BACKGROUND: Pediatric cardiomyopathies are a clinically and genetically heterogeneous group of heart muscle disorders associated with high morbidity and mortality. Although knowledge of the genetic basis of pediatric cardiomyopathy has improved considerably, the underlying cause remains elusive in a substantial proportion of cases.

METHODS: Exome sequencing was used to screen for the causative genetic defect in a pair of siblings with rapidly progressive dilated cardiomyopathy and death in early infancy. Protein expression was assessed in patient samples, followed by an in vitro tail-anchored protein insertion assay and functional analyses in zebrafish.

RESULTS: We identified compound heterozygous variants in the highly conserved ASNA1 gene (arsA arsenite transporter, ATP-binding, homolog), which encodes an ATPase required for post-translational membrane insertion of tail-anchored proteins. The c.913C>T variant on the paternal allele is predicted to result in a premature stop codon p.(Gln305*), and likely explains the decreased protein expression observed in myocardial tissue and skin fibroblasts. The c.488T>C variant on the maternal allele results in a valine to alanine substitution at residue 163 (p.Val163Ala). Functional studies showed that this variant leads to protein misfolding as well as less effective tail-anchored protein insertion. Loss of asna1 in zebrafish resulted in reduced cardiac contractility and early lethality. In contrast to wild-type mRNA, injection of either mutant mRNA failed to rescue this phenotype.

CONCLUSIONS: Biallelic variants in ASNA1 cause severe pediatric cardiomyopathy and early death. Our findings point toward a critical role of the tail-anchored membrane protein insertion pathway in vertebrate cardiac function and disease.

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Key Words: cardiomyopathies ◼ endoplasmic reticulum ◼ exome ◼ membrane proteins ◼ zebrafish

ORIGINAL ARTICLE

Biallelic Variants in ASNA1, Encoding a Cytosolic Targeting Factor of Tail-Anchored Proteins, Cause Rapidly Progressive Pediatric Cardiomyopathy

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Dilated cardiomyopathy (DCM) is defined by otherwise unexplained ventricular dilatation and impaired systolic function, that can result in progressive heart failure, arrhythmias, and premature death. To date, disease-causing variants in over 30 genes have been reported in DCM; the majority encoding structural proteins of cardiomyocytes such as TTN (titin), LMNA (lamin A/C), and MYH7 (myosin heavy chain 7). The same genes that are involved in adult-onset DCM also contribute to pediatric DCM, although the exact frequencies are unclear. De novo variants or a combination of multiple inherited variants may explain early-onset and severe disease presentation. Pediatric DCM can also be part of numerous syndromes and neuromuscular or metabolic disorders. However, the underlying cause remains unknown in ≈50% of cases.

Here, we used family-based exome sequencing and subsequent functional validation to identify compound heterozygous variants in ASNA1 in 2 siblings with early infantile-onset, rapidly progressive DCM. ASNA1, also known as TRC40 or GET3, is a ubiquitously expressed cytosolic chaperone that mediates insertion of TA (tail-anchored) proteins into the endoplasmic reticulum (ER) membrane. TA proteins are membrane proteins characterized by a single hydrophobic transmembrane domain near the C-terminus which serves as both a targeting signal and a membrane anchor. TA proteins constitute ≈5% of integral membrane proteins and are involved in a variety of cellular processes, such as protein translocation, vesicle trafficking, and apoptosis. Previous animal studies have implicated ASNA1-mediated membrane insertion of TA proteins in early embryonic development. This study offers the first evidence for its role in human disease, and provides new insight into the molecular mechanisms in DCM.

METHODS
The authors declare that all supporting data are available within the article and its in the Data Supplement. Affected individuals were recruited from 3 clinical genetic centers in the Netherlands. All samples were collected after obtaining informed consent in compliance with local institutional review boards. Zebrafish (Danio rerio) were raised and maintained under standard conditions. All zebrafish experiments were performed in compliance with Dutch animal welfare legislation. The authors declare that all supporting data are available in the Data Supplement (including Tables I and II and Figures I and II in the Data Supplement).

RESULTS
Clinical Presentation
The proband (Figure 1A: II:2) was the second child of nonconsanguineous white parents, born at term after an uneventful pregnancy. At age 2 weeks, she presented with severe tachypnea and feeding difficulties. No dysmorphic features were observed. Echocardiography revealed a small muscular ventricular septal defect, an ostium secundum atrial septal defect, and impaired left ventricular (LV) contractility (LV ejection fraction 41%; Figure IIA in the Data Supplement). ECG recordings showed sinus rhythm with broad QRS complexes (range 124–264 ms; Figure IVA in the Data Supplement). After rapid clinical deterioration with brief circulatory arrest, she was transferred to a tertiary referral hospital for extracorporeal membrane oxygenation. LV function remained poor without any signs of improvement (Figure 2A). In addition, a large LV thrombus developed refractory to medical therapy (Figure 2B, Movie I in the Data Supplement). The patient died at age 7 weeks.

Her younger sister (II:3) was born at term after an uneventful pregnancy with normal second-trimester advanced ultrasound examination. Because of the family history, echocardiography was performed at the first day postpartum showing a small midmuscular ventricular septal defect but otherwise normal size and function of the heart (Figure 2C, Figure IIB in the Data Supplement). She was reexamined after a week because of tachypnea. Echocardiographic findings were essentially unchanged (Movie II in the Data Supplement). However, only 3 days later (age 12 days), she presented with cardiorespiratory failure necessitating resuscitation. Echocardiography now showed dilatation of the heart chambers with poor contractility (Figure 2D and Movie III in the Data Supplement). ECG recordings in the resuscitation setting were severely abnormal (Figure IVB in the Data Supplement). The resuscitation attempt was terminated after 20 minutes.

In both siblings, extensive biochemical, hematologic, viral, and metabolic testing was unremarkable except for slightly abnormal serum transferrin and apolipoprotein C-III isoelectric focusing profiles, indicative of a combined defect in N-linked and O-linked glycosylation. Cardiac screening in both parents (aged 36 and 37 years) and the elder brother (aged 34 months), consisting of physical examination, 12-lead ECG and echocardiography, revealed no abnormalities. A full 3-generation family history was negative for cardiomyopathy, heart failure and sudden cardiac death.

Exome Sequencing
Targeted next-generation sequencing of 48 genes implicated in cardiomyopathy revealed no potentially deleterious variants. Exome sequencing in the affected proband (II:2) and her healthy parents identified 3 novel heterozygous variants in ASNA1 (NM_004317.2): 2 variants c.867C>G (p.(Cys289Trp) and c.913C>T (p.(Gln305*)) in cis configuration on the paternal allele, and a nonsense variant c.488T>C (p.(Val163Ala)) on the maternal allele (Figure 1A and 1B). No other potentially deleterious
variants were detected. We confirmed that the affected sister (II:3) carried all 3 \textit{ASNA1} variants. The unaffected brother (II:1) had inherited only the maternally derived \textit{ASNA1} variant (Figure 1A). All variants were absent from public databases, including the nearly 140,000 alleles in gnomAD v2.0.2. The high pLI score (0.92) indicates that \textit{ASNA1} is extremely intolerant to loss-of-function variants. Both missense variants were predicted to be deleterious (CADD >20 and M-CAP >0.025). The c.913C>T variant introduces a premature stop codon, likely resulting in the loss of the last 42 amino acids. In silico analysis did not predict an effect on splicing using the nearby splice site. Reverse transcription-polymerase chain reaction analysis showed equal amounts of wild-type and mutant products. Hence, no evidence was found for nonsense-mediated decay. No alternatively spliced transcripts were detected (data not shown).

**Cohort Screening**

To find additional cases, we sequenced 70 children with idiopathic cardiomyopathy for \textit{ASNA1} variants using either Sanger sequencing or filtering of exome sequencing data. No biallelic variants were found. In one patient, presenting with severe DCM requiring heart transplantation at age 16 years, we identified one paternally inherited, heterozygous missense variant c.547G>A p.(Val183Met) in \textit{ASNA1}. Genome-wide microarray analysis excluded a large deletion of the second allele. However, in addition, a de novo disease-causing variant c.473T>C p.(Leu158Pro) was found in \textit{LMNA} (NM_17070.2), generally associated with adult-onset DCM. Although the \textit{ASNA1} variant is rare and assigned to the top 1\% most deleterious substitutions possible in the human genome (CADD score 23.1), it is predicted to be tolerated by Sorting Intolerant From Tolerant and PolyPhen-2, and classified as likely benign by M-CAP. Nevertheless, given the relatively early-onset and severe disease presentation, it cannot be excluded that this \textit{ASNA1} variant acted as a modifier of the \textit{LMNA}-related cardiomyopathy. A second search aiming to identify further patients was performed in Centogene’s internal database, which contains next-generation sequencing data from a heterogeneous cohort of 19,144 index patients with suspected genetic diseases and a total of 33,762 samples (as per July 2018). However, no additional patients were identified with rare biallelic variants in \textit{ASNA1}.

**Histopathologic Examination**

In both siblings, postmortem examination revealed an increased heart weight to body size and severe dilata-
tion of the LV (Figure 3A). No other gross abnormalities were observed. Microscopic examination of the myocardium showed prominent subendothelial fibrosis. In age-matched controls, ASNA1 was predominantly localized to the cytoplasm and intercalated discs. Though subcellular localization of ASNA1 appeared unchanged, expression was markedly reduced in both patients compared with controls (Figure 3B). As demonstrated by N-cadherin labeling (Figure 3B) and electron microscopy (Figure 3C), in both patients, intercalated discs were irregular in appearance and intercellular space was increased. Desmin staining confirmed myofibrillar disorganization (Figure 3B). We examined the subcellular localization of the TA protein emerin in myocardium using immunofluorescence staining. Emerin correctly localized to the nuclear membrane. However, nuclei had an irregular shape (Figure 3D). Microscopic examination of other visceral organs did not reveal any obvious abnormalities (data not shown).

Biochemical Analysis of ASNA1 Protein
As expected from the reduced ASNA1 expression by immunohistochemistry (Figure 3B), Western blot experiments confirmed that ASNA1 was decreased in fibroblasts of patient II:2 (Figure 4A), suggesting that mutant ASNA1 protein in this patient is unstable. This is to be expected for the (Cys289Trp;Gln305*) double mutant. The Cys289 variant is part of an essential zinc-binding site; residues downstream of Gln305 would be essential for structural integrity of ASNA1.19 The other mutated residue, Val163, is universally conserved from yeast Get3 to human ASNA1 and forms part of the hydrophobic domain,19 suggesting that substitution of this residue might also lead to reduced stability and function of the protein. To explore this possibility, we investigated the consequences of the Val163Ala variant in vitro using recombinant zebrafish ASNA1 protein.

Although Val163Ala mutant ASNA1 was expressed equally well as wild-type ASNA1 in E. coli, the mutant was mostly insoluble indicating its inefficient folding (Figure VA in the Data Supplement). The folded population of mutant ASNA1 was purified (Figure VB in the Data Supplement) and shown to display comparable thermal stability as wild-type ASNA1 (Figure 4B). Recombinant mutant ASNA1 was also comparably efficient as wild-type ASNA1 in capturing a TA protein substrate (Figure 4C) using a previously established in vitro assay.20 However, TA protein in complex with mutant ASNA1 was very poorly inserted into ER microsomes compared with TA protein in complex with wild-type ASNA1 (Figure 4D). Thus, the Val163Ala variant has 2

Figure 2. Cardiac ultrasound examination.
Patient II:2 (A) parasternal long-axis view during extracorporeal membrane oxygenation (ECMO) showing mild dilatation of the left ventricle; (B) intracardiac thrombus formation. Note: images from the echocardiogram made before ECMO were not available. Patient II:3 (C) 4-chamber view at first day postpartum showing a midmuscular ventricular septal defect. D, Parasternal long-axis view during cardiopulmonary resuscitation showing dilatation of the heart chambers. Ao indicates aorta; LA, left atrium; LV, left ventricle; RA, right atrium; and RV, right ventricle.
consequences. First, it reduces the production of folded ASNA1 due to aggregation. Second, properly folded mutant ASNA1, while competent for TA protein interaction, is inefficient in facilitating TA protein insertion into the ER membrane.

