HEART DISEASE

Titin mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy

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Human mutations that truncate the massive sarcomere protein titin (TTN-truncating variants (TTNtvs)) are the most common genetic cause for dilated cardiomyopathy (DCM), a major cause of heart failure and premature death. Here we show that cardiac microtissues engineered from human induced pluripotent stem (iPS) cells are a powerful system for evaluating the pathogenicity of titin gene variants. We found that certain missense mutations, like TTNtvs, diminish contractile performance and are pathogenic. By combining functional analyses with RNA sequencing, we explain why truncations in the A-band domain of TTN cause DCM, whereas truncations in the Z band are better tolerated. Finally, we demonstrate that mutant titin protein in iPS cell–derived cardiomyocytes results in sarcomere insufficiency, impaired responses to mechanical and β-adrenergic stress, and attenuated growth factor and cell signaling activation. Our findings indicate that titin mutations cause DCM by disrupting critical linkages between sarcomerogenesis and adaptive remodeling.

Dilated cardiomyopathy (DCM) is characterized by progressive left ventricular dilation; systolic dysfunction; and, ultimately, heart failure. Occurring in 1 of 250 adults (1), DCM arises from underlying cardiovascular conditions or as a primary genetic disorder. We recently identified dominant mutations that truncate the sarcomere protein titin (TTN-truncating variants (TTNtvs)) as the most common genetic cause of DCM, occurring in ~20% of familial or sporadic cases (2). TTN is a massive protein that spans half of the sarcomere (1 μm) and includes ~94,000 amino acids within four functionally distinct segments (Fig. 1A): (i) an N-terminus that is anchored at the Z disk; (ii) a distensible I band (~1 MD) composed of repeating immunoglobulin-like domains and disordered regions; (iii) an inextensible, thick filament–binding A band (~2 MD); and (iv) a carboxyl M band with a kinase domain. TTNtvs have been identified in each protein segment, but TTNtvs in DCM patients are markedly enriched in the A band (2, 3). In addition, numerous rare missense variants in TTN have been identified, most with unknown medical importance (2, 3). Both TTN’s size and a general incomplete knowledge of the protein’s function in cardiac myocyte biology have hindered traditional approaches for elucidating why some TTN mutations produce clinical phenotypes. To address this, we harnessed recent advances in stem cell reprogramming (6), gene editing (5), and tissue engineering (6) to produce human cardiac microtissue (CMT) models of TTNtvs.

We generated human induced pluripotent stem cell–derived cardiomyocytes (iPS-CMs) from patients (labeled with “p” preceding genotype) (see supplementary materials and methods). Cryopreserved blood samples from one unaffected and three DCM patients with dominant TTN mutations (Fig. 1A and table SIA) were reprogrammed, and high-quality iPS cell clones (fig. S1. A to C) were expanded, differentiated (7), and enriched by metabolic selection (8) to achieve cultures with >90% iPS-CMs (fig. S2, A to C). We produced iPS-CMs with two A-band TTNtvs (pA23352fs+ or pP22582fs+ or pC21282fs+ or pA23352fs+ or pP22582fs+ or pA21282fs+) and a missense mutation (pW976R+ or pW976R+ or pW976R+ or pW976R+ or pW976R+). The Z/I junction missense mutation demonstrates that W976R+ is a pathogenic TTN missense mutation.

To ensure that the observed functional abnormalities did not reflect background genetic differences in patient-derived iPS-CMs, we also introduced TTNtvs into an independent, isogenic iPS cell using scarless, CRISPR (clustered regularly interspaced short palindromic repeats)/CRISPR (clustered regularly interspaced short palindromic repeats)/CAS9 technology (5) to target the I- or A-band exons (Fig. 1A and table SIB). Mutant isogenic iPS cell lines (labeled with “c” preceding genotype) were differentiated into iPS-CMs and incorporated into CMTs. Cn22577fs+ creates an A-band TTNtv in exon 322 (similar to patient-derived pP22582fs+) (Fig. 1A). Cn22577fs+ CMTs had significantly reduced contractile force (2.19 μN) compared with isogenic CWT-CMTs (Fig. 1E) (P < 0.003), but not to the extent observed in pA23352fs+ or pP22582fs+ CMTs (0.767 and 1.001 μN, respectively; P < 0.02). Tissue stress was similarly reduced in TTNtvs CMTs compared to WT CMTs (fig. S2D). These data confirm that A-band TTNtvs markedly reduced contractile function in both patient-derived and isogenic CMTs and raise the possibility that the genetic background can modify the functional severity of TTNtvs.

