**NMNAT1** mutations cause Leber congenital amaurosis

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Leber congenital amaurosis (LCA) is an infantile-onset form of inherited retinal degeneration characterized by severe vision loss1,2. Two-thirds of LCA cases are caused by mutations in 17 known disease-associated genes3 (Retinal Information Network (RetNet)). Using exome sequencing we identified a homozygous missense mutation (c.25G>A, p.Val9Met) in **NMNAT1** that is likely to be disease causing in two siblings of a consanguineous Pakistani kindred affected by LCA. This mutation segregated with disease in the kindred, including in three other children with LCA. **NMNAT1** resides in the previously identified LCA9 locus and encodes the nuclear isoform of nicotinamide mononucleotide adenyltransferase, a rate-limiting enzyme in nicotinamide adenine dinucleotide (NAD+) biosynthesis4,5. Functional studies showed that the p.Val9Met alteration decreased **NMNAT1** enzyme activity. Sequencing **NMNAT1** in 284 unrelated families with LCA identified 14 rare mutations in 13 additional affected individuals. These results are the first to link an **NMNAT1** isoform to disease in humans and indicate that **NMNAT1** mutations cause LCA.

Inherited retinal diseases, such as LCA, represent a heterogeneous group of early-onset blindness disorders that are characterized by progressive dysfunction and death of the rod and cone retinal photoreceptor cells6. Despite the identification so far of more than 180 different inherited retinal disease–associated genes, the genetic etiology remains uncertain in 40–50% of individuals with inherited retinal disease7 (RetNet). Additional loci have been identified at which disease-associated genes have not yet been identified, such as the LCA9 locus mapped to chromosome 1p36 (ref. 4). Identifying the genetic basis of inherited retinal diseases is essential to guide the development of potential therapies, as highlighted by the recent success of clinical gene therapy trials for RPE65-related LCA6–12. Here, we used whole-exome sequencing in a large consanguineous Pakistani family, including five children affected with LCA who did not have mutations in known LCA-causing genes.

Two Pakistani siblings, an 11-year-old girl and her 3-year-old brother (Fig. 1a, family 047, subjects IV-1 and IV-3, respectively), initially came to the Ophthalmology-Genetics Clinic at the Children’s Hospital of Philadelphia for evaluation of LCA because of severe vision impairment, congenital nystagmus and no detectable (<10 µV) retinal function by full-field electroretinography (ERG) testing in early infancy (see Supplementary Note for additional clinical details). Both children also had global developmental delay, nonverbal autism with stereotypes, hypotonia with joint hypermobility and dysmorphic faces. Severe-to-profound bilateral sensorineural hearing loss was present in the 11-year-old proband (IV-1) and in her 8-year-old brother (IV-2), who had a normal eye exam and normal development but exhibited clinical features consistent with mucolipidosis (Supplementary Note).

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Their parents were first cousins who were visually and developmentally normal (Fig. 1a, subjects III-4 and III-5). Notably, the parents’ siblings had married one another (Fig. 1a, subjects III-3 and III-6) and together had two children with similar vision and nonverbal autism phenotypes as the proband (subjects IV-7 and IV-8) and one child with isolated LCA (IV-6). Clinical genetic diagnostic testing identified a homozygous mutation in *GJB2* (c.71G>A, p.Trp24*) as the cause of sensorineural hearing loss in subject IV-1, but no mutation was identified in any of the known LCA-causing genes (Supplementary Note). Additional sequencing analyses verified that the homozygous mutation encoding p.Trp24* in *GJB2* segregated with the hearing loss phenotype in the larger kindred (Fig. 1a).

To search for the genetic cause of LCA in this family, we performed whole-exome sequencing of the nuclear family of the 11-year-old proband (Fig. 1a, subjects IV-7 and IV-8) and one child with isolated LCA (IV-6). Clinical genetic diagnostic testing identified a homozygous mutation in *GJB2* (c.71G>A, p.Trp24*) as the cause of sensorineural hearing loss in subject IV-1, but no mutation was identified in any of the known LCA-causing genes (Supplementary Note). Additional sequencing analyses verified that the homozygous mutation encoding p.Trp24* in *GJB2* segregated with the hearing loss phenotype in the larger kindred (Fig. 1a).

