

NAD(P)HX dehydratase (NAXD) deficiency: a novel neurodegenerative disorder exacerbated by febrile illnesses

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Physical stress, including high temperatures, may damage the central metabolic nicotinamide nucleotide cofactors [NAD(P)H], generating toxic derivatives [NAD(P)HX]. The highly conserved enzyme NAD(P)HX dehydratase (NAXD) is essential for intracellular repair of NAD(P)HX. Here we present a series of infants and children who suffered episodes of febrile illness-induced neurodegeneration or cardiac failure and early death. Whole-exome or whole-genome sequencing identified recessive NAXD variants in each case. Variants were predicted to be potentially deleterious through *in silico* analysis. Reverse-transcription PCR confirmed altered splicing in one case. Subject fibroblasts showed highly elevated concentrations of the damaged cofactors S-NADHX, R-NADHX and cyclic NADHX. NADHX accumulation was abrogated by lentiviral transduction of subject cells with wild-type NAXD. Subject fibroblasts and muscle biopsies showed impaired mitochondrial function, higher sensitivity to metabolic stress in media containing galactose and azide, but not glucose, and decreased mitochondrial reactive oxygen species production. Recombinant NAXD protein harbouring two missense variants leading to the amino acid changes p.(Gly63Ser) and p.(Arg608Cys) were thermolabile and showed a decrease in V_{max} and increase in K_M for the ATP-dependent NADHX dehydratase activity. This is the first study to identify pathogenic variants in NAXD and to link deficient NADHX repair with mitochondrial dysfunction. The results show that NAXD deficiency can be classified as a metabolite repair disorder in which accumulation of damaged metabolites likely triggers devastating effects in tissues such as the brain and the heart, eventually leading to early childhood death.

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Abbreviations: NAD = nicotinamide adenine dinucleotide; NADP = nicotinamide adenine dinucleotide phosphate; NAXD = NAD(P)HX dehydratase; NAXE = NAD(P)HX epimerase; OXPHOS = oxidative phosphorylation

Introduction

Metabolism is traditionally viewed as an efficient and precise system, supported by enzymes that are highly specific for their substrate and the type of reaction through which they convert the substrate. This view has been challenged over recent years by the identification of a growing list of enzymes that function to repair or remove metabolic side products, the latter of which are generated by side reactions of main metabolic enzymes. Metabolic side products can also be produced intracellularly by unwanted spontaneous chemical reactions. These non-canonical or 'damaged' metabolites can inhibit key metabolic reactions if they are left to accumulate. It is to precisely prevent the accumulation of potentially toxic small molecules that all organisms of all domains of life have most likely evolved a panoply of metabolite repair systems (Linster *et al.*, 2013; Van Schaftingen *et al.*, 2013).

The nicotinamide adenine dinucleotides (NAD) (reduced form NADH, oxidized form NAD⁺) and nicotinamide adenine dinucleotide phosphate (NADP) (reduced form NADPH, oxidized form NADP⁺) have essential roles in many cellular functions. NAD is involved in a series of

catabolic reactions and in mitochondrial energy production, whereas NADP is a key component of numerous biosynthetic processes as well as cellular antioxidant protection systems (Ying, 2008; Houtkooper *et al.*, 2010). The nicotinamide ring within these cofactors is prone to hydration, forming NADHX or NADPHX, which can be present as *R* or *S* epimers and which can further degrade irreversibly to cyclic NAD(P)HX (Yoshida and Dave, 1975). NADHX can be slowly produced from NADH by GAPDH (Rafter *et al.*, 1954); NAD(P)HX can also form spontaneously from the normal reduced cofactors under 'stress' conditions such as increased temperature or acidic pH (Rafter *et al.*, 1954; Yoshida and Dave, 1975). The damaged cofactors cannot act as electron carriers and have been shown *in vitro* to inhibit several key dehydrogenase enzymes (Yoshida and Dave, 1975; Prabhakar *et al.*, 1998). In *Saccharomyces cerevisiae*, *in vitro* and *in vivo* evidence for an inhibitory effect of NADHX on 3-phosphoglycerate oxidoreductase, catalysing the initial step of the serine biosynthesis pathway, has been obtained recently (Becker-Kettern *et al.*, 2018). Therefore, NAD(P)HX can be expected to be toxic to cells, and detoxification by a metabolite repair system is critical.

The nicotinamide nucleotide repair system consists of two partner enzymes: NAD(P)HX epimerase (NAXE, formerly APOA1BP; OMIM: 608862), which converts R-NAD(P)HX to S-NAD(P)HX, and NAD(P)HX dehydratase (NAXD, formerly CARKD; OMIM: 615910), which converts S-NAD(P)HX back to NAD(P)H in an ATP-dependent manner (Marbaix *et al.*, 2011). Both these enzymes are targeted to several subcellular compartments, including the mitochondrion (Marbaix *et al.*, 2014). They also have a ubiquitous tissue distribution (Marbaix *et al.*, 2014) and are conserved across all taxa (Marbaix *et al.*, 2011). The presence of the NAD(P)HX repair enzymes across all tissues and species, combined with the central metabolic roles of the cofactors that they function to preserve, suggest that the repair system is critical to sustain life. More specifically, it can be predicted that the brain, which has a very high and constant demand for energy supply generated by mitochondria, would be particularly vulnerable to impaired NAD(P)HX repair and as such, the NAXE and NAXD enzymes would be critical to support normal brain function. In apparent contradiction with those predictions, however, knocking out NAD(P)HX repair enzymes in other organisms such as yeast, bacteria and plants, has so far only revealed subtle growth phenotypes, if any, at least under standard growth conditions (Breslow *et al.*, 2008; Hillenmeyer *et al.*, 2008; Nichols *et al.*, 2011; Colinas *et al.*, 2014; Niehaus *et al.*, 2014; Becker-Ketterer *et al.*, 2018).

