Aberrant accumulation of TMEM43 accompanied by perturbed transmural gene expression in arrhythmogenic cardiomyopathy

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Abstract
Arrhythmogenic cardiomyopathy (ACM) caused by TMEM43 p.S358L is a fully penetrant heart disease that results in impaired cardiac function or fatal arrhythmia. However, the molecular mechanism of ACM caused by the TMEM43 variant has not yet been fully elucidated. In this study, we generated knock-in (KI) rats harboring a Tmem43 p.S358L mutation and established induced pluripotent stem cells (iPSCs) from patients based on the identification of TMEM43 p.S358L variant from a family with ACM. The Tmem43-S358L KI rats exhibited ventricular arrhythmia and fibrotic myocardial replacement in the subepicardium, which recapitulated the human ACM phenotype. The four-transmembrane protein

Abbreviations: ACM, arrhythmogenic cardiomyopathy; CHOP, C/EBP homologous protein; DEG, differentially expressed gene; ECG, electrocardiography; ER, endoplasmic reticulum; iPSCs, induced pluripotent stem cells; KI, knock in; NE, nuclear envelope; ProK, proteinase K; TM, transmembrane; TMEM43, transmembrane protein 43; WT, wild type; ΔGapp, free energy differences.

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INTRODUCTION

Arrhythmogenic cardiomyopathy (ACM) is an inherited cardiomyopathy characterized by lethal ventricular arrhythmias and progressive ventricular dysfunction. Patients typically develop initial symptoms associated with arrhythmias between adolescence and mid-adulthood. The representative histological characteristic of ACM is myocardial replacement with fibrofatty tissue progressing from the epicardium to the endocardium. More than 15 genes have been implicated in ACM, and ACM pathogenesis is assumed to be different for each gene locus. The gene variants involved in ACM are mostly inherited in an autosomal dominant pattern with low penetrance, although highly penetrant variants also exist. The highly penetrant causative variants are considered to be closely associated with the pathogenesis of the disease. However, as most ACM patients with highly penetrant causative variants do not develop cardiac phenotype in childhood, certain stress conditions may also contribute to the development of ACM.

Transmembrane protein 43 (TMEM43) has four-transmembrane (TM) domains, and its gene is reportedly causative of ACM. A heterozygous variant (c.1073C>T, p.S358L) of TMEM43 with the p.S358L variant (TMEM43S358L) was found to be modified by N-linked glycosylation in both KI rat cardiomyocytes and patient-specific iPSC-derived cardiomyocytes. TMEM43S358L glycosylation increased under the conditions of enhanced endoplasmic reticulum (ER) stress caused by pharmacological stimulation or age-dependent decline of the ER function. Intriguingly, the specific glycosylation of TMEM43S358L resulted from the altered membrane topology of TMEM43. Moreover, unlike TMEM43WT, which is mainly localized to the ER, TMEM43S358L accumulated at the nuclear envelope of cardiomyocytes with the increase in glycosylation. Finally, our comprehensive transcriptomic analysis demonstrated that the regional differences in gene expression patterns between the inner and outer layers observed in the wild type myocardium were partially diminished in the KI myocardium prior to exhibiting histological changes indicative of ACM. Altogether, these findings suggest that the aberrant accumulation of TMEM43S358L underlies the pathogenesis of ACM caused by TMEM43 p.S358L variant by affecting the transmural gene expression within the myocardium.

KEYWORDS
arrhythmogenic right ventricular dysplasia, glycosylation, TMEM43 protein

MATERIAL AND METHODS

Reagents and antibodies

Reagents in this report were purchased as follows: tunicamycin (Sigma), thapsigargin (Sigma), MG132 (Sigma), PS-341 (Selleck Chemical), chloroquine (Sigma), ammonium chloride (NH₄Cl, Wako), phenylephrine (Sigma), and Hoechst33342 (Thermo, H3570). Antibodies were purchased as follows: CD3 (Nichirei, 713591), CD68 (Bio RAD, MCA341R), Sun2 (abcam, ab124916), KDEL (Stressgen, ADI-SPA-827), Lamin A/C (Cell Signaling Technology, 4777), Emerin (Leica, NCL-EMERIN), GAPDH (Merck Millipore, MAB374), C/EBP homologous
proteins (CHOP; Santa Cruz, sc-575), α-tubulin (abcam, ab11304), Calnexin (Stressgen, SPA-860), GRP78 (BD Biosciences, 610978), LDHB (Abnova, H00003945-M01), histone H3 (Cell Signaling Technology, 9715), vimentin (abcam, ab24525), α-actinin (abcam, ab9465), GSK (Cell Signaling Technology, 9315), Phospho-GSK-3β (Ser9) (Cell Signaling Technology, 5558), AKT (Cell Signaling Technology, 9272), and Phospho-AKT (Cell Signaling Technology, 4060). The TMEM43-specific antibodies were generated according to a previous report with minor modifications. Briefly, the TMEM43 antisera were raised by immunizing rabbits with recombinant C-terminal 6× His-tagged mouse TMEM43 (a.a. 53-309). TMEM43-specific antibodies were obtained by affinity-purification of TMEM43 antisera using recombinant protein used for immunization.

2.2 Study subjects and whole-exome sequencing

A family affected by inherited ACM was enrolled. Whole-exome sequencing was performed on four affected family members (II:11, II:17, III:9, III:13) and two clinically unaffected individual (II:15 and II:22). For whole-exome sequencing, DNA was extracted from peripheral blood leukocytes and fragmented enzymatically or by ultrasonic irradiation. Fragmented DNA was hybridized with a biotin-labeled probe specific to the target region using the SureSelect Human All Exon V6 kit (Agilent). Hybridized samples were enriched with streptavidin beads, and captured DNA was amplified by on-bead polymerase chain reaction (PCR). Prepared genome libraries were sequenced as 100-bp paired-end reads on a HiSeq 3000 system (Illumina). Image analysis, base calling, and demultiplexing were performed using the Illumina bcl2fastq2 conversion software, v2.20. FASTQ files were quality checked using FASTQC, and low-quality reads were removed using the Trimmomatic-0.36 software. Read alignment was performed using standard parameterized BWA v0.7.17 software for human genome assembly hg19 (GRCh37). Single-nucleotide variants and small insertions and deletions were identified using the GATK best practice (GATK4.0.3) software. Called variants were filtered using GATK VariantFiltration, and variants that met the conditions “QD < 2.0, FS > 60.0, MQ < 40.0, MQRankSum < −12.5, ReadPosRankSum < −8.0, and SQR > 4.0” were analyzed. Annotation information was then added to the obtained variant list using ANNOVAR. The mutation list was narrowed using the original script by Python3.6. The type and position of variants on genes were determined via annotation of UCSC Known Genes, RefSeq genes, and Ensemble genes. Filtering of detected variants to be considered as candidates for analysis was performed by applying the following inclusion criteria (Table S2): (1) heterozygous variants; (2) exonic variants; (3) variants other than synonymous ones; and (4) variants with a minor allele frequency <0.5% in multiple variation databases. The remaining variant was finally confirmed by Sanger sequencing.

