Stx4 is required to regulate cardiomyocyte Ca^{2+} handling during vertebrate cardiac development

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Summary

Requirements for vesicle fusion within the heart remain poorly understood, despite the multitude of processes that necessitate proper intracellular trafficking within cardiomyocytes. Here, we show that Syntaxin 4 (STX4), a target-Soluble N-ethylmaleimide sensitive factor attachment receptor (t-SNARE) protein, is required for normal vertebrate cardiac conduction and vesicular transport. Two patients were identified with damaging variants in STX4. A patient with a homozygous R240W missense variant displayed biventricular dilated cardiomyopathy, ectopy, and runs of non-sustained ventricular tachycardia, sensorineural hearing loss, global developmental delay, and hypotonia, while a second patient displayed severe pleiotropic abnormalities and perinatal lethality. CRISPR/Cas9-generated stx4 mutant zebrafish exhibited defects reminiscent of these patients’ clinical presentations, including linearized hearts, bradycardia, otic vesicle dysgenesis, neuronal atrophy, and touch insensitivity by 3 days post fertilization. Imaging of Vamp2+ vesicles within stx4 mutant zebrafish hearts showed reduced docking to the cardiomyocyte sarcolemma. Optical mapping of the embryonic hearts coupled with pharmacological modulation of Ca^{2+} handling together support that zebrafish stx4 mutants have a reduction in L-type Ca^{2+} channel modulation. Transgenic overexpression of zebrafish Stx4^{R241W}, analogous to the first patient’s STX4^{R240W} variant, indicated that the variant is hypomorphic. Thus, these data show an in vivo requirement for SNAREs in regulating normal embryonic cardiac function and that variants in STX4 are associated with pleiotropic human disease, including cardiomyopathy.

Introduction

Soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) proteins bridge the vesicle and plasma membrane and are crucial for processes involving intracellular membrane fusion, including neurotransmitter release.1 In humans, variants in the loci of the SNARE proteins SNAP25 (MIM: 600322), VAMP1 (MIM: 185880), VAMP2 (MIM: 185881), and several of their cognates have been reported to result in synaptopathies, a spectrum of diseases that typically manifest in an encephalopathy phenotype composed of severe neural defects, including hypokinesia and epilepsy.2-13 While these syndromes have recently been collectively termed “SNAREopathies,”3 SNAREs ubiquitously regulate vesicle fusion in virtually all cell types by mediating the fusion and subcellular localization of various transmembrane and exocytic components to their membrane targets.14 Therefore, many other extra-neuronal disease pathologies may manifest as “SNAREopathies.” Cardiomyocytes (CMs) are among the most specialized cell types owing to a high degree of organization and the dynamic requirements for their diverse functions. Remarkably, while it has long been known that CMs heavily rely on intracellular membrane trafficking, few bona fide trafficking proteins have been identified as having specific functions in the heart.15-16 SNAREs are a promising candidate for the elucidation of how cardiac intracellular trafficking is regulated. Notably, previous in vitro experiments suggest that SNAREs may regulate cardiac ion channel vesicle fusion, sensitivity, or gating behavior.17-23 Furthermore, all voltage gated Ca^{2+} channels (VGCCs) are reported to physically interact with the...
vesicle fusion machinery via common, conserved SNARE-interacting “synprint” domains, \(^\text{17,19}\) although to date an interaction with cardiac VGCCs has not been examined. \(^\text{19}\)

Here, we report an association between SNARE function within the heart and cardiovascular disease. We identified two patients with unique biallelic pathogenic variants in \textit{Syntaxin 4} (\textit{STX4} [MIM: 186591]) that showed pleiotropic defects, including one patient with early-onset biventricular dilated cardiomyopathy (DCM) that ultimately necessitated heart transplant. Engineered zebrafish \textit{stx4} mutant embryos also have pleiotropic defects, including profound myocardial dysfunction and bradycardia. Within CMs, we found that zebrafish \textit{stx4} mutants exhibit a significant reduction in their number of docked vesicles. Mechanistically, optical mapping demonstrated that \textit{stx4} mutants have aberrant Ca\(^{2+}\) handling. Concordantly, \textit{stx4} mutant hearts are highly sensitized to pharmacological inhibition of L-type Ca\(^{2+}\) channels (LTCCs), while agonism of LTCCs can rescue the bradycardia. Transgenic lines expressing the zebrafish \textit{Stx4R421W} variant (analogous to patient 1’s variant) were unable to fully rescue \textit{stx4} mutants, suggesting that the patient’s \textit{Stx4R240W} allele is hypomorphic. Collectively, these data provide evidence for a conserved requirement for \textit{Stx4} variants in vertebrate cardiac conduction, which may provide mechanistic insights into the etiology of the cardiomyopathies found in humans with damaging \textit{STX4} variants.

Material and methods

Ethics statement

Approval of research on human subjects was obtained from the institutional review boards at Cincinnati Children’s Hospital Medical Center and the Ethics Committee of the University Children’s and Women’s Center/University Hospital Carl Gustav Carus at the Technische Universität Dresden performed in compliance with the Declaration of Helsinki, and conformed to the laws and regulations of the respective countries and institutions. Written consent was obtained for all patient images. All zebrafish husbandry and experimental procedures were performed in accordance with approved Institutional Animal Care and Use Committee protocols at the Cincinnati Children’s Hospital Medical Center and the Harvard Medical Area Standing Committee on Animals.

Genetic investigations

For the index patient (patient 1), a clinical SNP microarray (Infinium CytoSNP-850K v1.1 BeadChip [Illumina]) was performed following local protocols by the Cincinnati Children’s Hospital Medical Center cytogenetics lab. Mitochondrial DNA sequencing and clinical whole-exome sequencing (WES) was subsequently performed using a trio-based strategy by MNG Laboratories (Atlanta, GA). WES data processing, sequence alignment to GRCh37, variant filtering, prioritization by allele frequency, prediction of functional impact, and inheritance models were performed using previously reported analysis pipelines. \(^\text{24}\)

Targeted variant testing was performed on patient 1’s unaffected sibling. Testing was approved by the Cincinnati Children’s Hospital Medical Center internal Institutional Review Board (protocol #2020-0390). DNA was extracted from the sibling’s saliva using a DNeasy Blood & Tissue Kit (Qiagen, 69,504). Extracted DNA from patient 1 and DNA from an unaffected individual served as positive and negative controls, respectively. Forward and reverse primers were designed to flank patient 1’s variant (Table S3). PCR products were concentrated using a DNA Clean & Concentrator 5 (Zymo Research). Amplicons were confirmed by Sanger sequencing. Chromatograms were manually reviewed.

For patient 2, prenatal trio-WES was performed clinically on the fetus and on the parents. Trio analysis: Variants found in the patient and in the patient’s parents were compared and filtered for three cases: \textit{de novo} in the patient, patient is compound heterozygous, patient is homozygous, and the parents are heterozygous. The coding and flanking intronic regions were enriched using in solution hybridization technology and were sequenced using the HiSeq/NovaSeq system (Illumina). Copy number variations (CNV) were computed on uniquely mapping, non-duplicate, high-quality reads using an internally developed method based on sequencing coverage depth. Briefly, reference samples were used to create a model of the expected coverage that represents wet-lab biases as well as inter-sample variation. CNV calling was performed by computing the sample’s normalized coverage profile and its deviation from the expected coverage. Genomic regions are called as variant if they deviate significantly from the expected coverage. Illumina bcl2fastq2 was used to demultiplex sequencing reads. Adapter removal was performed with Skewer. \(^\text{25}\) The trimmed reads were mapped to the human reference genome (hg19) using the Burrows Wheeler Aligner. \(^\text{26}\) Reads mapping to more than one location with identical mapping scores were discarded. Read duplicates that likely resulted from PCR amplification were removed. The remaining high-quality sequences were used to determine sequence variants (single nucleotide changes and small insertions/deletions). Only variants (single nucleotide variants [SNVs]/small indels) in the coding region and the flanking intronic regions (±8 base pairs [bp]) with a minor allele frequency (MAF) <1% were evaluated. Known disease-causing variants (according to the Human Gene Mutation Database) were evaluated in up to ±30 bp of flanking regions and up to 5% MAF. All variants with an MAF <1% (in genes with autosomal recessive heredity) or <0.1% (in genes with dominant heredity) were evaluated, not including variants classified as benign or likely benign according to current literature. \textit{In silico} prediction of variants was calculated on the basis of the output of the program’s Mutation Taster, \(^\text{27}\) fathmm/fathmm-MKL coding, \(^\text{28}\) Mutation Assessor, \(^\text{29}\) SIFT, \(^\text{30}\) LRT, \(^\text{31}\) and PROVEAN \(^\text{32}\) according to the following criteria: 100% consensus = pathogenic/benign, ≥75% consensus = mostly pathogenic/benign, consensus <75% or no prediction possible = inconsistent. SpliceAI was used to evaluate the consequence of variants on splicing (default [high precision] thresholds: 0.8-1 "splice effect," 0.6-0.8 "possible splice effect," <0.6 "no splice effect"). \(^\text{33}\) For variants within a 0.5 delta score range cutoff with functional analysis unavailable to confirm a predicted splice effect, additional \textit{in silico} predictions were employed, including MaxEntScan, \(^\text{34}\) Combined Annotation Dependent Depletion (CADD), \(^\text{35}\) and MutationTaster. \(^\text{27}\) All variants were classified and reported based on American College of Medical Genetics and Genomics (ACMG)/Association for Clinical Genomic Sciences-2020v4.01 guidelines. \(^\text{36}\)

Zebrafish husbandry/mutant and transgenic lines used

Adult zebrafish (\textit{Danio rerio}) were maintained under standard laboratory conditions. \(^\text{37}\) The zebrafish \textit{stx4R1016} mutant allele was used. The following zebrafish transgenic lines were used: \textit{Tg(-5.1myl7:dsRed2-NLS)57,58 TgBAC(neurod:EGFP)57,59}\n
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Generation of transgenic lines
The Tg(actb2:stx4-IRES-EGFP) transgenic line was created using standard Gateway cloning methods and Tol2 mediated transgenesis. 

Generation of stx4 mutant line
The stx4 mutant allele was created with CRISPR-Cas9, using standard methods. 

In situ hybridization
Whole-mount in situ hybridization (ISH) was performed using NBT/BCIP (Roche, 11383213001 and 11383221001) according to previously established protocols. 

