Genetic resiliency associated with dominant lethal *TPM1* mutation causing atrial septal defect with high heritability

**Graphical abstract**

**Highlights**

- *TPM1* mutation linked to atrial septal defect causes embryonic death with no heartbeat
- *TLN2* identified as protective variant suppressing the deleterious *TPM1* mutation
- CRISPR Tpm1/Tln2 double-KI mice show rescued heart beating and atrial septal defects
- Functional annotation of variants of unknown significance with CRISPR founder analysis

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**In brief**

Pathogenic mutations known to cause severe disease can be found in healthy individuals, but the mechanism remains unknown. Teekakirikul et al. show suppression of embryonic lethality of a rare *TPM1* mutation by suppressor variant in *TLN2*. Rescue of embryonic lethality also uncovers a role for *TPM1* in atrial septal defect.

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Genetic resiliency associated with dominant lethal TPM1 mutation causing atrial septal defect with high heritability

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SUMMARY

Analysis of large-scale human genomic data has yielded unexplained mutations known to cause severe disease in healthy individuals. Here, we report the unexpected recovery of a rare dominant lethal mutation in TPM1, a sarcomeric actin-binding protein, in eight individuals with large atrial septal defect (ASD) in a five-generation pedigree. Mice with Tpm1 mutation exhibit early embryonic lethality with disrupted myofibril assembly and no heartbeat. However, patient-induced pluripotent-stem-cell-derived cardiomyocytes show normal beating with mild myofilament defect, indicating disease suppression. A variant in TLN2, another myofilament actin-binding protein, is identified as a candidate suppressor. Mouse CRISPR knock-in (KI) of both the TLN2 and TPM1 variants rescues heart beating, with near-term fetuses exhibiting large ASD. Thus, the role of TPM1 in ASD pathogenesis unfolds with suppression of its embryonic lethality by protective TLN2 variant. These findings provide evidence that genetic resiliency can arise with genetic suppression of a deleterious mutation.

INTRODUCTION

Recent large-scale acquisition of human next-generation sequencing data has led to rapid progress in the identification of monogenic causes of Mendelian diseases.1 This has yielded increasing evidence for the incomplete penetrance of many heritable diseases, with some striking examples noted in a study reporting 13 individuals with pathogenic mutations known to cause severe Mendelian diseases, but without evidence of disease.2 Such genetic resiliency is suggested to...
reflect the suppression of pathogenic mutations by protective variants in other genes. While this concept is appealing, no study has yet provided experimental evidence documenting such genetic buffering by protective variants. In the present study, we obtained evidence for such disease suppression in studies investigating the genetic etiology of atrial septal defect (ASD), a congenital heart disease (CHD) comprising a hole in the heart.

CHD is one of the most common birth defects, affecting 1% of live births. A genetic etiology is indicated by the increased recurrence risk among family members and also the association of CHD with chromosomal anomalies in syndromic disorders. In ASDs, a hole in the atrial septum allows shunting of blood from the left (LA) to the right atrium (RA) and is one of the most common CHDs. Most ASDs involve a defect in the septum secundum derived from infolding of the roof of the atria. Secundum ASD was one of the first CHDs shown to have high recurrence risk and also the first CHD for which a disease-causing gene was identified, NKX2.5. Several other transcription factors and also sarcomeric genes (ACTC1, MYH6) have been identified or implicated to cause ASDs. However, the genetic etiology for the vast majority of ASDs remain unexplained, with both recessive and dominant inheritance patterns of transmission reported. Studies conducted in inbred mice showed the presence of genetic modifiers for ASD and other CHDs. Interestingly, this was shown to mostly buffer the CHD-causing mutation against disease. Quantitative trait loci (QTL) mapping in mice also successfully identified multiple independent loci for patent foramen ovale, a phenotype closely related to ASD. However, recovery of the genes contributing to genetic buffering or underlying the QTL is still challenging.

In the present study, we document a multigenerational, non-consanguineous Caucasian pedigree exhibiting secundum ASD transmitted with complete penetrance. Using genomic approaches and animal modeling, we showed the highly heritable ASD phenotype has a digenic etiology involving an embryonic lethal pathogenic variant in TPM1 and a protective variant in TLN2, two proteins with important roles in sarcomeric actin assembly.

RESULTS

We recruited a family of 11 individuals spanning five generations in which 8 family members have large ASDs (Figure 1A). While small ASDs can close spontaneously, the large secundum ASDs in this family required surgical patch repair at birth (Figure 1B). The brother (VI-2) of the index patient also exhibited transient dilated cardiomyopathy (DCM) that resolved. Two of the affected family members, IV-2 and IV-4, suffered sudden death in mid- to late adult life (Figure 1A). The ASD phenotype showed complete penetrance in every generation. Notable is the union of one parent with two different unaffected partners in generations IV and V, yielding two offspring with ASD in generations V and VI, respectively (Figure 1). Together with the overall pattern of inheritance observed over the five generations, this would suggest a dominant genetic model of disease.

Linkage analysis and WGS identifies rare TPM1 pathogenic variant

To investigate the genetic etiology of the ASDs in this multigenerational family, we conducted genome-wide linkage analysis on the proband, as well as seven affected and three unaffected family members (denoted by asterisks in Figures 1A and S1). This
analysis yielded three regions, with the maximum peak log-of-odds (LOD) score (LOD = 1.806, not significant due to limited sample size) at chromosomal intervals 8p11.23-q11.23, 15q22.2-q26.1, and 18q21.2 (Data S1; Figure S1B). Three haplotypes were constructed over these three linkage intervals, none of which were detected in the general population. All three linkage regions segregated with ASDs in this family. Only the 18q21.2 interval was previously reported to be associated with diverse CHDs.22

To recover candidate genes in the linkage intervals, whole genome sequencing (WGS) analysis was conducted for the proband, three affected, and one unaffected family member from generations III, IV, and V (Figure 1A; Data S1). Filtering for rare variants with minor allele frequency (MAF) < 0.01 with predicted protein coding changes yielded seven variants shared by all four affected individuals, but not by the unaffected family member (Data S1). Of these, only one variant was found within one of the three chromosome intervals identified in the linkage analysis: a heterozygous 3-base in-frame deletion variant (c.13_15del; p.K5del) causing loss of a highly conserved N-terminal lysine (Human Genome Variation Society annotation of variant as c.20_22del; p.K7del) in TP1 encoding α-tropomyosin (Figures 2A and 2B).