The genes harboring these four variants had known retinal expression, which was determined from mouse retina RNA sequencing (RNA-seq) analyses. Only one of these variants was predicted to damage protein function by SIFT, PolyPhen-2 and other programs: c.25G>A (p.Val9Met) in *NMNAT1*. (refs. 16–20) (NM_022787). Sanger sequencing of the c.25G>A variant in *NMNAT1* validated its segregation with the LCA phenotype in the original nuclear kindred and in the proband’s similarly affected cousins, including the one with isolated LCA (Fig. 1a,b). Only the mutant M1 allele encoding p.Val9Met NMNAT1 was detected in the five children with LCA in generation IV, whereas the four unaffected parents of these children in generation III carry both the mutant and wild-type alleles, and their three children with normal vision harbor only the wild-type allele (Fig. 1b). The p.Val9Met variant was not present in 501 controls or in any public databases.

No clearly pathogenic mutations were identified that were likely to be the cause of developmental delay, nonverbal autism, hypotonia and dysmorphic facies in family members IV-1, IV-3, IV-7 and IV-8. These presentations likely have a separate genetic etiology from that of LCA and deafness in this family, as individual IV-6 has LCA alone, individual IV-2 has deafness alone and additional NMNAT1 mutations were identified in individuals with non-syndromic LCA, as described below.
Table 1 Identified NMNAT1 mutations

| Ancestry or nationality | DNA Alterations | Protein Alterations | EVS | PolyPhen-2 | SIFT |
|------------------------|-----------------|---------------------|-----|------------|------|
| CHOP/MEEI              |                 |                     |     |            |      |
| LCA-047                 | c.250>G        | p.Val9Met            | Novel | PoD | D     |
| LCA-007                 | c.196>C>T      | p.Arg66Trp           | Novel | PrD | D     |
| LCA-053                 | c.709>C>T      | p.Arg237Cys          | 1/7,019 | PrD | D     |
| LVPEI                  |                 |                     |     |            |      |
| LCA-73                 | c.250>A       | p.Val9Met            | Novel | PoD | D     |
| LCA-79                 | c.98>A         | p.Asp33Gly           | Novel | PoD | D     |
| LCA-100                | c.709>C>T      | p.Arg237Cys          | Novel | PrD | D     |
| LCA-128                | c.215>T>A     | p.Leu72His           | Novel | PrD | D     |
| UCL                    |                 |                     |     |            |      |
| LCA-1                 | c.205>A>G     | p.Met69Val           | Novel | PrD | D     |
| LCA-2                 | c.679>G>A     | p.Glu257Lys          | 13/10,745 | B | T     |
| LCA-3                 | c.370>A       | p.Ala13Thr           | 1/10,757 | PrD | D     |
| LCA-4                 | c.723>delA    | p.Pro241Profs*45    | Novel | N/A | N/A   |
| LCA-5                 | c.597>A       | p.Ile20Asn           | 13/10,745 | B | T     |
| LCA-6                 | c.552>A>G     | p.Ile184Met          | Novel | PoD | D     |
| LCA-7                 | c.466>G>C     | p.Gly156Arg          | Novel | PrD | D     |
| CHOP, Children’s Hospital of Philadelphia; LVPEI, LV Prasad Eye Hospital; MEEI, Massachusetts Eye and Ear Infirmary; UCL, University College London.

aData from Exome Variant Server, number of times variant detected/number of exomes analyzed. PolyPhen-2 Hum-Var score: PoD, probably damaging; PoD, possibly damaging; B, benign. SIFT: D, damaging; T, tolerated; N/A, not applicable.

To determine whether NMNAT1 mutations cause LCA in other families, we sequenced NMNAT1 in 56 unrelated probands with LCA evaluated at The Children’s Hospital of Philadelphia (CHOP) and the Massachusetts Eye and Ear Infirmary (MEEI). We found rare compound heterozygous mutations in NMNAT1 that segregated with disease in family 007, in which the proband was a 5-year-old girl with isolated LCA and there was no family history of the disease (Fig. 1c and Table 1). We also identified compound heterozygous variants in NMNAT1 that segregated with disease in family 053, in which the proband was a 20-year-old man with LCA (Fig. 1d and Table 1). The four NMNAT1 variants identified in these subjects were not identified in 501 control samples.