Recently, whole exome sequencing identified pathogenic variants in the epimerase NAXE, which were associated with cases of a lethal neurometabolic disorder of early childhood (Kremer *et al.*, 2016; Spiegel *et al.*, 2016). In these subjects, it appeared that episodes of febrile illness aggravated the consequences of an already compromised metabolite repair system, resulting in rapid neurological deterioration and decomposition of other tissues with clinical observation of ataxia, muscular hypotonia, respiratory insufficiency and/or respiratory failure, nystagmus and skin manifestations followed by premature death. This provided the first cases of subjects with pathogenic variants in a key enzyme of the nicotinamide nucleotide repair pathway. Here we report on the first known cases with pathogenic variants in the dehydratase NAXD.

Materials and methods

Full materials and methods are available in online Supplementary material.

Subjects and variant analysis

All procedures were approved by human research ethics committees. Primary cultures of fibroblasts were established from skin biopsies.

Mitochondrial analysis

Fibroblast extracts were analysed by immunoblotting using antibodies against representative oxidative phosphorylation

(OXPHOS) subunits. The complex I and IV activity was determined using commercially available dipstick assays (MitoSciences). Mitochondrial stress was induced by culturing fibroblasts galactose media and determining cell growth rates. Mitochondria reactive oxygen species production was measured by dihydroethidium fluorescence.

LC-MS analysis of NAD(H), NADP(H) and damaged derivatives

Wild-type NAXD including the mitochondrial targeting signal (mNAXD) or not (cNAXD starting at Met3) or a GFP control construct was introduced into patient fibroblasts by lentiviral delivery. Intracellular metabolites were extracted from cultured fibroblasts and concentrations were measured with an HRAM-*RP*-LC-MS (high resolution accurate mass reversed phase liquid chromatography mass spectrometry) method compared against chemically pure standards.

NADHX dehydratase activity assays and thermostability testing

Missense variants were introduced into wild-type cNAXD and proteins expressed and purified from a bacterial overexpression system as detailed in the Supplementary material. The S-NADHX substrate was synthesized and purified as previously described (Becker-Ketterer *et al.*, 2018) and NAXD enzyme kinetic properties determined using a spectrophotometric assay (Marbaix *et al.*, 2011). Protein thermostability was tested by pre-incubation of purified protein at different temperatures (30–47°C).

Statistical analysis

Statistical analyses were carried out using either a two-tailed Student's *t*-test or one-way ANOVA corrected for multiple comparisons as appropriate (GraphPad Prism® Software). Error bars represent the standard deviation of the mean [\pm standard deviation (SD)]. A *P*-value < 0.05 was considered to be statistically significant.

Data availability

Derived data supporting the findings of this study are available from the corresponding author upon request.

Results

In the present study we report six unrelated individuals with homozygous or compound heterozygous variants in NAXD. The predominant clinical features included repeated episodes of regression often triggered by episodes of mild fever or infection, an infantile onset neurodegenerative condition (Fig. 1A–M) and skin lesions (Fig. 1O and P), ultimately leading to early death in all cases. The clinical features of our NAXD subjects therefore resembled those of the previously published cases of NAXE deficiency (Table 1), and detailed clinical reports for all cases are available in Supplementary material. The six subjects with