2.3 Generation of Tmem43-S358L KI rats by CRISPR/Cas9 technology

Tmem43-S358L knock in (KI) rats were generated using the CRISPR-Cas9 genome-editing technology as previously reported. Using plasmid (ID #72602; www.addgene.org/CRISPR) as the template, Cas9 mRNA was transcribed in vitro using a mMESSAGE mMACHINE T7 Ultra Kit (Life Technologies) from linearized plasmid and was purified using a MEGAClear kit (Life Technologies). gRNAs were transcribed in vitro using a MEGAscript T7 Transcription Kit (Life Technologies) from synthetic double-stranded DNA obtained from IDT (Integrated DNA Technologies, IA, USA) or Life Technologies. Single-stranded oligo DNA (ssODN) as the KI donor was synthetic oligo DNA purchased from IDT. For electroporation, pronuclear embryos were placed in 42 µl of serum-free media (Opti-MEMI, Thermo) containing 400 ng/µl Cas9 mRNA, 200 ng/µl gRNA and 300 ng/µl ssODN. The media and embryos were put into a 5-mm gap electrode (CUY505P5 or CUY520P5 Nepa Gene) and electroporated using a NEPA21 Super Electroporator (Nepa Gene). The condition of electroporation is below; poring pulses 225 V, pulse width 2.0–2.5 ms, pulse interval 50 ms, number of pulses 4, decay rate 10% and the polarity +. Transfer pulses 20 V, Pulse width 50 ms, Pulse interval 50 ms, number of pulses 5, decay rate 40% and the polarity +/−. Embryos that developed to the two-cell stage after the introduction of RNA and ssODN were transplanted into the oviducts of foster mothers. The mutation of Tmem43 in F0 rats was confirmed by DNA sequencing of the PCR product for the exon 12 of the Tmem43 gene. A total of 28 rats of Jcl:SD background were born and two F0 rat lines with the p.S358L coding variant were identified. The F0 rats were backcrossed with Jcl:SD rats for one generation before intercrossing heterozygotes to generate homozygote mutant rats. All experimental procedures were performed on male rats.

2.4 Electrocardiography and echocardiography for rats

Electrocardiographic monitoring was performed in conscious non-anesthetized rats using telemetric recordings
in 1.5-year-old wild type (WT) and Tmem43-S358L KI rats. Transmitters (Primetech) were implanted in the abdominal cavity with subcutaneous electrodes in lead II configuration under anesthesia by intraperitoneal injection of a mixture of 0.15 mg/kg medetomidine, 2.0 mg/kg midazolam, and 2.5 mg/kg butorphanol. Telemetry was recorded >48 h after surgery. For echocardiography, the rats were anesthetized by 2% sevoflurane inhalation. The end-diastolic and end-systolic diameters (LVEDD and LVESD, respectively) of the left ventricles were measured at the level of the papillary muscles in M-mode echocardiography.

### 2.5 Histological studies

1.5-year-old WT and KI rats were euthanized by intraperitoneal injection of over-dose sodium pentobarbital. Their hearts were rapidly harvested, fixed overnight in a 20% formalin neutral buffer solution (Wako), embedded in paraffin, and sectioned on a microtome using standard techniques. To detect the fibrosis area in tissues, sections were stained with Masson’s Trichrome staining following the manufacturer’s protocol. Images were obtained with a microscope (BZ-X-700, Keyence), and the cardiac fibrosis area was calculated as the ratio of the total fibrosis area to the cardiac cross-sectional area of a cardiac section by BZ-X700 and BZ-X analysis application (Keyence).

### 2.6 Immunohistochemistry

For immunohistochemistry, heart tissue of 1.5-year-old rats was frozen in isopentane in liquid nitrogen and embedded in the Tissue Tek OCT compound. Sections were cut 5-μm thick and mounted on silane-coated slide glasses. Frozen sections were dried and fixed in acetone at −20°C for 5 min. After permeabilization with 0.1% Triton X-100, sections were placed in blocking buffer (5% goat serum and 3% bovine serum albumin in PBS) for 30 min, then labeled with primary antibodies overnight in blocking buffer in a humidified chamber at 4°C. For secondary reactions, Alexa 488-, 568-, or 647-labeled secondary antibodies (Invitrogen). Fluorescence images were recorded with an Olympus FV1000D confocal microscope (Olympus).

Immunoperoxidase staining on formalin-fixed paraffin-embedded sections was also performed using conventional techniques to detect inflammatory cell infiltrates in the heart tissue sections from WT and Tmem43-S358L KI rats. Anti-CD3 and anti-CD68 were used to detect T lymphocytes and macrophages, respectively. The sections were counterstained with hematoxylin and observed using light microscopy.

### 2.7 Oil red staining

For oil red staining, heart tissue from 1.5-year-old WT and Tmem43-S358L KI rats was frozen in isopentane in liquid nitrogen and embedded in the Tissue Tek OCT compound. Sections cut to 5 μm thickness were fixed in 10% formalin. The sections were then stained using Oil Red O solution (0.3% w/v in propylene glycol) for 15 min at 37°C. They were then washed with 60% propylene glycol and distilled water and counterstained with hematoxylin.

### 2.8 Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes (NRCMs) were prepared as previously described. In brief, harvested hearts were incubated in 0.25% trypsin/EDTA (Sigma) at 4°C overnight and then digested with collagenase type II (Worthington). The cardiomyocyte fraction was collected after differential plating for 70 min at 37°C, seeded and incubated with DMEM (Dulbecco’s Modified Eagle Medium, Gibco) containing 10% fetal bovine serum (FBS, Gibco) and penicillin, streptomycin, and glutamine (Gibco), unless otherwise indicated.

### 2.9 Prediction of the apparent free energy (ΔG\text{app}) for insertion of TM helices

Predictions of the free energy differences (ΔG\text{app}) for helix insertions for TMEM43\text{WT} and TMEM43\text{S358L} were acquired from full amino acid sequence scans using the DG prediction server v1.0 (www.dgpred.cbr.su.se). In this analysis, more negative ΔG\text{app} values indicated more energetically favorable membrane integration, whereas more positive ΔG\text{app} values indicated more unfavorable integration.

### 2.10 Isolation of adult rat cardiomyocytes

Isolated rat hearts were Langendorff-perfused as described previously. In brief, 3-month-old rats were anesthetized with pentobarbitone sodium (140 mg/kg body weight, intraperitoneally). Hearts were rapidly excised, and the aorta was cannulated. The hearts were then perfused with oxygenated (95% O₂ + 5% CO₂) Krebs-Henseleit (K-H) buffer at 37°C (pH7.4). The K-H buffer contained 120 mM NaCl, 5.4 mM KCl, 1.2 mM NaH₂PO₄, 20 mM NaHCO₃, 1.6 mM MgCl₂, 5.6 mM glucose, and 5.0 mM taurine. The enzymatic digestion was performed with collagenase (1.5 mg/ml) containing perfusion solution. After
enzymatic digestion, the heart was minced and filtered through nylon mesh. Isolated myocytes were obtained followed by four cycles of gravity sedimentation for 10 min with low Ca²⁺ (0.1–1 mM CaCl₂) containing buffer. Then, isolated cardiomyocytes were cultured in the dishes pre-coated with 10 μg/ml mouse laminin in 2% CO₂ incubator at 37°C for myocyte attachment.

2.11 | Subcellular fractionation

Subcellular fractionation was performed using previous methods with modifications. Heart tissues were resuspended in homogenization buffer comprising 250 mM sucrose, 10 mM tris-HCl pH 8.0, 25 mM KCl, 0.1% Triton X-100, 1 μM dithiothreitol, and a protease inhibitor cocktail (Nacalai Tesque) and homogenized on ice using a tight-fitting Teflon pestle attached to a Potter S homogenizer (AS ONE Co., Osaka, Japan) set to 1000 rpm. The homogenate was filtered through a 100 and 40 μm cell strainer (Corning) sequentially to remove undigested myocardial fragments. After centrifugation (500 g, 5 min, 4°C), the supernatant labeled S₀ was collected, and the pellet labeled P₀ was resuspended in a storage buffer comprising 4% bovine serum albumin in PBS. After S₀ was centrifuged (20 000 g, 20 min, 4°C), the pellet was solubilized with sample buffer, which was then used as the endoplasmic reticulum (ER) fraction. The nuclei of resuspended P₀ cells were further sorted using fluorescence-activated cell sorting with propidium iodide staining. After the sorted solution was centrifuged (1000 g, 5 min, 4°C), the pellet was solubilized with sample buffer, which was then used as the nuclear fraction.

