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Molecular properties and regulation of NAD^+ kinase (NADK)

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ABSTRACT

Nicotinamide adenine dinucleotide (NAD^+) kinase (NADK) phosphorylates NAD^+, thereby producing nicotinamide adenine dinucleotide phosphate (NADP). Both NADK genes and the NADP(H)-producing mechanism are evolutionarily conserved among archaea, bacteria, plants and mammals. In mammals, NADK is activated by phosphorylation and protein-protein interaction. Recent studies conducted using genetically altered models validate the essential role of NADK in cellular redox homeostasis and metabolism in multicellular organisms. Here, we describe the evolutionary conservation, molecular properties, and signaling mechanisms and discuss the pathophysiological significance of NADK.

1. Introduction

Nicotinamide adenine dinucleotide (NAD^+) phosphate (NADP(H)) plays a crucial role in redox homeostasis and metabolism [1]. Phosphorylation of NAD^+ by NAD kinase (NADK) is the only known mechanism by which NADP is produced *de novo* [2]. NADK plays an essential role in NADP(H) production and subsequent biological processes. Importantly, however, the pathophysiological significance and regulation of NADK remain underinvestigated, particularly in mammals and in the context of human disease. In this review, we describe the evolutionary conservation, molecular properties, and posttranslational regulation of NADK. We also discuss recent studies that demonstrate the pathophysiological significance of NADK in human diseases.

2. NADP(H) may mediate the beneficial effects of NAD^+

NAD^+ plays a crucial role in a wide range of metabolic processes, including energy production in mitochondria. NAD^+ is synthesized *de novo* or through the Preuss-Hander or salvage pathway (Fig. 1A). Dietary tryptophan is converted to NAD^+ in the *de novo* pathway, whereas nicotinic acid is used in the Preuss-Hander pathway [3]. NAD^+ is degraded to nicotinamide and ADP-ribose derivatives, such as O-acetyl-ADP-ribose and poly-ADP-ribose, by the NAD^+ consuming enzymes, sirtuins and poly-ADP-ribose polymerases (PARPs), respectively [4]. In addition, CD38, also known as cyclic ADP ribose hydrolase, a glycoprotein found on the surface of immune cells, degrades NAD^+ to produce nicotinamide and ADP-ribose [5]. In the salvage pathway, NAD^+ is synthesized from nicotinamide and ADP-ribose, which is produced either from poly-ADP-ribose by poly-ADP-ribose glycohydrolases (PARG) [6] or by conversion of O-acetyl-ADP-ribose [7]. Nicotinamide phosphoribosyltransferase (Nampt) is the rate limiting enzyme of the salvage pathway of NAD^+ production. NAD^+ can accept two electrons to form NADH. The levels of NAD^+ decline in multiple tissues, including muscle, adipose, brain, skin, liver and pancreas, in rodents during aging [8]. This aging-dependent decrease in NAD^+ is also observed in the brain, liver and blood plasma in humans [9,10]. Depletion of NAD^+ promotes diabetes [11], fatty liver diseases [12], neurodegenerative disorders [13–15] and heart disease [16,17]. Thus, the decline in NAD^+ may induce functional impairment in cells and tissues during aging. Indeed, replenishment of NAD^+ prolongs life span in worms and mice [18,19]. In contrast, recent studies demonstrated that the replenishment of NAD^+ prolongs heath span, but not life span, in mice [20,21]. Replenishment of NAD^+ also ameliorates diabetes, obesity, cardiac disease, a neurodegenerative disorder and renal degeneration [16,22,23]. Thus, NAD^+ may contribute to longevity and prolonged health span.

Interventions to increase NAD^+ levels include 1) supplementation of NAD^+ precursors, such as nicotinamide mononucleotide (NMN), nicotinamide ribose (NR), nicotinic acid (NA) and nicotinamide, 2) reduction of NAD^+ consumption through inhibition of NAD^+ consuming enzymes, 3) manipulation of the NAD^+ biosynthesis pathways, including activation of Nampt and 4) interventions that improve the bioavailability of NAD^+, including calorie restriction and exercise [24]. Among the NAD^+ consuming enzymes, sirtuins play a major role in the beneficial effects of NAD^+ [25]. For instance, Sirt1 mediates the salutary effect of NAD^+ in axonal degeneration, and cardiac ischemia reperfusion and acute kidney injuries [26–28]. However, given that NAD^+ is the sole

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List of abbreviations

AGC	(cAMP- and cGMP-dependent protein kinases and protein kinase C (PKC))
BAD	benzamide adenine nucleoside
CaM kinase	Ca^{2+}/calmodulin-dependent protein kinase
DHFR	dihydrofolate reductase
EMKA	Element conserved in mitochondrial kinases of animals
G6PD	glucose-6-phosphate dehydrogenase
GLUD	glutamate dehydrogenase
IDH	isocitrate dehydrogenase
IR	ischemia reperfusion
KRAS	Kirsten rat sarcoma viral oncogene homolog
ME	malic enzyme
NA	nicotinic acid
NAD^+	NADH: nicotinamide adenine dinucleotide, oxidized and reduced forms
NADK	NAD kinase
NADP	NADPH: nicotinamide adenine dinucleotide phosphate, oxidized and reduced forms
NADPS	thionicotinamide adenine dinucleotide phosphate
Nampt	nicotinamide phosphoribosyltransferase
NCBI	National Center for Biological Information
NMN	nicotinamide mononucleotide
NNT	nicotinamide nucleotide transhydrogenase
Nox	NADPH oxidase
NR	nicotinamide ribose
PARG	poly-ADP-ribose glycohydrolase
PARP	poly (ADP-ribose) polymerase
PDAC	pancreatic ductal adenocarcinoma
PKC	protein kinase C
ROS	reactive oxygen species
TN	Thionicotinamide
Trx	Thioredoxin
6PGD	6-phosphogluconate dehydrogenase

precursor of NADP(H) and that the NADP(H)-dependent antioxidant system exerts protective effects, including prevention of aging and disease, NADK-induced NADP(H) production may also account for the salutary actions of NAD^+. Indeed, we have shown recently that the salutary effect of NAD^+ in diabetic cardiomyopathy is partly mediated by NADK-dependent NADP(H) production [29] (Fig. 1B).

