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Mitochondrial NAD kinase in health and disease

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ABSTRACT

Nicotinamide adenine dinucleotide phosphate (NADP), a co-enzyme and an electron carrier, plays crucial roles in numerous biological functions, including cellular metabolism and antioxidation. Because NADP is subcellular-membrane impermeable, eukaryotes compartmentalize NAD kinases (NADKs), the NADP biosynthetic enzymes. Mitochondria are fundamental organelles for energy production through oxidative phosphorylation. Ten years after the discovery of the mitochondrial NADK (known as MNADK or NADK2), a significant amount of knowledge has been obtained regarding its functions, mechanism of action, human biology, mouse models, crystal structures, and post-translation modifications. NADK2 phosphorylates NAD(H) to generate mitochondrial NADP(H). NADK2-deficient patients suffered from hyperlysinemia, elevated plasma C10:2-carnitine (due to the inactivity of relevant NADP-dependent enzymes), and neuronal development defects. Nadk2-deficient mice recapitulate key features of NADK2-deficient patients, including metabolic and neuronal abnormalities. Crystal structures of human NADK2 show a dimer, with the $NADP^+$-binding site located at the dimer interface. NADK2 activity is highly regulated by post-translational modifications, including S188 phosphorylation, K76 and K304 acetylation, and C193 *S*-nitrosylation; mutations in each site affect NADK2 activity and function. In mice, hepatic Nadk2 functions as a major metabolic regulator upon increased energy demands by regulating sirtuin 3 activity and fatty acid oxidation. Hopefully, future research on NADK2 will not only elucidate its functional roles in health and disease but will also pave the way for novel therapeutics for both rare and common diseases, including NADK2 deficiency and metabolic syndrome.

1. Introduction

Nicotinamide adenine dinucleotide phosphate (NADP) plays crucial roles in numerous biological functions, including serving as a substrate and cofactor for numerous enzymes and metabolic processes, such as biosynthesis of cholesterol, production of fatty acids, and fatty acid oxidation [1–5]. NADP exists in oxidized ($NADP^+$) or reduced (NADPH) form, known as a "redox couple" which is collectively denoted as NADP or NADP(H). Redox reactions involve the gain or loss of electrons. NADPH, in particular, is an essential reducing equivalent that provides a reservoir of electrons for defense against oxidative stress, because it powers critical redox systems to regenerate antioxidants including glutathione (GSH) and thioredoxin to neutralize reactive oxygen species (ROS) [1–5].

NAD kinase (NADK) phosphorylates NAD to generate NADP, and because of the essential roles of NADK, it is found in all life kingdoms. Bacteria have a single NADK to synthesize NADP. Because NADP is not subcellular membrane-permeable, eukaryotic cells have dedicated NADKs to maintain NADP levels in separate compartments [3,5,6]. Mitochondria are a major source of ROS and are critical for energy metabolism, highlighting the crucial role of mitochondrial NADP. Because NADP cannot pass through the mitochondrial membrane, eukaryotic cells need compartment-specific NADKs for the cytosol and mitochondria.

In yeast, *Pos5* encodes mitochondrial NADK whereas *Utr1* and *Yef1* encode cytosolic NADKs [7]. In *Arabidopsis thaliana*, atNADK1, atNADK2, and atNADK3, are localized to cytosol, chloroplasts, and peroxisomes, respectively [8]. The human NADK was identified in 2001, and it is localized exclusively in cytosol [9,10]. However, the human mitochondrion-localized NADK remained unidentified until more than 10 years later, as discussed below [11–13].

2. NADK2 discovery

Dr. Kousaku Murata pioneered the research on NADKs by firstly identifying multiple NADK-encoding genes [14,15], stimulating subsequent discoveries of many other NADK genes in various species. To

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Abbreviations

AASS	aminoadipate-semialdehyde synthase
ETC	electron transport chain
FAO	fatty acid oxidation
GSSG	glutathione disulfide
GSH	glutathione
HFD	high-fat diet
IMPC	international mouse phenotyping consortium
KO	knockout
NADK2	mitochondrial NAD kinase (human)
Nadk2	mitochondrial NAD kinase (mouse)
MNADK	mitochondrial NADK
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NNT	nicotinamide nucleotide transhydrogenase
DECR	peroxisomal 2,4-dienoyl-CoA reductase
P5CS	pyrroline-5-carboxylate synthase
ROS	reactive oxygen species
SIRT3	sirtuin 3
TG	triglyceride

identify the human mitochondrial NADK, Murata's group performed a BLASTP search using atNADK3 and unexpectedly detected its homolog (C5ORF33) in the human genome. His group then performed a complementation experiment using yeast, showing that deleting three yeast NADKs, Utr1, Yef1 and Pos5, was lethal, and the lethality was rescued by expression of C5ORF33, suggesting that C5ORF33 generates NADP(H). *In vitro*, a recombinant C5ORF33 exhibited NADK activity using ATP as a phosphate donor. Notably, C5ORF33 also phosphorylates NADH, despite the activity being 10 times lower than that for NAD^+. In HEK293 cells, a human embryonic kidney cell line, C5ORF33 was located within mitochondria [13].