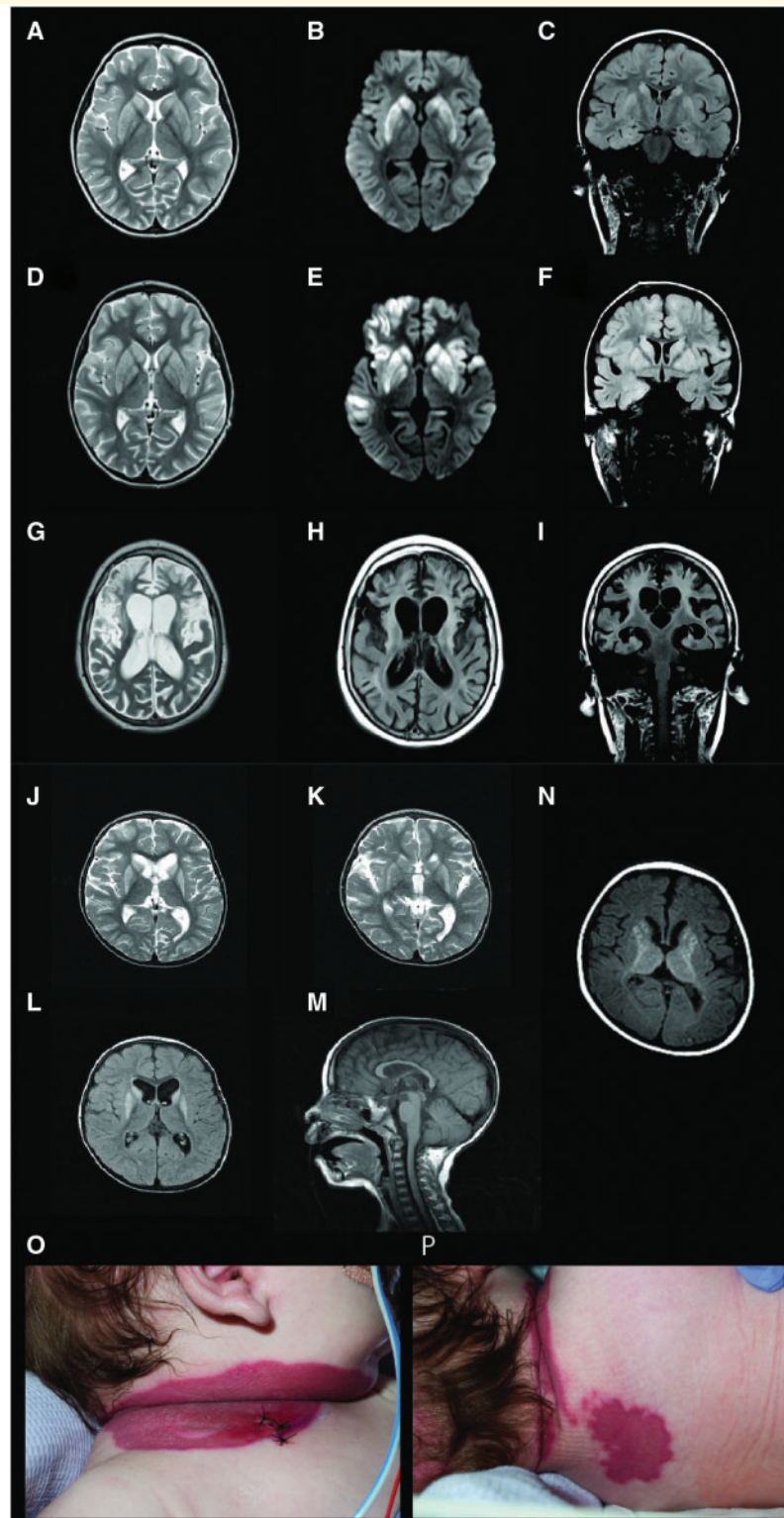


Figure 1 Neuroimaging findings and skin manifestations in children with NAXD mutations. Brain MRI scans for Case 1 at the age of 3 years and 7 months, 3 months after normal MRI scans: (A) axial T₂, (B) diffusion (DWI) and (C) coronal T₂ FLAIR showed bilateral and symmetrical T₂ high signal and cytotoxic oedema of the basal ganglia and focal areas of cortical involvement in the temporal lobes. Progress MRI after 3 weeks: (D) axial T₂, (E) DWI and (F) coronal T₂ FLAIR showed bilateral and symmetrical T₂ high signal and cytotoxic oedema of the basal ganglia persist with increasing areas of asymmetrical cortical involvement in the temporal lobes and frontal lobes. Follow-up MRI 3 years later: (G) axial T₂, (H) axial and (I) coronal T₂ FLAIR showed generalized cerebral atrophy, most marked in the frontal lobes and basal ganglia with exvacuo-dilatation of the lateral ventricles. The high signal is consistent with gliosis. Case 2 MRI (J–M) showed bilateral hyperintensity of striatal nuclei, which remained unchanged in subsequent scans. Case 4 brain MRI (N) showed bilateral basal ganglia changes suggestive of a mitochondrial disorder. Extensive skin lesions in Case 4 (O and P).

Table 1 Clinical findings in individuals with variants in *NAXD* or *NAXE*

Clinical presentation	<i>NAXD</i> cases (present study)						<i>NAXE</i> (previously published)	
	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Spiegel <i>et al.</i> (2016)	Kremer <i>et al.</i> (2016)
Gender	M	F	F	M	F	F	3F, 2M	2F, 4M
Episodes of fever/illness prior to deterioration	Y	Y ^a	Y	Y	Y	Y	5/5	4/6 (2 unclear)
Neurodegeneration	Y	Y	N	Y	? ^b	Y	5/5	4/6 (2 unclear)
Skin lesions	Y	Y	N	Y	N	Y	Not reported	4/6
Cardiac presentation	N	N	N	N	Y	N	Not reported	2/6 ^c
Early death	Y	Y	Y ^d	Y	Y	Y	4/5	6/6

^aFever was associated with some, but not all episodes of deterioration.

^bAbnormal MRI scan.

^cTwo *NAXE* patients died from cardiovascular failure.

^dSibling had mild developmental delay, mild anaemia, recurrent episodes of fever, and died after an episode of vomiting and lethargy at 1 year 7 months. N = no; Y = yes.

