regulate multiple facets of mitochondrial metabolism (reviewed in (Houtkooper et al., 2012; Pirinen et al., 2012; Verdin et al., 2010)). Surprisingly, mice deficient in either of the mitochondrial sirtuins do not develop an overt metabolic phenotype under basal non-challenged conditions (Fernandez-Marcos et al., 2012; Haigis et al., 2006; Haigis and Sinclair, 2010; Hirschey et al., 2010; Lombard et al., 2007; Nakagawa et al., 2009). SIRT6 deacetylates both histones and DNA polymerase β, a DNA repair protein. As a result, deletion of SIRT6 in mice results in a severe premature aging phenotype associated with defects in DNA repair (Mostoslavsky et al., 2006). Additionally, *Sirt6*$^{-/-}$ mice have reduced IGF1 levels and are severely hypoglycemic (Mostoslavsky et al., 2006), possibly mediated by the HIF1α-dependent activation of glycolysis and subsequent decreases in glucose levels (Zhong et al., 2010). No *in vivo* molecular deacetylation targets have been described for the nucleolar SIRT7, but knockdown or overexpression of SIRT7 resulted in decreased or increased RNA polymerase I-mediated transcription, respectively (Ford et al., 2006). A thorough characterization of *Sirt7*$^{-/-}$ mice has not been performed but mice deficient for SIRT7 display hyperacetylation of p53, develop cardiomyopathy and die young (Vakhrusheva et al., 2008).

## Other $NAD^+$ consumers: PARPs and CD38/CD157

$NAD^+$ is not only consumed by sirtuins, but also by the members of the poly(ADP-ribose) polymerase (PARPs) family and the NAD glycohydrolases CD38 and CD157 (figure 2). The nuclear PARP1 accounts for most of the PARP activity in vivo and is the best studied family member, but critical functions for other PARPs are emerging (Luo and Kraus, 2012; Schreiber et al., 2006). PARPs are best characterized for their role in DNA damage

pathways, but more generally PARPs regulate adaptive stress responses, including inflammatory, oxidative, proteotoxic, and genotoxic stresses (Luo and Kraus, 2012). For example, when protein translation is stalled, PARP13 localizes to cytosolic stress granules and regulates microRNA expression and activity, alleviating the protein stress (Leung et al., 2011). More recently, however, the role of the different PARPs role in metabolism has become more apparent, as *Parp1* and *Parp2* knockout mice are protected against high-fat diet induced obesity (Bai et al., 2011a; Bai et al., 2011b; Bai et al., 2007). Based on the functional links between PARPs and sirtuins, it was tempting to speculate that the levels of $NAD^+$, the joint co-substrate, could in fact dictate these functions. The in vivo characterization of mutant mice for PARP1—the major PARP isoform—further corroborated this hypothesis (Bai et al., 2011b). *Parp1*$^{-/-}$ mice were protected from high-fat diet induced obesity and showed overall improved fitness compared to control littermates. The effects of *Parp1* deletion were due to elevation of $NAD^+$ levels and subsequent SIRT1-dependent activation of mitochondrial metabolism in brown adipose tissue and muscle (Bai et al., 2011b). Importantly, this genetic evidence was confirmed by pharmacological studies using PARP inhibitors (Bai et al., 2011b), as will be further discussed in section "Pharmacological control of $NAD^+$ levels". Interestingly, *Parp2*$^{-/-}$ mice were also protected against diet-induced obesity, but this effect was not mediated through changes in $NAD^+$ levels and activation of SIRT1 as the case in *Parp1*$^{-/-}$ mice, but rather through the induction of muscle *SIRT1* expression (Bai et al., 2011a). While PARPs are stress response proteins, the $NAD^+$-consuming CD38 is an ubiquitous, but still quite enigmatic enzyme, involved in maintaining calcium homeostasis (Lee, 2012). Although CD38 is often referred to as an ectoenzyme, it may also have intracellular activity (Lee, 2012), although its full potential as an $NAD^+$ consumer is yet to be discovered. Still, even if most CD38 activity occurs outside the cell, the resultant metabolites can be transported inside, most likely in the form of NR (Nikiforov et al., 2011). Similar to PARP deficient mice, *CD38*$^{-/-}$ mice display highly elevated $NAD^+$ levels that are accompanied by SIRT1 activation and, at the organismal level, increased energy expenditure (Barbosa et al., 2007). The role of CD157, also known as Bst1, is not characterized in the context of metabolic disease. It is interesting to note, however, that a recent study demonstrated a role for CD157 in the response of intestinal Paneth cells to CR (Yilmaz et al., 2012). When Paneth cells are exposed to caloric restriction (CR), CD157 is activated to produce cyclic ADP-ribose, which in turn signals to intestinal stem cells to switch on maintenance programs rather than differentiation (Yilmaz et al., 2012). Whether or not CD157 is involved in metabolic regulation in other tissues as well remains to be investigated.

## 3. Modulation of NAD levels by physiological processes

### Fasting and exercise

SIRT1 activity is generally increased during restrictive metabolic conditions, and decreased in situations of caloric excess. Complying with the fact that SIRT1 activity is regulated by $NAD^+$, these observations shed light on the potential role of $NAD^+$ as a metabolic sensor in stress conditions, where the levels of $NAD^+$ are generally affected. During fasting and exercise, the level of $NAD^+$ increases (Canto et al., 2009; Canto et al., 2010). Interestingly, this increase in $NAD^+$ levels is linked with sirtuin activation. In a similar way, CR in mouse models leads to an increase in the level of $NAD^+$ in different tissues, such as muscle, liver and white adipose tissue (Canto et al., 2010; Chen et al., 2008). Conversely, caloric excess by means of a high-fat diet (Kim et al., 2011), but also aging (Braidy et al., 2011; Yoshino et al., 2011), lead to reduced $NAD^+$ levels. Several studies have revealed through the prism of the sirtuin family the potential involvement $NAD^+$ in longevity modulation during CR, and in a larger extent in the physiological aging mechanism.