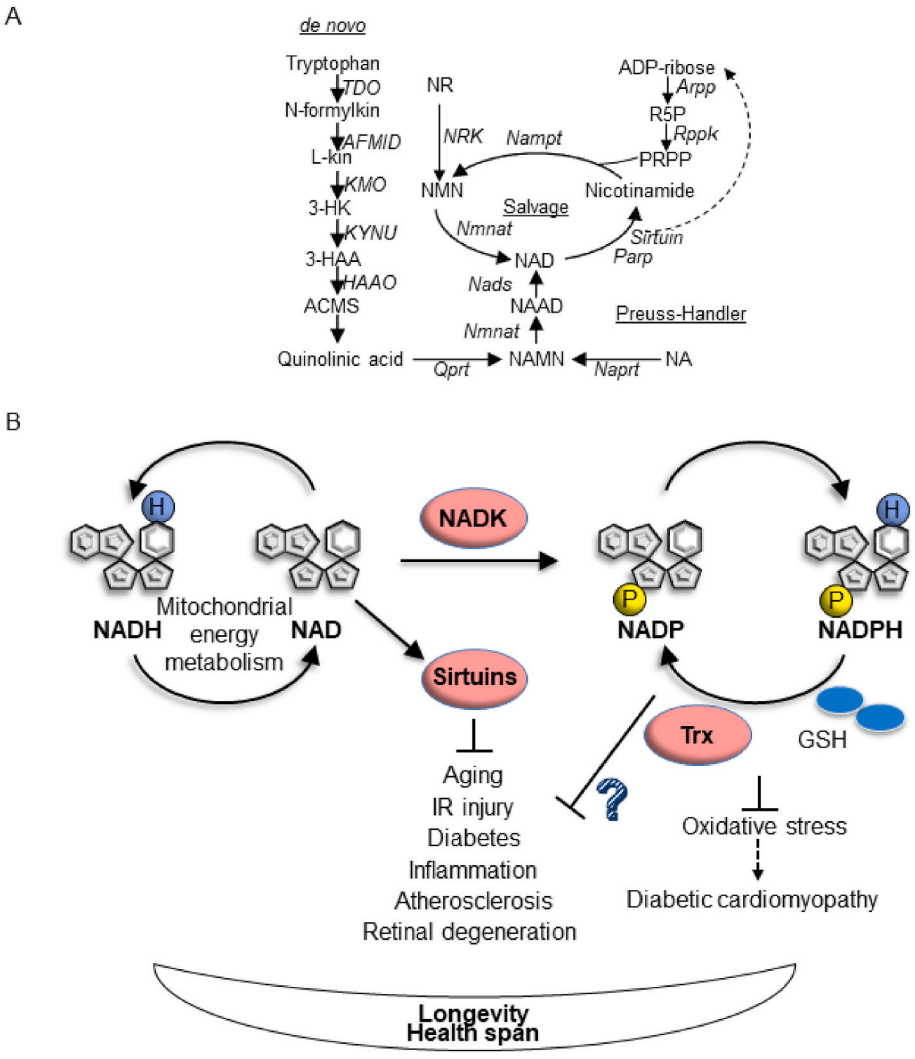

Fig. 1. Cellular functions of NAD^+. (A) A schematic representation of the NAD^+ synthetic pathways. NAD^+ is synthesized from tryptophan via the *de novo* pathway, nicotinic acid via the Preuss-Handler pathway, or nicotinamide and ADP-ribose via the salvage pathway. Abbreviated metabolites and enzymes are as follows. L-Kyn: L-kynurenine, 3-HK: 3-hydroxykynurenine, 3-HAA: 3-hydroxyanthranilic acid, ACMS: 2-amino-3-carboxymuconic semialdehyde, NAMN: nicotinic acid mononucleotide, NAAD: nicotinic acid adenine dinucleotide, NMN: nicotinamide mononucleotide, NR: nicotinamide ribose, NA: nicotinic acid, R5P: ribose 5 phosphate, PRPP: 5-phosphoribosyl-1-pyrophosphate, TDO: tryptophan 2,3-dioxygenase, AFMID: arylformamidase, KMO: Kynurenine 3-monooxygenase, KYNU: Kynureinase, HAAO: 3-hydroxyanthranilate 3,4-dioxygenase, Qprt: quinolinate phosphoribosyl transferase, Naprt: nicotinate phosphoribosyltransferase, Nmnat: nicotinamide nucleotide adenylyltransferase, NADS: NAD^+ synthase, Nampt: nicotinamide phosphoribosyltransferase, Parp: poly ADP ribose polymerase, Arpp: ADP-ribose pyrophosphatase, Rppk: ribose phosphate pyrophosphokinase and NRK: nicotinamide ribose kinase. (B) Cell protective and adaptive effects of NAD^+. NAD(H) functions as an electron carrier that contributes to metabolic processes, including mitochondrial energy metabolism. NAD^+ also functions as a co-substrate of NAD^+-consuming enzymes, including sirtuins and Parps. Sirtuins mediate beneficial effects of NAD^+ in aging and disease conditions. Although NAD^+ is a precursor of NADP, which plays an essential role in cellular redox homeostasis and metabolic processes, the extent to which NADK-induced NADP production contributes to the salutary effects of NAD^+ is not fully understood.

3. Evolutionary conservation of NADK

NADK genes are evolutionarily conserved from archaea to mammals (Fig. 2). NADK phosphorylates the 2' position of the ribose ring connected to the adenine moiety in NAD(H) (Fig. 3A). Because NADP and NADPH are membrane impermeable, the presence of organelle-specific NADP(H) synthesis systems is critical [30]. Plants possess three NADK genes, localized in the cytoplasm (NADK1), chloroplasts (NADK2) and peroxisomes (NADK3) [31]. In mammals, NADK is predominantly localized in the cytoplasm, whereas NADK2 is found in mitochondria [31,32]. The NCBI conserved domain database indicates that NADK contains a bifunctional NADP phosphatase/NAD^+ kinase domain, while NADK2 contains a diacylglycerol kinase catalytic domain (Fig. 3B). In addition, phylogenetic trees indicate that NADK and NADK2 developed independently during evolution (Fig. 3C). Whether or not eukaryotes possess other organelle specific NADKs, including in peroxisomes and the endoplasmic reticulum, remains unknown.

Some NADKs found in lower organisms such as bacteria and archaea use not only ATP but also polyphosphate to generate NADP. Polyphosphate is a negatively charged linear or cyclic homopolymer of orthophosphate (PO_4^{-3}), ranging in size from a few to several hundred subunits. It may have served as an energy carrier in ancient primitive organisms and is referred to as a fossil molecule or bioenergy fossil [33]. In contrast, in eukaryotes such as humans, NADK specifically uses ATP but not polyphosphate [31]. Interestingly, however, human mitochondrial NADK2 retains the ability to use polyphosphate to generate NADP [34]. This might be because mitochondria are derived from ancient organisms, even though the NADK2 gene is encoded in the nuclear genome. In addition, some NADKs in lower organisms phosphorylate not only NAD^+ but also NADH, whereas those in higher organisms, such as human NADK, phosphorylate only NAD^+ but not NADH. On the other hand, human NADK2 phosphorylates both NAD^+ and NADH, although NAD^+ is the preferred substrate [31,32]. Thus, NADK2 might be a more ancient type of enzyme than NADK in multicellular organisms.

