Functional Characterisation of Three Human Nicotinamide Mononucleotide Adenylyltransferases

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CONTENTS

List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

Paper I:

Berger F*****, **Lau C***, Dahlmann M, and Ziegler M (2005). *Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms.* J Biol Chem: 280, 36334-36341 *******The authors contributed equally to the work.*

Paper II:

Lau C and Ziegler M (2008). *Golgi localisation of the human NAD biosynthetic enzyme NMN Adenylyltransferase 2 is mediated by palmitoylation within an isoform-specific domain.* Manuscript

Paper III:

Berger F, **Lau C**, and Ziegler M (2007). *Regulation of poly(ADP-ribose) polymerase 1 activity by the phosphorylation state of the nuclear NAD biosynthetic enzyme NMN adenylyl transferase 1*. Proc Natl Acad Sci U S A: 104, 3765-3770

Abbreviations

2-BP	2-bromopalmitate, 2-bromopalmitoic acid
ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
ATP	Adenosine triphosphate
BFA	Brefeldin A
EGCG	(-)-epigallocatechin 3-gallate
ER	Endoplasmic reticulum
HPLC	High performance liquid chromatography
NAD	Collective for NAD^+ and NADH
NAAD	Nicotinic acid adenine dinucleotide
NADP	Collective for $NADP^+$ and NADPH
NaMN	Nicotinic acid mononucleotide
NMN	Nicotinamide mononucleotide
NMNAT	Nicotinamide mononucleotide adenylyltransferase
NNT	nicotinamide nucleotide transhydrogenase
NZ	Nocodazole
PAR	Poly(ADP-ribose)
PARG	Poly(ADP-ribose) glycohydrolase
PARP	Poly(ADP-ribose) polymerase
PKC	Protein kinase C
PPi	Inorganic pyrophosphate

Abstract

Nicotinamide adenine dinucleotide, NAD, is an essential redox factor in many, primarily catabolic, hydrogen transfer reactions. While the redox reactions are not accompanied by a net loss of the pyridine nucleotides, NAD^+ is actively degraded in a variety of ADP-ribosyl transfer reactions which are implicated, for examples, in cellular stress responses, calcium signalling and genomic stability. Thus, a continuous biosynthesis of NAD is essential for cell viability and metabolism. The final step in NAD biosynthesis, the reversible transfer of the AMP moiety from ATP onto a pyridine mononucleotide (NMN or its acidic form) is catalysed by nicotinamide mononucleotide adenylyltransferases (NMNATs) and is common to both the *de novo* and the known salvage pathways. In humans, three NMNAT isoforms have been described and partially characterised. The present study was aimed at a comprehensive characterisation of the human NMNATs, in particular, with regard to isoform-specific functions in order to gain further insight into the cellular NAD homeostasis and its regulation.

Kinetic studies revealed that NMNAT1 possesses the highest specific activity both in the forward and reverse reactions, while NMNAT3 exhibited the lowest substrate selectivity. Importantly, NMNAT3 readily used the reduced nicotinamide substrates (NMNH or NADH in the forward and reverse reaction, respectively) thereby indicating that besides NAD^+, NADH may be directly formed from a reduced precursor. No significant inhibition by physiological intermediates including nucleotides and nucleosides as well as poly(ADP-ribose), PAR, was found. However, we observed a considerable activation, particularly of NMNAT2, by the green tea polyphenol epigallocatechin 3-gallate (EGCG).

Investigations of the subcellular distribution established distinct localisations of the three NMNAT isoforms in the nucleus, the Golgi complex and the mitochondria. Structural analyses revealed that all human NMNATs have insertions in their primary structures which are absent from the bacterial counterparts. While in NMNAT1 this region of the protein

contains a known nuclear localisation signal, we revealed a targeting function of a corresponding domain in NMNAT2. The stretch of amino acids 109-192 was shown to direct the protein to the Golgi complex. Deletion of this domain resulted in a catalytically active, cytosolic protein. Moreover, we demonstrated that cysteine palmitoylation within this targeting domain is essential to anchor NMNAT2 at the cytosolic surface of the Golgi complex. The isoform NMNAT1 is known to be targeted to the nucleus by a nuclear localisation signal, which we highlight to reside within the corresponding domain. An important additional function of this targeting domain in NMNAT1 was also established. We found that this region (amino acids 102-149) mediates a phosphorylation-dependent association with poly(ADP-ribosyl)ated PARP1 which substantially enhances the poly(ADP-ribosyl)ating activity of PARP1. Interestingly, the catalytic activation of PARP1 was independent of the ability of NMNAT1 to synthesise NAD, as revealed using a catalytically dead mutant of NMNAT1.

Collectively, our results suggest that the inserts into the sequences of the human NMNATs mediate subcellular targeting and interactions with other cellular factors. Therefore, we propose to denote this region as isoform-specific targeting and interaction domain (ISTID). It will be important to establish this function also for the corresponding domain in the mitochondrial isoform, NMNAT3. Interestingly, its amino acid sequence indeed lacks a classical N-terminal targeting sequence implying another mode of mitochondrial import.

1. INTRODUCTION

1.1 Metabolism, pools, and signalling functions of the redox cofactor nicotinamide adenine dinucleotide

NAD collectively stands for nicotinamide adenine dinucleotide in its oxidised, NAD^+, and reduced form, NADH (Fig.1). NAD is a well known cofactor in mostly catabolic reduction/oxidation (redox) reactions in for example glycolysis, citric acid cycle and oxidative phosphorylation. Phosphorylation of NAD^+ by NAD kinase yields NADP (Fig.1). The redox couple $NADP^+$/NADPH serves mainly in reductive hydrogen transfer reactions catalysed by for example glucose 6-phosphate dehydrogenase in the pentose phosphate pathway. In general, the ratios of NAD^+/NADH and $NADP^+$/NADPH reflect the redox state of a cell (Pollak et al., 2007). Under physiological conditions, cellular NAD levels exceed those of NADP. Furthermore, NAD is mostly present in its oxidised form with a ratio of NAD^+/NADH ~ 3-10 (Zhang et al., 2002; Ido Y, 2007). The concentration of total cellular NAD is estimated to be in the submillimolar range (Pollak et al., 2007), and the majority of the nucleotide is suspected to be protein-bound (Williamson et al., 1967). The intracellular pool of NAD is commonly subdivided into the mitochondrial and the nucleo-cytosolic pool. This is because under normal cellular conditions, the inner mitochondrial membrane is assumed to be impermeable for NAD and NADP (DiLisa&Ziegler, 2001), whereas the nucleotides most likely freely diffuse through the pores of the nuclear envelope (Zhang et al., 2002). In contrast to yeast (Todisco et al., 2006), mitochondrial NAD transporters have not been found in mammals.