Our group identified C5ORF33 as a mitochondrial NADK from an RNA-seq experiment. To identify novel nutritionally-regulated genes in the liver and fat, we performed RNA-seq screening, using samples from mice that were fasted or on a high fat diet (HFD). 24-hour fasting significantly increased C5ORF33 expression levels in both the liver and white adipose tissue. Quantification of C5ORF33 expression across mouse tissues showed that the expression was highest in the liver, but relatively high in mitochondrion-rich tissues, including brown fat, heart, kidney, muscle, and brain [12]. Indeed, C5ORF33 harbors a mitochondrial localization signaling peptide. Using fluorescence imaging, we demonstrated that C5ORF33 localized to mitochondria in Hep G2 cells, a human liver cell line, because C5ORF33 co-localized with mitochondrial markers. Bioinformatic analysis showed that C5ORF33 harbored a conserved NADK domain. We then expressed C5ORF33 in *E. coli* and purified the recombinant protein. Indeed, this recombinant protein phosphorylated NAD^+ and generated $NADP^+$, as demonstrated by mass spectrometry. Because of the NADK activity and mitochondrial localization, we named the protein mitochondrial NAD kinase (MNADK) [12]. These results collectively suggest a model that NADK and MNADK *de novo* generate cytosolic and mitochondrial NADP, respectively (Fig. 1). We then contacted the HUGO gene nomenclature committee, which named the gene "*NADK2*, mitochondrial" with *MNADK* as a synonym. In this review, we use NADK2, the official name of this protein.

3. NADK2-deficient patients

The first NADK2-dificient human patient was identified by Dr. Houten's lab [16]. The patient, a Hispanic male, presented at 8 weeks of age with failure to thrive, microcephaly, and central hypotonia. Metabolic evaluation revealed hyperlysinemia and elevated plasma

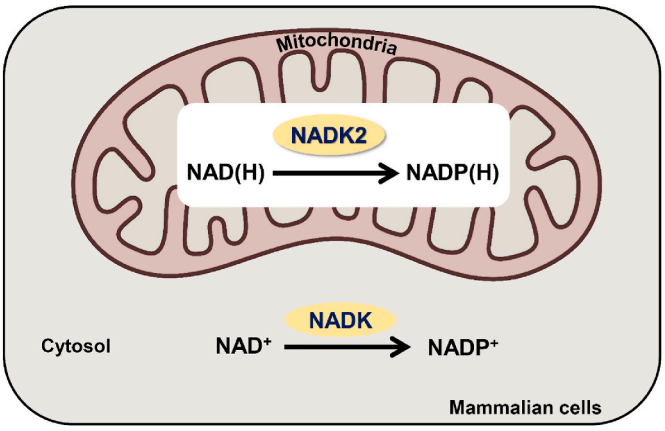

Fig. 1. In mammalian cells, NADK2 phosphorylates NAD(H) to generate mitochondrial NADP(H). NAD(H) represents NAD^+ and NADH. NADP(H) represents $NADP^+$ and NADPH. NADK phosphorylates NAD^+ to generate cytosolic $NADP^+$.

C10:2-carnitine. With age, neurological features increased with encephalopathy, dystonia, spastic quadriplegia, and epilepsy. The patient died at 5 years of age. Because of the elevated plasma lysine and C10:2-carnitine, Sanger sequencing was performed to sequence the coding region and intron/exon boundaries of the most likely candidate genes, including aminoadipate-semialdehyde synthase (AASS), peroxisomal 2,4-dienoyl-CoA reductase (DECR2), and *trans*-2-enoyl-CoA reductase (MECR). Surprisingly, subsequent sequence analysis excluded mutations in these candidate genes [16].

Due to the exclusion of these candidate genes, whole-exome sequencing was performed, and a homozygous nonsense mutation was found in exon 10 of *NADK2*. The mutation leads to a premature stop codon at position 340 (R340X). Sanger sequencing confirmed that the mutation was homozygous in the patient and heterozygous in his parents. Expression analysis using patient fibroblasts showed that the nonsense allele was expressed at reduced levels compared to controls, indicating that the mutated mRNA was subject to nonsense-mediated decay [16].

The identification of a MNADK deficiency immediately suggested an explanation for the patient's symptoms. That is, lack of NADK2 resulted in a deficit of mitochondrial NADP, which is the cofactor for AASS and DECR2. The lack of the cofactor caused the low activity of the two enzymes, resulting in elevated plasma levels of lysine and C10:2 carnitine. Subsequent analysis indeed showed that patient fibroblasts had lower mitochondrial NADP and lower DECR activity [16].

