Systems genetics analysis defines importance of TMEM43/LUMA for cardiac- and metabolic-related pathways

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Abstract

Broad cellular functions and diseases including muscular dystrophy, arrhythmogenic right ventricular cardiomyopathy (ARVC5) and cancer are associated with transmembrane protein43 (TMEM43/LUMA). The study aimed to investigate biological roles of TMEM43 through genetic regulation, gene pathways and gene networks, candidate interacting genes, and up- or downstream regulators. Cardiac transcriptomes from 40 strains of recombinant inbred BXD mice and two parental strains representing murine genetic reference population (GRP) were applied for genetic correlation, functional enrichment, and coexpression network analysis using systems genetics approach. The results were validated in a newly created knock-in Tmem43-S358L mutation mouse model (Tmem43358L) that displayed signs of cardiac dysfunction, resembling ARVC5 phenotype seen in humans. We found high Tmem43 levels among BXDs with broad variability in expression. Expression of Tmem43 highly negatively correlated with heart mass and heart rate among BXDs, whereas levels of Tmem43 highly positively correlated with plasma high-density lipoproteins (HDL). Through finding differentially expressed genes (DEGs) between Tmem43358L mutant and wild-type (Tmem43WT) lines, 18 pathways (out of 42 found in BXDs GRP) that are involved in ARVC, hypertrophic cardiomyopathy, dilated cardiomyopathy, non-alcoholic fatty liver disease, Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease were verified. We further constructed Tmem43-mediated gene network, in which Ctnna1, Adcy6, Gnas, Ndufs6, and Uqcrsc2 were significantly altered in Tmem43358L mice versus Tmem43WT controls. Our study defined the importance of Tmem43 for cardiac- and metabolism-related pathways, suggesting that cardiovascular disease-relevant risk factors may also increase risk of metabolic and neurodegenerative diseases via TMEM43-mediated pathways.

BXD family; cardiomyopathy; gene network; systems genetics; TMEM43/LUMA

INTRODUCTION

Transmembrane protein 43 (TMEM43/LUMA), a highly conserved integral membrane protein throughout all vertebrates, insects, unicellular eukaryotes, some plants, and several bacteria, is expressed in cellular inner nuclear membrane (INM) and endoplasmic reticulum (ER) (1, 2). TMEM43 is also found in adherens and composite junctions of cardiac myocytes and epithelial cells in humans and other mammals (3). Moreover, TMEM43 is found in many organs, including small intestine, thymus, prostate, and testis in humans (4), suggesting important cellular functions for TMEM43. Structurally, the protein consists of four transmembrane spanning domains, several phosphorylation sites, a transactivation domain, SUMO attachment site, and an O-glycosylation site that may play
important biological functions (4). TMEM43 has been shown to interact with emerin, lamins A/C, and B1 (4), and SUN2 (Sad1 and UNC84 Domain Containing 2) protein (5); all are the members of LINC (linker of nucleoskeleton and cytoskeleton) complex that provides physical and biological bridging between nucleus and cytoskeleton (6). LINC functions as a mechanosensing unit that translates extracellular and intracellular mechanical forces into biochemical signals in the nuclei, regulating diverse cellular processes such as cytoskeletal organization, nuclear architecture, chromatin dynamics, and gene expression (7).

Arrhythmogenic right ventricular cardiomyopathy (ARVC), an inherited cardiac muscle disease characterized by malignant arrhythmias, is associated with p.R28W, p.E142K, p.R312W, p.S358L, p.V89M, and p.R299T mutations (8, 9). Particularly, the S358L mutation causes a fully penetrant cardiac death (10, 11). Mutations p.E85K and p.I91V in TMEM43 have been reported in patients with Emery–Dreifuss muscular dystrophy type 7 (EDMD7) (5, 12). Hereditary serrated polyposis syndrome, a high-risk disorder of colorectal cancer, has shown significant segregation with rare TMEM43 variants (13). Moreover, a substantial phenotype variability in humans and animals associated with TMEM43 expression is shown in tumor malignancy via NF-κB activation (14), with innate immune signaling via stimulator of interferon genes (STING) pathway (15) resulting in severe disease, mild presentation, or be entirely asymptomatic (19). The cogent reason behind variable penetrance is limited in human cohorts (due to ethical and their encoded proteins combined with environmental genetic and epistatic interactions involving numerous genes (16). Despite that, the biological roles of Tmem43 in murine heart remain controversial (17, 18).

Many Mendelian diseases with the same mutation may result in severe disease, mild presentation, or be entirely asymptomatic (19). The cogent reason behind variable presentations is that a gene or disease-causing mutation does not entirely determine the phenotype or disease onset and progression. The final phenotype is characterized by complex genetic and epistatic interactions involving numerous genes and their encoded proteins combined with environmental factors. The power to detect those complex genetic and epistatic interactions is limited in human cohorts (due to ethical concerns) or in genetically modified animal models, because animal modeling is commonly created on a fixed genetic background (20). Thus, systems genetics approach linking complex genotypes to variable phenotypes, gene-gene, and gene-environment interactions at the genetic and epigenetic levels, pathogenic networks, and pathways are designed to identify biological processes and understand their regulation (21). Utilization of animal recombinant inbred (RI) genetic reference populations (GRP), particularly, offers a significant power to boost effective heritability, because each isogenic RI line and its stable genome can be replicated many times in a controlled environment.

In this study, we applied systems genetics analysis of BXD RI strains descended from cross between C57BL/6J (B6) and DBA/2J (D2) parental strains to determine biological roles of TMEM43 by identifying Tmem43-mediated pathways in murine GRP. Then, we validated those pathways by identifying differentially expressed genes (DEGs) between mutant knock-in S358L Tmem43 mice and WT littermate controls created on B6 background and constructed Tmem43-mediated genetic network (22). The identified Tmem43-correlated genes are predicted to perturb cardiac and metabolic related homeostasis and function pathways.