4. Molecular properties of NADK

The crystal structure of NADK in *Listeria monocytogenes* and *Mycobacterium tuberculosis* has been determined, and the critical residues and domains for NADK function have been proposed [35,36]. These are evolutionarily conserved from bacteria to mammals. Thus, the molecular properties and catalytic mechanisms are likely also preserved in mammalian NADK. Based on this assumption, we describe the residues and domains critical for NADK function in mouse NADK.

There are three conserved domains: the GGDG motif, NE/D short motif and conserved region II (Fig. 4A). In addition, mammalian NADK possesses serine residues at 44, 46 and 48 that are phosphorylated by Akt and PKC [37,38]. The NAD^+ binding and oligomerization domains are located at 280–347 and 286–380, respectively (Fig. 4A). As shown in Fig. 4B, D185 in the GGDG motif serves as a catalytic center that interacts with the phosphorylation site of NAD^+, namely the 2' position of ribose. N280 and E281 in the NE/D short motif recognize adenine and ribose connected to nicotinamide, respectively. T321 and Y323 in the conserved region II recognize adenine and nicotinamide, respectively. H347 recognizes the phosphate group. Site directed mutagenesis of NADK in *Mycobacterium tuberculosis* demonstrated that D185, N280, E281, T321 and Y323 are critical for NADK function [35,39]. In addition, several residues have been demonstrated to play a role in the substrate specificity of NADK. NADKs that possess arginine corresponding to Q308, serine corresponding to A325 and arginine corresponding to H347 tend to use NADH as a substrate [35,40]. Thus, the region from 280 to 347 is involved in substrate binding and selectivity.

Mammalian NADK forms a homotetramer, whereas NADK2 forms a homodimer [31,34]. The region critical for the oligomerization partly overlaps with the NAD^+ binding region, from 286 to 380. Residues that may be responsible for oligomerization include R286, D310, T321, Y323, H347 and G380 [35]. Several of the residues necessary for oligomerization in bacteria are not conserved in mammalian NADK [35]. Given the high homology among species, the molecular properties are likely evolutionarily conserved but the significance of these residues and domains should be validated in mammalian NADKs.

5. Validation of the NADP(H) producing mechanism in multicellular organisms

Phosphorylation of NAD^+ by NADK is the only known mechanism of *de novo* NADP production. Yeast possesses three NADK genes. An NADK triple mutant was shown to be lethal, suggesting that NADK-induced NAD(H) phosphorylation is the primary mechanism of NADP(H) production [41]. Similarly, systemic NADK knockout mice exhibit embryonic and preweaning lethality (http://www.informatics.jax.org/marker/phenotypes/MGI:2183149). Phenotypes of organisms where NADK is genetically-modified are summarized in Table 1. Recent studies have validated NADK-induced NAD^+ phosphorylation as a major pathway and mechanism responsible for NADP production in multicellular organisms. Knockdown of NADK significantly downregulates NADP in HEK293 cells [37,42], the fat body in flies [43], and cancer cells [38]. Inhibition of Nampt in HEK293 cells with FK866 significantly downregulates not only NAD^+ but also NADP [37]. Similarly, systemic Nampt heterozygous knockout downregulates not only NAD^+ but also NADP in the heart [29]. Conversely, cardiac specific Nampt overexpression upregulates not only NAD^+ but also NADP [29]. However, Nampt-induced NADP(H), but not NAD^+, upregulation is inhibited by knockdown of NADK in cardiomyocytes [29]. Taken together, these studies show that NADK-induced NAD^+ phosphorylation is a major mechanism responsible for NADP production in multicellular organisms. Furthermore, NAD^+ production through Nampt appears to be coupled to NADK-induced NADP production in some mammalian cells.

6. Redox cycle of NADP(H)

6.1. NADPH dependent cellular mechanisms

NADP is subject to two-electron reduction at the nicotinamide ring, thereby forming NADPH. NADPH functions as an electron donor for redox regulating enzymes, including NADPH oxidase (Nox) and the thioredoxin and glutathione systems [1], and metabolic enzymes, including those involved in fatty acid, proline and cholesterol synthesis [43,44] (Fig. 5A and B). In addition, NADPH donates electrons to aspartate semialdehyde dehydrogenase in the aspartate catabolism pathway and to dihydrouracil dehydrogenase in the nucleotide catabolism pathway [45,46].

6.2. Production of NADPH from NADP

Oxidized NADP is reduced by multiple enzymes (Fig. 5C). In the cytosol, NADP is reduced by glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) in the pentose phosphate pathway, cytosolic isocitrate dehydrogenase (IDH1) and cytosolic malic enzyme (ME1). Of these, G6PD is the major mechanism responsible for the production of NADPH [47]. In mitochondria, mitochondrial isocitrate dehydrogenase (IDH2), glutamate dehydrogenase (GLUD), mitochondrial malic enzyme (ME3) and nicotinamide nucleotide transhydrogenase (NNT) reduce NADP to NADPH [47]. Of these, IDH2 plays a major role in NADPH production [48]. Among the NADPH producing enzymes, only NADK has been demonstrated to have an indispensable role in fatty acid and proline syntheses *in vivo* [43,44]. Although the involvement of NADK is highly likely, whether *NADK--mediated* production of NADP(H) is also essential for other enzymatic reactions, including those mediated by IDHs and MEs, or in the pentose phosphate pathway and cholesterol synthesis has yet to be clearly demonstrated *in vivo*. Further investigation with targeted metabolomic