2.12 | Immunoblotting

For immunoblotting of heart tissue samples, frozen heart tissues were ground into a fine powder by using a Multi-Beads Shocker (Yasui Kikai). The tissue powders were lysed with Triton buffer (50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 5 mM EDTA), rotated at 4°C for 15 min. Lysates were centrifuged at 15 000 g at 4°C for 15 min, supernatants were collected as samples. For immunoblotting of NRCMs, cells were washed with cold PBS and lysed with SDS buffer (50 mM Tris-HCl (pH 7.4), 2% SDS, 5 mM EDTA) to inhibit proteinases during cell lysis. Proteins were separated by SDS-PAGE and transferred to PVDF membrane. After blocking with 3% nonfat milk for 30 min, the transferred membrane was incubated with primary antibody at 4°C overnight and with secondary antibody at room temperature for 30 min. The membrane signals were detected by chemiluminescence using ECL or ECL prime reagent (GE Healthcare). GAPDH or α-tubulin was used as a control for equal loading and transfer. The intensity of the protein band was quantified by ImageQuantTL (GE Healthcare).

2.13 | Immunocytochemistry of NRCMs and iPSC-derived cardiomyocytes

Cells were seeded on collagen-coated 35-mm glass dishes (Asahi Techno Glass). The cells were fixed with 4% paraformaldehyde (Wako) for 15 min. The cells were permeabilized with 0.1% Triton X-100 for 5 min and then immunostained with primary antibodies. For secondary reactions, Alexa 488-, 594-, or 647-labeled secondary antibodies (Invitrogen) were used. Fluorescence images were recorded with an Olympus FV1000D confocal microscope (Olympus) or with a SpinSR10 for super-resolution imaging.

2.14 | siRNA transfection

siRNAs (5 nM, SilencerSelect pre-designed siRNA, Thermo) were transfected into the precultured cardiomyocytes using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s instructions 3–5 h after primary culture. As a negative control, cells were transfected with siControl Non-Targeting siRNA (Thermo). Twenty-four hours after transfection, media were changed to DMEM (Gibco) supplemented with 10% FBS, 1% penicillin-streptomycin to avoid the toxicity of transfection reagent. Cells were incubated for an additional 48 h and used for immunoblotting or immunocytochemistry experiments. The sequence of siRNA for rat Tmem43 used in this study was as follows: tcactgacactgttgactg.

2.15 | Immunocytochemistry of adult rat cardiomyocytes

Adult rat cardiomyocytes isolated from heart tissue of 3-month-old rats as described above were seeded on mouse laminin-coated 35-mm glass dishes (Asahi Techno Glass). After 2 h of seeding, the cells were fixed with 4% paraformaldehyde for 15 min. The cells were permeabilized with 0.5% Triton X-100 for 5 min. After washing, cells were placed in blocking buffer (5% goat serum and 3% bovine serum albumin in PBS) for 1 h, then labeled with primary antibodies overnight at 4°C. For secondary reactions, Alexa 488- or 594- labeled secondary antibodies (Invitrogen). Fluorescence images were recorded with an Olympus FV1000D confocal microscope (Olympus).
2.16 | Quantification of immunostained images

CellSens (Olympus) was used to measure fluorescence profiles in super-resolution images. TMEM43 fluorescence signal merged with lamin A/C at the nuclear envelope (NE) was measured every 0.04 μm. The length of the NE is about 40–50 μm per cell, and the fluorescence intensity was measured at approximately 1000 points. To compare the variation of fluorescence signal between WT and KI, the coefficient of variation (CV) was calculated from the fluorescence intensity values at some 1000 points per cell.

2.17 | Generation and characterization of human iPSCs

iPSCs were generated from peripheral blood mononuclear cells using episomal vectors as previously reported. The vectors were introduced to the mononuclear cells by Nucleofector 2b (Lonza) using the Amaxa Human CD34 Cell Nucleofector Kit (Lonza) and plated on cell culture plates coated with Laminin 511-E8 (iMatrix-511 silk, Nippi). Approximately two weeks later, colonies with iPSC-like morphologies were picked up and cultured by changing the medium with StemFit (Ajinomoto). Pluripotent stem cell markers of iPSCs were confirmed by immunofluorescent staining with Oct4 Antibody (Stemgent) and SSEA-4 Antibody (Stemgent). The ability of iPSCs to differentiate into each of the three germ layers was verified by the Human Pluripotent Stem Cell Functional Identification Kit (R & D systems) according to the manufacturer’s instructions. Karyotyping of iPSCs were performed by chromocenter (Tottori) by Q-band analysis.

2.18 | Cardiac differentiation of iPSCs and purification of differentiated cardiomyocytes

iPSCs were differentiated into cardiomyocytes using an embryoid body formation protocol as described previously with slight modification using the AscleStem cardiomyocyte differentiation medium kit (Nacalai Tesque). Differentiated cardiomyocytes were purified by metabolic selection using no glucose DMEM (Nacalai Tesque) supplemented with 4 mM L-lactate (Wako) and 0.5% bovine serum albumin (Wako). The purified cardiomyocytes were subjected to analysis between days 50 and 60 post-differentiation.

2.19 | RNA-seq and analysis

RNA libraries from heart tissues of 6-month-old rats were prepared for sequencing using a TruSeq Stranded mRNA sample prep kit (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. Whole transcriptome sequencing was applied to RNA samples using the Illumina HiSeq 3000 platform in a 100 bp paired-end mode. Sequenced reads were mapped to the rat reference genome sequence (rn6) using TopHat ver. 2.0.13 in combination with Bowtie2 ver. 2.2.3 and SAMTools ver. 1.0. Each BAM file was converted integrated read count file using featureCounts (subread ver. 1.5.0). Read count file was normalized by the CPM (counts per million) method. Genes expressed with >1 CPM in at least one of the samples of any condition were only used for the analysis. Dimensional reduction analysis, principal component analysis (PCA) and t-Distributed Stochastic Neighbor embedding (t-SNE), was performed using integrated Differential Expression and Pathway analysis tool. A list of differentially expressed gene (DEG; genes with significantly different expression levels) was obtained by group comparison, and expression level comparison by MA-plotting was performed for each combination to see the relationship of gene expression. In gene ontology (GO) analysis using ShinyGO ver. 0.61, only genes with FDR-adjusted p < .05 were selected.

2.20 | Statistics

Statistical analysis and graph generation were performed using GraphPad Prism. Data are presented as the mean ± standard deviation, unless indicated otherwise. For two-group comparisons, the two-tailed Student’s t-test was used. For multiple-group comparisons, a one-way analysis of variance with Tukey-Kramer’s test was used. p values of less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Identification of the TMEM43 p.S358L variant in a family with autosomal dominant ACM

In this report, we describe the members of a Japanese family who presented with ventricular arrhythmia and cardiac dysfunction at young ages and were diagnosed with ACM (Figure 1A). This ACM family exhibited an autosomal dominant inheritance pattern and a total of ten affected individuals in this family had died suddenly. All surviving patients with available clinical data received an implantable
cardioverter-defibrillator as a consequence of syncope caused by ventricular arrhythmia or resuscitated cardiac arrest (Table 1 and Figure S1A). Most of these patients also exhibited ventricular systolic dysfunction in echocardiography or cardiac magnetic resonance (Table 1 and Figure S1B).

Using family-based whole-exome sequencing of four affected and two unaffected members, we identified one exonic variant, TMEM43 c.1073C>T, p.S358L (Table S1). Sanger sequencing of DNA from 15 of the family members confirmed that the TMEM43 c.1073C>T variant perfectly co-segregated with the ACM phenotype (Figure 1A,B). TMEM43 is known to be a ubiquitously expressed four-TM protein consisting of 400 amino acids. Serine (Ser) 358 of TMEM43 exists within the third TM domain and is highly conserved among vertebrates (Figure 1C). Based on a previous report, the membrane topology of TMEM43 was predicted, as shown in Figure S2. However, the biological function of TMEM43 and molecular pathogenesis caused by TMEM43 protein with the p.S358L variant (TMEM43S358L) remain largely unknown.

