Bi-allelic Mutations in *NADSYN1* Cause Multiple Organ Defects and Expand the Genotypic Spectrum of Congenital NAD Deficiency Disorders

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Birth defects occur in up to 3% of all live births and are the leading cause of infant death. Here we present five individuals from four unrelated families, individuals who share similar phenotypes with disease-causal bi-allelic variants in *NADSYN1*, encoding NAD synthetase 1, the final enzyme of the nicotinamide adenine dinucleotide (NAD) *de novo* synthesis pathway. Defects range from the isolated absence of both kidneys to multiple malformations of the vertebrae, heart, limbs, and kidney, and no affected individual survived for more than three months postnatally. NAD is an essential coenzyme for numerous cellular processes. Bi-allelic loss-of-function mutations in genes required for the *de novo* synthesis of NAD were previously identified in individuals with multiple congenital abnormalities affecting the heart, kidney, vertebrae, and limbs. Functional assessments of *NADSYN1* missense variants, through a combination of yeast complementation and enzymatic assays, show impaired enzymatic activity and severely reduced NAD levels. Thus, *NADSYN1* represents an additional gene required for NAD synthesis during embryogenesis, and *NADSYN1* has bi-allelic missense variants that cause NAD deficiency-dependent malformations. Our findings expand the genotypic spectrum of congenital NAD deficiency disorders and further implicate mutation of additional genes involved in *de novo* NAD synthesis as potential causes of complex birth defects.

Nicotinamide adenine dinucleotide (NAD) is an essential metabolite functioning as a coenzyme in over 400 cellular redox reactions,[1] and it has key signaling roles in mitochondrial function and metabolism, DNA repair, cell division, immune response and inflammation, circadian rhythm, protein-protein signaling, and epigenetics.[2] Mammalian cells lack the ability to import NAD, and so it must be synthesized *de novo* from L-tryptophan via the kynurenine pathway or from dietary vitamin B3; "vitamin B3" collectively refers to the NAD precursors nicotinamide, nicotinic acid, and nicotinamide riboside (NR).[2,3] These precursors can be converted to NAD through the salvage pathway, contributing to the vast majority of the reserve NAD pool in postnatal tissue.[4] Although the relative contribution of *de novo* and salvage-pathway-converted NAD levels during embryogenesis is currently unknown, NAD deficiency caused by *de novo* pathway perturbation during embryogenesis manifests in defective development of the vertebrae, heart, kidney, palate, and limbs.[5] Recessive loss-of-function (LoF) mutations in two genes that encode sequential enzymes of the *de novo* pathway (*HAAO* [MIM: 617660] and *KYNU* [MIM: 617661]) were identified in individuals with NAD deficiency causing defects of the heart, kidney, and vertebrae. Given the lack of genetic redundancy of *HAAO* and *KYNU*, individuals with *de novo* pathway blockage due to defects in other nonredundant genes might also manifest NAD deficiency causing Congenital NAD Deficiency Disorder.

Here we report five individuals from four unrelated families (identified via GeneMatcher[6]) with deleterious bi-allelic variants in NAD synthetase 1 (*NADSYN1*; HGNC: 29832), a gene which encodes the final enzyme of the *de novo* pathway. Individuals presented with multiple overlapping congenital malformations reminiscent of Congenital NAD Deficiency Disorder (Table 1). In Family 1 (F1), individuals F1.II.1 and F1.II.2 were siblings, born to nonconsanguineous parents (Figure 1). These individuals are homozygous for a missense variant in *NADSYN1* (RefSeq accession number NM_018161.5) (c.1717G>A [p.Ala573Thr]), and in Family 2 (F2), individual F2.II.1 harbors one copy of this variant and an additional truncating variant (c.1819del [p.Val607Trpfs*30]). In Family 3 (F3), individual F3.II.4 was the fourth pregnancy, following the birth of a healthy son (who was age 5 years at the time of the study), a

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Table 1. Clinical Summary of Individuals with *NADSYN1* Mutations