# MATERIALS AND METHODS

## Animals

Animal studies were approved by Institutional Animal Care and Use Committee (IACUC) at Cincinnati Children's Hospital Medical Center (CCHMC) and University of Tennessee Health Science Center (UTHSC). All animals had free access to standard laboratory diet and water. The total of 40 BXD strains, their B6 and D2 parental strains at age of 6–7 mo and 3 strains of Tmem43-knock-in (KI) heterozygous (HET), homozygous (HOMO), and wild-type (WT) mice at age of 3–4 mo (n > 5/strain) were used in this study.

## Creation of Tmem43-S358L Knock-In Mouse

To generate a murine model of Tmem43-S358L mutation, we targeted exon 12 in the Tmem43 gene and knocked-in the p.Ser358Leu substitution by replacing two nucleotides at c.1072 T → C and c.1073C → T into murine genome using a homologous recombination method (Supplemental Material; all Supplemental material is available at https://doi.org/10.6084/m9.ﬁgshare.14794596). Briefly, an MCI retrieval vector was created with AB and AR arms for homologous recombination corresponding to 548 and 526 bp sequences within intron 11 and 12, respectively, to target I3537 bp region (Supplemental Fig. S1A). Targeting vector comprised large and small homology arms (LHA and SHA, respectively), a removable neomycin resistance cassette (Neo) flanked with frt sites, and thymidine kinase negative selection cassette (TK). After NotI digestion to linearize the complete targeting construct, 40 μg of DNA was electroporated into 6 million Embryomax 129/SVEV embryonic stem cells (Millipore, Billerica, MA). Cells resistant to G418 were selected by culturing for 9 days in medium containing 0.26 mM G418 and 2 μM gancyclovir. Recombined embryonic stem cell clones were identified using PCR-based screening with primers, forward specific to Neo-cassette (5′-TGGCTGTGTGGCGGAATATCATGCTGTTGAA-3′) and reverse specific to genomic region downstream to SHA (5′-CTCGCTTAGATAAAATTCGTTCCA-3′). Clones that were conﬁrmed to be correct using direct sequencing were injected into C57BL/6 blastocysts at the Transgenic Mouse Core Facility of CCHMC (Cincinnati, OH). Mice were genotyped by PCR. All genotyping PCR was performed using LongAmp PCR mastermix (New England Biolabs, Ipswich, MA). Mouse colonies were maintained under barrier conditions with free access to standard laboratory diet and water. Chimeric animals were bred and backcrossed seven times with B6 mice and then the homozygote Tmem43S358LS358L mice were obtained from crossing the Tmem43WT/S358L heterozygotes. The correct mRNA as well as insertion of two nucleotides was confirmed by direct sequencing of total cDNA and levels of Tmem43 mRNA and TMEM43 protein expression in the heart were confirmed by quantitative real-time PCR (qRT-PCR) and Western blotting, respectively, using generic protocols.

## Cardiac Magnetic Resonance Imaging in Tmem43 Mice

Cardiac magnetic resonance imaging (cMRI) was used to evaluate heart function in 3-mo-old Tmem43S358L and
control Tmem43\textsuperscript{WT} littermate mice from heterozygous intercrosses (n = 5 animals per group). For all functional studies, mice were anesthetized by oxygenated 1.5% isoflurane and core temperatures were maintained using a heated platform set at 37°C. Cardiac magnetic resonance imaging (cMRI) was performed using a Bruker 7 T scanner. Image acquisition was prospectively ECG-gated using pediatric ECG probes attached to the paws. A bolus of gadopentetic acid (Gd-DTPA, 0.3–0.6 mmol/kg) was given intraperitoneally while the mouse was placed in the scanner bore. Delayed enhancement MRI was performed using a T1-weighted cine sequence. Cine imaging was performed in the short axis using a segmented fast low angle shot (FLASH) sequence. Slice thickness = 1.0 mm, matrix size = 256 × 256, in-plane resolution = 117 × 117 μm², echo time/repetition time (TE/TR) = 3/5.2 ms, flip angle = 20°, segments = 1. Approximately 15–20 cine frames were acquired during the cardiac cycle. Tagged images were acquired in the middle, basal, and apical planes of the left ventricle. The left ventricular (LV) maximum circumferential strain (Ecc) was calculated using HARP software (Diagnosoft Plus, Diagnosoft Inc., CA). Atrial and ventricular end diastolic volumes and LV and right ventricular (RV) ejection fractions (LVEF, RVEF) were calculated using freely available software Segment (http://segment.heiberg.se). Diastolic function was quantified as the rate of change of Ecc in diastole, \( \frac{d(Ecc)}{dt} \) using the mid ventricular tagged images. The tagged images were also used to quantify maximum apical twist, T, rate of change of T in diastole, \( \frac{dT}{dt} \). The LV sphericity was defined as the ratio of LV maximum width (approximately at the midlevel) to the LV base-to-apex length at end diastole in the four-chamber view.

**Electrocardiography in Tmem43 Mice**

Noninvasive in vivo ECG recordings were performed in conscious mice (n > 3/group/sex). Animals were placed in a restraining tunnel on top of four electrode pads (emka TECHNOLOGIES) and continuous signals were recorded for ~10 min using the accompanying IOX2 software. Raw ECG signals were exported into MATLAB 2017a for analysis of heart rate. A 60 Hz notch filter was applied, and heart rate variability and Poincare plots were generated to illustrate beat-to-beat variation.

**Mouse Blood and Tissue Harvest**

The mice were euthanized under isoflurane anesthesia after an overnight fast. Plasma and heart tissue were collected from mice per protocols published previously (23). Briefly, blood obtained from cardiac puncture was collected into EDTA tubes (10 mM final concentration) and the plasma was separated by centrifugation (1,500 g, 15 min) and stored in −80°C until further use. The hearts were removed and perfused with cardioplegic solution for histology or alternatively snap-frozen in liquid nitrogen for genetic molecular studies. Small intestine (jejunum and ileum) was removed from the gastrointestinal tract and gently flushed with iced saline. Tissue samples were snap frozen in liquid nitrogen and stored at −80°C until use. Primers used for qRT-PCR are shown in Supplemental Table S1. For histological evaluation, perfused mouse hearts were fixed in 10% formalin. After 48 h in formalin, hearts were embedded in paraffin, then sectioned and applied to H&E staining.