3.2 | *Tmem43*-S358L KI rats display ventricular arrhythmia and fibrotic myocardial replacement

To investigate the precise molecular mechanism underlying ACM, we generated genetically modified rats harboring
the Tmem43 p.S358L mutation using CRISPR/Cas9 (Figure 2A). Homozygous KI rats (<1 year of age) showed no overt abnormalities in cardiac function, cardiac dimension, cardiac rhythm, or histology. Intriguingly, through an extended period of observation, homozygous KI rats exhibited non-sustained ventricular tachycardia based on electrocardiographs without echocardiographic changes at 1.5 years of age (Figures 2B,C and S3). Although these homozygous KI rats showed no apparent sudden cardiac death, histological analysis of the heart showed myocardial degeneration and marked fibrotic replacement, both of which were especially observed in the subepicardium (Figure 2D,E). Quantitative analysis of cardiac fibrosis based on Masson’s trichrome staining confirmed that the fibrotic areas were significantly increased in homozygous KI rats compared to those in WT control rats (Figures 2E and S4A). Inflammatory cell infiltration, a histological feature of ACM 21,22 was also observed particularly in the border region between myocardial and fibrous tissues in KI rats (Figure S4B). However, no obvious lipid accumulation in the subepicardial layer was observed in these KI rats (Figure S4C). In a series of these analyses, heterozygous KI rats exhibited no overt dysfunctional cardiac phenotypes. Therefore, we used homozygous KI rats for the following examinations and hereafter referred to the homozygous KI rats simply as KI rats. Overall, although homozygous Tmem43-S358L KI rats showed no apparent sudden cardiac death, long-term observation of these animals revealed ventricular arrhythmia and tissue degeneration primarily in the subepicardium, which were also reported in humans with ACM. 1

### 3.3 TMEM43<sup>S358L</sup> is modified by N-linked glycosylation

To analyze in detail the upstream mechanisms involved in ACM pathology, we examined the protein expression of TMEM43 in the heart tissue of neonatal and adult KI rats. Intriguingly, we identified a slower-migrating band of TMEM43, which was seen exclusively in KI but not in WT rats (Figure 3A, arrowheads). Based on the difference in molecular weight of the novel band relative to those of the other bands, we assessed TMEM43 glycosylation using PNGase F, which cleaves N-linked glycosylation. 23 Treatment of immunoprecipitated TMEM43 protein from rat heart with PNGase F revealed that a portion of TMEM43<sup>S358L</sup> molecules was post-translationally modified by N-linked glycosylation (Figure 3B). As the amount of glycosylation was clearly higher in adults than in neonatal rats (Figure 3A), we examined age-dependent changes in the TMEM43 glycosylation of KI rats (Figure 3C). Quantitative analysis revealed that the

### Table 1 Clinical and laboratory features of seven family members

| Subject | Sex | Age, y | TMEM43 p.S358L variant | Symptoms | ECG findings | Echocardiography | ICD | Comment |
|----------|-----|--------|------------------------|----------|--------------|------------------|-----|---------|
| II:11    | M   | 64     | Carrier                | Heart failure | RRWP, non-specific | LVDD/LVDs = 35/35 | No | NSVT episodes |
| II:15    | F   | 67     | Non-carrier            | Asymptomatic | WNL | IVCD = 44/34 | No | NSVT episodes |
| II:17    | F   | 62     | Carrier                | Heart failure | RRWP | PRWP | Abnormal Q in V1-3, | No | NSVT episodes |
| III:9    | M   | 43     | Carrier                | Palpitation, | Syncope | Epsilon wave | No | NSVT episodes |
| III:10   | M   | 44     | Carrier                | Sudden cardiac arrest | PRWP | WNL | PRWP | No | NSVT episodes |
| III:13   | F   | 37     | Carrier                | Palpitation | PRWP | WNL | PRWP | No | NSVT episodes |
| III:14   | M   | 45     | Non-carrier            | Asymptomatic | PRWP | WNL | PRWP | No | NSVT episodes |

Abbreviations: ECG, electrocardiogram; ICD, implantable cardioverter-defibrillator; IVCD, intraventricular conduction disturbance; IVS, interventricular septum; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; LVEF, left ventricular ejection fraction; NA, not available; NSVT, non-sustained ventricular tachycardia; PRWP, poor R-wave progression; PW, posterior wall; RRWP, reversed R-wave progression; WNL, within normal limits.
amount of glycosylation of TMEM43_S358L gradually increased from birth to 2 months of age and retained at a high level after that (Figure 3D).

Next, we aimed to determine the glycosylation site of TMEM43_S358L. N-glycosylation occurs at the asparagine (Asn) residue in the consensus sequence Asn-X-Ser/threonine (Thr). The TMEM43 protein has this sequence only at Asn 4 (N4) (Figure 3E, upper panel). To examine whether N4 of TMEM43_S358L was glycosylated, we generated adenoviruses encoding FLAG-tagged TMEM43_WT, TMEM43_S358L, and TMEM43_S358L/N4A. Based on immunoblotting analysis of NRCMs expressing these viruses, glycosylation was observed for TMEM43_S358L but not TMEM43_WT or TMEM43_S358L/N4A (Figure 3E, lower panel).
These results indicated that TMEM43<sup>S358L</sup> was modified by N-linked glycosylation at N4.

### 3.4 Glycosylated TMEM43<sup>S358L</sup> shows aberrant membrane topology

Glycosylation of TMEM43 at the N4 position occurred only in TMEM43<sup>S358L</sup> even though both TMEM43<sup>WT</sup> and TMEM43<sup>S358L</sup> have the N4 N-glycosylation acceptor site. It is well known that the modification of glycosylation acceptor sites occurs in a compartment-specific manner; glycosylation of membrane proteins occurs only when the acceptor sites are located in the ER lumen. Thus, although the N terminus of TMEM43<sup>WT</sup> was reported to reside in the cytoplasm, we hypothesized that the N terminus of the glycosylated TMEM43<sup>S358L</sup> inverted from the cytoplasm to the ER lumen as a result of altered membrane topology.
To further consider the topology of TMEM43, we performed protease protection assays using membrane fractions extracted from neonatal cardiomyocytes of WT and KI rats (Figure 3F). In the membrane fractions treated with proteinase K (ProK), the molecular weight of TMEM43WT and unglycosylated TMEM43S358L decreased slightly, whereas the molecular weight of glycosylated TMEM43S358L was unchanged (Figure 3F, lane 2 and 5). This indicated that the ProK-digested N terminus of TMEM43WT and unglycosylated TMEM43S358L resided in the cytoplasm, whereas the ProK-resistant N terminus of glycosylated TMEM43S358L resided in the ER lumen. In the experimental controls, ProK almost completely digested the single TM protein calnexin, for which the antibody recognition site resides in the cytoplasm, whereas the luminal protein GRP78, which resides in the ER lumen, was not digested. Simultaneous ProK digestion and membrane solubilization with sodium decyl sulfate resulted in an almost complete loss of these proteins (Figure 3F, lanes 3 and 6). These results suggested an aberrant membrane topology of the glycosylated form of TMEM43S358L.

To further consider the topology of TMEM43, we predicted the apparent $\Delta G_{\text{app}}$ of individual TM helices, which can be used to verify the TM insertion efficiencies of TM helices. The predicted $\Delta G_{\text{app}}$ of the TM helices of TMEM43 indicated that only the TM4 helix was energetically favorable to integrate into the membrane, whereas the other helices were not (Table S2), suggesting the importance of the TM–TM interaction for correct topogenesis. Although the alteration of Ser358 to leucine (Leu) in TMEM43 resulted in only a slight change in the $\Delta G_{\text{app}}$ of the TM3 helix (Table S2), this change might affect the mutual interactions among the TM helices and alter the orientations of several TM helices in TMEM43 (Figure 3G).