Individual [Variant]	Gender	Gestation at Birth	Birth Weight (g)	Vertebral	Cardiac	Renal	Limb	Other
Family 1								
II.1 c.1717G>A (p.Ala573Thr) and c.1717G>A (p.Ala573Thr)	Male	38 w, 2 d	2,665	thoracic vertebral defect	hypoplastic mitral valve with borderline hypoplastic left ventricle, small bicuspid aortic valve and tubular hyperplasia of the left aortic arch with coarctation, anomalous origin of the left coronary artery from the right pulmonary artery	absent left kidney	bilateral shortening of humeri and femora	sacral dimple
II.2 c.1717G>A (p.Ala573Thr) and c.1717G>A (p.Ala573Thr)	Female	23 w, 4 d	410	multiple segmentation and formation defects of the thoracic, lumbar, and sacral spine [a]	absent left ventricle and pulmonary trunk, right ventricular outlet to the aorta	bilateral hypoplastic kidneys	short humeri and femora	nil
Family 2								
II.1 c.1717G>A (p.Ala573Thr) and c.1819del (p.Val607Trpfs*30)	Male	39 w, 5 d	3,380	scoliosis with multiple malformed vertebral anomalies and rib abnormalities	DORV, TGA in side by side orientation, doubly committed VSD, bidirectional PDA, left aortic arch	mild hyperechoic renal cortex	birth length: 45.5 cm, short proximal long bones, bowing of lower extremities	closed sacral dimple with tuft of hair
Family 3								
II.4 c.145T>C (p.Cys49Arg) and c.395G>T (p.Trp132Leu)	Male	TOP 16 w	na	na	na	oligohydramnios, bilateral renal agenesis	na	na
Family 4								
II.1 c.735T>A (p.Cys245*) and c.1839C>G (p.Tyr613*)	Male	TOP 16 w	na	na	na	left renal and ureter agenesis	micromelia, bilateral club feet	hydrocephalus, small thorax, echogenic bowel, flat nose and low set ears, edema, polysplenia, pulmonary hypoplasia

d—days, DORV—double outlet right ventricle, PDA—patent ductus arteriosus, na—not assessed, nil—no abnormality detected, TGA—transposition of the great arteries, TOP—termination of pregnancy, w—weeks, VSD—ventricular septal defect[a]See Figure S2

