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Regulation of NAD biosynthetic enzymes modulates NAD-sensing processes to shape mammalian cell physiology under varying biological cues

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[§]Abbreviations:

ADPR, ADP ribose; ART, mono-ADP ribose transferase; PARP, poly-ADP ribose transferase ; NAADP, nicotinic acid adenine dinucleotide phosphate; CR, calorie restriction; PAR, poly (ADP ribose); PGC-1, peroxisome proliferator-activated receptor- γ coactivator; Nam, nicotinamide; HFD, high-fat diet; NamPRT, nicotinamide phosphoribosyltransferase; PRPP, phospho-ribosyl pyrophosphate ; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; NMNAT, NMN adenylyltransferase; NRK, nicotinamide riboside kinase; PnP, purine nucleoside phosphorylase; NAR, nicotinate riboside; NAMN, nicotinate mononucleotide; NA, nicotinic acid; NAPRT, nicotinate phosphoribosyltransferase; NAAD, nicotinic acid adenine dinucleotide; NADS, NAD synthetase; KP, kynurenine pathway; ACMS, 2-amino-3-carboxymuconate semialdehyde; ACMSD, ACMS decarbosylase; QA, quinolinic acid; QAPRT, quinolinate phosphoribosyltransferase; PA, picolinic acid; CLL, chronic lymphocytic leukemia; HIF, hypoxia-inducible factor; FOXO, O family members of the forkhead transcription factors; AMPK, AMP-activated protein kinase; MIBP, Muscle Integrin Binding Protein; IDO, indoleamine 2,3-dioxygenase; IK, interleukin; AP, activator protein; NF, nuclear factor.

ABSTRACT

In addition to its role as a redox coenzyme, NAD is a substrate of various enzymes that split the molecule to either catalyze covalent modifications of target proteins or convert NAD into biologically active metabolites. The coenzyme bioavailability may be significantly affected by these reactions, with ensuing major impact on energy metabolism, cell survival, and aging. Moreover, through the activity of the NAD-dependent deacetylases sirtuins, NAD behaves as a beacon molecule that reports the cell metabolic state, and accordingly modulates transcriptional responses and metabolic adaptations. In this view, NAD biosynthesis emerges as a highly regulated process: it enables cells to preserve NAD homeostasis in response to significant NAD-consuming events and it can be modulated by various stimuli to induce, via NAD level changes, suitable NAD-mediated metabolic responses. Here we review the current knowledge on the regulation of mammalian NAD biosynthesis, with focus on the relevant rate-limiting enzymes.

Keywords: NAD biosynthesis; enzyme regulation; Nicotinamide phosphoribosyltransferase; Nicotinamide riboside kinase; Nicotinate phosphoribosyltransferase; Quinolinate phosphoribosyltransferase

1. INTRODUCTION

NAD(H) and NADP(H) are ubiquitous and essential redox coenzymes taking part to most cellular reactions in both catabolism and anabolism: NAD(H) mainly participates in ATP production, whereas NADP(H) is utilized in anabolism and for modulating the cellular redox status [1]. NADP is formed from existing NAD by the ATP-dependent NAD kinase catalyzed reaction, and the intracellular ratio NAD/NADP is largely in favor of NAD. As electron carriers, the coenzymes molecules shuttle between their oxidized and reduced form, while total coenzymes concentration is not altered. On the contrary, several enzymes are known that split the molecule at its Nglycosidic bond, thus rendering its continuous resynthesis indispensable (Figure 1). These enzymes include *i*) mono- and poly-ADP ribose (ADPR)[§] transferases (ARTsand PARPs, respectively) which cleave NAD and transfer ADPR, either as a single moiety or as a polymer, to acceptor proteins, resulting in their covalent modification and modulation of their function. ADP ribosylation is involved in a wide range of cellular processes, including DNA damage response, telomere maintenance, transcriptional regulation, control of immune response and cell death [2, 3]; ii) the multifunctional NAD glycohydrolase (CD38) that generates the NAD derivatives nicotinic acid adenine dinucleotide phosphate (NAADP), ADPR and cyclic-ADPR, all of them with a well recognized role in calcium signaling [4, 5]; iii) sirtuins, which through the NAD-dependent deacetylation of transcription factors and histones, mediate cell adaptation to various kinds of stress, like fasting, exercise and calorie restriction (CR). In this way, they have been shown to affect energetic metabolism, proliferation, DNA repair, apoptosis, senescence, endocrine signaling, and lifespan [6, 7].

PARPs activity represents the major NAD catabolic process, which consumes cellular NAD at sustained rate under basal conditions [8] and, even more significantly, following oxidative and genotoxic damage [9], thereby forcing cells to continuously synthesize NAD to maintain cell viability. A substantial role in the consumption of intracellular NAD has also been ascribed to CD38 activity [10], that consumes several NAD molecules while synthesizing single molecules of cyclic-ADPR, thus reinforcing the notion that intensive and continuous NAD regeneration is needed. On the other hand, in the last decade mounting evidence indicate that intracellular NAD levels are significantly affected by nutritional and environmental stimuli, and that changes in the NAD content are readily reflected into sirtuins activity modulation. This, in turn, leads to the up- or down-regulation of sirtuin-controlled genes that are central to energetic metabolism and stress response. In this view, it has been shown that maintenance of a proper intracellular NAD

concentration is critical for protecting against diet- and age-induced disorders [11, 12]. Accordingly, manipulation of NAD biosynthesis appears very promising for therapeutic benefits and, indeed, accumulating data point to enhancement of NAD synthesis as having protective effects in metabolic and neurodegenerative diseases and cancer [13].