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## Caloric restriction, NAD+ and aging

Aging is characterized by a progressive accumulation of molecular, cellular and organ damage, leading to dys- or malfunction of many metabolic processes and a generalized physiological decline. If this decline is uncompensated, it can result in the development of age-associated diseases, like neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, metabolic disorders, such as type 2 diabetes and atherosclerosis, or cancer. Despite the complexity of the aging process, many studies have demonstrated over the last two decades that aging is subject to regulation by common signaling pathways, transcription factors and their co-regulators. Among the different proposed mechanisms that impact on and modulate longevity, CR is by far the most consistent and reproducible intervention that increases lifespan and protects against the decline of biological functions with age in many different species.

CR is defined as a moderate limitation of food intake below the *ad libitum* level, without malnutrition. It was already known for ages that moderation and composition of diet can influence the aging process (Schafer, 2005), but the modern day founder was Clive McCay, who put its benefits in the scientific spotlight in 1935 (McCay et al., 1989). CR remains the most effective and reproducible intervention to extend lifespan and delay the development of age-associated diseases in divergent species, from yeast to monkeys (Houtkooper et al., 2010b; Koubova and Guarente, 2003). The concept that enhanced mitochondrial function upon CR contributes to its beneficial effects on lifespan was extended to humans, in which a general improvement in metabolic health occurs during CR ((Civitarese et al., 2007) and reviewed in (Holloszy and Fontana, 2007)). The implication of $NAD^+$ in aging is closely intertwined with the major role proposed for the $NAD^+$-dependent sirtuin enzymes in CR.

The role of $NAD^+$ in CR emerged from studies in yeast, where pioneering work revealed that longevity mediated by CR requires the $NAD^+$ biosynthesis pathway and the activity of the yeast sirtuin homolog Sir2 (Lin et al., 2000). It was proposed that increased Sir2 activity leads to the repression of recombination events at the homologous repeats present in the ribosomal DNA, preventing as such the formation of extra-chromosomal ribosomal DNA circles, which is one of the causes of replicative aging in yeast. Thus, the increased dosage of *SIR2* in yeast prevents the formation of extra-chromosomal ribosomal DNA circles and prolongs lifespan, whereas its inhibition has the opposite effect, reducing the replicative life span by 50% (Kaeberlein et al., 1999). Interestingly, mimicking CR by reducing glucose concentration of the growth medium from 2% to 0.5% is sufficient to extend lifespan to a similar level as by overexpressing Sir2, and these effects were dependent on the Sir2 gene or the nicotinate phosphoribosyltransferase 1 (NPT1) gene, which is involved in the biosynthesis of NAD (Lin et al., 2000).

The worm genome comprises four genes sharing homology with Sir2, with *sir-2.1* being the closest homolog of Sir2 (Frye, 2000), and *sir-2.1* is required for the lifespan extension in response to the CR-mutation *eat-2* (Wang and Tissenbaum, 2006). In the fruitfly *Drosophila melanogaster*, which expresses five homologs of Sir2 (Frye, 2000), CR extends lifespan and increases dSir2 mRNA expression, but was unable to mediate lifespan extension in flies where *dSir2* had been deleted (Rogina and Helfand, 2004). As discussed before, the mammalian genome encodes seven sirtuins and within this family of proteins, SIRT1 is the most extensively studied in the context of the lifespan regulation. Several *in vivo* studies assign a role for SIRT1 to explain the longer life in mice under CR. In fact, in *Sirt1*$^{-/-}$ mice the beneficial effects on metabolism and longevity induced by a CR diet are attenuated, although it should be noted that these mice are very sick to start with (Boily et al., 2008; Chen et al., 2005). Conversely, transgenic mice, constitutively overexpressing the *Sirt1* gene, exhibit a range of features that are reminiscent of the phenotypes seen in CR mice, as they are lighter and metabolically more active, show improved glucose homeostasis, and

develop less cancer (Banks et al., 2008; Bordone et al., 2007; Herranz et al., 2010; Pfluger et al., 2008).

The beneficial effects mediated by SIRT1 under CR conditions have been proposed to be due to an improvement of the mitochondrial function and biogenesis (Guarente and Picard, 2005), but also to a global increase in stress resistance and maintenance of the cellular and mitochondrial homeostasis (figure 3). Recent studies in worms added a new layer of complexity in this dynamic mechanism, by showing that an early burst of ROS is required for the induction of the ROS defense pathway and for the lifespan extension under CR conditions (Mouchiroud et al., 2011; Schulz et al., 2007). Moreover, compelling new evidence suggest that other pro-longevity pathways, induced by CR and/or stress conditions, could also potentially involve SIRT1, such as mitophagy, mitochondrial dynamics (fission/fusion) and mitochondrial unfolded response (Durieux et al., 2011; Egan et al., 2011; Yang et al., 2011). Further studies are needed to decipher the exact role of $NAD^+$ and SIRT1 in lifespan regulation through these mechanisms.

However, the requirement of sirtuin proteins in longevity modulation is not without controversy and still the object of an intense debate. Initial work reported that increased expression of the yeast protein Sir2 and of related sirtuin proteins in *Caenorhabditis elegans* and *Drosophila melanogaster* extends lifespan (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). These observations were recently challenged by showing that the effect of overexpression of worm *sir-2.1* and fly SIR2 on lifespan is, at best, limited or even absent (Burnett et al., 2011; Viswanathan and Guarente, 2011). In view of the predominant role of SIRT1 in metabolic homeostasis in mammals, we speculate that SIRT1 is rather a major keystone in health maintenance and stress response, instead of being crucial for the determination of lifespan *per se*. As such, $NAD^+$ serves as a central metabolite that communicates the metabolic state under such stressful conditions and activates the sirtuins to trigger adaptive and protective responses. Further studies are needed to elucidate the role of the others sirtuin family members in longevity regulation.