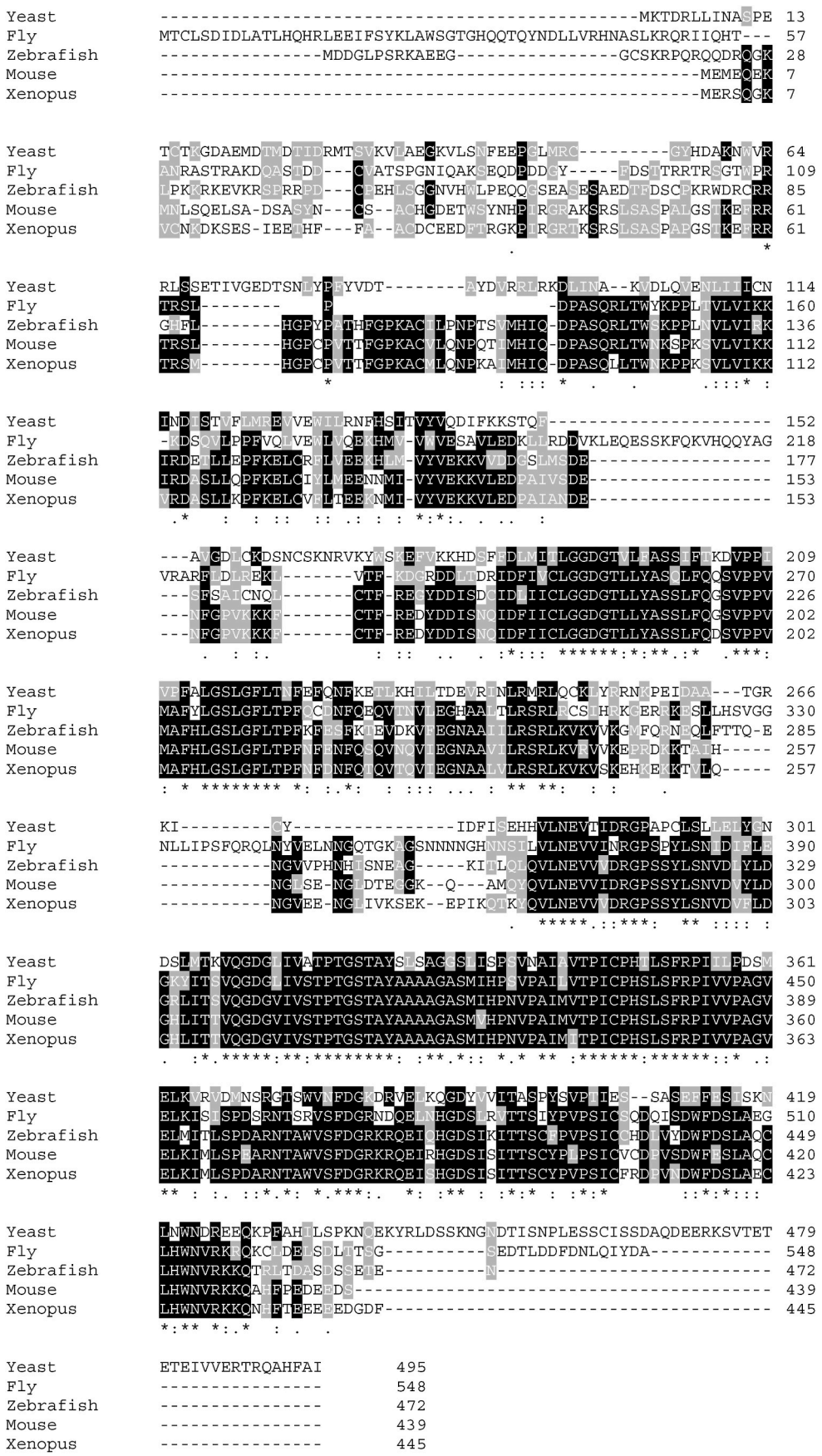

Fig. 2. Conservation of NADK in eukaryotes. Alignment of NADK proteins derived from yeast, fly, zebrafish, xenopus and mouse was conducted with the Clustal Omega program.

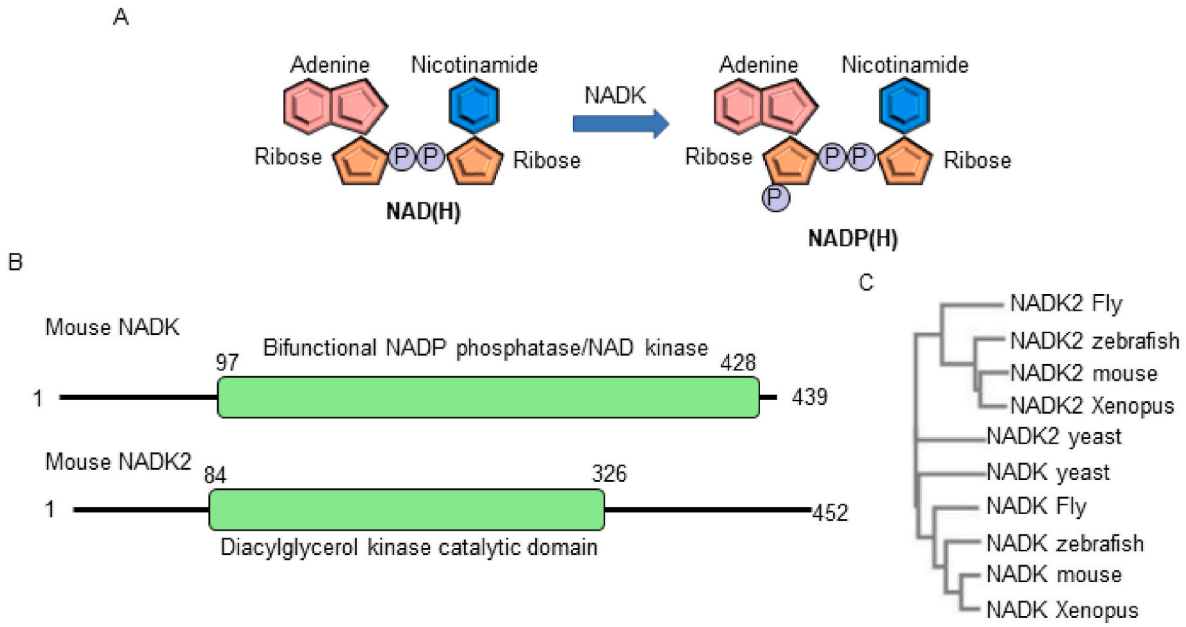

Fig. 3. Structure of NADK and NADK2. (A) NADK phosphorylates the 2′ position of the ribose ring connected to the adenine moiety in NAD(H). (B) The catalytic domains of mouse NADK and NADK2, as assigned by the NCBI conserved domain database. (C) Phylogenetic trees of NADK and NADK2 provided by the Clustal Omega program.

