SUPPLEMENTAL DATA

Detailed Methods

Study Population

Principal investigators evaluated U.S. patients at the University of Colorado Hospital in Aurora, Colorado, and Italian patients at Ospedali Riuniti in Trieste, Italy, as part of the Familial Cardiomyopathy Registry, a multi-center, three-decade-long, ongoing project studying hereditary human cardiomyopathies: 319 U.S. and European DCM families were investigated. Clinical information gathered from enrolled patients included medical history, family history, physical examination, and clinical investigations including laboratory, electrocardiogram (ECG), and echocardiogram evaluations.[1] Skeletal muscle was systematically evaluated by cardiologists using a comprehensive physical examination protocol and routine serum creatine kinase (CK) testing, as previously described.[2],[1] Criteria for DCM diagnosis were the presence of left ventricular (LV) fractional shortening <25% and/or an ejection fraction <45%, and LV end-diastolic diameter >117% of the predicted value by the Henry's formula. Skeletal muscle was systematically evaluated by cardiologists using a comprehensive physical examination protocol and routine serum creatine kinase (CK) testing, as previously described.[2],[1]

Registry exclusion criteria included the following conditions: blood pressure >160/110 mmHg, obstruction >50% of a major coronary artery branch, alcohol intake >100 g/day, persistent high-rate supraventricular arrhythmias, systemic diseases, pericardial diseases, congenital heart diseases, cor pulmonale, or myocarditis. Medical records from deceased subjects were reviewed when available.[2]

Next-generation Sequencing and Bioinformatic Analysis

DNA was extracted from whole blood in standard fashion. Twenty larger families were evaluated by whole-exome sequencing, as previously described.[2] In 299 smaller families, individual probands were evaluated using the Illumina TruSight One-Sequence panel (Illumina, Redwood City, California, U.S.), which queries 4,813 genes associated with known clinical phenotypes, as previously described.[3] Briefly, patient DNA samples had DNA regions of interest captured with the panel and sequenced on a HiSeq 2500 Sequencing System (Illumina) with v4 chemistry at the Genomics and Microarray Core at the University of Colorado Denver.

The sequenced reads were aligned to the human genome build GRCh37hg19 using Genomic Short-read Nucleotide Alignment Program (GSNAP; version 07/20/2012, Thomas Wu/Genentech Inc., South San Francisco, California, U.S.)[4]. The variants were called with the Genome Analysis Toolkit (GATK; version 2.1-8-g5efb575; Broad Institute, Cambridge, Massachusetts, U.S.) and annotated using Annotate Variation (ANNOVAR, version 2012-07-28, QIAGEN, Hilden, Germany) software[5]. Variants that changed an amino acid or altered splice sites were selected. After filtering, the database for nonsynonymous single-nucleotide polymorphism functional predictions (dbNSFP, version 2.0, Xiaoming Liu, University of Texas Health Science Center at Houston, Houston, Texas, U.S.)[6] was employed to provide predictions for all nonsynonymous single-nucleotide polymorphisms (SNPs) based on the widely used functional prediction algorithms, as previously described.[7] Variants predicted to be damaging in at least one of the prediction algorithms were retained, while missense and truncation variants present in greater than 1% in the 1000 Genomes Project were discarded.[2,8] All variants were confirmed by Sanger sequencing.

Variant frequency information obtained from the 6500 National Human, Lung, and Blood Institute (NHLBI) Exome Sequencing Project[9] and the Exome Aggregation Consortium (ExAC,

Cambridge, MA)[10] (accessed 10/17/2016) was used to assess the minor allele frequency (MAF) in the general population. The MAF in our study population was determined by dividing the number of variants by number of patients in our study population. *FLNC* variants were cross-referenced to the ClinVar[11] database to identify any clinical significance previously reported. Gene variant locations are provided in reference to *FLNC* transcript NM_001458. Cosegregation analysis was performed when DNA from additional biological relatives was available. Variants in other cardiomyopathy-related genes were also identified using the Illumina TruSight One Sequencing Panel, as described above, and confirmed using Sanger sequencing.