## 4. Pharmacological control of $NAD^+$ levels

### Resveratrol and STACs

As briefly mentioned above, various compounds can modulate the levels of $NAD^+$ and thereby activate sirtuin enzymes. One of the best described is the polyphenol 3,5,4′-trihydroxystillbene, which was originally isolated in 1939 from the roots of the plant white hellebore (*Veratrum grandiflorum O. Loes*) (Takaoka, 1939). This fact is reflected in the common name, of the compound, i.e., resveratrol, a combination of *res* (from the fact that it is a resorcinol or dihydroxy benzene), *veratr* (Veratrum) and *ol* (for the alcoholic groups) (figure 4). A few decades later, in 1963, resveratrol was extracted from the roots of another plant, Japanese knotweed (*Reynoutria japonica*), commonly used in traditional Chinese and Japanese medicine (reviewed in (Baur and Sinclair, 2006)). In the wild, resveratrol is found in many edible fruits, such as grapes, blueberries, cranberries or peanuts (Baur and Sinclair, 2006). It is also present in red wine at concentration ranging from 0.1 to 14.3 milligrams per liter (Soleas et al., 1997). Interestingly, higher levels of resveratrol are made by plants in response to infection or nutrient stress, thereby qualifying it as a phytoalexin. Resveratrol has attracted the attention of the scientific community when it was demonstrated that this molecule could be the origin cardioprotective effects specific to red wine which is commonly referred to as «the French paradox » (Kopp, 1998; Pace-Asciak et al., 1995). Since then resveratrol has been shown to be effective in preventing and delaying the progression of various diseases such as cancer (Jang et al., 1997), cardiovascular disease and glucose intolerance (Timmers et al., 2011) and ischemic stroke (Sinha et al., 2002; Wang et al., 2002). In 2003, an *in vitro* high throughput screening of small chemical compounds

identified resveratrol as the most potent activator of SIRT1, able to extend the yeast lifespan (Howitz et al., 2003). As in yeast, treatment with resveratrol increases lifespan of worms and fly in a SIRT1 (*sir-2.1* or Sir2p, respectively) dependent manner (Howitz et al., 2003; Wood et al., 2004), although this is controversial (Bass et al., 2007; Kaeberlein et al., 2005). In mammals, resveratrol supplementation in mice fed with a high fat diet (HFD) improved physiological parameters, as these mice showed a decrease in HFD-induced weight gain, an improved glucose metabolism, and less damage to the pancreas and heart, all features associated with increased activity of AMPK and PGC-1α, culminating in increased mitochondrial number and function (Baur et al., 2006; Lagouge et al., 2006). Ultimately, this beneficial metabolic profile leads to a longer life expectancy (Pearson et al., 2008). This effect on longevity is, however, only observed when mice are fed with a HFD. Under chow diet, treatment with resveratrol does not extend mice lifespan, although it seems to improve their overall health (Barger et al., 2008; Pearson et al., 2008). In chow fed mice, resveratrol significantly attenuated several hallmarks of aging, such as reduced inflammatory and apoptotic events in vascular endothelium, limiting the formation of cataracts, preservation of bone density and conservation of motor activity with age (Barger et al., 2008; Baur et al., 2006; Lagouge et al., 2006; Pearson et al., 2008). These resveratrol treated mice also exhibit a transcriptional profile in heart, liver and muscle that is similar to that seen in animals under CR, supports the idea that resveratrol mimics the effects of food limitation in ad libitum fed individuals. The fact that the effects of the CR mimetic, resveratrol, also depend on the diet contributed to the controversy that CR could work only in animals maintained under "regular" laboratory conditions. Indeed, it has been questioned whether the beneficial effects observed under CR were not due to a simple "rescue" of the deleterious effects brought by the state of overnutrition specific of the artificial diets prepared in laboratories (Harper et al., 2006; Longo and Finch, 2003; Martin et al., 2010; Prentice, 2005). These observations could explain the conflicting results obtained with CR in non-human primates, where two independent studies—both of which using a different control diet and feeding regimen—showed a different extent of CR health benefits (Colman et al., 2009; Mattison et al., 2012). Since it was known that resveratrol acts as an inhibitor of the ATP synthase complex in the oxidative phosphorylation (Zheng and Ramirez, 2000), it was hypothesized that the effects of resveratrol could be mediated by activation of the AMP-activated protein kinase (AMPK), rather than direct SIRT1 activation (Beher et al., 2009; Canto et al., 2010; Pacholec et al., 2010; Um et al., 2010). Indeed, AMPK is activated upon resveratrol treatment and resveratrol's effects are lost in cells or tissues devoid of AMPK (Canto et al., 2010; Um et al., 2010) (figure 5). Following AMPK activation, expression of NAMPT increased, leading to increased $NAD^+$ levels and activation of SIRT1 (Canto et al., 2009; Canto et al., 2010; Fulco et al., 2008). It should be noted that the mode of action of resveratrol is still subject of debate. Recent reports suggest that resveratrol may act through phosphodiesterase 4 inhibition, thereby mobilizing calcium stores and activating AMPK (Park et al., 2012), or through allosteric activation of SIRT1 that is dependent on structural hydrophobic motifs in SIRT1 substrates (Hubbard et al., 2013), but further work is needed to clarify whether this also plays a physiological role.

Regardless of these issues, resveratrol treatment in mice induces mitochondrial biogenesis and energy expenditure (Baur et al., 2006; Lagouge et al., 2006). The required dose (200-400 mg/kg/day), however, was in a range that is normally incompatible with human consumption (15-30 g per day for a 75 kg person). Importantly and reassuring from a clinical point of view, resveratrol supplementation in obese humans reached beneficial effects at a far lower dose (150 mg per day) (Timmers et al., 2011). It should be noted, however, that resveratrol failed to exert beneficial effects in non-obese female subjects (Yoshino et al., 2012), in line with mouse data where resveratrol is particularly effective in high-fat diet fed mice (Baur et al., 2006; Lagouge et al., 2006).