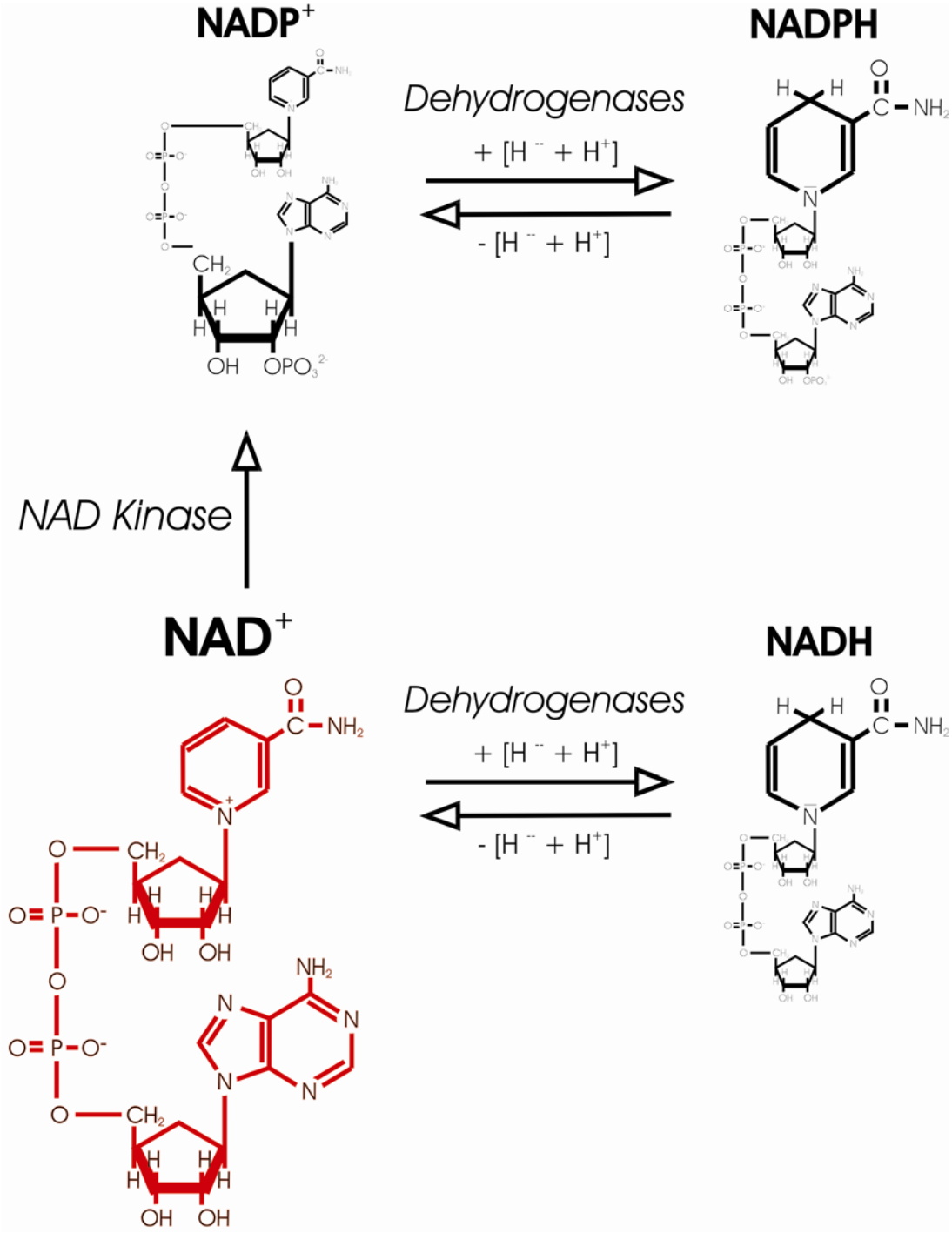

Figure 1: Electron transfer reactions of NAD(P). The NAD^+/NADH redox couple exerts a vital function as cofactor of dehydrogenases in several metabolic redox reactions. NAD^+ can be phosphorylated at the 2'-hydroxyl group of the adenosine ribose by NAD kinase. The product of this reaction, $NADP^+$, also serves as electron acceptor in redox reactions.

Redox equivalents are exchanged between cytosol and mitochondria by dehydrogenase dependent NADH shuttles (McKenna et al., 2006) and the activity of mitochondrial nicotinamide nucleotide transhydrogenase (NNT) (Jackson JB, 2003). Reduced nucleotides could have a particular impact on mitochondrial NAD metabolism. In mitochondria, the ratio of free NAD^+/NADH is only ~7-8, while the level of free cytosolic NADH is ~700 times lower than that of NAD^+ (Williamson et al., 1967; Zhang et al., 2002). Although, the mitochondrial NAD pool is considered to contain significant amounts of the total cellular NAD, uncertainty exists about how it is established (Pollak et al., 2007). In 1996, Barile et al. reported an ATP dependent NAD synthesising activity in rat liver mitochondria (Barile et al., 1996). Apart from this, no other NAD biosynthetic activity could clearly be associated with the mitochondria.

The utilisation of NAD in energy metabolism as essential electron carrier does not result in a net consumption of the nucleotide. The need for continuous NAD synthesis has only been recognised following the recent discoveries of a variety of NAD catabolising processes (Berger et al., 2004). Commonly, pyridine nucleotides degrading reactions include the transfer of ADP-ribose from NAD^+ or $NADP^+$ onto an acceptor molecule under release of nicotinamide (Fig.2) which, in turn, regulates most of these reactions in a negative feedback fashion (Pollak et al., 2007). ADP-ribose transfer reactions and their products are involved in the regulation of calcium signalling, genome stability, ageing/longevity, oxidative stress response and cell death (Virág L., 2005; Belenky et al., 2007; Beneke&Bürkle, 2007; Dali-Youcef et al., 2007; Fliegert et al., 2007; Pollak et al., 2007; Ying W., 2008). Thus, the absolute amounts of NAD and NADP, and not only their redox ratios, are crucial for the maintenance of cellular viability. $NADP^+$ serves as substrate of the multifunctional enzyme CD38 to generate NAADP, the most potent calcium releasing compound in the cell (Lee H.C., 2000; Yamasaki et al., 2005). Apart from this, most of the transfer reactions strictly depend on NAD^+ as substrate. For example, the classes of ADP-ribose transferases (ARTs)

and poly(ADP-ribose) polymerases (PARPs) transfer either a single ADP-ribose molecule or up to 200 units, respectively, from NAD^+ onto acceptor proteins (Hassa et al., 2006; Koch-Nolte et al., 2008) (Fig.2).