To investigate the frequency of NMNAT1 mutations in LCA, we screened additional populations of affected individuals from varying ancestry groups. This analysis included 228 additional probands ascertained at Institut de la Vision in Paris, LVPEI in India and University College London (UCL). These analyses identified homozygous or compound heterozygous mutations in NMNAT1 in 11 additional families with LCA (Table 1 and Supplementary Fig. 2). All of the mutations detected are rare, and all, with the exception of one encoding the p.Glu257Lys variant, were predicted to be damaging by PolyPhen-2 and/or SIFT (Table 1). None of the NMNAT1 variants identified in these subjects were identified by Sanger sequencing in 501 control samples. Review of available clinical information for individuals in whom NMNAT1 mutations were identified as the cause of LCA (Supplementary Note) indicated that the majority have atrophic macular lesions (Fig. 2).

NMNAT1 encodes a rate-limiting enzyme that generates NAD+ from nicotinic acid mononucleotide (NaMN) and in a biosynthetic pathway from nicotinamide mononucleotide (NMN) (Supplementary Fig. 3). Three functionally nonredundant mammalian NMNAT isoforms encoded by different genes have been identified within distinct cellular compartments, with NMNAT1, NMNAT2 and NMNAT3 localizing to the nucleus, Golgi complex and mitochondria, respectively. The mitochondrial isoform, NMNAT3, regenerates NAD+ for cellular energetics, whereas NMNAT1 is involved in the nuclear NAD+ homeostasis that is necessary for both DNA metabolism and cell signaling. Of interest, Nmnat1 is the principal component of the mouse Wallerian degeneration fusion protein (Wld), which also includes a 70-residue N-terminal sequence from the Ube4b multiquitination factor, and has been shown to have neuroprotective activity. Homozygous Nmnat1-knockout mice are embryonic lethal, whereas heterozygous Nmnat1-knockout mice have normal development. Loss of nmnat in Drosophila melanogaster photoreceptors leads to photoreceptor cell degeneration.

Figure 2 Retinal image from individual with LCA due to mutations in NMNAT1. Composite fundus image of the right eye of subject II-1 from LVPEI family LCA-100, showing pallor of the optic disc, attenuation of the retinal blood vessels, pigment disruption, atrophic changes in the macula (arrow) and scattered pigment clumping in the peripheral retina. The optic disc is ~1.75 mm in diameter.
We assessed the potential deleterious effects of the novel missense variants p.Val9Met, p.Arg66Trp and p.Arg237Cys on the NMNAT1 protein. These altered residues are located in conserved regions of the protein (Supplementary Fig. 4) and are predicted to damage NMNAT1 protein structure and stability by several prediction programs\(^{21,27}\). All three of these mutant proteins showed correct nuclear localization and normal expression levels following expression of recombinant NMNAT1 proteins in heterologous cells (Supplementary Fig. 5a,b). In addition, the p.Val9Met mutant correctly localized to the nucleus of a fibroblast cell line obtained from the proband with LCA in family 047 (Fig. 1a, subject IV-1, and Supplementary Fig. 5c)\(^{22}\).