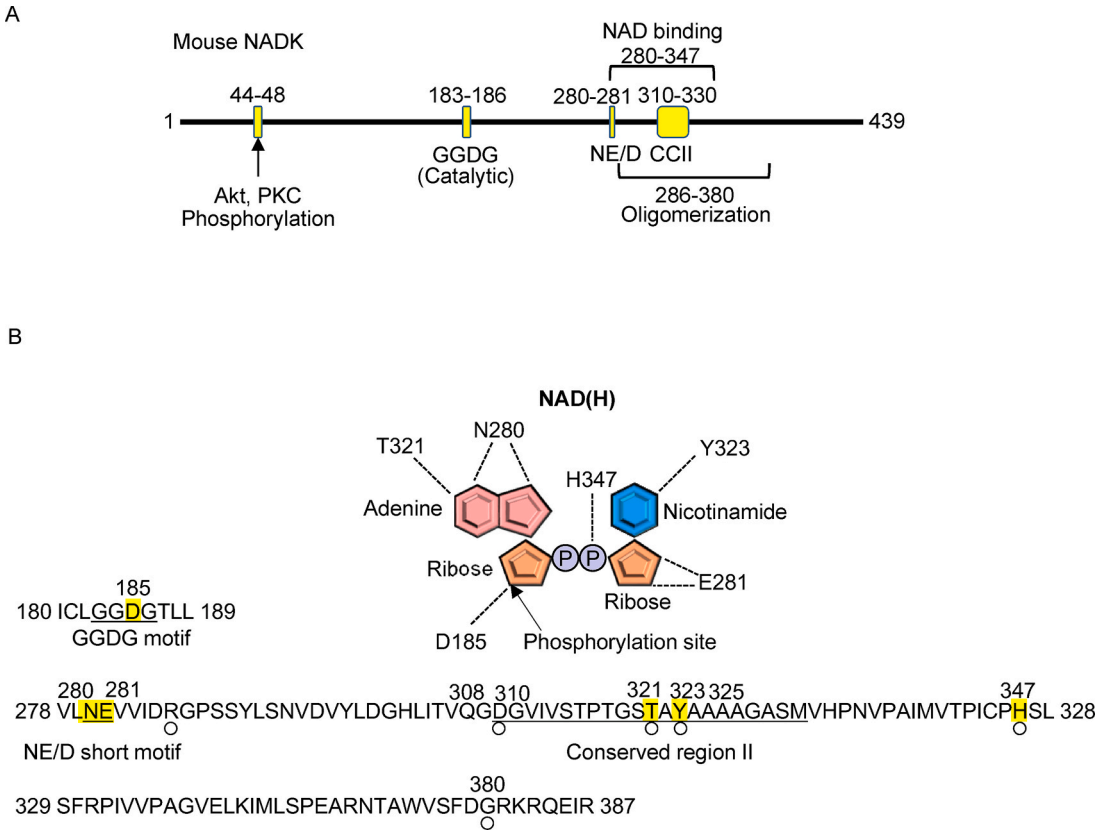

Fig. 4. Structure and function of NADK. (A) A schematic representation of the NADK domains, including phosphorylation sites, GGDG motif, NE/D short motif, conserved region II (CCII), NAD^+ binding region and oligomerization region. (B) Critical amino acid residues for catalysis, NAD^+ recognition and oligomerization. Underlines indicate the GGDG motif, NE/D short motif, and conserved region II. Highlighting indicates residues involved in the kinase reaction and binding to NAD^+. Open circles indicate residues involved in oligomerization.

Table 1
Genetic models of NADK and NADK2 and their phenotype.

Organism	Genotype	Phenotype	References
Saccharomyces cerevisiae	POS5, UTR1 and YEF1	● Triple mutant: Lethal ● POS5 mutant: Sensitive to oxidative stress, defects in iron homeostasis and amino acid biosynthesis ● UTR1 mutant: Retarded growth in iron deficient medium	[41,82]
Drosophila melanogaster	CG6145/NADK RNAi	Lower lipid storage in fat body, reduced mitochondrial mass and ROS (mitochondrial) level, mitochondrial cristae disruption	[43]
Mus musculus	C57BL/6N-Nadkem2 (IMPC)Bay (Endonuclease-mediated mutation 2, Baylor College of Medicine)	● Homozygotes: Embryonic/ preweaning lethality and complete penetrance ● Heterozygotes: Low total body fat amount ● Elevated lean body mass and bone mineral content	Nadk Mouse Gene Details | NAD kinase | International Mouse Phenotyping Consortium (mousephenotype.org) and Nadk < em2 (IMPC)Bay > Endonuclease-mediated Allele Detail MGI Mouse (MGI:6257722) (jax.org)
	Chemically-induced NADK2 point mutations S326L and S330P	Severe neuromuscular disease Short lifespan	[83]
	NADK2-KO (C57BL/6NTac background)	● Increased serum concentrations of lysine and C10:2 carnitine ● Development of non-alcoholic fatty liver disease under atherogenic high fat diet ● Increased ROS in liver ● Fatty acid oxidation defects under starvation	[65]
Homo sapiens (Clinical studies)	a homozygous nonsense mutation, c.1018C > T (NM_001085411.1), in exon 10 of NADK2, which leads to a premature stop codon at position 340 (p.R340X) and start loss mutation in the NADK2 gene	Developmental defects, hyperlysinemia, severe mitochondrial dysfunction, metabolic defects, metabolic acidosis, CNS anomalies, ataxia, and incoordination	[66,75]
	Gain-of-function mutation: NADK (I90F)	Pro-tumor effects due to increased kinase activities and induction of NADP(H) level-dependent enhanced antioxidant defense response	[62]

analyses is needed to address these issues.

6.3. NADK-dependent upregulation of NADPH

Overexpression of NADK upregulates NADPH, but not NADP, in HEK293 cells [42]. Similarly, overexpression of Nampt upregulates NADPH, but not NADP, in primary cultured cardiomyocytes [29]. NADP produced by NADK can be immediately converted to NADPH by the NADP-dependent dehydrogenases. Feedback regulation may prevent NADK from functioning except when NADP-dependent dehydrogenases are functional, thereby limiting accumulation of NADP when dehydrogenase activity is limited. Indeed, G6PD binds to and stimulates NADK in pancreatic cancer cells [49]. Alternatively, NADK may directly phosphorylate NADH in cells. NADK specifically phosphorylates NADH when NAD^+ and NADH concentrations are low and the NAD^+/NADH ratio is high *in vitro* [42]. Further investigation is required to clarify the mechanism through which NADK produces NADPH without NADP upregulation.

7. Redox regulation via NADK

NADPH plays a crucial role in redox homeostasis as an electron donor. Since NADPH donates electrons not only to antioxidant systems, including thioredoxin and glutathione, but also to Noxs to produce superoxide, in theory, NADK can potentiate both the antioxidant defense and oxidative stress. However, recent studies have demonstrated the significance of NADK in the antioxidant defense rather than in oxidative stress. Overexpression of NADK attenuates, whereas knockdown of NADK enhances, H_2O_2-induced oxidative stress in HEK293 cells [42], and increased oxidative stress is observed in HEK293 cells with NADK2 knockdown [34]. NADK knockdown also promotes high sugar diet-induced oxidative stress in fly fat body [43] and promotes glutathione oxidation in cancer cell lines [38]. In addition, we have shown that cardiac-specific Nampt overexpression potentiates the antioxidant defense partly through NADK-mediated NADP(H) production [29]. These observations suggest that NADK plays an essential role in the antioxidant defense via NADPH production.

In contrast, whether NADK is essential for Nox-induced ROS production has yet to be fully investigated. Although NADPH is the preferred electron donor, Nox enzymes also utilize NADH [50]. Interferon-γ (INFγ) induces NADP(H) and oxidative stress in a Nampt-dependent manner, but the extent to which NADP(H) and NADK are required for oxidative stress has not been demonstrated [51]. Thus, NADK and NADPH may not be essential for Nox function. In addition, Nox-induced ROS production is tightly regulated by cellular signal transduction [52]. For instance, Nox4 is negatively regulated by Fyn kinase via direct phosphorylation [53]. Thus, an NADK-induced increase in NADPH may not be sufficient to activate Nox enzymes.