In this review, we will first emphasize the importance for the cell to maintain a physiological NAD homeostasis, by presenting some of the evidence on how deeply the intracellular NAD availability can influence mammalian physiology. We will then focus on the NAD biosynthetic enzymes that are known to control the maintenance of intracellular NAD levels, with particular attention to the current knowledge on their regulation at transcriptional, post-transcriptional and enzymatic level.

2. THE IMPORTANCE OF CONTROLLING INTRACELLULAR NAD LEVELS

NAD homeostasis is the result of the balance between a number of NAD cleaving reactions and NAD biosynthetic routes. In this section we focus first on the influence of NAD splitting enzymes on the intracellular NAD availability, and then on the role of NAD as a messenger modulating cellular transcriptional responses and metabolic adaptations.

2.1 PARPs activity

PARPs comprise a number of enzymes that hydrolyze the N-glycosidic bond of NAD, releasing Nam and transferring the ADP-ribosyl moiety to target acceptor proteins. By adding further ADPR units, PARPs are able to elongate the protein-bound chain to create a polymer, termed poly (ADP ribose) (PAR). The most studied among the several known PARPs isoforms is PARP1, which is activated in response to specific signaling pathways, and by DNA breaks, leading to the covalent poly-ADP ribosylation of target proteins, thus regulating several processes such as replication, transcription, DNA repair, and metabolism [2]. PARP1 is a significant contributor to NAD consumption under basal conditions [8], and it can profoundly affect intracellular NAD content under conditions leading to its hyperactivation. As example, the hyperactivation of the enzyme in a neuronal culture model of acute acquired epilepsy leads to severe NAD loss, energy failure, translocation of the apoptosis-inducing factor from mitochondria to nucleus and neuronal death [14]. Likewise, the permanent activation of PARP1 in Nijmegen Breakage Syndrome cells unable to repair DNA double strand breaks causes a dramatic decrease in NAD levels leading ultimately to a loss of the antioxidative capacity [15]. Whether NAD depletion and/or PAR accumulation

following PARP1 hyperactivation are the causal event of cell energy failure and death is still matter of debate [16]. Nevertheless, in several *in vitro* [17, 18] and *in vivo* [19, 20] models of PARP1 hyperactivation, NAD repletion has been shown to prevent the cell death. In many studies, the NAD protective effect has been shown to depend on sirtuins [14, 21], leading to the suggestion that the decline of sirtuins activity due to NAD depletion might play a key role in the PARP1mediated cell death. Indeed, experimental evidence has been provided that SIRT1-catalyzed deacetylation is lowered in situations of PARP1 hyperactivation [21].

2.2 NAD glycohydrolase activity

CD38, originally identified as a lymphocyte antigen, is a multifunctional transmembrane enzyme responsible for the synthesis of the intracellular second messengers cyclic- ADPR and ADPR (using NAD as the splitted substrate) and NAADP (from NADP, in a peculiar pyridine baseexchange reaction), all relevant Ca²⁺ mobilizers [22]. A recent study showed that the enzyme can be found on the membrane in two opposing orientations, with the catalytic domain facing either the outside or the inside of the cell, which might explain the intracellular formation of its second messengers products [23]. Notably, NAD hydrolysis seems to be the enzyme's major catalytic activity, as the reaction appears to yield approximately one molecule of cyclic-ADPR every 100 molecules of NAD hydrolyzed [24]. Conflicting reports on the enzyme localization are available in the literature: it is still debated whether CD38 is only localized in the cell plasma membrane, or also in the intracellular membranes of mitochondria, nuclei and sarcoplasmic reticulum [25]. Increasing evidence indicate that the enzyme is a significant contributor to NAD homeostasis. Indeed, CD38 deficient mice show 10- to 20-fold higher tissue NAD levels than wild-type, implying that its enzymatic activity is physiologically very sustained in vivo [10]. Accordingly, it has been demonstrated that CD38 is able to modulate SIRT1, as CD38-deficient mice show increased activity of SIRT1 in several tissues, and a consequent activation of the peroxisome proliferator-activated receptor-y coactivator (PGC-1) α in liver, which might explain the resistance of these mice to dietinduced obesity [26, 27]. On the other hand, a recent study demonstrates that cultured cells overexpressing CD38 show a severe decrease in NAD levels, with a consequent down-regulation of the synthesis of enzymes involved in energetic metabolism and antioxidative defense [28]. In line with these results, the increase in CD38 expression which is observed in HL-60 cells induced to differentiate into neutrophil-like cells, is accompanied by a drastic decrease in intracellular NAD levels and increased oxidative damage [29].

2.3 The NAD signaling role through sirtuins activity

Sirtuins are NAD-dependent protein deacetylases that are considered diet-sensitive, antiaging enzymes: under conditions of DNA damage, oxidative stress, energy stress and low nutrient availability their activity modulates enzymes of energetic metabolism and stress response, resulting in cell adaptation, health promotion and lifespan extension. Among the seven known sirtuin isoforms, the best studied is SIRT1; its activity is generally upregulated under restrictive metabolic conditions, as well as oxidative stress, while it is decreased under conditions of nutritional excess. Known SIRT1 targets comprise histones and a number of transcription factors that are key regulators of glucose and lipid metabolism, mitochondrial respiration, mitochondrial biogenesis, oxidative stress resistance, apoptosis and autophagy. Their SIRT1-mediated regulation promptly tunes cell energetic metabolism to diet variations, and enhances antioxidant [30]. SIRT1 mediates the beneficial effects of CR on health and lifespan, and many studies connect the increase of its activity with prevention of aging and age-associated diseases, including diabetes, cancer, cardiovascular and neurological disorders [31, 32].