### 3.5 ER stress enhances N-glycosylation of TMEM43S358L and translocates TMEM43S358L to the NE in rat cardiomyocytes

The onset of human ACM phenotypes in mid-adulthood suggests that not only genetic factors but also age-related factors contribute to the development of ACM. This, together with the finding that the glycosylation level of TMEM43S358L in KI rat heart increased with age, led us to examine the conditions under which glycosylation increased in cultured cardiomyocytes. Following treatment of KI NRCSMs with various drugs (Figure S5A), glycosylation of TMEM43S358L was especially increased by treatment with the SERCA inhibitor thapsigargin and proteasome inhibitors MG132 and PS-341. We further examined TMEM43 protein glycosylation over 24 h under treatment with thapsigargin or MG132 (Figure 4A). The glycosylation level of TMEM43S358L gradually increased over time after these treatments, whereas no glycosylation of TMEM43WT was detected (Figure 4B). Increased expression of the ER stress marker CHOP, as reported previously, indicated that both thapsigargin and MG132 treatment induced ER stress (Figure 4A). The increase in the glycosylation of TMEM43S358L was observed in NRCSMs treated with MG132 even at a low concentration at which ER stress was slightly induced (Figure S5B).

We then sought to investigate whether ER stress could affect the localization of TMEM43 in cardiomyocytes. First, we performed immunofluorescence staining of TMEM43 in WT NRCSMs without ER stress. Partially in contrast to previous reports of TMEM43 localization enriched at the NE in other cell types, TMEM43 mainly localized to the ER in WT NRCSMs, although signals in the...
NE were also detected in some cardiomyocytes (Figures 4C and S6). In KI NRCMs, the amount of TMEM43 signals increased at the NE and diminished in the ER, especially after treatment with MG132 or thapsigargin (Figure 4C, arrowheads). Accumulation of TMEM43S358L at the NE in KI NRCMs was also confirmed in XZ and YZ sections of the 3D image (Figure S7).

Using the images obtained by super-resolution confocal microscopy, we created fluorescence intensity line profiles for the signals at the NE of TMEM43 and lamin A/C, which is a major structural component of the nuclear lamina (Figures 4D, E and S8). Quantitative analysis using these line profiles revealed that the TMEM43 intensity was increased in KI cardiomyocytes compared to that in WT cardiomyocytes under steady-state conditions and was enhanced by MG132 treatment (Figure 4F). Under the same conditions, the lamin A/C intensity was essentially unchanged in all the groups. We also confirmed that the nuclear envelope ratio (NE ratio), which is a measure of the fluorescence signals of TMEM43 in the...
Figure 4  N-glycosylation of TMEM43<sub>S358L</sub> is enhanced by endoplasmic reticulum (ER) stress and accumulates at the nuclear envelope (NE) in a non-uniform and punctate manner. (A) Representative immunoblot of cell lysates from neonatal cardiomyocytes of WT or Tmem43<sup>-S358L</sup> knock-in (KI) rats treated with MG132 (2.5 μM, upper panel) or thapsigargin (Tg) (0.5 μM, lower panel) at the indicated times. (B) Quantified glycosylation rates of TMEM43<sup>WT</sup> and TMEM43<sup>S358L</sup> in neonatal rat cardiomyocytes (NRCMs) treated with MG132 (upper panel) or thapsigargin (Tg; lower panel). Glyco. rate (%) represents the percentage of the glycosylated form relative to total glycosylated and total unglycosylated TMEM43<sup>S358L</sup>. These rates were calculated from three independent experiments. Data are presented as the mean ± SD. *p < .05 and **p < .01 based on two-way ANOVA with Tukey’s test. (C) NRCMs were treated with DMSO, MG132 (2.5 μM), or Tg (0.5 μM) for 24 h and then immunostained using TMEM43 antibody. Nuclei were stained with Hoechst 33342 (blue). Arrowheads indicate the accumulation of TMEM43 at the NE. Scale bars, 10 μm. (D) High-resolution images of immunostained NRCMs treated with MG132 (2.5 μM) for 24 h. The images were acquired using a super-resolution confocal microscope. Scale bars, 5 μm. (E) Fluorescence intensity line profiles traced on the signals of TMEM43 and lamin A/C at the nuclear envelope (NE) in (D) (see also Figure S8). AU, arbitrary units. (F) The signal intensity of TMEM43 and lamin A/C at the NE of NRCMs. Each dot indicates the average fluorescence intensity obtained from a line profile, as in (E) (n = 30). Data are presented as the mean ± SD. **p < .01 according to one-way ANOVA. **p < .01 based on two-way ANOVA with Tukey’s test. (G) Nuclear envelope ratio (NE ratio) of WT or KI NRCMs treated with DMSO or MG132, calculated by dividing the NE fluorescence signal by the cytoplasmic fluorescence signal. Each dot was calculated from a single immunostained cell (n = 30). Data are presented as the mean ± SD. *p < .05 and **p < .01 based on one-way ANOVA with Tukey’s test. (H) Coefficient of variation of TMEM43 and lamin A/C fluorescence signals used to analyze the distribution of TMEM43 and lamin A/C at the NE. Each dot was calculated from a line profile, as in (D) (n = 30). Data are presented as the mean ± SD. **p < .01 based on one-way ANOVA with Tukey’s test. **p < .01 based on two-way ANOVA with Tukey’s test.
pluripotency and normal karyotypes of the iPSCs, we successfully differentiated them into cardiomyocytes (Figure S12). In these patient-specific iPSC-derived cardiomyocytes (iPSC-CMs), additional slower-migrating bands showing N-glycosylation were detected after MG132 treatment (Figure 6A,B). The immunofluorescence study showed that the MG132 treatment resulted in the accumulation of TMEM43 at the NE in patient-specific iPSC-CMs, whereas the localization was almost unchanged in the control iPSC-CMs (Figure 6C). Furthermore, quantitative analysis of the fluorescence intensity line profile, using high-resolution images of immunostained iPSC-CMs, showed that MG132 treatment markedly increased the TMEM43 NE ratio in patient-specific iPSC-CMs (Figure 6D–F). In contrast, no such differences were observed in control iPSC-CMs, regardless of the treatment. The CV of the TMEM43 fluorescence intensity was strikingly increased in patient-specific iPSC-CMs treated with MG132 (Figure 6G), indicating that TMEM43 non-uniformly accumulated at the NE under conditions of ER stress. In summary, our analysis using iPSC-CMs derived from patients of the affected family suggested that glycosylation of TMEM43S358L and its aberrant accumulation at the NE contribute to the pathogenesis of ACM in humans.

3.7 Reduction in regional differences in gene expression profiles in Tmem43-S358L KI rat myocardium

As GSK3β and AKT signaling has recently been reported to be involved in the pathogenesis of ACM caused by the Tmem43 p.S358L variant, we examined the phosphorylation status of GSK3β and AKT in the heart tissues of WT and KI rats (Figure S11B). However, no obvious differences were observed between them. Therefore, a more
comprehensive analysis was considered needed to investigate the effect of the accumulation of TMEM43\textsuperscript{S358L} at the NE on cardiac phenotypes in KI rats.

In Tmem43-S358L KI rats, TMEM43\textsuperscript{S358L} was highly glycosylated after 2 months of age. Although the appearance of arrhythmias in KI rats was not observed until one year of age, KI rats showed histological changes of degeneration, especially in the subepicardium, but not in the whole heart. Therefore, to examine the upstream mechanism, we analyzed gene expression profiles of 6-month-old rats, when KI rats still showed no overt myocardial degeneration. We collected tissue from the outer (Out) and inner (In) layers of the left ventricular myocardium and conducted RNA-seq analysis for four groups: WT\textsubscript{Out}, WT\textsubscript{In}, KI\textsubscript{Out}, and KI\textsubscript{In} (Figure 7A). The normalized transcripts of each replicate sample were highly correlated in any group ($r = .984$ for WT\textsubscript{Out}, $r = .985$ for WT\textsubscript{In}, $r = .988$ for KI\textsubscript{Out}, and $r = .988$ for KI\textsubscript{In}; Figure 7A and Excel File 1).