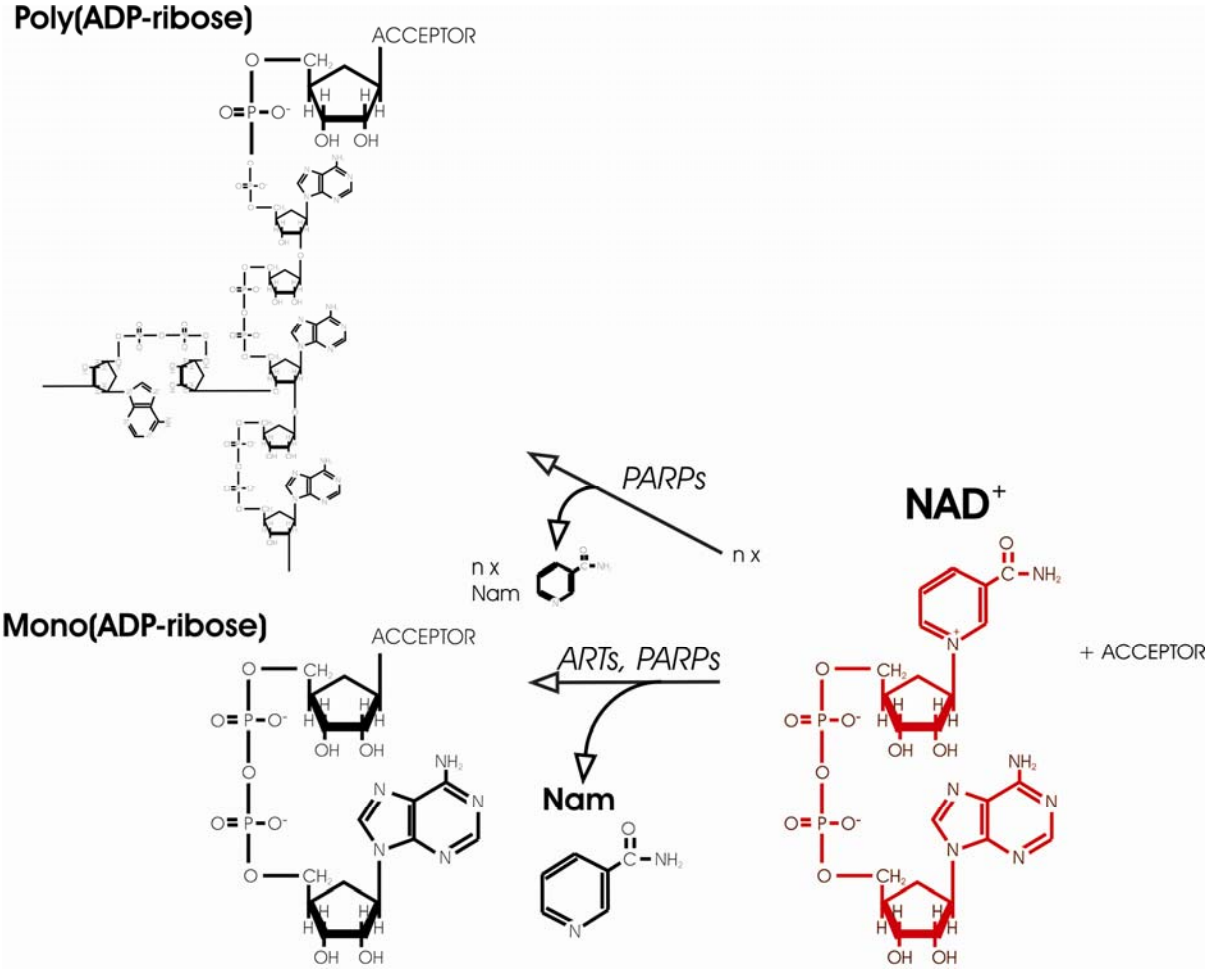

Figure 2: NAD consumption. NAD^+ serves as substrate for ADP-ribose transfer reactions like mono and poly(ADP-ribosyl)ation of acceptor molecules which are catalysed by ADP-ribosyl transferases (ARTs) and poly(ADP-ribose) polymerases (PARPs) under release of nicotinamide (Nam).

Both, ADP-ribose and poly(ADP-ribose) (PAR) can be cleaved off the acceptor by specific hydrolases, ADP-ribose-protein hydrolases and PAR glycohydrolase (PARG). The liberated PAR has been reported to directly mediate apoptotic cell death, although the exact mechanism

is not yet understood completely (Yu et al., 2006; Andrabi et al., 2006; Li et al., 2007). In fact, all free NAD derivatives are proposed to exhibit signalling functions. For example, the metabolite of NAD^+-dependent protein deacetylation, O-acetyl ADP-ribose (OAADPR), influences calcium homeostasis through its binding to TRPM2 calcium channels (Grubisha et al., 2006). The comprehensive role of NAD in cellular signalling is possible due to the localisation of the respective enzymatic activities to subcellular compartments like mitochondria, nucleus, cytosol and the extracellular space. Therefore, gaining insight into to the mechanism and distribution of NAD biosynthesis should be of utmost concern.

1.2 NAD biosynthesis

NAD biosynthesis proceeds via several different routes and has been extensively studied (Magni et al., 2008). In general, the *de novo* synthesis can be distinguished from the recycling salvage pathway (Fig.3). *De novo* NAD synthesis in eukaryotes utilises the essential amino acid L-tryptophan as precursor and proceeds via quinolinic acid with the generation of nicotinic acid mononucleotide (NaMN), one of two possible mononucleotide intermediates. Precursors of the salvage pathway are nicotinic acid (NA), nicotinamide (Nam) and nicotinamide riboside (NR) which are either recycled by pyridine nucleoside and nucleotide hydrolases activities and ADP-ribosylation reactions (Noctor et al., 2006; Magni et al., 2008) or taken up from food sources. The deficiency of NA and Nam (together niacin) causes severe degenerative symptoms which eventually classified them as Vitamin B3 (Elvehjem C.A., 1939). As substrates for nicotinic acid phosphoribosyltransferase (NAPRT) and nicotinamide phosphoribosyltransferase (NamPT), they are metabolised to their mononucleotide equivalents, NaMN and nicotinamide mononucleotide (NMN), respectively, by the transfer of the phosphoribose moiety of phosphoribosyl pyrophosphate (PRPP) onto the pyridine ring (Fig.3).

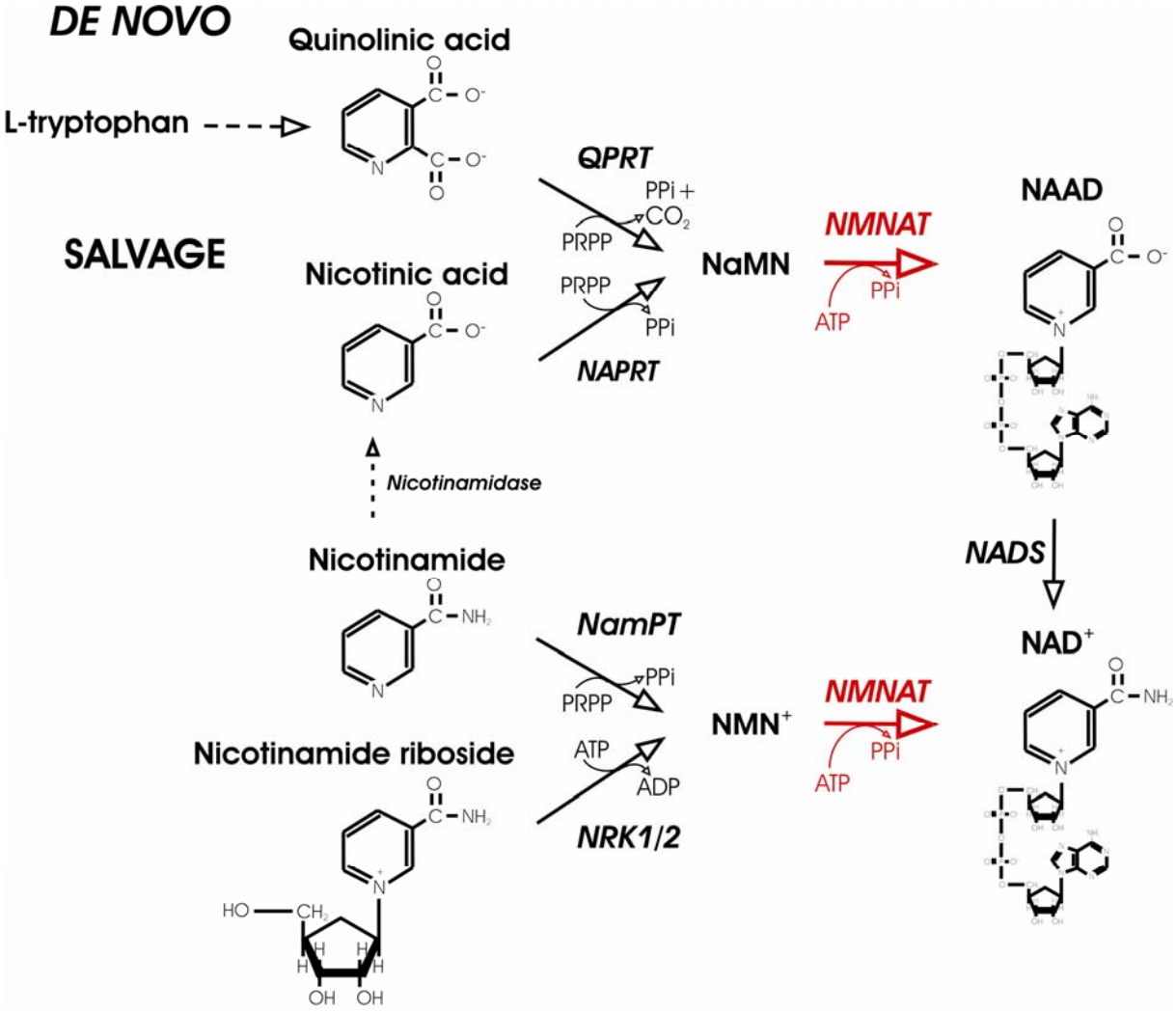

Figure 3: NAD biosynthesis in humans. Human NAD biosynthesis is established by *de novo* synthesis from the amino acid tryptophan and three salvage pathways. In the *de novo* synthesis, quinolinic acid phosphoribosyltransferase (QPRT) catalyses the transfer of the phosphoribose moiety from PRPP onto quinolinic acid. The product of this reaction is NaMN. In the salvage pathway, NAPRT and NamPT synthesise NaMN and NMN (nicotinamide mononucleotide), from nicotinic acid and nicotinamide, respectively. Nicotinamidase activity has not been identified in vertebrates, yet. It is present in lower organisms which lack NamPT. Nicotinamide riboside (NR) kinases, NRK1 and 2, phosphorylate NR to generate NMN. All routes eventually merge at the ATP-dependent step of dinucleotide generation by NMNAT activity. NAAD (Nicotinic acid adenine dinucleotide) is amidated to NAD^+ by NAD synthase (NADS).