NAXD variants were identified individually by whole exome or whole genome sequencing as part of either independent international gene discovery cohorts, National Health Services diagnostic testing or through the Genematcher/Matchmaker database (Philippakis *et al.*, 2015; Sobreira *et al.*, 2015).

Sanger sequencing confirmed all variants identified through next generation sequencing (Supplementary Fig. 1). The human *NAXD* gene is predicted to generate four coding transcripts, leading to the expression of cytosolic, mitochondrial and endoplasmic reticulum (ER) protein isoforms, as explained in detail in the Supplementary material (see also Supplementary Figs 2 and 3). For initial interpretation we focused on the most abundant transcript RefSeq isoform c (NM_001242882.1; Supplementary Table 1) whose existence is most strongly supported by EST (expressed sequence tag) analysis and from which mitochondrial and cytosolic *NAXD* protein forms can be translated (Supplemental material). *In silico* analyses predicted the variants to be pathogenic to each of the four *NAXD* transcript isoforms, and affect the structure and function of the *NAXD* protein (Supplementary Table 2, Supplementary Figs 2 and 3). By HSF3 analysis (Desmet *et al.*, 2009) the splicing variant in Case 1 was predicted to lead to disruption of the wild-type donor site, which was confirmed by RT-PCR studies, revealing aberrant splicing and skipping of Exon 9 (Supplementary Fig. 4).

We then used patient fibroblasts to determine the intracellular concentrations of NAD(P), NAD(P)H, S-NAD(P)HX, R-NAD(P)HX and cyclic-NAD(P)HX using HRAM RP-LC-MS with comparison against chemically pure standards (Supplementary Fig. 5). We detected no significant differences in NAD, NADH and NADP levels between control and subject-derived fibroblasts (NADPH levels were below the limit of quantification). Importantly, the damaged cofactor derivatives S-NADHX, R-NADHX and cyclic-NADHX were only detected in fibroblasts from Cases 1, 2 and 4, but not in any of our control cell lines (Fig. 2A and B). Quantification of these metabolites revealed a similar level of accumulation of S-NADHX

and R-NADHX in Cases 1, 2 and 4 (Fig. 2C). Interestingly the levels detected in Case 3 were much lower than for Cases 1, 2 and 4 and only just above the detection threshold for LC-MS analysis (Fig. 2B). Lentiviral transduction with either the cytosolic (c*NAXD*) or mitochondrial (m*NAXD*) wild-type *NAXD* cDNA completely prevented the accumulation of any of the NADHX derivatives in fibroblasts from Cases 1 and 2 (Fig. 2D) whereas a control GFP construct had no effect (Fig. 2D).

To address whether mitochondrial function is affected by *NAXD* deficiency in our subjects, we examined the expression of representative protein subunits of the mitochondrial respiratory chain and enzyme activity of complexes I and IV in fibroblasts from Cases 1 and 2. There was a marked reduction in the expression of both NDUFB8 (complex I membrane subunit) and COXII (complex IV subunit) in Cases 1 and 2 compared to four paediatric control fibroblast extracts (Fig. 3A and Supplementary Fig. 6). We found a significant reduction in cytochrome *c* oxidase activity in Cases 1 and 2 compared to controls (Fig. 3B), whilst complex I NADH oxidation activity was not affected (data not shown). Mitochondrial superoxide production revealed a significant decrease in rotenone-inhibited complex I superoxide production in both Cases 1 and 2 compared to controls (Fig. 3C). There was a significant decrease in the growth rate of fibroblasts in glucose-free, galactose-azide medium to induce mitochondrial stress from Cases 1 and 2 compared to control fibroblasts (Fig. 3D).

The *NAXD* missense variants [p.(Arg308Cys) and p.(Gly63Ser)] were introduced by site-directed mutagenesis into human *NAXD* cDNA, then expressed and purified as recombinant proteins. For the p.(Gly63Ser) variant, we found a 3.4-fold decrease in V_{max} and a 3.3-fold increase in K_M compared to the wild-type protein. For the p.(Arg308Cys) variant, we determined a 2.5-fold decrease in V_{max} and a 2.2-fold increase in K_M compared to wild-type (Supplementary Table 3). Thermostability analyses of recombinant *NAXD* revealed that both the p.(Gly63Ser) and the p.(Arg308Cys) proteins lost enzymatic activity