Histology, Immunohistochemistry and Transmission Electron Microscopy

For transmission electron microscopy (TEM), frozen human heart muscle tissue samples were thawed and fixed in cacodylate buffered 2.5% glutaraldehyde, followed by 2.0% osmium tetroxide, dehydrated through a graded ethanol series, and then embedded in epoxy resin. Ultrathin sections (90nm) were post-stained with uranyl acetate and lead citrate. The ultrastructural observations were performed using a Hitachi H-7650 TEM (Hitachi, Tokyo, Japan) machine equipped with an AMT XR-11 digital camera system (Advanced Microscopy Techniques, Woburn, Massachusetts, U.S). Sample preparation and imaging were performed by the Electron Microscopy Lab at Children's Hospital Colorado.

Preparation and Immunostaining of Buccal Mucosa Smears

As previously described,[12] briefly, the slides were sprayed with 70% ethanol and, after air-drying, were stored in plastic bags containing N_2 gas. Then the slides were simultaneously permeabilized and blocked by incubation in phosphate-buffered saline (PBS) containing 1% Triton X-100, 3% normal goat serum, and 1% bovine serum albumin. They were then incubated with a primary antibody (overnight at 4°), then with indocarbocyanine (Cy3)-conjugated goat

anti-rabbit or anti-mouse IgG secondary antibodies (2 hrs at room temperature), and counter-stained with DAPI to label nuclei. Primary antibodies included: rabbit polyclonal anti-E-cadherin (AbCam ab15148; 1:400), mouse monoclonal anti-plakoglobin (Sigma-Aldrich P8087; 1:1,500), rabbit polyclonal anti-Cx43 (AbCam ab11370; 1:1000), mouse monoclonal anti-desmoplakin (Fitzgerald 10R-D109A; 1:50), mouse monoclonal anti-SAP97 (LSBio, LS-B8156; 1:100), and mouse monoclonal anti-GSK3β (Sigma-Aldrich SAB5300175; 1:100).

Immunostaining of Myocardial Tissue

In preparation for immunofluorescence microscopy, deparaffinized, rehydrated slide-mounted sections were heated in citrate buffer (10mmol/L, pH 6.0), and after being cooled to room temperature, were simultaneously permeabilized and blocked in PBS containing 1% Triton X-100, 3% normal goat serum and 1% bovine serum albumin. The sections were then incubated with a primary antibody O/N at 4°. Antibodies included: mouse monoclonal anti-plakoglobin (Sigma-Aldrich P8087; 1:1000), mouse monoclonal anti-connexin 43 (Cx43) (Millipore MAB3068; 1:400), mouse monoclonal anti-N-cadherin (Sigma-Aldrich C3865; 1:400), mouse monoclonal anti-desmoplakin (Fitzgerald 10R-D109A; 1:10), mouse monoclonal anti-SAP97 (Santa Cruz sc-9961; 1:100), and rabbit polyclonal anti-glycogen synthase kinase 3 β (GSK3β) (Cell Signaling 27C10, 9315; 1:80). The slides were thereafter incubated with indocarbocyanine-conjugated goat anti-mouse or anti-rabbit IgGs (1:400, Jackson ImmunoResearch). Immunohistochemistry staining for FLNC was performed on frozen heart tissues, fixed in formalin and processed for paraffin embedded sections in the UCDAMC Histology Shared Resource laboratory. Immunoperoxidase staining for FLNC was performed using anti FLNC rabbit monoclonal antibody (Abcam EPR14498(B), Cambridge, MA) (1:500). Counterstaining for nuclei was performed with hematoxylin. Images were obtained with Leica Aperio in the

UCDAMC Biorepository Shared resource laboratory by eSlide Manager with ImageScope software.

RNA-sequencing of explanted heart tissue

The sample was DNase treated (TURBO DNA-free Kit, Thermo Fisher Scientific), RNA integrity was measured at 7.3 (Agilent 2100 Bioanalyzer, Agilent Technologies), polyA transcripts were isolated from 1µg total RNA using oligo-dT beads, a cDNA library was constructed (TruSeq Stranded mRNA Library Prep Kit, Illumina), and the library was sequenced 1x50 (Illumina HiSeq 2500).