Premature protein truncation at any location within a molecule is generally assumed to result in loss of function and comparable deleterious consequences. However, I-band TTNtvs have been identified in healthy individuals and in the general population without DCM (2, 3). Two models have been proposed to explain this dichotomy: (i) Alternative splicing excludes I-band exons from most mature TTN transcripts and thereby reduces the functional consequences of I-band TTNtvs, whereas the inclusion of A-band exons with TTNtvs is deleterious. (ii) A-band TTNtv transcripts produce longer, stable mutant proteins with dominant negative effects on sarcomere biology. To distinguish these models, we compared the functional consequences of isogenic
heterozygous (cV6382fs<sup>+</sup>) and homozygous (cV6382fs<sup>+</sup>) I-band TTNTs in exom 66 to A-band TTNTs (cN225776<sup>+</sup>-cT33520fs<sup>-</sup>). We used RNA sequencing (RNA-seq) analyses to assess TTNT RNA splicing in IPS-CMs and to compare our findings with normal adult left ventricle (LV) tissue. TTNT transcripts from IPS-CMs incorporated more I-band exons than did adult LV tissue (fig. S3, A and B). More than 80% of IPS-CM TTNT transcripts included exon 66, five times more than adult LV (in which 18% of TTNT transcripts contained exon 66).

eV6382fs<sup>-/-</sup> CMTs had markedly impaired force generation, similar to the deficits observed in isogenic CMTs with A-band (cN225776<sup>-/-</sup>) or patient-derived CMTs with A-band (pP22582fs<sup>-/-</sup>) (Fig. 1, D and E). Functional comparisons of CMTs with homoygous TTNTs in the I or A band were also similar. Homozygous A-band CMTs (cN225776<sup>-/-</sup> and cT33520fs<sup>-/-</sup>) produced no force; whereas homoygous I-band CMTs (cV6382fs<sup>-/-</sup>) generated small but demonstrable force (0.47± μN), presumably due to 18% of TTNT transcripts that excluded exon 66 and the TTNT (fig. S3A). On the basis of these functional and RNA-seq data, we suggest that alternative exon splicing is the predominant mechanism for reduced penetrance of I-band TTNTs.

As TTNT is responsible for sensing and responding to myocardial stress (18), we posited that TTNT mutations would exhibit aberrant stress responses. To model low and high mechanical load, we assessed contractile performance of pWT and pP22582fs<sup>-/-</sup> CMTs grown on flexible (0.2 μN/μm) and rigid (0.45 μN/μm) cantilevers (Fig. 1F), pP22582fs<sup>-/-</sup> CMTs produced less force than pWT CMTs at low load. At higher load, pWT CMTs produced a twofold increase in force, greater than four times that produced by pP22582fs<sup>-/-</sup> CMTs. In response to isoproterenol treatment to mimic β-adrenergic stimulation, force and beating rate were increased in pWT CMTs, but pP22582fs<sup>-/-</sup> CMTs had markedly blunted responses (Fig. 1G and H). Together, these data demonstrate that TTNTs had both basal and stress-induced inotropic and chronotropic deficits that are expected to impair cardiac adaptation to increased mechanical load and β-adrenergic signalling.

We considered whether A-band TTNTs affected the organization of the sarcomere, where TTNT is localized, which could cause or contribute to functional defects. Using antibodies to TTNT (9D10 recognizes the N-terminus of TTNTs) (Fig. 2A) and α-actinin (Fig. 2B and C), we identified well-formed parallel arrays of repeating sarcomeres in myofibrils from WT IPS-CMs. pP22582fs<sup>-/-</sup> IPS-CMs had fewer myofibrils and abnormal, irregular sarcomeres, a phenotype that was even more pronounced in homozygous cT33520fs<sup>-/-</sup> IPS-CMs (Fig. 2, A and D, and fig. S4A). Sarcomere disorganization was observed regardless of whether the IPS-CMs were aligned on micro-patterned lines (Fig. 2B) or 3D CMTs (Fig. 2C). In addition, sarcomere length was shorter in pP22582fs<sup>-/-</sup> CMTs compared with pWT CMTs (Fig. 2E), indicating both a quantitative and qualitative defect in sarcomereogenesis. Analogous to these results, LV tissue from the patient with the P22582fs<sup>-/-</sup> mutation showed disorganized myofibrils compared with control tissue (Fig. 2, G and H, and fig. S4B).

