The effect of \textit{Tmem135} overexpression on the mouse heart

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Abstract

Tissues with high-energy demand including the heart are rich in the energy-producing organelles, mitochondria, and sensitive to mitochondrial dysfunction. While alterations in mitochondrial function are increasingly recognized in cardiovascular diseases, the molecular mechanisms through which changes in mitochondria lead to heart abnormalities have not been fully elucidated. Here, we report that transgenic mice overexpressing a novel regulator of mitochondrial dynamics, transmembrane protein 135 (\textit{Tmem135}), exhibit increased fragmentation of mitochondria and disease phenotypes in the heart including collagen accumulation and hypertrophy. The gene expression analysis showed that genes associated with ER stress and unfolded protein response, and especially the pathway involving activating transcription factor 4, are upregulated in the heart of \textit{Tmem135} transgenic mice. It also showed that gene expression changes in the heart of \textit{Tmem135} transgenic mice significantly overlap with those of aged mice in addition to the similarity in cardiac phenotypes, suggesting that changes in mitochondrial dynamics may be involved in the development of heart abnormalities associated with aging. Our study revealed the pathological consequence of overexpression of \textit{Tmem135}, and suggested downstream molecular changes that may underlie those disease pathologies.

Introduction

Tissues with high-energy demand, such as the retina, brain, skeletal muscle, and heart, are rich in the energy-producing organelles, mitochondria, and sensitive to mitochondrial dysfunction
Heart abnormalities caused by Tmem135 overexpression

In the heart muscle, 35% of the volume of each cardiomyocyte is composed of mitochondria [2]. Thus, global disruptions to mitochondrial function have detrimental effects on the function of the heart and lead to a number of heart disorders including cardiomyopathies, arrhythmias and heart failure [3,4]. Decline in the mitochondrial function is also observed in the aging heart [5,6], which is thought to reduce the ability of cardiomyocytes to counter pathological stress. It has been shown that mitochondria are dynamic organelles undergoing fission and fusion (mitochondrial dynamics). Balanced mitochondrial dynamics are particularly important in terms of the quality control of this organelle, where fusion enables compensation for mitochondrial damage by joining neighboring mitochondria, and fission allows for elimination of mitochondria with unrecoverable damage through autophagy (mitophagy) [7]. Accordingly, balanced mitochondrial dynamics are essential for tissue homeostasis. Indeed, dysregulation of mitochondrial dynamics in the heart has been shown to result in abnormalities including cardiomyopathy and heart failure [8,9]. Ablation of a mitochondrial fission factor, dynamin-related protein 1 (Drp1) in the adult mouse heart lead to mitochondrial enlargement and dilated cardiomyopathy [10]. On the other hand, impairment of mitochondrial fission through combined ablation of mitochondrial fusion factors, mitofusin (Mfn) 1 and Mfn2, in the adult mouse heart caused mitochondrial fragmentation and eccentric cardiac hypertrophy [10]. These observations indicate that proper regulation of mitochondrial dynamics is essential for heart health. However, the molecular mechanisms through which changes in mitochondrial dynamics lead to heart abnormalities have not been fully explored.

Recently, we discovered a novel regulator of mitochondrial dynamics, transmembrane protein 135 (TREM135) [11] which is highly conserved across species [12]. TREM135 regulates mitochondrial morphology and dynamics, presumably through promoting mitochondrial fission [11]. A mouse mutation in Tmem135, a point mutation which results in an early stop codon, causes elongated mitochondria with defective respiratory functions, which leads to age-dependent retinal abnormalities with early onset and faster progression [11]. On the other hand, overexpression of Tmem135 transgene under the control of chicken beta-actin promoter (Tmem135 TG) leads to fragmented mitochondria with reduced basic oxygen consumption rate and ATP production in fibroblasts [11]. Here, we report that Tmem135 TG mice show disease phenotypes in the heart including hypertrophy and collagen accumulation. To identify key molecules and pathways affected in Tmem135 TG hearts, we examined gene expression changes in the Tmem135 TG heart combined with gene ontology analysis. Our study identifies a pathway through which abnormal mitochondrial dynamics, particularly excessive mitochondrial fission, may lead to disease phenotypes in the heart.