**Lipid-Level Assays in Plasma and Feces of Mice**

Plasma lipids levels were measured using cholesterol, HDL, and LDL assay kit (Abcam, Cambridge, UK) according to the provided protocols (n > 5 mice/group). Fecal samples were collected from 3-mo-old Tmem43 mice fed with standard laboratory chow (n = 10 mice per genotype). Briefly, 1 g of fecal pellets was collected and powered using a mortar and pestle. Total lipids were extracted via evaporation using saline and chloroform (Thermo Fisher Scientific, Waltham, MA) and the lipid mass (mg) per 1 g of feces (mg/g) was obtained.

**Extraction of Total RNA from Mouse Myocardium and Intestine and Microarray Analysis**

In this study, we used transcriptome data set of cardiac tissue across 40 strains of BXD and two parental C57BL/6J (B) and DBA/2J (D2) strains that we generated previously and deposited as “EPFL/LISP BXD CD Heart Affy Mouse Gene 2.0 ST (Jan14) RMA” in GeneNetwork (www.genenetwork.org) (24). GeneNetwork is a web resource of multi-omic data sets for BXD GRP, including genome, transcriptome, proteome, metabolome, and phenome data. Total RNA was extracted from the ventricular myocardium of BXDs, B6 and D2 parental strains, and Tmem43\textsuperscript{WT} and Tmem43\textsuperscript{SSSL} animals euthanized under isoflurane anesthesia after an overnight fast using QIAGEN RNA extraction kits (https://www.qiagen.com) as per the manufacturer’s instructions. To reduce the inhomogeneous nature of tissues due to the presence of different segments of the heart, the individual RNA sample from five mice at same strain were pooled evenly (by μg of RNA) into a single RNA sample and then purified using RNEasy kit. The RNA integrity number (RIN) for all samples were evaluated using Agilent 2100 Bioanalyzer. The RNA integrity value between 1.8 and 2 as well as the RIN values greater than 8 were considered for passing as quality control. The Affymetrix Mouse Gene 2.0 ST arrays were used to generate the gene expression data. The transcriptome quantification of ventricular myocardium including RNA extraction, array platform, data normalization, and validation was performed in adult Tmem43\textsuperscript{WT} and Tmem43\textsuperscript{SSSL} mice as aforementioned. Five animals per group were used. Comparisons between groups were conducted with the Limma package (25). Differential expression analysis between groups was examined by t test, and genes with P values <0.05 were defined as differentially expressed genes (DEGs). Candidate cardiac DEGs and genes involved in intestinal lipid absorption were validated by qRT-PCR using a Quant Studio 6 Flex (ThermoFisher) and Eva-Green master mix (Bio-Rad). All samples were assayed in triplicate at least two times, and the average value was used for quantification. In all experiments, \( Gapdh \) or \( b\)-actin was used as housekeeping genes. The data were analyzed using the comparative ACT method (ΔΔCT method) and results are expressed in fold changes related to that in Tmem43\textsuperscript{WT} mice.

**Microarray Data Set Analysis**

Raw microarray data was normalized using the Robust Multichip Array (RMA) method. The expression data were
then renormalized using a modified Z-score described previously (26). We calculated the log base 2 of normalized values above, computed Z-scores for each array, multiplied the Z-scores by 2, and added an offset of 8 units to each value. The reason for this transformation was to produce a set of Z-like scores for each array that have a mean of 8 and standard deviation of 2. The advantage of this modified Z-score is that a twofold difference in expression corresponds approximately to a 1-unit change.

Genetic Correlation Analysis

We computed genetic correlations between expression of Tmem43 and expression of all other probe sets across the genome using heart gene expression data sets of BXD mice. All genetic correlations were computed through the Pearson’s product-moment correlation using Genenetwork tools (27). The Pearson’s product correlations value $P < 0.05$ was used for indicating significant correlations. Literature correlation examines the $r$ value for genes that are described by similar terminology in published papers (28). Genes with literature correlation value $r > 0.1$ were selected for further analysis.

Gene Set Enrichment Analysis and Gene Network Construction

Gene set enrichment analysis for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway was done with ShinyGO (http://bioinformatics.sdstate.edu/go/) (29). The $P$ values generated from the hypergeometric test were automatically adjusted to account for multiple comparisons using Benjamini and Hochberg correction (30). The categories with false discovery rate (FDR) $< 0.1$ indicated that the set of submitted genes are significantly overrepresented in those categories. The genes that were significantly enriched in cardiomyopathy-related pathways were used for gene network analysis. Spring Model Layout Network Graphs were constructed using online Genenetwork tools. Each node in a graph represents an individual transcript and interconnecting lines illustrate ranges of Pearson’s correlation coefficient values. Other public search engines such as PubMed, OMIM, DAVID, GeneCard, BioGrid, and Cytoscape were used for function evaluation of members in the Tmem43 network.

Western Blot Analysis

For protein expression quantification, Western blotting analysis was performed. Whole heart tissue was homogenized in T-PER reagent (Thermo Fisher Scientific) with a protease inhibitor mixture (Roche Applied Science). Total proteins were quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and 25 $\mu$g of total protein was applied on Nupage 4%–12% Bis-Tris gel (Invitrogen). Blotting on PVDF membrane was done for 2 h at 30 V while cooled on ice. After 2 h of blocking in Tris-buffered saline (0.1% Tween-20 and 5% nonfat dry milk), membranes were incubated with primary Tmem43 antibody (Abcam) overnight at 4$^\circ$C on a rocking platform. The membranes were subsequently incubated with secondary antibodies for an hour, and proteins were detected using Li-Cor Odyssey. Levels were quantified in relative optical density (OD) units using Image Studio Lite software. GAPDH was used as a reference.

Statistical Analysis

All results, except gene expression data, are presented as means $\pm$ SD and statistical analysis was achieved with Student’s $t$ test or ANOVA using GraphPad Prism6 (GraphPad Software, La Jolla, CA). A $P$ value $< 0.05$ was considered significant after Mann–Whitney tests with appropriate Bonferroni correction.