If NADPH originating from the NAD^+-NADK pathway is utilized by both the antioxidant system and Nox, the cellular mechanism regulating the coupling remains to be elucidated. Since the antioxidant system can be located in the cytosol and Noxs are membrane-associated enzymes, it is possible that the NAD^+ synthesis system, NADP-dependent dehydrogenases and electron acceptors are compartmentalized, thereby forming a signaling complex. Alternatively, their coupling may be regulated through unknown mechanisms. Further investigation is required to address this issue.

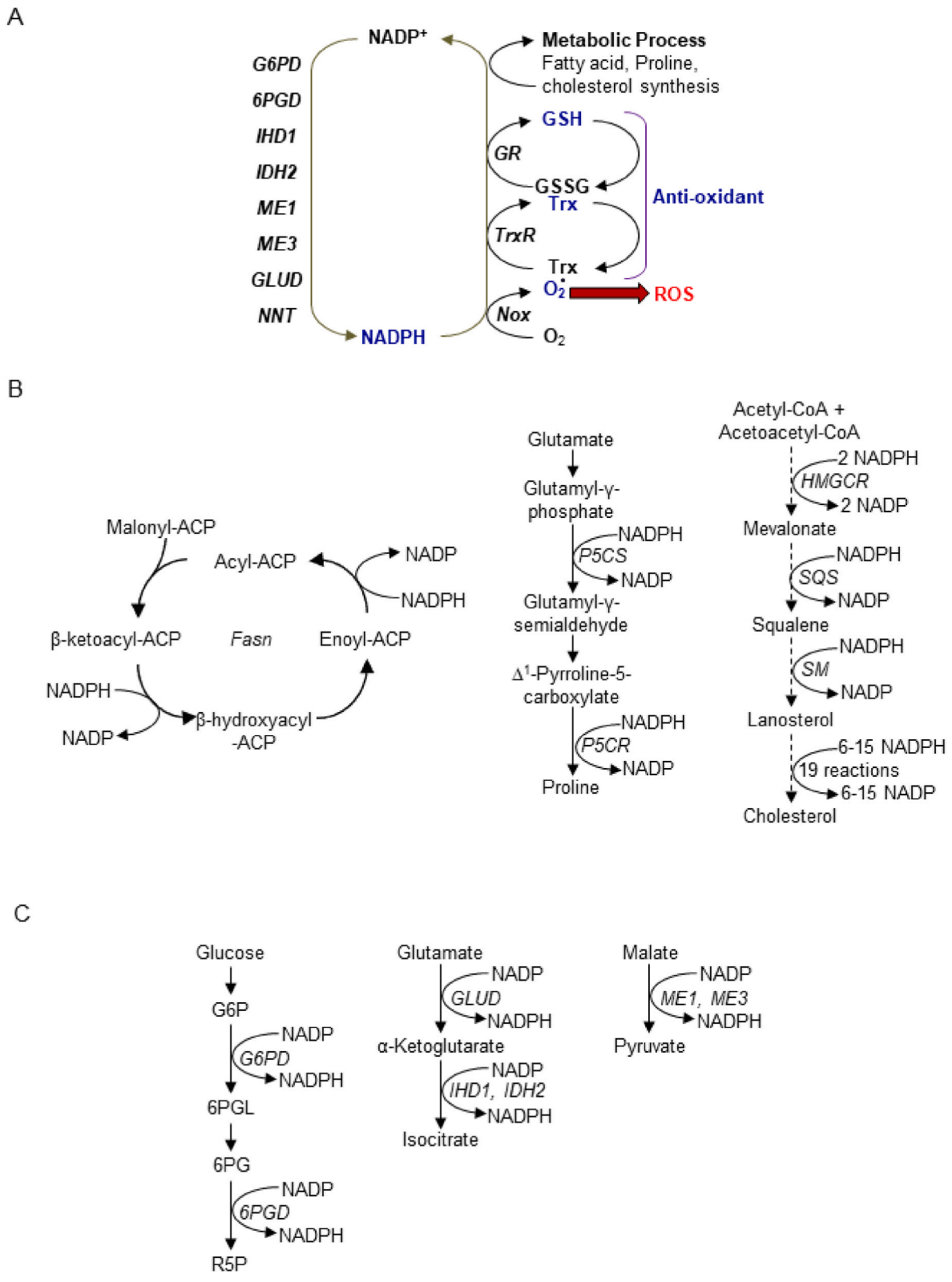

Fig. 5. The redox cycle of NADP(H) couples with cellular redox homeostasis and metabolism. (A) NADP is reduced by multiple enzymes, whereas NADPH serves as an electron donor for Nox enzymes, the Trx and Glutathione systems, and metabolic processes. (B) Metabolic reactions in which NADPH donates electrons, including fatty acid (left), proline (middle) and cholesterol (right) syntheses. In the last 19 reactions of cholesterol synthesis, 6 reactions require NADPH and 9 reactions redundantly use NADPH and NADH. As a result, 6–15 NADPH can be used for cholesterol synthesis from lanosterol. (C) Metabolic reactions in which NADP accepts electrons, including catabolic processes of glucose, glutamate, and malate. Abbreviated metabolites and enzymes are as follows. ACP: acyl carrier protein, G6P: Glucose 6 phosphate, 6PGL: 6-phosphogluconolaconase, 6 PG: 6-phosphogluconate, R5P: ribose 5 phosphate, G6PD: glucose 6 phosphate dehydrogenase, 6PGD: 6 phosphogluconate dehydrogenase, IDH: isocitrate dehydrogenase, ME: malic enzyme, GLUD: glutamate dehydrogenase, NNT: nicotinamide nucleotide transhydrogenase, Fasn: fatty acid synthase, P5CS: pyrroline 5 carboxylate synthase, P5CR: pyrroline 5 carboxylate reductase. HMGCR: HMG-CoA reductase, SQS: squalene synthase and SM: squalene monooxygenase.

8. Cellular signaling regulating NADK

NADKs are highly important in the regulation of anabolic processes and the antioxidant defense in cells. NADK affects the NAD^+/NADH pool for both catabolic processes leading to ATP production and anabolic processes, including maintenance of the antioxidant system. However, the mechanisms by which NADK maintains or changes the balance between the catabolic and anabolic processes are not well understood.

8.1. Upregulation of endogenous NADK

In the liver and white adipose tissue (WAT), fasting upregulates NADK2, and the opposite occurs in response to high fat diet consumption [32]. In WAT, downregulation of CD38, cyclic ADP ribose hydrolase, upregulates NADK and NADP(H) levels [54].