Given the normal nuclear localization and expression of the mutant NMNAT1 proteins, we postulated that the deleterious effect of the p.Val9Met, p.Arg66Trp and p.Arg237Cys variants might be on NMNAT1 enzymatic function. We therefore measured the NAD\(^+\) biosynthetic activity of wild-type and mutant purified recombinant NMNAT1 proteins (Fig. 3a). Despite variability in enzyme rates between experimental days, the protein activity of the NMNAT1 p.Val9Met variant was reproducibly and significantly lower than that of wild-type protein on the same day (63.4% median reduction, interquartile range 31.4–88.7; Wilcoxon rank-sum test \(P = 0.0015\)). The enzyme activity of the p.Arg66Trp mutant was also significantly lower (99.5% median reduction, interquartile range 0.01–0.11; Wilcoxon rank-sum test \(P = 0.0014\)), although we were not able to achieve effective purification of the Flag-tagged version of this mutant (Supplementary Fig. 6), despite its clearly normal expression and nuclear localization in CHO and mIMCD3 cells (Supplementary Fig. 5). The enzyme activity of the p.Arg237Cys mutant was only marginally lower than that of wild-type protein (18.9% reduction, interquartile range 41.1–90.1; Wilcoxon rank-sum test \(P = 0.034\)) (Fig. 3a), raising the question of how this mutation causes disease. It has been observed that NMNAT1 forms functional homo-oligomers and that amino acids 234–238 participate in these protein interactions\(^5\). To evaluate whether the pathogenic effect of the p.Arg237Cys alteration could be related to its location in a region of NMNAT1 that is involved in protein multimerization, we measured NMNAT1 activity in recombinant protein purified from cells cotransfected with constructs for both the p.Arg66Trp and p.Arg237Cys variants that were identified in family 007. We observed notably lower enzyme activity (18% of wild-type control rate; data not shown) in the combined protein preparation. Additional studies using extracts from the fibroblast cells of the proband with the p.Val9Met alteration (Fig. 1a, subject IV-1) showed 73% lower total cellular NMNAT enzyme activity (two-tailed \(t\) test \(P = 0.016\)) relative to wild-type control (Fig. 3b). These data suggest that the pathogenic effects of these mutations are related, at least in part, to significantly reduced NMNAT1 enzyme activity. It will be of interest to investigate the function of the mutant NMNAT1 proteins in retinal cells, given the isolated retinal phenotype of LCA.

The total NAD\(^+\) concentration of human cells has many contributing determinants\(^{21,28}\). We measured NAD\(^+\) in the fibroblast cell line from the proband with LCA (Fig. 1a, subject IV-1) to determine whether the p.Val9Met alteration in the nuclear-localized NMNAT1 protein significantly affected total cellular NAD\(^+\) content. Fibroblasts from the proband with LCA had 16% less NAD\(^+\) content than wild-type controls, although this difference was not statistically significant (two-tailed \(t\) test \(P = 0.067\); Fig. 3c). These data suggest that the reduction in NMNAT1 enzyme activity caused by the p.Val9Met alteration may be sufficient to affect total cellular NAD\(^+\) content.

Cellular NAD\(^+\) concentrations can be directly increased by nicotinic acid, which requires NMNAT activity for its conversion to NAD\(^+\) (Supplementary Fig. 3)\(^{29}\). We therefore asked whether cellular NAD\(^+\) concentration in the fibroblasts expressing the NMNAT1 p.Val9Met mutant (from subject IV-1) was altered by exposure to 10 mM nicotinic acid for 24 h. Notably, whereas nicotinic acid significantly increased the total cellular NAD\(^+\) content by 53% in control cells (two-tailed \(t\) test \(P = 0.021\)), it had no effect on NAD\(^+\) content in the fibroblasts from the proband with LCA (Fig. 3c; two-tailed \(t\) test \(P > 0.05\)). The inability of nicotinic acid to increase the NAD\(^+\) content in NMNAT1 p.Val9Met mutant fibroblasts provides further evidence that they have a substantial deficiency in cellular NMNAT enzymatic activity.

In summary, we report here the first instance of disease association with an NMNAT isoform. NMNAT1 mutations cause LCA and are the likely pathogenic basis for disease previously linked to the LCA9 locus, although the family in which disease was originally linked to this locus was not available for analysis in this study\(^4\) (C. Toomes and C. Inglehearn, personal communication). Through exome sequencing in a consanguineous Pakistani kindred with LCA and subsequent Sanger sequencing of NMNAT1 in 284 additional unrelated probands with LCA, we identified mutations in 14 unrelated...
families (14/285 = 4.9% of unrelated cases). This work suggests that mutations in *NMNAT1* are a relatively common cause of LCA3. However, because the cohorts of individuals used for these studies are enriched for subjects without mutations in known LCA-causing genes, the proportion of all LCA cases caused by *NMNAT1* mutations is likely to be overestimated by these data.