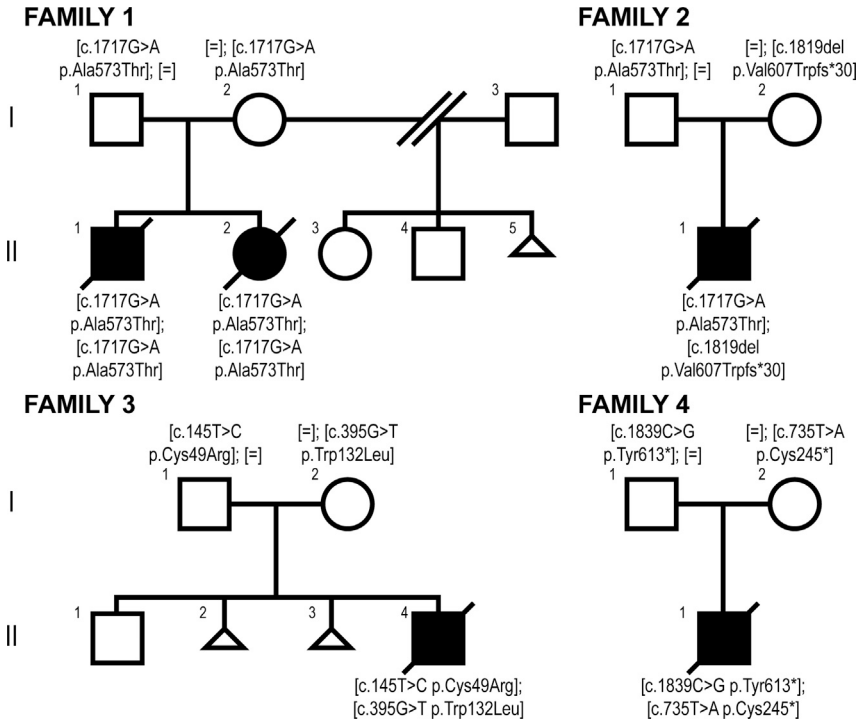

Figure 1. Pedigrees of Four Unrelated Families Harboring Pathogenic Bi-Allelic *NADSYN1* Mutations in Individuals with Congenital NAD Deficiency Disorder
Phenotypes of affected individuals are presented in Table 1 and the Supplemental Note. In Family 3, F3.II.2 was a blighted ovum, while F3.II.3 was a first-trimester miscarriage.

first-trimester miscarriage, and a blighted ovum; individual II.4 carries two *NADSYN1* missense variants (c.145T>C [p.Cys49Arg] and c.395G>T [p.Trp132Leu]). In Family 4 (F4), individual F4.II.1 carries two truncating variants (c.735T>A [p.Cys245*] and c.1839C>G [p.Tyr613*]). All variants are ranked >20 by CADD-PHRED,[7] placing them in the top 1% of deleterious variants, and all except for p.Cys49Arg are predicted pathogenic by PolyPhen-2 HVAR[8] (Table 2). All affected residues are highly conserved, as reflected in GERP[9] rejected substitution scores >4, which are suggestive of intolerance to mutation. Finally, all variants are rare with respect to healthy individuals, and none of them are present in the homozygous state in gnomAD.[10]

All individuals exhibited renal abnormalities (5/5) (Table 1) ranging from a mild hyperechoic renal cortex to the complete absence of both kidneys. The limbs were the next most commonly perturbed (4/5) with individuals exhibiting short proximal long bones or micromelia. Equally common were cardiac (3/5) and vertebral abnormalities (3/5). Vertebral defects included scoliosis with multiple malformed vertebrae and ribs, and segmentation defects of the thoracic, lumbar, and sacral spine. The heart was either hypoplastic due to underdevelopment of the left ventricle or compromised by malformation of the aorta and pulmonary artery. Extra abnormalities were also apparent (Table 1). Due to the severity of their phenotypes, individuals F3.II.4 and F4.II.1 were terminated at 16 weeks of gestation, while neither individual F1.II.1, F1.II.2, nor F2.II.1 survived more than three months postnatally. Complete phenotypes of *NADSYN1* variant carriers and their respective genetic assessments are presented in the Supplemental Note (see Supplemental Information).

The pathway for the *de novo* synthesis of NAD from tryptophan is largely conserved from bacterium to human. In the case of vertebrate NADSYN1, yeast orthologous protein Qns1 (Qns1p) functions equivalently as the final enzyme in this pathway, converting the precursor nicotinic acid adenine dinucleotide (NaAD) to NAD. Qns1p shares 58% amino acid sequence identity with human NADSYN1 and similarly catalyzes NAD generation through a coupled glutaminase-NAD synthetase fused-domain system. In addition, the rat homolog of NADSYN1, sharing 86% homology with the human sequence, can rescue function of inactivated Qns1p in yeast.[12] Therefore, we functionally assessed human *NADSYN1* variants via a genetic complementation approach in yeast. *QNS1* is an essential gene in yeast, however, metabolic block as a result of gene inactivation may be overcome through exogenous supplementation of NR.[13] Therefore, we first generated a haploid yeast line by replacing *QNS1* with a geneticin resistance cassette, as previously described,[14] sustained on media supplemented with NR (Supplemental Methods). *Δqns1* yeast were subsequently transformed with plasmids expressing HA-tagged human wild-type (WT) *NADSYN1*, *NADSYN1 Ala573Thr* mutant, or untagged *HAAO* as a control.