**Extreme rarity of the TP1 mutation**

α-tropomyosin is a highly conserved sarcomeric actin-binding protein expressed in the heart, skeletal, and smooth muscles.
and in the cytoskeleton of non-muscle cells. Sanger sequencing confirmed this TPM1 mutation is present in all affected, but not in any unaffected, family members (Figure S1). This mutation was found in heterozygosity throughout the pedigree, supporting a dominant model of disease. It was not observed in any public databases (Figure 2C; Data S1) and is reported in only three other individuals worldwide as a variant of unknown significance (VUS) in ClinVar (VCV000181682.4; see Table S1). This mutation was not detected in further screening of >900 unrelated simple and complex CHD cases (all white), 111 of whom have isolated ASDs and 94 inherited cardiomyopathy cases.

**TPM1 mutation may exert dominant-negative effects**

The α-tropomyosin protein forms an α-helical coiled-coiled dimer assembled in head-to-tail fashion, helping to stabilize actin filaments and regulate actomyosin cross-bridge formation. Modeling using a 4-helix bundle showed the N-terminal region spanning the K5del mutation is stabilized by a hydrophobic core (Met1, Ile4, Leu274, Leu278) with an interchain salt bridge between Lys5 and Asp280 (Figure 2D, left). Lys5 loss would predict rotation of the Met1 and Ile4 away from the helical bundle center and replacement with a single Ala (Ala3; Figure 2D, middle). As a result, Ile4 would occupy the position normally occupied by Lys5, causing loss of the interchain salt bridge. These structural changes either in heterozygosity (TPM1_13_15del/+) or homozygosity (TPM1_13_15del/K5del) (Figures 2D and 2E, middle and right) are likely to destabilize the dimeric complex and, thus, alter its interactions with troponin T. This would predict a disruption of myofilament assembly and actomyosin cross-bridge formation that could lead to loss of contractility. Consistent with this, the Tpm1 knockout (KO) mice have been shown to suffer early embryonic lethality with non-beating hearts. As the TPM1_13_15del variant is observed in heterozygosity in all individuals of the pedigree, this suggests the TPM1 heterodimeric assembly could exert dominant-negative effects to perturb wild-type (WT) TPM1 protein function.

**CRISPR gene editing in Xenopus embryos**

The early embryonic lethality of the Tpm1 KO mouse embryos precluded the assessment of its role in ASD pathogenesis in mice. However, ASDs have been observed with tpm1 morpholino (MO) gene knockdown (KD) in chick embryos. To further assess experimentally how TPM1 deficiency may affect atrial septation, we conducted tpm1 MO KD in Xenopus laevis embryos (Figures S2 and S3). Xenopus have three chamber hearts with distinct left and right atri, and Xenopus embryos can be obtained in large numbers and their development and viability can be visually tracked. We observed that 81% of the MO-injected Xenopus laevis embryos have severe pericardial edema, indicating heart failure with tpm1 deficiency, and the heart rate was significantly reduced (Figures 3A and 3B). It should be noted that the tpm1 gene is duplicated in Xenopus laevis, and the two closely homologous tpm1-related genes are both targeted by the same MO used in these experiments (Figure S3). Similar analysis with tpm1 gene KO generated by CRISPR-Cas9 gene editing replicated these findings in X. tropicalis embryos, with 64% of the CRISPR-targeted embryos exhibiting pericardial edema with reduced heart rate (Figures 3C and 3D). Analysis of the CRISPR-targeted embryos by western blotting confirmed the absence of TPM1 protein expression (Figure 3E). Histological reconstruction using episcopic confocal microscopy (ECM) showed large ASDs in some of the embryos after tpm1 KD (X. laevis) or KO (X. tropicalis) (Figures 3F and S2D; Table S1).

**Embryonic lethality with no heartbeat in Tpm1 CRISPR-gene-edited mouse embryos**

To further assess the functional effects of the Tpm1_13_15del mutation, we used CRISPR-Cas9 gene editing to pursue knock-in
Given the known lethality of the Tpm1 KO mouse, we conducted direct analysis of the CRISPR-gene-edited embryos referred to as founder embryo analyses. Mouse oocytes that were CRISPR targeted were transferred to surrogate mothers for development in utero, and noninvasive fetal ultrasound imaging was conducted daily from E7.5 onward to track embryo viability, growth, and development and to assess cardiac function. The number and viability of the embryos were recorded, and heart beating—which is initiated at E8.5—was tracked. We noted that embryos that failed to initiate heart beating at E8.5 were dead or resorbed by E9.5. This was observed for 64 of 67 Tpm1 CRISPR-treated mouse embryos. (Table S2; Video S1). Whole-mount examination of these embryos harvested at E9.5 showed hydrops with severe pericardial effusion and abnormally looped heart tube (Figure 4A; Video S1). These results are reminiscent of those seen in the Tpm1 KO mice that also showed no heartbeat with early embryonic lethality. 28

K5del TPM1 exerts dominant-negative effects on WT TPM1 protein function

To assess the efficiency of CRISPR gene editing, E9.5 founder embryos were harvested, and DNA was extracted to assess the efficiency of CRISPR gene editing. The site of gene editing was PCR amplified and sequenced, and the Synthego Inference of CRISPR Edits (ICE) algorithm was used to analyze the sequencing data to determine the efficiency of CRISPR KI and KO events from nonhomologous end joining. This analysis revealed an average KI efficiency of 59%, with range of 28.2% to 98.9%, and an average KO of 21.6%, with range of 0% to 46% (Figure 4B; Table S3). In embryos with lower Tpm1 KI efficiency, higher KO events were observed. As none of these embryos initiated heart beating, these findings would suggest the Tpm1 K5del mutant protein can exert dominant-negative effects on WT TPM1 protein function. This is supported by observations from whole-mount embryo immunostaining of the CRISPR-targeted embryos for TPM1, α-actinin, and phosphohistone H3. Scale bar, 500 μm. (D–F) Quantification of the ratio of α-actinin/TPM1 immunostaining (D), phosphohistone H3 (E), and ratio of anaphase-telophase per total mitotic cells (F) in the Tpm1 KI versus WT control embryos (N > 2,000 cells). In (B) and (D–F), the number of CRISPR mouse embryos analyzed in each experiment is indicated. Data for (D–F) are represented as mean ± SEM and analyzed using unpaired Student’s t test.

Figure 4. Severe pericardial effusion and early embryonic lethality of CRISPR Tpm1 KI mouse embryos

(A) Representative example of E8.5 Tpm1 CRISPR-Cas9 KI mouse embryo showed severe pericardial effusion with abnormal heart (H) looping seen in whole-mount view (left panel) and in a histological section (right panel) showing the outflow tract (OFT) and primitive ventricle (V). Scale bar, 0.1 mm. (B) The frequency of Tpm1 KI and KO in mouse embryos generated by CRISPR gene editing. (C) Representative whole-mount confocal imaging of E8.5–9.0 WT and CRISPR-Cas9 Tpm1 KI mouse embryos immunostained for TPM1, α-actinin, and phosphohistone H3. Scale bar, 500 μm. (D–F) Quantitation of the ratio of α-actinin/TPM1 immunostaining (D), phosphohistone H3 (E), and ratio of anaphase-telophase per total mitotic cells (F) in the Tpm1 KI versus WT control embryos (N > 2,000 cells). In (B) and (D–F), the number of CRISPR mouse embryos analyzed in each experiment is indicated. Data for (D–F) are represented as mean ± SEM and analyzed using unpaired Student’s t test.