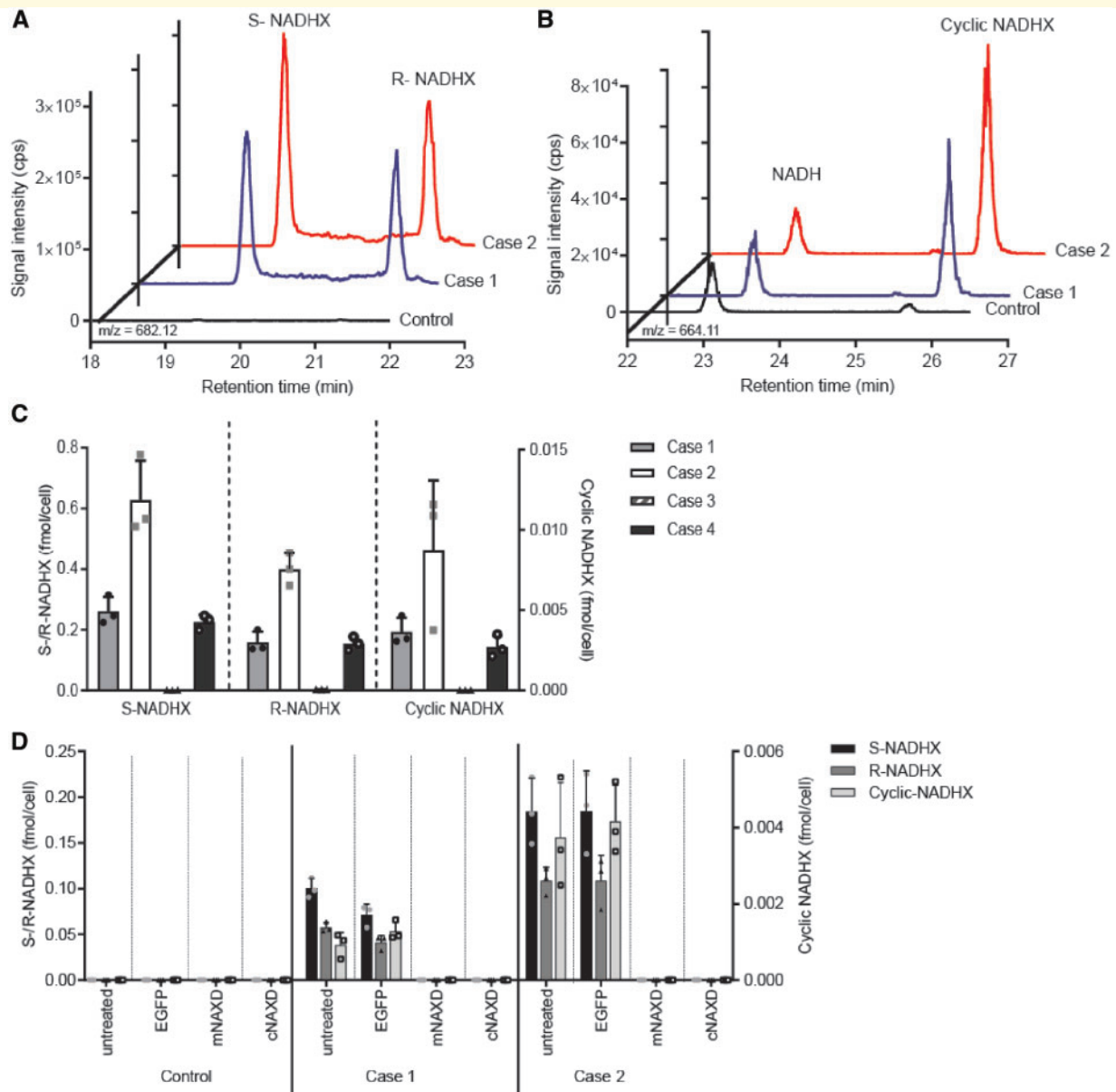


Figure 2 NADHX accumulation in subject fibroblasts and phenotypic rescue by lentiviral gene delivery of wild-type NAXD. Representative examples of LC-MS extracted ion chromatograms of (A) S- and R-NADHX metabolites (XIC = 682.12) and (B) NADH and cyclic NADHX (XIC = 664.11). The chromatograms clearly show peaks of S-, R- and cyclic NADHX in Cases 1 and 2, and not in a control. (C) Quantitative results of the NADHX measurements after cultivation of fibroblasts from Cases 1–4 under standard conditions at 37°C for 96 h. Note that these metabolites were not detected in four control fibroblast lines. Data are mean \pm SD, $n = 3$. (D) Quantitative results of the NADHX measurements in lentiviral-rescued cells. There was a clear accumulation of S-, R- and cyclic NADHX in Cases 1 and 2, independently of transduction with GFP-only vectors. S-, R- and cyclic NADHX were not detected in control fibroblasts. Lentiviral gene rescue with either the mNAXD or cNAXD construct completely prevented accumulation of S-, R- and cyclic NADHX in subject cells. Data are mean \pm SD, $n = 3$.

upon pre-incubation at temperatures higher than 30°C while the wild-type protein activity resisted exposure to temperatures up to 47°C (Fig. 3E). This thermolability was most pronounced for the p.(Arg308Cys) variant, with a more than 90% decrease in activity above 45°C, while for the p.(Gly63Ser) variant this same treatment resulted in a ~40% activity loss (Fig. 3E).