Immunohistochemistry and CM quantification
Immunohistochemistry (IHC) and CM quantification were performed as previously described. 

Quantification of Vamp2 vesicles
To quantify Vamp2 positive vesicles, embryos were labeled with anti-Vamp2, anti-Alcama, and DAPI and mounted between two coverslips, before being imaged using an inverted, motorized Nikon Eclipse Ti microscope equipped with a GaAsP PMT, an HD dual resonant/galvanometric scanner, and an excitation range from 405 to 640 nm, using a Plan Apo 10x/0.45 DIC L; Apo dual resonant/galvanometric scanner, and an excitation range. 

inside each surface that overlap (Vamp2-labeled vesicles and Alcama-labeled membrane). The resulting new surface generated from these overlapping regions was generated from this Xten- sion and tabulated, as a surrogate for vesicle docking.

O-dianisidine heme staining
Larval zebrafish were developed in heme staining solution (2.5 mM o-dianisidine, 10 mM sodium acetate pH 5, 0.65% hydrogen peroxide [v/v %], 40% ethanol [v/v %] in water) for 15 min, washed with PBST, subsequently fixed overnight at 4°C in 4% paraformalde- hyde, and washed and stored in PBST, as previously described,54 before being imaged by brightfield using a Zeiss M2BioV12 stereomicroscope.

Heart rate measurements
For heart rate measurements, 72 h postfertilization (hpf) larvae were anesthetized with 0.16 mg/mL tricaine (MS-222) and immo- bilized in 15-well angiogenesis µ-Slides using 1% low melt agarose in embryo water (Milli-Q [Millipore] water supplemented with 0.0001% methylene blue [w/w %] and 0.03% Instant-ocean [w/v %]). Larvae were assayed in embryo water or with drugs at concentrations described below. Hearts were imaged by phase contrast using a Plan Fluor 20x/0.5 Ph1 DDL objective on an inverted, motor- ized Nikon Ti-2 SpectraX Widefield microscope equipped with an Andor Xyla 4.2 megapixel, 16-bit sCMOS monochromatic camera. Larvae were equilibrated for 5 min at thermoneutral conditions (28.5°C) in a temperature-controlled chamber prior to high-speed time-lapse imaging. For each acquisition, 500 frames were captured at 2x2 binning with a 17- to 20-ms exposure, using a 12-bit rolling shutter and a 540 MHz readout. Heart rates were quantified as beats per minute from kymographs obtained from ventricular regions of interest of time measurements that were re- corded using NIH Elements.

Drug treatments
At 72 hpf, larvae were treated with the following: 500 µM (±)-isopropenyl hydrochloride (Sigma Aldrich, I5627); 100 nM, 1 µM, 5 µM, or 10 µM of the L-type Ca²⁺ channel blocker nifedipine (Sigma Aldrich, N7634); 20 µM of LTCC agonist (±)-Bay K-8644 (AG Scientific, B-1019); 10 µM thapsigargin (Sigma Aldrich, T9033) and 10 mM of Ca²⁺ channel antagonist NNC 55-0396 dihydrochloride (Tocris, 2268). All stocks were made with DMSO prior to diluting at the indicated concentrations in embryo water. Control larvae were treated with 1% DMSO in embryo water. Except for isoproterenol treatments, all larvae were treated prior to assaying heart rates for 45 min at 28.5°C, similar to what has been reported.53 For isopro- terenol treatments, heart rates were assayed both at baseline and following 30 min of treatment at 28.5°C, as previously reported.53

Calcium imaging
Embryonic hearts were explanted with calcium imaging using ra- diometric dyes and analysis performed as previously described.53 Briefly, hearts were stained for 20 min with 50 µM Fura-2,AM (Invitrogen) and washed with Normal Tyrode’s solution (NTS; 136 mM Na⁺, 5.4 mM K⁺, 1.0 mM Mg²⁺, 0.3 mM PO₄³⁻, 1.8 mM Ca²⁺, 5.0 mM glucose, and 10.0 mM HEPES, pH 7.4) at room temperature for 45 min. Individual hearts were transferred into perfusion chambers (Warner Instruments, RC-49FPS), con- taining NTS supplemented with 1 mM Cytochalasin D to inhibit contraction. Perfusion chambers were mounted on a Nikon TE- 2000 inverted microscope equipped with a 120 W metal halide lamp (X-Cite 120, Exfo). A high-speed monochromator (Opto- scan, Cairn Research Ltd, UK) was used to rapidly switch the excitation wavelength between 340 nm and 380 nm, with a bandwidth of 20 nm and at a frequency of 500 Hz. The excitation light was reflected by a 400-nm cutoff dichroic mirror with the fluores- cence emission collected through a 510/580-nm emission filter using a high-speed 80x80 pixel charge-coupled device (CCD) camera (CardioCCD-SMQ, RedShirtImaging) at 14-bit resolution to obtain four frames per acquisition, resulting in a final ratio rate of 125 Hz. For quantification, images were analyzed with MATLAB 2018b (MathWorks) using customized software, as previously described.53

Conventional histopathology and transmission electron microscopy
All biopsy tissue was processed and imaged in a CAP/CLIA-certifi- ed laboratory in the Division of Pathology at Cincinnati Child- ren’s, or in the Institute of Pathology at the University Hospital Carl Gustav Carus. For the succinic dehydrogenase staining (SDH), fresh striated muscle biopsy tissue was snap-frozen in 2-methylbutane, sectioned at a thickness of 10 µM, and stained according to standard methodologies.57 For H&E staining, tis- sues were formalin-fixed, imbedded in paraffin, sectioned to a thickness of 4 to 5 µM, and stained according to standard method- odologies.57 Brightfield images were acquired using an ScanScope XT Slide Scanner (Aperio, Leica Biosystems). For ultra- structural examination, muscle tissues were fixed in 3% glutaraldehyde in cacodylate buffer, post-fixed in 1% osmium te- xitrode, dehydrated in graded ethanol, epon LX112 resin (Ladd Research Industries Inc) embedded, semithin and ultrathin cut, stained with uranyl acetate and lead citrate, and examined on a Hitachi transmission electron microscope (H-7650; Hitachi High Technologies) equipped with a TEM CCD camera (Advanced Microscopy Techniques), in accordance with standard protocols.58

RT-qPCR
RNA was extracted from whole WT (non-transgenic) and actb2:stx4²⁴¹W-tdTomato;stx4²⁴¹W::IRES-EGFP embryos at 72 hpf using TRIzol (Invitrogen, 15596026), as previously described.59 cDNA was prepared using a SuperScript IV kit (Invitrogen, 18091200) and amplified with oligo d(T)₉₀ primers. RT-qPCR was performed using SYBR green PCR master mix (Applied Biosystems, 4368706) was performed under standard PCR conditions using a Bio-Rad CFX PCR system. Relative expres- sion levels of endogenous stx4 and transgenic stx4²⁴¹W::IRES-EGFP were run in technical triplicates, standardized to actb2 (β-actin), and quantified using the 2−ΔΔC₅₇ Livak Method.59 Primers used for stx4 (to endogenous stx4) and GFP (to stx4²⁴¹W::IRES-EGFP) are listed in Table S3.

Statistical analysis
All statistical analyses were performed using GraphPad Prism (version 9.2.0). Data are represented as mean ± SEM, unless otherwise reported in the figure legend. A minimum of four bio- logical replicates were performed for each experiment. For com- parisons between two groups, a 2-tailed Student’s t test was per- formed with the exception of the survival curve, which employed a Log rank (Mantel-Cox) test. For comparisons
Figure 1.  *STX4* germline variants in patients

(A) Left to right: Photographs of patient 1 taken before and after onset of DCM, and pre- and post-OHT.

(B) Echocardiograms at onset of patient's admission due to heart failure. Left: Apical 4-chamber view at end-diastole showed severely dilated, thin-walled left ventricle and severely dilated left and right atria. Right: parasternal short-axis view at the level of the mitral valve at end-diastole showed severely dilated, thin-walled left ventricle. The patient had severe biventricular systolic dysfunction, and evidence for diastolic dysfunction (biatrial dilation). ALPM, anterolateral papillary muscle; LA, left atrium; LV, left ventricle; PMPM, posteromedial papillary muscle; RA, right atrium; RV, right ventricle.

(legend continued on next page)
involving more than two groups, a one-way ANOVA was performed with Tukey's multiple comparison test for post hoc analysis to estimate significance of differences from multiple comparisons. For heart rate assays, each arm represents 72 hpf larvae pooled from two to four technical replicates. For all data, p ≤ 0.05 was considered significant. Within graphs p values are indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Results

Clinical phenotypes of individuals with STX4 variants

Patient 1 with a prior medical history significant for congenital sensorineural hearing loss, hypotonia, and global developmental delays initially presented with progressive fatigue, dyspnea, hypotension, and severe acidosis at 3 years of age (Figure 1A). Echocardiography showed DCM and severely depressed biventricular systolic function (Figure 1B); patient 1 developed frequent ectopy and ventricular tachycardia. Ultimately, his heart failure and arrhythmia were refractory to medical therapy, and he received an orthotopic heart transplant (OHT) within 2 months from the onset at his initial admission. Consistent with a diagnosis of DCM, post-OHT the explanted heart showed interstitial fibrosis, diminished myocardial cross-striations, loss of myofibrillar volume hypertrrophic fibers, and marked variation in fiber size (Figures S1A and S1B). Patient 1’s muscular weakness was progressive after OHT, although no other significant post-operative complications were observed. Skeletal muscle biopsy demonstrated myopathy with fibrosis (Figures S1C and S1D). A cochlear implant installed at age 6 has helped with communication and resulted in marked improvement over patient 1’s previous hearing aids. Additional details of patient 1’s clinical description are provided in “Supplemental note: Case Report for Patient 1.”