Patient-induced pluripotent-stem-cell-derived CMs show mild defects

To assess the impact of the TPM1 mutation on the patients, we generated induced pluripotent stem cells (iPSCs) from the index ASD subject (VI:1) and a healthy control subject and differentiated the iPSCs into cardiomyocytes (iPSC-CMs) using standard CM differentiation protocol. The efficiency of CM differentiation was unchanged, and the CMs generated were of normal size

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with grossly normal myofilament organization (Figures 5A and S4). Western immunoblotting showed no change in the level of TPM1 protein expression compared to control (Figures 5B and 5C), and quantification of myofibrillar organization showed no evidence of myofibrillar disarray (Figure 5D). However, α-actinin expression was reduced (Figure 5A), resulting in lower α-actinin/TPM1 ratio (~20%) (Figure 5E). This change was relatively modest compared to the change observed in the Tpm1 patient iPSC-CMs. (F) MYH6/MYH7 ratio in iPSC-CMs. (G and H) Analysis of TPM1K5del/+ iPSC-CMs showed no difference in beating rate (G) or calcium spark frequency (H), indicating preserved intrinsic contractility. (I and J) Ki67 (I) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) labeling (J) in ASD patient and control iPSC-CMs. Data for (C), (E), (F), (I), and (J) are represented as mean ± SEM and analyzed using unpaired Student’s t test. Data for (G) and (H) are represented as boxplots with median and minimum-maximum shown and analyzed statistically using non-parametric Mann-Whitney test (two-tailed). The data for (C)–(J) are derived from three independent experiments. For (D), (E), (I), and (J), each experiment included analysis of >2000 iPSC-CMs.

**Recovery of TNL2 variant as a candidate protective variant**

The rarity of the TPM1K5del mutation in the human population is in line with the observed early embryonic lethality of the Tpm1K5del mouse embryos. This is in striking contrast to the heritability of this mutation in eight members of the same extended family. This suggests buffering of the TPM1K5del mutation by another variant in the genetic background of this family. To investigate this, we reanalyzed the WGS data using a less stringent frequency filter (MAF < 0.1), hypothesizing protective variants may be more common. Focusing on the same three chromosomal intervals coinherited by all eight ASD family members, we recovered 11 additional variants, of which 9 were found in proximity to the TPM1K5del mutation (Data S1). Single-cell RNA sequencing data on human fetal hearts from 6.5 to 21 postconceptional weeks (PCWs) showed among the genes harboring the 11 candidate suppressor variants, only TNL2 was co-expressed with TPM1 in CMs (Figure 6A), making the heterozygous variant in TNL2 (c.G6716A;p.R2239H;MAF = 0.01; CADD = 22.8) as the most likely suppressor gene (Figure 6B). TNL2 is not only highly expressed in CMs, but it also plays a critical role in cardiac...
contractility. It encodes a large costameric protein bound to α-actinin and mediates extracellular matrix engagement by anchoring the actin myofilaments to integrins. This TLN2 variant was coinherited with the TPM1K5del mutation in all eight ASD subjects (Figure 6C), consistent with a possible role as a genetic modifier suppressing the severe deleterious effects of the TPM1 mutation.

Tpm1/Tln2 KI cause ASDs with rescue of both heart beating and embryonic lethality

To functionally assess the TLN2 (c.G6716A:p.R2239H) variant for a potential role in buffering the embryonic lethality of the TPM1K5del mutation, we conducted founder embryo analysis with mouse CRISPR-Cas9 double KI of the Tpm1K5del/Tln2R2239H variants. Previous studies showed Tln2 KO mice are viable with only mild late-onset skeletal myopathy. We observed that with CRISPR Tpm1/Tln2 double KI, 50% of the E8.5 embryos had beating hearts (27 of 56 embryos). This compares with only 4.4% of embryos with CRISPR gene editing directed at Tpm1 alone (Table S2), yielding an OR of 19.35 (5.33–107.81, 95% CI) (p = 9.63 × 10⁻²⁵) that indicated significant rescue of heart beating by the Tln2 variant (Figure 7A).

To assess whether the rescued Tpm1/Tln2 double-CRISPR-targeted embryos may have ASDs. 19 of these founder embryos were harvested at E16.5–17.5, the late-term stage when atrial septation is near complete, allowing for assessment of ASDs. This was conducted using 3D digital reconstructions of ECM-generated serial image stacks. It showed three of the double-targeted embryos had a large ASD (Figure 7B; Video S2). Double immunofluorescent staining for TPM1 and α-actinin showed normal sarcomere organization (Figure 7C). Quantification of the staining intensity showed TPM1 protein expression was unchanged, but α-actinin was decreased. This resulted in a modest reduction (~25%) in the α-actinin/TPM1 ratio, similar to the ASD patient iPSC-CMs, but markedly different from the severe depression observed in the Tpm1 KO embryos (Figure 7C; compare to Figure 5C).

To quantitatively assess the KI efficiency in these 19 late-stage double-Tpm1/Tln2-CRISPR-targeted embryos, DNA was recovered from sections spanning the heart for analysis of CRISPR gene editing events. Analysis using the Synthego ICE algorithm to quantify the frequency of KI/KO events showed a paucity of Tpm1 KI/KO events in these late-term fetuses, consistent with the early embryonic lethality with no heartbeat seen in the Tpm1 KO/KI embryos. Only three embryos showed significant Tpm1 KI events (56%–76%). These were also the three embryos found to have ASDs. Significantly, these three embryos also showed high levels of Tln2 KI (81%–98%), supporting the role of the Tln2 variant in suppression of the severe deleterious impact of the Tpm1K5del mutation (Figure 7D). These three embryos exhibited only low levels of Tln2 KO (81%–98%), supporting the role of the Tln2 variant in suppression of the severe deleterious impact of the Tpm1K5del mutation (Figure 7D). These three embryos exhibited only low levels of KO events in Tpm1 (2%–16%) and Tln2 (0.82%–2%) (Table S4). In contrast, among the 16 embryos without ASDs, there was little or no Tpm1 KO, but 5 had high levels of Tpm1 KO (38%–57%). The latter embryos also showed significant levels of Tln2 KO (30%–90%) (Figure 7D). Together, these findings support the role of Tln2 in rescuing the embryonic lethality associated with Tpm1 deficiency and with the rescue, uncovering the possible role of Tpm1 in causing ASDs (Figure 7D; Table S4).
DISCUSSION