In contrast to eukaryotes, bacteria do not express NamPT and are, therefore, not capable to directly synthesise NMN from Nam and PRPP. The conversion of Nam to NA by a nicotinamidase activity, however, allows for the utilisation of Nam in bacterial and plant NAD synthesis (Rongvaux et al., 2003; Wang&Picherski, 2007). This activity has not been identified in mammals. An alternative pathway, the phosphorylation of NR to NMN, has recently regained interest due to the identification of two human nicotinamide riboside kinases (NRK1 and NRK2) in humans (Bieganowski&Brenner, 2004). NR has been found as nutrient in milk which suggests a vitamin character for the nucleoside, similar to niacin. Common to all pathways is the final generation of respective pyridine adenine dinucleotides by the reversible transfer of AMP onto NMN or NaMN (Fig.3). This essential step in NAD biosynthesis is catalysed by nicotinamide mononucleotide adenylyltransferases (NMNATs).

1.3 The family of nicotinamide mononucleotide adenylyltransferases

NAD synthesis by NMNATs has been extensively studied since the 1970ies (Magni et al., 1999; Magni et al., 2004; Lau et al., *In press*). However, the first NMNAT activity was already described 20 years before by Sir Arthur Kornberg in his reports on the reverse reaction of NAD synthesis, namely the pyrophosphorylysis of NAD and NADH (Kornberg&Lindberg, 1948; Kornberg A., 1950) (Fig.4). NMNATs are globular proteins of 20 to 50 kDa and mostly assemble into homo-oligomers of two to six subunits. The overall tertiary fold of NMNATs is highly conserved and resembles the dinucleotide binding fold of α/β phosphodiesterases which is established by two structurally related βαβαβ mononucleotide binding Rossmann folds (Rossmann et al., 1975; Raffaelli et al., 1997). The NMNAT-specific catalytic and substrate binding motif GxFxPx[H/T]xxH can be found at the N-terminus of the enzymes. A second, C-terminal motif, ISSTxxR, is implicated in substrate binding (Emanuelli et al., JBC2001; Saridakis&Pai, JBC2003) (Fig.5).

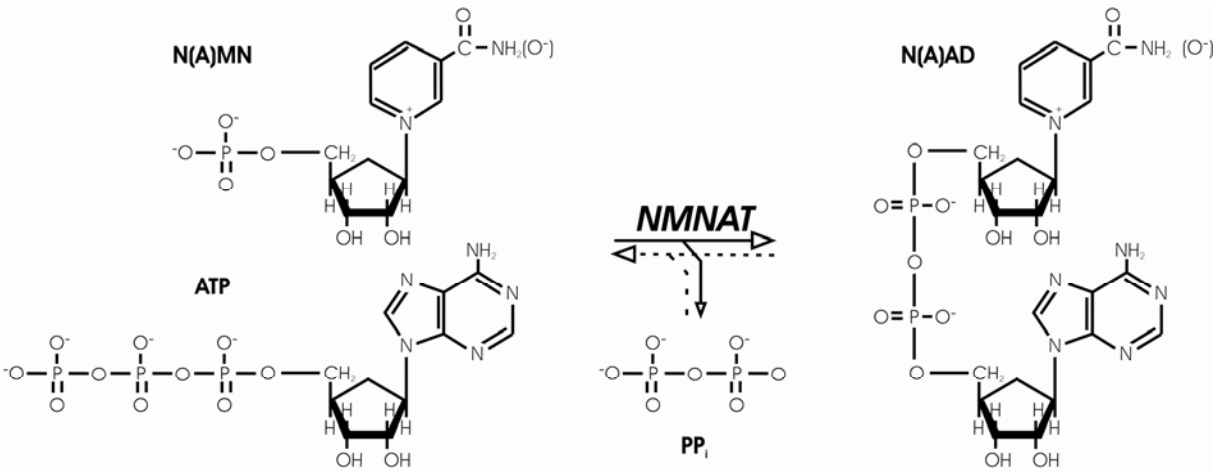

Figure 4: The NMNAT reaction. The reversible adenylyltransfer catalysed by NMNATs utilises ATP for the generation of either nicotinamide adenine dinucleotide (NAD) or its deamidated form nicotinic acid adenine dinucleotide (NAAD) from the respective mononucleotides (NMN or NAMN) and under release of inorganic pyrophosphate (PPi).

Substrate specificities of NMNATs with respect to NMN or NaMN are particularly mediated by structural water molecules (Lau et al., *In Press*). Prokaryotic NMNATs display strong preference towards either NaMN or NMN, whereas human NMNATs are capable of utilising both nucleotides *in vitro* (Magni et al., 2004; Lau et al., *In press*). In an alternative pathway, reduced NMN, NMNH, has been suggested to serve as substrate for NMNATs (Kornberg&Pricer, 1951). Moreover, NMNH may serve as cofactor for certain redox reactions (Fischer&McGregor, 1969). It remains to be established whether NADH could indeed be synthesised directly, for example via the NRK/NMNAT route.

Intriguingly, overexpression of NMNAT results neither in elevated NAD levels nor in a change of the redox state in mammalian cells (Anderson et al., 2002). In contrast, it stimulates NAD dependent processes like nuclear histone deacetylation which indicates increased NAD turnover (Sasaki et al., 2006). These observations suggest that NAD biosynthesis may specifically fuel NAD-dependent signalling processes rather than affect cellular NAD homeostasis.