Zebrafish Mutants

The zebrafish gene and protein share 82% and 95% sequence identity with their respective human counterparts. To confirm the role of ASNA1 variants in cardiac disease, we generated asna1-deficient mutant zebrafish by Clustered Regularly Interspaced Short Palindromic Repeats/Cas9-mediated genome editing. Incrossed heterozygous asna1 mutants (asna1\(\Delta7/\Delta++\)) resulted in Mendelian ratios of progeny. On gross examination, asna1\(\Delta7/\Delta7\) embryos had impaired swim bladder inflation and smaller body size compared with their wild-type and heterozygous clutchmates (Figure 5A). From 5 dpf, asna1\(\Delta7/\Delta7\) embryos had impaired swim bladder inflation and smaller body size compared with their wild-type and heterozygous clutchmates (Figure 5A). From 5 dpf, asna1\(\Delta7/\Delta7\) embryos displayed abnormal cardiac contractions and decreased blood flow velocity in the dorsal aorta and cardinal vein (Movies IV and V in the Data Supplement). Fractional shortening was significantly reduced in asna1\(\Delta7/\Delta7\) mutants compared with wild-type and heterozygous clutchmates (\(P=0.0349\)). Mean heart rate was not significantly different between all groups, even after cessation of blood flow (Figure 5B), pointing towards a primary defect in cardiac contractility and not the electrical system. Compatible with the findings in our family, heterozygous mutants (asna1\(\Delta7/\Delta++\)) did not show any overt phenotype. In contrast, none of the homozygous mutants (asna1\(\Delta7\Delta7\)) survived past 8 dpf.

On microscopic examination, hearts of asna1\(\Delta7/\Delta7\) zebrafish were irregular in shape and had thinner walls compared with wild-type and heterozygous clutchmates. In addition, electron microscopic examination revealed less organized Z-lines (plate-like structures that anchor actin filaments) and irregular intercalated discs in asna1\(\Delta7\Delta7\) zebrafish (Figure 5C). Injection of wild-type human ASNA1 mRNA into asna1\(\Delta7/\Delta7\) zebrafish embryos significantly rescued the phenotype at each time point examined (\(P<0.0005\); Figure 5D). For example, at 8 dpf only 38% of fish that were injected with wild-type human ASNA1 mRNA had died or showed absent blood flow compared with 75% of noninjected fish. This rescue effect seems to disappear over time, likely due to mRNA degradation. In contrast, to rescue observed with wild-type ASNA1 mRNA, injection of
either the paternal or maternal mutant ASNA1 mRNA failed to rescue the disease phenotype (Figure 5D), supporting their pathogenicity.

Key Candidate Proteins

Inspection of the list of predicted human TA proteins (Table III in the Data Supplement) revealed 7 proteins of interest that have been associated with cardiomyopathy in humans: DMPK (myotonin-protein kinase; Q09013), DYSF (dysferlin; O75923), EMD (emerin; P50402), JPH2 (junctophilin-2; Q9BR39), PPLA (cardiac phospholamban; P26678), SYNE1 (nesprin-1; Q8NF91), and SYNE2 (nesprin-2; Q8WXH0).

DISCUSSION

Our results show that biallelic loss-of-function variants in ASNA1 cause a rapidly progressive cardiomyopathy resulting in acute heart failure and death in early infancy. We report that asna1 deficiency in zebrafish also causes cardiac defects and early lethality, which implies that, in vertebrates, the TA protein insertion pathway is specifically critical to development and function of the heart. ASNA1 binds to the transmembrane segment of newly synthesized TA proteins and delivers them to the WRB/CAML receptor complex for insertion into the ER membrane.21 Together, this complex is essential for efficient and proper targeting of a wide range of TA proteins.22 Thus far, the corresponding genes ASNA1 (MIM 601913), WRB (MIM 602915), and CAMLG (MIM 601118) have not been associated with disease in humans.

The nucleotide and amino acid sequences of ASNA1 are highly conserved across vertebrate species (Figure 1C). The mouse Asna1 gene and corresponding protein share 90% nucleotide identity and 100% amino acid identity with its human counterparts. Homozy-
Heterozygous Asna1 knockout mice, though apparently normal at the blastocyst (E3.5) stage, displayed early embryonic lethality. Contrary, were viable and showed no apparent abnormalities. These findings underscore that Asna1 plays a crucial role in embryonic development, and that one
functional copy of the gene is sufficient for normal development. The prevalence of ASNA1-related cardiomyopathy is probably low, given the negative results upon cohort screening (n=70) and the low rate of protein-altering variants in population data sets. Considering the rapidly fatal disease course, additional patients may be found in cases of sudden unexpected infant death, or, assuming that severe impairment of ASNA1 is incompatible with life, families with recurrent miscarriage or fetal death.

We explored the role of asna1 in cardiac development in the zebrafish. Unlike mice, zebrafish embryos are not dependent on a functional cardiovascular system for sufficient supply of oxygen but rely on passive diffusion. Embryos with severe cardiovascular defects can, therefore, be studied past the initial stages of embryonic development. Here, we used Clustered Regularly Interspaced Short Palindromic Repeats/Cas9-mediated genome editing to generate a loss-of-function model for ASNA1 in zebrafish. This strategy resulted in an early cardiac phenotype. Clustered Regularly Interspaced Short Palindromic Repeats-mediated asna1 knockout displayed decreased blood flow in the dorsal aorta, impaired cardiac contractility, and premature lethality, recapitulating the heart failure phenotype observed in our patients.

Previous studies in vertebrate models of the WRB-CAMLG receptor complex also point toward a role in cardiac development and disease (Table IV in the Data Supplement). Morpholino knockdown of wrb in clawed frogs (Xenopus tropicalis) and medaka fish (Oryzias latipes) induced cardiac looping defects and abnormal chamber differentiation. Of note, microscopic analysis in wrb-deficient frogs revealed large intercellular gaps between cardiomyocytes, reminiscent of the intercalated disc abnormalities observed in our family. These findings suggest that genes encoding other components of the TA protein insertion pathway may be good candidate genes for cardiovascular disease as well.

The exact mechanism by which ASNA1 variants result in cardiomyopathy remains to be determined. Several TA proteins have been linked to cardiomyopathy (including dysferlin, emerin, juncrophilin-2, phospholamban, nesprin-1, and nesprin-2), and failure to correctly localize one or more of these proteins, due to defective ASNA1-mediated membrane insertion, may be responsible for the cardiac phenotype observed in both patients and zebrafish. Intriguingly, variants in the gene EMD, which cause a progressive skeletal muscle weakness and cardiomyopathy known as X-linked Emery-Dreifuss muscular dystrophy (MIM 310300), result in mislocalization of the protein due to impaired ASNA1-mediated nuclear targeting. Though emerin staining showed apparently normal localization of the protein in our patients, it did reveal the characteristic abnormal nuclear morphology previously described in Emery-Dreifuss muscular dystrophy. Similarly, variants in the PLN (phospholamban) gene, which can result in various types of cardiomyopathy (MIM 609909 and 613874), impair proper localization of cardiac phospholamban to the ER membrane. Interestingly, patients with PLN-associated heart disease also exhibit intercalated disc abnormalities. Taken together, we hypothesize that defective ASNA1-mediated targeting affects several cardiomyopathy-related TA proteins, which together may explain the early-onset and severity of disease in our patients.

Given the ubiquitous expression of ASNA1 and the fundamental cellular processes TA proteins are involved in, one might expect that biallelic loss-of-function variants in ASNA1 would have more pleiotropic effects. Indeed, zebrafish mutant for Asna1 or for the Asna1 receptor Wrb have visual function defects and reduced touch response (Table IV in the Data Supplement). In addition, both mouse and zebrafish Wrb mutants have hearing defects due to mislocalization of the TA protein otoferlin, indicating the ASNA1-mediated TA protein insertion is critical in hearing. Moreover, in the nematode Caenorhabditis elegans, reduced asna1 activity causes exocytosis defects leading to defective insulin secretion, which was confirmed in pancreatic mouse Asna1 knockouts. While no extra-cardiac abnormalities were found in the siblings it is possible that other abnormalities have gone unnoticed, did not yet develop at this early age, or were masked by the low but detectable functionality of the Val163Ala mutant protein. Of note, both siblings had passed the newborn hearing screening.

A distinct subset of TA proteins are involved in vesicular trafficking between the ER and Golgi and the secretory machinery, including several SNAREs (SNAP-receptors) and VAMPs (vesicle-associated membrane proteins) essential for intracellular membrane fusion (Table III in the Data Supplement). Glycosylation of proteins and lipids, a complex process which starts in the ER and continues in the Golgi, highly depends on intracellular vesicular trafficking. Therefore, it is possible that the abnormal isoelectric focusing profiles of transferrin and apolipoprotein C-III in both our patients result from defective membrane targeting of TA proteins involved in vesicular trafficking and exocytosis. On the contrary, as isolectric focusing profiles were only slightly abnormal, these results should not necessarily be considered pathogenic.

TA proteins do not solely rely on ASNA1 for insertion into the ER membrane. A subset of TA proteins with moderately hydrophobic transmembrane domains can integrate in the ER membrane via an alternative route dependent on the highly conserved EMC (ER membrane protein complex). Although other routes have been demonstrated in vitro, their functional contribution to TA protein insertion in mammalian cells remains unclear.
at present. These alternative routes might not be effective or sufficient for all TA proteins, in particular strongly hydrophobic TA proteins (such as the vesicle-associated membrane protein 2), or in all cell types, suggesting why certain proteins or tissues might be more severely affected by defective ASNA1-mediated targeting.

Taken together, our study shows that biallelic variants in ASNA1, encoding a cytosolic targeting factor for TA proteins, cause severe pediatric DCM with early-onset and rapid progression. We hypothesize that this phenotype is caused by mislocalized TA proteins, either by toxic aggregation or reduced levels of functional protein. Our findings point toward a critical role of the TA protein insertion pathway in vertebrate heart function and disease.

ARTICLE INFORMATION
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Disclosures
None.

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SUPPLEMENTAL METHODS

Clinical evaluation. The diagnosis of DCM was made based on current practice guidelines.\textsuperscript{1,2} Biochemical analysis in both affected siblings included quantitative analysis of lactate, amino acids, organic acids, carnitine and acylcarnitines, oligosaccharides, and isoelectric focusing of transferrin and apolipoprotein C-III. Family members who participated in this study underwent cardiac screening with electrocardiogram and echocardiography.

Exome sequencing. Genomic DNA was extracted from peripheral blood samples using standard protocols, and fragmented by sonication. Exons were captured using the SureSelect Human All Exon V4 (Agilent Technologies). Sequencing was performed on a Hiseq 2000 system (Illumina) for 101 base pair paired-end runs. Reads were mapped to the human reference genome GRCh37/hg19 using the Burrows-Wheeler Aligner (BWA).\textsuperscript{3} Variants were called using the Genome Analysis Toolkit (GATK),\textsuperscript{4} and filtered using Cartagenia Bench Lab software. We selected for rare variants, defined as variants with a minor allele frequency <0.1% in public variant databases, including the Genome Aggregation Database (gnomAD), the NHLBI GO Exome Sequencing Project (ESP), and the Genome of the Netherlands (GoNL). We only included non-synonymous coding and splice site (±10 bp from exon-intron boundaries) variants with a minimum coverage of 10 reads. Apparent de novo, homozygous and compound heterozygous variants were considered for further analysis.