We identified an extremely rare pathogenic TPM1 13_15del mutation as the underlying cause for ASDs in a five-generation pedigree comprising eight individuals with ASD. This variant causing the deletion of an N-terminal lysine residue (p.K5del) is predicted to disrupt assembly of the α-helical coiled-coil dimer stabilizing actin filaments and facilitating actomyosin cross-bridge formation (Figure 7E). As this perturbation would affect homodimers and heterodimers comprising mutant and WT proteins, dominant-negative effects would be expected that could contribute to disease. Consistent with this, our analysis of Tpm1 CRISPR KI mouse embryos showed a severe defect in myofilament assembly. Thus, despite no change in TPM1 protein expression level, the Tpm1 KO embryos showed a marked reduction in α-actinin and the ratio of α-actinin/TPM1 protein expression in the heart. These CRISPR Tpm1 KO embryos also never initiated heart beating, a phenotype similar to that of the Tpm1 KO mouse embryos. The pathogenicity of this TPM1 mutation is supported by observation of an identical mutation in TPM2, a β-tropomyosin closely homologous to TPM1 and known to cause rare autosomal dominant congenital skeletal myopathies. The rarity of the K5del TPM1 mutation in the human population is likely accounted for by its dominant-negative effects blocking heartbeat initiation. While this mutation has been reported in only three individuals worldwide, its remarkable transmission in eight individuals of the same family over five generations suggested possible coinheritance of a protective genetic modifier. We identified this genetic modifier as a more common variant in TLN2, another sarcomeric actin-related gene coinherited with the TPM1 mutation. TLN2 is co-expressed with TPM1 in embryonic and fetal CMs and is a component of the costamere structure anchoring sarcomeres to the membrane (Figure 7E). Significantly, double-CRISPR KI of the Tpm1 and Tn2 mutations in mice rescued heart beating and allowed survival with late-term fetuses also exhibiting ASDs. This was associated with the restoration of an α-actinin/Tpm1 ratio to more normal levels that are
similar to those observed in the beating iPSC-CMs derived from the index patient. Together, these findings suggest co-segregation of the TLN2 variant underlies the high heritability of an otherwise rare embryonic lethal TPM1 mutation. The TLN2 variant may restore actomyosin cross-bridge formation and costamere anchoring, thus restoring CM contractility and heart beating (Figure 7E). The meiotic segregation of the TLN2 suppressor variant from the embryonic lethal TPM1 13_15del mutation may explain the high incidence of miscarriages reported in this family. TPM1 mutations have been associated with cases of hypertrophic cardiomyopathy (HCM) (5%) and DCM (1%-2%). Their role in ASDs has not been demonstrated but has been implicated by a screen for TPM1 mutations in CHD patients. However, the c.13_15del mutation was not recovered in this screen or in the screening of over 900 patients with CHDs in this study. Two other sarcomeric genes, ACTC1 and MYH6, were previously identified to cause ASDs. The role of the TPM1 13_15del mutation in ASDs was uncovered by suppression of its early embryonic lethality by the TLN2G6716A variant. Our finding of ASDs with tpn1 CRISPR-mediated KO or MO KD in Xenopus embryos would suggest ASDs can arise from TPM1 deficiency. Although the mechanism for ASD pathogenesis is not well understood, the involvement of a cell proliferation defect is suggested by the reduced cell proliferation in the heart tissue of the Tln2/Tpn1 double-CRISPR KI mouse embryo and in the iPSC-CMs of the index patient. It will be of interest to examine whether misoriented cell division planes may play a role, given this has been observed for ventricular septation defects.

These findings provide a paradigm for how protective variants can increase genetic resiliency. Oligogenic inheritance of multiple pathogenic variants has been shown to cause CHDs and cardiomyopathies. While such studies showed multiple pathogenic variants can contribute to disease, our study showed pathogenic and protective variants in combination can provide the stable heritable transmission of an otherwise lethal mutation. It is worth noting that a study of genetic modifiers impacting ASD penetrance in heterozygous Nkx2.5 KO mice indicated p13q in a heterogeneous population, and the predominant modifiers are likely to be suppressors buffering against the pathogenic mutations. Together, these findings raise the possibility that protective and pathogenic variants interact in oligogenic combinations to contribute to the complex genetics of human CHDs.

In summary, our study identified a critical role for a severe lethal TPM1 mutation in the genetic etiology of ASDs. Importantly, we further showed emergence of the ASD phenotype was made possible by the coinheritance of a protective variant suppressing the embryonic lethality of the pathogenic TPM1 mutation. These findings provide a paradigm for how individuals harboring severe disease-causing mutations can be without disease. Our findings also indicate the possible use of founder mouse modeling with CRISPR gene editing to assess variant pathogenicity. Not requiring the breeding of mice, the founder analysis is rapid and cost effective and can accelerate the testing of variant contribution to human disease. This may be especially critical for variants likely to cause dominant embryonic lethal phenotypes difficult to functionally assess otherwise, as demonstrated by the K5del TPM1 mutation. Based on the American College of Medical Genetics criteria for variant classification, our familial segregation analysis and functional studies with patient iPSCs and animal modeling would support reclassification of the K5del TPM1 variant from VUS to likely pathogenic.

Limitations of the study
There are several limitations in our study to note. One relates to the animal modeling for assessing the biological impact of the TPM1 variant. The mouse CRISPR gene editing showed the Tpn1 mutation causes embryonic lethality associated with a lack of heart beating, but tpn1 MO KD or KO in Xenopus embryos caused marked reduction but not extinction of heart beating. This difference could reflect species differences. Alternatively, Xenopus, unlike mice, are not inbred. Thus, genetic modifiers in its heterogeneous genetic background may provide more robust rescue of tpn1-dependent sarcomere function. Second, we tested only one variant for suppressor function, the TLN2 variant. We note TLN2 is the only candidate gene from the linkage interval expressed in the embryonic heart, other than TPM1, and both genes functionally converge on the regulation of sarcomeric actin. In addition, strong support for the Tln2 variant acting as a suppressor can be seen in the significant rescue of heart beating in mouse embryos with Tpn1/Tln2 double KI, but not with Tpn1 KI alone. However, TLN2 suppressor function in the human context will need to be further investigated with CRISPR correction of the TLN2 variant in the patient iPSCs and examining its impact on iPSC-CM contractility, analyses beyond the scope of the present study. A third limitation is the discrepant finding of three unrelated individuals with the TPM1 pathogenic variant but without ASDs. As these are the only three individuals worldwide with the TPM1 mutation outside of the ASD pedigree, these are ultra-rare individuals that may have fortuitously coinherited multiple suppressor mutations, allowing more robust suppression of the pathogenic effects of the TPM1 mutation. Finally, another limitation is the fact that CRISPR mouse modeling with Tpn1/TLN2 double KI yielded ASDs in only 3 of 19 late-term fetuses. However, we note all three fetuses with ASDs had substantial TPM1 (>50%) KI, supporting its role in ASD pathogenesis. Additionally, all three fetuses also showed high-level Tln2 KI, consistent with the role of Tln2 in suppression of the early embryonic lethality of the K5del Tpn1 variant. Overall, these limitations do not detract from the main conclusion that genetic resiliency can arise from genetic suppression of a deleterious mutation, findings that suggest both pathogenic and protective variants contribute to the genetic architecture of human diseases.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
AUTHOR CONTRIBUTIONS