### RESULTS

**TMEM43 Is Highly Expressed in the Heart of BXD Mice**

To determine cardiac Tmem43 expression in murine GRP of BXD strains, we analyzed microarray data set of ventricular myocardium deposited in the Genenetwork, a web-based multi-omic resource of GRP, including genome, transcriptome, proteome, metabolome, and phenoome data (24), and found the range of Tmem43 expression showed the highest in BXD81 (10.68) and the lowest in BXD100 (9.77), a difference of $>1.88$-fold (Fig. 1A), presenting a broad variability among BXDs. The average Tmem43 expression was $10.27 \pm 0.03$ (log$_2$ scale, means $\pm$ SE), which is approximately eight times higher compared with average expression of all other genes, suggesting important roles for Tmem43 in heart function and Tmem43 expression is affected by genetic background.

**Cardiac Tmem43 Expression Is Highly Negatively Correlated with Heart Weight and Heart Rate in BXD Strains**

To determine association of Tmem43 with cardiac phenotypes, we first performed an unbiased correlation between levels of Tmem43 in the heart and cardiac traits in BXDs that we collected in previous study and archived in our Genenetwork database (www.genenetwork.org). Considerable strain differences in heart weight (HW) and heart rate (HR) have been reported among BXDs (31). This phenomenon is associated with genetic background of individual lines as the BXDs are maintained in controlled laboratory environment. We found the levels of cardiac Tmem43 highly negatively ($P < 0.01$) correlated to HW and HR in BXDs (Fig. 1B), indicating that higher cardiac Tmem43 expression was observed in BXD strains with lower HW or slower HR, suggesting important roles for Tmem43 in heart morphology and function.

**Cardiac Tmem43 Expression Is Highly Correlated with Metabolic-Related Phenotypes in BXD Strains**

Further, we found Tmem43 expression in the heart highly significantly correlated with the levels of high-density lipoproteins (HDL, Fig. 1Ca) in plasma of BXD mice fed with normal chow diet indicating that higher cardiac Tmem43 expression was observed in BXD strains with higher HDL levels in plasma. Next, we correlated Tmem43 with phenotypes of BXDs fed with high-fat diet (HFD). Although the significant correlation between Tmem43 and plasma HDL was lost on HFD (Fig. 1Cb), we found highly significant correlation between cardiac Tmem43 and fat gain (Fig. 1Cc, $P < 0.05$, phenotype ID 15000) as well as fat weight (Fig. 1Cd, $P < 0.05$, phenotype ID 14835) in BXD
mice fed a HFD. These results suggested that Tmem43 may also play important roles in fat absorption, metabolism, and storage, especially under environmental modifiers such as diet.

**Enrichment Analysis Reveals Involvement of Tmem43 in the Development of Cardiac Diseases**

We further hypothesized that Tmem43 interacts with other candidate genes involved in pathogenesis of cardiac diseases and we could find those genes by identifying the genetic correlates of Tmem43 and enriching with similar biological functions, pathways, and phenotypes. Thus, we performed genetic correlation analysis and identified ~1,600 transcripts/probe sets whose expression levels were significantly (average expression > 7, \( P < 0.05, r > 0.1 \)) correlated with Tmem43 expression and uploaded those transcripts to the ShinyGO website (http://bioinformatics.sdstate.edu/go/) for gene function enrichment analysis. We then identified 42 significantly enriched pathways (FDR < 0.1) that were mainly enriched in cardiac-related pathways such as hypertrophic cardiomyopathy (HCM, 25 genes, FDR = 9.03E-04), dilated cardiomyopathy (DCM, 25 genes, FDR = 1.39E-03), and ARVC (17 genes, FDR = 5.93E-02). Other significant pathways included metabolic pathways (MP, 203 genes, FDR = 5.43E-03), nonalcoholic fatty liver disease (NAFLD, 39 genes, FDR = 2.28E-04), oxidative phosphorylation (40 genes, FDR = 3.15E-06), protein processing in endoplasmic reticulum (32 genes, FDR = 3.47E-02), neurodegenerative, and cancer-related pathways (Fig. 2A).
Analysis of Cardiac Phenotype and Tmem43 Expression in Tmem43-KI Mice

A heterozygous TMEM43-S358L mutation causes a lethal autosomal dominant ARVC5 in humans (10). To validate results obtained in BXD strains, we created a mutant knock-in Tmem43 S358L mouse model by conventional genetic targeting and crossed to B6 mice for genetic background concurrence with BXDs. All lines of Tmem43-KI mice were born and developed normally. To confirm expression of Tmem43 mRNA free of insertions such as the Neo-cassette and frt sites used for targeted knock-in, we performed direct sequencing of RT-PCR product of total RNA isolated from the heart of Tmem43-KI mice and the presence of two nucleotide knocked-in substitutions TCC > CTC, resulting in Ser to Leu substitution has been confirmed (Supplemental Figs. S1 and S2). Quantitative RT-PCR analysis of the heart demonstrated significantly reduced levels of mutant Tmem43 expression in the heart, which we also validated on a protein level by Western blot analysis (Fig. 2B).

Further, cardiac function was evaluated in 3-mo-old mice by cMRI, which revealed significant decrease in LVEF and RVEF, demonstrating biventricular systolic dysfunction in Tmem43S358L mutant strains compared with Tmem43WT controls (Fig. 3A). Volume of RV was significantly (*P < 0.05) increased in Tmem43 S358L compared with that in Tmem43WT littermate mice, suggestive for RV dilation. We also observed a thinning of the RV walls in Tmem43S358L mice and related aneurysm on cMRI and histology that are often reported in ARVC patients as the “triangle of dysplasia” (32) (Fig. 3B, arrows). Taken together, these results suggested a direct causal effect of TMEM43-S358L mutation resulting in biventricular cardiomyopathy phenotype.

We next validated correlations between Tmem43 expression and HW and HR in Tmem43-KI mouse hearts using microarray. Similar to qRT-PCR analysis, cardiac microarray revealed significantly downregulated Tmem43 in Tmem43S358L mice.
compared with that of Tmem43WT controls. In contrast, HW and ratio of HW to body weight (HW/BW) were significantly increased in Tmem43S358L mice, corroborating similar negative trends between Tmem43 expression and HW seen in BXDs (Fig. 3C).