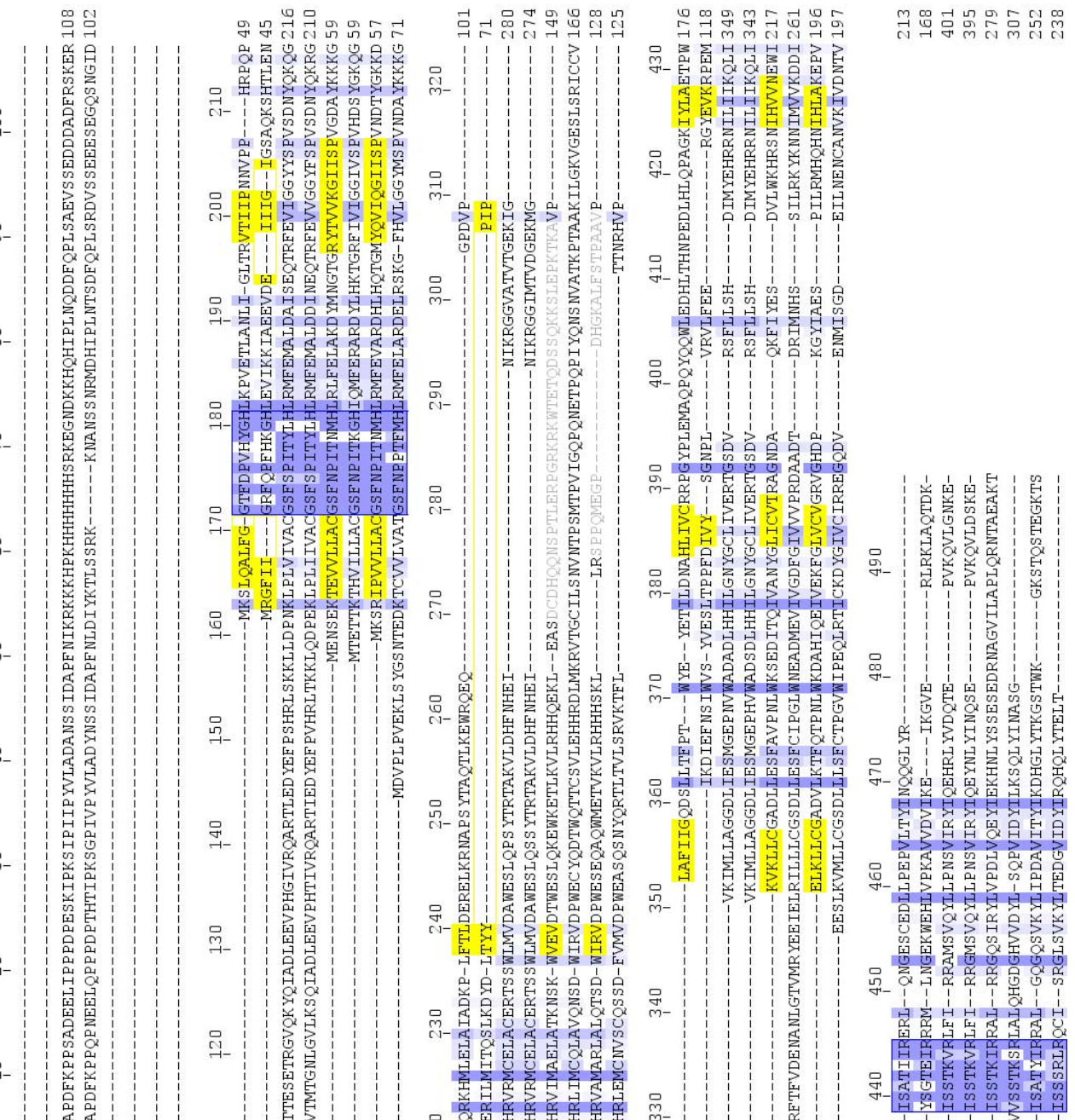

Figure 5: Multiple sequence alignment of NMNATs. The multiple sequence alignment of selected NMNAT homologues was generated using Muscle (Edgar R.C., 2004). Regions which fold as β-sheets in known crystal structures are coloured in yellow. Increasing significance of the Blossum 62 matrix scores in the alignment is reflected by successively darkening blue. *Lau et al., In press*

1.4 Three NMNAT isoforms in humans

In humans, three NMNAT gene products are known to date. Despite their relative high sequence identity, the isoforms display a number of differences (Tab.1). For example, the major activity, NMNAT1, locates to the nucleus (Schweiger et al., 2001), whereas the exact localisation of NMNAT2 and 3 has not been as definite. NMNAT2 was found to locate to cytosol and nucleus (Zhang et al., 2003; Yalowitz et al., 2004). NMNAT3 has been detected in the cytosol and possibly mitochondria (Zhang et al., 2003). This means this isoform could contribute to the mitochondrial NAD pool (1.1). The primary structures of human NMNATs differ from that of their prokaryotic homologues particularly by a variable insertion upstream of β-sheet 4 (Fig.5). Its implication in catalysis might be questionable due to its lack in bacterial NMNATs. Interestingly, human NMNAT1 harbours its nuclear localisation signal here (Schweiger et al., 2001). Yeast NMNATs have also been proposed to locate to the nucleus (Emanuelli et al., 1999; Emanuelli et al., 2003). Yet, the targeting of these enzymes and, therefore, a possible implication of this insertion has not been studied. Interestingly, NMNAT2 and 3 are tissue-specific, while nuclear NMNAT1 is considered to be ubiquitously expressed (Emanuelli et al. 2001; Raffaelli et al., 2002; Zhang et al., 2003; Yalowitz et al., 2004) (Tab.1). It appears important to reveal whether the three human NMNAT isoforms exhibit redundant or isoform-specific functions in the living cell.

Tumour cells are known to exhibit lower NMNAT activity than normal cells (Branster&Morton, 1956; Emanuelli et al., 2001), which prompted the search for substrate analogues as exogenous inhibitors (Franchetti et al., 2005; Sorci et al., 2007). Despite recently established isoform-specific substrate and product inhibition (Sorci et al., 2007) (Tab.1), all tested inhibitors failed to efficiently affect any NMNAT activity. That several pyridine or purine nucleotide derivatives might indeed serve as alternative substrates has been reported for the yeast homologues (Magni et al., 2004), but has not been investigated in case of the human NMNATs. In addition, sufficient information on isoform-specific catalytic

mechanisms in humans was lacking until recently (Sorci et al., 2007), and this study. Substrate accommodation and catalytic efficiency are influenced by the capability to provide hydrogen bonds (Zhang H. et al., 2002) but also by the enzymes quaternary structure (D'Angelo et al., 2000; Werner et al., 2002; Zhou et al., 2002). Indeed, human NMNAT isoforms exhibit different quaternary structures (Tab.1) (Lau et al., *In press*). NMNAT1, for example, assembles into a homo-hexamer, which may account for a more efficient catalysis.

Table 1: Three human NMNAT isoforms.

		NMNAT1	NMNAT2	NMNAT3
Ensembl: ENSG00000		**173614**	**157064**	**163864**
Chromosome		1p36.2[(1)]	1q25[(8,9)]	3q23[(11) 1]
Tissue mRNA levels	**High**	Skeletal muscle, heart, liver, kidney[(5)]	Brain[(8,10)]	Lung, spleen[(7)]
	Low	Brain[(1)] cancer cells[(5)]	Heart, muscle[(10)]	Placenta, kidney, cancer cells[(7)]
CDS (bp)		840	924	756
Theoretical molecular mass [2] (Dalton)		31 932	34 439	28 322
UniProtKB accession number		Q9HAN9	Q9BZQ4-1	Q96T66-1
Tertiary structure		Hexamer[(2-4)] tetramer[(5)]	Monomer [(8)]	Tetramer [(7)]
Substrate specificity[(6)]		NMN	NMN/NaMN	NMN/NaMN
Product inhibition[(6)]	**Competitive**	NAD, NaAD vs. ATP	NAD, NaAD vs. ATP	NAD, NaAD vs. NMN
	Non-competitive	PPi vs. NMN and ATP	None	None
Protein sequence identity [3] %	**NMNAT2**	36; 37[(7)]		
	NMNAT3	49; 50[(7)]	39; 34[(7)]	

(1) The corresponding annotated gene product, Q96T66-2, lacks the essential NMNAT-specific ATP binding motif at its N-terminus, and has, so far, not been subject of experimental investigations. Splice variant 1, Q96T66-1, is alternatively annotated in the RefSeq database as gene product FKSG76 (AAK52726) encoded on chromosome 8. (2) ExPASy server tool Compute pI/Mw, average resolution mode (3) extracted from *ClustalW* (V1.83) pairwise alignment

1.5 Nuclear poly(ADP-ribosyl)ation by PARP1, and cell viability

The catalytic activity of poly(ADP-ribose) polymerases, PARPs, modifies acceptor molecules with ADP-ribose moieties from NAD^+ and eventually generates protein-bound, branched poly(ADP-ribose) (PAR) chains (Bürkle et al., 2005) (Fig.2). PAR-sylation is a post-translational modification that is involved in a variety of physiological and pathophysiological events (Schreiber et al., 2006; Hassa&Hottiger, 2008). The major cellular PARP activity is represented by the nuclear localised PARP1 which, under physiological conditions, detects DNA strand breaks and recruits DNA repair proteins, thereby regulating e.g. genome maintenance and ageing (Dantzer et al., 2000; Oei et al., 2005; Kim et al., 2005; Bürkle A., 2006) (Fig.6). Besides the modification of target proteins like p53 and histone H1, PARP1 serves as major acceptor itself (Adamietz P., 1987; Mendoza-Alvarez&Alvarez-Gonzalez, 2001; Kun et al., 2002).