Study design, P.T., W.Z., X.X., C.B.Y., and C.W.L.; recruitment of research participants, P.T., W.Z., X.X., C.B.Y., and C.W.L.; sample collection, clinical data curation, P.T., M.Z., T.C.W., F.A., J.-h.L., M.-L.F., B.K., E.F., B.F., and C.W.L.; WGS and linkage analysis, W.Z., H.G., and A.S.B.; single-cell RNA sequencing analysis, W.Z.; iPSC modeling, X.X.; CM analysis, X.X., C.B.Y., and T.F.; Xenopus MO and CRISPR/Cas9 experiments, A.M.S., M.S.W., C.W., and H.Z; mouse CRISPR experiments, X.X.; Phenotyping and genotyping CRISPR-Cas9 mouse embryos, O. Phenotyping and genotyping CRISPR-Cas9 mouse embryos, O. Episcopic confocal microscopy histological analysis, O. Assessment of CRISPR gene editing efficiency in mouse embryos, O. Immunostaining, O. Human iPSC and iPSC-CM production, O. Analysis of human iPSC-CM, O. Transcript and protein analysis, O. Single-cell RNA-sequencing data analysis, O.

DEVELOPMENT OF THE VENTRICULAR MYOCARDIUM AND SUBJECT DETAILS

- Human tissue
- Human cell lines
- Xenopus embryo
- Mouse strain
- Animal study ethics approvals

METHOD DETAILS

- Genome-wide linkage analysis
- Whole genome sequencing analysis, variant assessment, and variant confirmation
- Generation of antisense-morpholino gene knockdown in Xenopus embryos
- Generation of CRISPR-Cas9 gene-edited tpm1 Xenopus mutant embryos
- Generation of CRISPR-Cas9 gene-edited mouse embryos
- Phenotyping and genotyping CRISPR-Cas9 mouse embryos
- Episcopic confocal microscopy histological analysis
- Assessment of CRISPR gene editing efficiency in mouse embryos
- Immunostaining
- Human iPSC and iPSC-CM production
- Analysis of human iPSC-CM
- Transcript and protein analysis
- Single-cell RNA-sequencing data analysis

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2021.100501.

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AUTHOR CONTRIBUTIONS

Study design, P.T., W.Z., X.X., C.B.Y., and C.W.L.; recruitment of research participants, P.T., W.Z., X.X., C.B.Y., and C.W.L.; sample collection, clinical data curation, P.T., M.Z., T.C.W., F.A., J.-h.L., M.-L.F., B.K., E.F., B.F., and C.W.L.; WGS and linkage analysis, W.Z., H.G., and A.S.B.; single-cell RNA sequencing analysis, W.Z.; iPSC modeling, X.X.; CM analysis, X.X., C.B.Y., and T.F.; Xenopus MO and CRISPR/Cas9 experiments, A.M.S., M.S.W., C.W., and H.Z; mouse CRISPR experiments, K.A.P., S.M., Y.S., and K.O.; mouse ultrasound phenotyping, T.T.; Sanger sequencing and genotyping, P.T., A.M.B., E.F., T.-P.A., M.X.W., and B.J.G.; imaging analysis, P.T., X.X., C.B.Y., S.H., Y.L.W., G.C.G., and D.A.S.; molecular modeling, W.J.L. and M.J.R.; statistics, P.T. and C.W.L.; manuscript preparation, C.W.L., P.T., W.Z., X.X., C.B.Y., and G.C.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.
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### Key Resources Table