The identification of *NMNAT1* as an LCA-causing gene raises the intriguing question of how mutations in a widely expressed NAD+ biosynthetic protein lead to a retina-specific phenotype. The data presented suggest that the retinal degeneration phenotype observed in individuals with *NMNAT1* mutations results from decreased NAD+ biosynthetic activity. This hypothesis is consistent with findings from studies of the *Wld* protein in mice, which showed that the neuroprotective effect of the *Wld* protein required both the Ube4b component and an enzymatically active *NMNAT1* portion of the chimeric protein10. However, it seems that, in some systems, such as *Drosophila*, *nmnat* alone has a neuroprotective role that may be independent of its NAD+ biosynthetic activity25,31. Thus, it remains to be determined whether retinal degeneration caused by mutations in *NMNAT1* results primarily from the loss of a potentially novel neuroprotective effect of *NMNAT1* or a previously unappreciated role of NAD+-mediated signaling in retinal health and disease. In either case, *NMNAT1* mutations represent a new pathophysiological cause of LCA, further underscoring the genetic heterogeneity of inherited retinal diseases3,5. We postulate that pharmacologic and/or genetic therapies directed at restoring cellular NAD+ homeostasis in retinal cells may offer a therapeutic strategy for *NMNAT1*-related LCA.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Exome sequence data for family 047 is available at the NCBI Sequence Read Archive (SRA) under accession SRP013517.