Materials and methods

Mice

All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison. Tmem135 TG mice were generated at University of Wisconsin-Madison as previously described [11]. We replaced the EGFP sequence in the pCX-EGFP vector (kindly provided by Dr. Junichi Miyazaki [13]) with the full length Tmem135 cDNA and named it pCX-Tmem135. We used pCX-TEM135 for the transgene construct after linearization with HindIII and SalI (New England Biolabs, Ipswich, MA). The construct was micro-injected into pronuclei of FVB/NJ embryos at the Transgenic Facility of the University of Wisconsin-Madison Biotechnology Center. Transgene-positive founders were crossed to C57BL/6J mice for one generation and subsequently maintained by intercrossing (FVB/NJ—C57BL/6J mixed genetic background). For the purpose of examining retinal
phenotypes in separate studies, we removed the Ped6b\textsuperscript{rd1} mutation in the FVB/NJ background known to cause retinal degeneration during this process [11]. The Tmem135 transgene maps to chromosome 19 between SNPs 19-025120427-M and 19-049914266-M. For experiments, Tmem135\textsuperscript{TG} mice as well as age-matched, littermate non-transgenic control (wild-type [WT]) mice were used.

**Heart weight/ body weight ratio**

Body weights were measured in grams using an Ohaus CS 200 scale (Ohaus corporation, NJ). Heart weights were measured in mg immediately following dissection using a Pinnacle Balance P-114 scale (Denver Instruments, Bohemia, NY). Ratios were calculated by dividing the heart weight by the body weight (n = 6 WT, n = 9 TG).

**Masson’s Trichrome Staining**

Following asphyxiation by CO\textsubscript{2} administration, hearts were immediately removed and immersion fixed in Bouin’s fixative overnight at 4˚C. Hearts were then rinsed and dehydrated, and embedded in paraffin. Paraffin blocks were sectioned 6 \( \mu \)m thick on an RM 2135 microtome (Leica Microsystems, Wetzlar, Germany) and mounted on glass slides. Three non-consecutive, central sections of each heart were chosen for staining. Central sections contained all 4 chambers. The slides were stained with Masson’s Trichrome Stain Kit (American MasterTech, Lodi, CA) to distinguish heart muscle cells, nuclei and collagen according to the manufacturer’s protocol. Masson’s Trichrome-stained sections were imaged on an Axio Imager 2 Microscope and camera (Carl Zeiss MicroImaging, Thornwood, NY). Collagen positive areas were quantified using the threshold function of ImageJ 1.46r [14], and represented as % blue area of total area measured, which were averaged for each genotype (n = 4 WT, n = 4 TG).

**Immunohistochemistry**

For immunohistochemistry on cryostat sections, 6-\( \mu \)m-thick sections were blocked in PBS with 0.5% Triton X-100 and 2% normal donkey serum for 20 min at room temperature. Next, sections were incubated at 4˚C overnight in primary antibody solution. Primary antibodies against Collagen III (Abcam, Cambridge, MA) and myosin (Sigma, St. Louis, MO) were used. Sections were rinsed in PBS and incubated with a secondary antibody in block solution (1:200 dilution) for 2 hours at room temperature. Following PBS rinse, sections were stained with 4’,-6-diamidino-2-phenylindole (DAPI). All sections were imaged on the Nikon A1R+ confocal microscope (Nikon, Tokyo, Japan) equipped with high sensitivity GaAsP detectors; high-speed resonant scanner; six lasers at 405, 440, 488, 514, 561, and 640 nm. NIS-Elements AR software (Nikon) was used for image acquisition and image analysis.

**Quantification of cardiomyocyte size**

Following asphyxiation by CO\textsubscript{2} administration, hearts were immediately dissected and rinsed with PBS. Hearts were then fixed in 4% paraformaldehyde by cannulation and immersion for two hours and dehydrated before embedding in paraffin. Paraffin blocks were sectioned 6 \( \mu \)m thick on an RM 2135 microtome (Leica Microsystems, Wetzlar, Germany) and mounted on glass slides. Three nonconsecutive, central sections of each heart were chosen for staining. Sections were rehydrated to PBS and heated in citric acid buffer for antigen retrieval. Sections were then stained with FITC-labeled wheat germ agglutinin (WGA) (Sigma, L4895) in PBS (1:200 dilution) to distinguish the cell membrane of cardiomyocytes. The size of cardiomyocytes was measured as areas outlined by WGA staining. The surface area of at least 250
transverse sections of cardiomyocytes were measured per heart section and averaged for each animal (n = 5 WT, n = 6 TG).