Increasing SIRT1 activity through the use of synthetic SIRT activating compounds or STACs, such as SRT1720, also prevents diet-induced obesity and delays the onset of associated metabolic abnormalities in mice models (Feige et al., 2008; Milne et al., 2007). Similar to resveratrol, the small molecule SIRT1 activator SRT1720, which is 1,000-fold more potent than resveratrol, also extends both mean and maximum mouse lifespan in mice fed with HFD (Minor et al., 2011). This effect is potentially explained by the improved metabolic homeostasis, as typified by insulin sensitization and increased mitochondrial and locomotor activity (Feige et al., 2008; Milne et al., 2007). It is important to mention that there is, however, still some debate whether SRT1720, as well as other SIRT1-activators, are targeting SIRT1 in a specific manner (Beher et al., 2009; Dai et al., 2010; Pacholec et al., 2010), although recent evidence suggests that this may be dictated by specific hydrophobic residues in SIRT1 substrates (Hubbard et al., 2013). Human efficacy studies with such synthetic SIRT1 activators should be reported in the near future.

## $NAD^+$ boosters

A more specific approach to modify $NAD^+$ levels involves the supplementation of $NAD^+$ precursors or $NAD^+$-consumption inhibitors (figure 3). The precursors NA, NMN, and NR, but also PARP or CD38 inhibitors increase NAD levels in various cell types and tissues of mice (Bai et al., 2011b; Barbosa et al., 2007; Canto et al., 2012; Yoshino et al., 2011).

NA, also called niacin, has been used to treat dietary tryptophan deficits (pellagra) and hyperlipidemia (Elvehjem et al., 1937; Karpe and Frayn, 2004; Sauve, 2008). Along another line, reduced NAMPT expression in *Nampt*$^{+/-}$ mice, which decreased plasma NMN levels and lowered $NAD^+$ levels in brown adipose tissue, at least in female mice, was shown to impair glucose-stimulated insulin secretion (Revollo et al., 2007). This effect can be rescued by NMN supplementation, which indicates that the maintenance of $NAD^+$ levels is crucial for pancreatic function (Revollo et al., 2007). A recent study has confirmed this observation by demonstrating that NAMPT activity is compromised by HFD and aging, and could contribute to the pathogenesis of type 2 diabetes (Yoshino et al., 2011). Enhancing $NAD^+$ biosynthesis by intraperitoneal injection of NMN indeed improved glucose homeostasis in obese mice (Yoshino et al., 2011). NR is another potent naturally occurring $NAD^+$ precursor. Dietary NR supplementation increases $NAD^+$ levels in brown adipose tissue, muscle and liver, but not in brain and white adipose tissue (Canto et al., 2012). In responsive tissues, NR activates both SIRT1 and SIRT3 activity, improves mitochondrial function and thereby alleviates metabolic dysfunction associated with HFD-induced obesity (Canto et al., 2012). These observations indicate that NR could also be used to prevent and/or treat the decline in mitochondrial function observed upon age-associated diseases.

Another attractive angle to modulate $NAD^+$ levels consists in targeting the activity of other (non-sirtuin) $NAD^+$-consuming enzymes, such as PARPs and CD38 (figure 3). Following the hypothesis that $NAD^+$ is the rate-limiting factor for the activation of SIRT1, SIRT1 activity is reduced when PARP1 is activated. Conversely, genetic or pharmacological inactivation of PARP1 (Bai et al., 2011b) or CD38 (Barbosa et al., 2007; Dong et al., 2011) function increases $NAD^+$ levels resulting in SIRT1 activation and the induction of a gene expression program that stimulates mitochondrial metabolism. In line with this premise, *Parp1* or *Cd38* knockout mice show improved metabolic function and are protected against diet-induced obesity (Bai et al., 2011b; Barbosa et al., 2007). It is important to note that in *Parp1*$^{-/-}$ mice only SIRT1 is activated (Bai et al., 2011b), which is in contrast to the dual SIRT1 and SIRT3 activation observed with the $NAD^+$ precursor NR. This is, however, consistent with the nuclear localization and activity of PARP1, raising $NAD^+$ levels in this compartment, while NR can be converted to $NAD^+$ in mitochondria as well (Canto et al., 2012). While PARP inhibitors have not yet been tested for metabolic effects, their clinical

development for cancer therapy could provide interesting opportunities in this direction (Audeh et al., 2010; Gelmon et al., 2011). In fact, we expect that boosting oxidative metabolism through modulating $NAD^+$ levels could in itself prove to be a powerful anti-cancer regimen and actually inhibit the "Warburg effect". A potential issue could be the pancreatic dysfunction observed in *Parp2*$^{-/-}$ mice (Bai et al., 2011a) that may be replicated when mice are treated with pan-PARP inhibitors. Small molecule CD38 inhibitors (or CD157 inhibitors if identified) may circumvent this problem, but further insights in the $NAD^+$ accumulation and trafficking are required (Sauve et al., 1998; Sauve and Schramm, 2002). Additionally, prolonged studies are required to exclude potential long-term adverse effects of both the PARP and CD38 inhibitors.

## 5. Conclusions and future perspectives

Following the description of the sirtuin enzyme family, both allosteric sirtuin activating compounds and small molecules that modulate $NAD^+$ levels took center stage as a potential ways to therapeutically target sirtuin signaling for the treatment of diseases linked with mitochondrial dysfunction. Research on $NAD^+$ modulators led to the identification of various physiological and pharmaceutical interventions that resulted in an increase in $NAD^+$ levels and an activation of SIRT1. These include direct biosynthesis precursors, e.g. NR and NMN, inhibitors of its utilization, e.g. PARP and CD38/CD157 inhibitors, and indirect effectors, e.g. resveratrol. Improving on this relatively rich pharmacology, we recently identified a compound (NR) that activates both SIRT1 and SIRT3, since it is metabolized throughout the cell, not only in the nucleus. It is furthermore likely that novel pharmacophores, which affect the activity of other enzymes in the NAD biosynthesis pathway, will emerge as potential tools to modulate $NAD^+$ levels (e.g. targeting NR kinase). This highlights the importance of understanding the basic biochemistry underlying $NAD^+$ homeostasis. Despite these specificities, all compounds that increase $NAD^+$ levels improve metabolic homeostasis and protect against metabolic diseases, although to various degrees. Future work on the differences between these distinct classes of compounds that modulate $NAD^+$ levels and affect sirtuin signaling—for instance defining their molecular targets and/or tissue-specific effects—will elucidate how the compounds can be optimally employed clinically to treat both common polygenic forms of metabolic diseases, such as type 2 diabetes, as well as rare monogenic diseases that cause metabolic dysfunction, such as inherited mitochondrial diseases.