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**AUTHOR CONTRIBUTIONS**

Experiments were designed by Q.Z., M.J.F., X.G. and E.A.P. LCA case samples and controls were provided by I.A., A.D.B., E.L.B., S.S.B., C.K., M.J.F., S.J., A.T.M., E.P., S.M.-S., J.-A.S., A.R.W. and E.A.P. Pedigrees were compiled by A.D.B., C.C., C.K., S.J. and E.P. Individuals III-4, III-5, IV-1, IV-2 and IV-3 were clinically evaluated by M.I.J. and E.A.P. (individuals III-3, III-6 and IV-4 to IV-7 were not clinically evaluated by the authors). Exome sequencing was performed by M.C. Bioinformatics pipeline development was performed by X.G., M.J.F. and E.A.P. and M.C. Exome data analyses were performed by J.C.P., X.G., Z.F.-K. and E.A.P. *NMNAT1* sequencing and segregation analyses were performed by I.A., C.C., M.C., Z.F.-K., D.S.M., L.P., R.S., N.H.W., C.Z. and Q.Z. High-performance liquid chromatography (HPLC)-based *NMNAT* enzyme activity assay and NAD+ content assay development was performed by E.N.-O., with data analysis performed by E.N.-O., M.J.F., E.A.P. and R.X. In vitro functional studies were carried out by Q.Z., E.N.-O., J.O., Q.L. and M.S. The manuscript was written by M.J.F., Q.Z., X.G. and E.A.P.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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1. Weleber, R.G. Infantile and childhood retinal blindness: a molecular perspective. *Ophthalimic Genet.* 23, 71–97 (2002).
2. Michaelides, M., Hardcastle, A.J., Hunt, D.M. & Moore, A.T. Progressive cone and cone-rod dystrophies: phenotypes and underlying molecular genetic basis. *Surv. Ophthalomol.* 51, 232–258 (2006).
3. den Hollander, A.I., Black, A., Bennett, J. & Cremers, F.P. Lighting a candle in the dark: advances in genetics and gene therapy of recessive retinal dystrophies. *J. Clin. Invest.* 120, 3042–3053 (2010).
4. Keen, T.J. et al. Identification of a locus (LCA9) for Leber’s congenital amaurosis on chromosome 1p36. *Eur. J. Hum. Genet.* 11, 420–423 (2003).
5. Lau, C., Niere, M. & Ziegler, M. The NMN/NaMN adenylyltransferase (NMNAT) protein family. *Front. Biosci.* 14, 410–431 (2009).
6. Pierce, E.A. Pathways to photoreceptor cell death in inherited retinal degenerations. *Bioessays* 23, 605–618 (2001).
7. Daiger, S.P., Bowne, S.J. & Sullivan, L.S. Perspective on genes and mutations causing retinitis pigmentosa. *Arch. Ophthalomol.* 125, 151–158 (2007).
8. Maguire, A.M. et al. Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. *Proc. Natl. Acad. Sci. USA* 105, 15112–15117 (2008).
9. Maguire, A.M. et al. Age-dependent effects of RPE65 gene therapy for Leber’s congenital amaurosis: a phase 1 dose-escalation trial. *Lancet* 374, 1597–1605 (2009).
10. Jacobson, S.G. et al. Gene therapy for Leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years. *Arch. Ophthalomol.* 130, 9–24 (2012).
11. Sherry, S.T. dbSNP: the NCBI database of genetic variation. *Nature Genet.* 23, 410–431 (2009).
12. Jacobson, S.G. et al. Gene therapy for Leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years. *Arch. Ophthalomol.* 130, 9–24 (2012).
13. Berry, J.M. et al. Identification of a locus (LCA9) for Leber’s congenital amaurosis on chromosome 1p36. *Eur. J. Hum. Genet.* 11, 420–423 (2003).
14. Keen, T.J. et al. Identification of a locus (LCA9) for Leber’s congenital amaurosis on chromosome 1p36. *Eur. J. Hum. Genet.* 11, 420–423 (2003).
15. Lau, C., Niere, M. & Ziegler, M. The NMN/NaMN adenylyltransferase (NMNAT) protein family. *Front. Biosci.* 14, 410–431 (2009).
16. Pierce, E.A. Pathways to photoreceptor cell death in inherited retinal degenerations. *Bioessays* 23, 605–618 (2001).
17. Daiger, S.P., Bowne, S.J. & Sullivan, L.S. Perspective on genes and mutations causing retinitis pigmentosa. *Arch. Ophthalomol.* 125, 151–158 (2007).
18. Maguire, A.M. et al. Safety and efficacy of gene transfer for Leber’s congenital amaurosis. *N. Engl. J. Med.* 358, 2240–2248 (2008).
19. Bainbridge, J.W. et al. Effect of gene therapy on visual function in Leber’s congenital amaurosis. *N. Engl. J. Med.* 358, 2231–2239 (2008).
20. Cideciyan, A.V. et al. Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. *Proc. Natl. Acad. Sci. USA* 105, 15112–15117 (2008).
21. Maguire, A.M. et al. Age-dependent effects of RPE65 gene therapy for Leber’s congenital amaurosis: a phase 1 dose-escalation trial. *Lancet* 374, 1597–1605 (2009).
22. Jacobson, S.G. et al. Gene therapy for Leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years. *Arch. Ophthalomol.* 130, 9–24 (2012).
23. Berry, J.M. et al. Identification of a locus (LCA9) for Leber’s congenital amaurosis on chromosome 1p36. *Eur. J. Hum. Genet.* 11, 420–423 (2003).
24. Keen, T.J. et al. Identification of a locus (LCA9) for Leber’s congenital amaurosis on chromosome 1p36. *Eur. J. Hum. Genet.* 11, 420–423 (2003).
18. Sullivan, L.S. et al. Prevalence of disease-causing mutations in families with autosomal dominant retinitis pigmentosa: a screen of known genes in 200 families. Invest. Ophthalmol. Vis. Sci. 47, 3052–3064 (2006).

19. Stone, E.M. Leber congenital amaurosis—a model for efficient genetic testing of heterogeneous disorders: LXIV Edward Jackson Memorial Lecture. Am. J. Ophthalmol. 144, 791–811 (2007).

20. Wang, M. & Marín, A. Characterization and prediction of alternative splice sites. Gene 366, 219–227 (2006).

21. Belenky, P., Bogan, K.L. & Brenner, C. NAD+ metabolism in health and disease. Trends Biochem. Sci. 32, 12–19 (2007).

22. Berger, F., Lau, C., Dahlmann, M. & Ziegler, M. Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms. J. Biol. Chem. 280, 36334–36341 (2005).

23. Coleman, M.P. & Freeman, M.R. Wallerian degeneration, wld+, and nmnat. Annu. Rev. Neurosci. 33, 245–267 (2010).

24. Conforti, L. et al. Reducing expression of NAD+ synthesizing enzyme NMNAT1 does not affect the rate of Wallerian degeneration. FEBS J. 278, 2666–2679 (2011).