Due to patient 1’s clinical course, to rule out a known genetic etiology, normal chromosomal microarray, karyotype, Duchenne Muscular Dystrophy (DMD [MIM: 310200]) genetic sequencing, mitochondrial genome sequencing, and metabolic investigations, including amino acids, urine organic acids, and plasma acylcarnitine profiles were performed. As all these assays were negative, an SNP microarray was performed; this identified a 24.8-Mb region of homozygosity, DGV (GRCh 37: Feb 2009) (hg19): arr[hg19] 16p12.1q12.1(24,848,663-49,683,420)x2 Hmz. To refine whether a novel genetic lesion in this region was associated with patient 1’s phenotype, WES was performed. Exome trio analysis detected several variants, including a homozygous missense variant (c.718C > T; p.R240W) in exon 9 of the target-SNARE (t-SNARE) STX4 locus (Table S1), which was confirmed with Sanger sequencing (Figure S2); the trio was of American-European (non-Finnish) descent. Among the detected variants, the STX4 variant was the only candidate locus detected in the region of homozygosity found on the SNP array (Figure 1C). In addition, all other variants were either not concordant with patient 1’s phenotype or zygosity, were predicted to be benign, or failed single-gene deletion/duplication testing by comparative genomic hybridization (Table S1). Notably, this variant is located within the coiled-coil SNARE homology domain of STX4 that is highly conserved among vertebrates (Figure 1D) and was predicted to be damaging by in silico analyses (ACMG Criteria: PM2 PP3). 36

A second individual (patient 2) was also identified via GeneMatcher.62 Upon fetal ultrasound at 25 + 0 weeks of gestation, multiple anomalies were detected, including frontal edema, dilated ductus arteriosus, oligohydramnios, hypoplastic kidneys, dilated echogenic small bowel loops, duodenal atresia, and overlapping fingers. Prenatal trio-WES analysis of the fetus showed compound heterozygous STX4 variants, consisting of two variants (Table S2): c.89_90delGC; p.G30Dfs*28 and c.232+4A > C. While the former variant is predicted to cause an early truncation, the latter variant occurred at a highly conserved position and is predicted to affect splicing using several in silico analyses, including SpliceAI (donor gain with score of 0.5), 34 MaxEntScan (likely disrupting: decreases splicing efficiency as predicted by a MaxEntScan score decrease of 47% [from 7.36 to 3.85]; likely effect: alternative splicing/insertion leading to a frameshift/3’ exon extension), 34 CADD (deleterious with a score of 23.7), 35 and MutationTaster (disease causing) (Figure 1C). This second trio was of European (non-Finnish) descent. No other pathogenic or likely pathogenic variants fitting patient 2’s phenotype were detected (Table S2). Patient 2 was subsequently delivered at 30 + 4 weeks of pregnancy by secondary cesarean delivery due to complications of anhydramnios and subsequently died 5 days after birth due to multi-organ failure. Additional details of patient 2’s clinical description are provided in Figure S3 and “Supplemental note: Case Report for Patient 2.” Collectively, these clinical data suggest a requirement for STX4 during normal human development and imply a role in cardiac physiology and neuromuscular function.

(C) Pedigree segregating the homozygous STX4 c.718C > T; p.R240W variant in family 1 and the c.89_90delGC; p.G30Dfs*28 and c.232+4A > C alleles in family 2. m, variant allele; ?, unknown carrier; SAB, spontaneous abortion without further data.

(D) Alignment of portion of the STX4 sequences containing the human variant. There is a high degree of conservation overall for STX4 homologues. For instance, mouse STx4 and zebrafish stx4 respectively share 95.3% and 75.3% amino acid conservation with human STX4. Dashed-magenta line indicates position of p.G30Dfs*28 allele. Dashed-green line indicates the position of the p.R240W variant and expanded residue sequence indicates the conservation of this residue among the selected vertebrates. The residue positions indicate domain boundaries of the 297 amino acid human STX4 protein. 61 Black highlights indicate amino acids that are conserved, gray highlights residues that are chemically similar (conservative), and white highlights display non-conservative variants. Habc, antiparallel three-helix bundle stabilization domain, H3abc, coiled-coil SNARE homology domain; NP, N-terminal peptide, TM, transmembrane.
Zebrafish stx4 mutants have pleiotropic defects analogous to the clinical presentation observed in human patients

Global Stx4 knockout (KO) mice are early embryonic lethal, precluding analysis without conditional alleles. In combination with the perinatal lethality of the biallelic variants of patient 2, this suggests that homozygous loss of STX4 function is incompatible with survival in mammals; therefore, we examined the requirements for stx4 in zebrafish, which often can overcome loss-of-function of genes essential for early development due to maternal deposition of RNA and protein, and can survive without a functional heart up to 5 days post fertilization (dpf). IHC for Stx4 showed restricted localization in the lateral mesoderm at the 8 somite stage (ss) (Figures S4A and S4A’), and the ventral vasculature, blood progenitors, and endoderm by the 18 ss (Figures S4B and S4B’). From 24 to 48 hpf, Stx4 progressively became enriched in the spinal cord, the axonal tracts, and heart (Figures S4C and S4F’). By 72 hpf, Stx4 was expressed in neurons throughout the nervous system (Figures S4H and S4H’).

To determine the requirements for stx4 in zebrafish, we generated a stx4 mutant zebrafish allele using CRISPR-Cas9. The allele identified and analyzed has a 38-bp deletion that eliminates the splice donor site of exon 3 (Figure 2A). Sequencing of RNA from the stx4 mutants showed that the resulting allele produces three alternatively spliced transcripts due to the introduction of cryptic splice variants, which are all predicted to generate severely truncated proteins in the Habc stabilization domain (Figure 2B) similar to the earlier truncation observed due to allele 1 of patient 2 (Figure 1D).

Although overt defects like bradycardia and pericardial edema are already evident in a small proportion of stx4 mutants at 48 hpf, by 72 hpf all stx4 mutants exhibit a complex of defects, including myocardial dysfunction with linearized hearts, pericardial edema, bradycardia, microcephaly, loss of the midbrain/hindbrain boundary, otic vesicle dysgenesis, neuronal cell death, and touch insensitivity (Figures 2C and S5A–S5H, Videos S1 and S2). Interestingly, a small proportion of the stx4 mutants (estimated at <10%) have hearts that cease to beat entirely by 72 hpf. In addition, ~20% of stx4 mutants exhibit hemorrhaging of the intersegmental and cranial vasculature (Figures S5I–S5M). IHC showed diminished expression of Stx4 in the stx4 mutants (Figures 3A–3D’) and stx4 mutants do not survive past 5 dpf (Figure S5N), presumably due to defects in multiple organs, further supporting that this allele results in loss of Stx4. Heterozygous carriers of the zebrafish stx4 allele were equivalent to WT siblings, indicating the allele is recessive. Collectively, the pleiotropic defects found in stx4 mutant zebrafish larvae appear to be reminiscent of the syndromic features observed in patient 1, particularly the cardiac dysfunction, sensorineural hearing loss, global developmental delay, and hypotonia, but also are also consistent with the perinatal lethality observed in patient 2.

stx4 mutant CMs have abrogated vesicle fusion

Given the cardiac abnormalities shared by the index patients and zebrafish stx4 mutants, we next examined Stx4 in the hearts of 72 hpf WT and stx4 mutant larvae in greater detail. As a target-SNARE (t-SNARE), Stx4 mediates vesicular fusion at the cell membrane. As might be expected for a t-SNARE, we found that in CMs, Stx4 predominantly localized in a punctate pattern in WTs, while its expression was significantly diminished in stx4 mutants (Figures 3C and 3D’). We next assayed vesicle localization by IHC for Vamp2, the cognate v-SNARE partner of Stx4 (Figures 3E–3H). Whereas stx4 mutant CMs have a similar number of vesicles as WT (Figures 3I and 3J), fewer vesicles appear “docked” (colocalized) to the CM sarcolemma, despite vesicle “clustering” similar to WT levels as measured by Vamp2+ vesicle proximity to the sarcolemma (Figures 3G, 3H, 3K, and 3L). Collectively, these data support a requirement for Stx4 in promoting vesicle fusion in CMs.

stx4 mutant hearts have marked bradycardia despite normal adrenergic function

As the hearts of 72 hpf zebrafish stx4 mutant larvae are morphologically more linear than WT (Figures S6A–S6D), we assessed if this aberrant morphology reflects a difference in CM number. To quantify CMs, IHC for chamber-specific myosin heavy chains Myh6 and Myh7, which respectively label atrial and ventricular CMs, was used with the Tg(-5.1myl7:dsRed2-NLS) transgene, which is expressed in all CM nuclei. However, no difference in overall or chamber-specific CM number was detected in the hearts of stx4 mutant or WT zebrafish at 72 hpf (Figures S6E–S6G). Similarly, despite the marked bradycardia observed in the mutants (Figures 4A–4C, Videos S1 and S2), the amount of Isl1+ pacemaker CMs at the venous pole of the stx4 mutant hearts appeared similar to WT (Figures S6H and S6I). A previous report suggested that zebrafish hearts are not yet innervated by 72 hpf. Consistent with this, we failed to detect innervation to the hearts of 72 hpf WT (or stx4 mutant) zebrafish using a-Bungarotoxin (Figures S7A and S7B), acetylated a-Tubulin (Figures S7C and S7D), or the vagus nerve reporter transgene Tg(BAC(neurod:EGFP)) (Figure S7E). However, challenging stx4 mutants with isoproterenol at 72 hpf indicated that they were just as competent to respond to adrenergic stimulation as WT clutch-mates (Figures S7F and S7G). Collectively, these data imply that adrenergic dysfunction does not account for the bradycardia observed in stx4 mutants.

stx4 mutants exhibit Ca2+ handling defects and increased sensitization to Ca2+ modulation

As adrenergic function appeared to be preserved by 72 hpf, we explored Ca2+ handling as a potential mechanism for the bradycardia using Fura-2,AM Ca2+ imaging. Imaging of 48 hpf explanted hearts extrinsically paced for comparison between stx4 mutant hearts and WT clutch-mates did
not reveal any significant differences, consistent with the limited penetrance of pericardial edema and bradycardia at this stage. However, Ca^{2+} imaging at 72 hpf indicated that the Ca^{2+}-transient amplitude in stx4 mutant atria was significantly diminished compared with WTs (Figures 4D–4G). In addition, the Ca^{2+}-transient duration in paced 72 hpf stx4 mutant ventricles was significantly shorter than in WTs (Figures 4H and 4I). Thus, the cardiac dysfunction observed in stx4 mutants is at least partially due to defects in Ca^{2+} handling in CMs.