| **REAGENT or RESOURCE**                              | **SOURCE**                      | **IDENTIFIER** |
|------------------------------------------------------|----------------------------------|----------------|
| **Antibodies**                                       |                                  |                |
| TPM1 Antibody                                        | Abcam                           | Cat# ab55915; RRID:AB_883154 |
| Anti-Sarcomeric Alpha Actinin antibody                 | Sigma-Aldrich                    | Cat# A7811; RRID:AB_476766 |
| pH3                                                  | Millipore                        | Cat# 06-570; RRID:AB_310177 |
| AF555 goat anti-rabbit IgG                           | ThermoFisher                     | Cat# A-21428; RRID:AB_2535849 |
| AF647 goat anti-mouse IgG1                           | ThermoFisher                     | Cat# A-21240; RRID:AB_2535809 |
| Anti-Sarcomeric Alpha Actinin antibody                 | abcam                            | RRID:AB_137346 |
| ki67                                                 | Abcam                            | Cat# ab15580; RRID:AB_443209 |
| human iPSC-CM: goat anti-rabbit IgG                   | ThermoFisher                     | Cat# A-21429; RRID:AB_2535850 |
| human iPSC-CM: goat anti-mouse IgM                    | ThermoFisher                     | Cat# A-21042; RRID:AB_2535711 |
| TropinonT, Cardiac Isoform Ab-1, Mouse Monoclonal Antibody | Lab Vision                      | Cat# MS-295-P1; RRID:AB_61808 |
| ph3Anti-HistoneH3 (phosphoS10) antibody               | abcam                            | Cat# ab14955; RRID:AB_443110 |
| **Chemicals, peptides, and recombinant proteins**    |                                  |                |
| Alt-R S.p. Cas9 Nuclease 3NLS                         | Integrated DNA Technologies      | RRID: #1074181 |
| **Critical commercial assays**                        |                                  |                |
| e-Mycoplus Mycoplasma PCR Detection Kit               | bocascientific                   | 25235          |
| Matrigel® hESC-Qualified Matrix, *LDEV-free*          | Corning                          | 354277         |
| mTeSR                                                | STEMCELL Technologies            | 85850          |
| Nucleofector Kit for Human Dermal Fibroblast (NHDF)   | Lonza                            | VPD-1001       |
| RPMI 1640 Medium, no glucose                         | Thermofisher                     | 11879020       |
| KSOM medium                                          | Millipore                        | #MR-121-D      |
| DNA extraction kit—QIAamp DNA FFPE tissue kit         | QIAGEN                           | #56404         |
| RNA extraction kit—Trizol reagent                    | Invitrogen                       | #12183555      |
| MAXIscript T7 transcription kit                       | ThermoFisher                     | #AM1312        |
| TUNEL assay kit                                      | Sigma Millipore                  | #12156792910 Roche |
| **Deposited data**                                   |                                  |                |
| Raw sequencing reads of WGS and genotype              | This paper                       | PRJNA624983    |
| data of SNP Array                                     |                                  |                |
| Single cell RNA sequencing data at 6.5 PCW            | Asp et al.                       | [30]           |
| Single cell RNA sequencing data at 12 PCW             | Miao et al.                      | [31]           |
| Single cell RNA sequencing data at 19-21 PCW          | Suryawanshi et al.               | [32]           |
| **Experimental models: Cell lines**                   |                                  |                |
| iPSC-CM from patient with ASD and healthy subject    | This paper                       | N/A            |
| **Experimental models: Organisms/strains**            |                                  |                |
| B6D2F1 strain mouse                                   | Jackson Laboratory               | 100006         |
| CD1 strain mouse                                      | Jackson Laboratory               | 003814         |
| X. laevis Xenopus                                     | Chinese University of Hong Kong  | N/A            |
| X. tropicalis Xenopus                                 | and Indiana University School    |                |
|                                                     | Chinese University of Hong Kong  | N/A            |
|                                                     | and Indiana University School    |                |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Oligonucleotides** | | |
| Human TPM1 primers for variant confirmation Forward: | This paper | N/A |
| GTCATATCCACCGTCAACTGG | | |
| Human TPM1 primers for variant confirmation Reverse: | This paper | N/A |
| ACCGTGCTTGTGCTTGTCTC | | |
| Human TNN2 primers for variant confirmation Forward: | This paper | N/A |
| GAGTCTGGGACTGGCAAT | | |
| Human TNN2 primers for variant confirmation Reverse: | This paper | N/A |
| CCGAGACAGCTAGAGGGAAC | | |
| Mouse Tpm1 Primers for mouse CRISPR genotyping Forward: | This paper | N/A |
| TCCCTAAGGAGACTGGCTGCC | | |
| Mouse Tpm1 Primers for mouse CRISPR genotyping Reverse: | This paper | N/A |
| AGTCCAGAAGGGGCCAGGACG | | |
| Mouse Tnn2 Primers for mouse CRISPR genotyping Forward: | This paper | N/A |
| AGACCGAGATACCGAAAGTTC | | |
| Mouse Tnn2 Primers for mouse CRISPR genotyping Reverse: | This paper | N/A |
| CCGAGACAGCTAGAGGGAAC | | |
| X. tropicalis Tpm1 CRISPR target region genotyping Forward: | This paper | N/A |
| CCGAGGCGCTGACTGAGGAA | | |
| X. tropicalis Tpm1 CRISPR target region genotyping Reverse: | This paper | N/A |
| TTGAGACAGCTGCTTGGGG | | |
| X. laevis Tpm1 Morpholino oligo sequence: | This paper | N/A |
| CATCCCTTCTTGTAGGCGTACCAG | | |
| X. tropicalis Tpm1 CRISPR sgRNA 1 Forward: | This paper | N/A |
| TAGGCCCTTGGACAGACAAAGGAC | | |
| X. tropicalis Tpm1 CRISPR sgRNA 1 Reverse: | This paper | N/A |
| AAACGTTCTGCTCTGGCAATGG | | |
| Mouse Tmp1 CRISPR sgRNA: | This paper | N/A |
| CTTCTTCTGAGGCGCTCCATGG | | |
| Mouse Tmp1 CRISPR single-stranded donor: | This paper | N/A |
| TCCGTGCCCTAGG | | |
| GGGCCCTGGCCACGCCCATTTGGAGCCATCAGTACAGAAGAGCAC | | |
| AAGAAGATGACATGCTGAAAGTGAAGCACAAGAAGAAGAC | | |
| CTTGGATGTCGAGCTGAGCAAGCGGAGGCTGATAAGGCGCC | | |
| Mouse Tn2 CRISPR sgRNA: | This paper | N/A |
| AGGTGCGAACCAGCTGACTGCTCACTGCGCC | | |
| Mouse Tn2 CRISPR single-stranded donor: | This paper | N/A |
| CACCCACCTGCCCACCTCCTATACCAACACCAGACGGTGTCTGAGCTGAGTCGAGCTGAGG | | |
| ATGGAAGGCGCTTGGACCTTGACACTGACATCGGTA | | |
| Recombinant DNA | | |
| DR274 vector | Addgene | #42250 |
| pCXLE-hOCT3/4-shp53-F | Addgene | #27077 |
| pCXLE-hSK | Addgene | #27078 |
| pCXLE-hUL | Addgene | #27080 |
| pCXLE-EGFP | Addgene | #27082 |
| Software and algorithms | | |
| GraphPad Prism | GraphPad Software | GraphPad Prism, RRID:SCR_002798 |
| ZiFI T targeter | Sander et al. | http://zifit.partners.org/ZiFI/ |
| ImageJ | ImageJ | ImageJ, RRID: SCR_003070 |
| LASX | Leica Application Suite X | Leica Application Suite X, RRID:SCR_013673 |
| R (v3.6.0) | R Core | R Project for Statistical Computing, RRID: SCR_001905 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Cecilia W. Lo (cel36@pitt.edu).

Materials availability
Additional materials are available from the lead contact upon request and without restriction.

Data and code availability
All raw sequencing data and genotype data generated in this paper have been deposited to the Sequence Read Archive (PRJNA624983) and are publicly available as of the date of publication. Publicly available single cell RNA sequencing data of human embryonic hearts were downloaded from https://www.spatialresearch.org/resources-published-datasets/doi-10-1016-j-cell-2019-11-025/, the single cell portal under accession number SCP1021 and NCBI database under BioProject: PRJNA576243, respectively. Accession numbers are listed in the Key resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this work paper is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human tissue
Blood for DNA extraction were obtained from patients at the University of Pittsburgh and University of Manchester with informed consent under human study protocols approved by the University of Pittsburgh and University of Manchester Institutional Review Board. The ASD family comprising 5 female and 6 male subjects were recruited from the Children’s Hospital of Pittsburgh of UPMC with informed consent under a human study protocol approved by the University of Pittsburgh Institutional Review Board. An inhouse cohort of > 900 simple and complex CHD cases, including 94 inherited cardiomyopathy individuals (University of Pittsburgh) and 111 isolated ASD cases previously described (University of Manchester) were screened for the TPM1 mutation by either Sanger sequencing or via analysis of whole exome sequencing data if available.