Both WT and mutant enzymes were detected via immunoblot in lysates of *Δqns1* yeast cultured for 48 h (Figure 2A and B). Protein was generated by both yeast lines at equivalent amounts. To next assess the capacity of human NADSYN1 to compensate for loss of Qns1p, *Δqns1* yeast generating WT NADSYN1 (*Δqns1* WT) were grown in liquid cultures in the absence of NR for 48 h (Figure 2C). *Δqns1* WT yeast grew equivalently well in the presence or absence of NR (Figure S1); this result suggests that human NADSYN1 expression rescues loss of Qns1p as efficiently as does bypassing the metabolic block via supplementation with NR. Similar growth to *Δqns1* WT was seen for *Δqns1* yeast generating the NADSYN1 p.Ala573Thr (*Δqns1* p.Ala573Thr) mutant in supplemented conditions (Figure S1). However, in the absence of NR, *Δqns1* p.Ala573Thr yeast grew at a compromised rate, a result significantly different from that for *Δqns1* WT (Figure 2C). The control *Δqns1* yeast generating HAAO (*Δqns1* HAAO) did not grow in the absence of NR supplementation.

Table 2. *NADSYN1* Variants Identified in Affected Individuals and Their Predicted Pathogenicity

Individual	cDNA Change	Protein Change	PolyPhen-2 HVAR[11]	CADD-PHRED[7]	GERP++[9]	Population Frequency (with Respect to gnomAD[10])
F1.II.1, F1.II.2, F2.II.1	1717G>A	Ala573Thr	1	24.3	4.83	7.40E-04
F2.II.1	1819del	Val607Trpfs*30	na	23	na	7.07E-06
F3.II.4	145T>C	Cys49Arg	0.553	33	5.63	7.97E-06
F3.II.4	395G>T	Trp132Leu	0.989	24.5	4.95	4.65E-04
F4.II.1	735T>A	Cys245*	na	35	na	Novel
F4.II.1	1839C>G	Tyr613*	na	41	na	Novel

PolyPhen-2 HVAR score ≥ 0.909—probably damaging; 0.908 ≤ score ≤ 0.447—possibly damaging; score ≤ 0.446—benign; CADD-PHRED scaled CADD score ≥ 15—damaging; GERP++, >—evolutionarily constrained; na—not applicable

To determine if NAD deficiency is the cause of altered growth between *Δqns1* WT or p.Ala573Thr yeast, we compared total NAD (NAD^+ and NADH)[15] in cell extracts. *Δqns1* WT, p.Ala573Thr, or HAAO yeast were grown in minimal media supplemented with NR. Yeast were subsequently transferred to media that lacked NR, then were grown and lysed and their NAD concentration was determined (Supplemental Methods). Levels of NAD in *Δqns1* p.Ala573Thr or HAAO yeast were significantly lower than in *Δqns1* WT yeast (Figure 2D), a result which suggests that reduced growth for yeast-generating mutant in non-supplemented conditions was a result of compromised enzymatic activity. Total cellular NAD for *Δqns1* HAAO yeast was baseline because this protein cannot rescue functionality of yeast Qns1p.

Having shown that mutation of Ala573 in the synthetase domain results in reduced yeast viability as a result of deficient cellular NAD, we questioned if glutaminase mutations also reduced total NAD levels via impaired enzymatic activity. WT NADSYN1, p.Ala573Thr, and p.Trp132Leu mutant proteins were found in equivalent levels in transfected COS-7 cells, and p.Cys49Arg protein levels were significantly reduced (Figure 3A). p.Cys49Arg could not be purified, despite efforts to scale up sample