Sanger sequencing. Bidirectional Sanger sequencing of the entire coding region and exon-intron boundaries of the candidate genes was performed using PCR primers designed by Primer3 software (Supplemental Table 1). PCR products were purified and subsequently sequenced using the BigDye Terminator v3.1 kit on an ABI 3730xl DNA Analyzer (Life Technologies). Sequence data was analyzed using SeqScape v2.5 software. For annotation of DNA and protein changes, the mutation nomenclature recommendations from the Human Genome Variation Society were followed. To describe variants at the cDNA level, the A of the translation initiation codon of the reference sequence was designated as position +1.
**Histology and immunostaining.** Paraffin-embedded, hematoxylin and eosin (H&E) stained myocardial tissue from both affected siblings was examined using standard techniques. For immunohistochemistry, sections were deparaffinized and rehydrated before antibody retrieval. Primary antibodies included: full-length rabbit polyclonal anti-TRC40 (non-commercial, dilution 1:400), mouse monoclonal anti-N-cadherin (Agilent Technologies #M3616, dilution 1:200), and mouse monoclonal anti-desmin (Ventana Medical Systems #760-2513, prediluted). The slides were counterstained with hematoxylin II for 8 minutes and bluing reagent for 8 minutes according to the manufacturer’s instructions (Ventana Medical Systems). Immunostained preparations were analyzed by light microscopy. Glutaraldehyde-fixed myocardial tissue was prepared for electron microscopy. Immunolabeling was performed on cryosections as described previously. Primary antibodies included: mouse monoclonal anti-N-cadherin (Sigma-Aldrich #C3865, 1:800) and mouse monoclonal anti-emerin (Novocastra Laboratories #NCL-EMERIN). Secondary labeling was performed with appropriate Texas Red (N-cadherin) or fluorescein isothiocyanate–conjugated antibodies (emerin). After immunolabeling, sections were analyzed with a Nikon Eclipse 80i epifluorescence microscope, and images were taken using a DS-2BWc digital sight camera and NIS-Elements BR 3.0 software (Nikon Instruments).

**Western blotting.** Cultured skin fibroblasts from patient II:2 and three control individuals were lysed in 100 µL TNE buffer [50 mM Tris-HCl (pH 7.6), 100 mM NaCl, 50 mM NaF, 1% (v/v) Triton X-100] and cOmplete Protease Inhibitor Cocktail tablets (Roche Applied Science). Lysates were centrifuged for 10 minutes at 10,000 rpm to remove small cell debris. Equal amounts (20 µg or 40 µg) of protein were separated on a 4-15% precast polyacrylamide gel (Bio-Rad Laboratories). Rabbit polyclonal antibodies against the full-length (non-commercial; dilution 1:2000) and N-terminus (non-commercial; dilution 1:2000) of human ASNA1 were used for detection. Results were normalized to the GAPDH loading control. Note: these experiments were only performed in patient II:2, as we did not have access to cultured skin fibroblasts from patient II:3.

**In vitro synthesis of mRNA.** Total RNA was extracted from human skin fibroblasts using the RNeasy Mini Kit (QIAGEN), and converted into cDNA using the iScript Reverse Transcription
Supermix (Bio-Rad Laboratories, #1708840). Products were ligated into the pCMV6-entry vector with C-terminal Myc-DDK tag, and subsequently transformed into XL10-Gold ultracompetent cells (Stratagene). All constructs were verified by DNA sequencing. Expression of recombinant proteins was checked after transfection into human embryonic kidney (HEK) 293 cells using previously described rabbit polyclonal antibodies raised against the full-length and N-terminal peptide of human ASNA1. Linearized constructs were used as a template for *in vitro* synthesis of capped mRNA using the mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific).

**Purification of recombinant ASNA1.** The construct for wild-type zebrafish ASNA1 (TRC40) expression in *E. coli* has been described previously. It contains an N-terminal 6xHis tag and tobacco etch virus (TEV) protease cleavage site, followed by the full-length ASNA1 open reading frame. The Val163Ala variant was introduced into this construct by site-directed mutagenesis and verified by sequencing. Expression and purification from *E. coli* used minor modifications of previously published methods. Briefly, the Rosetta BL21(DE3) pLysS strain of *E. coli* (Novagen) was transformed with the plasmid for wild-type or mutant ASNA1, and a single colony was used to grow an overnight starter culture. This was expanded to either 1 L or 6 L (for the wild-type and mutant cultures, respectively), and when the absorbance at 600 nm was between 0.4 to 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM. After 3 hours at 37°C, the cells were collected by centrifugation at 4°C, washed once in ice cold PBS supplemented with 250 mM NaCl, and re-collected by centrifugation. The washed cells were resuspended in 35 mL of ice cold lysis buffer [PBS with 250 mM NaCl, 5 mM 2-mercaptoethanol, and 1X cComplete EDTA-free Protease Inhibitor Cocktail mix (Roche)]. After lysis by sonication, the insoluble material was sedimented by centrifugation at 18,000 rpm for 30 minutes at 4°C. The soluble extract was adjusted to 20 mM imidazole, then passed over a 3 mL column of Ni-NTA resin, washed three times in 10 mL of lysis buffer supplemented with 20 mM imidazole, and eluted with lysis buffer supplemented with 250 mM imidazole. The peak fractions (identified by absorbance at 280 nm) were pooled, mixed with TEV protease (at a protein ratio of 1:100), and dialyzed overnight against dialysis buffer (150 mM KAc, 50 mM HEPES, pH 7.4, 2 mM MgCl2, 10% glycerol, 7 mM 2-mercaptoethanol). Insoluble material was
removed by centrifugation, and the dialyzed sample was passed over a 3 mL column of Ni-NTA to remove the cleaved tag and TEV protease. The flow-through was collected and concentrated to ~4 mg/mL by centrifugal concentrators (Amicon). The protein was snap-frozen in liquid nitrogen and stored in aliquots at -80°C.

**Analysis of ASNA1 protein function in vitro.** Thermal stability of the purified wild-type and Val163Ala mutant ASNA1 protein was analyzed using the Prometheus NT.48 system (NanoTemper Technologies). Purified protein at 0.8 mg/mL was monitored for intrinsic tryptophan fluorescence during a temperature ramp from 20°C to 95°C. A change in the ratio of emission at 330 nm and 350 nm was used to measure unfolding. The ability of ASNA1 protein to capture TA protein was assayed exactly as described previously. In short, 35S-labeled TA protein containing the transmembrane domain of VAMP2 (referred to simply as VAMP2 hereinafter) was assembled with the upstream chaperone SGTA. The TA protein contained a photo-crosslinking residue within the transmembrane domain to monitor its interactions. The SGTA-VAMP2 complex was then mixed with the bridging cBAG6 complex and either wild-type or mutant ASNA1. After incubating at 32°C for 90 seconds, the reaction was transferred to ice and irradiated with UV to induce crosslinking for 10 minutes. The samples were analyzed by SDS-PAGE and autoradiography to determine whether VAMP2 was successfully transferred from SGTA to ASNA1. To test the functionality of ASNA1 for TA protein insertion, a complex between ASNA1 and VAMP2 was assembled as before, and incubated with ER microsomes for between 0 to 15 minutes at 32°C. The samples were then analyzed by SDS-PAGE and autoradiography. Insertion of the TA protein was monitored by its glycosylation at a site located near the C-terminus. The ER microsomes used for this assay were derived from HEK293 cells and were prepared as described before.

**CRISPR/Cas9 targeting of zebrafish asna1.** Zebrafish asna1 (ENSDARG00000018190) was targeted by Cas9/gRNA complex injection as described previously. The online program CRISPRscan (www.crisprscan.org) was used to design a single-stranded guide RNA (gRNA) targeting exon 5 in asna1 (5’-CCAAACTGGAGGAGACGCTGC-3’), approximately in between the variants identified in the parents. The gRNAs were obtained by *in vitro* transcription of synthetic
oligonucleotides containing a minimal T7 RNA polymerase promoter using the mMESSAGE mmACHINE T7 Ultra Kit (Thermo Fisher Scientific). SP-Cas9 plasmid was a gift from Niels Geijssen (Addgene plasmid #62731). A mix of 100 pg of either gRNA and 650 pg Cas9 protein was injected into single-cell stage zebrafish embryos. Injected embryos were raised to adulthood (F0) and analyzed for genomic modifications at the target site by Sanger sequencing and the online tool Tracking Indel by DEcompensation (TIDE). In two individual F0 founder fish, ~30% of the mapped reads contained indels at the target site in exon 5 (Supplemental Figure 1). We screened their offspring (F1) for germline transmission using PCR followed by restriction enzyme digestion (Supplemental Table 2), and identified three fish (25%) that carried a heterozygous 7 base pair deletion (Δ7). These fish were used for further breeding to create a stable mutant line. For rescue experiments, 300 pg wild-type or mutant human ASNA1 mRNA (see above) was injected in the yolk at the single-cell stage. Expression of MYC-tagged human ASNA1 was confirmed by Western blot analysis with an anti-MYC primary antibody.

Phenotypic analysis of mutant zebrafish. Zebrafish were anesthetized with tricaine methanesulfonate (MS-222) and imaged using a Leica M165 FC stereo microscope connected to a Leica DFC550 digital camera. Zebrafish were positioned horizontally in 5% methylcellulose to obtain a lateral view of the ventricle (Supplemental Figure 2). Heart rate (beats/minute) was calculated by three independent counts of the number of beats in 15 second intervals. Fractional shortening (%) was derived from linear measurements of the ventricle at end-diastole and end-systole. Blood flow rate, that can be used an indirect measure of cardiac function, was determined by visual inspection of circulating red blood cells passing through the dorsal aorta and classified as “normal”, “decreased” or “absent”. For microscopic analysis, zebrafish larvae (n=4 for each group) were anesthetized, fixed in Karnovsky fixative (PBS containing 2% paraformaldehyde and 3% glutaraldehyde), and embedded in Epon. Semithin sections (1 µm) were stained with toluidine blue and studied under a light microscope. Ultrathin sections (70 nm) were stained with 5% uranyl acetate and 2.5% lead citrate, and photographically recorded using a JEOL 1200-EX II transmission electron microscope.
**Bioinformatics.** In order to find proteins that might be affected by defective ASNA1-mediated membrane insertion, we obtained a list of all human single-pass membrane proteins from UniProt.\(^{14}\) We first removed all proteins that contain an N-terminal signal sequence, and from the remainder, selected for proteins that contain a transmembrane domain within the last 50 residues from the C-terminus. The final list contained 286 predicted human TA proteins (Supplemental Table 3). We investigated the potential association between the corresponding genes and cardiomyopathy using the Online Mendelian Inheritance in Man database (https://www.omim.org).

**Statistical analysis.** Statistical analyses were performed using Microsoft Excel or GraphPad Prism software. Continuous variables were expressed as means ± standard deviation, and compared using the Student’s \( t \)-test. Categorical variables were expressed as counts and percentages, and compared using the Fisher’s exact test. An asterisk (*) indicates \( p \)-values lower than 0.05.
## SUPPLEMENTAL TABLES

Supplemental Table 1. List of primer sequences used for Sanger sequencing.

| Target      | Direction | Primer sequence (5’ - 3’) | Product size (bp) |
|-------------|-----------|---------------------------|-------------------|
| ASNA1 exon 1| F         | tcctaaaaggaagcaataatgagga | 367               |
|             | R         | gtggaaagcccggtccttg       |                   |
| ASNA1 exon 2| F         | ctgcctcagggaacctacc       | 389               |
|             | R         | tggtcccttgtgatgtggt       |                   |
| ASNA1 exon 3| F         | ccccttgtttttgacccctt      | 470               |
|             | R         | AAGTTTCATGCCCCCTCCACCA    |                   |
| ASNA1 exon 4| F         | ATCGATGAGGCCATGAGCTA      | 375               |
|             | R         | tggaaaggaagagaattgt       |                   |
| ASNA1 exon 5| F         | ccacctggaggtacaggag       | 599               |
|             | R         | caggagctagagggcagag       |                   |
| ASNA1 exon 6| F         | TCAAGGGACCCCTgtgatgtg     | 400               |
|             | R         | caggagctagagggcagag       |                   |
| ASNA1 exon 7| F         | cactctgtctgtccctcctg      | 299               |
|             | R         | GGCTCCCCCCTGTATTATGG       |                   |

F: forward; R: reverse.
Supplemental Table 2. List of oligonucleotide sequences used in zebrafish studies.

| Target    | Direction | Primer sequence (5’ - 3’) | Product size (bp) |
|-----------|-----------|---------------------------|-------------------|
| asna1 exon 5 | F         | TAAAGCCCATTCTGAGTGC       | 404               |
|           | R         | TTGAAGTGGATGGATGATGG      |                   |

F: forward; R: reverse.