To further characterize the Ca^{2+} handling abnormalities in stx4 mutants, we exploited pharmacological manipulations of both sarcolemmal Ca^{2+} uptake and Ca^{2+}-induced-Ca^{2+} release from the sarcoplasmic reticulum of CMs. We challenged 72 hpf larvae with pharmacological modulators of Ca^{2+} handling and assayed heart rates only of stx4 mutants with observable heart rates for this analysis. Consistent with the ability of Syntaxins to regulate LTCCs in other tissues,68 stx4 mutants treated with nifedipine, a selective LTCC blocker, were sensitized in a dose-dependent manner and exhibited asystole at concentrations below the threshold for bradycardia in WT clutch-mates (Figure 5A).55 Conversely, Bay K-8644, an LTCC agonist,55 was able to partially rescue the bradycardia phenotype, as

Figure 2. Generation of stx4 mutant zebrafish
(A) CRISPR-Cas9 was used to generate a zebrafish stx4 loss-of-function allele that creates a 38-bp deletion (red underlined cytosine marks the endonuclease cut site), eliminating the splice donor site of exon 3 (protoscaler denoted by purple arrow and codons; the last three codons at the splice junction are eliminated in stx4 mutants).
(B) Schematic of transcripts showing the three alternative splice variants generated from this allele. All three transcripts are predicted to be out-of-frame (OOF; left) and to produce truncation products (translated peptides; right). H_{abc}: antiparallel three-helix bundle stabilization domain; H3_{abc}: coiled-coil SNARE homology domain; NP, N-terminal peptide, TM, transmembrane. Red polygon represents a stop codon in the 298 amino acid sized zebrafish Stx4 protein, resulting in early truncation products.
(C) Bright field images of 72 hpf WT/stx4^+/− sibling and stx4 mutant larvae. Images of WT sibling embryos in all figures are stx4^+/−/C0 sibling embryos. Black arrow indicates pericardial edema and linear heart. Yellow arrowheads indicate hemorrhages. stx4 heterozygotes are overtly indistinguishable from WT. Scale bar, 200 μm.
the *stx4* mutant treated group mean heart rate is restored to ~90% of untreated WTs, though still statistically different from the untreated WT embryos (Figure 5B). We also challenged larvae with a cocktail of thapsigargin and caffeine (TgC), which blocks the sarco/ER Ca\(^{2+}\)-ATPase (SERCA) or stimulates Ryanodine receptors, respectively, thereby depleting sarcoplasmic Ca\(^{2+}\) stores. TgC produced marked bradycardia in both WT larvae and

Figure 3. *stx4* mutants exhibit loss of Stx4 expression and function

(A and B) Confocal images of 72 hpf WT or *stx4* mutant larvae labeled using IHC markers for Mhc (striated muscle - red), Myh6 (atrial CMs - yellow) and Stx4 (purple). (A’ and B’) Single channel confocal images of Stx4. Scale bars, 100 μM.

(C and D) Confocal images of 72 hpf WT and *stx4* mutant CM membranes labeled with Alcama (red), and Stx4 (purple). Scale bars, 10 μM. (C’ and D’) Single channel confocal images of Stx4. Membranes (red lines) from (C and D) are indicated.

(Figure continues)
the majority of the stx4 mutant larvae (a 72.4% reduction in heart rate in WT versus a 76.8% reduction in stx4 mutants versus controls; Figure 5C), suggesting that baseline SERCA activity is a less likely contributor to the basal bradycardia. We also challenged stx4 mutants with NNC55-0396 (NNC), a selective T-type Ca$^{2+}$ channel (TTCC) antagonist.69 However, two different concentrations of NNC, including one previously reported to abolish the cardiac action potential in larval zebrafish, 69 were not sufficient to significantly affect the heart rate of WT or stx4 mutant larvae at 72 hpf (Figure 5D), consistent with previous reports regarding the contribution of LTCCs (and to a lesser extent SERCA) to intrinsic cardiac pacing in zebrafish and other vertebrates.70 Together, these data imply that stx4 mutants have reduced sarcolemmal LTCC function, and strongly support this mechanism as a substantive contributor to the cardiac dysfunction observed in the setting of Stx4 loss-of-function.

The analogous zebrafish Stx4R241W allele is functionally hypomorphic

Given the discordance between the predominantly cardiac manifestations emerging in the third year of life observed with the homozygous STX4R240W variant of patient 1 and the perinatal lethality of patient 2 (with the premature truncation variant in trans with a splice variant resulting in a donor gain in intron 3), we next investigated whether features of the pleotropic stx4 mutant syndrome might be rescued and whether patient 1’s STX4R240W variant functions as a hypomorphic allele. Thus, we generated stable transgenic lines ubiquitously expressing WT zebrafish Stx4, or Stx4 with a R241W variant, which is the analogous point-mutation in zebrafish to that observed in patient 1 (Figure 6A). Importantly, hemizygous expression of either transgene in WT or stx4 heterozygous larvae does not cause gain-of-function or dominant negative phenotypes (Figures 6B and 6D). Hemizygous expression of the actb2:stx4-IRES-EGFP transgene was sufficient to rescue the pleiotropic stx4 mutant defects (Figures 6B and 6C), including the bradycardia (Figure 6F). However, hemizygous expression of the actb2:stx4R241W-IRES-EGFP transgene was not able to rescue the mutants (Figures 6D and 6E), despite being expressed approximately at the levels of one WT stx4 allele (Figure S8). In contrast to heterozygous stx4 larvae and stx4 mutant larvae hemizygous for the actb2:stx4-IRES-EGFP transgene, stx4 mutant larvae hemizygous for the actb2:stx4R241W-IRES-EGFP transgene showed a high degree of variability in their bradycardia (Figure 6G), which suggested that the variant likely produces a hypomorphic protein, consistent with patient 1’s survival into childhood and the progressive onset of their syndrome. Finally, we treated stx4 mutants bearing hemizygous
Figure 5. Stx4 regulates L-type voltage gated Ca²⁺ channel activity
(A) Heart rates (HRs) determined from ventricular regions of interest of 72 hpf WT and stx4 mutant larvae following a dose-response treatment with 1% DMSO/embryo water (control), 100 nM nifedipine, 1 μM nifedipine, 2 μM nifedipine, 5 μM nifedipine, or 10 μM nifedipine for 45 min. Data are represented as the mean ± SEM, n = 10–21 larvae/group, one-way ANOVA, *p < 0.05, ****p < 0.0001.
(B) HRs determined from 72 hpf WT and stx4 mutant larvae treated with either 1% DMSO/embryo water (control) or 20 μM Bay K-8644. Control stx4 mutants exhibit an ~40% reduction in mean HR versus WT (WT control mean HR: 174.2 bpm, stx4 mutant control mean HR: 104.1 bpm). By contrast, stx4 mutants treated with 20 μM Bay K-8644 (mean HR: 153.1 bpm) are rescued to ~90% of the WT control HR and ~150% of the untreated stx4 mutant HR, while HR of WT treated with 20 μM Bay K-8644 are similar to untreated WT controls (mean HR: 179.3 bpm). Data are represented as the mean ± SEM, n = 20–34 larvae/group, one-way ANOVA, *p < 0.05, ****p < 0.0001.
(C) HRs determined from WT and stx4 mutant larvae treated with either 1% DMSO/embryo water (control) or a mixture of 10 μM thapsigargin and 10 mM caffeine (TgC). Data are represented as the mean ± SEM, n = 9–10 larvae/group, one-way ANOVA, *p < 0.05, ****p < 0.0001.
(D) HRs determined from a dose-response of WT and stx4 mutant larvae treated with either 1% DMSO/embryo water (control), 10 μM NNC 55-0396, or 20 μM NNC 55-0396. Data are represented as the mean ± SEM, n = 10 larvae/group, one-way ANOVA, ****p < 0.0001.

expression of actb2:stx4R241W-IRES-EGFP with Bay K-8644 to ascertain whether this allele might be responsive to modulation of LTCC function. Remarkably, treatment with Bay K-8644 was able to fully rescue the bradycardia of stx4 mutant larvae with hemizygous expression of the actb2:stx4R241W-IRES-EGFP, as their heart rates were indistinguishable from WT (Figure 6H). Therefore, together these data support that the variant is hypomorphic and present the possibility that an LTCC agonist may therapeutically rescue some aspects of the dysfunction due to the R240W allele.

Discussion
In the present study, we report an index patient (patient 1) with a complex syndrome consisting of global developmental delays, sensorineural hearing loss, hypotonia, frequent ectopy, and biventricular DCM that were refractory to treatment and necessitated heart transplant. WES revealed a homozygous, non-conservative missense variant in STX4 that was predicted by Polyphen-2 and SIFT to be probably damaging and deleterious, respectively.30,71 Although three heterozygous alleles of the same residue detected in patient 1 are readily identifiable in the gnomAD database (single nucleotide variants: 16-31050877-C-T [GRCh37] and 16-31039556-C-T[GRCh38], gnomAD v.2.1.1 and v.3.1.2, respectively),72 no additional homozygous variants are available, which is unsurprising, given that this allele is rare with a global MAF of 0.00000795. Recently, an association between Silver-Russell syndrome (SRS [MIM: 180860]) and other SNVs in STX4 has also been suggested73; however, we did not detect SRS features in patient 1, such as characteristic facies, nor analogous features in stx4 mutant zebrafish, aside from a general failure to thrive. Thus, to the best of our knowledge, we are reporting the first direct association of STX4 in human disease and an SNARE protein affecting cardiac function in vivo.