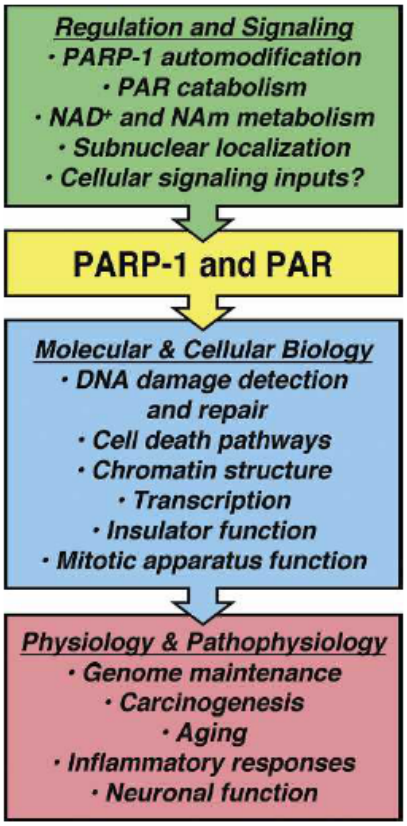

Figure 6: Cellular effects of PAR and PARP1 function. PARP1 and the generation of nuclear poly(ADP-ribose) (PAR) is activated by DNA strand breaks, which causes PARP1 automodification under NAD^+ utilisation and nicotinamide (here: NAm) release. The subsequent cellular effects are diverse and play crucial roles in the regulation of several physiological and pathophysiological processes, e.g. ageing. PARP and PAR related effects are regulated by, amongst others, substrate (NAD^+) supply, nicotinamide feedback inhibition and the degree of PARP1 automodification. *Kim et al., 2005*

Pathophysiologic events such as ischaemia-reperfusion or inflammation result in the release of reactive oxygen species (ROS) which causes extensive DNA damage (Virág L., 2005) (Fig.7). As a response, PARP1 activity rises to more than 100-fold which eventually leads to depletion of cellular NAD^+ pools, energy depletion and necrotic cell death (Juarez-Salinas et al., 1979; Shie et al., 1998; Meli et al., 2003). Further, PARP1 hyperactivation induces apoptotic cell death by mediating translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus (Yu et al., 2002). In fact, PAR directly triggers AIF translocation in a dose dependent manner (Yu et al., 2006; Li et al., 2007) and has been identified as cell death signal in neuronal excitotoxicity (Andrabi et al., 2006). The second product of poly(ADP-ribosyl)ation, Nam, inhibits nuclear NAD^+-dependent histone deacetylation by SIRT1. SIRT1 is the founding member of sirtuins, the mammalian counterpart of yeast Sir2, and regulates gene expression and cell stress response (Dali-Youcef et al., 2007). Elevated NAD biosynthesis by overexpression of nuclear NMNAT1 has been shown to delay neurodegeneration, supposedly by the mediation of SIRT1 function (Wang et al., 2005; Araki et al., 2004). Also, an impact of NMNAT1 on PARP1 activity has been suggested, but remains to be demonstrated (Schweiger et al., 2001; Uhr&Smulson, 1982; Bouchard et al., 2003). In line with such interplay between NAD metabolic events, it would be interesting to know whether the products of PARP1 and SIRT1, PAR, Nam and OAADPR may, in turn, exert regulatory effects on NMNAT activity.

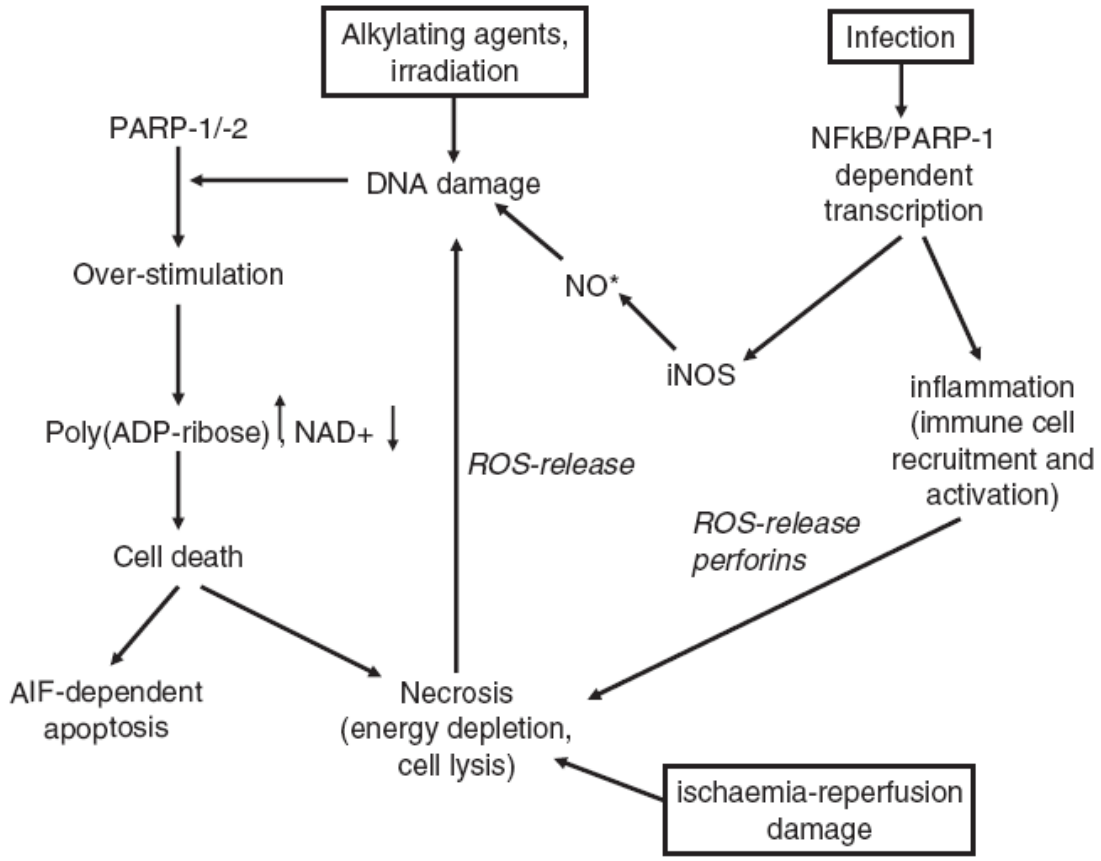

Figure 7: Cell death cycle via nuclear poly(ADP-ribosyl)ation. Hyperactivation of nuclear PARP1 (and 2) by excessive DNA damage can experimentally be induced by, e.g. alkylating agents like MNNG, but also by reactive oxygen species (ROS) including hydrogen peroxide. As a result, the cellular pool of NAD, which serves as substrate for the poly(ADP-ribosyl)ation reaction, becomes depleted, and cell death pathways are initiated, e.g. via nuclear translocation of apoptosis inducing factor (AIF) or energy depletion. These processes also play a crucial role in pathophysiological states such as inflammation and ischaemia-reperfusion damage. *Beneke & Bürkle, 2007*

2. AIMS OF STUDY

Efficient biosynthesis of nicotinamide adenine dinucleotide (NAD) is vital to all organisms. It supplies a variety of NAD(P) dependent and degrading signalling processes which are involved in, amongst others, cellular stress response, genomic stability, calcium signalling and viability (Pollak et al., 2007; Ying W., 2008). The final step in the generation of the dinucleotide is catalysed by nicotinamide mononucleotide adenylyltransferases (NMNATs). This essential enzymatic activity has been studied since 1950, and was initially thought to fuel a general cellular NAD pool. However, the recent identification of three human NMNAT isoforms with differential tissue expression profiles appears to challenge this view. In order to establish the possibility of non-redundant functions of the human NMNATs, we aimed to examine their sub-cellular localisation and catalytic properties, also with regard to regulation by NAD metabolites. NMNAT1 is known to reside in the cell nucleus. Therefore, we wanted to elucidate an earlier proposed functional relationship to the cells major NAD^+-degrading activity owned by the nuclear enzyme poly(ADP-ribose) polymerase 1 (PARP1). To further examine the proposed extra-nuclear distribution of NMNAT2 and 3 promised to reveal information about the establishment of extra-nuclear NAD pools and was, therefore, part of this study. NMNAT1 harbours its localisation signal within an apparently inserted loop region within the conserved dinucleotide binding fold. Intriguingly, all human NMNATs can be distinguished from their bacterial homologues by this feature. Its high flexibility and structurally external residence encouraged us to establish whether and how it could allow for mediating post-translational modification, sub-cellular localisation or intermolecular interactions of human NMNATs.