Discussion

Here we report on the first known pathogenic NAXD variants, which affected six families, leading to a fever-induced severe multisystem disease and death within the first decade of life. To explore the consequences of NAXD deficiency, quantification of NAD(P)HX metabolites revealed a similar

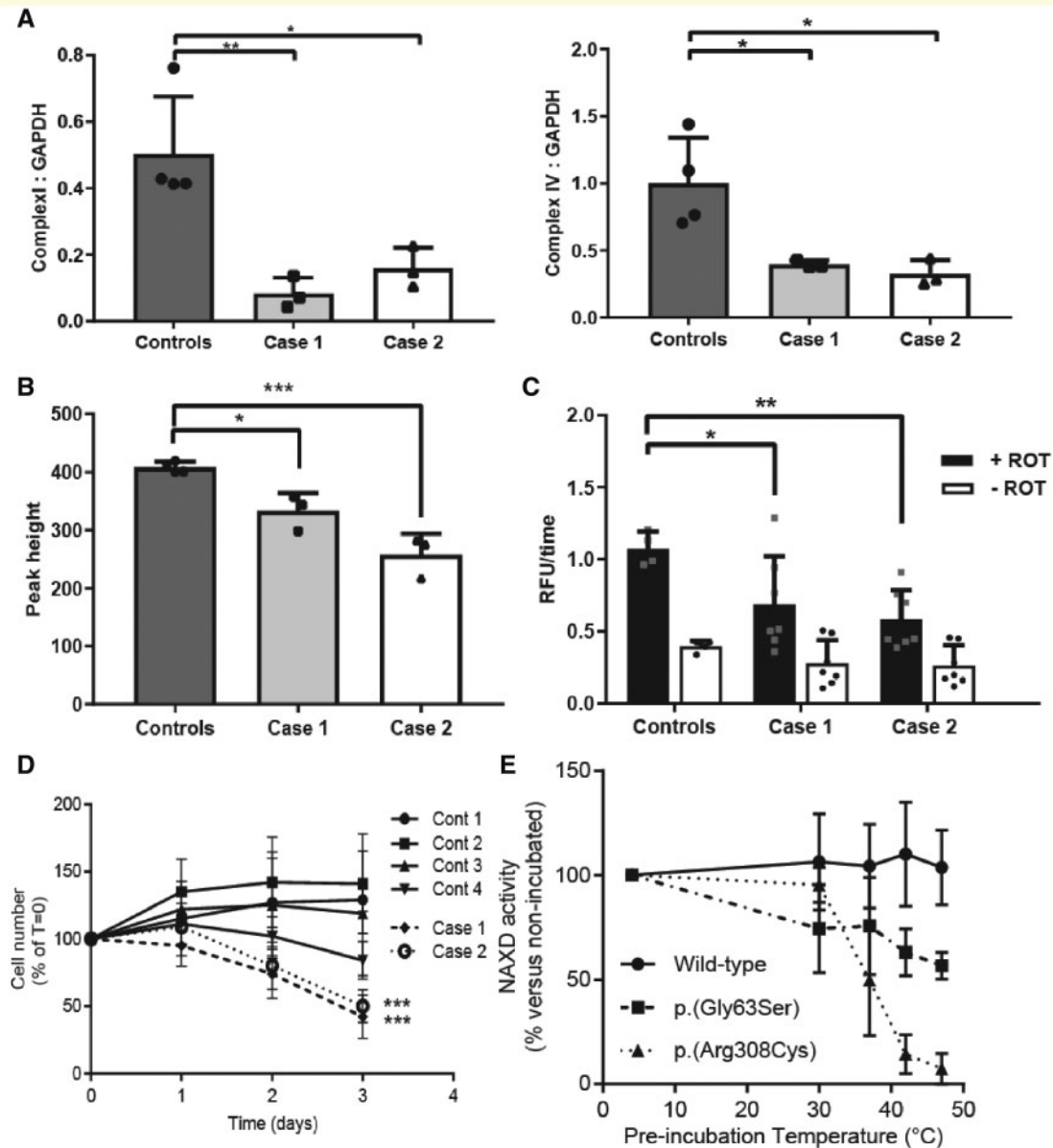


Figure 3 Mitochondrial impairment in NAXD subject fibroblasts and thermostability of recombinant NAXD protein variants. (A) Mitochondrial OXPHOS proteins were separated by SDS-PAGE, probed for relative expression levels of key OXPHOS subunits by immunoblotting, and expression levels normalized to GAPDH as a loading control. There was a significant decrease in the expression of complex I and complex IV proteins expression in Cases 1 and 2 compared to controls. (B) Mitochondrial OXPHOS enzyme activity was measured in cell extracts by immunocapture dipstick assays. There was a significant decrease in complex IV activity [monoclonal antibodies (mAbs)/mg protein] in Cases 1 and 2 relative to controls. (C) Relative rates (relative fluorescent units/time) of mitochondrial superoxide production in fibroblasts was measured with the superoxide sensitive probe dihydroethidium in the presence or absence of the complex I inhibitor rotenone. (D) Growth rate in medium devoid of glucose but containing 5 mM galactose and 50 μ M sodium azide was normalized to growth rate in basal medium to account for variation in the baseline growth rate of each cell line. The normalized growth rate in Cases 1 and 2 was significantly lower than controls. (E) Purified recombinant NAXD protein, without or with missense mutations p.(Gly63Ser) and p.(Arg308Cys), was pre-incubated at the indicated temperature for 30 min prior to addition to a reaction mixture for the spectrophotometric assay of NADHX dehydratase activity. Data are mean \pm SD, $n > 3$ per measurement from at least two independent experiments. Statistical significance was determined using one-way ANOVA with Bonferroni correction for multiple comparisons; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

level of accumulation of S-NADHX and R-NADHX in Cases 1, 2 and 4 in the expected 60:40 *S* to *R* epimer ratio (Marbaix *et al.*, 2011). This was completely reversed by lentiviral transduction with either cytosolic (cNAXD) or