The PCR product was subjected to restriction enzyme digestion by Bsrl. As a result of the 7 bp deletion induced by CRISPR/Cas9, one Bsrl enzyme restriction site will be lost and the mutant allele will only be cut once. Subsequent gel electrophoresis will reveal three bands in wild-type (199, 131 and 74 bp), four bands in asna1^Δ/+ (266, 199, 131 and 74 bp), and two bands in asna1^Δ/Δ (266 and 131 bp) zebrafish.
Supplemental Table 3. List of predicted human tail-anchored proteins.

| Entry   | Protein names                                                                 | TMD sequence                  |
|---------|------------------------------------------------------------------------------|-------------------------------|
| E0CX11  | Short transmembrane mitochondrial protein 1                                  | GFTLGNVVGMYLAQNYD             |
| Q8NDB6  | Protein FAM156A/FAM156B (Transmembrane protein 29/29B)                       | WETLVQGLSGLTSLGT              |
| Q9H7X2  | Uncharacterized protein C1orf115                                             | VVIGLQGFAAAYSAPFAVATSVV       |
| Q8TCY0  | Small integral membrane protein 11B                                          | MEFPLCGCLSILHHFA              |
| Q96PS6  | Putative uncharacterized protein GAFA-1 (Gene associated with FGF-2 activity protein 1) | IHLVYMASAMSSSIPIFFFFQ         |
| O75438  | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 1 (Complex I-MNLL) (CI-MNLL) | HWVHLVPMGFVIGCYL              |
| Q9H1C7  | Cysteine-rich and transmembrane domain-containing protein 1                  | LGPSTCLTACWTALCCCC            |
| Q9HDD0  | Phospholipid-metabolizing enzyme A-C1 (EC 2.3.1.-) (EC 3.1.1.-) (HRAS-like suppressor 1) (HRSL1) | ISTVETFVTAAVGVSFLGLFKGPQ      |
| L0R6Q1  | SLC35A4 upstream open reading frame protein                                  | ASAVLGFAVGTCTGIYAAQAYAV       |
| Q96I36  | Cytochrome c oxidase assembly protein COX14                                   | FSTSMMLLLTVYGGYLSVRVYHY       |
| P21397  | Amine oxidase [flavin-containing] A (EC 1.4.3.4) (Monoamine oxidase type A) (MAO-A) | VSGLLKJGFSTSVATALGFVL         |
| O75452  | Retinol dehydrogenase 16 (EC 1.1.-) (Microsomal NAD(+)‐dependent retinol dehydrogenase 4) (RoDH-4) (Short chain dehydrogenase/reductase family 9C member 8) (Sterol/retinol dehydrogenase) | LLYLPMSYMPTFLVDAIMYWV         |
| Q9BVW6  | Small integral membrane protein 2                                             | GHAISILFGFWTSFICDITYIVLA      |
| Accession | Description                                                                 | Sequence |
|-----------|-----------------------------------------------------------------------------|----------|
| Q75NE6    | Putative microRNA 17 host gene protein (Putative microRNA host gene 1 protein) | LNVPKLVLILQLQSHFVLFFFSMC |
| Q9UMX3    | Bcl-2-related ovarian killer protein (hBOK) (Bcl-2-like protein 9) (Bcl2-L-9) | WLVAALCSFGRKLAAFFVLL |
| Q8TCP9    | Protein FAM200A                                                             | ILLLLPFTTLYLCELGFSIL |
| O95167    | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3 (Complex I-B9) (CI-B9) (NADH-ubiquinone oxidoreductase B9 subunit) | LVVSFVVGGLAVILPPLSPYF |
| Q07812    | Apoptosis regulator BAX (Bcl-2-like protein 4) (Bcl2-L-4)                    | TVTIFVAGVLTASLTIWKKMG |
| Q8WXE9    | Stonin-2 (Stoned B)                                                          | IWLMLPTPFVHPTTLPLLFLLLAM |
| Q9NX95    | Syntabulin (Golgi-localized syntaphilin-related protein) (Syntaxin-1-binding protein) | SFLVDDLAVAAPVPTLVAF |
| Q6ZSY5    | Protein phosphatase 1 regulatory subunit 3F (R3F)                            | VLAGLVVVPVALNSGVSLLVL |
| Q3KP22    | Membrane-anchored junction protein                                           | AATGFFGFLSSLFPFRYFF |
| A8MTT3    | Protein CEBPZOS (CEBPZ antisense RNA 1) (CEBPZ opposite strand)              | GVLVAELVGFGAYFLFS |
| Q07817    | Bcl-2-like protein 1 (Bcl2-L-1) (Apoptosis regulator Bcl-X)                 | FNRWFLTGMTVAGVLL |
| P56378    | 6.8 kDa mitochondrial proteolipid                                            | VYQEIWIGMLMGFIYKI |
| O15079    | Syntaphilin                                                                 | YIVDLLAVVVPAVPTAVAWLC |
| Q9NRY6    | Phospholipid scramblase 3 (PL scramblase 3) (Ca(2+)-dependent phospholipid scramblase 3) | VKAQLLGATFLIDYMFF |
| Q9NUB4    | Uncharacterized protein C20orf141                                            | LLLLMGLGPLLRACGMPHTLL |
| O95139    | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6 (Complex I-B17) (CI-B17) (NADH-ubiquinone oxidoreductase B17 subunit) | SIFVFTHLVLPVWIHYYM |
| Accession | Description                                                                 | Protein Sequence |
|-----------|------------------------------------------------------------------------------|------------------|
| O43676    | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3 (Complex I-B12) (CI-B12) (NADH-ubiquinone oxidoreductase B12 subunit) | VFFKGFKWGFAAFVVAVGAEOYYL |
| Q8NCU8    | Uncharacterized protein encoded by LINC00116                                 | LQLSVLVAFAVGVLGLGW |
| Q8N4H5    | Mitochondrial import receptor subunit TOM5 homolog                           | SIRNFIYVALLRVPFFIL |
| Q9NRY7    | Phospholipid scramblase 2 (PL scramblase 2) (Ca(2+)-dependent phospholipid scramblase 2) | MKAVMIGACFLIDYMFF |
| Q8N7S6    | Uncharacterized protein ARIH2OS (Ariadne-2 homolog opposite strand protein) | CILTALLAVSFHSIGVIMTS |
| Q9UL19    | Retinoic acid receptor responder protein 3 (EC 3.1.1. -) (HRAS-like suppressor 4) (HRSL4) (RAR-responsive protein TIG3) (Retinoid-inducible gene 1 protein) (Tazarotene-induced gene 3 protein) | KVEVGVATALGILVAGCSFAI |
| A2RU48    | Single-pass membrane and coiled-coil domain-containing protein 3              | IGASLLGSIGVAVGLGIDMI |
| P03928    | ATP synthase protein 8 (A6L) (F-ATPase subunit 8)                             | VWPTMIPMMLTLFLIT |
| P0DJ07    | Protein PET100 homolog, mitochondrial                                        | IFRMIYLTFFVAMFWS |
| Q5TGZ0    | MICOS complex subunit MIC10 (Mitochondrial inner membrane organizing system protein 1) | AVVKGITGFGLGVFSFLFF |
| O15162    | Phospholipid scramblase 1 (PL scramblase 1) (Ca(2+)-dependent phospholipid scramblase 1) (Erythrocyte phospholipid scramblase) (MmTRA1b) | MKAVMIGACFLIDFMFF |
| O15239    | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1 (Complex I-MWFE) (CI-MWFE) (NADH-ubiquinone oxidoreductase MWFE subunit) | MWFEIIPGLSVMGVCCLIPGL |
| Accession | Description                                                                 | Sequence                                      |
|-----------|------------------------------------------------------------------------------|-----------------------------------------------|
| A6NCl5    | Putative transmembrane protein encoded by LINC00862 (Small integral membrane | IMALILMPSLHCIGNILILLF                        |
|           | protein 16)                                                                   |                                               |
| Q9NRQ2    | Phospholipid scramblase 4 (PL scramblase 4) (Ca(2+)-dependent phospholipid    | MKAMIFGACFLIDFMYF                             |
|           | scramblase 4) (Cell growth-inhibiting gene 43 protein) (TRA1)                  |                                               |
| Q9BSJ5    | Uncharacterized protein C17orf80 (Cell migration-inducing gene 3 protein)     | GFGGITMLFTGYFVLCCWSWF                        |
|           | (Human lung cancer oncogene 8 protein) (HLC-8)                                 |                                               |
| P08574    | Cytochrome c1, heme protein, mitochondrial (Complex III subunit 4) (Complex   | MLMMMAALLVPLVYT                            |
|           | III subunit IV) (Cytochrome b-c1 complex subunit 4) (Ubequiol-cytochrome-c    |                                               |
|           | reductase complex cytochrome c1 subunit) (Cytochrome c1)                      |                                               |
| Q9HD87    | Putative uncharacterized protein C6orf50 (Nasopharyngeal carcinoma-associated | IISLLAIIFKMCWLWKOQFL                        |
|           | gene 19 protein)                                                              |                                               |
| P60602    | Reactive oxygen species modulator 1 (ROS modulator 1) (Epididymis tissue     | GFVMGCAVGAAGALFCLTSCLR                      |
|           | protein Li 175) (Glyrichin) (Mitochondrial targeting GxxxG motif protein) (MTGM) |                                               |
|           | (Protein MGR2 homolog)                                                       |                                               |
| Q9P0U1    | Mitochondrial import receptor subunit TOM7 homolog (Translocase of outer     | FAIRWGFPLVIYLGFL                            |
|           | membrane 7 kDa subunit homolog)                                               |                                               |
| P0DMW3    | Small integral membrane protein 10-like protein 1                             | FFYFYILASVILNVHLQVY                         |
| Q96HG1    | Small integral membrane protein 10                                             | FFYFYILASVILNVHLQVY                         |
| Q96IX5    | Up-regulated during skeletal muscle growth protein 5 (Diabetes-associated     | TLTGRMNCVLATYGSIALIVLF                      |
|           | protein in insulin-sensitive tissues) (HCV F-transactivated protein 2)       |                                               |
| Accession | Description                                                                 | Sequence |
|-----------|------------------------------------------------------------------------------|----------|
| O95237   | Lecithin retinol acyltransferase (EC 2.3.1.135) (Phosphatidylcholine--retinol O-acyltransferase) | VLAVSLASIVCTGLVSYT |
| E9PQ53   | NADH dehydrogenase [ubiquinone] 1 subunit C2, isoform 2 (NDUFC2-KCTD14 readthrough transcript protein) | GLHRQLLYITAFFAGYYLYV |
| O95298   | NADH dehydrogenase [ubiquinone] 1 subunit C2 (Complex I-B14.5b) (CI-B14.5b) (Human lung cancer oncogene 1 protein) (HLC-1) (NADH-ubiquinone oxidoreductase subunit B14.5b) | GLHRQLLYITAFFAGYYLYV |
| P56134   | ATP synthase subunit f, mitochondrial                                         | ISGTMVLACYVLFSYSFSY |
| A0A5B9   | T-cell receptor beta-2 chain C region                                        | TILYEILLGKATLYAVLVSALVL |
| P53816   | HRAS-like suppressor 3 (HRSL3) (EC 3.1.1.32) (EC 3.1.1.4) (Adipose-specific phospholipase A2) (AdPLA) (Group XVI phospholipase A1/A2) (H-rev 107 protein homolog) (H-REV107) (HREV107-1) (HRAS-like suppressor 1) (HREV107-3) (Renal carcinoma antigen NY-REN-65) | V1IAAVSAGMLAAMSLIGVMFS |
| Q7Z412   | Peroxisome assembly protein 26 (Peroxin-26)                                 | FFSLPFKKSLAALILCCLLVV |
| Q9NS69   | Mitochondrial import receptor subunit TOM22 homolog (hTom22) (1C9-2) (Translocase of outer membrane 22 kDa subunit homolog) | ALWIGTTSFMLVLPVFET |
| Q9GZY8   | Mitochondrial fission factor                                                 | VMYSITVAFWLLNSWLWF |
| Q13505   | Metaxin-1 (Mitochondrial outer membrane import complex protein 1)           | ILSVLAGLAAMGVYALLSGIV |
| P01848   | T-cell receptor alpha chain C region                                        | VIGFRILLLKVAGFNLLMTL |
| P27338   | Amine oxidase [flavin-containing] B (EC 1.4.3.4) (Monoamine oxidase type B) (MAO-B) | PGLRLILGTTIFSATALGFLAHKRGL |
| Accession | Description                                                                 | FASTA Sequence         |
|-----------|-----------------------------------------------------------------------------|------------------------|
| O00198    | Activator of apoptosis harakiri (BH3-interacting domain-containing protein 3) (Neuronal death protein DP5) | WPWLCAAQAALAAWLLG     |
| B7Z8K6    | T-cell receptor delta chain C region                                        | LGLRMLFAKTVAVNFLLTAKLFF |
| O95168    | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4 (Complex I-B15) (CI-B15) (NADH-ubiquinone oxidoreductase B15 subunit) | LMGALCGFGPILIYYII     |
| Q16611    | Bcl-2 homologous antagonist/killer (Apoptosis regulator BAK) (Bcl-2-like protein 7) (Bcl2-L-7) | ILNVLVVGLVVLGLGQFVV   |
| O60238    | BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like (Adenovirus E1B19K-binding protein B5) (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3A) (NIP3-like protein X) (NIP3L) | VFIPSLFLSHVLALGLGQFVV |
| Q96N68    | Putative uncharacterized protein C18orf15                                   | MCVCVHCACVYVCMCVCVCM  |
| Q07820    | Induced myeloid leukemia cell differentiation protein McI-1 (Bcl-2-like protein 3) (Bcl2-L-3) (Bcl-2-related protein EAT/mcl1) (mcl1/EAT) | IRNVLLAFAGVAGVAGL QAYL |
| Q09013    | Myotonic protein kinase (MT-PK) (EC 2.7.11.1) (DM-kinase) (DMK) (DM1 protein kinase) (DMPK) (Myotonic dystrophy protein kinase) | LLLFAVYLSRAALGCGIGLVA |
| Q9Y3D6    | Mitochondrial fusion 1 protein (FIS1 homolog) (hFis1) (Tetratricopeptide repeat protein 11) (TPR repeat protein 11) | LVGMAIVGGMALGAGLAGL |
| Q96JJ6    | Junctophilin-4 (JP-4) (Junctophilin-like 1 protein)                          | LVVGAVALDLSLAFLFQLL LT |
| Q96K12    | Fatty acyl-CoA reductase 2 (EC 1.2.1.84) (Male sterility domain-containing protein 1) | NIHYLFNTALFMLIAWRLLLIA |
| Accession | Protein Name                                                                 | Description                                                                 | Sequence                                                                 |
|-----------|-----------------------------------------------------------------------------|----------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Q9H0X9    | Oxysterol-binding protein-related protein 5 (ORP-5) (OSBP-related protein 5) (Oxysterol-binding protein homolog 1) | SWFLLCVFLACQLFINHIL                                                        |
| F7VJQ1    | Alternative prion protein (AltPrP)                                           | WWWLGAASWWWLGAAPWWWL                                                        |
| Q96KF7    | Small integral membrane protein 8                                            | PVMAFGLVTLSLCVAYIGYLHAI                                                    |
| Q8WV1O    | Small integral membrane protein 4                                            | FGIYRFLPFFFVLGGTMEWIMI                                                    |
| P37268    | Squalene synthase (SQS) (SS) (EC 2.5.1.21) (FPP:FPP farnesyltransferase) (Farnesyl-diphosphate farnesyltransferase) | PIYLSFVMLAALSWQYLTTL                                                      |
| Q3B7S5    | Small integral membrane protein 21                                           | HIRFFTLLVLFHVMVLL                                                          |
| P10415    | Apoptosis regulator Bcl-2                                                    | FSWLSLKTLLSLALVGACITLG                                                    |
| H7C350    | Coiled-coil domain-containing protein 188                                    | LLLGALLVWTAAYVYVV                                                         |
| Q14318    | Peptidyl-prolyl cis-trans isomerase FKBP8 (PPIase FKBP8) (EC 5.2.1.8) (38 kDa FK506-binding protein) (38 kDa FKBP) (FKBP-38) (hFKBP38) (FK506-binding protein 8) (FKBP-8) (FKBPR38) (Rotamase) | WLFGATAVALGGVALSVVIAA                                                    |
| Q8IVJ8    | AP20 region protein 1                                                        | IALALAGPGAILILELSWFLG                                                      |
| P0DMT0    | Myoregulin                                                                  | VGRLLKILFVIFVDLISIYV                                                      |
| Q8N326    | Uncharacterized protein C10orf111                                           | MSLLLLPASFGLTWAPFLFLF                                                      |
| P60059    | Protein transport protein Sec61 subunit gamma                                | FQKIAMATAIGFAIMGFIGFFVKLIHPI                                               |
| Accession  | Description                                                                                           | Sequence                                      |
|-----------|--------------------------------------------------------------------------------------------------------|-----------------------------------------------|
| Q12983    | BCL2/adenovirus E1B 19 kDa protein-interacting protein 3                                               | VFLPSLLSHLLAIGLGIYIG                         |
| O43677    | NADH dehydrogenase [ubiquinone] 1 subunit C1, mitochondrial (Complex I-KFYI) (CI-KFYI) (NADH-ubiquinone oxidoreductase KFYI subunit) | WLKVGFHTLGTTVFLWIYLI                         |
| Q8N2K1    | Ubiquitin-conjugating enzyme E2 J2 (EC 2.3.2.23) (E2 ubiquitin-conjugating enzyme J2) (Non-canonical ubiquitin-conjugating enzyme 2) (NCUBE-2) | GLLGGALANLFVIVGFAAFAY                        |
| O96011    | Peroxisomal membrane protein 11B (Peroxin-11B) ( Peroxisomal biogenesis factor 11B) (Protein PEX11 homolog beta) (PEX11-beta) | GIVGLCGLVSSILLSLTLIYPWL                      |