To assess heart rate, ECG tracings of 3-mo-old conscious mice were applied. ECG tracing revealed similar HR in all groups, and no association between Tmem43 and HR is observed (Fig. 3D). Of note, HR asymmetry was observed in Tmem43-KI mice. A significant increase in HW (a, *P < 0.001) and HW/BW ratio (b, *P < 0.05) found in Tmem43S358L (white columns) compared with Tmem43WT (black columns) and Tmem43WT/S358L (gray columns) mice. D: representative Poincare scatterplots from ECG recording of conscious 3-mo-old WT female (a), Tmem43S358L female (b), and Tmem43S358L male (c) mice (n = 3). No association between Tmem43 expression and heart rate was found in Tmem43-KI mice. d, diastole; EF, ejection fraction; KI, knock-in; LV, left ventricular; RV, right ventricular; s, systole; SV, stroke volume; WT, wild type; Vol, volume; RR, interval between two heart beats; SD1, short-term standard deviation; SD2, long-term standard deviation.

Figure 3. Cardiac phenotypes of Tmem43-KI mice. A: results of cardiac MRI presented in median ranges. *P < 0.05, significant difference from WT littermates. B: representative images of longitudinal view of mouse hearts on cardiac MRI (top) and histology (bottom) demonstrate aneurism of the RV wall (arrow) and RV dilation in Tmem43S358L mutants compared with WT littermate mice. C: heart weight (HW) and heart and body weight (HW/BW) ratio in Tmem43-KI mice. A significant increase in HW (a, *P < 0.001) and HW/BW ratio (b, *P < 0.05) found in Tmem43S358L (white columns) compared with Tmem43WT (black columns) and Tmem43WT/S358L (gray columns) mice.
Correlation between Cardiac Tmem43 Expression and Plasma Lipid Levels in Tmem43-KI Mice

As significant correlation between Tmem43 expression and HDL was found in BXDs, we next measured levels of plasma lipids in Tmem43 mice (Fig. 4A) using ELISA, which revealed significantly ($P < 0.05$) higher total cholesterol and LDL levels in mutants compared with Tmem43$^{WT}$ controls. HDL was also increased in mutants as well, but it did not reach a statistical significance compared with Tmem43$^{WT}$ groups.

Elevated HDL and LDL levels in plasma have been previously reported in parental D2 mice, and this phenomenon was associated with increased expression of genes controlling cholesterol absorption (33). Thus, we next examined fecal lipid content to expose whether increased plasma lipids in Tmem43 mutants was associated with increased lipids absorption. Compared with Tmem43$^{WT}$ ($n = 10$), feces from mutants contained 35% less lipid ($P < 0.05$), suggesting that the latter animals more efficiently absorbed lipids and fatty acids (FA) from the gut lumen, providing the first evidence of Tmem43$^{S358L}$ association with a decrease in fecal lipid excretion and reciprocal hyperlipidemia. Subsequently, we examined expression levels of genes involved in absorption and reesterification of lipids in the intestine of Tmem43 mice using qRT-PCR (Fig. 4C). Expression of Npc1, Npc1l1, Abcg5 (cholesterol transporters), Asbt, Fabpt (bile acid and FA transporters), Mogat2, Dgat1, Dgat2 (triglyceride reesterification), Lrpat1 (inhibiting LDL degradation), and Ppara and Pparg (transcription factors that regulate lipid metabolism) was significantly increased in the gut mucosa of Tmem43$^{S358L}$ compared with Tmem43$^{WT}$ littermates. These results suggested enhanced fat and macronutrient uptake from the gut in Tmem43$^{S358L}$ mice and likely exposing similar origin of hyperlipidemia-reported parental D2 mouse (33).

Validation of Tmem43 Gene Pathways

To further validate the Tmem43 pathways that were identified using systems genetics in BXD GRP, we conducted microarray analysis of the myocardium to compare expression differences between Tmem43$^{WT}$ and Tmem43$^{S358L}$ strains. We identified ~3,200 differentially expressed transcript/probe sets ($P < 0.05$) in the LV between Tmem43$^{WT}$ and Tmem43$^{S358L}$ mice, and 3,000 in the RV and 3,400 in the LV and RV combined. After excluding unannotated probe sets, a total of ~4,200 cardiac DEGs were identified between Tmem43$^{WT}$ and Tmem43$^{S358L}$ strains (Supplemental Table S2). Further, KEGG pathway enrichment analysis found 48 enriched terms (FDR < 0.1), 18 of those were overlapped with the KEGG pathways enriched for the Tmem43 covariates found in BXDs, including cardiomyopathy-, metabolic- and neurodegenerative-related pathways (Table 1), suggesting that those pathways could be directly disturbed by change in Tmem43 expression or induced by the S358L mutation.

We further identified five genes (Cacng6, Igta2, Ttn, Sgcg, and Tpm3) that are also involved in HCM-related pathways, seven genes (Adcy6, Gnas, Ttn, Cacng6, Igta2, Sgcg, and Tpm3) in DCM-related pathways, and four genes (Cacng6, Ctnn1a, Igta2, and Sgcg) in ARVC-related pathways identified in BXD strains had a significant expression change between Tmem43$^{WT}$ and Tmem43$^{S358L}$ strains. In addition, we also found 16 overlapping genes between BXDs and Tmem43-KI mice involved in oxidative phosphorylation and 9 genes in NAFLD (Table 1). Collectively, the results of comparative microarray analyses demonstrated that Tmem43$^{S358L}$ mutation may disturb all these putative Tmem43-mediated pathways through Tmem43-correlated genes.

Construction and Validation of Tmem43-Mediated Gene Expression Network in the Myocardium of Tmem43-KI Mice

As we found a significant correlation between Tmem43 with cardiomyopathy- and metabolic-related traits in BXD strains and Tmem43 mutants, we performed genetic correlation analysis for the genes that have significant genetic correction with Tmem43 in HCM, DCM, ARVC, NAFLD, and metabolic pathway (MP) and constructed a Tmem43-mediated gene network that is involved in the development of cardiomyopathy and metabolic diseases. We included 70 genes including Tmem43 itself in this analysis and identified 990 gene pairs out of the 2,415 pairwise comparisons that had a significant Pearson’s correlation ($P < 0.05$). Out of 70 genes analyzed, we found 17 DEGs between Tmem43$^{WT}$ and Tmem43$^{S358L}$ strains (Fig. 5A, red nodes), suggesting that Adcy6, Cacng6, Cox7a2, Ctnn1a, Gnas, Itch, Igta2, Nduf5a3, Ndufb3, Ndufb9, Ndufs1, Ndufs6, Sgcg, Tpm3, Ttn, and Uqrc2 may be the interacting genes with Tmem43 according to the putative interactions identified.