To determine whether sarcomere deficits reflected insufficient TTNT levels, we performed RNA-seq (14) and protein analyses. CMT and cN225776<sup>-/-</sup> IPS-CMs showed comparable levels of TTNT transcripts [fragments per kilobase of transcript per million mapped reads (FPKM)] and similar patterns of TTNT splicing (fig. S3), B

![Fig. 1. Engineered IPS-CM microtissues with TTNT mutations have impaired intrinsic contractility and responses to stress.](http://science.sciencemag.org/)
and C). RNA-seq (table S1D) and Sanger sequencing of reverse transcriptase polymerase chain reaction products (fig. S5) demonstrated equal amounts of mutant and WT transcripts. Consistent with RNA-seq data, protein gels of iPSC-CM extracts showed expression of the larger fetal TTN isoforms, which include more 1-band exons than the adult TTN isoforms (Fig. 2F). We next sought to identify truncated TTN protein (Fig. 2F and fig. S6, A to I) in protein lysates from iPSC-CMs with an A-band (pP22582fs<sup>101</sup>, pA2335fs<sup>101</sup>) or 1-band (pS6394fs<sup>101</sup> (benign variant)) TTNtv. Both iPSC-CMs and adult LV contained N2BA (~3300 to 3700 kD) and N2B TTN isoforms (~3000 kD); however, iPSC-CM lysates also contained the larger fetal N2BA isoforms (~3700 kD). In addition, a smaller fragment (~2500 kD) was present in pP22582fs<sup>101</sup> iPSC-CM extracts, but not in pWT (Fig. 2F), pA2235fs<sup>101</sup>, or pS6394fs<sup>101</sup> iPSC-CMs (fig. S6, B and C). This smaller fragment reacted with TTN T12 antibody and, on the basis of size and immunoreactivity, is probably a stable truncated TTN protein. Given both the detection of mutant TTNtv protein and the paucity of sarcomeres in heterozygous pP22582fs<sup>101</sup> and homozygous cT33520fs<sup>101</sup> iPSC-CMs (fig. 2, A to C), we deduced that TTNtv protein, even if stable within CMs, is unable to promote sarcomereogenesis.

RNA-seq analyses of eWT, cN22577fs<sup>101</sup>, and cN22577fs<sup>101</sup> iPSC-CMs also indicated that TTNtvfs affected CM signaling and RNA expression. The significantly altered transcripts in iPSC-CMs with TTNvts (Fig. 3A and table S1C) suggested increased activity of three critical upstream microRNA (miRNA) regulators: miR-124 (15), miR-16 (16), and miR-1 (17). Consistent with this prediction, cN22577fs<sup>101</sup> and cN22577fs<sup>101</sup> iPSC-CMs had diminished expression of miR-124 targets (15), including lower MYH7/MYH6 transcript and protein ratios (Fig. 3, B and C, and fig. S6, J and K) and reduced atrial (NPPA) and brain (NPPB) natriuretic peptide transcript levels compared with WT iPSC-CMs (Fig. 3, B and C).

Pathway analysis of RNA-seq data on cN22577fs<sup>101</sup> and cN22577fs<sup>101</sup> compared with WT iPSC-CMs (fig. S3E) also implied diminished activation of factors regulating growth [transforming growth factor β1 (TGFβ1)], vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), and basic fibroblast growth factor (FGF2)], responses to hypoxia [hypoxia-inducible factor 1 (HIF1A) and EPAS1 or HIF2A], and mitogen-activated protein kinases.