mitochondrial (mNAXD) lentiviral constructs. Supposing an intracellular fibroblast volume of 2 pL, the approximate intracellular concentrations of S-NADHX (100–300 μ M) and R-NADHX (70–210 μ M) were quantitatively similar

to values induced by a complete loss of function for NAXD in an experimental cell line (Becker-Ketterer *et al.*, 2018). The levels in Case 3 were only just above the detection threshold, which may be due to the frameshift mutation in this patient preventing expression of the mitochondrial and endoplasmic reticulum targeted NAXD isoforms (early truncation), but allowing for expression of the cytosolic NAXD protein from the ATG residing in exon 2 (Met3 in Supplementary Fig. 3). The same reasoning would also apply to Case 5, for which fibroblasts were, however, not available for measurements. These results provide strong support that the NADHX accumulation is specifically caused by NAXD mutations.

NAD(P)H are essential cofactors for many cellular reactions, particularly in the mitochondria, therefore accumulation of the non-canonical NAD(P)HX derivatives may impede multiple cellular functions including key mitochondrial dehydrogenases (Yoshida and Dave, 1975; Prabhakar *et al.*, 1998). Subjects with pathogenic variants in NAXE had decreased complex I activity (Kremer *et al.*, 2016) indicating impaired mitochondrial function as a consequence of a deficiency in one of the NAD(P)HX repair enzymes. Respiratory chain activity was impaired in NAXD patients; fibroblasts had a significant decrease in the expression of complex I and IV, and activity of complex IV and muscle enzymology showed reduced respiratory chain activity (complex II + III, Case 2; complex I, Case 4; complex I and IV, Case 6). We also demonstrated reduced superoxide production in NAXD subjects, which may be explained by decreased expression of specific complex I subunits subsequently affecting holocomplex stability, but partial enzyme activity may still remain because of preservation of NADH oxidation in the matrix arm of complex I. Mitochondrial dysfunction can also be revealed by culturing cells under galactose growth conditions (Robinson *et al.*, 1992) in the presence of the complex IV inhibitor sodium azide (Swalwell *et al.*, 2011), which limits ATP production by glycolysis, forcing cells to rely on mitochondrial OXPHOS. There was a significant decrease in the growth rate of fibroblasts from Cases 1 and 2 compared to control fibroblasts under galactose conditions, further supporting that mitochondrial function in fibroblasts from NAXD subjects is compromised.

To determine the effect of the NAXD missense variants [p.(Arg308Cys) and p.(Gly63Ser)] on NADHX dehydratase activity, the missense variants were introduced by site-directed mutagenesis into the cytosolic NAXD cDNA since similar kinetic properties were previously obtained for both mitochondrial and cytosolic NAXD/Carkd (mouse homologue of human NAXD) (Marbaix *et al.*, 2011) and the cytosolic protein gave greater yield. Analysis of the kinetic properties for the two missense variants revealed significantly reduced V_{\max} and increased K_M compared to the wild-type protein. Thermostability analysis revealed loss of enzymatic activity upon pre-incubation temperatures above 30°C, and the thermostability was more pronounced for the p.(Arg308Cys) variant. The Gly63 residue is highly

conserved from bacteria to humans (Marbaix *et al.*, 2011); the Arg308 residue is also conserved in the mouse and yeast homologs of NAXD, but not in the *Escherichia coli* homologue (Marbaix *et al.*, 2011). This arginyl residue is relatively close to the C-terminus of the protein, as are the splicing and frameshift mutations found in Cases 1 and 2, respectively, suggesting that this may be a critical domain for NAXD protein stability. In addition, the thermostability of the NAXD missense variants found in Cases 1 and 2 may at least in part explain the coincidence of deterioration in the subjects sometimes occurring after episodes of fever. Those individuals who had infections may have had unreported fevers secondary to the viral infection, which likely precipitated rapid decompensation. In summary, our *in vitro* analyses of recombinant NAXD demonstrated that the missense variants, while retaining residual enzyme activity, show a markedly decreased stability especially at higher temperatures.

This report highlights the importance of the NAD(P)HX repair system to preserve cellular and overall health in humans. We could show high intracellular NADHX accumulation for three of the four individuals where fibroblast lines were available for analysis, and impaired mitochondrial function. The missense variants found in Cases 1 and 2 led to partial loss of enzyme activity and a significant decrease in thermostability. More particularly, NAXD deficiency appears to have devastating effects in key tissues such as the brain, which are critically dependent on efficient energy metabolism and are exquisitely sensitive to abnormal metabolite accumulation. After 2-hydroxyglutaric aciduria (Van Schaftingen *et al.*, 2009) and NAXE deficiency (Kremer *et al.*, 2016), NAXD deficiency represents now the third known disorder of metabolite repair. We also suggest that NAXD deficiency should be included in the growing list of genetic disorders associated with fever-induced neurological deterioration (Powers and Scheld, 1996; Longo, 2003).