In the hierarchy of the gene expression pattern, clustering by region (i.e., the outer and inner layers of the myocardium) was surprisingly greater than clustering by genotype (i.e., WT and KI), which suggests that the differences in gene expression patterns between regions for each genotype are more easily distinguishable than those between genotypes (Figure 7B). PCA and t-SNE also showed a strong statistical separation from tissues from the outer layer (WT\textsubscript{Out} and KI\textsubscript{Out}) and the inner layer (WT\textsubscript{In} and KI\textsubscript{In}) (Figure S13). Based on this result and the documented myocardial degeneration in the subepicardium of KI rats, we next performed DEG analysis to compare the gene expression profiles of inner and outer layers of the WT myocardium. Of the total 11 189 genes detected, the expression levels of 196 or 266 genes were significantly upregulated or downregulated in the inner (WT\textsubscript{In}) versus the outer layer (WT\textsubscript{Out}), respectively (FDR < 0.05; Figure 7C, left panel and Excel File 2), suggesting that regional (i.e., Out vs. In) differences in gene expression patterns exist within the myocardium. Indeed, some of these genes (i.e., Irx3, Irf5, Etv1, and Foxp2) with high differential expression have been shown to be expressed with regional differences in the myocardium.\textsuperscript{31} In contrast, when focusing on KI rats, the expression of 61 or 192 genes was found to be significantly upregulated or downregulated in the inner (KI\textsubscript{In}) versus the outer layer (KI\textsubscript{Out}), respectively (Figure 7C, right panel, and Excel File 2). This suggested that regional differences in gene expression patterns observed in the WT myocardium were moderately reduced. Furthermore, these DEGs in the KI partially overlapped with those in the WT. Of 61 upregulated and 192 downregulated DEGs in the KI, 33 and 104 were also found in the WT, respectively (Figure 7D). However, there were 28 and 88 upregulated and downregulated DEGs, respectively, detected only in the KI.

Therefore, to reveal the characteristics of genes with reduced expression differences in KI rats, we used GO analysis for the DEGs. First, in GO analysis for DEGs detected only in the KI, significant KEGG pathways were detected for neither upregulated nor downregulated DEGs. Thus, when we performed GO analysis in combination with DEGs detected in the WT, four KEGG pathways were found specifically in WT but not KI rats (Figure 7E, and Excel File 3). Particularly, regional differences for nine genes from the pathway “Adrenergic signaling in cardiomyocytes” were markedly reduced in KI versus WT rats. These data imply that the intrinsic nature of the regional differential gene expression patterns in the WT myocardium was beginning to be lost in the Tmem43-S358L KI myocardium (Figure 7F), at least in the early stage of ACM showing no apparent phenotypes.

4 | DISCUSSION

In this study, following the identification of the variant TMEM43 c.1073C>T, p.S358L in a family with ACM, we successfully created Tmem43-S358L KI rats, which exhibited similar phenotypes to those of humans with ACM. Using the KI rats and iPSC-CMs from patients with ACM, we determined that TMEM43\textsuperscript{S358L} underwent glycosylation as a result of its altered membrane topology and accumulated at the NE, especially under conditions of ER stress induced by pharmacological stimulation or aging. Furthermore, transcriptome analysis showed that regional differences in gene expression between the inner and outer layers of the myocardium were partially reduced in KI rats. These results suggest that the accumulation of aberrant TMEM43\textsuperscript{S358L} at the NE contributes to the pathogenesis of ACM through changes in a characteristic gene expression pattern within the myocardium.

Creating appropriate animal models is crucial for clarifying the pathogenesis of human diseases. Whereas mouse models in which the Tmem43 gene is manipulated (including the p.S358L variant) have been recently generated and analyzed, their phenotypes are highly inconsistent.\textsuperscript{30,32,33} The reason for the discrepancies remains unclear but might be attributed to differences in the mouse strains used or the short-term observations of cardiac phenotypes (within 1 year). In the current study, our observation of Tmem43-S358L KI rats for more than 1 year allowed us to determine that KI rats exhibited ventricular arrhythmia and fibrotic myocardial replacement in the subepicardial layer. The fact that patients with the TMEM43 p.S358L variant in the family included in our study developed symptoms of ACM during mid-adulthood is consistent with the late onset of ACM phenotypes in the KI rats. Recapitulation of ACM
disease progression over time using the KI rats enabled us to analyze the mechanism of disease development.

In terms of molecular pathogenesis, we presented novel biochemical findings using the KI rats, suggesting that TMEM43S358L is glycosylated owing to its perturbed membrane topology. We further utilized the predicted ΔG_{app} of individual TM helices to determine how the topology of glycosylated TMEM43 was altered. Several studies have shown that some disease-causing mutations perturb the membrane topology of multi-spanning TM helices.
proteins, which is accompanied by changes in the energetics of the TM helices.\textsuperscript{26,34–36} Although S358L mutations were not predicted to destabilize integration of the TM3 helix, many pathogenic mutations in the TM region are also predicted to cause little change in the $\Delta G_{\text{app}}$.\footnote{26} Furthermore, a certain mutation in the helix is also reported to cause topological alterations, despite only slight changes in the predicted energetics.\footnote{35} $\Delta G_{\text{app}}$ analysis of TMEM43 also revealed that the $\Delta G_{\text{app}}$ of the TM4 helix was clearly lower than that of the other three helices. This suggests that the TM4 helix is more thermodynamically stable than the other three helices. Given our current findings in conjunction with those of previous reports, the substitution of Ser358 to Leu in the TM3 helix might have affected interactions among the TM helices rather than the TM efficiency of the TM3 helix alone. This likely led to a disturbance in the conformation of TMEM43 (Figure 3G), which has energetically delicate TM helices.

As noted previously herein, the Ser358Leu substitution itself might affect the conformation of TMEM43. However, during steady-state conditions, the majority of TMEM43\textsuperscript{S358L} molecules of cardiomyocytes existed in an unglycosylated form, which indicates that they retained their correct membrane topology. Considering that TMEM43 presumably adopted an intrinsically unstable membrane topology based on the predicted energetics of the TM helices (Table S2), the correct topology of most TMEM43\textsuperscript{S358L} molecules might be maintained by ER chaperones, which assist in proper protein conformation.\footnote{27} The function of the ER is perturbed when the influx of nascent and unfolded or misfolded proteins exceeds the ER folding capacity, which results in ER stress.\footnote{37} Therefore, in cardiomyocytes exposed to conditions of ER stress induced by pharmacological stimulation or aging, the topology of TMEM43\textsuperscript{S358L} is likely perturbed, which might have resulted in the increase in N-glycosylation. Given that the increase in the glycosylation of TMEM43\textsuperscript{S358L} was also observed with a slight increase in CHOP expression in NRCM (Figure S5B) or heart tissue (Figure 5A), even low levels of ER stress might alter the membrane topology of TMEM43\textsuperscript{S358L} harboring intrinsic structural instability. In addition, ER stress induced the accumulation of TMEM43\textsuperscript{S358L} at the NE in a non-uniform and punctate manner, as well as an increase in its glycosylation. This was in contrast to that seen for TMEM43\textsuperscript{WT}, suggesting that glycosylated TMEM43\textsuperscript{S358L} molecules with aberrant topology accumulate at the NE, where they might form local aggregation-like structures.