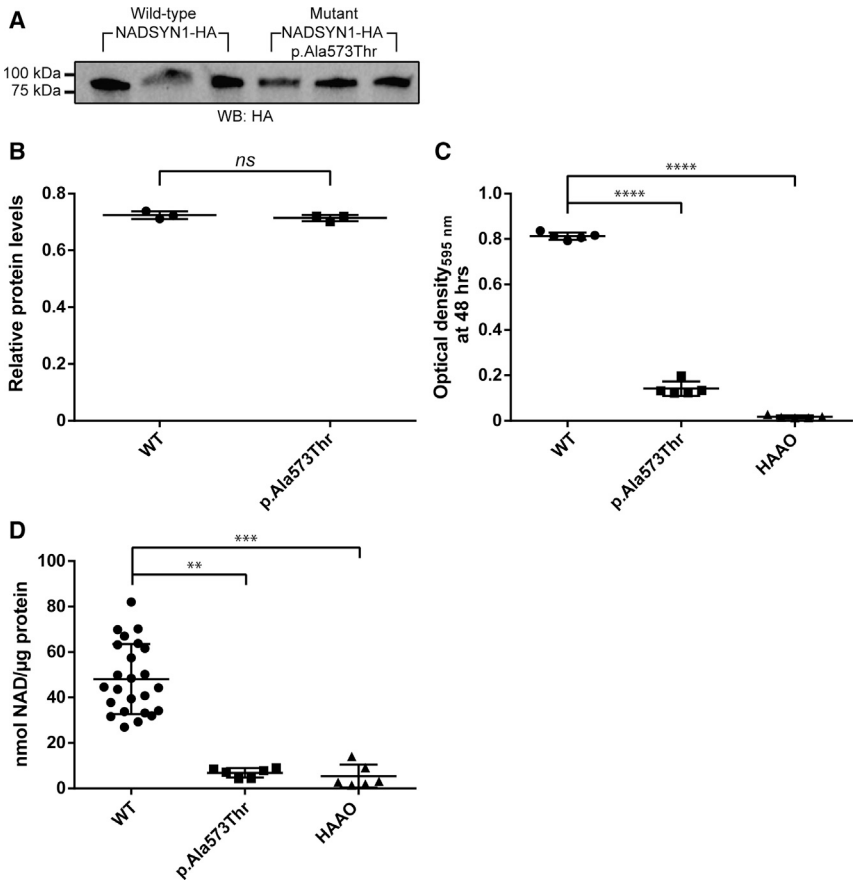

Figure 2. Genetic Complementation of Yeast Qns1p with Human NADSYN1

(A) Protein lysates from *Δqns1* yeast generating wild-type (WT) NADSYN1-HA (*Δqns1* WT) or p.Ala573Thr (*Δqns1* p.Ala573Thr) mutant, grown in nicotinamide riboside (NR)-supplemented conditions in triplicate, detected with an anti-HA antibody. WB—western blot.

(B) Quantification of detected protein, normalized to total lane protein.

(C) *Δqns1* WT, p.Ala573Thr mutant, or HAAO control yeast, seeded at $OD_{595\ nm}$ 0.01, were grown in the absence of NR with $OD_{595\ nm}$ measurements taken at 48 h. ****$p < 0.0001$. One-way ANOVA with Tukey's multiple comparisons test. Horizontal bars indicate mean and standard deviation.

(D) NAD^+/H concentrations in yeast cultures grown for 48 h with NR, then without NR for 8 h, normalized to protein lysate. **$p = 0.0012$, ***$p = 0.0002$. Horizontal bars indicate mean and standard deviation. Kruskal-Wallis with Dunn's multiple corrections test.