To date, there has been limited understanding of the mechanism by which STX4 proteins might affect cardiac function. Although Stx4 has been proposed to affect release of atrial natriuretic factor in cultured CMs,74,75 this role is unlikely to account for patient 1’s cardiac defects, given their complex physiology.76 In addition to the aforementioned early embryonic lethality of Stx4 global-KO mice, which is reminiscent of the compound heterozygous truncating allele that we identified in patient 2 (perinatally lethal, with even more severe, pleotropic abnormalities), heterozygous Stx4 mice merely exhibit impaired glucose tolerance due to reduced Glut4 translocation in skeletal muscle.63 While this is consistent with STX4’s role in GLUT4 trafficking in adipocytes 77-79 similar to heterozygous stx4 mutant zebrafish, these mice display no other overt defects.63 Additionally, a recent study that
aimed to uncover the role of SNAREs in cardiac insulin resistance and Glut4 trafficking failed to detect differential expression of Stx4 between control, C57BL/KsJ-leprdb/leprdb, or high-fat diet fed mice.80 Conditional Stx4 KO mice have also been used to investigate Stx4’s requirement in bone matrix deposition.81,82 Despite these proposed roles for STX4, patient 1 did not exhibit metabolic or bone mineralization defects, the former of which is another common feature of SRS. Because cardiac dysfunction was the major feature of patient 1’s acute presentation and because reduced-LV function was also identified perinatally in patient 2, indicating that progressive loss of STX4 function may present along a spectrum manifesting in cardiac dysfunction, we aimed to understand the requirement for Stx4 in the heart. While decreased cardiac function has been associated as a potential morbidity in synaptopathies, including Otahara and West Syndrome,83,84 the regulation of proper cardiac

Figure 6. The zebrafish Stx4R241W variant is hypomorphic
(A) Schematic of constructs used to generate transgenic zebrafish expressing WT Stx4 (actb2:stx4-IRES-GFP) and the zebrafish Stx4R241W variant (actb2:stx4R241W-IRES-GFP), which is equivalent to the patient 1 STX4R240W variant.
(B–E) Images of 72 hpf WT and stx4 mutants hemizygous for the actb2:stx4-IRES-GFP and actb2:stx4R241W-IRES-GFP transgenes. Scale bar, 200 μM.
(F and G) Heart rates determined from ventricular regions of interest of 72 hpf WT and stx4 mutant larvae lacking and hemizygous for actb2:stx4-IRES-GFP or actb2:stx4R241W-IRES-GFP. Data are represented as the mean ± SEM, n = (F) 4–14 and (G) 10–18 larvae/group, respectively, one-way ANOVA, ****p < 0.0001.
(H) Heart rates from ventricular ROIs determined from 72 hpf WT and stx4 mutant larvae lacking and hemizygous for actb2:stx4R241W-IRES-GFP treated with either 1% DMSO/embryo water (control) or 20 μM Bay K-8644. Data are represented as the mean ± SEM, n = 8–10 larvae/group, one-way ANOVA, ***p < 0.001, ****p < 0.0001.
function by SNARE proteins had not been previously established in vivo. Importantly, in both of these reports, one a clinical case report and the other a zebrafish model study, the effects on cardiac function were secondary due to neuronal input. In vitro, studies conducted in HEK 293 cells and *Xenopus laevis* oocytes have suggested that SNARE proteins play a role in regulating ion channel vesicle fusion as well as can exhibit independent allosteric interactions with ion channels to modulate sensitivity or gating behavior. For instance, Syntaxin 1A is reported to modulate different steps of the exocytosis and gating of several cardiac ion channels, including Cav (L-, N-, and T-type), Kv (Kv2.1, Kv4.2, Kv4.3, Kv7.1, and Kv11.1), and KATP (KATP6.2), by direct interaction. Notably, although some of these proteins are implicated as causes of cardiac channelopathies, many have extracardiac functions. While these previous studies suggested that SNARE proteins may play a role in normal cardiac function, we investigated potential mechanistic links between the variants and the relevant cardiac outcomes. In contrast to previous *stx4* loss-of-function models, *stx4* mutant zebrafish develop overt pleiotropic abnormalities that are analogous to both of the patients (a cardiac syndrome and early developmental lethality by 5 dpf, respectively), suggesting a highly conserved requirement for STX4 during vertebrate development. Notably, the syndromic phenotype becomes fully penetrant by 72 hpf in zebrafish *stx4* mutant larvae, indicating that maternal *stx* mRNA likely accounts for a delay in the onset of the syndrome. The expression of Stx4 in neural tissues and the heart is consistent with the conservation of the predominantly neuromuscular/neuro-sensory and cardiac syndrome of the zebrafish *stx4* mutants and both index patients.

In addition to modeling features of both identified patients, loss of Stx4 leads to severe bradycardia in zebrafish larvae, at least partially due to aberrations in Ca$^{2+}$ handling that are directly attributable to reduced LTCC activity. These results indicate at least a functional interaction between Stx4 and LTCCs within CMs. Evidence for this is bolstered by our finding that vesicle docking is significantly reduced in CMs of *stx4* mutants, concomitant with Ca$^{2+}$ handling defects. Notably, it has been reported that approximately 80% of early CM Ca$^{2+}$ in zebrafish is mediated by Ca$^{2+}$ influx via LTCCs, which are known to play a crucial role in zebrafish heart development and human disease. We also observed a significant decrease in the ventricular Ca$^{2+}$ transient duration of *stx4* mutants. The changes in Ca$^{2+}$ transient amplitude and duration that we observed imply substantial reduction in total Ca$^{2+}$ flux in each cardiac cycle, which would be predicted to affect not only pacemaker CM rates but also atrial and ventricular CM contractility, as observed in the clinic. These data also support the possibility that STX4 functionally interacts and affects LTCCs in a conserved fashion among vertebrates. Interestingly, a recent report has implicated an association between *CACNA1C* and neurodevelopmental abnormalities and epilepsy (including West Syndrome), suggesting a possible common axis of interaction between SNAREs and LTCCs, given the parity of the syndromic features observed from both protein classes.

Despite the effects on Ca$^{2+}$ handling in the *stx4* zebrafish mutants, we presently cannot rule out that aspects of the cardiac phenotype and dysfunction are non-cell autonomous. For example, pericardial edema, a common defect observed in zebrafish mutants with cardiovascular dysfunction, may contribute to the morphologically linearized hearts in the *stx4* mutants. In addition, a cardiac specific *myl7:stx4-IREs-EGFP* transgenic line that we created failed to rescue the overt cardiac defects in the *stx4* mutants (data not shown). Although one interpretation of this result is that it supports there may be non-autonomous effects contributing to the cardiac defects observed in the *stx4* mutants, there are caveats to using this transgenic CM-specific rescue approach and line, such as whether the timing and levels of transgenic *stx4* expression reflect endogenous *stx4* within CMs. Unfortunately, we could not determine if there was rescue of Ca$^{2+}$ transients, despite the lack of morphological rescue, as the spectral emission of EGFP and Fura-2,AM overlap. Thus, the variables involved with this experiment prevent us from definitively concluding there are non-autonomous cardiac defects from *stx4* loss. Nevertheless, the *stx4* zebrafish mutants also have defects in endothelial integrity, as indicated by hemorrhages, suggesting vascular defects as a possibility that could non-autonomously contribute to the cardiac dysfunction. However, while negative modulation of LTCC activity may reflexively increase heart rate due to vasodilation of the coronary arteries and peripheral vasculature in humans, zebrafish lack coronary vasculature until ~7 weeks post fertilization. In addition, *stx4* mutants exhibit asystole upon treatment with nifedipine, suggesting that the effect of LTCC modulation is predominantly chronotropic and acts specifically in the heart. Therefore, our data are consistent with at least the cardiac Ca$^{2+}$ handling defects observed in *stx4* mutants being cell autonomous within CMs; however, further analysis is needed to discern if there are additional cell non-autonomous influences of *stx4* on the heart.

Our data also support that patient 1’s *STX4* R240W variant functions as a hypomorph, given that ubiquitous expression of zebrafish *STX4* R241W is not sufficient to rescue the syndromic phenotype of *stx4* mutant zebrafish. However, treatment with Bay K-8644 is sufficient to rescue the bradycardia of *stx4* mutants hemizygous for the *actb2:stx4* R241W, IRES-EGFP transgene to rates indistinguishable from WT, while *stx4* homozygous mutants treated with Bay K-8644 is only sufficient to restore their heart rate to ~90% of WT’s, supporting some functionality of the variant. Collectively these data suggest that arrhythmias due to loss or partial abrogation of *STX4* function may be ameliorated by modulation of Ca$^{2+}$ levels in CMs. While the heterozygous *stx4* larvae do not have overt defects, a caveat of the current analysis using the hemizygous *actb2:stx4* R241W-IRES-EGFP transgenic line is that it may be expressed at slightly lower.
levels than predicted for one endogenous WT allele of stx4 (Figure S8), suggesting that higher levels of this variant may confer greater compensatory functionality in the absence of WT stx4 alleles. It should also be noted that variants in other genes that stabilize or traffic ion channels, such as Ankrin-B (ANK2 [MIM: 106410]) and Caveolin-3 (CAV3 [MIM: 601253]) have been implicated in channelopathies, suggesting that monogenic lesions, such as the one observed in patient 1, might plausibly be linked to arrhythmic disorders. This is notable as congenital heart diseases (CHDs), which are the most common birth defects and account for nearly one-third of all major congenital anomalies, often lead to arrhythmia by adulthood. As such disorders often present as sudden cardiac arrest and are typically lethal, the collective impact of congenital arrhythmic disorders (CADs) is significant. While further study of the role of Stx4 in the heart may provide additional mechanistic information, our data modeling previously unreported human disease variants using zebrafish demonstrate a conserved requirement for SNARE proteins in vertebrate heart development, and highlight new potential avenues targeting SNARE proteins in the treatment of CHD and CADs.

Data and code availability
This study did not generate/analyze [datasets/code].

Supplemental information
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Declaration of interests
The authors declare no competing interests.

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Web resources
The following web-based resources were used:
Burrows-Wheeler Aligner. http://bio-bwa.sourceforge.net.
CADD. https://cadd.gs.washington.edu/
ChopChop. http://chopchop.cbu.uib.no.
Developmental Studies Hybridoma Bank. https://dshb.biology.uiowa.edu.
Ensembl. http://useast.ensembl.org/index.html.
Fathmm. http://fathmm.biocompute.org.uk.
GeneMatcher. https://genematcher.org.
gnomAD/ExAC. https://gnomad.broadinstitute.org.
ImageJ. https://imagej.nih.gov/ij/download.html.
Imaris Open. https://imaris.oxinst.com/open.
MaxEntScan. http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html.
MutationAssessor. http://mutationassessor.org/r3.
MutationTaster. www.mutationtaster.org.
The Zebrafish Information Network. https://zfin.org.