| Q86T96    | E3 ubiquitin-protein ligase RNF180 (EC 2.3.2.27) (RING finger protein 180) (RING-type E3 ubiquitin transferase RNF180) | MVIIYIYSNVWVGIFIYFCFL                        |
| O95169    | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial (Complex I-ASHI) (CI-ASHI) (NADH-ubiquinone oxidoreductase ASHI subunit) | LFGLAFMIFMCWVGDVYPVY                         |
| O94966    | Ubiquitin carboxyl-terminal hydrolase 19 (EC 3.4.19.12) (Deubiquitinating enzyme 19) (Ubiquitin | FVLGTVAAALVALVNLNFYPLV                      |
| Accession | Description                                                                 | Sequence                      |
|-----------|-----------------------------------------------------------------------------|-------------------------------|
| P01850    | T-cell receptor beta-1 chain C region                                       | ILLGKATLYAVLVSAVLVMLAM        |
| Q96A26    | Protein FAM162A (E2-induced gene 5 protein) (Growth and transformation-dependent protein) | ISYLMIALTVVGCFIMVI            |
| Q969F0    | Fetal and adult testis-expressed transcript protein (Cancer/testis antigen 43) (CT43) (Tumor antigen BJ-HCC-2) | TLIIAVLVSAIANLWLWM            |
| Q8N5G0    | Small integral membrane protein 20 (Mitochondrial translation regulation assembly intermediate of cytochrome c oxidase protein of 7 kDa) (MITRAC7) | TALIFGGFISLIGAAFYPIYF         |
| Q86UQ5    | Gilles de la Tourette syndrome chromosomal region candidate gene 1 protein   | AICMEVFLFLWIFIAPIYACVC        |
| P00167    | Cytochrome b5 (Microsomal cytochrome b5 type A) (MCB5)                       | WWTNWVIPAISAVAVALMYRLYM       |
| Q9NPU4    | Uncharacterized protein C14orf132                                           | AVLLWIAIATLGNIVVGVV           |
| P60468    | Protein transport protein Sec61 subunit beta                                | VPVLVMSSLFIASVFMLHIWG         |
| Q9NWV9    | HRAS-like suppressor 2 (EC 2.3.1.-) (EC 3.1.1.-)                             | AVTTVGVAAGGLAAAASLVGILLA      |
| Q7Z3B0    | Small integral membrane protein 15                                          | YGFLTTVILALTPLFLASAVL         |
| Q9Y5L2    | Hypoxia-inducible lipid droplet-associated protein (Hypoxia-inducible gene 2 protein) | LYLLGVVLTLLSIFVRV             |
| Q96FB5    | Protein RRNAD1 (Ribosomal RNA adenine dimethylase domain-containing protein 1) | VVAFFSIALLLAPLNETLILL         |
| Accession | Description                                                                 | Uniprot ID |
|-----------|------------------------------------------------------------------------------|------------|
| B2RUZ4    | Small integral membrane protein 1 (Vel blood group antigen)                  | LGIAMKVLGGVALFWIIFILG |
| Q9Y2R0    | Cytochrome c oxidase assembly factor 3 homolog, mitochondrial (Coiled-coil domain-containing protein 56) (Mitochondrial translation regulation assembly intermediate of cytochrome c oxidase protein of 12 kDa) | IVTGLGIGALVLAIYGYTFYS |
| Q8IX11    | Mitochondrial Rho GTPase 2 (MIRO-2) (hMir-2) (EC 3.6.5.-) (Ras homolog gene family member T2) | GLLGVVGAĀAAVLSFSLYRVVLV |
| P0C6T2    | Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 4 | VQLAIFANMLGVSLFLVVLY |
| Q14BN4    | Sarcolemmal membrane-associated protein (Sarcolemmal-associated protein)    | WMPMLAALVAATAIYVPGL |
| Q8WVX9    | Fatty acyl-CoA reductase 1 (EC 1.2.1.84) (Male sterility domain-containing protein 2) | IRYGFNTILVILIWRIFI |
| Q14D33    | Receptor-transporting protein 5 (3CxxC-type zinc finger protein 5) (CXXC-type zinc finger protein 11) | FWIWSMTVCVFWMCM |
| Q8NI28    | Putative uncharacterized protein encoded by LINC01006 (Long intergenic non-protein coding RNA 1006) | WIPLLLVAGCVSCFVLAVCV |
| I3L115    | Putative uncharacterized protein LOC100996504 | VLSIILGSLLMCASSFCFAL |
| A4D256    | Dual specificity protein phosphatase CDC14C (EC 3.1.3.16) (EC 3.1.3.48) (CDC14 cell division cycle 14 homolog C) | ILLPSPLATFTLCSVVIIWWIV |
| Q9Y385    | Ubiquitin-conjugating enzyme E2 J1 (EC 2.3.2.23) (E2 ubiquitin-conjugating enzyme J1) (Non-canonical ubiquitin-conjugating enzyme 1) (NCUBE-1) (Yeas ubiquitin-conjugating enzyme UBC6 homolog E) (HsUBC6e) | DHGGSAVLIVTLALAALIF |
| ID     | Description                                                                                     | Description                                                                                     |
|--------|------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| Q6ZS62 | Colorectal cancer-associated protein 1                                                          | LYGCFCVGLVSGMAISVLLLA                                                                        |
| A6NFE2 | Single-pass membrane and coiled-coil domain-containing protein 2                                 | IFIMFDVLTVTGLLCYILFFG                                                                        |
| Q9NS64 | Protein reprimao                                                                             | VVQIAVMCVLSLTVVFGVFFL                                                                        |
| Q16821 | Protein phosphatase 1 regulatory subunit 3A (Protein phosphatase 1 glycogen-associated regulatory subunit) (Protein phosphatase type-1 glycogen targeting subunit) (RG1) | YFLLFLIFLITVVHYDLMIGL                                                                        |
| Q9NQG1 | Protein MANBAL                                                                                | YGLFLGAIFQLICVLAIIVPI                                                                        |
| P58511 | Small integral membrane protein 11A                                                              | PLLLYILAAKTTLILCLTFAGVKM                                                                      |
| Q9Y228 | TRAF3-interacting JNK-activating modulator (TRAF3-interacting protein 3)                       | WLPVLMVVAAILAVFLA                                                                           |
| Q9BXU9 | Calcium-binding protein 8 (CaBP8) (Calneuron I) (Calneuron-1)                                 | LICAFAHAFIISVMLIAANQI                                                                         |
| A6NL05 | Protein FAM74A7                                                                               | LSLLHLHAVFLWIIIAINFSN                                                                         |
| Q4VXF1 | Putative protein FAM74A3                                                                       | LSLLHLHAVFLWIIIAINFSN                                                                         |
| Q5RGS3 | Protein FAM74A1                                                                               | LSLLHLHAVFLWIIIAINFSN                                                                         |
| Q5T6X4 | Protein FAM162B                                                                               | VKACYIMIGLTIIACFAVIVS                                                                        |
| Q9NXE4 | Sphingomyelin phosphodiesterase 4 (EC 3.1.4.12) (Neutral sphingomyelinase 3) (nSMase-3) (nSMase3) (Neutral sphingomyelinase III) | LLLAFFVASLFCVGPLPCTLL                                                                        |
| P51648 | Fatty aldehyde dehydrogenase (EC 1.2.1.3) (Aldehyde dehydrogenase 10) (Aldehyde dehydrogenase family 3 member A2) (Microsomal aldehyde dehydrogenase) | LGLLLLTLFLGIVAAVLV |
| Accession | Description                                                                 | Sequence                                      |
|-----------|----------------------------------------------------------------------------|-----------------------------------------------|
| Q8IXI2    | Mitochondrial Rho GTPase 1 (hMiro-1) (EC 3.6.5.-) (Rac-GTP-binding protein-like protein) (Ras homolog gene family member T1) | WLRASFGATVFAVLGFAMYKALL                      |
| Q8N4K4    | Reprimo-like protein                                                         | VAQIAVLCVLSTTVFGVFFF                        |
| Q8N6R1    | Stress-associated endoplasmic reticulum protein 2 (Ribosome-associated membrane protein RAMP4-2) | GPWLLALFVFFVCGSAIFQII                       |
| P58549    | FXYD domain-containing ion transport regulator 7                             | TVQTVGMLTALILFLLLGILIVIS                    |
| Q86V35    | Calcium-binding protein 7 (CaBP7) (Calneuron II) (Calneuron-2)               | LICAFIAFIISVMLIAANQV                        |
| Q6ZNB6    | NF-X1-type zinc finger protein NFXL1 (Ovarian zinc finger protein) (hOZFP)   | YYLISVCGVVVVFAWYI                           |
| O75056    | Syndecan-3 (SYND3)                                                           | AVIVGGVGAALFAFLVTLLI                        |
| P03986    | T-cell receptor gamma-2 chain C region (T-cell receptor gamma chain C region PT-gamma-1/2) | MYLLLLLKSVVYFAITCCLL                        |
| A1L1A6    | Immunoglobulin superfamily member 23                                        | LLAAGILGAGALIAGMCFIII                       |
| Q8N5Y8    | Mono [ADP-ribose] polymerase PARP16 (EC 2.4.2.30) (ADP-ribosyltransferase diphtheria toxin-like 15) (Poly [ADP-ribose] polymerase 16) (PARP-16) | SHWFTVMISYLLLLLLLVSVI                     |
| P61266    | Syntaxin-1B (Syntaxin-1B1) (Syntaxin-1B2)                                   | IMIIIICCVVVLGVLASSIGGTGL                    |
| Q9BZF1    | Oxysterol-binding protein-related protein 8 (ORP-8) (OSBP-related protein 8) | YFIIFLLLLLQVIINFMF                         |
| A6NGB0    | Transmembrane protein 191C                                                  | VLGALQVLLTPLLLFLGLSLL                      |
| P0C7N4    | Transmembrane protein 191B                                                  | VLGALQVLLTPLLLFLGLSLL                      |
| Q7Z419    | E3 ubiquitin-protein ligase RNF144B (EC 2.3.2.-) (IBR domain-containing protein 2) (RING finger) | VVGILVGLIGIALVTSPPLL                        |
| Accession | Description                                                                                                                                                                                                 | Identity |
|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|
| Q9UPX6    | UPF0258 protein KIAA1024                                                                                                                                                                                  | IAALIAAAACTVILVIVVPIC |
| P54710    | Sodium/potassium-transporting ATPase subunit gamma (Na(+)/K(+) ATPase subunit gamma) (FXYD domain-containing ion transport regulator 2) (Sodium pump gamma chain)                                                   | GGLIFAGLAFIVGLLILL |
| Q96LL3    | Uncharacterized protein C16orf92                                                                                                                                                                             | PGLFHHILVGLLVVAFSLF |
| Q9Y6X1    | Stress-associated endoplasmic reticulum protein 1 (Ribosome-attached membrane protein 4)                                                                                                                                 | GPWLALIFIVVCGSAIFQII |
| Q16623    | Syntaxin-1A (Neuron-specific antigen HPC-1)                                                                                                                                                                  | IMIIICCVILGIVIASTVGGI |
| P60509    | Endogenous retrovirus group PABL B member 1 Env polyprotein (Endogenous retrovirus group PABL B member 1) (Envelope polyprotein) (HERV-R(b) Env protein) (HERV-R(b)_3p24.3 provirus ancestral Env polyprotein) [Includes: Surface protein domain (SU); Transmembrane protein domain (TM)] | ILIVLATWSVGIALCCGLYF |
| P50876    | E3 ubiquitin-protein ligase RNF144A (EC 2.3.2.-) (RING finger protein 144A) (UbcM4-interacting protein 4) (Ubiquitin-conjugating enzyme 7-interacting protein 4)                                                      | VVGIFAGFGLLLLVASPFLL |
| Q96DX8    | Receptor-transporting protein 4 (28 kDa interferon-responsive protein) (3CxxC-type zinc finger protein 4)                                                                                                       | PLNICVFILLLFIVVKCFTS |
| Q12846    | Syntaxin-4 (Renal carcinoma antigen NY-REN-31)                                                                                                                                                                | IAICVSITVVLLAVIGTVV |
| Q9UEU0    | Vesicle transport through interaction with t-SNAREs homolog 1B (Vesicle transport v-SNARE protein)                                                                                                              | LSIIILELAILGGLVYYKFF |
| Accession Number | Description                                                                 | Accession Number | Description                                                                 |
|------------------|------------------------------------------------------------------------------|------------------|------------------------------------------------------------------------------|
| A8MYB1           | Vti1-like 1) (Vti1-rp1) Transmembrane and coiled-coil domain-containing protein 5B | YFQYLTFMVLFIRLLAYVIFHL |
| Q9BXX5           | Bcl-2-like protein 13 (Bcl2-L-13) (Bcl-rambo) (Protein Mil1)                 | ILLFGGAAAVALAVAIGVAL |
| Q6PJW8           | Consortin                                                                    | CILLVLLCIATVFLSVGGTAL |
| H3BV60           | Transforming growth factor-beta receptor type 3-like protein (TGF-beta receptor type-3-like protein) (TGFR-3L) (Transforming growth factor-beta receptor type III-like protein) (TGF-beta receptor type III-like protein) | VVALVLAAFVLGAALAAGLGL |
| P17706           | Tyrosine-protein phosphatase non-receptor type 2 (EC 3.1.3.48) (T-cell protein-tyrosine phosphatase) (TCPTP) | ILTKMGFMSVILVGAFVGWTLFF |
| Q8N111           | Cell cycle exit and neuronal differentiation protein 1 (BM88 antigen)         | LVAGGVAVAIALILGVAFLV |
| E7ERA6           | RING finger protein 223                                                      | LVSALLMLFCVALWPVQCAL |
| O14653           | Golgi SNAP receptor complex member 2 (27 kDa Golgi SNARE protein) (Membrin)   | YFMIGGMLTCCVVMFLVVQYL |
| Q9BZ97           | Putative transcript Y 13 protein                                              | LLGWDLNLSLFLGLCLMLLLA |
| Q9P0L0           | Vesicle-associated membrane protein-associated protein A (VAMP-A) (VAMP-associated protein A) (VAP-A) (33 kDa VAMP-associated protein) (VAP-33) | LPSLLVVIAIFIGFFLGKFI |
| Q8N8J7           | Uncharacterized protein C4orf32                                              | VIVIFFWVMLWFLGLQALGLV |
| P37287           | Phosphatidylinositol N-acetylglicosaminyltransferase subunit A (EC 2.4.1.198) (GlcNAc-PI synthesis) | PVTGYIFALLAVFNFLFLIFL |
| Accession | Description |
|-----------|-------------|
| Q5VV42    | Threonylcarbamoyladenosine tRNA methylthiotransferase (EC 2.8.4.5) (CDK5 regulatory subunit-associated protein 1-like 1) (tRNA-\(t(6)A37\) methylthiotransferase) |
| Q8WWG1    | Pro-neuregulin-4, membrane-bound isoform (Pro-NRG4) [Cleaved into: Neuregulin-4 (NRG-4)] |
| Q96NA8    | t-SNARE domain-containing protein 1 |
| Q68G75    | LEM domain-containing protein 1 (Cancer/testis antigen 50) (CT50) (LEM domain protein 1) (LEMP-1) |
| P0CF51    | T-cell receptor gamma chain C region 1 |
| Q9NX14    | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial (Complex I-ESSS) (CI-ESSS) (NADH-ubiquinone oxidoreductase ESSS subunit) (Neuronal protein 17.3) (Np17.3) |
| Q8IUY3    | GRAM domain-containing protein 2A |
| Q9UNK0    | Syntaxin-8 |
| Q9GZT6    | Coiled-coil domain-containing protein 90B, mitochondrial |
| O95249    | Golgi SNAP receptor complex member 1 (28 kDa Golgi SNARE protein) (28 kDa cis-Golgi SNARE p28) (GOS-28) |
| Q9P0B6    | Coiled-coil domain-containing protein 167 |
| Accession | Description                                                                                   | Peptide                                      |
|---------|------------------------------------------------------------------------------------------------|----------------------------------------------|
| Q9HDC5 | Junctophilin-1 (JP-1) (Junctophilin type 1)                                                   | IMIVLVM[LNI]GLAILFVHFL                       |
| Q8NF91 | Nesprin-1 (Enaptin) (KASH domain-containing protein 1) (KASH1) (Myocyte nuclear envelope protein 1) (Myne-1) (Nuclear envelope spectrin repeat protein 1) (Synaptic nuclear envelope protein 1) (Syne-1) | AALPLQLLLLLLIGLACLVPM                       |
| Q8TBA6 | Golgin subfamily A member 5 (Cell proliferation-inducing gene 31 protein) (Golgin-84) (Protein Ret-II) (RET-fused gene 5 protein) | VFVIYMALLHLWVMIVLLTY                        |
| Q53EP0 | Fibronectin type III domain-containing protein 3B (Factor for adipocyte differentiation 104) (HCV NS5A-binding protein 37) | IIVLGFATLSILFAILQYFL                        |
| Q13323 | Bcl-2-interacting killer (Apoptosis inducer NBK) (BIP1) (BP4)                                  | VLLALLLLLALLLPLLSGGLH                       |
| Q9Y6F6 | Protein MRVII (Inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate) (JAW1-related protein MRVII) | WQVIWMMAAVMLVTVVLGLY                       |
| Q8N912 | Nutritionally-regulated adipose and cardiac enriched protein homolog                          | GGSLLLQLCVCVLLVLALGLY                       |