Sirtuins-mediated protein deacetylation involves binding of NAD and the acetyl-lysine moiety of protein targets, generating deacetylated lysine, acetyl-ADPribose and nicotinamide (Nam) [33]. Nam acts as an inhibitor of the reaction, and provides negative feedback inhibition of sirtuins activity *in vivo* [34]. It is widely accepted that the major regulator of sirtuins is their substrate NAD, as the enzymes activity readily responds to changes in intracellular NAD concentrations [11].

It is well documented that NAD levels are significantly affected by a number of conditions, like circadian rhythm, diet, exercise, and aging. In particular, in mice liver NAD levels display circadian oscillations that are regulated by the core clock machinery [35, 36]. Intracellular NAD content increases in skeletal muscle during fasting, exercise and glucose starvation [37-39]. NAD levels also increase in liver of fasted mice and return to control levels after refeeding [40]. On the other hand, both high-fat diet (HFD) [41, 42] and aging [42, 43] have been shown to reduce NAD availability in different tissues of mice models. Such fluctuations in NAD content are readily reflected in changes in sirtuins activity. This has been clearly demonstrated in a very recent study showing that the SIRT2-controlled acetylation state of tubulin is NAD level-dependent [44]. Also, in models of aging it has been shown that a reduced NAD availability compromises SIRT1 activity and severely impairs mitochondrial metabolism [43, 45]. Notably, in cultured vascular smooth muscle cells, the increase in NAD levels following up-regulation of the NAD biosynthetic enzyme NamPRT, increases

sirtuins activity that is required for cells maturation [46]. Here, since activation of NamPRT implies the increase of intracellular NAD content as well as the decrease of the sirtuins inhibitor Nam, both effects might contribute to the increase in sirtuins activity.

In keeping with the above observations, several strategies aimed at increasing in vivo NAD bioavailability lead to the enhancement of different sirtuins activity, thus promoting health benefits superimposable to those observed in animal models overexpressing the same enzymes. As examples, boosting intracellular NAD levels through inactivation of the NAD consumer enzymes PARP1 [47] or CD38 [27, 48] has been shown to protect mice, via SIRT1 activity increase, against obesity and many of the metabolic complications induced by HFD diet. Enhancing NAD biosynthesis through supplementation of the NAD precursor nicotinamide mononucleotide (NMN) also improves impaired glucose tolerance and glucose-stimulated insulin secretion in diabetic model mice via SIRT1 activity stimulation [42]. Notably, mice overexpressing SIRT1 have the same positive glucose-responsive phenotype, which is lost with aging but can be restored by NMN [49]. NMN also exerts positive effects on β -cells exposed to chronic inflammation via SIRT1 and SIRT3 activation [50, 51]. Likewise, supplementation of the NAD precursor nicotinamide riboside (NR) to mice models increases NAD levels in a tissue-specific manner and, via SIRT1 and SIRT3 activation, protects against HFD-induced metabolic abnormalities and increases oxidative capacity and energy expenditure, by enhancing mitochondrial biogenesis [52]. A significant improvement of mitochondrial function has also been reported in a mouse model of mitochondrial myopaty following NR administration: a marked increase in mitochondrial biogenesis was shown in skeletal muscle and brown adipose tissue, concomitant with a decrease in the level of the markers of disease progression [53]. NMN supplementation also significantly protects against the decline in mitochondrial function in aged mice [45], and NR treatment markedly attenuates cognitive deterioration in the Alzheimer disease mouse model [54]. Supplementation of NAD itself was shown to protect cardiomyocytes from hypertrophy and cell death, both in vitro and in vivo, via SIRT3 activity increase [55].

Altogether these findings clearly indicate that NAD behaves as a central messenger, reporter of the metabolic state under stressful conditions. As a SIRT1 substrate, it increases its deacetylating activity to trigger adaptative and protective responses. Given the beneficial effects of SIRT1 activity increase in aging and age-related diseases, boosting NAD biosynthesis represents a promising therapeutic means.

3. OVERVIEW OF NAD BIOSYNTHESIS

Given the central role of NAD and therefore the crucial requirement for the cell to guarantee its homeostasis, it is not surprising that cells harbor alternative metabolic routes leading to its regeneration. Figure 2 shows a schematic overview of the major mammalian NAD biosynthetic routes. The most studied is the route recycling back to NAD the Nam released by all the NAD-splitting enzymes described above. It involves two consecutive steps: Nam phosphoribosylation to NMN, by the enzyme NamPRT, which uses phospho-ribosyl pyrophosphate (PRPP) and releases pyrophosphate, and NMN adenylylation to NAD, by the enzyme NMN adenylyltransferase (NMNAT), which uses ATP as the adenyl donor, releasing pyrophosphate. Three NMNAT isoenzymes have been identified, differing in their catalytic and structural properties, as well as in the subcellular localization, which supports compartmentalized NAD pools in different organelles [56]. The same route salvages circulating dietary Nam, and might be also exploited in salvaging the Nam released in the extracellular space by the ecto-enzyme CD38 acting on extracellular NAD and/or NMN [22], and by the activity of ecto-ARTs (Figure2). While the presence of NAD in the extracellular space is well documented and several mechanisms for its origin have been described [57], the presence of NMN in the extracellular milieu remains controversial [58, 59].