The second patient was a 10-year-old Spanish girl when reported [17]. Whole exome sequencing identified a homozygous splice site mutation in *NADK2*, leading to skipping of exon 9, thus producing a truncated protein (W319X). In fact, *NADK2* mRNA and the corresponding protein were nearly absent in the patient fibroblasts, suggesting a nonsense-mediated decay.

At birth, axial hypotonia, uncoordinated movements, microcephaly, and generalized cerebellar atrophy were detected. Metabolically, the patient revealed high plasma levels of lysine and 2-trans, 4-cis-decadienoylcarnitine. To alleviate the symptoms, a lysine-restricted diet was started when she was 1 month old. The patient's clinical symptoms have been much improved by the lysine-restricted diet together with cofactors and pyridoxal phosphate administration [17]. Overall, the patient presented with metabolic and developmental abnormalities similar to those of the first patient.

In the third patient, a 9-year-old girl when reported, a homozygous Met1Val change in NADK2 was found, which is expected to affect the start of protein translation [18]. Consistently, the patient's fibroblasts revealed residual protein, suggesting that this allele is hypomorphic. She was observed at 9 years of age to have normal intelligence but decreased

visual acuity, optic atrophy, nystagmus and peripheral neuropathy. Hyperlysinemia was present, but C10:2 carnitine levels were normal. Notably, the patient received NADH supplementation as a treatment [18]. The milder phenotypes suggest that this is a hypomorphic allele of NADK2, producing protein through an unknown mechanism.

Overall, studies based on human NADK2-deficient patients have confirmed that in humans, NADK2 deficiency results in reduced mitochondrial NADP(H), leading to consistent metabolic and developmental abnormalities.

4. Mouse models to study NADK2

Mouse models have been critical to study human disease and health. One important feature is that mice and humans are strikingly similar in physiology, pathology, and genetic makeup. Indeed, the mouse genome encodes homologous genes to human NADK2 and NADK. Therefore, the mouse models have been especially important to study the physiology of NADK2 and pathology of its deficiency.

The International Mouse Phenotyping Consortium (IMPC) is an international effort to generate null alleles across the mouse genome [19]. IMPC mice are produced and maintained on a C57BL/6N genetic background, which is suitable for NADK2 research, because the gene encoding nicotinamide nucleotide transhydrogenase (NNT) is wild type, while the strain C57BL/6J harbors NNT mutations, which may complicate phenotype interpretation [20,21]. IMPC generated two independent null alleles, *Nadk2*$^{tm1b\ (EUCOMM)Wtsi}$ (MGI:5637034) and em1 (IMPC)J (MGI:5689888). Tm1b homozygotes had preweaning lethality, with complete penetrance. The em1J null alleles are embryonically lethal with complete penetrance; all homozygotes died at E12.5 [19]. The lethality phenotype of independent *Nadk2* null alleles suggests the essential functions of Nadk2.

The mouse model that we have been using is the *Nadk2* tm1a allele. The *Nadk2* tm1a allele on regular chow can survive with no obvious morphological phenotypes. The reason why tm1a allele, but not the other two null alleles, can survive is likely that a limited amount of Nadk2 can still be produced [22].

The tm1a allele was originally designed to be a knockout by splicing the cDNA to a LacZ cassette. The cassette was then inserted upstream of a critical exon to create a null allele of the gene. However, it is possible that, to some extent, the mouse genome can skip over the LacZ cassette to restore gene expression. This is indeed the case for the *Nadk2* tm1a allele, as we detected a limited expression of Nadk2 in the liver [22], indicating that this allele is hypomorphic. However, this hypomorphic feature is, in fact, highly useful for studying NADK2, because the tm1a allele is a whole-body KO, analogous to the situation in NADK2-deficient patients.

We found that *Nadk2* KO mice metabolically phenocopy the patients' symptoms. Specifically, the plasma levels of lysine and C10:2 carnitine were elevated [23], and additionally, the KO mice generally had elevated plasma carnitines, as well as much higher liver lysine levels [23]. When placed on a high fat diet (HFD), the mice developed fatty liver quickly, largely due to reduced fatty acid oxidation (Fig. 2). The liver ROS was also much elevated with an increased GSSG:GSH ratio [23]. Notably, the tm1a allele has the potential to be converted into a floxed allele, enabling the generation of tissue specific KO mice. Indeed, we were able to generate flox-Nadk2 mice, and after infecting the mice with AAV8-Cre, we generated liver-specific KO mice. The hepatocytes of these mice showed reduced mitochondrial NADP(H) and reduced oxygen consumption rate as determined by the Seahorse system [22].