25. Zhai, R.G. et al. Drosophila NMNAT maintains neural integrity independent of its NAD synthesis activity. PLoS Biol. 4, e416 (2006).

26. Siepel, A. et al. Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. Genome Res. 15, 1034–1050 (2005).

27. Worth, C.L., Preissner, R. & Blundell, T.L. SDM—a server for predicting effects of mutations on protein stability and malfunction. Nucleic Acids Res. 39, W215–W222 (2011).

28. Bogan, K.L. & Brenner, C. Nicotinic acid, nicotinamide, and nicotinamide riboside: a molecular evaluation of NAD+ precursor vitamins in human nutrition. Annu. Rev. Nutr. 28, 115–130 (2008).

29. Nikiforov, A., Dölle, C., Niere, M. & Ziegler, M. Pathways and subcellular compartmentation of NAD biosynthesis in human cells; from entry of extracellular precursors to mitochondrial NAD generation. J. Biol. Chem. 286, 21767–21778 (2011).

30. Conforti, L. et al. Wld5 protein requires Nmnat activity and a short N-terminal sequence to protect axons in mice. J. Cell Biol. 184, 491–500 (2009).

31. Avery, M.A., Sheehan, A.E., Kerr, K.S., Wang, J. & Freeman, M.R. Wld5 requires Nmnat1 enzymatic activity and N16-VCP interactions to suppress Wallerian degeneration. J. Cell Biol. 184, 501–513 (2009).

32. Berger, F., Lau, C. & Ziegler, M. Regulation of poly(ADP-ribose) polymerase 1 activity by the phosphorylation state of the nuclear NAD biosynthetic enzyme NMN adenylyltransferase 1. Proc. Natl. Acad. Sci. USA 104, 3765–3770 (2007).

33. den Hollander, A.I., Roepman, R., Koenekoop, R.K. & Cremers, F.P. Leber congenital amaurosis: genes, proteins and disease mechanisms. Prog. Retin. Eye Res. 27, 391–419 (2008).

34. Chun, S. & Fay, J.C. Identification of deleterious mutations within three human genomes. Genome Res. 19, 1553–1561 (2009).

35. Wei, Q., Wang, L., Wang, Q., Kruger, W.D. & Dunbrack, R.L. Jr. Testing computational prediction of missense mutation phenotypes: functional characterization of 204 mutations of human cystathionine β synthase. Proteins 78, 2058–2074 (2010).
Cell culture. CHO-K1 and wild-type mIMCD3 cell lines were purchased from the American Type Culture Collection (ATCC). mIMCD3 cells were maintained in DMEM:F12 medium supplemented with 10% FBS and 0.5 mM sodium pyruvate. CHO cell culture was performed in F12 medium supplemented with 10% FBS. Transfection was performed with Lipofectamine 2000 (Invitrogen), and cells were processed for immunocytochemistry 48–72 h after transfection. Human skin fibroblast cells obtained from two siblings with LCA (Fig. 1a, subjects IV-1 and IV-3) and their parents (Fig. 1a, subjects III-IV and III-V) were maintained in Medium 106 (Invitrogen) with low-serum growth supplement and were grown to confluence before undergoing immunofluorescence and immunoblot analyses.

Immunofluorescence analyses. Cells were fixed in 4% paraformaldehyde, permeabilized and then blocked with 1% BSA and 0.2% Triton X-100 in PBS, as previously described40. Cells were then stained with antibody to V5 (Invitrogen, 46-0705; 1:1,000 dilution) and then with Alexa Fluor 555–conjugated goat secondary antibody to mouse IgG (Invitrogen, A21127; 1:1,000 dilution)40. Fluorescent signals were visualized using a Nikon Eclipse fluorescent microscope.