NAD can also be synthesized starting from NR, a recently identified vitamin, following its phosphorylation to NMN by the ATP-dependent NR kinase (NRK), and adenylation of the formed NMN to NAD by NMNAT [60]. In yeast, NR can be converted to NAD through an alternative route whereby it is first splitted into Nam by the enzyme purine nucleoside phosphorylase (PnP), and then the formed Nam is converted to NAD via the Nam salvage enzymes [61]. It has been also shown in yeast that the deamidated form of NR, nicotinic acid riboside (NAR), can be used as NAD precursor via both NRK- and PnP-dependent pathways [61]. The *in vitro* findings that NR can be phosphorolysed by bovine PnP [61], and that NAR can be used as a substrate by human NRK [62] and bovine PnP [61], might suggest that also mammalian cells can salvage NR via a PnP-dependent route, and can use NAR as an alternative NAD precursor; however direct evidence is still missing. While in yeast NR and NAR have demonstrated to be intracellular metabolites, deriving from the respective mononucleotides via NMN- and nicotinate mononucleotide (NAMN)- 5'-nucleotidase activities [63], to our knowledge, only one study investigated their presence in mammalian cells and their level was reported to be undetectable under the growth conditions described [64]. The available information indicate that NR mainly derives from the diet, both as such (its presence has been assessed in milk [60]) and from partial digestion of NAD and NMN [65]. NR can also be

formed in the extracellular space from NAD and/or NMN by the ecto-enzyme CD73 [66], and the role of CD73 in mediating intracellular NAD synthesis from exogenous NR has been demonstrated [67].

Nicotinic acid (NA) is a third form in which the pyridine ring can be used to synthesize NAD. It can derive directly from the diet, as well as from the efficient activity of Nam deamidase of the gut microbial community [65]. Inside the cell NA is converted to NAD through the Preiss-Handler pathway, which involves three consecutive steps: phosphoribosylation of NA to NAMN by the enzyme NA phosphoribosyltransferase (NAPRT), NAMN adenylation to nicotinic acid adenine dinucleotide (NAAD) by NMNAT, and NAAD amidation to NAD by the glutamine-dependent NAD synthetase (NADS).

In addition to the routes that enable salvaging of the pyridine ring, a *de novo* biosynthetic pathway is operative in mammals, that allows NAD formation starting from tryptophan (Figure 3). The first reactions of the *de novo* pathway, which comprise the so called kynurenine pathway (KP), transform tryptophan into 2-amino-3-carboxymuconate semialdehyde (ACMS), which undergoes spontaneous cyclization to quinolinic acid (QA). QA is then converted to NAMN by the enzyme QA phosphoribosyltransferase (QAPRT), which commits tryptophan catabolism towards NAD synthesis, irreversibly diverting ACMS from either complete oxidation or conversion to picolinic acid (PA). NAMN is in fact transformed to NAD via the two last steps of the Preiss-Handler pathway (Figure 3).

4. REGULATORY PROPERTIES OF THE NMN- AND NAMN-SYNTHESIZING ENZYMES

The various metabolic routes described in section 3 can contribute to the overall NAD pool with different efficiencies and in different combinations depending on the tissue, cell type and metabolic status. This has been ascribed to a differential expression and regulation of the recognized rate-limiting enzymes of the pathway, *i.e.* the enzymes mediating the conversion of the NAD precursors, QA, NAM, NR and NA, into the two mononucleotides NMN and NAMN (Figure 2).

In this section, the features of these enzymes will be outlined, with particular emphasis on the currently available knowledge on their regulation. The reader is referred to specific reviews for their structural and molecular properties [68, 69].

4.1 Nicotinamide phosphoribosyltransferase (NamPRT, EC 2.4.2.12)

4.1.1 A pleiotropic enzyme

NamPRT represents the rate-limiting enzyme of the recycling of the Nam generated by all the reactions leading to NAD consumption (Figure 2) [70]. Under basal conditions the rate of this route is sustained, mostly due to the necessity of replenishing cells with the NAD consumed by a constitutive PARP activity [8]. Under altered conditions, like diet variation, caloric restriction, exercise, stress and aging, variations in the NamPRT expression and activity occur, that are directly responsible for the changes in NAD levels described in section 2.3.

The enzyme is upregulated under conditions of nutrient restriction, both in cultured cells and in the liver of animal models [39, 71]; on the other hand, it is downregulated in specific organs by HFD feeding, a feature that is likely to contribute to the pathogenesis of type 2 diabetes [42]. Indeed, the enzyme's catalytic activity has been demonstrated to be required for the maintenance of glucose homeostasis [58]. Exercise significantly increases NamPRT levels in the skeletal muscle of young rats, and, notably, a regular exercise is able to reverse the age-associated decline in both the enzyme and the NAD levels in aged animals [72]. Also in humans, skeletal muscle NamPRT is upregulated in response to exercise [73, 74], and the protein expression correlates with mitochondrial content, as well as mitochondrial ATP synthesis and aerobic capacity [73].

NamPRT is upregulated both *in vivo* and *in vitro* models of cerebral ischemia and plays a key role in neuron survival [75-77]. Recent data link the enzyme's neuroprotective action to its catalytic activity, which is required to reduce both the mitochondrial dysfunction and to increase mitochondrial biogenesis [77]. In keeping with these results, enzyme's synthetic activators have been shown to block neuronal cell death and to improve the neurological outcome in several rodent models of both neurodegenerative diseases and injury [78, 79]. On the other hand, in transected mammalian axons where active transport from neuronal cell bodies cannot replenish the short lived cytoplasmic NMNAT2 isoform leading to NMN accumulation, NamPRT inhibition seems to protect from axonal degeneration by locally limiting NMN formation [80]. Thus, whether NamPRT is neuroprotective or pro-degenerative seems to depend on the presence of an NMNAT activity able to scavenge NMN towards NAD biosynthesis.

NamPRT is also considered a longevity protein as, parallel to the decrease in NAD levels, NamPRT protein levels also decrease in several organs of aged mice [42, 81]. Indeed, NamPRT overexpression in cultured human vascular smooth muscle cells, besides conferring resistance to acute oxidative stress, delays senescence and increases replicative lifespan via SIRT1 activation

[82]. Likewise, a modest enzyme overexpression is able to increase cell proliferation and to extend the replicative lifespan of human endothelial cells following glucose overload [83].