We next validated the expression levels of all 17 DEGs identified in the myocardium of Tmem43 mice using qRT-PCR (Fig. 5B). The results demonstrated a significant ($P < 0.05$) upregulation of Adcy6, Ctnn1a, and Gnas, whereas Nduf56 and Uqrc2 were significantly decreased in the heart of Tmem43$^{S358L}$ mice compared with Tmem43$^{WT}$ littermates, corroborating the involvement of cardiac- and metabolic-related pathways in the phenotypes seen in Tmem43$^{S358L}$ mice and partly exposing the underlying mechanisms of deleterious ARVC5 in humans.

Discussion

Mutations in TMEM43 are associated with varied diseases in humans including ARVC5, a severe form of cardiomyopathy with malignant arrhythmias and sudden cardiac death (5, 10–12). In particular, the TMEM43-S358L mutation causes a fully penetrant lethal ARVC5 in humans (10). Several mouse models carrying the Tmem43 S358L mutation were reported including Tmem43-KI mouse of SV129 background (34), humanized transgenic mice overexpressing human mutation (35), and KI mouse created by CRISPR/Cas9 approach (18). In this study, we used a high-power and high-precision systems genetics approach to explore Tmem43 gene function and identified novel TMEM43-induced mechanisms, pathways, and networks relevant to heart function and cardiovascular diseases. For systems genetics studies, we utilized murine GRP consisting of 40 BXD R1 lines and their parental B6 and D2 strains for genotype-phenotype association and compared the results with newly created knock-in Tmem43$^{S358L}$ mouse on B6 background using
conventional targeting, Tmem43S358L mutants recapitulated some of human traits including biventricular systolic dysfunction, RV dilation, and thinning of the RV walls with aneurysm revealed by cMRI.

Among BXD strains maintained in laboratory standard conditions with minimal environmental influences and fed with chow diet, Tmem43 was highly expressed in the heart with a broad expression variability between BXD lines, suggesting that Tmem43 levels are affected by a genetic background. In contrast, levels of cardiac TMEM43 expression were significantly reduced in Tmem43S358L mutants compared with Tmem43WT controls, suggesting a

Figure 4. Plasma and fecal lipids and expression of lipid absorption-related genes in the intestine of Tmem43-KI mice. A: levels of plasma cholesterol, HDL, and LDL in plasma and fecal lipids (n = 10) of Tmem43-KI mice. *P < 0.05, significant difference compared with WT mice. B: results of qRT-PCR indicate significant increase of genes involved in lipid absorption in intestine of Tmem43S358L mice (white columns) compared with that in Tmem43WT (black columns) controls, n = 5 mice per group. C: expression of genes is shown in fold change. Gapdh was used as a reference. *P < 0.05, significant difference compared with WT mice. KI, knock-in; WT, wild type.
To leading to senescence-associated cardiomyopathy in vivo 

Interestingly, haplo-insufficiency of Tmem43 has been shown to activate the DNA damage response pathway leading to senescence-associated cardiomyopathy in vivo (16). To find genes that could share the genetic regulator and have similar biologic function with Tmem43, we performed genetic correlation and functional enrichment analysis in BXDs and identified 42 Tmem43-mediated pathways, 18 of those (42.8%) including cardiomyopathy-related (HCM, DCM, and ARVC), and metabolic-related (NAFLD) pathways have been confirmed through analysis of DEGs between Tmem43S358L and Tmem43WT mice.

Further, cardiac Tmem43 expression significantly negatively associated with heart weight and heart rate as well as HDL levels in plasma of BXD mice, suggesting important roles for Tmem43 not only in murine heart morphology and function but also in lipid metabolism. Moreover, cardiac Tmem43 highly significantly correlated with fat gain and fat weight in BXDs fed HFD. Intriguingly, Tmem43S358L mutant mice also had hyperlipidemia due to increased lipids absorption from the gut lumen, emphasizing the importance of assessing levels of plasma lipids in patients with ARVC5 under environmental modifier such as HFD. NAFLD is the most common liver disease worldwide with the prevalence of 25%–30% among general adult population in Western countries and of

Table 1. List of the overlapped KEGG pathways, Tmem43 covariates in BXDs, and DEGs in Tmem43-KI mice

| KEGG Pathway                                      | Covariates | DEGs | No. of Overlapped Genes |
|--------------------------------------------------|------------|------|-------------------------|
| Acute myeloid leukemia (AD)                       | 0.01       | 19   | 0.10                    | 22          | 7 |
| Alzheimer’s disease (AD)                          | 3.19E-07   | 51   | 1.0E-03                 | 59          | 20 |
| Apelin signaling                                  | 0.07       | 27   | 0.09                    | 39          | 10 |
| ARVC                                             | 0.06       | 17   | 0.07                    | 24          | 4 |
| Cellular senescence                              | 0.05       | 34   | 0.10                    | 47          | 11 |
| Dilated cardiomyopathy (DCM)                     | 1.39E-03   | 25   | 0.10                    | 27          | 7 |
| Human papillomavirus infection                   | 0.03       | 62   | 0.10                    | 87          | 21 |
| Human T-cell leukemia virus 1 infection          | 0.01       | 47   | 0.02                    | 68          | 16 |
| Huntington’s disease (HD)                        | 4.42E-04   | 45   | 0.01                    | 60          | 19 |
| Hypertrophic cardiomyopathy (HCM)                | 9.03E-04   | 25   | 0.10                    | 26          | 5 |
| Kaposi sarcoma-associated herpesvirus infection  | 0.05       | 39   | 0.02                    | 60          | 11 |
| Nonalcoholic fatty liver disease (NAFLD)         | 2.28E-04   | 39   | 0.07                    | 43          | 9 |
| Oxidative phosphorylation                        | 3.15E-06   | 40   | 0.03                    | 41          | 16 |
| Parkinson’s disease (PD)                         | 1.20E-06   | 43   | 0.05                    | 42          | 15 |
| Protein processing in endoplasmic reticulum     | 0.03       | 33   | 0.07                    | 46          | 7 |
| Prostate cancer                                  | 0.04       | 52   | 0.07                    | 73          | 6 |
| Thermogenesis                                    | 3.63E-04   | 52   | 1.0E-03                 | 73          | 6 |