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Supplemental information

Stx4 is required to regulate cardiomyocyte Ca^{2+}

handling during vertebrate cardiac development

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Supplemental Information

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Supplemental note: Case Report for Patient 1

Patient 1 presented with progressive fatigue at three years of age. Initial evaluation in the emergency room was significant for increased work of breathing, hypotension, and severe acidosis. Previous medical history was significant for congenital profound sensorineural hearing loss, hypotonia, and global developmental delays. Echocardiography showed Patient 1 to have dilated cardiomyopathy (DCM) with severely depressed biventricular systolic function, and he was electively intubated and managed with calcium and milrinone infusion to augment cardiac output. Patient 1 developed frequent ectopy (premature atrial and ventricular contractions) with runs of non-sustained ventricular tachycardia (VT). Subsequently, he was placed on extracorporeal membrane oxygenation (ECMO) and continued to have hypotension and episodes of VT. Patient 1 was initially managed with propranolol but required an esmolol drip due to the progressive arrhythmia. Due to his instability, a transeptal puncture with bladed and serial balloon atrial septoplasty was performed and resulted in substantial left atrial decompression with overall improvement of right atrial and ventricular filling and systolic function. Patient 1 was subsequently placed on a Berlin Heart EXCOR® left ventricular assist device (LVAD) and continued to have prolonged episodes of VT, requiring amiodarone and atrial single chamber (AAI) pacing for a period of time. The LVAD was changed to a CentriMag™ Extracorporeal Blood Pumping System to better unload the left ventricle. He was then placed on a biventricular assist device (BiVAD), which improved LVAD filling and cardiac output but did not affect his continuous VT. Ultimately, Patient 1 received an orthotopic heart transplant (OHT) two months from the onset of his initial symptoms. Patient 1’s muscular weakness was progressive after OHT, and he required a tracheostomy with ventilatory support for the inability to tolerate coming off of mechanical ventilation. His course since transplant has been notable for multiple infections, likely related to chronic immunosuppression, as well as poor somatic growth on enteral feeds. He remains ventilator dependent. The transplanted heart shows normal function without evidence for graft coronary artery disease at five years of age, and there have been no significant episodes of rejection. Despite atrial tachycardia shortly after transplant, he has been weaned off beta blocker therapy and has had no further ectopy, to date. Patient 1 is nine years of age at the time of publication and has
a sibling who is regularly screened for DCM and is asymptomatic as of five years of age. The sibling is homozygous for the reference (WT) STX4 allele (Figure S2).

**Supplemental note: Case Report for Patient 2**

Patient 2 presented upon fetal ultrasound at 25+0 weeks of gestation with multiple anomalies including frontal edema, persistent ductus arteriosus, oligohydramnios, hypoplastic kidneys, severely dilated echogenic small bowel loops, duodenal atresia, and overlapping fingers. At 30+4 weeks of pregnancy, Patient 2 was delivered via secondary cesarean section due to complications of anhydramnios and induction of premature labor. TORCH complex was excluded prenatally, and there were no signs of other noxious agents during pregnancy. At delivery, Patient 2 exhibited normal birth measurements including, size, weight, and head circumference; however, a short umbilical cord was noted. Upon postnatal evaluation, Patient 2 exhibited multiple malformations, including pulmonary hypoplasia, hepatomegaly, duodenal atresia, renal dysplasia, a small urinary bladder, scoliosis, clubfoot, and musculoskeletal contractures of the right hand, elbow, and foot (Figure S3). Postnatal echocardiography showed normal heart arrangement, a patent foramen ovale with aneurysms of the septum, a persistent ductus arteriosus, and T1 1-2° and moderately reduced left ventricular function. The patient also displayed facial dysmorphia, including retrognathia, a tent-shaped mouth and small, dysplastic low-set, posteriorly rotated ears. Due to the prenatal WES, chromosomal analysis was performed after birth but was normal. In addition to the multiple congenital anomalies, Patient 2 had massive ubiquitous edema and required ventilatory support; he subsequently died five days after birth. Upon autopsy, the cause of death was determined to be consistent with multi-organ failure. A cerebral MRI performed post-mortem indicated a subarachnoid hemorrhage, but was unremarkable for neuronal migration or gyration disorders, although partial pachygyria was noted upon autopsy. Other pertinent findings included renal hypoplasia, pulmonary hypoplasia, and malposition of the vermiform appendix. Calcifications on the posterior wall of the heart were also noted; the patent foramen ovale and persistent ductus arteriosus were documented to be consistent with the early gestational birth. Aspects of the pleotropic phenotypes listed were differentially noted to possibly be secondary to the anhydramnios/oligohydramnios or due to gestational
age at delivery. However, the etiology of the anhydramnios was not clinically resolved. Patient 2 has one sibling who is healthy as of four months of age. Notably, the mother’s first pregnancy terminated as a spontaneous abortion in the first trimester. Genetic testing of the living sibling was unavailable at time of publication (Figure 1C).
Figure S1. Histopathology of heart and skeletal muscle biopsy of Patient 1. (A,B) Cross-sections from a biopsy of the explanted heart of Patient 1 stained with hematoxylin-eosin. (A) Diminished myocardial cross-striations, interstitial fibrosis, hypertrophic fibers, loss of myofibrillar volume, and marked variation in fiber size (circled) are observed. Scale bar: 100 μM. (B) Micrograph of the explanted heart at high magnification shows numerous mainly rounded atrophic fibers (arrowhead), both single and in small groups. Scale bar: 50 μM. (C) Succinic acid dehydrogenase staining of skeletal muscle biopsy shows a normal quantity and distribution of mitochondria in most fibers and the occasional ragged blue fiber (orange arrowheads). Scale bar: 50 μM. (D) Transmission electron micrographs of skeletal muscle biopsy show endomysial fibrosis, increased mitochondrial aggregates (circled), with multiple scattered fibers showing regeneration or degeneration. No significant inflammatory infiltrates are detectable, nor is there appreciable vacuolization within the myofibers. Scale bar: 2 μM.
Figure S2. Targeted STX4 variant testing of Patient 1 and sibling. Chromatogram traces of the amplified complementary STX4 sequence in both Patient 1 (II-1) and their sibling (II-2) compared to an unaffected control individual (reference). Arrow indicates the position of the c.718C>T variant. Patient 1 is homozygous for the variant, while their sibling is homozygous for the reference allele as compared to the unaffected individual.
Figure S3. Autopsy and gross histology of patient with compound heterozygous truncating STX4 variants. (A,B) External malformations of patient 2 depicting hyperflexion of the right hand and clubfoot, respectively. (C,D) Pachygyria of the cerebral cortex and cerebellar subarachnoid hemorrhage, identified upon autopsy. (E,F). Persistent ductus arteriosus (PDA) and patent foramen ovale, respectively, are noted with probes demarcating each defect. (G) Lung lobes depicting grossly normal appearance. (H,I) Stenotic ileum with presstenotic dilatation of the duodenum/ileum as well as stomach. (J-M) Cross-sections from a biopsy of the explanted heart of Patient 2 stained with hematoxylin-eosin. (J) Anterior wall of the heart is grossly normal (Scale bar: 50 µM), and (K) without significant signs of hypertrophy (Scale bar: 10 µM). (L,M) Dystrophic calcification was observed on the posterior wall of the heart, without significant signs of hypertrophy. Scale bars: 20 µM and 5 µM, respectively. (N,O) Cross-sections from lung shows partially developed, somewhat delayed lung parenchyma. Scale bars: 50 µM and 10 µM, respectively. (O) Parenchyma at saccular stage of development with acute blood congestion. (P,Q) Cross-sections from kidney show slightly hypoplastic kidneys with regularly developed kidney parenchyma. 50 µM and 10 µM, respectively (R) Prenatal ultrasound of patient 2 depicts abnormal, severely dilated intestinal loop.
Figure S4. Stx4 expression is temporally refined during zebrafish development. (A-H') Confocal max intensity projections (MIPs) of embryos at the 8-somite stage (ss), 18 ss, 24 hpf, 48 hpf, and 72 hpf. IHC for Mhc (striated muscle - red), Myh6 (atrial cardiomyocytes (CMs) - yellow), and Stx4 (purple). (A'-H') Single channel micrographs of Stx4 in A-H. (A,A') At the 8 ss, Stx4 is enriched in the posterior lateral mesoderm (yellow asterisk). (B,B') At the 18 ss, Stx4 becomes refined to the ventral vasculature (white arrowhead), blood progenitors (yellow arrowhead), and pronephros (white asterisk). (C,C') At 24 hpf, Stx4 is enriched in ventral vascular cells (asterisk) and spinal cord (white arrowhead). (D,D') By 48 hpf, Stx4 becomes predominantly neuronally-enriched in several forebrain white matter tracts (including the habenula, posterior, and post-optic commissures (white arrowheads), and spinal cord (yellow arrowhead)). (E,E') Confocal image of Stx4 expressed in the heart at 48 hpf (white puncta). (F,F') MIP of a partial Z-stack of the heart shown in E. Stx4 signal intensity was enhanced relative to E,G and E',G', respectively, to show clear demarcation of Stx4 expression in the heart, including in the myocardium (outlined). (G,G') 48 hpf zebrafish heart stained for Mhc (red) and secondary antibody used for Stx4 antibody alone (anti-rabbit IgG(H+L) Alexa Fluor®-647) (negative control) demonstrates the specificity of the Stx4 antibody staining in the heart at 48 hpf. A: atrium; V:
ventricle. (H,H') At 72 hpf, Stx4 is expressed predominantly in the peripheral nervous system (yellow asterisk indicates motor neurons in the axial myomeres). (A-D,H) Scale bar: 100 µM. (E-G) Scale bars: 50 µM.
Figure S5. Stx4 is required for neurodevelopment and vasculogenesis. (A,B) In situ hybridization (ISH) showing tbx2b expression in 72 hpf WT and stx4 mutant larvae. Stx4 mutants exhibit a loss of the midbrain-hindbrain transition. Mb: midbrain, Hb: hindbrain. Scale bar: 200 μM. (C,D) Brightfield images of 72 hpf WT and stx4 mutant larvae otic vesicles. Stx4 mutant have atrophic otic vesicles (outlined) with smaller otoliths. OV: otic vesicle. Scale bar: 200 μM. (E,F) Confocal images of 72 hpf WT and stx4 mutant larvae labelled with IHC for Mhc (magenta) and neurod:EGFP (yellow). (E',F') stx4 mutants with the neurod:EGFP transgene showing disorganized cerebellum (arrowhead) and fewer, less-organized retinal progenitor cells (asterisk). Scale bar: 100 μM. (G,H) Confocal max intensity projections (MIPs) of IHC on 72 hpf WT and stx4 mutant larvae labelled with cleaved-Caspase 3. Stx4 mutants exhibit a significant amount of cell death, particularly in the brain and eye. Scale bar: 200 μM. (I-K) 72 hpf WT and stx4 mutant larvae labelled with o-dianisidine, which labels hemoglobin in the erythrocytes (red). Asterisks indicate hemorrhaging from the craniopharyngeal or intersegmental vasculature observed in ~20% of mutant larvae (n = 9/44 zebrafish). Scale bar: 200 μM. (L,M) Confocal MIPs of 72 hpf WT or stx4 mutant larvae carrying the Tg(kdrl:EGFP); Tg(gata1:dsRed) transgenes. (L',M') Single channel images showing kdrl:EGFP expression. Asterisk in K' indicates enlarged primordial mid/hindbrain channels and the agenesis of central arteries observed in stx4 mutants. (L'',M'') Single channel images of gata1:dsRed. Arrowhead indicates hemorrhaging observed from central arteries in the forebrain of stx4 mutant. Scale bar: 100 μM. Views in images are lateral with anterior up or to the left. (N) Survival curve of stx4 mutant zebrafish. WT/stx4+/- zebrafish exhibit a 93.33% survival rate past 96 hpf, by contrast 30.77% of stx4 mutants survive past 96 hpf and all die by 120 hpf. Data are represented as percent survival of n = 30 WT/stx4+/- and n = 26 stx4-/- mutant zebrafish, Log-rank (Mantel-Cox) test, ****p < 0.0001.
Figure S6. Stx4 is dispensable for CM differentiation. (A,B) Whole-mount ISH for myl7 in 72 hpf WT and stx4 mutant larvae. Frontal views. Scale bar: 100 µM. (C,D) IHC for Myh7 (ventricular CMs – magenta) and Myh6 (yellow) in hearts of 72 hpf WT and stx4 mutant larvae. Scale bar: 50 µM. (E,F) IHC of representative hearts from 72 hpf WT and stx4 mutant larvae carrying the myl7:DsRed2-NLS transgene and labeled for Myh6 (yellow) used for CM quantification. Scale bar: 50 µM. (G) Quantification of atrial and ventricular CMs hearts from 72 hpf WT and stx4 mutant larvae. Data are represented as the mean ± SEM, n = 10 larvae/group, Student’s t-test. (H,I) Confocal images of IHC for Mhc (magenta), Myh6 (red), and Isl1 (pacemaker CMs – yellow) in hearts of 72 hpf WT and stx4 mutant larvae. (H’,I’) Single channel images of Isl1 at the venous pole. Scale bar: 50 µM. A: atrium; V: ventricle.
Figure S7. Stx4 is dispensable for autonomic innervation or stimulation by 72 hpf. (A,B) Confocal images of hearts from 72 hpf WT and stx4 mutant larvae labelled for Mhc (white), Mhy6 (yellow), and conjugated α-Bungarotoxin (α-Btx), a nicotinic acetylcholine receptor neurotoxin (magenta). α-Btx labelling does not label cells at the venous pole of the hearts (yellow arrowheads) at 72 hpf, suggesting there is not parasympathetic input yet at this stage. Scale bar: 50 μM. (A’,B’) Single channel of α-Btx staining. White vesicles around the pericardial sac are hatching gland granules. (C,D) Confocal images of 72 hpf WT and stx4 mutant larvae labelled for Myh6 (red), Myh7 (blue) and acetylated tubulin (acTub; purple). (C’,D’) Single channel image of acTub. AcTub, marking axonal projections was not detected at the venous poles of the heart (yellow arrowheads). Scale bar: 50 μM. (E) Confocal image of 72 hpf WT larvae carrying the TgBAC(neurod:EGFP) transgene, which labels cranial nerves including the vagus nerve, and labelled for Mhc (magenta). CN X: vagus nerve nuclei. (E’) Single channel confocal image of TgBAC(neurod:EGFP). No parasympathetic input from the vagus nerve is detected at 72 hpf at the base of the heart (arrowhead). Scale bar: 100 μM. (F,G) Heart rates determined from ventricular ROIs of 72 hpf WT and stx4 mutant larvae captured by high-speed imaging and quantified as beats per minute (bpm) and the rate of change upon isoproterenol treatment relative to baseline (iso-BL/BL), imaged both before and after 30 minutes of treatment with 500 μM isoproterenol. Data are represented as the mean ± SEM, n = 16 larvae/group, Student’s t-test. BL: baseline; iso: isoproterenol. A; atrium; V: ventricle.
Figure S8. Transgenic expression of \textit{stx4}^{R241W-\text{IRES-EGFP}}. RT-qPCR for \textit{stx4}^{R241W-\text{IRES-EGFP}} expression from zebrafish hemizygous for the \textit{actb2:stx4}^{R241W-\text{IRES-EGFP}} transgene relative to endogenous \textit{stx4} in WT zebrafish at 72 hpf. Endogenous \textit{stx4} was amplified using primers internal to \textit{stx4} cDNA; transgenic \textit{stx4}^{R241W-\text{IRES-EGFP}} was amplified using primers for GFP. \( n = 3 \) biological replicates of pooled (\( n = 25-30 \) embryos/pool) embryos assayed as technical triplicates. Student’s t-test, **** \( p < 0.0001 \).
## Supplemental Tables