Recently, two mouse lines carrying two distinct *Nadk2* mutations were discovered in the Jackson Laboratory, and both lines showed consistent neuromuscular symptoms [24]. Both mutations alter serine residues near the C-terminal end of the catalytic domain of Nadk2 and are partial loss-of-function alleles. Histological examination of the Nadk2 hypomorphic mice indicated that there were neuromuscular and neurodegeneration phenotypes, with muscle atrophy and denervation, as well as loss of cerebellar Purkinje cells. Further analysis showed that the Nadk2 hypomorphic mice recapitulate major hallmarks of the patients, including hyperlysinemia and deficiencies in beta-oxidation of fatty acids, mediated partially by DECR [24]. Therefore, these two lines not only have metabolic changes consistent with NADK2-deficient patients, but also show consistent neurological abnormalities [24].

Therefore, complete *Nadk2* deletion is lethal, but *Nadk2* homophors (the tm1a allele and two recently described Jackson Laboratory lines) phenocopy the main features of NADK2 deficiency in humans in terms of metabolism and neuronal developmental abnormality. Therefore, these hypomorphic Nadk2 models are highly suitable to the study NADK2 physiology and the pathology of its deficiency, as well as pre-clinical studies to validate potential therapeutics.

5. NADK2 determines mitochondrial NADP(H) levels and functions as a major metabolic regulator upon increased energy demand

It has been clear that NADK2 activity is crucial to maintaining mitochondrial levels of NADP(H). It should be noted that, in addition to phosphorylating NAD^+, NADK2 also phosphorylates NADH. Using phylogeny analysis we showed that NADK2 is within the same clade as atNADK3 and Pos5, both having NADH kinase activity [11]. Recently, Du et al. examined NADK2 activity using a direct method, which monitors the formation of the radiolabeled product by incubating NADK2 with radiolabeled ATP. The authors demonstrated that NADK2 can use

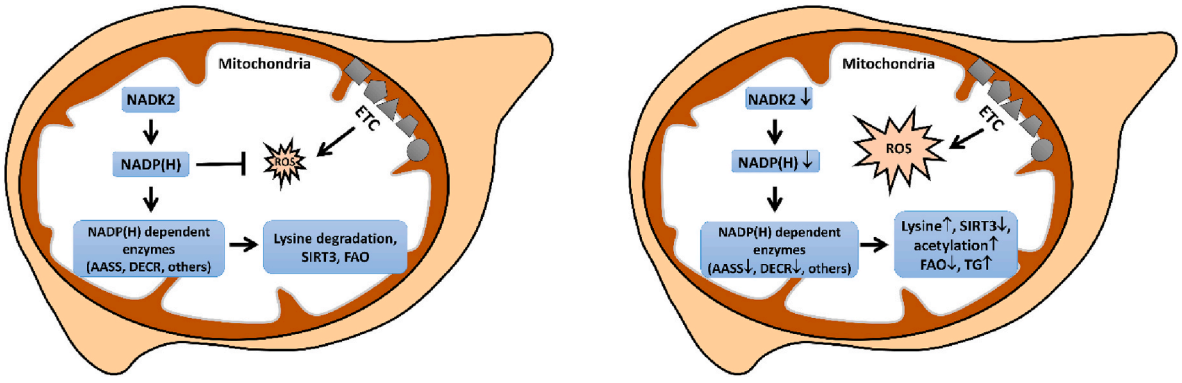

Fig. 2. Hepatic NADK2 determines mitochondrial NADP(H) levels, maintains SIRT3 activity, and regulates fatty acid oxidation. In hepatocytes, NADK2 generates mitochondrial NADP(H), which is crucial to maintain the activities of NADP(H) dependent enzymes, such as AASS, DECR, and to regenerates antioxidant systems (left panel). Reduced level or activity of NADK2 decreases mitochondrial NADP(H) levels, activities of NADP(H) dependent enzymes, and SIRT3 activity, leading to increased levels of lysine, acetylation of SIRT3 targets and ROS. Overall, reduced NADK2 decreases FAO, leading to TG accumulation (right panel).

both NAD^+ and NADH as equivalent substrates with comparable catalytic activity [25]. Although the exact degree of catalytic efficiency using substrates NAD^+ *vs.* NADH by NADK2 varies in different studies, they all consistently show NADK2's NADH kinase activity [13,25]. This result is significant, because it shows that NADK2 generates not only $NADP^+$, but also NADPH, representing a novel pathway for NADPH generation.

Consistently, in *Nadk2* KO mice, we found that NADP(H) levels were significantly reduced in the liver mitochondria, but not in cytosol [23]. In NADK2-deficient humans, NADP(H) levels are specifically reduced in mitochondria but not the cytosol of patient fibroblasts [16]. Consistent results were obtained in other systems such as human cell lines and various mouse tissues, showing that NADK2 deficiency significantly reduces mitochondrial NADP(H) [16,24,26–28]. Therefore, NADK2 determines mitochondrial NADP(H) levels.

Mitochondrial NADP(H) is crucial to the activity of many mitochondrial enzymes as a cofactor. AASS, highly liver-enriched, is involved in the breakdown of lysine [29], and thus when AASS is inhibited, lysine accumulates in the liver and blood. Consistently, in NADK2-deficient patients, plasma lysine is elevated. *Nadk2* KO mice have elevated lysine in both the liver and the circulation [23].