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**Fig. 2. Sarcomere abnormalities in TTNtv iPSC-CMs.** (A) pWT, pP22582fs<sup>101</sup>, and cT33520fs<sup>101</sup> iPSC-CMs stained with TTN specific antibody (9D10, green) and nuclei (DAPI, blue). Magnification, 40×; scale bar, 20 μm. (B) pWT and pP22582fs<sup>101</sup> iPSC-CMs were patterned on 25-μm by 25-μm grids and stained for Z discs (α-actinin A, green) and nuclei (DAPI, blue). Magnification, 40×; scale bar, 20 μm. (C) pWT, pP22582fs<sup>101</sup>, and cT33520fs<sup>101</sup> CMs stained for α-actinin A (green) and F-actin (red). Staining, 40×; scale bar, 20 μm. (D) Sarcomere organization (κ) quantified by 2D fast Fourier transform (FFT) analysis of pP22582fs<sup>101</sup>, and cT33520fs<sup>101</sup> iPSC-CMs (N > 5 per genotype). (E) Sarcomere length (in micrometers) measured by intensity profiles of α-actinin in pWT and pP22582fs<sup>101</sup> iPSC-CMs (N > 22). (F) Protein electropherograms of lysates from pP22582fs<sup>101</sup> and pWT iPSC-CMs and human LV stained with Coomassie Blue (for additional blots, see fig. S6). Sizes of TTN isoforms and fragments are as follows: N2BA fetal, ~3700 kD; N2BA adult, ~3300 kD; N2B, ~3000 kD; TTNvts, ~2500 kD; degraded TTN, ~1800 to 2200 kD. Obscurin size is ~700 kD. Western blots (WB) were probed with N-terminal TTN antibody (T12 in fig. S6). TTNtv protein (~2500 kD) was detected in pP22582fs<sup>101</sup> iPSC-CMs. (G) Representative micrographs of hematoxylin-and-eosin-stained tissue from the LV of control and P22582fs<sup>101</sup> patients. Scale bars, 20 μm. (H) Sarcomere organization quantified by FFT analysis of LV tissue from control and pP22582fs<sup>101</sup> patients (n > 15 regions per genotype). Significance was assessed by Student’s t test [(D), (E), and (H)]; data are means ± SEM (error bars) [(D), (E), and (H)].
(MAPKs) MAPK kinase (MEK) and extracellular signal-regulated kinase (ERK). Quantification of transcripts, proteins, and phosphorylation levels confirmed that TTNv IPS-CMs exhibited significant attenuation (all P < 0.01) in the levels or phosphorylation of TGFβ, VEGF, MAPKs, and AKT (Fig. 3, D to H) but not HGF, EGF, or FGFR2 (Fig. S3F). To determine whether these signaling deficits contributed to force deficits, we pretreated pP22582fs+/- and pW976R+/- IPS-CMs with VEGF (50 ng/mL) or TGFβ (0.5 ng/mL) for 4 days before studying CMTs. In contrast to the failed augmentation in response to mechanical load (Fig. 1F) or β-adrenergic stimulation (Fig. 1G), supplementation with VEGF, but not TGFβ (Fig. S3F), improved force production in pP22582fs+/- and pW976R+/- CMTs (Fig. 3I).

Coupled with engineered biomimetic culture systems, the use of patient-derived or gene-editing technologies to produce iPSC-CMs provides robust functional genomic insights. Our studies of iPSC-CM with different TTN mutations revealed that some missense variants like TTNv are pathogenic, whereas comparisons of contractile function in patient-derived and isogenic CMTs implied a role for genetic modifiers in clinical manifestations of TTNv. We found that both I- and A-band TTNv can cause substantial contractile deficits but that alternative exon splicing (fig. S3, A and B) mitigates the pathogenicity of I-band TTNv. Additional factors—such as IPS-CMs not fully recapitulating the cell biology of adult cardiomyocytes and CMT tissues lacking in vivo compensatory responses—may also account for different degrees of contractile deficits in IPS-CM and human hearts with TTNv. Surprisingly, some TTNv produced stable truncated protein, but this mutant peptide failed to assemble with other contractile proteins into

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**Fig. 3 TTN regulates IPS-CM signaling and RNA expression.** (A) Upstream transcriptional regulators were identified by Ingenuity pathway analysis (IPA) of differentially regulated genes (normalized ratio >1.2 and <0.8 and P < 0.01) (table S1C) using RNA-seq from cWT, cN22577fs+/-, and cN22577fs+/- IPS-CMs. Data are plotted as z score of enrichment (z score cutoff: ±3.5 and ±3.5). (B to D) Comparison of cWT, cN22577fs+/-, and cN22577fs+/- IPS-CMs normalized expression (FPKM) of (B) β(MYH7) and α(MYH6) myosin heavy-chain ratios; (C) atrial (NPPA) and brain natriuretic (NPRB) peptides; and (D) TGFβ1 and VEGF-A in cWT, cN22577fs+/-, and cN22577fs+/- IPS-CMs. (E) Densitometry of Western blots (N = 4 lanes) of pWT and pP22582fs+/- lysates, normalized for protein loading and probed with antibodies to phosphorylated c-Jun N-terminal kinase (JNK) (p46, p54), ERK, p38, and AKT. (F) Representative Western blots of pWT and pP22582fs+/- IPS-CMs lysates probed for (α) JNK (183/185), p-ERK (1202/1204), p-p38(1810/1812), and p-AKT (1208), as well as total JNK, ERK, p38, and AKT. (G) Densitometry of Western blots (N = 4 lanes) normalized to protein loading of TGFβ-3 and VEGF. (H) Representative lanes from Western blots from pWT and pP22582fs+/- IPS-CMs probed for TGFβ3, TGFβ2, TGFβ1, VEGF, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (I) Mean twitch force (in micronewtons) generated by pP22582fs+/- IPS-CMs pretreated with 50 ng/mL VEGF (N = 4 CMTs). Significance was assessed by Bayesian P values [(B) to (D)] or Student’s t test [(E), (G), and (I)]; data are means ± SEM (error bars) [(E), (G), and (I)].
well-organized functional sarcomeres. The resultant sarcomere insufficiency (fig. S7) caused both profound baseline contractile deficits and attenuated signaling that limited cardiomyocyte reserve in response to mechanical and adrenergic stress, parameters that are critical to DCM pathogenesis. The consequences of TTN truncation are markedly different from the effects of truncating mutations in another sarcomere protein, myosin-binding protein C (MYBPC). truncation of MYBPC causes enhanced contractile power (18). Our findings also suggest potential therapeutic targets for TTNVs, including strategies to enhance TTN gene expression, diminish mRNAs that inhibit sarcomereogenesis (15, 19), or stimulate cardiomyocyte signals that improve function (20).