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

References

- Becker-Kettern J, Paczia N, Conrotte JF, Zhu C, Fiehn O, Jung PP, et al. NAD(P)HX repair deficiency causes central metabolic perturbations in yeast and human cells. *FEBS J* 2018; 285: 3376–401.
- Breslow DK, Cameron DM, Collins SR, Schuldiner M, Stewart-Ornstein J, Newman HW, et al. A comprehensive strategy enabling high-resolution functional analysis of the yeast genome. *Nat Methods* 2008; 5: 711–8.
- Colinas M, Shaw HV, Loubery S, Kaufmann M, Moulin M, Fitzpatrick TB. A pathway for repair of NAD(P)H in plants. *J Biol Chem* 2014; 289: 14692–706.
- Desmet FO, Hamroun D, Lalande M, Collod-Beroud G, Claustres M, Beroud C. Human splicing finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Res* 2009; 37: e67.
- Hillenmeyer ME, Fung E, Wildenhain J, Pierce SE, Hoon S, Lee W, et al. The chemical genomic portrait of yeast: uncovering a phenotype for all genes. *Science* 2008; 320: 362–5.
- Houtkooper RH, Canto C, Wanders RJ, Auwerx J. The secret life of NAD⁺: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* 2010; 31: 194–223.
- Kremer LS, Danhauser K, Herebian D, Petkovic Ramadza D, Piekutowska-Abramczuk D, Seibt A, et al. NAXE mutations disrupt the cellular NAD(P)HX repair system and cause a lethal neurometabolic disorder of early childhood. *Am J Hum Genet* 2016; 99: 894–902.
- Linster CL, Van Schaftingen E, Hanson AD. Metabolite damage and its repair or pre-emption. *Nat Chem Biol* 2013; 9: 72–80.
- Longo N. Mitochondrial encephalopathy. *Neurol Clin* 2003; 21: 817–31.
- Marbaix AY, Noel G, Detroux AM, Vertommen D, Van Schaftingen E, Linster CL. Extremely conserved ATP- or ADP-dependent enzymatic system for nicotinamide nucleotide repair. *J Biol Chem* 2011; 286: 41246–52.
- Marbaix AY, Tyteca D, Niehaus TD, Hanson AD, Linster CL, Van Schaftingen E. Occurrence and subcellular distribution of the NADPHX repair system in mammals. *Biochem J* 2014; 460: 49–58.
- Nichols RJ, Sen S, Choo YJ, Beltrao P, Zietek M, Chaba R, et al. Phenotypic landscape of a bacterial cell. *Cell* 2011; 144: 143–56.
- Niehaus TD, Richardson LG, Gidda SK, ElBadawi-Sidhu M, Meissen JK, Mullen RT, et al. Plants utilize a highly conserved system for repair of NADH and NADPH hydrates. *Plant Physiol* 2014; 165: 52–61.
- Philippakis AA, Azzariti DR, Beltran S, Brookes AJ, Brownstein CA, Brudno M, et al. The matchmaker exchange: a platform for rare disease gene discovery. *Hum Mutat* 2015; 36: 915–21.
- Powers JH, Scheld WM. Fever in neurologic diseases. *Infect Dis Clin North Am* 1996; 10(1): 45–66.
- Prabhakar P, Laboy JJ, Wang J, Budker T, Din ZZ, Chobanian M, et al. Effect of NADH-X on cytosolic glycerol-3-phosphate dehydrogenase. *Arch Biochem Biophys* 1998; 360: 195–205.
- Rafter GW, Chaykin S, Krebs EG. The action of glyceraldehyde-3-phosphate dehydrogenase on reduced diphosphopyridine nucleotide. *J Biol Chem* 1954; 208: 799–811.
- Robinson BH, Petrova-Benedict R, Buncic JR, Wallace DC. Nonviability of cells with oxidative defects in galactose medium: a screening test for affected patient fibroblasts. *Biochem Med Metab Biol* 1992; 48: 122–6.
- Sobreira N, Schiettecatte F, Valle D, Hamosh A. GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. *Hum Mutat* 2015; 36: 928–30.
- Spiegel R, Shaag A, Shalev S, Elpeleg O. Homozygous mutation in the APOA1BP is associated with a lethal infantile leukoencephalopathy. *Neurogenetics* 2016; 17: 187–90.
- Swalwell H, Kirby DM, Blakely EL, Mitchell A, Salemi R, Sugiana C, et al. Respiratory chain complex I deficiency caused by mitochondrial DNA mutations. *Eur J Hum Genet* 2011; 19: 769–75.
- Van Schaftingen E, Rzem R, Marbaix A, Collard F, Veiga-da-Cunha M, Linster CL. Metabolite proofreading, a neglected aspect of intermediary metabolism. *J Inherit Metab Dis* 2013; 36: 427–34.
- Van Schaftingen E, Rzem R, Veiga-da-Cunha M. L: -2-Hydroxyglutaric aciduria, a disorder of metabolite repair. *J Inherit Metab Dis* 2009; 32: 135–42.
- Ying W. NAD⁺/NADH and NADP⁺/NADPH in cellular functions and cell death: regulation and biological consequences. *Antioxid Redox Signal* 2008; 10: 179–206.
- Yoshida A, Dave V. Inhibition of NADP-dependent dehydrogenases by modified products of NADPH. *Arch Biochem Biophys* 1975; 169: 298–303.