| Q13948 | Protein CASP                                                                                  | IGFFYTLFLHCLVFLVLYKLA                       |
| Q8WXI7 | Mucin-16 (MUC-16) (Ovarian cancer-related tumor marker CA125) (CA-125) (Ovarian carcinoma antigen CA125) | FWAVILG[LLG]VTCLIC                         |
| Q14789 | Golgin subfamily B member 1 (372 kDa Golgi complex-associated protein) (GCP372) (Giantin) (Macrogolgin) | VPLLAAIYFLM[II]LICFT                      |
| Accession | Description                                                                 | Sequence                        |
|-----------|------------------------------------------------------------------------------|---------------------------------|
| Q8WXH2    | Junctophilin-3 (JP-3) (Junctophilin type 3) (Trinucleotide repeat-containing gene 22 protein) | LVVMVILLNIGVAILFINFFI          |
| Q8TC41    | Probable E3 ubiquitin-protein ligase RNF217 (EC 2.3.2.-) (IBR domain-containing protein 1) (RING finger protein 217) | LIMVLGLALGAIAVVIGLFVF           |
| Q9Y6H6    | Potassium voltage-gated channel subfamily E member 3 (MinK-related peptide 2) (Minimum potassium ion channel-related peptide 2) (Potassium channel subunit beta MiRP2) | YMYILFVMFLFAVTGSLILG            |
| Q8NCQ3    | Putative uncharacterized protein encoded by LINC00301                         | SFGLAIIGILLIACEIILFLT           |
| P0DN84    | Sarcoplasmic/endoplasmic reticulum calcium ATPase regulator DWORF (SERCA regulator DWORF) (Dwarf open reading frame) (DWORF) | VIILVVCVLFLFLVLTGMPMMF          |
| P0DL12    | Small integral membrane protein 17                                            | IVLVVCVLFLFLVLTGMPMMF           |
| Q86Z14    | Beta-klotho (BK) (BetaKlotho) (Klotho beta-like protein)                      | LIFLGCFFSTLVLILLSIAIF           |
| P59025    | Receptor-transporting protein 1 (3CxxC-type zinc finger protein 1)             | IPWCLFWATVLLLIIYLQFSF           |
| Q5QGT7    | Receptor-transporting protein 2 (3CxxC-type zinc finger protein 2)             | LSLRWCLFWASLCLLVVLQYF           |
| Q6ZS82    | Regulator of G-protein signaling 9-binding protein (RGS9-anchoring protein)   | ALAAILFGAVLLAVALAVCV            |
| Q9NYM9    | BET1-like protein (Golgi SNARE with a size of 15 kDa) (GOS-15) (GS15) (Vesicle transport protein GOS15) | LLCGMAVGLIVAFFILSYFLS           |
| Q9NRQ5    | Single-pass membrane and coiled-coil domain-containing protein 4 (Protein FN5) | TVVLPVVVLLLVVVFVYVA             |
| Q0VAQ4    | Small cell adhesion glycoprotein (Small transmembrane and glycosylated protein) | IAVVITVVFLLTLLSVVIIIYFF        |
| Accession | Description                                                                 | Sequence                                                                 |
|-----------|------------------------------------------------------------------------------|-------------------------------------------------------------------------|
| Q96AG4    | Leucine-rich repeat-containing protein 59 (Ribosome-binding protein p34) (p34) | WAVLKLPLLLLLFGVAGGLVA                                                   |
| Q9BR39    | Junctophilin-2 (Junctophilin type 2)                                          | ILICMVILLNIGLAILFVHLL                                                   |
| P23763    | Vesicle-associated membrane protein 1 (VAMP-1) (Synaptobrevin-1)              | MMIMLGAICAIIVVVIVIYF                                                   |
| Q96JN2    | Coiled-coil domain-containing protein 136 (Nasopharyngeal carcinoma-associated gene 6 protein) | IFSLPLVGLVVISALLWCWWA                                                  |
| P51809    | Vesicle-associated membrane protein 7 (VAMP-7) (Synaptobrevin-like protein 1) (Tetanus-insensitive VAMP) (Ti-VAMP) | LTIIIIIVSVIFIIYIIVSPLC                                                |
| Q9BQQ7    | Receptor-transporting protein 3 (3CxxC-type zinc finger protein 3) (Transmembrane protein 7) | SIFCCCVILIVIVVIVKVTAI                                                |
| O00631    | Sarcolipin                                                                    | LFLNFTIVLITVIJMWWLL                                                   |
| A6NCQ9    | RING finger protein 222                                                       | LITLIAVVAVVAAILPWVLL                                                   |
| Q8WWP7    | GTPase IMAP family member 1 (Immunity-associated protein 1) (hIMAP1)           | SWRLGLALLLGALLFWVLL                                                   |
| Q96D05    | Uncharacterized protein C10orf35                                              | ILLLFLLMLGVRGLLLVGLV                                                  |
| Q9Y2H6    | Fibronectin type-III domain-containing protein 3A (Human gene expressed in odontoblasts) | ILVLFADFSILIAIIQYFVI                                                  |
| P04921    | Glycophorin-C (Glycoconnectin) (Glycophorin-D) (GPD) (Glycoprotein beta) (PAS-2') (Sialoglycoprotein D) (CD antigen CD236) | DIVVIAGVIAAVAILVLSLLFVML                                               |
| Q8N8N0    | E3 ubiquitin-protein ligase RNF152 (EC 2.3.2.27) (RING finger protein 152) (RING-type E3 ubiquitin transferase RNF152) | SGVCTVILVACVLFLLGIVL                                                 |
| Accession | Description                                                                 | Seq                             |
|-----------|-----------------------------------------------------------------------------|---------------------------------|
| A6NNC1    | Putative POM121-like protein 1-like                                          | LGLLFLVSFFLLTTWASFSF            |
| Q12912    | Lymphoid-restricted membrane protein (Protein Jaw1) [Cleaved into: Processed lymphoid-restricted membrane protein] | ALWLSIAFIVLFAALMSFLTG           |
| P61566    | Endogenous retrovirus group K member 24 Env polyprotein (Envelope polyprotein) (HERV-K101 envelope protein) (HERV-K_22q11.21 provirus ancestral Env polyprotein) [Cleaved into: Surface protein (SU); Transmembrane protein (TM)] | IGSTTIINLILILVCLFCLLL           |
| P61567    | Endogenous retrovirus group K member 7 Env polyprotein (Envelope polyprotein) (HERV-K(III) envelope protein) (HERV-K102 envelope protein) (HERV-K_1q22 provirus ancestral Env polyprotein) [Cleaved into: Surface protein (SU); Transmembrane protein (TM)] | IGSTTIINLILILVCLFCLLL           |
| Q8N6L0    | Protein KASH5 (Coiled-coil domain-containing protein 155) (KASH domain-containing protein 5) | LIPAPVLGLLLLLLSSVLLLGL         |
| Q8N6Q1    | Transmembrane and coiled-coil domain-containing protein 5A                   | IFCCLFFITLFFIRLLSYMFF           |
| Q12981    | Vesicle transport protein SEC20 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 1) | TDKLLIFLALALFLATVLYIV           |
| P59773    | UPF0258 protein KIAA1024-like                                               | GLILLVVISILVTIVTIITFF           |
| Q8WXH0    | Nesprin-2 (KASH domain-containing protein 2) (KASH2) (Nuclear envelope spectrin repeat protein 2) (Nucleus and actin connecting element protein) (Protein NUANCE) (Synaptic nuclear envelope protein 2) (Syne-2) | AALPLQLLLLLLLLACLLPS           |
| Accession | Description                                                                 | Sequence                                      |
|-----------|------------------------------------------------------------------------------|-----------------------------------------------|
| Q8WVX3    | Uncharacterized protein C4orf3 (Hepatitis C virus F protein-transactivated protein 1) (HCV F-transactivated protein 1) | WLDLWFLFDVVVFVFVYFL                           |
| P32856    | Syntaxin-2 (Epimorphin)                                                      | WIIAVSVVLVAIALIIGLSVGK                       |
| Q6IEE8    | Schlafen family member 12-like                                               | IFLFVCLFRFCFLVCWFVCFF                        |
| Q8NHP6    | Motile sperm domain-containing protein 2                                     | LLLLSTMLLAFVTSFFYL                            |
| O95292    | Vesicle-associated membrane protein-associated protein B/C (VAMP-B/VAMP-C) (VAMP-associated protein B/C) (VAP-B/VAP-C) | RLLALVVLFFIVGVIIGKIAL                        |
| Q96QK8    | Small integral membrane protein 14                                           | GISVTMILVAWMVIALILFLL                        |
| Q96JQ2    | Calmin (Calponin-like transmembrane domain protein)                          | MMYFILFLWLTVYCLLFLPQL                       |
| Q71RC9    | Small integral membrane protein 5                                            | IVAFSVIILFTATVLLLLLIA                        |
| A2A2Y4    | FERM domain-containing protein 3 (Band 4.1-like protein 4O) (Ovary type protein 4.1) (4.1O) | LLVVGLGLLFLVFPLLLE                           |
| O42043    | Endogenous retrovirus group K member 18 Env polyprotein (Envelope polyprotein) (HERV-K(C1a) envelope protein) (HERV-K110 envelope protein) (HERV-K18 envelope protein) (HERV-K18 superantigen) (HERV-K_1q23.3 provirus ancestral Env polyprotein) (IDDMK1.2 22 envelope protein) (IDDMK1.2 22 superantigen) [Cleaved into: Surface protein (SU); Transmembrane protein (TM)] | IRSTMIIILIVVCLFCLL |
| P50402    | Emerin                                                                       | VPLWGQLLLLFLVFIVLFFIY                        |
| Q96D59    | Probable E3 ubiquitin-protein ligase RNF183 (EC 2.3.2.27)                    | IFAYLMAVILSVTLLLIFSIF                       |
| Accession | Description                                                                 | Sequence            |
|-----------|------------------------------------------------------------------------------|---------------------|
| Q9P2W9    | Syntaxin-18 (Cell growth-inhibiting gene 9 protein)                          | AGFRVWILFFLVMCSFSLFL |
| Q01629    | Interferon-induced transmembrane protein 2 (Dispanin subfamily A member 2c) | IWALILGIFMTILLIIPVLI |
| Q86Y82    | Syntaxin-12                                                                 | KKMCILVLVLSVIIILILGII |
| Q01628    | Interferon-induced transmembrane protein 3 (Dispanin subfamily A member 2b) | IWALILGILMTILLIVIPVLI |
| P0C2S0    | Cortexin-2                                                                   | TGFAFVGILCIFLGLLIIRCFC |
| O75396    | Vesicle-trafficking protein SEC22b (ER-Golgi SNARE of 24 kDa)                | KLAAVAVFFIMLYVRFWWL |
| P42167    | Lamina-associated polypeptide 2, isoforms beta/gamma (Thymopoietin, isoforms | IPVWIKILLFVVAVFLFLVYQAM |
|           | beta/gamma) (Thymopoietin-related peptide isoforms beta/gamma) (TP isoforms |                                                                  |
|           | beta/gamma) (Thymopoietin-related peptide isoforms beta/gamma) (TP isoforms |                                                                  |
|           | beta/gamma)                                                                   |                                                                  |
| P13164    | Interferon-induced transmembrane protein 1 (Dispanin subfamily A member 2a) | IWALILGILMTIGFILLLVFG |
| Q0VDE8    | Adipogenin                                                                   | FSFLVFWFCLPVGLLLLIIW |
| Q9BZL3    | Small integral membrane protein 3 (NGF-induced differentiation clone 67 protein) (Small membrane protein NID67) | IIVIVLIIATIVMTSLLLLCC |
| Accession | Description                                                                 | Sequence                       |
|-----------|-----------------------------------------------------------------------------|--------------------------------|
| Q96AJ9    | Vesicle transport through interaction with t-SNAREs homolog 1A (Vesicle transport v-SNARE protein Vti1-like 2) (Vti1-rp2) | ILLVILGIIIVVITILMAITFS         |
| O75379    | Vesicle-associated membrane protein 4 (VAMP-4)                               | IKAIMALVAAIILLVIILIV           |
| O95159    | Zinc finger protein-like 1 (Zinc finger protein MCG4)                         | LLLLLLGFLALLALMSRLG            |
| Q86Y07    | Serine/threonine-protein kinase VRK2 (EC 2.7.11.1) (Vaccinia-related kinase 2) | VYYYRIIPVWLMLVFLALFF           |
| Q86W74    | Ankyrin repeat domain-containing protein 46 (Ankyrin repeat small protein) (ANK-S) | LGFWRVLLLIFVIALSLGIA          |
| Q629K1    | Triple QxxK/R motif-containing protein (Triple repetitive-sequence of QXXK/R protein homolog) | VGLVLAAILALLAFYAFFYL           |
| P0DKX4    | Small integral membrane protein 18                                          | CFVIIILLFIITVSVLVLAFL          |
| Q8N8F7    | Leucine-rich single-pass membrane protein 1                                  | VGLLIVLIVSLALVFFVIFLI          |
| O15155    | BET1 homolog (hBET1) (Golgi vesicular membrane-trafficking protein p18)       | KLLCYMMLFLFVFIIYWI             |
| P63027    | Vesicle-associated membrane protein 2 (VAMP-2) (Synaptobrevin-2)             | MIIIILGVICAIIIIVYF             |
| O15400    | Syntaxin-7                                                                   | CIIILILVIGVAAISLIIWGL          |
| A2A2V5    | Serine-rich and transmembrane domain-containing protein 1                    | IYVSIFLSALALLLLLIIAL          |
| Q6ZMZ3    | Nesprin-3 (KASH domain-containing protein 3) (KASH3) (Nuclear envelope spectrin repeat protein 3) | VALPLQLLLLLFLLLLLFLLPPI      |
| O60499    | Syntaxin-10 (Syn10)                                                          | WCAIAVLGVLLLVLILLFSL           |
| P60606    | Cortexin-1                                                                   | TVFAFVCLLVLVLVMVRCV            |
| Q13277    | Syntaxin-3                                                                   | LIIIIVLVVVLLGILALIIGL         |
| Accession | Description                                                                 | Sequence |
|-----------|------------------------------------------------------------------------------|----------|
| O95183    | Vesicle-associated membrane protein 5 (VAMP-5) (Myobrevin)                   | VGLVVGVLLIIILIVLVVFL |
| Q7Z6J6    | FERM domain-containing protein 5                                             | LLLVMGLLVLLLIIILTE |
| Q15836    | Vesicle-associated membrane protein 3 (VAMP-3) (Cellubrevin) (CEB) (Synaptobrevin-3) | MWAIGITVLIIFIIIIIVVV |
| Q4LDR2    | Cortexin-3 (Kidney and brain-expressed protein)                              | MTFVFVILLFIFGLILIVRCF |
| A9Z1Z3    | Fer-1-like protein 4                                                         | LVLLLVLLTVFLLLVFYHIP |
| Q9HCU5    | Prolactin regulatory element-binding protein (Mammalian guanine nucleotide exchange factor mSec12) | VPVVLLLLLCVGLIIVTILL |
| Q8N112    | Leucine-rich single-pass membrane protein 2                                  | GFLLLLALLVLTCLVLALLAV |
| Q13190    | Syntaxin-5                                                                   | WLMVKIFLILIVFIIFVFL |
| P0DI80    | Small integral membrane protein 6                                            | LAVIIIFMATAVLLLILFAIVF |
| Q96F15    | GTPase IMAP family member 5 (Immunity-associated nucleotide 4-like 1 protein) (Immunity-associated nucleotide 5 protein) (IAN-5) (hIAN5) (Immunity-associated protein 3) | IFVFLLLCSILFIIFLIFIFH |
| Q9NX77    | Endogenous retrovirus group K member 13-1 Env polyprotein (Envelope polyprotein) (HERV-K_16p13.3 provirus ancestral Env polyprotein) [Cleaved into: Surface protein (SU); Transmembrane protein (TM)] | GSLLLLALLILVCLCLLLVL |
| Q8N205    | Nesprin-4 (KASH domain-containing protein 4) (KASH4) (Nuclear envelope spectrin repeat protein 4) | FLLILFLLLLVGAMFLLPA |
| P26678    | Cardiac phospholamban (PLB)                                                  | FINFCLILICLLLICIIVMLL |
| O14662    | Syntaxin-16 (Syn16)                                                          | MLVILILFVIIIVLIVVVGLV |
| Accession | Description                                                                 | Sequence                                                                 |
|-----------|------------------------------------------------------------------------------|--------------------------------------------------------------------------|
| Q9BV40    | Vesicle-associated membrane protein 8 (VAMP-8) (Endobrevin) (EDB)             | MIVLICVIFIIIIFIVLFAT                                                     |
| Q9NZ43    | Vesicle transport protein USE1 (Putative MAPK-activating protein PM26) (USE1-like protein) (p31) | WLLWAMLIIICFIISMILFI                                                     |
| O43752    | Syntaxin-6                                                                   | WCAIAILFAVLLVVLILFLVL                                                    |
| Q9NZM1    | Myoferlin (Fer-1-like protein 3)                                              | WVIIGLLFLILLLFVAVLLY                                                     |
| O75923    | Dysferlin (Dystrophy-associated fer-1-like protein) (Fer-1-like protein 1)    | IILFIIFILLFLAIFIYAF                                                      |
| Q2WGJ9    | Fer-1-like protein 6                                                          | IIIAFILIIFLVLFIYTL                                                       |
| Q9HC10    | Otoferlin (Fer-1-like protein 2)                                              | WLLKLLLLLLLLLLLLALFLY                                                    |