8.2. Posttranslational modification of NADK

At the post-translational level, NADK is activated via phosphorylation of its N-terminal regulatory domain by various AGC kinases (cAMP- and cGMP-dependent protein kinases and the protein kinase C (PKC) family of protein kinases) [43] and Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) [3]. In human neutrophils, calmodulin and PKC induce NADK phosphorylation and activation, triggering robust host defense mechanisms [55,56]. In pancreatic ductal adenocarcinoma (PDAC), oncogenic Kirsten rat sarcoma viral oncogene homolog (KRAS) signaling through PKC induces NADK phosphorylation at serines 46 and 48, leading to hyperactivation of NADK [38]. Growth factor-mediated Akt signaling similarly relieves the auto-inhibitory action of the regulatory N-terminal domain of NADK through phosphorylation of serines 44, 46 and 48 and activates NADK [37]. In humans, phosphorylation at serine 188 activates NADK2, but the responsible kinase and phosphatase have yet to be identified [57]. In addition, NADK2 may be regulated by acetylation at lysines 76 and 304 [44].

Ca^{2+}/calmodulin binds to the N-terminal region to activate NADK [58]. Furthermore, NADPH allosterically inhibits NADK by enhancing the tetramer formation [59]. NADK2 possesses a unique tetramer disruptor/dimerization element conserved in mitochondrial kinases of animals (EMKA), which prevents NADK2 oligomerization, promotes dimerization and maintains NADK2 in a constitutively active conformation [60]. Thus, mammalian NADKs are regulated by phosphorylation, acetylation and protein-protein interaction (Fig. 6). Further investigation is required to clarify the upstream regulatory mechanisms and the functional significance of post-translational modifications of NADKs.

9. Pathophysiological role of NADK

9.1. Cancer development

NADK contributes to cancer development in PDACs and colon cancers [38,61]. Through screening of patients with PDAC, NADK(I90F) has been identified as a gain-of-function mutation: NADK(I90F) possesses increased kinase activity and induces a greater antioxidant defense response through increased NADP(H) levels [62] compared to wild type NADK.

9.2. Redox homeostasis and metabolism

NADK plays an important role in regulating the intracellular redox homeostasis, thereby regulating insulin secretion in the pancreatic β-cell [63]. NADK also plays an essential role in mediating the antioxidant defense through NADP(H) production in cardiomyocytes, thereby ameliorating cardiomyopathy associated with obesity [29]. Loss of NADK in fly fat body impairs lipid storage, as well as mitochondrial function and mass [43]. Interestingly, however, inhibition of NADK protects against acetaminophen-induced acute liver injury by elevating NAD^+ levels, which improves the antioxidant capacity through

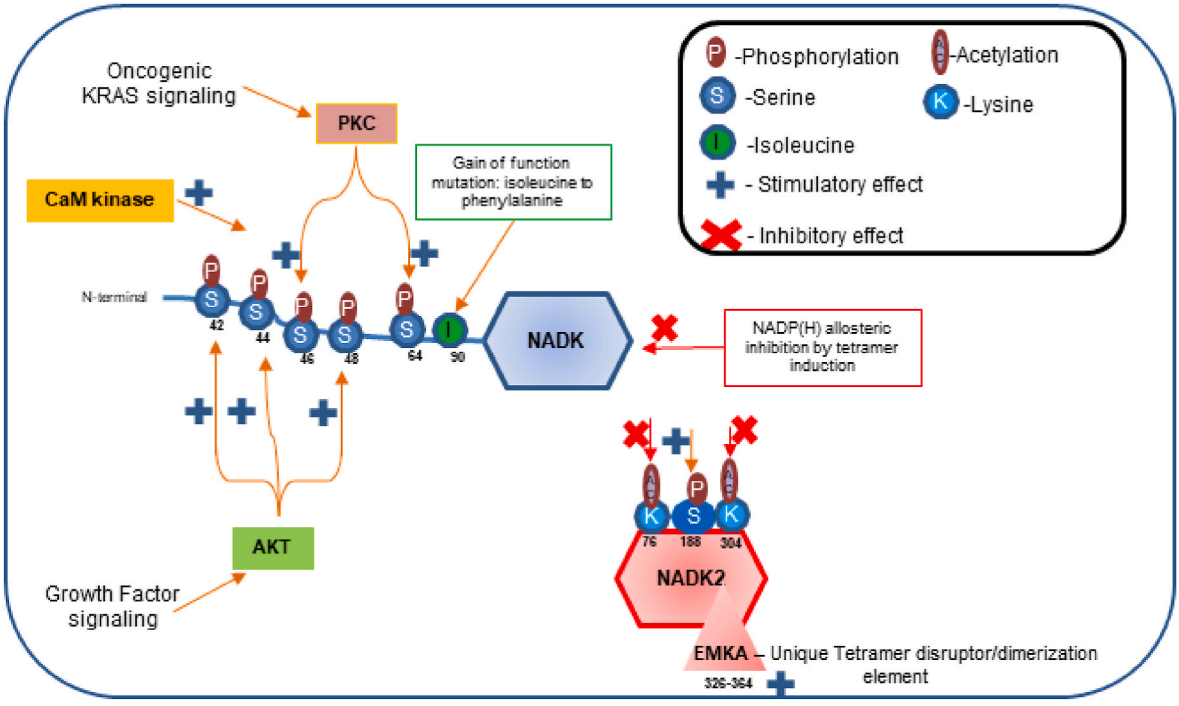

Fig. 6. Cellular signaling mechanisms regulating NADKs. KRAS signaling, growth factor signaling, and CaM kinase stimulate NADK. High NADP(H) levels exert allosteric feedback inhibition of NADK by inducing tetramerization. NADK2 has a unique tetramer disruptor/dimerization Element conserved in Mitochondrial Kinases of Animals (EMKA) that restricts its oligomerization and instead promotes dimerization to maintain a constitutively active conformation. Mutational studies have shown that acetylation of lysines 76 and 304 and phosphorylation of serine 188 regulate NADK2 activity. Amino acid positions are indicated with numbers at their sites.

unknown mechanisms [64]. On the other hand, loss of NADK2 promotes stress-induced liver steatosis in mice [65].

9.3. Nervous system and aging

Loss of NADK2 due to mutation of the start codon is responsible for early-stage lethality and central nervous system anomalies, ataxia, and loss of coordination in humans [66]. Furthermore, both NADK and Akt levels were upregulated in aging mice [67,68]. Thus, Akt-induced NADK activation may account for the decrease in NAD^+ during aging, although further investigation is necessary to confirm this hypothesis. In summary, although only a limited number of studies have been conducted, increasing lines of evidence suggest that endogenous NADKs play a significant role in regulating cancer, insulin secretion, heart disease, anabolic and catabolic processes, neuronal development, and aging (Fig. 7).

10. NADKs as therapeutic targets in cancer

Because of the differences in the active structure and substrates between prokaryotic and mammalian NADK, researchers have focused on the development of pharmacological inhibitors as a treatment strategy for pathogenic bacterial infections, particularly against multidrug-resistant bacteria [69–71]. The idea of targeting human NADK as a treatment strategy for inflammation and cancer has only recently gained traction [72].