### Table S1. Variants detected in Patient 1 by WES trio-analysis.

| Gene Symbol | Chromosome (Cytoband) | Positiona (Exon) | HGVS Nomenclature (Variant Type) | dbsNP RS ID | MIM | ACMG Criteria | Zygosity in Patient 1, Segregation (Known inheritance pattern of variant: disease manifestation) | Minor Allele Frequencyb |
|-------------|-----------------------|------------------|---------------------------------|-------------|-----|---------------|------------------------------------------------------------------------------------------------|--------------------------|
| **Pathogenic/Likely Pathogenic Variants** | | | | | | | | |
| **EXOSC8** | Chr13 (13q13.3) | 37583420 (Exon 11) | NM_181503.3: c.815G>C; p.Ser272Thr (SNV) | rs36027220 | 606019 | PS3 PP1 PP5 BP4 | Heterozygous, Maternal (AR: Pontocerebellar hypoplasia, type 1C [MIM: 616081]) | 0.00385 |
| **SLC26A4c** | Chr7 (7q22.3) | 107312690 (Exon 4) | NM_000441.2: c.412G>T; p.Val138Phe (SNV) | rs111033199 | 605646 | PS4 PM2 PM3 PP5 | Heterozygous, Maternal (AR: Deafness, autosomal recessive 4, with enlarged vestibular aqueduct [MIM: 600791]; AR: Pendred Syndrome [MIM: 274600]) | 0.000175 |
| **TREX1** | Chr3 (3p21.31) | 48508395 (Exon 1) | NM_016381.5: c.506G>A; p.Arg169His (SNV) | rs72556554 | 606609 | PS3 PM2 PM3 PP1 PP3 PP5 | Heterozygous, Paternal (AR/AD: Aicardi-Goutieres syndrome 1 [MIM: 225750]; AD: Chilblain lupus [MIM: 610448]; AD: Vasculopathy, retinal, with cerebral leukoencephalopathy and systemic manifestations [MIM: 192315]) | 0.000208 |
| **Variants of Uncertain Significance** | | | | | | | | |
| **DSG2d** | Chr18 (18q12.1) | 29104553 (Exon 7) | NM_001943.5: c.828+5C>T (SNV) | rs373286117 | 125671 | PM2 BP4 | Heterozygous, Maternal | 0.00000806 |
| **TNXBe** | Chr6 (6p21.33) | 32052313 (Exon 8) | NM_019105.8: c.332G>A; p.Val1108Met (SNV) | rs121912575 | 600985 | PM2 BP4 | Heterozygous, Paternal | 0.000827 |
| **STX4** | Chr16 (16p11.2) | 31050877 (Exon 9) | NM_004604.4: c.718C>T; p.Arg240Trp (SNV) | rs770931989 | 186591 | PM2 PP3 | Homozygous, Paternal/Maternal | 0.00000796 |
a. Sequence positions refer to human reference genome hg19.
b. MAFs were obtained from Varsome using gnomAD Exomes Version: 2.1.1 entries.
c. Targeted deletion and duplication analysis by comparative genomic hybridization was performed by the Genetics and Genomics Diagnostic Laboratory at Cincinnati Children’s and was confirmed negative.
d. Other variants in this gene are associated with the following phenotypes: Arrhythmogenic right ventricular dysplasia 10 (autosomal dominant; MIM: 610193) and Cardiomyopathy, dilated, 1BB (MIM: 612877); however, this variant is not predicted to affect exon splicing.
e. Other SNVs in TNXB are associated with Ehlers-Danlos syndrome, classic-like, 1 (autosomal recessive; MIM: 606408) and Vesicoureteral reflux 8 (autosomal dominant; MIM: 615963), which is not concordant with Patient 1’s phenotype. A clinical testing submission of this variant were reported as "Likely Benign" on Varsome and ClinVar.
Table S2. Variants detected in Patient 2 by WES trio-analysis.