High levels of NamPRT have been observed in several kinds of tumors, with a positive correlation between tissue levels and a more aggressive phenotype and poor prognosis [84]. Following the first study showing that inhibition of NamPRT activity induced apoptosis in tumor cells [85], several studies, both *in vitro* and *in vivo*, demonstrated that inhibition of the enzyme result in tumor growth suppression, and cell sensitization to oxidative stress and DNA damaging agents. Indeed, the first synthesized NamPRT inhibitors (FK866 and CHS828) have already entered clinical trials, and the interest in the synthesis of novel NamPRT-targeted molecules is increasing [86]. The mechanism by which NamPRT inhibition causes cancer cell death is linked to the decrease in NAD levels and the consequent impairment of the glycolitic pathway [87, 88] and/or the pathways mediated by SIRT1 activity [89] [90].

NamPRT is critically required for development of both T and B lymphocytes [91], and its expression is upregulated by inflammatory stimuli, like lipopolysaccharide and different kinds of cytokines, in several types of immune cells, including monocytes, macrophages, dendritic cells, T and B lymphocytes, as well as epithelial and endothelial cells [92]. In turn, its upregulation favors myeloid and lymphoid differentiation and increases the secretion of a number of inflammatory cytokines. Indeed, the enzyme's inhibition has anti-inflammatory effect in several inflammation-related diseases [93, 94].

NamPRT itsef can behave as a cytokine: it is released into the extracellular environment from several different cell types, including leukocytes, differentiated adipocytes (and thus it was early referred to as visfatin), macrophages, cardiomyocytes, and hepatocytes [95, 96]. Actually, NamPRT was first identified as a Pre-B cell colony-Enhancing Factor (PBEF), *i.e.* as a cytokine able to synergize with interleukin (IL)-7 and stem cell factor for the maturation of B cells precursors [97, 98]. Since then, a variety of effects have been demonstrated to be exerted by the extracellular form of NamPRT, including stimulation of angiogenesis, cell proliferation and chemotaxis, induction of the production of various inflammatory cytokines, like IL-6, IL-1 β , tumor necrosis factor α , and tumor growth factor β 1, as well as costimulatory molecules [95, 96, 99]. Intriguingly, in most such effects the enzyme catalytic activity seems to be not indispensable. However, the biological function of extracellular NamPRT and its mechanism of action, as well as the mechanism of secretion, have not been clarified yet. The extracellular enzyme levels are increased in various illnesses, including chronic inflammatory conditions, like rheumatoid arthritis, lupus, inflammatory

bowel disease, sepsis, infection, and in many tumors [100, 101]. We have recently demonstrated that NamPRT is secreted by chronic lymphocytic leukemia (CLL) cells, and contributes to the induction of a immunosuppressive and tumor-promoting microenvironment in CLL [102]. Levels of extracellular NamPRT have been reported to be high also in some metabolic disorders, like type 2 dyabetes, even though in this case contradictory results have appeared in the literature [86, 103].

4.1.2 NamPRT gene organization and regulation

In silico sequence analysis of the namprt gene reveals the presence of two distinct segments in the 5'-flanking region, which might act as a distal and a proximal promoter, and whose alternative use has been proposed to result in a tissue-specific expression of the gene [104]. Throughout this region, different and multiple transcription factors binding sites, including hormonally and chemical responsive elements, have been identified. Among them are the binding sites for nuclear factor (NF)-1, activator protein (AP)-1, AP-2, NF-kB, and signal transducer and activator of transcription (STAT), which are known to regulate cytokine expression, and whose presence suggests a role for NamPRT in innate and adaptive immunity [104, 105]. NF-kB is also known to be responsive to mechanical stimulation, and the presence of its binding sequence might explain the observed induction of NamPRT expression in fetal membranes during mechanical distension [106] and in lung endothelium during mechanical ventilation [107]. The analysis also revealed the presence of the binding sites for the nuclear factor NF-IL6, the cAMP response element binding protein, the glucocorticoid receptor and the corticotropin releasing factor. Indeed, several hormones are reported to affect NamPRT expression, like growth hormone, T3, insulin, progesterone and testosterone, which all suppress NamPRT mRNA expression in adipocytes [108, 109].

Despite these findings, only few studies have addressed the regulatory mechanism of NamPRT expression. In particular, it has been demonstrated that the gene is transcriptionally activated in response to hypoxia through binding of the hypoxia-inducible factor (HIF)-1 to functional HIF-responsive elements located within the proximal promoter region [110]. Interestingly, NamPRT is induced by hypoxia in breast cancer, where it stimulates tumor cell proliferation and invasion [110, 111]. Moreover, it has been shown in liver that the O family members of the forkhead transcription factors (FOXO) control triglyceride homeostasis via direct regulation of the NamPRT gene expression. In particular, FOXO1 binds to a functional insulin-responsive element located

within the proximal promoter of the gene, thus inducing NamPRT expression, which results in reduced lipogenesis and increased fatty acid oxidation [112].