DECR is an enzyme required for the full degradation of polyunsaturated fatty acyl-CoAs and for their oxidation [30]. Indeed, fibroblasts of NADK2-deficient patients showed reduced DECR activity and increased plasma C10:2-carnitine levels. Consistently, *Nadk2* KO mice showed increased plasma C10:2-carnitine levels. Pyrroline-5-carboxylate synthase (P5CS) is an enzyme critical for proline synthesis. In *NADK2* KO cells, P5CS activity was suppressed, and proline was absent, showing an essential role of NADK2 in proline synthesis [26–28]. NADPH is an essential reducing equivalent that provides a reservoir of electrons to defend against oxidative stress [2,4]. For example, NADPH reduces GSSG to GSH, a major antioxidant, catalyzed by glutathione reductase. Consistently, in *NADK2* KO hepatocytes, palmitate-induced ROS production was increased. In *Nadk2* KO mice, liver ROS levels were elevated in mice on HFD, and consistently, the GSSG to GSH ratio was increased [22,23]. Therefore, NADK2 maintains mitochondrial NADP(H) levels, supports enzymatic activities, and mediates antioxidant protection in the liver (Fig. 2). However, severe symptom manifestation of NADK2-deficient patients suggests that NADK2 functions include more than those discussed above.

Common variants of the human *NADK2* gene or its decreased expression were significantly associated with the occurrence of type-2 diabetes, non-alcoholic fatty liver disease (NAFLD), or hepatocellular carcinoma (HCC) [22]. Ablation of the *Nadk2* gene in mice led to decreased fat oxidation, especially when energy demand is high, such as during fasting or endurance exercise [22]. When placed on a high-fat diet (HFD), *Nadk2*-null mice exhibited hepatic insulin resistance and glucose intolerance, indicating a type-2 diabetes-like phenotype [22]. Nadk2 deficiency led to a decrease in mitochondrial NADP(H) but an increase in cellular reactive oxygen species (ROS) in mouse livers. In the livers of *Nadk2* KO mice, activity of SIRT3 is reduced, and consistently, acetylation of SIRT3 targets was increased [22] (Fig. 2). Feeding mice a HFD causes reduced activity of Nadk2 through *S*-nitrosylation (SNO), while reconstitution of an SNO-resistant NADK2 variant, NADK2-S193, into *Nadk2*-null mice mitigated the hepatic steatosis induced by HFD [22]. Overall, NADK2 also plays an important role in regulating fatty acid oxidation (FAO) especially upon increased energy demand, such as fasting.

6. Structural biology of NADK2

The crystal structures of human NADK2 have been solved by X-ray crystallography, and these include crystal structures of NADK2 in a ligand-free form, in a complex with NAD^+, and in a complex with $NADP^+$ [25,31]. Those structures determined by independent groups are strikingly consistent (Fig. 3A). The Nadk2 monomer reveals a two-domain architecture, an N-terminal domain (residues 66–219 and 436–442) and a C-terminal domain (residues 220–435) (Fig. 3B and C).