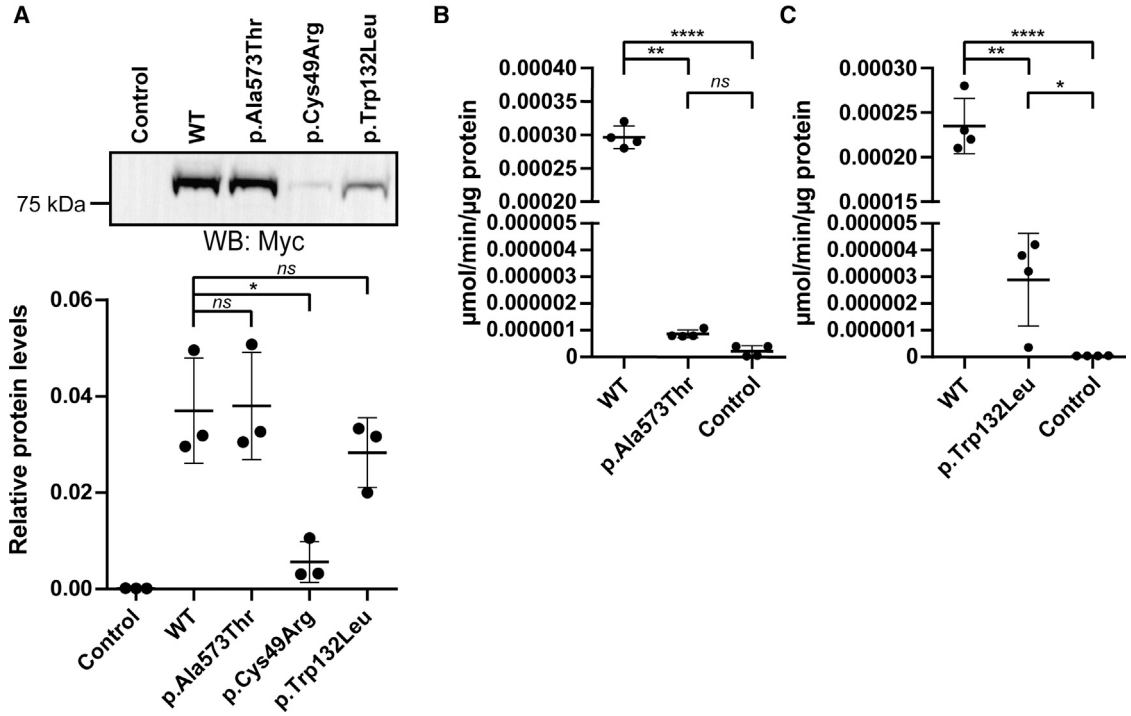

Figure 3. Functional Assessment of Mammalian-Expressed Human NADSYN1 Protein Compared to Pathogenic Missense Variants Identified in Affected Individuals

(A) Mammalian-expressed human NADSYN1 and mutant protein lysates and their levels relative to total lane protein, detected with an anti-Myc antibody. *p = 0.0417. ns—not significant, WB—western blot. Horizontal bars indicate mean and standard deviation. Brown-Forsythe and Welch one-way ANOVA.

(B) Nicotinamide adenine dinucleotide synthetase activity of purified p.Ala573Thr mutant compared to wild-type (WT) NADSYN1 protein. **p = 0.0026, ****p < 0.0001. ns—not significant. Horizontal bars indicate mean and standard deviation. Brown-Forsythe and Welch one-way ANOVA.

(C) Enzymatic activity of purified p.Trp132Leu mutant compared to WT NADSYN1 protein. *p = 0.0167, **p = 0.0096, ****p < 0.0001. Horizontal bars indicate mean and standard deviation. Brown-Forsythe and Welch one-way ANOVA.

preparation (Figure S3). This decrease in NADSYN1 p.Cys49Arg levels was independent of transfection efficiency (data not shown) and prevented downstream analysis of enzymatic activity. Finally, direct enzymatic assessment of NAD synthetase activity of purified proteins (adapted from Hara et al.,[16] Supplemental Methods) revealed that both synthetase mutant (p.Ala573Thr) and glutaminase mutant (p.Trp132Leu) exhibited limited capacity to generate NAD (Figure 3B and 3C). This confirms that reduced viability of the *Δqns1* p.Ala573Thr yeast, although sufficient for complementation, was a consequence of impaired NAD synthetase activity.