TMD: transmembrane domain.
### Supplemental Table 4. Animal models for ASNA1-mediated TA protein insertion related genes.

| Gene   | Synonyms | Species          | Genotype                     | Mechanism     | Phenotype                                                | Refs |
|--------|----------|------------------|------------------------------|---------------|----------------------------------------------------------|------|
| WRB    | CHD5, GET1 | *Mus musculus*   | Wrb<sup>tm1.1(KOMP)Vlcg</sup>/Wrb<sup>tm1.1(KOMP)Vlcg</sup> | homozygous ko | embryonic lethality (<E9.5)                              | 15   |
|        |          | *Mus musculus*   | Wrb<sup>tm1.1(KOMP)Vlcg</sup>/Wrb<sup>+</sup> | heterozygous ko | abnormal brain development                                | 13   |
|        |          | *Mus musculus*   | Wrb<sup>fl/fl</sup>:Vglut3-Cre | conditional ko | progressive hearing impairment, tonic-clonic seizures    | 16   |
|        |          | *Mus musculus*   | Wrb<sup>fl/fl</sup>:Vglut3-ires-Cre | conditional ko | progressive hearing impairment, tonic-clonic seizures    | 16   |
|        |          | *Danio rerio*    | wrb<sup>hi1482Tg</sup>          | homozygous ko | abnormal myocardial repolarization, bradycardia          | 17   |
|        |          | *Danio rerio*    | wrb<sup>hi1482Tg</sup>; nl1Tg   | homozygous ko | reduced auditory startle response, reduced visual evoked potentials | 18   |
|        |          | *Danio rerio*    | lri<sub>48Tg</sub>; wrb<sup>hi1482Tg</sup> | homozygous ko | photoreceptor synapse defects                            | 19   |
|        |          | *Danio rerio*    | q16aTg; q16bTg; wrb<sup>hi1482Tg</sup>; wrb<sup>hi1482Tg</sup> | homozygous ko | impaired hair cell exocytosis and hearing                | 16   |
|        |          | *Oryzias latipalis* | WT + MO chd5 (ATG)                        | knockdown     | cardiac looping defects, abnormal chamber differentiation, ocular abnormalities | 20   |
| Organism       | Gene Symbol(s) | Strain/Condition | Knockdown | Phenotype Description                                                                 |
|---------------|---------------|------------------|-----------|--------------------------------------------------------------------------------------|
| *Xenopus tropicalis* | Tg (actc1:GFP)$^{MO}$ + MO chd5 (ATG) | Tg (actc1:GFP)$^{MO}$ + MO chd5 (SB) | knockdown | cardiac looping defects, abnormal chamber differentiation                               |
| *CAMLG*       | *CAML, GET2*  | *Caml*tm1Rjb/Caml*tm1Rjb | homozygous ko | embryonic lethality (E4.5-E7.5)                                                      |
| *Mus musculus* |               | *Caml*tm1Rjb/Caml*tm2Rjb | conditional ko | abnormal T-cell development                                                           |
| *ASNA1*       | *TRC40, GET3* | *Asna1*tm1Hbha/Asna1*tm1Hbha | homozygous ko | embryonic lethality (E3.5-E8.5)                                                      |
| *Danio rerio* | q16aTg ; q16bTg + MO1 asna1 WT + MO1 asna1 (ATG) | WT + MO2 asna1 (SB) | knockdown | decreased visual perception, photoreceptor synapse defects, lack of swim bladder      |
| *Danio rerio* | asna1∆/∆    |                        | homozygous ko | impaired swim bladder inflation, decreased blood flow in dorsal aorta, impaired cardiac contractility, early lethality (6-8 dpf) |