Importantly, the potential therapeutic impact of changing $NAD^+$ levels is not limited to the metabolic realm discussed in this manuscript, but can be extended to other age-associated diseases such as neurodegenerative disorders and cancer. Indeed, CR permits to maintain neuronal plasticity with age (Adams et al., 2008; Halagappa et al., 2007; Patel et al., 2005) and reduces cancer risk (Kalaany and Sabatini, 2009), suggesting that increasing $NAD^+$ levels could also be interesting within this context (Speakman and Mitchell, 2011). In line with these beneficial effects of CR on age-associated pathologies, transgenic mice with SIRT1 overexpression are not only protected against HFD-induced metabolic pathology, but also show protection against tumor development (Herranz et al., 2010) and progression of neurodegenerative disease (Donmez et al., 2010; Jeong et al., 2012; Wareski et al., 2009). Importantly, some of the $NAD^+$ boosting compounds may display adverse effects that may preclude their use for relatively mild metabolic disturbances that can also be treated by changes in life style, while this may be acceptable for the severely debilitating inherited conditions (Houtkooper and Auwerx, 2012). Finally, although it is clear that increasing $NAD^+$ levels improves stress response and prevents development of metabolic disease, for instance upon HFD, it remains to be seen whether or not increasing $NAD^+$ levels may lead to increased lifespan in higher species.

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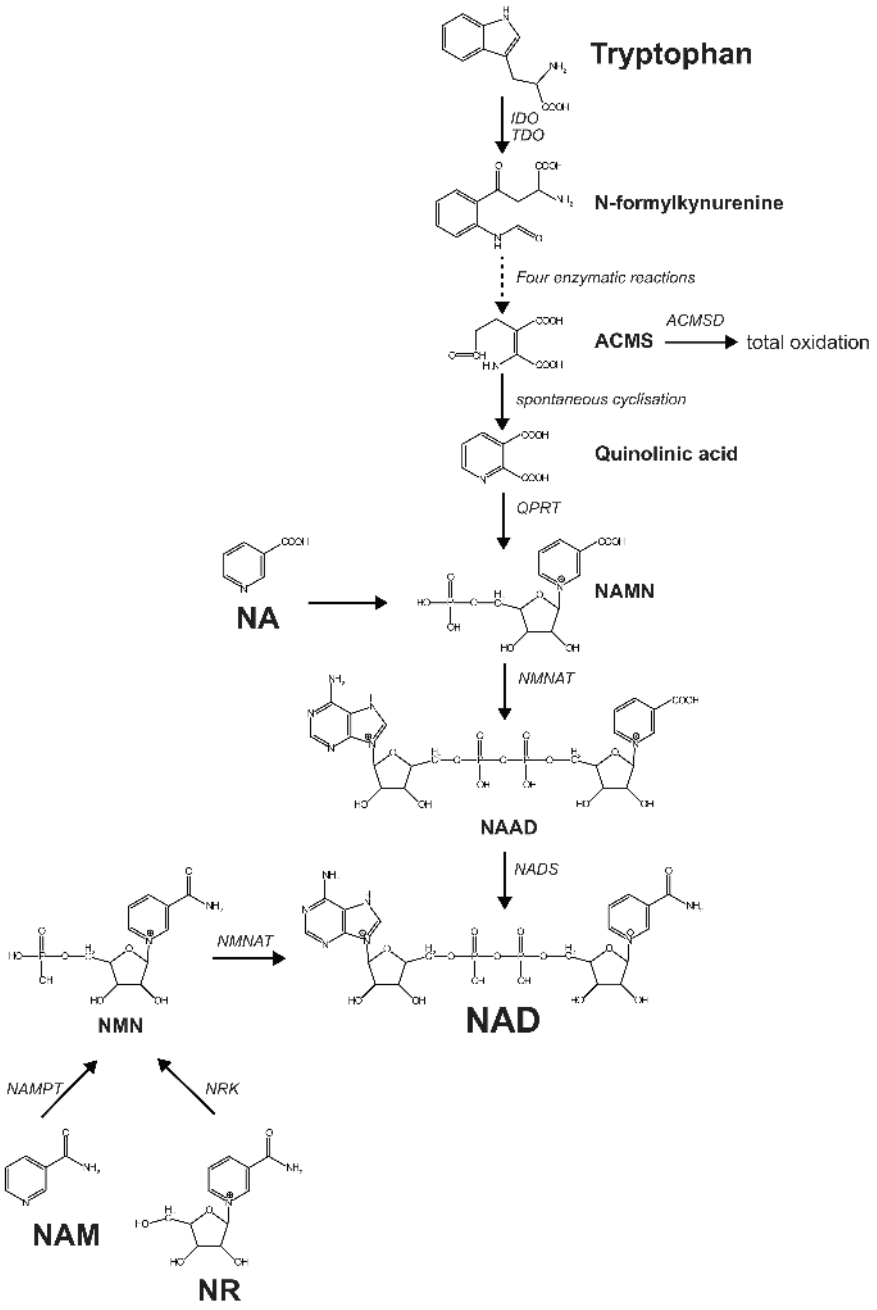


**Figure 1. *De novo* biosynthesis and salvage pathway of NAD$^+$**

The first step of the NAD$^+$ *de novo* biosynthesis is the conversion of tryptophan into N-formylkynurenine through an enzymatic reaction catalyzed by either indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase (TDO). N-Formylkynurenine is then converted in four successive enzymatic reactions into α-Amino-β-carboxymuconate-ε-semialdehyde (ACMS), which can undergo either enzymatic conversion directed to total oxidation or spontaneous cyclization to quinolinic acid. The following step is the formation of nicotinic acid mononucleotide (NAMN) through the quinolinate phosphoribosyltransferase (QPRT) activity. NAMN is then transformed to nicotinic acid adenine dinucleotide (NAAD) by the nicotinamide mononucleotide adenylyltransferase