3. SUMMARY OF RESULTS

The biochemical characterisation of three human NMN adenylyltransferases (NMNATs), in the study of paper I, could corroborate that the isoforms exert non-redundant activities through differential catalytic properties and sub-cellular distribution. In addition to the nuclear localisation of human NMNAT1, we established the distribution of isoform 2 and 3 to the Golgi complex and mitochondria, respectively. In addition to the distinct sub-cellular compartmentation, the human NMNAT isoforms also displayed differential catalytic properties. NMNAT1 is assumed to be ubiquitously expressed and proved to exhibit the highest specific activity in catalysing both the forward and reverse reaction of the reversible NAD synthesis, NMN + ATP $\rightleftharpoons$ NAD + PPi (Fig.4). We could further reveal restricted substrate specificity for this isoform. In contrast, NMNAT3 accepts a variety of substrate analogues. Strikingly, the reduced nicotinamide mononucleotide, NMNH, proved to serve as even better substrate than NMN. The discovery that also NMNAT1 and 2 can synthesise NADH from NMNH establishes a pathway to generate the reduced dinucleotide directly. In contrast to the apparent lack of exogenous inhibitors of NMNAT activity, we revealed the green tea polyphenol (-)-epigallocatechin 3-gallate (EGCG) to be capable of activating NAD synthesis catalysed by NMNAT2 and 3. However, nucleotide intermediates of the NAD metabolism failed to efficiently affect the activity of any NMNAT isoform. We could also demonstrate that all isoforms appear to co-exist at least in some cell types. Therefore, it remains to be elucidated whether and how this triplicate NAD synthesis by human NMNATs is endogenously regulated besides its tissue-specific occurrence and possibly in concert with its sub-cellular compartmentation.

The distribution of human NMNAT2 to the Golgi complex is not only puzzling with respect to the physiological importance of NAD synthesis at this compartment but also due to the lack of an N-terminal targeting sequence. In paper II, we could demonstrate that an isoform-specific variable domain, which is largest in case of NMNAT2 (Lys^{107}-Leu^{192}) and

absent in bacteria (Fig.5), facilitates the post-translational lipid anchoring of NMNAT2 at *cis*-Golgi membranes. Moreover, two adjacent cysteine residues (Cys^{164} and Cys^{165}) within this domain could be identified as target site for the palmitoylation, which is crucial for the enzyme's Golgi localisation. This indicates that NMNAT2 activity is most likely facing the cytosol. Importantly, the deletion of amino acids Val^{109} to Leu^{192} did not affect the catalytic activity of NMNAT2. Together, these findings suggest that this isoform-specific domain carries regulatory rather than catalytic functions.

In paper III, we demonstrate that the phosphorylation state of NMNAT1 regulates the activation of poly(ADP-ribosyl)ated PARP1. We could also prove a regulatory role of the newly identified isoform-specific domain in human NMNAT1 by our discovery that a PKC-specific phosphorylation site resides here (amino acid Ser^{136} within Gln^{107} - Pro^{149}, Fig.5). NMNAT is a homo-hexamer and, therefore, possesses six potential phosphorylation sites per assembled enzyme. Despite the close proximity to the enzyme's nuclear localisation signal, we could not observe an effect of phosphorylation on sub-cellular targeting. However, a negative charge at Ser^{136} diminished a physical interaction of this NAD biosynthetic enzyme with the major cellular NAD degrading activity, PARP1 via PAR, and, thereby, PARP1 stimulation in presence of NMNAT1. We further demonstrated that a physical interaction of NMNAT1 with PAR is essential for accelerating PARP1 activity. An additional substrate channelling effect could be important *in vivo.* A putative PAR binding motif for NMNAT1 could be predicted to reside close to Ser^{136} in the proteins tertiary structure. It remains to be elucidated, whether PAR binding and PARP1 activation is gradually regulated by successive phosphorylation of the homo-hexameric NMNAT1. Furthermore, we provide evidence that the functional interplay between NMNAT1 and PARP1 activity is of pathophysiological relevance. In cells overexpressing NMNAT1, in response to oxidative stress induced DNA damage, we observed an accelerated nuclear translocation of apoptosis inducing factor, which is a hallmark of PAR mediated cell death.

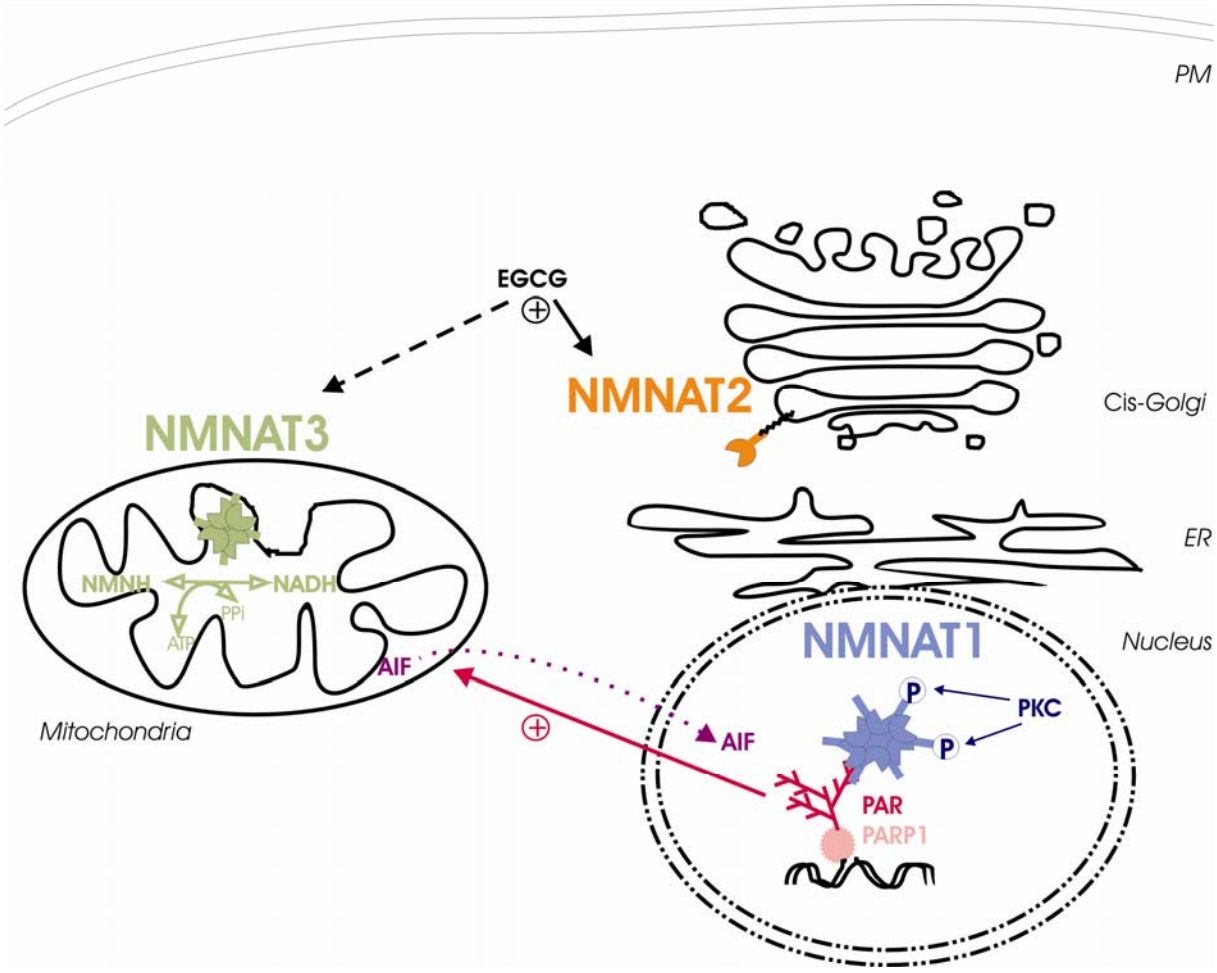

Figure 8: Summary of results Human NMNATs are localised to three distinct sub-cellular compartments, nucleus (NMNAT1), *cis*-Golgi complex (NMNAT2), and mitochondria (NMNAT3). The targeting is at least in case of NMNAT1 and 2 mediated by an isoform-specific loop domain which is dispensable for enzymatic function. NMNAT2 is membrane anchored by cysteine palmitoylation within this domain. The loop domain of NMNAT1 harbours a PKC specific phosphorylation site. Phosphorylation diminishes the interaction with and the stimulation of automodified PARP1, which would otherwise induce AIF release from the mitochondria. The activity of NMNAT3 and especially NMNAT2 is enhanced by EGCG. *In vitro*, NMNAT3 preferably catalyse the reversible synthesis of NADH. We propose non-redundant functions of the three human NMNAT isoforms. (ER, endoplasmic reticulum. PM, plasma membrane)