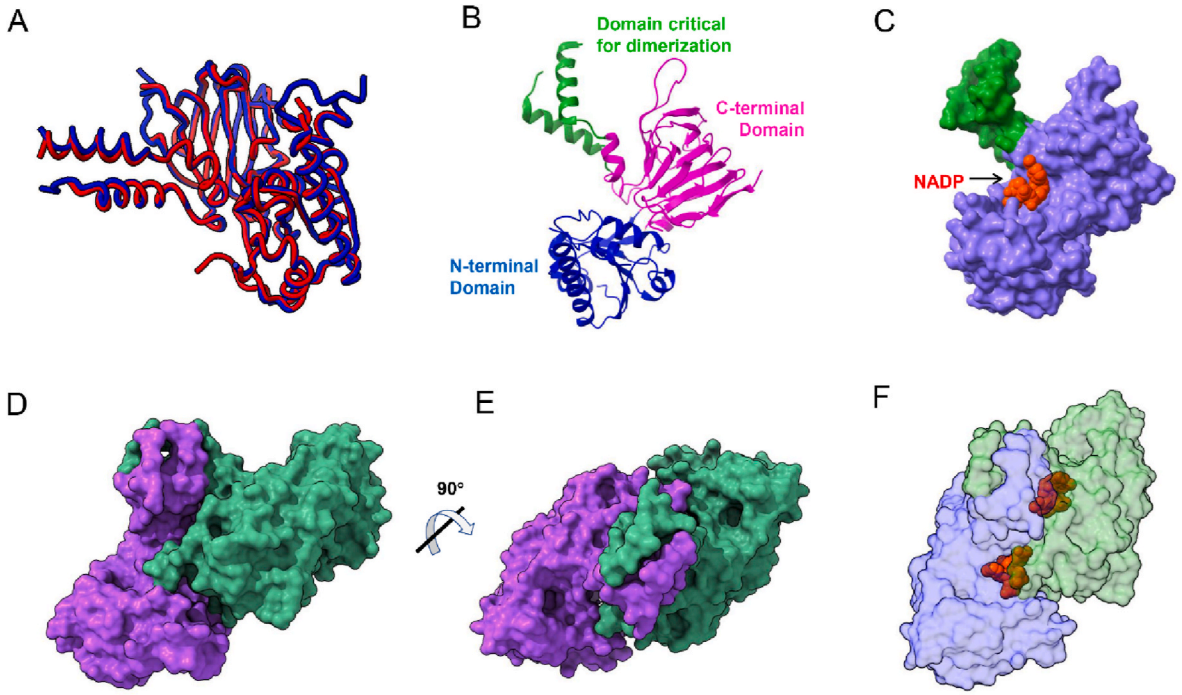

Fig. 3. Crystal structures of NADK2. A) alignment of NADK2 structures solved by independent studies. PDB codes: 7R4J and 7N29. B) NADK2 is organized in 2 domains, an N-terminal and a C-terminal domain. The extension, in green, located in the C-terminal domain, is critical for dimerization. C) surface plot of NADK2 structure, showing the critical extension and $NADP^+$ binding. D) and E dimer of NADK2. F) $NADP^+$ is located in dimer interface. PDB code: 7R4J. The protein structures are visualized by ChimeraX [45]. Also refer to the supplemental file for a video showing the 360° view. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Overall, NADK2 formed a dimer with a perfect 2-fold symmetry (Fig. 3D and E, Supplemental file for a video showing the 360° view), in contrast to the tetrameric structures observed in cytosolic NADKs. The $NADP^+$-binding site is located at the interface of the NADK2 dimer, specifically in a groove formed between the N-terminal domain of one monomer and C-terminal domain of the other monomer and vice versa (Fig. 3F) [25,31].

NADK2 has a unique extension (residue 325–365) that is required for its dimerization and function. This extension nearly doubles the homodimer interface size in NADK2 and is critical for dimerization (Fig. 3B–E) [25]. The mutant harboring a mutation, V331Y, sterically hinders the extension binding interface and destabilizes the NADK2 homodimer, and strongly inhibits NADK2 activity. Deletion of the extension (residue 325–365) resulted in monomeric and inactive NADK2, which, when expressed in NADK2-deficient cells, resulted in proline auxotrophy due to its inability to enable proline production. These results establish that the extension is required for stabilizing the catalytically active homodimer of NADK2. Interestingly, the extension is not only critical for dimer formation, but also prevents formation of tetramer [25,31].

Supplementary video related to this article can be found at https://doi.org/10.1016/j.redox.2023.102613

7. NADK2 activity and functions are regulated by post-translational modifications

Mitochondrial enzymes are highly regulated by post-translational modifications, including acetylation and phosphorylation, which modify their activity and functions [32,33]. For instance, the Akt serine/threonine kinase phosphorylates NADK on three serine residues (Ser44, Ser46, and Ser48), and this phosphorylation stimulates NADK activity to increase $NADP^+$ production [34]. Multiple types of NADK2 post-translational modifications have been identified in both humans and mice. Thus far, four specific NADK2 sites have been functionally characterized regarding post-translational modifications.

A study examined the *in vitro* kinase activity of recombinant NADK2 proteins and a series of phospho-deficient mutants. S188 phosphorylation was found to be especially critical for the kinase activity [35]. Substituting S188 with Ala completely abolished the catalytic activity, suggesting a critical role for S188. Functionally, another study showed that the S188 to Ala mutation also blocked proline production in the cells [31]. Phosphorylation at S188 has also been identified in a number of other independent studies [36,37].

The acetylation of NADK2 has been comprehensively examined, and K76 and K304 were found to be especially critical [31]. In *NADK2* KO cells, profiling of 10 putative K/Q acetylation-deficient mutants suggested that K76Q and K304Q mutants profoundly reduced proline synthesis. Moreover, NADK2-deficient cells expressing these mutants were unable to grow in the absence of proline, similar to NADK2-deficient cells. Indeed, cells harboring the mutations K76Q, K304Q, or S188A have reduced mitochondrial NADP(H). NADK2 mutants also exhibited reduced activity, shown by *in vitro* kinase assays. The K76Q, K304Q, and S188A mutations did not affect NADK2 dimerization. Consistently, according to NADK2 structures, these post-translational modifications are believed to affect binding of NADK2 to NAD^+ and ATP [31].