| Gene Symbol | Chromosome (Cytoband) | Positiona (Intron/Exon) | HGVS Nomenclature (Variant Type) | dbSNP RS ID | MIM | ACMG Criteria | Zygosity in Patient 2, Segregation (Known inheritance pattern of variant: disease manifestation) | Minor Allele Frequencyb |
|-------------|-----------------------|-------------------------|----------------------------------|-------------|-----|----------------|-----------------------------------------------------------------------------------|--------------------------|
| **Pathogenic/Likely Pathogenic Variants** | | | | | | | | |
| EIF3Fc | Chr11 (11p15.4) | 8016013 (Exon 5) | NM_003754.2: c.694T>G; p.Phe232Val (SNV) | rs141976414 | 603914 | PM2 PP3 PP5 | Homozygous, Paternal/Maternal (AR: Intellectual Developmental Disorder, 67 [MIM: 618295]) | 0.000701 |
| XDHf | Chr2 (2p23.1) | 31560605 (Exon 35) | NM_000379.3: c.3853C>T; p.Gln1285* (SNV) | rs761545629 | 607633 | PVS1 PM2 PM3 | Compound heterozygous, Maternal | 0.00000398 |
| **Variants of Uncertain Significance** | | | | | | | | |
| STX4 | Chr16 (16p11.2) | 31045392 (Exon 2) | NM_004604.4: c.89_90delGC; p.Gly30Aspfs*28 (DEL) | rs1301001687 | 186591 | PM2 | Compound heterozygous, Paternal | 0.0000000000301d |
| STX4 | Chr16 (16p11.2) | 31045650 (Intron 3) | NM_004604.4: c.232+4A>C (SNV) | rs922762463 | 186591 | PM2 PP3 | Compound heterozygous, Paternal | 0.00000399 |
| XDHf | Chr2 (2p23.1) | 31595130 (Exon 17) | NM_000379.3: c.1820G>A; p.Arg607Gln (SNV) | rs45442092 | 607633 | BP6 | Compound heterozygous, Paternal | 0.00203 |
| COL22A1 | Chr8 (8q24.23) | 139772485 (Intron 18) | NM_152888.3: c.1902+1G>A (SNV) | rs372694589 | 610026 | PM2 PP3 | Compound heterozygous, Paternal | 0.0000723 |
| COL22A1 | Chr8 (8q24.23) | 139629176 (Exon 53) | NM_152888.3: c.3851C>T; p.Ser1284Phe (SNV) | rs200631977 | 610026 | PM2 BP4 | Compound heterozygous, Maternal | 0.0000318g |
| DNAH2h | Chr17 (17p13.1) | 7674168 (Exon 27) | NM_020877.4: c.4279G>C; p.As1427His (SNV) | 603333 | PM2 PP3 BP1 | Compound heterozygous, Paternal | 0.0000000000192 |
| DYSF | Chr2 (2p13.2) | 71797041 (Exon 27) | NM_003494.4: c.2902A>T; | rs144636654 | 603009 | PM2 BP4 | Compound heterozygous, Paternal | 0.00140 |
| Gene | Chromosome (Location) | Sequence Position (Exon) | Transcript | Mutation | PM2 | PP3 | Concurrence | MAF |
|------|----------------------|--------------------------|------------|----------|-----|-----|-------------|-----|
| *TNK2* | Chr3 (3q29) | 195594879 (Exon 13) | NM_001010938.2: c.2479C>A; p.Pro827Thr (SNV) | 606994 | PM2 | PP3 | Compound heterozygous, Maternal | 0.00000000000155 |
| *TNS4* | Chr13 (17q21.2) | 38643441 (Exon 4) | NM_0032665.6: c.1135G>C; p.Gly379Arg (SNV) | 608385 | PM2 | PP3 | Compound heterozygous, Maternal | 0.00000000000200 |
| *OPRK1* | Chr8 (8q11.23) | 54142245 (Exon 3) | NM_000912.5: c.755G>A; p.Arg252His (SNV) | rs200672427 | 165196 | PM2 | De novo | 0.00000398 |
| *CACNG8* | Chr19 (19q13.42) | 54485817 (Exon 4) | NM_031895.6: c.992_994del; p.Gly331del (DEL) | rs769981108 | 606900 | PM2 | Mosaic, Maternal | 0.0000391 |

a. Sequence positions refer to human reference genome hg19.
b. MAFs were obtained from Varsome using gnomAD Exomes Version: 2.1.1 entries.
c. Previous associations were not concordant with Patient 2's phenotype; however, this variant was noted as an additional finding upon postnatal re-evaluation of the WES trio-analysis.
d. gnomAD MAF was not available. MAF computed from in-house global allele frequency.
e. Other variants in this gene are associated with Xanthinuria, type I (autosomal recessive; MIM: 278300) and Xanthinuria, type II (autosomal recessive; MIM: 603592), which are not concordant with patient 2's phenotype or zygosity.
f. This variant was associated with lowered activity in xanthine dehydrogenase in cell culture; however, two clinical testing submissions of this variant were reported as "Likely Benign" on Varsome and ClinVar.
g. gnomAD MAF was not available. MAF computed from in-house global allele frequency.
h. An additional heterozygous maternal SNV was synonymous. gnomAD MAF was not available. MAF computed from in-house global allele frequency. Other variants in this gene are associated with Spermatogenic failure 45 (autosomal recessive; MIM: 619094).
i. Conflicting interpretations of pathogenicity are reported for this variant in association with the listed conditions. Two additional heterozygous maternal alleles (NM_003494.4:c.3065G>A; p.Arg1022Gln [RS ID: rs34211915] and NM_003494.4:c.3992G>T; p.Arg1331Leu [RS ID: rs61742872]) were indicated as "Benign" by in silico prediction methods.
j. An additional heterozygous paternal SNV was filtered out due to quality. gnomAD MAF was not available. MAF computed from in-house global allele frequency.
k. An additional heterozygous paternal SNV was filtered out due high MAF and indication as "Likely Benign". gnomAD MAF was not available. MAF computed from in-house global allele frequency.
Table S3. List of primer sequences used.

| Name                              | Sequence                                                                 |
|-----------------------------------|--------------------------------------------------------------------------|
| **Patient sequencing primers:**   |                                                                          |
| R240W_F                           | 5’- CTTCATTTCCCTGAACCACCC-3’                                            |
| R240W_R                           | 5’- CTCACCTTTCTCGCCTTCTT-3’                                             |
| **In situ primers:**              |                                                                          |
| stx4-probe-F1                     | 5’- TCGCCCCCACACTGATCTCTA-3’                                            |
| stx4-probe-R1                     | 5’- GTCCACCATCTCACCCCTGTG-3’                                            |
| **gRNAs:**                        |                                                                          |
| stx4-t2 gRNA                      | 5’-GCTAGGAAGTTGCACTTCCAG-3’                                             |
| **Zebrafish sequencing primers:** |                                                                          |
| stx4-t2-F1                        | 5’- GAGATTCGAGAGGACTTGAAA-3’                                            |
| stx4-t2-R1                        | 5’- CTTTTTTTCATACCTGTGCTCAA-3’                                          |
| **Gateway cloning:**              |                                                                          |
| GFP-seq-F2                        | 5’- AGAAGAAACGGCATCAAGGTG-3’                                            |
| M13 Forward (-20)                 | 5’- GTAAAACGACGCGCAGT-3’                                                |
| M13 Reverse                       | 5’- CAGGAAACAGCTATGAC-3’                                                |
| stx4-attB1-F1                     | 5’-GGGGACAAGTTTGTACAAAAAGCAGGTTCACCATGCGGGACCGGACCAAAGAATGAC-3’          |
| stx4-attB2-R1                     | 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCAAGAGGACTTGCAAGGCA-3’               |
| stx4-RT-F2                        | 5’- GTTCAGGAGATTCGAGAGGACTTG-3’                                         |
| stx4-RT-R3                        | 5’- AACCATGCTGACCTCTCTCCTC-3’                                           |
| **QuikChange II primers:**        |                                                                          |
| stx4R241W-QC-F                     | 5’- GCACAGGGGTGAGTGATGGAGCTCGAATGAGTTC-3’                               |
| stx4R241W-QC-R                     | 5’- GGGCACATTAAAATGTTGGACTCAATCCAGTCACCATC-3’                           |
| **RT-qPCR primers:**              |                                                                          |
| stx4-RT-F4                        | 5’- CAGAGAAGCCAAATGTGGAG-3’                                             |
| stx4-RT-R4                        | 5’- ATCGTGTCGTGACTCAATC-3’                                              |
| gfp forward                       | 5’- CCAGATCCGACAACATCG-3’                                               |
| gfp reverse                       | 5’- GTCCATGCGAGGTAGTCACCG-3’                                            |
| actb2 forward                     | 5’- TACAGCTTCAACCACCAGAC-3’                                             |
| actb2 reverse                     | 5’- AGGAAAGGAAGGCTGGAAGAG-3’                                            |
Table S4. List of antibodies used.

| Name                                      | Host       | Clonality; Isotype | Manufacturer                                      | Catalogue number | Dilution |
|-------------------------------------------|------------|--------------------|---------------------------------------------------|-------------------|----------|
| **Primary antibodies**                    |            |                    |                                                   |                   |          |
| anti-acetylated Tubulin                   | Mouse      | Monoclonal; IgG2b  | Sigma Aldrich                                     | T7451             | 1:250    |
| anti-cleaved Caspase 3                   | Rabbit     | Polyclonal         | BD Biosciences                                    | 559565            | 1:250    |
| anti-Alcama                               | Mouse      | Monoclonal; IgG1   | University of Iowa Developmental Studies Hybridoma Bank (DSHB) | zn-8-s            | 1:10     |
| anti-DsRed2                               | Rabbit     | Polyclonal         | Clontech                                          | 632496            | 1:1000   |
| anti-Is1                                  | Rabbit     | Polyclonal         | Genetex                                           | GTX128201         | 1:00     |
| anti-Sarcomeric Myosin Heavy Chain (Mhc)  | Mouse      | Monoclonal; IgG2b  | DSHB                                              | MF20              | 1:10     |
| anti-Atrial Myosin Heavy Chain (Myh6)     | Mouse      | Monoclonal; IgG1   | DSHB                                              | S46               | 1:10     |
| anti-zebrafish Ventricular Myosin Heavy Chain (Myh7) | Rabbit | Polyclonal         | YenZym                                            |                   | 1:200    |
| anti-Syntaxin 4                           | Rabbit     | Polyclonal         | Sigma Millipore                                   | AB5330            | 1:400    |
| anti-Vamp2                                | Rabbit     | Polyclonal         | Genetex                                           | GTX132130         | 1:200    |
| **Secondary antibodies**                  |            |                    |                                                   |                   |          |
| anti-mouse IgG2b Alexa Fluor®-647         | Goat       | Polyclonal         | Southern Biotech                                  | 1091-31           | 1:250    |
| anti-rabbit IgG(H+L) Alexa Fluor®-647     | Goat       | Polyclonal         | Southern Biotech                                  | 4050-31           | 1:250    |
| anti-rabbit IgG (H+L) Cascade blue        | Goat       | Polyclonal         | Life Technologies                                 | C-2764            | 1:00     |
| anti-Mouse IgG1 DyLight™-405              | Goat       | Polyclonal         | Bio Legend                                        | 409109            | 1:00     |
| anti-mouse IgG1-FITC                      | Goat       | Polyclonal         | Southern Biotech                                  | 107002            | 1:100    |
| anti-rabbit IgG-FITC                      | Goat       | Polyclonal         | Southern Biotech                                  | 405002            | 1:100    |
| anti-mouse IgG1-TRITC                     | Goat       | Polyclonal         | Southern Biotech                                  | 107003            | 1:100    |
| anti-mouse IgG2b-TRITC                    | Goat       | Polyclonal         | Southern Biotech                                  | 109003            | 1:100    |
| anti-rabbit IgG-TRITC                     | Goat       | Polyclonal         | Southern Biotech                                  | 405003            | 1:100    |
Supplemental References:

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