Human cell lines
Human fibroblasts cells were generated from nasal scrape, which were obtained from one ASD proband (13-years-old female) and one healthy control (26-year-old male). iPSC lines were generated from fibroblasts cells. All the iPSC cell lines have been cultured in mTeSR medium. Informed consent was obtained from both control and patient with human study protocol approved by the IRB of the University of Pittsburgh.

Xenopus embryo
Two-cell stage X. laevis embryos and X. tropicalis embryos at the 1 cell stage were obtained by in vitro fertilization from wild-type adult frogs from Chinese University of Hong Kong and Indiana University School of Medicine separately. Embryos of the same age were randomly assigned to experimental groups.
Mouse strain
Mouse zygotes for CRISPR gene editing were obtained from B6D2F1 mouse strain. Pseudopregnant female are of CD1 outbred strain background. All mice were housed and handled according to approved animal study protocols from the University of Pittsburgh and the Jackson Laboratory and in full compliance with Public Health Service policy on Human Care and Use of Laboratory Animals.

Animal study ethics approvals
All animal procedures were performed according to study protocols approved by the Institutional Care and Use Committee of the University of Pittsburgh, The Jackson Laboratory, Chinese University of Hong Kong and Indiana University School of Medicine.

METHOD DETAILS

Genome-wide linkage analysis
High density genotyping was performed using the Illumina Omni2.5-8 v1.3 BeadChip SNP Array. The genotype data generated comprising ~2.3 million SNP markers underwent quality control, filtering criteria and genotype error checking. We removed SNPs with MAF < 0.01, call genotyping rate < 99%, Hardy-Weinberg equilibrium p value < 0.00001, duplicated locus, and being on non-autosome and chromosome 6:2847796-33448353. In addition, SNPs with missing locus frequency in public database, existence of multiple alleles (> 2) and high linkage disequilibrium (LD) were eliminated. Using MERLIN,43 parametric linkage analysis under autosomal dominant model with a penetrance of 1 and mutant allele frequency of 0.0001, and nonparametric linkage analysis were both implemented on a set of SNP genotypes. Focusing on candidate linkage regions, haplotypes were constructed using MERLIN.

Whole genome sequencing analysis, variant assessment, and variant confirmation
Library was constructed and sequenced to an average depth coverage of 43X per individual with 100-bp paired-end reads. Reads were aligned to the human reference genome (b37) using BWA-MEM algorithm.53 GATK Best Practices including marking duplication and base quality score recalibration was performed to ensure accurate variant calling. SNP and InDel were detected by GATK Haplotypercaller.53 Detection of copy number variants and structural variants were detected by CNVnator and Breakdancer. Variants were annotated using ANNOVAR. Damaging segregating variants including nonsynonymous, stoploss, stopgain, splicing, frameshift, insertion/deletions were filtered for < 0.01 MAF in GnomAD.55 The impact of variant was assessed using SIFT, PolyPhen-2, GERP++, and Combined Annotation Dependent Depletion (CADD).56 Variant prioritization pipeline is listed in supplementary sheet.

Generation of antisense-morpholino gene knockdown in Xenopus embryos
Antisense-morpholino oligonucleotide (MOs) were designed and obtained from Gene Tools. Tpm1 translation blocking MOs (250 nM/ cell) were injected into two-cell stage X. laevis embryos. The MO injected embryos and uninjected control embryos were observed on late stage 30’s to early stage 40’s under microscope for heart rate and edema. All embryos were harvested at mid stage 40’s and fixed in 4% paraformaldehyde overnight for further histopathological analyses.

Generation of CRISPR-Cas9 gene-edited tpm1 Xenopus mutant embryos
Tpm1 sgRNA sequences were designed by the online tool ZiFIT targeter (http://zifit.partners.org/ZiFIT/). To assemble sgRNA, two oligonucleotides were annealed and linked to the linearized DR274 vector (Addgene #42250). After DraI digestion, the sgRNA-DR274 vector was used as template for in vitro transcription (MAXIscript T7 transcription kit, ThermoFisher). Tpm1 sgRNA was purified and co-injected with Cas9 protein (PNA BIO Inc) into the X. tropicalis embryos at the 1 cell stage. To assess gene disruption efficiency, ten of the injected or un.injected embryos were harvested at stage 32 for western blot or T7E1 assay as previously described.57 The CRISPR-Cas9 target region was also amplified from the extracted genomic DNA and analyzed by sequencing. The CRISPR-Cas9 injected embryos were observed on stage 42 under microscope for heart rate and edema. The Cas9 injected embryos were used as control. All embryos were harvested for further histopathological analyses in a similar fashion to the MO experiments. Assessment for ASD was carried out using ECM imaging as described below.

Generation of CRISPR-Cas9 gene-edited mouse embryos
For mouse CRISPR-Cas9 experiments, Tpm1 and Tln2 sgRNA DNA templates were designed by overlapping PCR with a forward primer containing a T7 promoter and the guide sequence and a common reverse primer with the stem loop. B6D2F1 oocytes were collected in KSOM medium (Millipore, Cat # MR-121-D) and electroporation was conducted in Opti-MEM medium containing Cas9 protein 100 ng/µl (AlteR S.p, Cas9 Nuclease 3NL5 IDT Cat # 1074181), Tpm1 sgRNA 200 ng/µl, Tpm1 ssODN 200 ng/µl, Tln2 sgRNA 200 ng/µl, Tln2 ssODN 200 ng/µl. The electroporation was performed using the Super electroporator NEPA21 type II and CUY 501-1-1.5 electrode (NEPA GENE Co. Ltd, Chiba, Japan) using the following settings: poring pulse (voltage 40 V, pulse length 2.5 ms, pulse interval 50 ms, number of pulses 4, decay rate 10%, polarity +); transfer pulse (voltage 7V, pulse length 50 ms, pulse interval 50 ms, number of pulses 5, decay rate 40%, polarity +/+ ). After electroporation, the embryos were washed two times in KSOM...
medium, then cultured in KSOM medium overnight at 5% CO2 and 37°C. In the following day, the two cell stage embryos were transferred to the oviducts of pseudopregnant CD1 females (0.5 dpc). The pseudopregnant mothers were then examined by ultrasound imaging to track development and viability of the embryos start at E6.5-7.5 onward. All Cas 9 guide RNA and single stranded donor sequences are provided in Key resources table.