Detailed Results

Detailed description of the clinical phenotype in FLNC truncation carriers

In family DNFDC057, the proband II:2 presented with palpitations, polymorphic sustained VT and paroxysmal atrial fibrillation.[2] The ECG showed an atrioventricular ventricular conduction delay, while echocardiography showed a mildly dilated LV (5.6cm), with severe LV dysfunction (LVEF 10%), and right ventricular (RV) enlargement. The proband's brother (II:1), also a FLNC truncation carrier, similarly presented with arrhythmias, including non-sustained ventricular tachycardia (NSVT), premature ventricular contractions (PVCs), premature atrial contractions (PACs), and conduction disease.

Two families, DNFDC079 and DNFDC195, were noted for having pre-syncope and syncope. In family DNFDC079, proband III:1 presented with pre-syncope and DCM at 33 years old. Although III:1 did not present with arrhythmias, he had RV enlargement. In family DNFDC195, the proband (II:2) presented with syncope and was diagnosed at 39 years old with

polymorphic PVCs. The patient eventually required a pacemaker and automatic implantable cardioverter-defibrillator (AICD). Proband II:2 also had mildly dilated and mildly dysfunctional RV. His family history was remarkable for the sudden cardiac death of his father at 41 years old.

Families TSFDC029, TSFDC031 (as previously reported[2]), and TSFDC043 were also characterized by a strong arrhythmogenic trait. In family TSFDC029, individuals II:6 and III:6 had a history of SCD at the ages of 54 and 35, respectively, while the proband had frequent (>40/h) and repetitive premature ventricular contractions. In family TSFDC031, the proband II:2 died suddenly and unexpectedly at the age of 48, and his son III:1 presented with supraventricular arrhythmias (recurrent atrial fibrillation and atrial tachycardia) early in the disease at 21 years old (*Figure 1*).

In family TSFDC043, the proband (I:2) demonstrated an arrhythmogenic phenotype with frequent PVCs and atrial fibrillation. Her electrocardiogram (ECG) showed conduction disease with atrial ventricular block (AVB), right bundle branch block (RBBB), and left anterior fascicular block (LAFB). This proband also carried a secondary, possibly pathogenic gene variant, a unique loss of function variant in *SCN5A* (reference build GRch37 chr3.38592538, c.5270delT, p.F1757fs), which may have contributed to atrial fibrillation and a more severe and progressive conduction disease requiring permanent pacing, and severe LV dysfunction. This individual was the only proband found to carry a possibly pathogenic secondary variant, which was performed by screening variants in 54 other known cardiomyopathy-related genes.

Finally, in family TSSDC130, the proband (II:3) was diagnosed at 20 years old with NYHA functional class 2 symptoms, and presented with syncope, conduction delay, and recurrent sustained ventricular tachycardia (VT) requiring an AICD (*Figure 1B*). The ECG revealed sinus bradycardia, conduction delay, and non-specific ST changes.