SNO, a reversible post-translational modification, represses protein activities through covalent attachment of a nitrogen monoxide group to cysteine residues of target proteins. In obesity and HFD-related overnutrition, chronic metabolic inflammation leads to enhanced SNO modification of key metabolic regulators in the liver [38]. We found that both human and mouse NADK2 has a conserved SNO motif, RSEGHLCLPVRYT, characterized by a cysteine (C) residue surrounded by acidic or basic amino acids. We utilized a biotin-switch method to label S-nitrosylated proteins in liver tissue sections and found that a HFD enhanced levels of Nadk2 SNO modifications [22]. We previously demonstrated that Nadk2 deficiency leads to hepatic steatosis and the associated metabolic phenotypes in mice on a HFD [23]. Consistently, expression of an SNO-resistant form of Nadk2 in *Nadk2*-KO mouse livers mitigated hepatic steatosis caused by the HFD [22]. Mechanistically, Seahorse analysis showed that the primary hepatocytes expressing Nadk2-S193 exhibited higher oxygen consumption rates [22].

The four sites undergoing posttranslational modifications are highly evolutionarily conserved (Fig. 4A). Interestingly, all four sites that impact NADK2 activity are in the vicinity of the NADK2 binding pocket (Fig. 4B and C, Supplemental file for a video showing the 360° view). According to the NADK2 structure, it is expected that acetylation of K304 interferes with NAD^+ binding, acetylation of K76 interferes with ATP binding, and phosphorylation of S188 is expected to prevent the ATP to NAD^+ phosphotransfer [31]. Overall, NADK2 activity is highly regulated by phosphorylation, acetylation, and SNO, and this complex regulation likely reflects the need for the accurate control of mitochondrial NADP biosynthesis in the cell in response to various physiological and pathological conditions.

8. Future perspectives

One important direction for future research would be the dynamic regulation of NADK2 activity and its expression in different pathological conditions. NADK2 activity is highly regulated by post-translational modifications. We have shown that in the liver, a HFD and the presence of obesity enhanced SNO modification of Nadk2, inhibiting its activity, resulting in lower mitochondrial NADPH and FAO [22]. The next logical questions to ask include: a) How do obesity, HFD, insulin resistance, and diabetes affect other post-translational modifications, including S188 phosphorylation and K76 and K304 acetylation? b) How are these post-translational modifications altered by other pathological conditions, especially neuronal degeneration disease and atherosclerosis?

It has become evident that ROS overproduction of the antioxidant system is associated with inflammation, metabolic dysfunction, and cardiovascular disorders [39]. NADPH oxidases (NOX) represent a class of hetero-oligomeric enzymes whose primary function is the generation of ROS [39]. In the vasculature, NOX-derived ROS contribute to the maintenance of vascular tone and regulate important processes involved in vascular biology [40]. In pathological conditions, excessive NOX-dependent ROS formation induces dysregulation of redox control systems and promotes oxidative injury of cardiovascular cells. NADK2, by generating mitochondrial NADP(H), appears to have an opposing role to NOX, and therefore the involvement of NADK2 in the process of ROS overproduction and atherosclerosis is worthy of further investigation.

In addition to homozygous mice, IMPC also generated heterozygous mice for $Nadk2^{em1(IMPC)J}$ and $Nadk2^{tm1b(EUCOMM)Wtsi}$ alleles. These heterozygous mice display phenotypes in characterizations including eye morphology (persistence of hyaloid vascular system), auditory brain stem response, hematology (cell hemoglobin concentration, white blood cell count), and blood parameters (urea, potassium, thyroxine, calcium) [19]. The diverse phenotypes of Nadk2 heterozygous mice suggest the functional roles of Nadk2 in multiple tissues and systems. Indeed, although Nadk2 is mostly expressed in the liver, it is also abundant in other mitochondrion-rich tissues [12]. Therefore, it would be informative to characterize NADK2 functions in tissues other than the liver, such as the brain and muscle.

Another area of study inquires how NADK2 affects tumor development. We found that altered NADK2 expression is associated with human liver cancers [12,22]. NADK2 amplifications are found in 10% of the non-small cell lung cancer, and 6%–8% of other cancers, including bladder, esophageal, stomach, and ovarian [26,27]. NADK inhibitors have been suggested as promising anti-cancer therapeutics [41,42]. Indeed, previous work has indicated that loss of NADK2 results in proline auxotrophy and reduces tumor growth [31]. Therefore, the roles of NADK2 and its inhibition in tumor development are of interest for further investigation.