**Phenotyping and genotyping CRISPR-Cas9 mouse embryos**

Noninvasive in utero fetal ultrasound imaging with the Vevo2100 ultrasound system using previously described method was used to track development and viability of CRISPR-Cas9 targeted mouse embryos. Ultrasound scanning was initiated at day E7.5/8.5, with daily scans performed to track development and viability of the embryos. The number of conceptuses on initial scan is counted and tracked on successive days to determine long term viability. Heart beating is initiated on E8.5 and can easily detected by ultrasound imaging. Embryos without heart beat at E8.5, usually die and are resorbed between E9.5-10.5, with finding of severe pericardial effusion and hydrops indicating heart failure and imminent death. Litters were harvested at different stages for phenotype/genotype analyses. Embryos were fixed in 4% paraformaldehyde, with the E8.5-9.5 embryos processed for whole mount immunostaining. Embryos collected at later gestation were paraffin embedded for imaging by episcopic confocal microscopy for 2D and 3D histological reconstructions.

**Episcopic confocal microscopy histological analysis**

Paraformaldehyde fixed embryos were embedded in paraffin and then sectioned and imaged using episcopic confocal microscopy (ECM) as previously described. ECM imaging provided serial 2D histological image stacks that were then digitally resectioned in different imaging planes and 3D reconstructed in different view for assessing cardiovascular anatomy and diagnosis of structural heart defects. This served as the gold standard for CHD diagnosis. For mouse embryos, paraffin sections were also collected during ECM imaging for additional immunohistology and DNA extraction for assessing CRISPR gene editing efficiency.

**Assessment of CRISPR gene editing efficiency in mouse embryos**

For assessing the efficiency of CRISPR mediated KI/KO in E8.5-9.5 embryos, tissue was microdissected from the caudal region of the embryo 5-7 sections spanning the heart, including the atria, were processed for DNA extraction (QIAGEN, #56404, QIAamp DNA FFPE tissue kit), followed by PCR DNA amplification using mouse Tpm1 and Tnn2 genotyping primers (Key resources table). The DNA products were further analyzed by Sanger sequencing and the sequence trace files were analyzed using Inference of CRISPR Edits (ICE), an online CRISPR gene editing analysis tool from Synthego (https://design.synthego.com). This provided computational analysis of the recombination events, allowing determination of the KI and KO frequencies.

**Immunostaining**

For whole mount fluorescent immunostaining, embryos were fixed overnight in 4% paraformaldehyde at 4°C and processed as described previously. Entire embryos and/or paraffin-embedded tissues were incubated with primary antibodies to TPM1 (Abcam, #ab55915, 1:500), α-actinin (Sigma-Aldrich, #A7811, 1:500), and pH3 (Millipore, Ser10, #2664259, 1:100). Secondary antibodies used were AF555 goat anti-rabbit IgG (A-21428, 1:100) and AF647 goat anti-mouse IgG1 (A-21240, 1:100). DAPI was used to visualize nuclei. Whole mount embryos and sections were imaged with confocal microscopy and quantitatively analyzed with ImageJ.

**Human iPSC and iPSC-CM production**

Human fibroblasts or lymphoblastoid cells were transfected with four episomal plasmids (Addgene: #27077 (pCXLE-hOCT3/4-shp53-F), 27078 (pCXLE-hSK), 27080 (pCXLE-Hul), 27082 (pCXLE-EGFP)) using electroporation. After 7 days these cells are transfected with four episomal plasmids (Addgene: #27077 (pCXLE-hOCT3/4-shp53-F), 27078 (pCXLE-hSK), 27080 (pCXLE-Hul), 27082 (pCXLE-EGFP)) using electroporation. After 21-40 days, iPSC clone was formed and alkaline phosphatase staining positive colonies were picked up at day 40. The clone was chosen and expanded in 24-well plates coated with feeder cells or BD Matrigel with mTESR1 media. Pluripotency of iPSCs was identified by immunofluorescent staining and quantitative PCR analysis. The continuous culture of iPSC cells (15-45 passages) underwent monolayer cardiomyocyte differentiation method. Clusters of beating cardiac myocytes were observed after 8 days of differentiation. All the assays were then tested on the following days (day 18-22).

**Analysis of human iPSC-CM**

Cell proliferation was assessed by immunofluorescence staining of paraffin-embedded sections with antibodies to pH3 (1:100) and Ki67 (Abcam, #ab15580, 1:500). Secondary antibodies included goat anti-rabbit IgG (Invitrogen, #A21429, 1:1,000) and goat anti-mouse IgM (Invitrogen, #A21042, 1:1,000). DAPI staining was used for visualization of cell nuclei and cells in anaphase or telophase versus prometaphase or metaphase. Apoptosis was analyzed with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) with a TUNEL assay kit (Roche, #12156792910). Sections were imaged with confocal microscopy and quantitatively analyzed with ImageJ. All primer sequences are provided in Table S5.
**Transcript and protein analysis**

Total RNA was isolated from iPSC-CM using Trizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA), and subjected to real-time quantitative reverse transcription PCR according to the standard protocols. Each sample was performed in triplicate. Relative expression of RNA was normalized to GAPDH level. Primers used are listed above. Protein was extracted and separated on 10% SDS-PAGE gels, and western blot was performed as previously described. Primary antibody against TPM1 was used to detect the protein. Densitometry analysis was performed with ImageJ.50

**Single-cell RNA-sequencing data analysis**

Publicly available single-cell RNA sequencing (scRNA-seq) datasets of human embryonic hearts at 6.5, 12 and 19-21 postconceptional weeks (PCW) were analyzed for cardiomyocyte co-expression of TPM1 and candidate protective genes.30–32 Cell type annotation and gene-UMI count sparse matrix files of scRNA-seq datasets at 6.5 and 12 PCW were directly downloaded30 or obtained from the single cell portal under accession number SCP1021,31 respectively. Raw sequencing data from scRNA-seq dataset at 19-21 PCW were downloaded from NCBI database under BioProject: PRJNA576243 and re-processed as described in the original paper32 to generate cell type annotation and count matrix files, as these two files were not available. Downstream analyses were performed on all three independent scRNA-seq datasets. The sum of UMI counts for each cell was normalized to 10,000 and then log-transformed. Differentially expressed genes (DEGs) in human cardiomyocytes against all other cell types were identified with the Wilcoxon Rank Sum test using Seurat “FindAllMarkers” function49 with default parameters. An adjusted p < 0.0001 was considered statistically significant.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The animal experiments were not randomized, but the investigators were blinded to sample allocation. D’Agostino & Pearson normality test and Shapiro-Wilk normality test were used to test if the data had a Gaussian distribution. For Gaussian distribution, comparison was conducted with unpaired Student’s t test. For Non- Gaussian distribution, comparisons were conducted with non-parametric Mann-Whitney test (two-tailed). P values for t tests are reported in the respective figures, and p < 0.05 were considered statistically significant.