How the accumulation of TMEM43\textsuperscript{S358L} at the NE contribute to the pathogenesis of ACM? Some pathogenic mechanisms of ACM caused by the TMEM43 p.S358L variant have been previously proposed.\footnote{6,10,30,33} Among them, GSK3β activation in transgenic mice expressing human TMEM43\textsuperscript{S358L} has recently been reported.\footnote{30} However, no GSK3β activation and AKT inhibition were observed in our KI rats. This discrepancy might be due to the severity of the phenotype associated with the different expression levels of the TMEM43\textsuperscript{S358L} mutant in the heart, but the TMEM43 protein and/or species used as models might also affect these signals. In this study, since Tmem43-S358L KI rats showed marked fibrosis, especially in the outer layer of the heart, we examined comprehensive gene expression profiles focusing on regions within the myocardium. We first identified the distinct regional differences in gene expression patterns between the outer and inner layers of the WT myocardium. Gene expression analyses conducted so far showed that several genes were differentially expressed between regions within the myocardium.\footnote{31,38–40} Accordingly, this intrinsic characteristic in the myocardium may be a prerequisite for maintaining cardiac tissue homeostasis. Intriguingly, in Tmem43-S358L KI rats, these differences between outer and inner layers were moderately reduced. Indeed, loss of regional differences in gene expression patterns in cardiomyopathy has been shown to underlie susceptibility to fatal arrhythmia.\footnote{41} GO analysis showed that disruption in regional differences in...
distinct signaling pathways, including genes encoding ion channels or ion transporters, may be partially involved in the onset of ventricular arrhythmias in ACM. In contrast, a previous murine model study suggested that the NF-κB-TGFβ signal cascade was involved in the pathogenesis of cardiac fibrosis in ACM caused by TMEM43 p.S358L mutation. Changes in regional differences in two TGFβ genes (Tgfb2 and Tgfb3) within the myocardium were observed in KI rats in our transcriptome analysis. These changes may exert greater effects on the signal cascade in the late stage of the disease. However, not only these genes, but also many others showed reduced regional differences in KI rats compared to WT rats. This suggests that the overall reduction of regional differences in gene expression patterns, rather than specific gene clusters in specific pathways, may contribute to ACM development. Although further analysis is
needed to assess the reduction of these regional differences in detail, this may reflect the subtle perturbation of cardiac homeostasis in the early stage of the disease. Of note, even in this stage, TMEM43<sup>S358L</sup> was well glycosylated and had accumulated at the NE. These biochemical changes of TMEM43<sup>S358L</sup> prior to tissue degeneration may affect the intra-nuclear environment and contribute to subsequent alterations in gene expression profiles.

In our disease model, heterozygous <i>Tmem43</i>-S358L KI rats did not show overt dysfunctional cardiac phenotypes. Consistent with the results of our current study, some animal models of autosomal dominant human diseases do not show phenotypes when heterozygous but only when homozygous. Moreover, <i>Emd</i> (encoding emerin)-null mice, a model for Emery-Dreifuss muscular dystrophy, show no overt muscle pathology. This is possibly because the expression of emerin is lower in skeletal muscles of mice than in those of humans, whereas that of LAP1, a functionally related protein of emerin, is higher in mice than in humans, suggesting that LAP1 compensates for the function of emerin in <i>Emd</i>-null mice. Taken together, the differences in the genetic background and/or gene expression level of TMEM43 itself between humans and rodents might have affected the phenotype of heterozygous <i>Tmem43</i>-S358L KI rats. Furthermore, even homozygous KI rats showed no apparent sudden cardiac death and no obvious difference in prognosis compared with WT rats. A possible reason for this result is that electrically induced ventricular arrhythmias in rodents are often self-terminating after several cycles because of the small size of their hearts. Therefore, ventricular arrhythmias in KI rats may not have affected their prognosis. Another reason may be the contribution of environmental factors to the development of the disease. In fact, endurance sports and frequent exercise have been shown to increase the risk of ventricular arrhythmias and the occurrence of heart failure in ACM. Thus, long-term endurance training could exacerbate cardiac phenotypes in the KI rats. Despite such limitations, it is important to note that the phenotypes of ventricular arrhythmia and myocardial degeneration in the subepicardium shown in the animal model were similar to that of human diseases.

Our study highlights a novel molecular mechanism involved in the pathogenesis of ACM. Although further investigation of the functional consequences of TMEM43 topological perturbation in cardiomyocytes is needed, our findings suggest that the aberrant membrane topology of TMEM43<sup>S358L</sup> could become a novel therapeutic target to decrease the risk of sudden cardiac death and the exacerbation of heart failure associated with cardiomyopathy caused by the TMEM43 p.S358L variant. We hope that this study will be helpful to develop such new treatments.

ACKNOWLEDGMENTS

The authors thank the patient (proband) and his family members for their contribution to this study. The authors also thank S. Ishino for preliminary exome sequencing analysis; R. Ushioda at Kyoto Sangyo University (Kyoto, Japan) for fruitful discussions; S. Ikezawa, Y. Uegaki, S. Kondo, Y. Jinno and M. Yasui for technical assistance; S. Yamazaki for primer design and Sanger sequencing analysis; L. Bengtsson at Max Delbrück Center for Molecular Medicine in the Helmholtz Association (Berlin, Germany) for providing antibodies.

DISCLOSURES

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Yoshihiro Asano and Hisakazu Kato directed the entire project, and designed the experiments. Haruki Shinomiya mainly performed the experiments and analyzed the data. Yuki Kuramoto generated iPS cells and
analyzed the data. Haruki Shinomiya, Yoshihiro Asano, and Hisakazu Kato wrote the manuscript. Yoshihiro Asano, Nozomi Watanabe, Yoshihiro Tsuruda, and Tadaaki Arimura acquired and analyzed clinical data. Yohei Miyashita and Ayako Takuwa contributed to genome informatics analysis. Yoshiaki Miyasaka and Tomoji Mashimo contributed to generation of Tmem43-S358L KI rats. Yohei Miyashita, Daisuke Motooka, and Daisuke Okuzaki contributed to RNA seq analysis. Ken Matsuoka, Osamu Tsukamoto, Hideyuki Hakui, Noriaki Yamada, Jong-Kook Lee, and Hitetaka Kioka analyzed the data and revised the manuscript. Masafumi Kitakaze, Seiji Takashima, Yasushi Sakata, and Yoshihiro Asano supervised this project.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author on reasonable request.

ETHICS STATEMENT
This study was performed under the ethical code approved by the Ministry of Health, Labor, and Welfare of Japan, and written informed consent was obtained from all subjects before inclusion in the study. The genome research protocol was approved by the Human Genome Research Bioethical Committee at Osaka University and Miyazaki University. All procedures of rat studies were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th Edition, 2011) and were approved by the Animal Care and Use Committee of the Osaka University Graduate School of Medicine. For the generation of iPSCs, written informed consent was obtained from the patients according to the protocol approved by the Institutional Review Board of Osaka University.

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REFERENCES
1. Corrado D, Basso C, Judge DP. Arrhythmogenic cardiomyopathy. Circ Res. 2017;121:784-802.
2. James CA, Syrris P, van Tintelen JP, Calkins H. The role of genetics in cardiovascular disease: arrhythmogenic cardiomyopathy. Eur Heart J. 2020;41(14):1393-1400.
3. Austin KM, Trembley MA, Chandler SF, et al. Molecular mechanisms of arrhythmogenic cardiomyopathy. Nat Rev Cardiol. 2019;16:519-537.
4. Marian AJ. Causality in genetics: the gradient of genetic effects and back to Koch's postulates of causality. Circ Res. 2014;114:e18-e21.
5. Bengtsson L, Otto H. LUMA interacts with emerin and influences its distribution at the inner nuclear membrane. J Cell Sci. 2008;121:536-548.
6. Siragam V, Cui X, Masse S, et al. TMEM43 mutation p. S358L alters intercalated disc protein expression and reduces conduit velocity in arrhythmogenic right ventricular cardiomyopathy. PloS ONE. 2014;9:e109128.
7. Merner ND, Hodgkinson KA, Haywood AF, et al. Arrhythmogenic right ventricular cardiomyopathy type 5 is a fully penetrant, lethal arrhythmic disorder caused by a missense mutation in the TMEM43 gene. Am J Hum Genet. 2008;82:809-821.
8. Christensen AH, Andersen CB, Tybjaerg-Hansen A, Haunso S, Svendsen JH. Mutation analysis and evaluation of the cardiac localization of TMEM43 in arrhythmogenic right ventricular cardiomyopathy. Clin Genet. 2011;80:256-264.
9. Baskin B, Skinner JR, Sanatani S, et al. TMEM43 mutations associated with arrhythmogenic right ventricular cardiomyopathy in non-Newfoundland populations. Hum Genet. 2013;132:1245-1252.
10. Milting H, Klauke B, Christensen AH, et al. The TMEM43 Newfoundland mutation p. S358L causing ARVC-5 was imported from Europe and increases the stiffness of the cell nucleus. Eur Heart J. 2015;36:872-881.
11. Miyasaka Y, Uno Y, Yoshimi K, et al. CLICK: one-step generation of conditional knockout mice. BMC Genom. 2018;19:318.
12. Shintani Y, Drexler HC, Kioka H, et al. Toll-like receptor 9 promotes non-immune cells from stress by modulating mitochondrial ATP synthesis through the inhibition of SERCA2. EMBO Rep. 2014;15:438-445.
13. Hessa T, Meindl-Beinker NM, Bernal S, et al. Molecular code for transmembrane-helix recognition by the Sec61 translocon. Nature. 2007;450:1026-1030.
14. Eykyn TR, Aksentijevic D, Aughton KL, Southworth R, Fuller W, Shattock MJ. Multiple quantum filtered 23Na NMR in the Langendorff perfused mouse heart: ratio of triple/double quantum filtered signals correlates with [Na]i. J Mol Cell Cardiol. 2015;86:95-101.
15. Litvinukova M, Talavera-Lopez C, Maatz H, et al. Cells of the adult human heart. Nature. 2020;588:466-472.
16. Okita K, Yamakawa T, Matsumura Y, et al. An efficient nonviral method to generate integration-free human-induced pluripotent stem cells from cord blood and peripheral blood cells. Stem Cells. 2013;31:458-466.
17. Kuramoto Y, Naito AT, Tojo H, et al. Generation of Fabry cardiomyopathy model for drug screening using induced pluripotent stem cell-derived cardiomyocytes from a female Fabry patient. J Mol Cell Cardiol. 2018;121:256-265.
18. Tohyama S, Hattori F, Sano M, et al. Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. Cell Stem Cell. 2013;12:127-137.
19. Ge SX, Son EW, Yao R. iDEP: an integrated web application for differential expression and pathway analysis of RNA-Seq data. BMC Bioinformatics. 2018;19:534.
20. Ge SX, Jung D, Yao R. ShinyGO: a graphical gene set enrichment tool for animals and plants. Bioinformatics. 2020;36:2628-2629.
21. Chelko SP, Asimaki A, Lowenthal J, et al. Therapeutic modulation of the immune response in arrhythmic cardiomyopathy. *Circulation*. 2019;140:1491-1505.