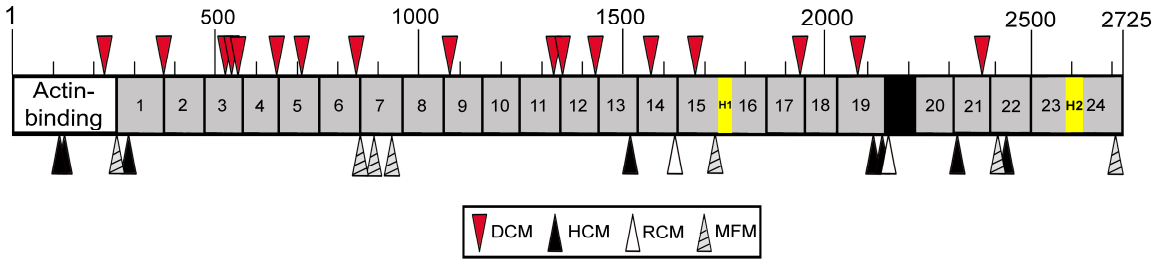
References for Supplemental Methods

1. Mestroni L, Maisch B, McKenna WJ, et al. Guidelines for the study of familial dilated cardiomyopathies. Collaborative Research Group of the European Human and Capital Mobility Project on Familial Dilated Cardiomyopathy. European heart journal. Jan 1999;20(2):93-102.
2. Begay RL, Tharp CA, Martin A, et al. FLNC Gene Splice Mutations Cause Dilated Cardiomyopathy. JACC. Basic to translational science. Aug 2016;1(5):344-359.
3. Rowland TJ, Graw SL, Sweet ME, Gigli M, Taylor MR, Mestroni L. Obscurin Variants in Patients With Left Ventricular Noncompaction. Journal of the American College of Cardiology. Nov 15 2016;68(20):2237-2238.
4. Wu TD, Nacu S. Fast and SNP-tolerant detection of complex variants and splicing in short reads. Bioinformatics (Oxford, England). Apr 01 2010;26(7):873-881.
5. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic acids research. Sep 2010;38(16):e164.
6. Liu X, Jian X, Boerwinkle E. dbNSFP: a lightweight database of human nonsynonymous SNPs and their functional predictions. Human mutation. Aug 2011;32(8):894-899.
7. Campbell N, Sinagra G, Jones KL, et al. Whole exome sequencing identifies a troponin T mutation hot spot in familial dilated cardiomyopathy. PloS one. 2013;8(10):e78104.
8. Abecasis GR, Auton A, Brooks LD, et al. An integrated map of genetic variation from 1,092 human genomes. Nature. Nov 01 2012;491(7422):56-65.
9. NHLBI Go Exome Sequencing Project (ESP). http://evs.gs.washington.edu/EVS/. Accessed October 17, 2016.

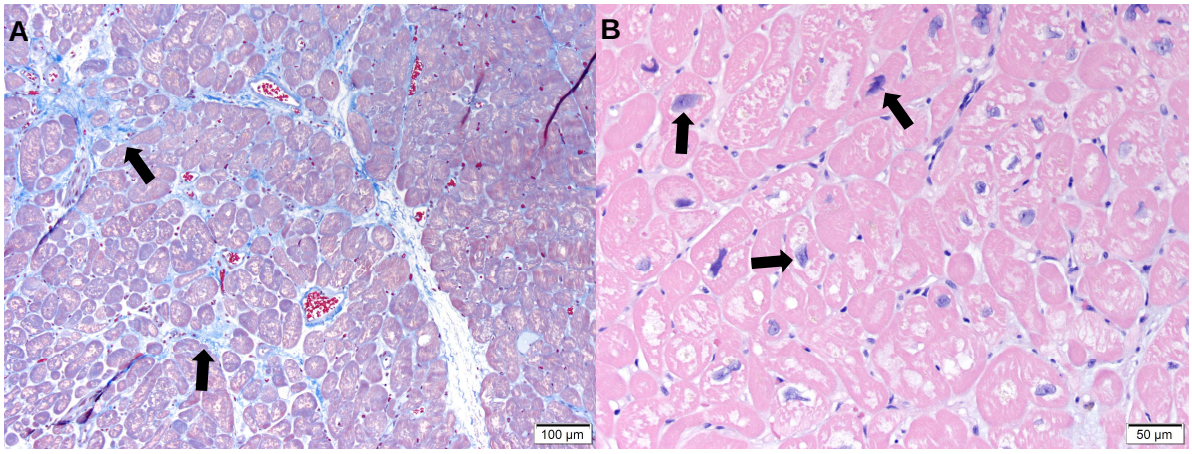
10. Lek M, Karczewski KJ, Minikel EV, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature. 08/18/print 2016;536(7616):285-291.
11. Landrum MJ, Lee JM, Benson M, et al. ClinVar: public archive of interpretations of clinically relevant variants. Nucleic acids research. Jan 04 2016;44(D1):D862-868.
12. Asimaki A, Protonotarios A, James CA, et al. Characterizing the Molecular Pathology of Arrhythmogenic Cardiomyopathy in Patient Buccal Mucosa Cells. Circulation. Arrhythmia and electrophysiology. Feb 2016;9(2):e003688.