(NMNAT) enzymes. The final step in the biosynthesis of $NAD^+$ is the amidation of NAAD by the NAD synthase enzyme. $NAD^+$ is also synthesized through the $NAD^+$ salvage pathway from its precursors NA, NAM, or NR. From NA, the first step in $NAD^+$ synthesis is catalyzed by nicotinic acid phosphoribosyltransferase (NAPT) and leads to the formation of NAMN. Similarly, NAM is converted by nicotinamide phosphoribosyltransferase (NAMPT), forming NMN, which is also the product of phosphorylation of NR by nicotinamide riboside kinase (NRK). Both NAMN and NMN are then converted by NMNAT, after which the NAMN-derived NAAD requires the final amidation through NAD synthase.



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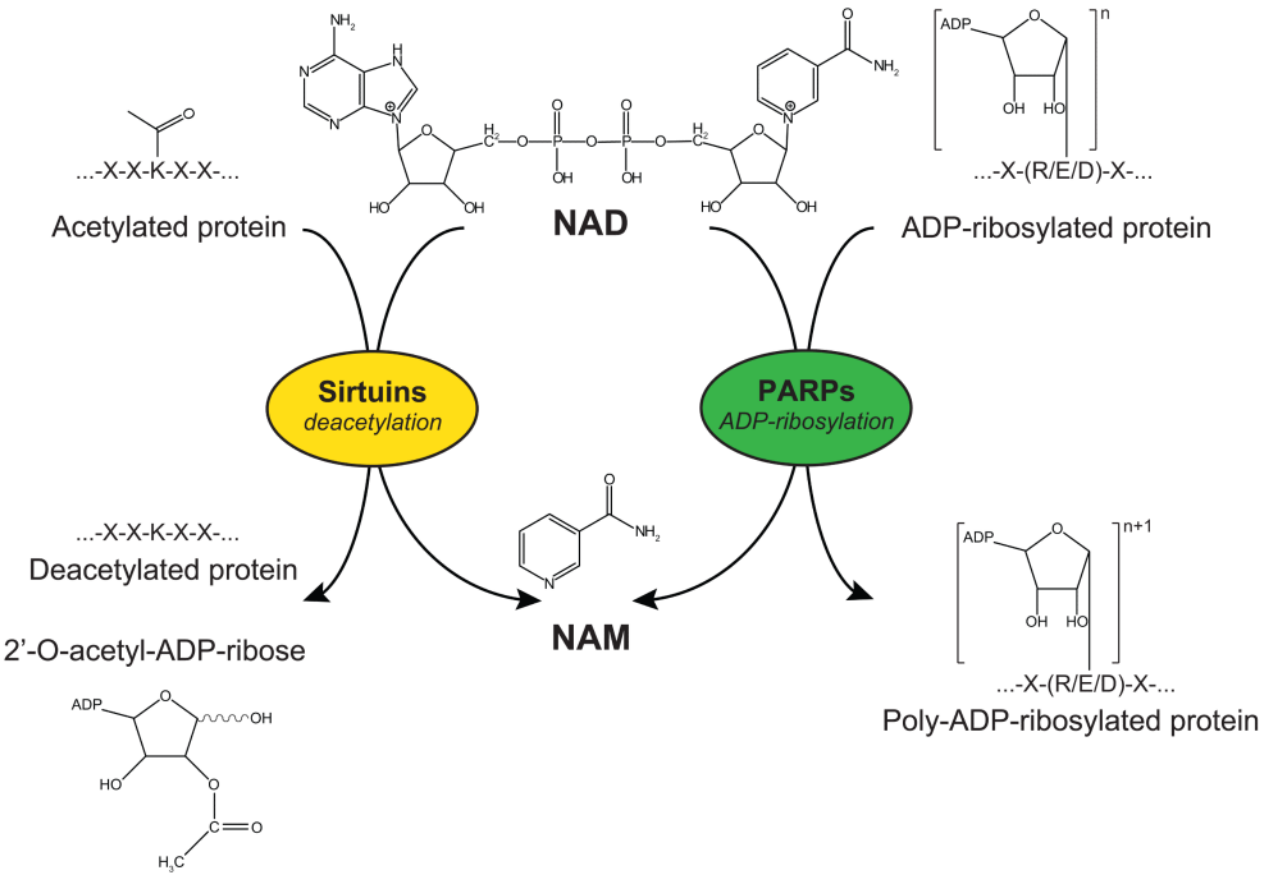


**Figure 2. Sirtuins and PARPs as competing NAD$^+$-consuming enzymes**

Sirtuins are NAD$^+$-consuming deacetylases, using NAD$^+$ to cleave acetyl groups from acetylated lysine residues of target proteins, in a reaction that generates NAM and 2′-O-acetyl-ADP-ribose. PARP family members are also NAD$^+$-consuming enzymes. They catalyze a reaction in which multiple ADP-ribose groups are transferred to a mono ADP ribosylated substrate protein, forming long chains and branches of ADP-ribosyl polymers.