Newborn screening for DECR deficiency is included in the

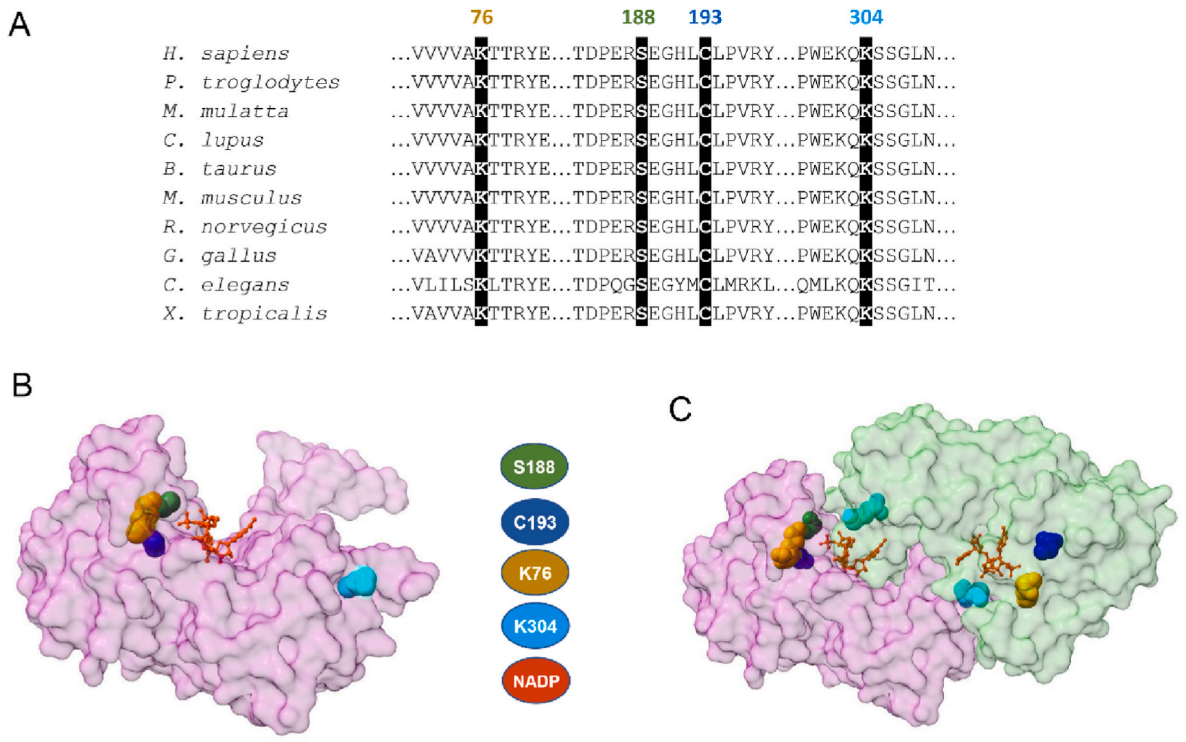

Fig. 4. NADK2 post-translational modifications. NADK2 is phosphorylated at S188, acetylated at K76 and K304, and S-nitrosylated at C193. All the post-translational modifications have been characterized, and it has been demonstrated that mutations in each of the 4 sites affect NADK2 functions. A) all four sites are highly evolutionarily conserved. Locations of the post-translational modification sites in NADK2 B) monomer and C) dimer. The protein structures are visualized by ChimeraX [45]. Also refer to the supplemental file for a video showing the 360° view.

Recommended Uniform Screening Panel, likely using C10:2-carnitine as the primary marker [16]. NADK2 deficiency causes reduced DECR activity and thus increased C10:2-carnitine [16]. Because DECR deficiency does not cause hyperlysinemia, including plasma lysine enables differentiation of the two conditions. Therefore, including lysine analysis and genotyping of NADK2 will enable better diagnostics and genetic counseling for related conditions.

From a personal perspective, the discovery of NADK2 represents an interesting scientific journey and career development for the first author of the current review. In early 2010, when Dr. Ren Zhang established his laboratory at Wayne State University, to find novel nutritionally regulated genes, his group utilized RNA-seq, a technology that was new at the time. The samples were white adipose tissue and livers from mice either fasted or on a HFD for 3 months. This RNA-seq experiment turned out to be an effective one, and two previously uncharacterized genes were found to be nutritionally regulated and metabolism relevant. One was Gm6484, named lipasin (later known as ANGPTL8), a feeding-induced gene that was specific to fat and the liver [43]. ANGPTL8 regulates triglyceride metabolism by being a critical regulator of triglyceride partitioning between fat and oxidative tissues [44]. The other was C5ORF33, named MNADK (later known as NADK2), which is a fasting-induced liver-enriched protein that generates mitochondrial NADP(H) [12]. The publication of the MNADK manuscript, however, was far from being smooth. Starting in early 2012, the manuscript was rejected by multiple journals including Biochemical and Biophysical Research Communications. The manuscript was also initially rejected by Open Biology, which, following a re-submission, eventually published it [12]. It is amazing that one decade after the discovery of MNADK (NADK2), a significant amount of knowledge has been obtained regarding its functions, mechanism of action, human biology, mouse models, crystal structures, and post-translation modifications. We hope that future research on NADK2 will not only elucidate its functional roles, but will also pave the way for new therapeutics for both rare diseases such as NADK2 deficiency and common diseases such as dyslipidemia, diabetes, and cardiovascular disease.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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