Supplemental Reference for the Main Text

31. van der Zwaag PA, van Rijsingen IA, Asimaki A, Jongbloed JD, van Veldhuisen DJ, Wiesfeld AC, Cox MG, van Lochem LT, de Boer RA, Hofstra RM, Christiaans I, van Spaendonck-Zwarts KY, Lekanne dit Deprez RH, Judge DP, Calkins H, Suurmeijer AJ, Hauer RN, Saffitz JE, Wilde AA, van den Berg MP, van Tintelen JP. Phospholamban R14del mutation in patients diagnosed with dilated cardiomyopathy or arrhythmogenic right ventricular cardiomyopathy: evidence supporting the concept of arrhythmogenic cardiomyopathy. Eur J Heart Fail. 2012;14(11):1199-207.



Supplemental Figure 1. Distribution of *FLNC* missense variants on FLNC protein (NP_001449.3). All *FLNC* missense variants detected by our group are labeled by protein position (1-2725) indicated by red triangles. Other variants previously reported are labeled as black, white, and diagonal triangles are HCM, RCM, and MFM respectively. Actin-binding refers to the actin-binding domain. Immunoglobulin domains are labeled 1-24. H1 and H2 are Hinge 1 and 2 respectively. Variant (Q233H), located in the actin-binding domain, is the only missense variant that cosegregated.



Supplemental Figure 2. Cardiac tissue analysis of DCM affected individual II:1 from family DNFDC057. A) Trichrome stain of left ventricle cardiac tissue shows moderate interstitial fibrosis (indicated by blue staining and black arrowheads), and **B)** H&E staining showed marked myocyte hypertrophy (see arrowheads) Scale bars in A-100µm, B-50µm.

Family	Function	Exon	Position	Nucleotide Change	Amino Acid Change	SIFT	GERP	ExAC Allele Frequency
TSSDC130	stopgain	4	128477557	c.C805T	p.R269X	-	-	8.29E-06
DNFDC079	stopgain	13	128481619	c.C2119T	p.Q707X	-	-	absent
DNFDC195	splicing	20	128484057	c.2930-1G>T	p.K977fs	0	5.05	absent
TSFDC043	splicing	22	128486043	c.3791-1G>A	p.G1264fs	0	5.07	absent
DNFDC057	splicing	34	128491508	c.5669-1delG	p.G1891Vfs61X	-	-	absent

Supplementary Table 1. *FLNC* truncation variants detected in five families. Variant location corresponds to transcript NM_001458 (reference build GRch37). Variants were not present in 1000 Genomes Project (accessed 10/17/2016) except for variant p.R269X. ExAC frequency was detected (accessed 10/17/2016) for variant p.R269X, but absent for all other variants. Variants were absent from the ClinVar database (accessed 10/17/2016) and the NHLBI Exome Sequencing Project Exome Sequencing Project (accessed 10/17/2016). Dashed lines indicate not available.

Position	Amino Acid Change	SIFT	GERP	ExAC Allele Frequency	ClinVar
chr7:128477311	p.Q233H	0	5.74	Absent	Absent
chr7:128478381	p.M370V	0.18	5.51	0.00005801	Absent
chr7:128480968	p.V586A	0.02	4.87	0.00002174	Absent
chr7:128482971	p.A838V	0.06	5.7	0.00001672	Absent
chr7:128481359	p.R650Q	0.25	5.2	0.00004141	Absent
chr7:128484766	p.F1083L	0	5.54	Absent	Absent
chr7:128486459	p.G1357R	0	4.19	Absent	Absent
chr7:128487763	p.R1434L	0.01	4.23	0.00006652 (rs143623535)	Absent
chr7:128491632	p.R1931L	0.04	5.7	0.00003303	Absent
chr7:128493531	p.G2073S	0.18	5.42	Absent	Absent
chr7:128494942	p.V2371I	0.08	4.27	0.00004143	Absent

Supplemental Table 2. List of detected *FLNC* missense variants. Variant location corresponds to transcript NM_001458 (reference build GRch37). ClinVar and ExAC databases were accessed 7/1/2016.