Significantly altered genes between Tmem43S358L mutant and Tmem43WT control mice are highlighted in bold and underlined. ARVC, arrhythmogenic right ventricular cardiomyopathy; DEGs, differentially expressed genes; FDR, false discovery rate; KI, knock-in.

| KEGG Pathway                                      | FDR   | No. of Genes | FDR | No. of Genes | Overlapped Genes |
|--------------------------------------------------|-------|--------------|-----|--------------|-----------------|
| Acute myeloid leukemia (AD)                       | 0.01  | 19           | 0.10| 22           | RUNX1 CCND1 STAT5B ZBTB16 MAPK3 DUSP6 |
| Apelin signaling                                  | 0.07  | 27           | 0.09| 39           | ADC6 CALM1 CCND1 GNB4 HDAC5 KLF2 PLAT |
| ARVC                                             | 0.06  | 17           | 0.07| 24           | CTNNA1 ITGA2 SGC6 CACNG6 |
| Cellular senescence                              | 0.05  | 34           | 0.10| 47           | TNC4 CALM1 CCND1 CDC25A MDM2 PTEN |
| Dilated cardiomyopathy (DCM)                     | 1.39E-03| 25    | 0.10| 27           | ITGA2 TTN SGC6 CACNG6 TPM3 |
| Human papillomavirus infection                   | 0.03  | 62           | 0.10| 87           | ATP6V1B2 ATP6V0D1 ATP6V1E1 ATP6V1G1 |
| Human T-cell leukemia virus 1 infection          | 0.01  | 47           | 0.02| 68           | CALM1 CCND1 CDC25A CYCS ADD8 GNAB4 HIFIA |
| Huntington’s disease (HD)                        | 4.42E-04| 45    | 0.01| 60           | COX7A2 CYCS ITCH NDUF51 NDUF56 |
| Hypertrophic cardiomyopathy (HCM)                | 9.03E-04| 25    | 0.10| 26           | NDUF9 NDUF38 NDUF3 |
| Kaposi sarcoma-associated herpesvirus infection  | 0.05  | 39           | 0.02| 60           | COX7A2 CYCS ADD8 GNAS MAGI3 ITGA2 JAK1 |
| Nonalcoholic fatty liver disease (NAFLD)         | 2.28E-04| 39    | 0.07| 43           | ATP6V1B2 ATP6V0D1 ATP6V1E1 COX7A2 CYCS ADH7 |
| Oxidative phosphorylation                        | 3.15E-06| 40    | 0.03| 41           | E1A1 ADD8 GNAS |
| Parkinson’s disease (PD)                         | 1.20E-06| 43    | 0.05| 42           | RAF1 ADD8 GNAS |
| Protein processing in endoplasmic reticulum     | 0.03  | 33           | 0.07| 46           | ITGA2 TTN SGC6 CACNG6 |
| Prostate cancer                                  | 0.04  | 52           | 0.07| 73           | ITGA2 TTN SGC6 CACNG6 |
| Thermogenesis                                    | 3.63E-04| 52    | 1.0E-03| 73         | ITGA2 TTN SGC6 CACNG6 |

**Gene set enrichment analysis of 42 Tmem43-mediated pathways in BXDs.** The enrichment analysis was performed through KOBAS software. We listed KEGG pathways (21) with FDR < 0.05 and No. of Genes > 15. PPIF, protein phosphatase interacting with F performance of the pathway.

**Gene expression analysis of Tmem43S358L mutant and Tmem43WT control mice.** The differential expression analysis was performed by DESeq2 package. Significant DEGs with FDR < 0.05 and Log2 fold change > 1 were defined as differentially expressed genes (DEGs). We listed genes with FDR < 0.05 and Log2 fold change > 1. ATP6V1B2, ATP6V0D1, ATP6V1E1, ATP6V1G1, and COX7A2 were validated through western blot analysis.
70%–75% among patients with type 2 diabetes mellitus (38), resulting from increased fat absorption from the gut similar to that we found in our Tmem43S358L mutants. There is a strong association between NAFLD and increased risk in death due to cardiac arrhythmias, cardiomyopathy, and coronary disease often seen in patients with NAFLD (39). Therefore, we used the genes that showed a significant genetic correction with Tmem43 in HCM, DCM, ARVC, or NAFLD pathways among BXDs and DEGs between Tmem43S358L and Tmem43WT mice to construct the Tmem43-mediated gene network shown in Fig. 5. Using qRT-PCR, we confirmed significant alterations of Ctnna1, Adcy6, Gnas, Ndufs6, and Uqcr2 between Tmem43S358L and Tmem43WT hearts.

Almost 50% of ARVC cases are associated with mutation(s) in genes encoding desmosomal and cell-adhesion proteins required for mechanoelectrical coupling in the heart (37). Tmem43-mediated genes identified by systems genetics approach in this study are rather uncommon. Encoded by CTNNAL, catenin-a1 (also known as aE-catenin) is highly expressed in adherens junctions of cardiomyocytes, functioning as a mediator of actin filaments anchorage to the cadherin-catenin-vinculin complex at the sarcolemma as well as to the desmosomes via its interactions with plakophilin-2 (40). Inducible cardiac-specific aE-catenin conditional knockout mice developed cardiomyopathy with tendency to ventricular free-wall rupture due to intercalated disk defects (41). The ADCY6 (also known as AC6) encodes adenylyl cyclase 6, a member of ubiquitously expressed family of enzymes that catalyze cyclic AMP (cAMP) from ATP, and regulates numerous cellular functions including cell growth, differentiation, apoptosis, and transcriptional regulation through β adrenergic receptor/G-protein signal transduction (42). The G-protein α-subunit (Gαs) that couples the G-protein receptors to adenylyl cyclase is a gene product of GNAS (43). Although g.2714G > T variant in ADcy6 is associated with