4.1.3 The regulatory loop NamPRT/SIRT1

Most of the effects deriving from the upregulation of intracellular NamPRT, described in section 4.1.1, are mediated by the increase of sirtuins' deacetylating activity on their protein targets. In this view, NamPRT is considered the major regulator of sirtuins activity: not only its catalyzedreaction provides the NMN substrate to start the recycling of Nam back to NAD, but it also acts as a scavenger of Nam itself, which is a sirtuins inhibitor. Several studies clearly show that sirtuins activity is directly modulated by the NamPRT activity. The two enzymes appear also transcriptionally linked: in liver, the core clock components of the circadian machinery CLOCK:BMAL1, together with SIRT1, bind to the E-boxes on the NamPRT promoter, increasing its expression. This is followed by the increase of NAD synthesis, which in turn enhances SIRT1 activity. In a negative feedback loop, SIRT1 catalyzes deacetylation of BMAL1 and represses the clock machinery, thereby blocking NamPRT expression [35, 36]. In this way NamPRT and SIRT1 work cooperatively in the circadian regulation of NAD biosynthesis, and thus in the circadian regulation of metabolism. Indeed, recently, it has been demonstrated that the clock-driven NAD oscillations are responsible for the circadian oscillation of SIRT3 activity in mitochondria, which in turn generates rhythms in the activity of mitochondrial oxidative enzymes, contributing to the maintenance of energy homeostasis during feeding-fasting cycles [113].

In liver, the regulatory loop NamPRT/SIRT1 has been shown to control lipid metabolism under nutrient-deprived conditions. During fasting, SIRT1 is recruited to both its own and NamPRT promoters, leading to genes transcription; the induced NamPRT raises NAD levels, which results in increased SIRT1 deacetylating activity with the consequent increase in lipid oxidation and inhibition of lipid synthesis [114]. Notably, in obese mice elevated levels of microRNA-34a inhibit both NamPRT and SIRT1 expression by directly binding to the 3' UTRs of their mRNAs, thus disrupting the loop and reversing the effects on lipid metabolism [114].

4.1.4 The regulatory axis AMPK-NamPRT-SIRT1

The best characterized cellular energy sensor is AMP-activated protein kinase (AMPK). Its upregulation under conditions of metabolic stress that cause an increase in the AMP/ATP ratio, like muscle contraction and glucose deprivation, shuts down energy-consuming pathways while

simultaneously switching on ATP-producing pathways [115]. Several lines of evidence suggest that the exercise- and fasting-induced increases in NamPRT mRNA and protein levels depend on a functional AMPK signaling cascade [38, 39, 74]. By increasing NamPRT expression and NAD levels, AMPK enhances SIRT1 activity, leading to activation of its downstream targets. Among the targets controlled by AMPK are PGC-1 α , FOXO1 and FOXO3 transcription factors: it is their AMPKmediated deacetylation that might explain many of the biological effects of the AMPK- NamPRT-SIRT1 axis on energy metabolism [37, 39]. AMPK is also activated in response of ischemia, and it has been shown that the neuroprotective effect of NamPRT during ischemic stress is AMPKdependent [75]. The AMPK-NamPRT-SIRT1 axis has been also implicated in the regulation of the circadian clock machinery in mice white adipose tissue, where a decrease in the axis signaling causes deregulation of lipid metabolism, leading to an obese phenotype [116]. It also regulates the expression of a circadian gene involved in glucagone release by α -pancreatic cells in response to changes in extracellular glucose levels [117].

The mechanism underlying the AMPK-mediated regulation of NamPRT is still unknown: some studies on the exercise-induced upregulation of the enzyme in skeletal muscle report that the AMPK-dependence might be mediated by both transcriptional and post-translational mechanisms [38, 74].

4.1.5 NamPRT localization

Intracellular and extracellular enzyme localizations have been reported. Consistent with its pivotal role in recycling Nam to NAD, intracellular NamPRT is an ubiquitous enzyme, with the highest expression found in peripheral blood lymphocytes [97, 118]. A systemic inhibition of the enzyme in mice leads to a significant decrease of the intracellular NAD content in several organs, with the brain showing the highest depletion [119]. Notably, in the brain the enzyme is mostly expressed in neurons, and at a much lower level in glial cells [76]. In pancreas, the enzyme is predominantly localized in β -cells, and in human islets it has been shown to be secreted in response to high glucose stimulation [120]. Serum levels of circulating NamPRT are reported to be around 5 ng/ml by western blot analyses [102]. Leukocytes and differentiated adipocytes are considered to be the major sources of circulating NamPRT [58, 118, 121], however also hepatocytes can constitutively release the enzyme [122]. While it has been demonstrated that the extracellular form of NamPRT is catalytically active, it is still unclear whether sufficiently high levels of its substrates, particularly PRPP and ATP, would be present in the extracellular space to sustain

the enzyme activity. Likewise, the mechanism of its secretion is still unknown: neither a classic endoplasmic reticulum- Golgi-, nor a microvescicle-dependent pathway seem to mediate the enzyme release [121]. The mechanism of its cytokine function and the nature of its putative receptor are also under investigation.

While it is accepted that the intracellular enzyme is localized in both the nucleus and the cytoplasm, its mitochondrial localization is still controversial. Under condition of nutrient restriction, Its expression was found to be induced in mitochondria of both cultured cells and mammalian tissues, leading to an increase of NAD levels specifically in such organelle [71]. Subsequent studies, however, did not confirm the presence of the enzyme in mitochondria [8, 123, 124] where the only NAD biosynthetic enzyme to be found was the isoform NMNAT3 [124]. The absence of the Nam salvage route in mitochondria, and the presence of an autonomous mitochondrial NAD pool which is maintained even upon massive depletion of the cytoplasmic pool [8, 71] raises questions on how these organelles can maintain their NAD content. The current model is that mitochondrial NMNAT3 synthesizes NAD from the cytoplasmic NMN that crosses the mitochondrial barrier by means of still unknown mechanism of transport [124].