22. van der Voorn SM, Te Riele A, Basso C, Calkins H, Remme CA, van Veen TAB. Arrhythmic cardiomyopathy: pathogenesis, pro-arrhythmic remodelling, and novel approaches for risk stratification and therapy. *Cardiovasc Res*. 2020;116:1571-1584.

23. Elder JH, Alexander S. Endo-beta-N-acetylglucosaminidase F: endoglycosidase from Flavobacterium meningosepticum that cleaves both high-mannose and complex glycoproteins. *Proc Natl Acad Sci U S A*. 1982;79:4540-4544.

24. Marshall R. The nature and metabolism of the carbohydrate-peptide linkages of glycoproteins. *Biochem Soc Symp*. 1974;40:17-26.

25. Cheung JC, Reithmeier RA. Scanning N-glycosylation mutagenesis of membrane proteins. *Methods*. 2007;41:451-459.

26. Schlebach JP, Sanders CR. Influence of pathogenic mutations on the energetics of translocon-mediated bilayer integration of transmembrane helices. *J Membr Biol*. 2015;254:371-381.

27. Oslowski CM, Urano F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. *Methods Enzymol*. 2007;41:451-459.

28. Buchwalter A, Kaneshiro JM, Hetzer MW. Coaching from the ER. *Nat Rev Genet*. 2005;280:4968-4974.

29. Brown MK, Naidoo N. The endoplasmic reticulum stress response in aging and age-related diseases. *Dev Cell*. 2012;3:263.

30. Padron-Barthe L, Villalba-Orero M, Gomez-Salino JM, et al. Severe cardiac dysfunction and death caused by arrhythmogenic right ventricular cardiomyopathy type 5 are improved by inhibition of glycogen synthase kinase-3beta. *Circulation*. 2019;140:1188-1204.

31. Rosati B, Grau F, McKinnon D. Regional variation in mRNA transcript abundance within the ventricular wall. *J Mol Cell Cardiol*. 2006;40:295-302.

32. Stroud MJ, Fang X, Zhang J, et al. Luma is not essential for murine cardiac development and function. *Cardiovasc Res*. 2018;114:378-388.

33. Zheng G, Jiang C, Li Y, et al. TMEM43-S358L mutation enhances NF-kappaB-TGFbeta signal cascade in arrhythmogenic right ventricular dysplasia cardiomyopathy. *Protein Cell*. 2019;10:104-119.

34. Choi MY, Partridge AW, Daniels C, Du K, Lukacs GL, Deber CM. Destabilization of the transmembrane domain induces misfolding in a phenotypic mutant of cystic fibrosis transmembrane conductance regulator. *J Biol Chem*. 2005;280:4968-4974.

35. Schlebach JP, Narayan M, Alford C, et al. Conformational stability and pathogenic misfolding of the integral membrane protein PMP22. *J Am Chem Soc*. 2015;137:8758-8768.

36. Coelho JPL, Stahl M, Bloemeker N, et al. A network of chaperones prevents and detects failures in membrane protein lipid bilayer integration. *Nat Commun*. 2019;10:672.

37. Schroder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem*. 2005;74:739-789.

38. Sharma S, Razeghi P, Shakir A, Keneson BJ 2nd, Clubb F, Taegtmeyer H. Regional heterogeneity in gene expression profiles: a transcript analysis in human and rat heart. *Cardiology*. 2003;100:73-79.

39. Gaborit N, Le Bouter S, Sztuts V, et al. Regional and tissue specific transcript signatures of ion channel genes in the non-diseased human heart. *J Physiol*. 2007;582:675-693.

40. McCormick ME, Manduchi E, Witschey WR, et al. Integrated regional cardiac hemodynamic imaging and RNA sequencing reveal corresponding heterogeneity of ventricular wall shear stress and endocardial transcriptome. *J Am Heart Assoc*. 2016;5:e003170.

41. Costantini DL, Arruda EP, Agarwal P, et al. The homeodomain transcription factor Irx5 establishes the mouse cardiac ventricular repolarization gradient. *Cell*. 2005;123:347-358.

42. Dallaz F, Ösinska H, Robbins J. Manipulating the contractile apparatus: genetically defined animal models of cardiovascular disease. *J Mol Cell Cardiol*. 2001;33:9-25.

43. Arimura T, Helbling-Leclerc A, Massart C, et al. Mouse model carrying H222P-Lmna mutation develops muscular dystrophy and dilated cardiomyopathy similar to human striated muscle laminopathies. *Hum Mol Genet*. 2005;14:155-169.

44. Mounkes LC, Kozlov SV, Rottman JN, Stewart CL. Expression of an LMNA-N195K variant of A-type lamins results in cardiac conduction defects and death in mice. *Hum Mol Genet*. 2005;14:2167-2180.

45. Shin JY, Mendez-Lopez I, Wang Y, et al. Lamina-associated polypeptide-1 interacts with the muscular dystrophy protein emerin and is essential for skeletal muscle maintenance. *Dev Cell*. 2013;26:591-603.

46. Bruegmann T, Boyle PM, Vogt CC, et al. Optogenetic defibrillation terminates ventricular arrhythmia in mouse hearts and human simulations. *J Clin Invest*. 2016;126:3894-3904.

47. Choy L, Yeo JM, Tse V, Chan SP, Tse G. Cardiac disease and arrhythmogenesis: mechanistic insights from mouse models. *Int J Cardiol Heart Vasc*. 2016;12:1-10.

48. Kirchhof P, Fabritz L, Zwiener M, et al. Age- and training-dependent development of arrhythmic right ventricular cardiomyopathy in heterozygous plakoglobin-deficient mice. *Circulation*. 2006;114:1799-1806.

49. James CA, Bhonsale A, Tichnell C, et al. Exercise increases age-related penetrance and arrhythmic risk in arrhythmogenic right ventricular dysplasia/cardiomyopathy-associated desmosomal mutation carriers. *J Am Coll Cardiol*. 2013;62:1290-1297.

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