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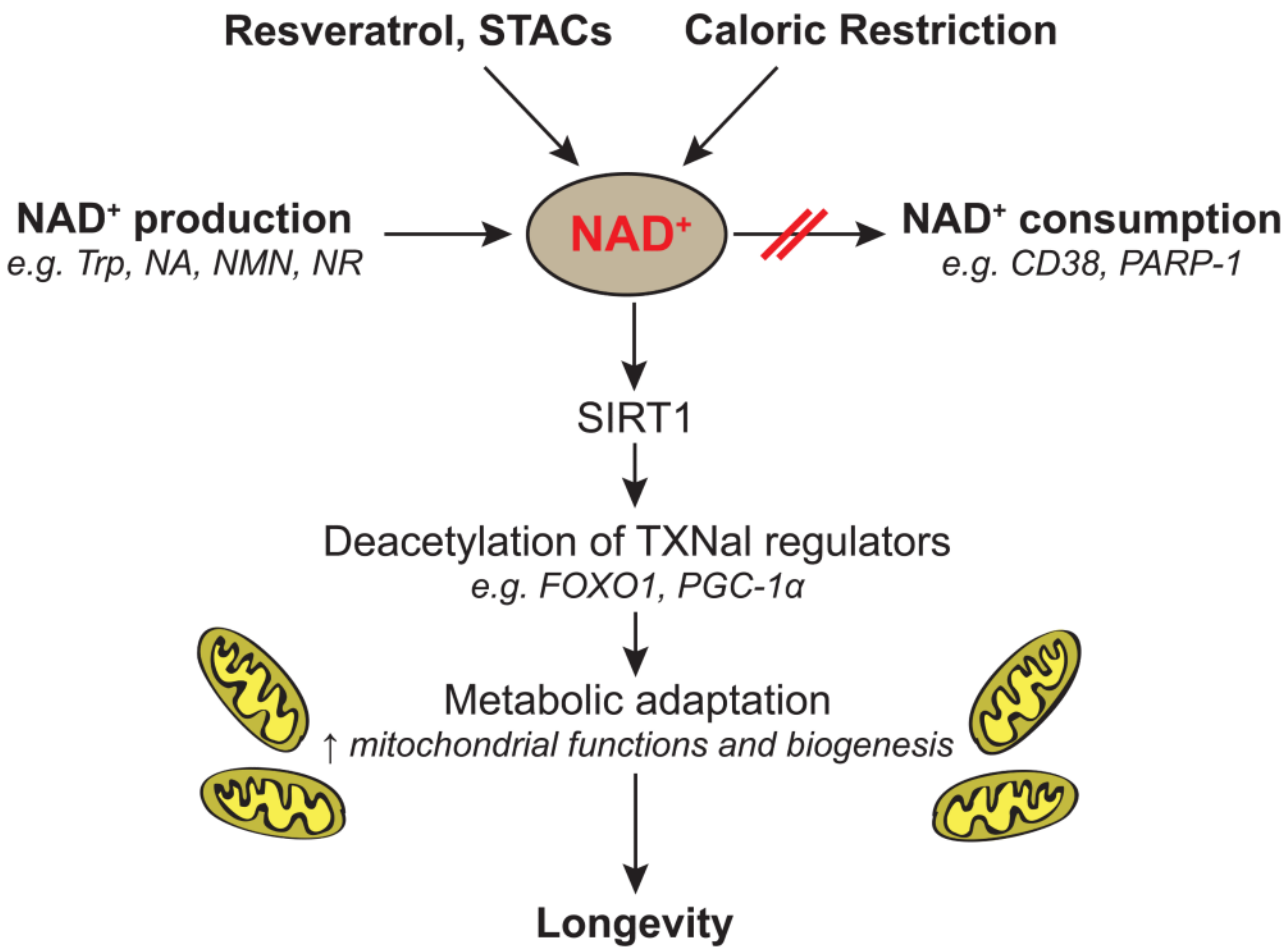


**Figure 3. NAD$^+$ as a keystone for mitochondrial regulation**

NAD$^+$ is a rate-limiting metabolite for the SIRT1 enzymatic activity. SIRT1 activity can be increased by different types of physiological or experimental interventions that increase NAD$^+$ levels, such as caloric restriction, treatment with STACs/resveratrol, enhancement of NAD$^+$ biosynthesis through supplementation with precursors (NA, NR, NMN), or through inhibition of NAD$^+$-consuming activities, such as the PARPs or CD38. This stimulation of the SIRT1 deacetylation activity leads to an improvement of the mitochondrial adaptation and ultimately to beneficial effects on health and lifespan.