At a protein level, Ala573 is a highly evolutionarily conserved residue within the P2 loop of the NAD synthetase domain (Figure 4). Crystallographic studies of bacterial NAD synthetase orthologs have revealed that the P2 loop adopts alternating conformations that regulate NaAD accessibility at the active site.[17–20] Within the catalysis state, amino acids corresponding to human Ala573 and Leu575 stabilize the positioning of the bound NaAD. A threonine substitution at Ala573, found in our individual cases, is thus likely to have caused the reduction in NADSYN1 enzymatic activity via steric hindrance at the active site. This mirrors the 86% reduction of total cellular NAD in *Δqns1* p.Ala573Thr yeast (Figure 2D), and the corresponding ~342-fold decreased NAD synthetase-specific activity of purified mutant protein (Figure 3B), compared to respective WTs. It is therefore likely that the *NADSYN1* variant in individuals F1.II.1 and F1.II.2 similarly culminate in reduced NAD levels *in vivo*, below a necessary threshold for normal embryonic development. NAD deficiency is also expected in individuals F2.II.1 and F4.II.1 who exhibit NAD-dependent organ malformations and are either compound heterozygous for the p.Ala573Thr variant and a frameshift (c.1819del [p.Val607Trpfs*30]) or carry two frameshifts (c.735T>A [p.Cys245*] and c.1839C>G [p.Tyr613*]), respectively (Table S1).

Mammalian NADSYN1 represents a catalytically coupled fused-domain system[21] whereby ammonia produced by glutamine hydrolysis at the glutaminase domain acts as substrate for NaAD amidation. The catalytic linkage between glutaminase and synthetase domains relies on a glutamine-ammonia channel bridging these two domains.[17,20,22,23] This connectedness exists due to multimeric arrangement of NADSYN1 monomers in a ring structure, connected centrally via glutaminase domains with synthetase domains extending outward.[20,22] Accordingly, missense mutation of key residues within the glutaminase domain

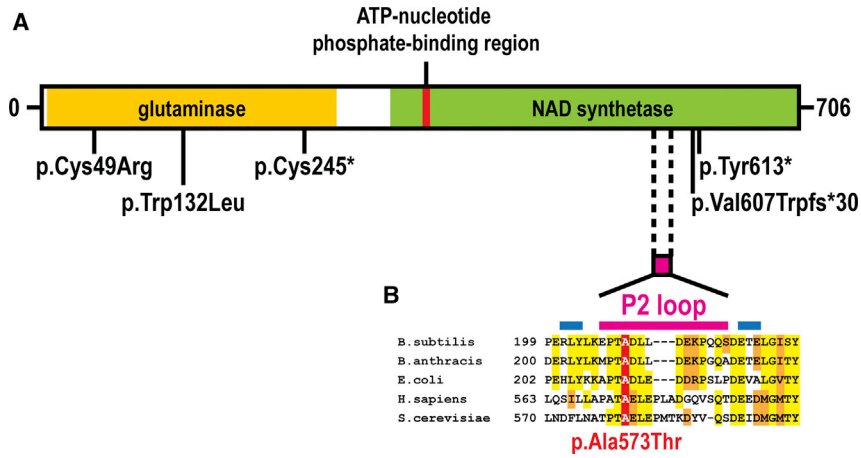

Figure 4. Protein Domain Diagram of Human NADSYN1 Highlighting the Position of Mutations Identified in Individuals

(A) Positions of missense and frameshift variants relative to protein domains identified in affected individuals.

(B) Position of the p.Ala573Thr variant (red) relative to an evolutionarily conserved region defined as the P2 loop (pink), alpha helices (blue). ClustalW sequence alignment adapted from Jauch et al.[19]

of Qns1p result in compromised synthetase activity incompatible with survival *in vivo*.[22,23]