4. DISCUSSION

4.1 Non-redundant functions of human NMNATs and possible individual impact on cell viability

Human NMNATs are not only tissue-specifically expressed but also distributed to distinct sub-cellular compartments, the nucleus (NMNAT1), the Golgi complex (NMNAT2) and the mitochondria (NMNAT3), as we could establish with paper I. The compartment-specific occurrence of human NMNATs might very well reflect non-redundant, tissue- but also cell-type specific functions. Evidence which supports this assumption is also provided by the fact that NMNAT2 expression was detected in pancreatic beta cells but not in pancreatic acinar cells (Yalowitz et al., 2004). Thus, it is tempting to propose that the NMNAT expression profile and their sub-cellular distribution determine a cell's response to metabolic changes and oxidative stress by directing the output of cellular NAD synthesis to particular response mechanisms. This could also partially account for cell type-specific phenomena such as metabolic dysfunction and ageing (Ramsey et al., 2008).

It has to be noted that the NMNAT catalysed step in NAD biosynthesis is not rate limiting (Magni et al., 2008), which indicates that NAD homeostasis is regulated beyond NMNAT activity. A general impact of NMNAT1 on NAD homeostasis has indeed been questioned since overexpression of the nuclear NAD biosynthetic enzyme fails to increase cellular NAD levels (Anderson et al., 2002). Its activity, however, stimulates NAD turnover by nuclear SIRT1 and has been proposed to mediate neuroprotection via SIRT1 regulated gene expression (Araki et al., 2004). NAD^+-dependent deacetylases are also found in mitochondria. For example, SIRT4 controls cellular insulin secretion by deacetylation of glutamate dehydrogenase (Haigis et al., 2006). It would be interesting to test whether NMNAT3 supports this process. We could demonstrate in paper I and II that NMNAT2 locates to the Golgi complex. This compartment is known to harbour two poly(ADP-ribose) polymerases, tankyrase 1 and 2, which are involved in glucose homeostasis (Yeh et al., 2007),

telomere function and ageing (Hsiao&Smith, 2008). A functional link between NAD^+ consumption and NAD synthesis here is indicated by the observation that NMNAT2 expression levels and activity might be associated with malignancy of cancer cells (Sorci et al., 2007). In this regard, it was intriguing to discover the activating effect of the cancer-preventive green tea polyphenol (-)-epigallocatechin 3-gallate (EGCG) on particularly NMNAT2 activity. EGCG is an effective scavenger of reactive oxygen species (ROS) that induces apoptosis in tumour cells but not normal cells (Shankar et al., 2007). It would be interesting to see whether some of its effects are related to NMNAT activity.

4.2 The phosphorylation state of NMNAT1 and PARP1 activity

In paper III, we could demonstrate a direct impact of an NMNAT1 on cellular DNA damage response and oxidative stress induced cell death. This nuclear enzyme accelerates PARP1 activation and AIF translocation upon oxidative stress and DNA damage. In contrast to the proposed impact of NMNAT1 on SIRT1 function (Araki et al., 2004), we found that the stimulation of PARP1 does not require NMNAT activity. This effect is regulated by the phosphorylation status of NMNAT1. A negative charge at Ser^{136} diminishes both the physical interaction of NMNAT1 with poly(ADP-ribosyl)ated PARP1 and PARP1 activation. Since NMNAT1 is a homo-hexameric enzyme (Garavaglia et al., 2002; Werner et al., 2002; Zhou et al., 2002), successive phosphorylation could gradually regulate the degree of PARP1 activity and, moreover, represent the decisive switch between AIF mediated apoptosis and NAD/energy depletion-induced necrotic cell death.

Importantly, Ser^{136} is target of protein C kinase (PKC), which has been reported to directly phosphorylate and, thereby, inhibit PARP1 itself (Hegedus et al., 2008). Nevertheless, the PKC isoform which modifies NMNAT1 *in vivo* has not yet been identified. Therefore, NMNAT1 and PARP1 could be targets of different PKC isoforms. Additionally, the identification of the protein phosphatase which dephosphorylates NMNAT1 should be of

great interest since it would further disclose the regulatory pathway upstream to the NMNAT1 effect.

4.3 Targeting of human NMNATs is mediated by an isoform-specific domain

The phosphorylation site of NMNAT1 resides within a structural loop which as well harbours the enzyme's nuclear localisation signal (Schweiger et al., 2001; Sasaki et al., 2006). Its flexibility and location at the outer surface of the homo-hexamer make this region accessible for modification and interaction. An extended equivalent in NMNAT2 mediates Golgi association and membrane anchoring by post-translational cysteine palmitoylation. Conclusively, in paper II, we propose this region to constitute an independent domain implicated in isoform-specific targeting and interaction. This is justified by three observations. (i), this domain is not part of the conserved active fold. (ii), NMNAT activity is not affected upon deletion of this domain. (iii), bacterial NMNATs are lacking this domain. Moreover, in the human homologues, we could find that the amino acids spanning this domain are encoded by individual exons. This observation implies that the domain could have inserted into an ancestral NMNAT for example by exon shuffling (Ponting&Russel, 2002). Unravelling the origin of this isoform-specific targeting and interaction domain (ISTID) might shed light on both the evolutionary development of NMNATs and the possible occurrence of similar regulatory strategies in other protein families.

We could demonstrate that human NMNAT isoform 3 is targeted to the mitochondria. We, however, did not investigate the role of the enzyme's ISTID in this process. NMNAT3 lacks a putative N-terminal targeting signal, most likely due to the immediate occurrence of the Rossmann fold's first β-sheet and the conserved NMNAT-specific active site. If mitochondrial localisation of this isoform were indeed facilitated by its ISTID, this would represent an alternative mitochondrial import mechanism.

4.4 Mitochondrial NAD synthesis

NMNAT3 is the only NAD biosynthetic activity which has been associated with the mitochondria. With regard to precursor availability this finding seems puzzling, because neither pyridine mono- nor dinucleotides are assumed to pass the inner mitochondrial membrane under physiological conditions. In addition, specific transporters have not been identified in mammals. Thus, the initial precursor of mitochondrial NAD biosynthesis has not been found. Interestingly, our results in paper I demonstrated that NMNAT3 exhibits rather low substrate selectivity with regard to purine and pyridine nucleotides in both, the forward and the reverse reaction. Most strikingly, NMNAT3 readily accepts and converts the reduced forms of NMN and NAD *in vitro*. A possibility to generate NMNH, besides reducing NMN, would be the phosphorylation of reduced nicotinamide riboside (NRH) by one of the recently discovered NRK activities. Together, this could allow for direct synthesis of NADH in mitochondria and, thus, contribute to the low NAD^+/NADH ratio in this organelle (Zhang et al., 2002). That mitochondrial NMNH may be of physiological relevance is indicated by our findings and supported by the observation that NMNH serves as substrate for mitochondrial nicotinamide nucleotide transhydrogenase, NNT (Hu et al., 1998). NNT generates NADPH by oxidation of NADH and, thereby, plays a fundamental role in thiol redox homeostasis (Rydström J., 2006). Moreover, NMNAT3 activity could directly supply reduced pyridine nucleotide equivalents to the mitochondrial respiratory chain.

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