4.1.6 Regulation of the NamPRT-catalyzed reaction

NamPRT enzyme is highly evolved to efficiently regulate NMN synthesis through a facultative ATP-dependent autophosphorylation process. This ATPase activity is non-stoichiometrically coupled with the intrinsic transferase activity through the formation of a phosphorylated enzyme intermediate at a conserved histidine residue in the active site [125]. This feature has remarkable effects on both the enzyme kinetics and the chemical equilibrium of the reaction [126]. In particular, the K_m of the enzyme for Nam decreases to 5 nM, and the *k*_{cat} for NMN synthesis increases 6-fold, leading to an enhancement of the catalytic efficiency of about 1,100-fold . Moreover, ATP hydrolysis thermodynamically shifts the unfavorable products/substrate ratio toward NMN accumulation. In this view, intracellular ATP appears to represent an *in vivo* determinant of the extent of Nam recycling, even at very low Nam intracellular concentrations, due to both kinetic and thermodynamic effects.

NAD and NADH are strong competitive inhibitors of the ATP-coupled reaction, with K_i values of 0.14 μ M and 0.22 μ M versus PRPP, and 2.1 μ M and 3.2 μ M versus Nam [126]. The ATP-uncoupled reaction is not affected. The enzyme activity is subject to substrate inhibition by high concentrations of Nam and the presence of NAD is able to remove such inhibition [126].

Remarkably, the enzyme inhibition by NAD and NADH provides a molecular basis for a regulatory feedback mechanism within the NAD biosynthetic pathway, capable of sensing the NAD(H) pool to maintain its homeostasis.

4.2 Nicotinamide riboside kinase (NRK, EC 2.7.1.22)

NRK allows utilization of NR towards NAD biosynthesis, by catalyzing its ATP-dependent phosphorylation to NMN (Figure 2). Two distinct human genes have been identified coding for two isoforms, NRK1 and NRK2, sharing 57% identity [60]. Both isoforms exhibit K_m values for NR in the low micromolar range, with the highest catalytic efficiency displayed by NRK1 [62, 127]. While NRK1 is ubiquitously expressed, a splice variant of NRK2, named Muscle Integrin Binding Protein (MIBP) has been demonstrated to be selectively expressed in heart and skeletal muscle, and to be translated only in the latter tissue, indicating a control of the NRK2 gene at both transcriptional and translational level [128]. It has been demonstrated that during myogenesis, MIBP levels decrease and the protein exerts an important role in the regulation of cell differentiation [128, 129]. In zebrafish, MIBP is a critical regulator of muscle fiber growth, and the interesting finding that treatment with NAD is able to rescue MIBP knockdown phenotypes of impaired muscle morphogenesis strongly indicates that the NRK2 catalytic activity is required for proper muscle development [130]. In keeping with the existence of a transcriptional control of NRK2, its mRNA levels, which are undetectable in the nervous system, significantly increase in rat dorsal root ganglia upon sciatic nerve injury [131].

4.3 Quinolinate phosphoribosyltransferase (QAPRT, EC 2.4.2.19)

QAPRT participates to NAD *de novo* synthesis from tryptophan by converting QA to NAMN (Figure 2). Five steps are necessary to generate QA from the aminoacid through the KP, a principal route of tryptophan catabolism producing several neurotoxic and neuroprotective metabolites. The intermediate (ACMS) is the last metabolite common to both the catabolic and anabolic fate of the aminoacid. In fact, as shown in Figure 3, it can be either decarboxylated to AMS by the enzyme ACMS decarboxylase (ACMSD) and thus channeled towards complete oxidation, or it can non-enzymatically cyclize to QA and thus be directed towards NAD synthesis [132]. Changes in ACMSD activity are known to be readily reflected in serum and tissue QA levels and in the rate of tryptophan-NAD conversion, clearly indicating the role of ACMSD activity in the *de novo* NAD biosynthesis regulation [133-135].

The significant contribution of the *de novo* pathway to the overall intracellular NAD availability is confirmed by several studies performed in cultured macrophages and nervous cells, showing that alteration in the activity of the rate-limiting enzyme of the KP, *i.e.* indoleamine 2,3-dioxygenase (IDO) (Figure 3), or inhibition of QAPRT result in changes in the intracellular NAD content [136-139]. Notably, the two enzymes activities are required to maintain SIRT1 function in cultured human neurons and astrocytes [139]. Even more striking, in vivo experiments dealing with the pharmacological inhibition of the KP in rats with pneumococcal meningitis show a reduction of NAD levels in the hippocampus [140]. These results suggest that the role of the induction of the KP that occurs during neuroinflammatory diseases might be to maintain NAD levels through *de novo* synthesis [139].

The nearly ubiquitous distribution of QAPRT mRNA, as revealed from inspection of the current gene expression databases, and the detection of an active enzyme in all the mammalian tissues examined recently by us [141], indicate that synthesis of NAD from QA is not confined to brain, kidney, liver and immune cells, in which the KP is known to be fully operative [65]. It is likely that NAD synthesis from QA might represent a significant route also in those tissues/cell-types that generate QA from an incomplete KP, or can take it up from the bloodstream [142].

Many reports suggest that QAPRT might be subjected to regulation at transcriptional level. In particular, the enzyme is reported to be expressed in follicular thyroid carcinoma at significantly higher levels than in adenoma [143], and it is also upregulated in gliomas and acute myeloid leukemia, with a high expression associated with an unfavorable prognosis [144, 145]. Notably, treatment of cultured glioma cells with temozolomide, irradiation or oxidative stress has been shown to induce the expression of the enzyme mRNA [144]. Accordingly, recurrent glioblastoma after the radiochemoterapy shows higher levels of QAPRT protein than the tumor tissue before the treatment. It is concluded that in glioma tissues, the enzyme would commit the microgliaderived QA towards NAD synthesis, thus raising NAD levels, which in turn would protect malignant cells from the therapy stress [144]. Due to the neurotoxic nature of the QA substrate, several studies have investigated the possible enzyme's involvement in the pathogenesis of neurodegenerative disorders characterized by a significant accumulation of QA deriving from KP activation. The increase of the enzyme activity in the brain of patients with Huntington disease [146] and with olivopontocerebellar atrophy [147], the increase of the enzyme level in glial cells of rat models of chronic epilepsy [148] and the increase of mRNA expression in the brain of Alzheimer disease mice [149] appear to suggest a neuroprotective function of the enzyme.