It is likely that the phenotype of individual F3.II.4, harboring missense variants in the glutaminase domain (p.Cys49Arg, p.Trp132Leu, Figure 4), is a result of NAD depletion. Within the glutaminase domain, according to our modeling, Cys49 resides close to Tyr51, a hydrophobic component of the first major bottleneck of the ammonia channel which interacts with the highly conserved YRE loop.[20] Mutation of Tyr51 results in reduced stability of the protein.[17] The p.Cys49Arg mutation might result in loss of a disulfide bond to Cys53, thereby maligning the interaction of Tyr51 with the YRE loop and similarly destabilizing the glutaminase domain. Trp132 likely forms a cation-π interaction with Lys114, which is part of the catalytic triad[24] that stabilizes the glutaminase active site. Corresponding p.Lys114Ala mutations in yeast and bacteria lack glutaminase activity and are inviable *in vivo*,[23,25] a result which suggests that p.Trp132Leu mutation might mimic this loss of interaction and destabilize the glutaminase domain. Our studies show that the p.Trp132Leu mutant exhibits ~81-fold decreased NAD synthetase-specific activity compared to WT NADSYN1 (Figure 3C) and, while the p.Cys49Arg mutant could not be similarly assessed enzymatically, its reduced expression and stability suggest a deleterious impact on NADSYN1 activity. Together, both missense variants contribute to the NAD deficit in individual F3.II.4.

In postnatal tissue, *Nadsyn1* is highly expressed in the small intestine, kidney, liver, and testis and weakly expressed in the heart and skeletal muscle.[16] This parallels enzymatic activity *in vivo,* when the kidney and liver exhibit the highest NADSYN1 activity, and so to a lesser extent do the small intestine, heart, and spleen.[4] However, it is currently unclear how NAD deficiency disrupts embryonic development. In mice, NAD deficiency causes the same complex abnormalities[5] as seen here in individuals with *NADSYN1* mutation. Moreover, in mice, disrupted development is apparent at embryonic day 9.5 (H.C., unpublished data; equivalent to human day 22), a stage that includes tissue induction and patterning, as well as progenitor cell development and deployment, both of which are key to organogenesis. It will be important to determine which of the NAD-dependent processes (metabolism, ATP production, biosynthesis, DNA repair, and stress responses) are individually or collectively responsible for the observed disruption of embryogenesis.

NADSYN1, like many non-redundant genes of the *de novo* NAD synthesis pathway from tryptophan (Table S2), appears tolerant to heterozygous missense mutation (0.91 observed/expected missense variants) in humans.[10] This is, however, expected of genes that cause recessive disease; a single deleterious allele may be compensated for by the other allele.[26] Nevertheless, here we expand the genotypic spectrum of Congenital NAD Deficiency Disorder to that of bi-allelic missense variants, and we expect individuals harboring deleterious bi-allelic variants in additional genes of this pathway (*AFMID, KMO*, and *QPRT*) to be similarly susceptible to NAD deficiency and congenital malformation. Through a combination of yeast complementation assays, observation of altered mutant protein levels, and enzymatic analysis of mammalian-expressed NADSYN1 protein, we show that disease-causal variants impair catalytic activity of the enzyme, resulting in impaired NAD synthesis. We further recommend that bi-allelic LoF variants in the *NADSYN1* gene be recognized as a cause of Congenital NAD Deficiency Disorder, and that additional nonredundant genes of this pathway be considered in unresolved cases characterized by defects in the vertebrae, heart, and kidney such as VACTERL association.[27] Furthermore, it is conceivable that defects induced by mutation of *NADSYN1* may be rescued via NAD precursor-rich dietary supplementation, as has been demonstrated for *Haao*- or *Kynu*-deficient mice.[5]

Accession Numbers

The *NADSYN1* variants reported in this manuscript are accessible in ClinVar with the submission number SUB6305474.

Supplemental Data

Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2019.12.006.

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Declaration of Interests

Baylor College of Medicine (BCM) and Miraca Holdings Inc. have formed a joint venture with shared ownership and governance of Baylor Genetics (BG), formerly Baylor Miraca Genetics Laboratories, which performs chromosomal microarray analysis and clinical exome sequencing. During preparation of this manuscript, R.X. was an employee of BCM and derived support through a professional services agreement with BG. The remaining authors declare that they have no competing interests.

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Web Resources

CADD score, https://cadd.gs.washington.edu/score
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
GnomAD, https://gnomad.broadinstitute.org/gene/ENSG00000172890
OMIM, https://omim.org/

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