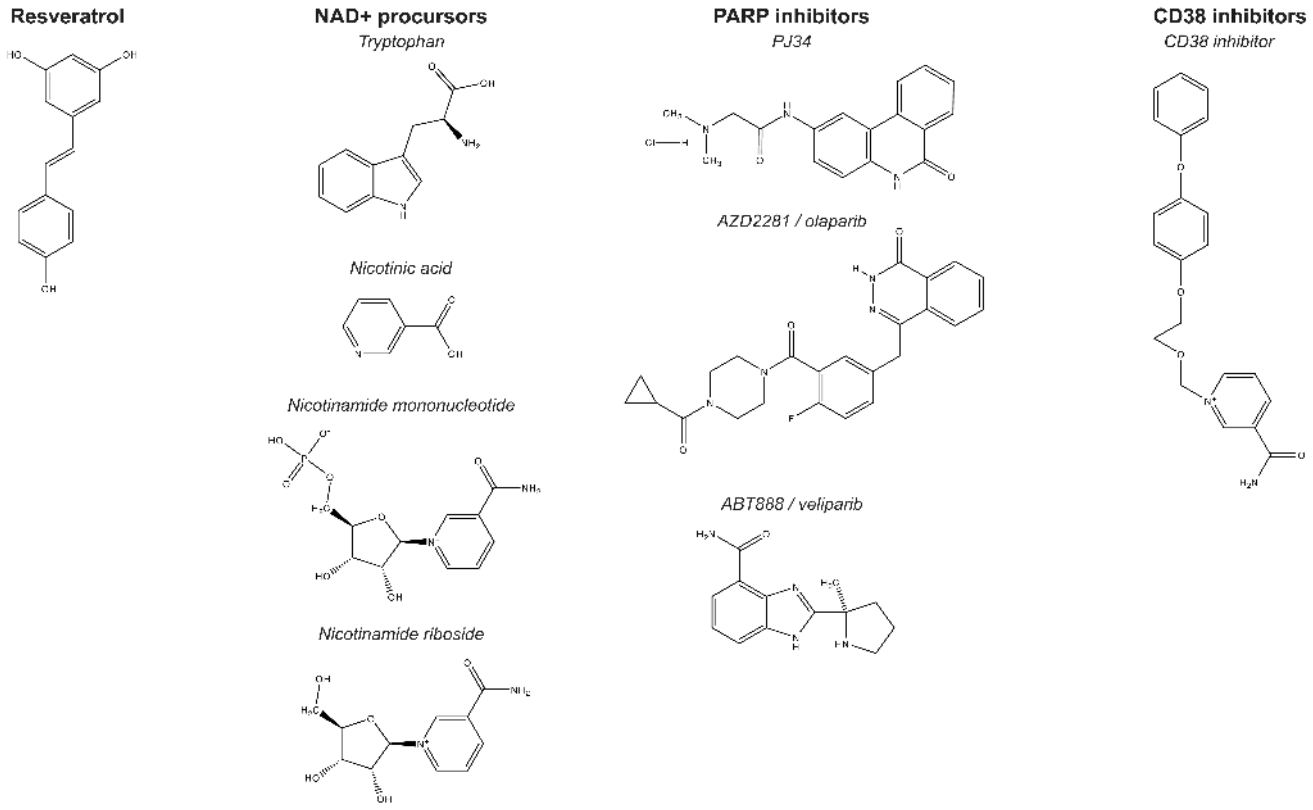


**Figure 4. Compounds increasing NAD$^+$ levels**

Chemical structures of compounds that increase NAD$^+$ levels. We distinguish four types of NAD$^+$ boosters, including resveratrol, the primary NAD$^+$ precursors NA, NMN and NR, the PARP inhibitors PJ34, olaparib and veliparib, and the CD38 inhibitor 1-{[2-(4-phenoxyphenoxy)ethoxy]methyl}-3-(aminocarbonyl)-pyridinium chloride (Dong et al., 2011).

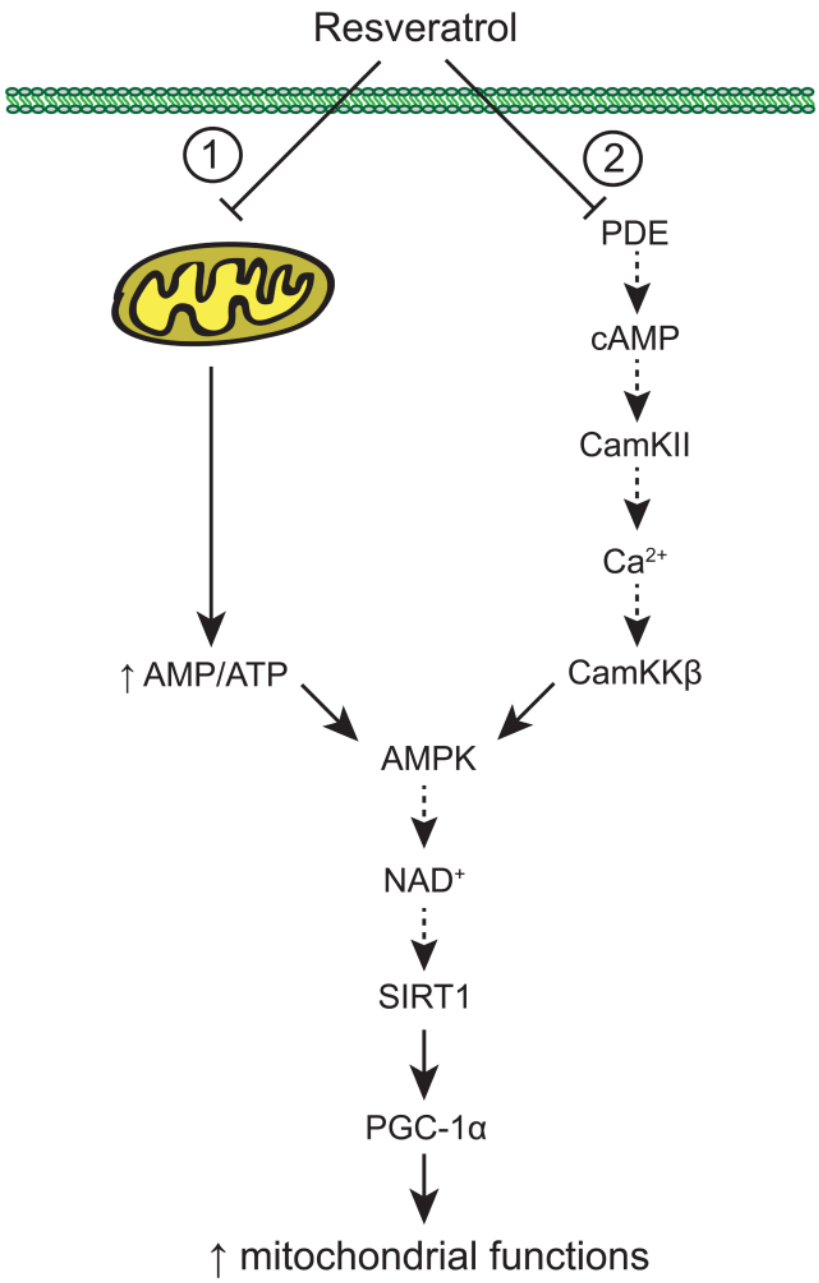


**Figure 5. Mechanisms of action of resveratrol**

Resveratrol promotes mitochondrial biogenesis and functions through indirect AMPK and SIRT1 activation. First, compelling evidence suggests that the metabolic actions of resveratrol are based on its ability to act as a mitochondrial poison by inhibiting ATP synthase activity (1). The resulting energy stress will in turn activate AMPK, leading to the stimulation of SIRT1 by increasing $NAD^+$ levels. SIRT1 will then activate downstream targets through deacetylation, ultimately leading to an improvement of mitochondrial function. Another potential explanation of how resveratrol acts is based on a recent report showing that resveratrol inhibits phosphodiesterase (PDE) 4 activity and induces cAMP

signaling resulting in $Ca^{2+}$ release and, ultimately the activation of the CamKKb-AMPK pathway (2).



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