The role of NADK in modulating cellular ROS through NADPH, knock-down or over-expression of cytosolic NADK has only a modest effect on levels of ROS [42,73]. However, mitochondrial NADK2 might also play a role in NADPH generation and protect mitochondria from ROS [34,74]. On the other hand, although NADK2 is possibly a better target in cancers, because of its critical role in protection against ROS in normal tissues like heart and brain, inhibition of NADK2 might have detrimental effects. Indeed, NADK2 deficiency due to a homozygous nonsense mutation was identified in a pediatric patient with

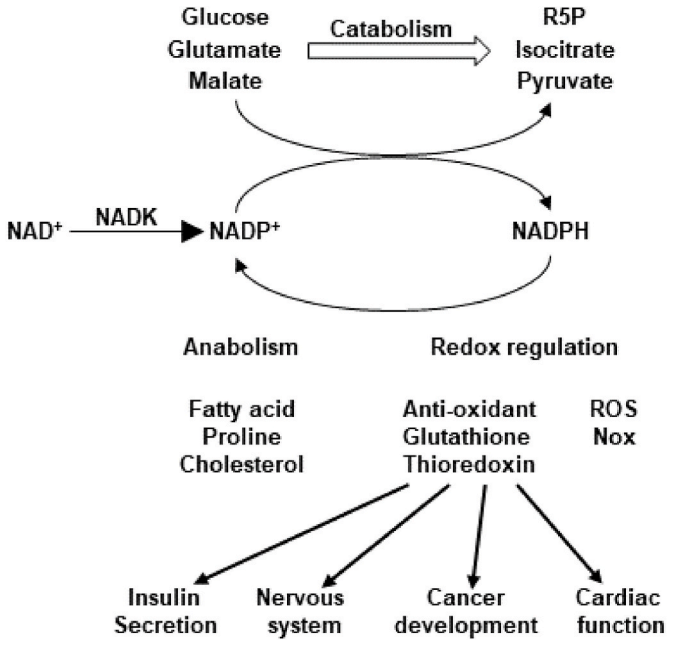

Fig. 7. Cellular effects and pathophysiological roles of NADKs. NADK is essential for NADP production. NADP is reduced to NADPH, which couples with catabolic processes, including the pentose phosphate pathway. NADPH donates electrons to anabolic pathways and enzymes for redox homeostasis. Anti-oxidant pathways play a role in insulin secretion, cancer development and the maintenance of nervous and cardiac function.

developmental defects and early death [75]. Thus, NADK might be a better target for the development of pharmacological inhibitors. To this end, various mutants of NADK, including NADK(I90F), have been isolated from PDAC that render pro-tumor effects by enhancing NADPH levels and ROS protection [62].

11. NAD analog as potential NADK inhibitors

Approximately 30 clinically approved NADK inhibitors targeting the ATP binding site have been developed thus far. However, as with many chemical inhibitors targeting ATP binding domains, their selectivity is not optimal [70,72,76]. Targeting the NAD^+ binding site instead could increase selectivity, since human NADK possesses a unique NAD^+ binding domain that differs from those of other NAD^+-binding enzymes [72]. Benzamide riboside is metabolized in cells to benzamide adenine nucleoside (BAD), an NAD^+ analog, thereby acting as a prodrug. BAD inhibits NADKs, thereby lowering NADPH levels and inhibiting dihydrofolate reductase (DHFR), an enzyme that uses NADPH as an electron donor [77]. Due to skeletal muscle loss and liver toxicity, however, benzamide riboside was not advanced in the drug development process. Screenings with small compound libraries for NAD^+ analogs showed that thionicotinamide adenine dinucleotide and thionicotinamide adenine dinucleotide phosphate (NADPS) exhibit cytotoxicity due to NADK inhibition [78]. Thionicotinamide (TN), another NAD^+ analog, is converted to NADPS upon cell entry and also exhibits cytotoxicity in various cancer cell lines due to NADK inhibition [79]. TN upregulates ROS in C85 cells through downregulation of NADPH [79]. TN also inhibits fatty acid synthesis and nucleotide synthesis pathways, both of which are NADPH-dependent [73]. TN effectively sensitizes cells to chemotherapeutic agents, including methotrexate, gemcitabine, docetaxel, and irinotecan, due to its ability to increase ROS levels and inhibit NADPH-dependent synthetic pathways [73]. TN administration as a single dose in human xenograft mouse models produced a promising result comparable to that of NADK downregulation (50%) with shRNA [73]. Another NAD^+ analog, 6-amino nicotinamide, showed promising pre-clinical anti-cancer activity but was associated with neurological toxicity [80]. The neurotoxicity of 6-amino nicotinamide was ascribed to its ability to block the pentose phosphate pathway via phosphogluconate dehydrogenase inhibition [81] and inhibit NADK2 phosphorylation [79]. Nicotinamide administration was found to reverse the cytotoxic effects of NAD^+ analogs [79]. On the other hand, TN does not show neurotoxic effects, suggesting that not all NAD^+ analogs are neurotoxic [79]. Aside from the neurotoxicity of 6-amino nicotinamide, interventions that inhibit NADPH synthesis via NADK inhibition demonstrate safe therapeutic anti-cancer activity by lowering NADPH levels, which hinders the protection against ROS and negatively affects NADP(H)-dependent metabolic pathways. However, the development of more selective inhibitors that can differentiate between cytoplasmic and mitochondrial forms of NADKs is important for the development of more effective cancer treatments.

12. Conclusions

NADKs are evolutionarily conserved enzymes that phosphorylate NAD^+ and produce NADP(H). NADK is the only known mechanism by which NADP(H) is produced *de novo*. In eukaryotes, both cytosolic and mitochondrial NADKs exist, but other organelle specific NADKs have yet to be identified. Recent studies using genetically modified mouse models validate NADKs as the major enzymes that produce NADP(H) across species. Moreover, these studies validate the significance of NADP(H) in cellular redox homeostasis. Although NADP(H) plays an essential role in many metabolic processes, its functional significance requires further validation *in vivo* with NADK loss-of-function models. In addition, the mechanisms by which NADK balances the NAD(H) and NADP(H) pools remain to be clarified, and how cells sense the status of the NAD(H) vs. NADP(H) pools and regulate NADK function is not currently well

understood. The molecular mechanisms that regulate NADK include protein-protein interaction and post-translational modifications, such as phosphorylation and acetylation. However, other than Akt, CaMK and PKC, the phosphatases, acetylases and deacetylases involved in post-translational modification of NADKs remain to be identified. How NADKs are differentially regulated in tissues, biological processes and disease conditions also remains unclear. Considering the wealth of knowledge about NAD^+ biology, investigation of NADKs would dramatically advance our understanding of how NAD^+ is utilized during disease and aging in humans.

Author contributions

Author contributions: S.O., A.S.T., D.Z. and J.S. wrote the manuscript, and the authors reviewed and approved the manuscript for publication.

Declaration of competing interest

The authors declare no competing interests.

Data availability

No data was used for the research described in the article.

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