Recombinant protein production and purification. Human NMNAT1 cDNA was amplified by RT-PCR from a cDNA clone (OpenBiosystem) and cloned into a pET30a vector (Novagen) with a hexahistidine N-terminal tag. Recombinant protein was expressed in BL21 cells (Novagen) and purified using a HisTrap FF column (GE Healthcare). Antibody binding was detected with an Odyssey infrared imager (LI-COR). The coding sequence was moved by recombination to a Gateway-compatible destination expression vector modified to encode a C-terminal His6 tag and was amplified in E. coli DH5α. Recombinant NMNAT1 (with Flag tag) was expressed in CHO cells and was purified using a FPLC M purification kit (Sigma) for subsequent enzyme activity assay.

NMNAT enzyme activity assay. NMNAT activity was measured by HPLC quantitation of the reaction product, NAD⁺. The assay mixture contained 1.5 mM ATP, 1 mM NMN and 10 mM MgCl₂, in 25 mM Tris-HCl (pH 7.4) and the appropriate amount of enzyme sample (typically 20 or 40 µl) to achieve a final volume of 0.2 ml. The reaction was started by addition of NMN substrate. After incubation of the reaction mixture for 10, 30 or 120 min at 37 °C, a 40-µl aliquot was removed and added to 20 µl of ice-cold 1.2 M perchloric acid (PCA) containing 20 mM EDTA and 0.15% sodium metabisulfite to stop the reaction. After a 15-min incubation at 4 °C, the mixture was centrifuged for 10 min at 16,000g in a Beckman microcentrifuge. A 55-µl aliquot of the supernatant was further neutralized by the addition of 20 µl of ice-cold 1 M K₂CO₃ and was centrifuged again. The supernatant was isolated and stored at −80 °C until HPLC analysis.

Sample preparation for HPLC analyses of NAD⁺. Harvested cells were rinsed with Hank’s balanced salt solution twice and were centrifuged at 2,150g for 5 min. The cell pellet was resuspended in argon-bubbled 20 mM Tris-HCl (pH 7.4) for analysis of the oxidized dinucleotides, including NAD⁺. The cell suspension was extracted with four volumes of argon-bubbled, ice-cold 1.2 M PCA containing 20 mM EDTA and 0.15% sodium metabisulfite. After vortexing, the suspension was placed on ice for 15 min and then centrifuged at 16,000g for 10 min. The supernatant was neutralized with 1 M potassium carbonate and was centrifuged to remove insoluble material. The pellet from the PCA extraction was used for protein estimation. Samples were stored at −80 °C and were subjected to HPLC analysis.
HPLC conditions for analyses of NAD⁺. Separation of the oxidized dinucleotides was carried out on a C18 column (5 µm, 4.6 × 250 mm, Adsorbosphere XL C18 90Å) preceded by a guard column at 40 °C. Flow rate was set at 0.5 ml/min. The mobile phase was initially 100% of mobile phase A (0.1 M sodium phosphate buffer (pH 6.0) containing 3.75% methanol). The methanol concentration was linearly increased with mobile phase B (0.1 M sodium phosphate buffer (pH 6.0) containing 30% methanol), increasing to 50% over 15 min. The column was washed after each separation by increasing mobile phase B to 100% for 5 min. UV absorbance was monitored at 260 and 340 nm with Shimadzu SPD-M20A. Pertinent peak areas were integrated using LabSolution software from Shimadzu and were quantified using standard curves.

Statistical analyses. For comparison of the activity rates of purified recombinant NMNAT1 proteins with that of wild-type protein, rates were normalized by the mean rate of the wild-type protein analyzed on the same day to account for variation in the absolute enzyme activity rates on different analysis dates. The significance of differences between groups was evaluated using a nonparametric Wilcoxon rank-sum test in SAS 4.3 because of skewness observed in the data and small sample size. For measurements of cellular NMNAT activity and NAD⁺ concentrations, statistical comparisons between groups were performed using Student’s two-tailed t-tests.

36. Gnirke, A. et al. Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. Nat. Biotechnol. 27, 182–189 (2009).
37. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760 (2009).
38. Li, H. et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079 (2009).
39. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Royal Stat. Soc. B (Methodological) 57, 289–300 (1995).
40. Davis, E.E. et al. TTC21B contributes both causal and modifying alleles across the ciliopathy spectrum. Nat. Genet. 43, 189–196 (2011).