REFERENCES AND NOTES
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SUPPLEMENTARY MATERIALS
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SYNTHETIC BIOLOGY
Emergent genetic oscillations in a synthetic microbial consortium
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A challenge of synthetic biology is the creation of cooperative microbial systems that exhibit population-level behaviors. Such systems use cellular signaling mechanisms to regulate gene expression across multiple cell types. We describe the construction of a synthetic microbial consortium consisting of two distinct cell types—an “activator” strain and a “repressor” strain. These strains produced two orthogonal cell-signaling molecules that regulate gene expression within a synthetic circuit spanning both strains. The two strains generated emergent, population-level oscillations only when cultured together. Certain network topologies of the two-circuit system were better at maintaining robust oscillations than others. The ability to program population-level dynamics through the genetic engineering of multiple cooperative strains points the way toward engineering complex synthetic tissues and organs with multiple cell types.

M ost synthetic gene circuits have been constructed to operate within single, isogenic cellular populations (1–4). However, synthetic microbial consortia could provide a means of engineering population-level phenotypes that are difficult to obtain with single strains (5). Indeed, several synthetic systems have been constructed to exhibit population-level phenotypes (6–9), including synthetic predator-prey systems (10), multicellular computers (11), and spatio-temporal pattern generators (12, 13). We constructed two genetically distinct populations of Escherichia coli to create a bacterial consortial that exhibits robust, synchronized transcriptional oscillations that are absent if either strain is grown in isolation. Specifically, we used two different bacterial quorum-sensing systems to construct an “activator” strain and a “repressor” strain that respectively increase and decrease gene expression in both strains. When cultured together in a microfluidic device, the two strains form coupled positive and negative feedback loops at the population level, akin to the circuit topology (i.e., how regulatory components within a circuit regulate each other) of a synthetic dual-feedback oscillator that operates within a single strain (14, 15). We used a combination of mathematical modeling and targeted genetic perturbations to better understand the roles of circuit topology and regulatory promoter strengths in generating and maintaining oscillations. The dual-feedback topology was robust to changes in promoter strengths and fluctuations in the population ratio of the two strains.

The two synthetic strains in our system were constructed to enzymatically produce and transcriptionally respond to intercellular signaling molecules (Fig. 1A). The activator strain produces C4–homoserine lactone (C4-HSL) (16), a signaling molecule that increases transcription of target genes within the synthetic circuits of both strains. The repressor strain produces 3-OH-C4-HSL (17), which decreases transcription in both strains through a synthetic transcriptional inverter (18, 19) mediated by the repressor ladC. These two signaling mechanisms jointly create coupled positive and negative feedback loops at the population level when the two strains are grown together (Fig. 1B). Additionally, each strain, when active, produces the enzyme AiIA, which degrades both signaling molecules, resulting in another layer of negative feedback.

To observe the dynamics of the synthetic consortial, we used a custom-designed microfluidic device in conjunction with time-lapse fluorescence microscopy to observe the two strains as they grew together in a small chamber in which the diffusion time of the HSLs was small (see supplementary materials) (20). Each strain contained a gene encoding a spectrally distinct fluorescent reporter (cfp, cyan fluorescent protein, in the activator; yfp, yellow fluorescent protein, in the repressor), driven by promoters that respond to both positive and negative signals in the network (Fig. 1A). After an initial transient time, synchronous, in-phase oscillations emerged in the fluorescent reporters of both strains (Fig. 1, C and D). Neither strain oscillated when cultured in isolation (fig. S1). Oscillations had a period of ~2 hours and persisted throughout the experiments (usually more than 14 hours).

The circuit topology of our synthetic consortial consisted of linked positive and negative feedback loops, similar to the topologies of many naturally occurring biological oscillators (21, 22).