ATG, translation-blocking; dpf, days post fertilization; E, embryonic day; ko, knock-out; MO, morpholino; SB, splice-blocking; WT, wild-type.
**SUPPLEMENTAL FIGURES**

**Supplemental Figure 1. CRISPR/Cas9-induced asna1 deletion in zebrafish.**

(A) Schematic representation of guide RNA target site (asna1 exon 5). Protospacer is highlighted in cyan; PAM in red. Bsrl recognition site used for genotyping is underlined. (B) Sequence and position of induced 7 bp deletion (∆7) predicted to result in a frameshift and premature stop codon. (C) Chromatogram of PCR-amplified DNA from F1 fish showing wild-type and mutant sequence (reverse complement). The arrow indicates the position of the deletion.

**A**

5’-GACATGAATGCAGATCAGCTGGCGTACTGGAAGACGCTGCTGTCATCCGCTCGTCAGCGAGCAG-3’

**B**

5’-GACATGAATGCAGATCAGCTGGCGTCCAAACTGGAGAGACGCTGCTGTCATCCGCTCGTCAGCGAGCAG-3’ WT

| D | M | N | A | D | Q | L | A | S | K | L | E | E | T | L | P | V | I | R | S | V | S | E | Q |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

5’-GACATGAATGCAGATCAGCTGGCGTGGGAGACGCTGCTGTCATCCGCTCGTCAGCGAGCAG-3’ ∆7

| D | M | N | A | D | Q | L | A | W | R | R | C | L | S | S | A | P | S | (missing 48 AA) |

**C**

G C A G C G T C T C T C C A S K Y Y G G A Y G A C C K G T G A T C
Supplemental Figure 2. Lateral views of the heart in wild-type and mutant zebrafish.

Microscopic images of the heart in wild-type and *asna1^{Δ7/Δ7}* zebrafish larvae. The atria and ventricles are marked as A and V, respectively. The bulbus arteriosus (outflow tract) is marked as BA.
Supplemental Figure 3. M-mode imaging in both patients.

M-mode image of the heart in parasternal long axis view in (A) patient II:2 and (B) patient II:3 showing severely reduced left ventricular contractility.

A

B
Supplemental Figure 4. Electrocardiography recordings of both patients.

(A) ECG of patient II:2 during hospital admission showing sinus rhythm at a rate of 130/min with extremely broad QRS complexes of 220 ms and normal QRS axis of 60 degrees. (B) ECG of patient II:3 during cardiopulmonary resuscitation (no prior ECG available).
**Supplemental Figure 5. Recombinant expression and purification of ASNA1 from E. coli.**

(A) Expression tests of *E. coli* transformed with plasmids encoding either wild-type ASNA1 or the Val163Ala mutant. In each case, equal numbers of cells harvested before or after induction with 1 mM IPTG (for 3 hours at 37°C) were analyzed by SDS-PAGE and staining with Coomassie Blue. Two individual isolates of wild-type and four of mutant ASNA1 all show comparable expression levels of recombinant ASNA1 (indicated by the arrow). (B) The cells from a larger scale induction of wild-type and mutant ASNA1 (as in panel A) were collected, lysed by sonication, and subjected to chromatography using Ni-NTA columns. The total cells, soluble lysate, flow through, and elution fractions are shown. Note that a substantially higher proportion of wild-type ASNA1 is produced as a soluble protein, and recovered by chromatography. This is a consistent effect observed in more than six independent trials. (C) Increasing amounts of purified wild-type or mutant ASNA1 (ranging from 100 ng to 1 µg protein) were analyzed by SDS-PAGE and Coomassie staining to document concentration and purity. (D) A model TA protein containing the transmembrane domain from VAMP2 was translated in a purified *E. coli*-based translation system. This system contains only recombinant translation factors and ribosomes, with no additional proteins. In addition, it contains 35S-methionine to label the newly synthesized TA protein, and the photo-crosslinking amino acid benzyl-phenylalanine (BPA) and components for its incorporation at amber codons. A single amber codon in the transmembrane domain of the TA protein is used to incorporate this photo-crosslinking amino acid. The translation was supplemented with either wild-type or mutant ASNA1, which forms a complex with the newly made TA protein. The successful formation of the TA-ASNA1 complex was verified by UV irradiation to induce a covalent crosslink between these two proteins (indicated by “x ASNA1”). These recombinant TA-ASNA1 complexes were used for the insertion assay shown in Figure 4D.
SUPPLEMENTAL VIDEOS

1. Cardiac ultrasound examination in patient II:2 showing poor contractility and thrombus formation in the left ventricle prior to death at age 7 weeks.

2. Cardiac ultrasound examination in patient II:3 showing minor abnormalities at age 9 days.

3. Repeat examination in patient II:3 on day 12 showing ventricular dysfunction and dilatation.

4. Microscopic imaging of blood flow velocity in wild-type and asna1Δ7/Δ7 zebrafish larvae.

5. Microscopic imaging of heart contractions in wild-type and asna1Δ7/Δ7 zebrafish larvae.
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