However, it is evident that QAPRT activity is not sufficient to fulfill the role of QA scavenger. On the other hand, the decrease of the enzyme activity observed in the brain of epileptic patients has been considered as a possible mechanism for the pathological accumulation of QA [150]. Notably, a recent study showed that the value of the tryptophan to Nam conversion ratio, which is zero in $qaprt^{-}/^{-}$ mice, is very similar between $qaprt^{+}/^{-}$ and $qapr^{+}/^{+}$ mice [151]. Overall, these results suggest that QAPRT may not to be rate-limiting in the NAD *de novo* biosynthesis under physiological conditions, but it may become limiting under pathological conditions.

Kinetic analysis of the human QAPRT-catalyzed reaction suggested a complex regulation via a PRPP substrate inhibition of mixed-type [152]. The authors speculated that, in addition to triggering the formation of a dead-end QAPRT-PRPP complex, the PRPP substrate would bind to a allosteric site of the enzyme, inducing a conformational change to be transmitted to the active site [152]. Differently from NamPRT and NAPRT, QAPRT activity seems to be ATP-insensitive, in keeping with the irreversible nature of the reaction, due to the decarboxylation step occurring along with the phosphoribosyl transfer. Contradictory data on the inhibitory effect of NAD on partially purified enzyme from different mammalian sources have been reported [153, 154].

4.4 Nicotinate phosphoribosyltransferase (NAPRT, EC 6.3.4.21)

NAPRT is responsible for salvaging the NA deriving from diet directly or after deamidation of Nam by intestinal flora [65]. Although the enzyme activity can be detected in most mouse tissues [141], classic feeding studies have shown that in mouse NA acts as a more efficient precursor than Nam in liver, intestine, heart and kidney [155, 156]. NA is also found to be more efficient than Nam in raising NAD levels in a cultured kidney cell line, and in protecting cells from the drop in NAD content induced by oxidative stress [157]. It is also able to restore cell viability in stressed and NAD-depleted atrocytes [144]. To our knowledge, no data on the enzyme regulation at the gene level are available in the literature, and also scarce are the studies on the regulation of the NAPRTcatalyzed reaction. It is reported that ATP behaves as an allosteric modulator of the enzyme [158-160]; at concentrations in the millimolar range it stimulates the enzyme activity by lowering the K_m for both substrates with either no effect [159, 160] or a lowering effect [158] on the maximal velocity. It is likely that the ATP activation mechanism is similar to that reported for the bacterial ortholog, *i.e.* the transient phosphorylation of a conserved histidine [161], since mutation of the corresponding residue in the human enzyme (His213) results in the loss of the ATP stimulatory effect [158]. Contrary to NamPRT, NAPRT is not inhibited by NAD, which explains its significantly

higher efficiency in raising NAD levels *in vivo* [157, 162]. Moreover, NAPRT is strongly activated by phosphate [158].

5. CONCLUDING REMARKS

In this review we have highlighted the importance of the NAD biosynthetic enzymes to maintain NAD homeostasis and to determine the cell metabolic strategy in order to efficiently respond to environmental and nutritional stimuli. The research of the past decade has been particularly focused on unraveling the diverse regulatory network that surrounds the enzyme NamPRT, likely because it is considered the major enzyme fueling NAD to sirtuins. Such studies clearly demonstrate the NamPRT role as a nutrient- and stress-responsive enzyme dictating, via sirtuins actuation, the regulation of mitochondrial biogenesis and energy homeostasis with profound impact on metabolism, cell survival, and aging. While much effort has been devoted to the investigation on the regulatory properties of the NamPRT-catalyzed reaction, the mechanisms underlying the enzyme regulation at transcriptional and post-translational level are still largely unknown. Likewise, a rather unexplored area is the regulation of the enzymes considered to be rate-limiting in those NAD biosynthetic routes that are alternative to Nam salvaging, i.e. NRK, NAPRT and QAPRT. They are differentially distributed among various tissues and cell types and their levels and/or enzymatic activities are reported to change in response to various stimuli, like malignant transformation, oxidative stress and cell differentiation. Although the studies on these enzymes are rather limited, this review evidenced the significance of their impact on the intracellular NAD content both in pathological and physiological conditions. This suggests that these enzymes might also be subject to regulation to drive suitable metabolic adaptations.

In this review, we sought to convey all the available evidence on the regulatory properties of the committed enzymes in NAD biosynthesis. This survey underscored several gaps, that should prompt a deeper investigation into missing features in order to clarify the contribution of individual enzymes to overall NAD biosynthesis, also in light of the exploitation of the NAD biosynthetic pathway as a source of therapeutic targets.

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FIGURE LEGENDS

Figure 1. The dual role of NAD(P) in redox metabolism and signaling.

Figure 2. Overview of mammalian NAD biosynthesis. *De novo and* salvage/recycling routes are represented. The release of Nam by all NAD consuming enzymes is highlighted. The extracellular formation of NAD precursors by CD73, CD38 and ARTs is also shown.

Figure 3. NAD *de novo* biosynthetic pathway. The alternative fates of the ACMS intermediate are represented.





NAD(P)H