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**Figure 5.** Tmem43-mediated gene coexpression network and expression of genes involved in this network. A: Tmem43-mediated gene coexpression network. The nodes in the network represent genes and edges represent genetic correlations (P < 0.05), respectively. Genes in this network are significantly correlated (P < 0.05) with Tmem43 in the heart of BXD mice and involved in KEGG pathways of HCM, DCM, ARVC, or NAFLD pathways. Red nodes represent genes differentially expressed in the heart between mutant Tmem43S358L and control Tmem43WT mice. B: results of qRT-PCR for genes differentially expressed between Tmem43WT and mutant Tmem43S358L myocardium. Black columns indicate Tmem43WT group and white columns indicate Tmem43S358L mutants (n = 4). Gapdh was used as a reference. *P < 0.05, significant difference in fold change compared with Tmem43WT mice. ARVC, arrhythmogenic right ventricular cardiomyopathy; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; NAFLD, nonalcoholic fatty liver disease.**
increased cardiac output, heart rate, and blood pressure at baseline conditions as well as in response to exercise (44), the common GNAS single nucleotide polymorphisms (SNPs), c.2273C > T and c.393C > T, are reported as predictors of ventricular tachyarrhythmias and sudden cardiac death (45).

Mitochondria are the site of fatty acid oxidation, a mechanism that protects cells and tissues against fatty acid accumulation, whereas reactive oxidative species (ROS) are freed during oxidation of FAs (46). Genes Ndufs6 and Uqrc2 are involved in mitochondrial oxidation significantly downregulated in Tmem43S358L mutant heart. Deficiency in mitochondrial complex I subunit NDUFS6 causes an excessive accumulation of ROS, underlying many cardiac, metabolic, and neurodegenerative disorders in humans (47). Wisloff et al. (48) reported markedly reduced UQCRC2 (ubiquinol-cytochrome c oxidoreductase core 2 subunit) protein levels in low-capacity runner rats compared with the high-capacity runners. Patients with ARVC5 respond to high-level physical activity with more than ninefold increase of malignant ventricular arrhythmias (1). Our results provide a genetic link between decreased mitochondrial oxidation and exercise-induced malignant arrhythmias, and thus, future mitochondrial functional studies in Tmem43-KI mice may aid to understanding the underlying mechanisms in ARVC of both TMEM43 and non-TMEM43 origins. In addition to cardiomyopathy- and metabolic-related pathways, we noted that Tmem43-associated mitochondrial genes, NDUFS6 and UQRC2, are enriched in neurodegenerative disease-related pathways, including Alzheimer’s disease (AD), Parkinson’s disease (PD), and Huntington’s disease (HD; Table 1). AD and its precursor mild cognitive impairment are progressive neurodegenerative disorders, which accounts for ~70% of all dementia cases (49). Similar to patients with AD, typical plaque-like amyloid aggregations are found in the hearts of patients with DCM carrying PSEN1 (Asp333Gly) and PSEN2 (Ser130Leu) mutations (50). Moreover, mitochondrial complex I activity defects are consistently observed in the substantia nigra and prefrontal cortex of patients with PD and dementia (51), suggesting that similar Tmem43-mediated biochemical and genetic triggers may exist for these two vital organs.

In conclusion, this is the first systems genetics analysis using murine GRPs of BXD strains and genetically modified Tmem43-KI mice to clarify biological roles of Tmem43 in murine heart. Analysis of transcriptome data sets of both BXDs and Tmem43-KI strains demonstrated that Tmem43 plays important roles not only in the heart but it is also an important gene involved in metabolic and mitochondrial oxidation processes, suggesting that same risk factors relevant to cardiovascular diseases may also increase person’s risk of developing metabolic and neurodegenerative diseases via TMEM43-mediated pathways. Although the study was not directed to validating human phenotypes, underlying molecular mechanisms, direct gene-gene, and gene-protein interactions in Tmem43S358L mice, our systems genetics approach by comparing with murine genetic reference population of RI strains determined the candidate genes that potentially interact with Tmem43, signifying the need for future investigations on cardiometabolic effects of Tmem43.

Study Limitations

We note limitations of this study. First, cardiac histological and ultrastructural phenotypes and mechanisms of how the Tmem43S358L mice develop biventricular phenotypes were not studied in depth. Second, ECG phenotypes in mutant Tmem43S358L mice do not fully recapitulate phenotypes (e.g., arrhythmias or sudden death) seen in patients with ARVC5. ARVC5 is a disease expressed often in young adults that progresses with aging and exercise. Thus, further studies in aging mice as well as under exercise are needed to fully recapitulate human phenotypes and our ongoing studies focused on all these aspects in Tmem43-KI mice are in progress.

DATA AVAILABILITY

The RNA-Seq data that support this study are available as follows: gene expression data sets of Tmem43 in BXD strains and computer code produced in this study are available at: https://genenetwork.org/show_trait?trait_id=17460918&dataset=EPFL-LISPBXDHeCD0114.

SUPPLEMENTAL DATA

Supplemental Figs. S1 and S2 and Supplemental Tables S1 and S2: https://doi.org/10.6084/m9.figshare.14794596.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

E.P. and L.L. conceived and designed research; Q.G., F.X., B.-O.O., Z.K., U.M., J.N.J., N.R.A., J.F.P., J.A.B., B.M.C., and I.R.E. performed experiments; Q.G., F.X., B.-O.O., J.N.J., J.F.P., J.A.B., B.M.C., I.R.E., E.P., and LL. analyzed data; F.X., D.D.B., I.R.E., E.P., and LL. interpreted results of experiments; Q.G., F.X., B.-O.O., Z.K., and E.P. prepared figures; F.X. drafted manuscript; Z.K., N.R.A., J.F.P., D.D.B., D.D., J.A.T., E.P., and LL. edited and revised manuscript; E.P. and LL. approved final version of manuscript.

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