**Quantification of mitochondrial size**
Following asphyxiation by CO$_2$ administration, hearts were immediately dissected into fixative (2% glutaraldehyde, 4% paraformaldehyde, 1% osmium tetroxide). The left ventricular tissue was cut into sections no larger than 1mm thick and fixed overnight at 4 degrees. The tissue was post-fixed in 1% osmium tetroxide and dehydrated to 100% ethanol. The tissue was then washed in propylene oxide before being embedded in 100% propylene oxide and baked to cure resin. Sections were then cut to 0.1 μm and stained with uranyl acetate. Sections were imaged using Philips CM120 STEM. Cardiomyocytes containing correct orientation of z lines were imaged for mitochondria. Surface area of mitochondria were measured using ImageJ software (n = 4 WT, n = 4 TG; 8655 WT mitochondria and 8950 TG mitochondria examined).

**Gene expression analysis**
We conducted RNA sequencing analysis to compare gene expression profiles between 6-month-old Tmem135 TG and WT mouse hearts (n = 3 WT, n = 3 TG). The left ventricular tissue was dissected and snap frozen in liquid nitrogen. The heart tissue was first homogenized using the Qiagen Tissuelyser II system with stainless steel beads (Qiagen USA, Germantown, MD). RNA was extracted in TRIzol (Thermo Fisher Scientific, Waltham, MA) and chloroform (Thermo Fisher Scientific) was used to separate the aqueous layer. Samples were purified using the Qiagen RNeasy Mini Kit protocol according to manufacturer’s instructions (Qiagen). Stranded mRNA was sequenced using the sequencing platform HiSeq2000 (Illumina, San Diego, CA) at University of Wisconsin-Madison Biotechnology Center. Raw, 100 bp reads were examined for quality using FastQC v0.10.1 [15]. Reads were trimmed with fastx-toolkit v 0.0.14, aligned with tophat v2.0.9 [16,17] and converted to bam files using samtools v0.1.19 [18]. Using the Cuffdiff [17] software (v2.1.1), we detected differentially expressed (DE) genes (q-value < 0.05) in the Tmem135 TG heart compared to WT controls. Gene expression data are available at the Gene Expression Omnibus (GEO), accession number GSE99522. Using the Database for Annotation, Visualization, and Integrated Discovery Functional Annotation Tool (DAVID) for gene ontology (GO) term analysis [19,20], we categorized differentially expressed (DE) genes in the Tmem135 TG heart. We obtained gene expression data of the heart of aged (25–28 month old) C57BL/6J mice (GSE12480), and a diet-induced obese C57BL/6J mice (17 week old) (GSE47022) [21] from a public database [22]. Then, we determined DE genes in the heart of each mouse model compared with control mice (young [4–6 month old] C57BL/6J mice and non-obese age-matched C57BL/6J mice on control diet, respectively). Overlap between DE gene sets was determined using GeneOverlap simulation in R and significance was determined using fisher’s exact test.

**Western blot analysis**
Following asphyxiation by CO$_2$, the heart was collected and stored at -80°C. The heart was homogenized with RIPA buffer (Thermo Fisher) containing protease/phosphatase inhibitor cocktail (Thermo Fisher) on ice. Proteins were separated by SDS-PAGE gel electrophoresis and transferred to a PVDF or PVDF-FL membrane. To detect each protein, the membrane was incubated with a primary antibody for ATF4 (Cell Signaling Technology [CST]), ATF6 (Novus Biologicals, Littleton, CO), IRE1alpha (CST), PERK (CST), Phospho-PERK (Thr981) (Elabscience Biotechnology), GRP78/Bip (CST), Pdi (CST), CHOP (CST), phospho-eIF2alpha (CST), eIF2alpha (CST), beta-actin (Hybridoma Bank, Iowa City, IA), and then with a
horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000 dilution) or LI-COR secondary antibody (LI-COR, Lincoln, NE) for fluorescent detection (1:10000). LI-COR secondary antibody was detected using the LI-COR Odyssey System. HRP-conjugated secondary antibodies were detected using enhanced chemiluminescent substrates for HRP (Thermo-Fisher Scientific, USA), followed by exposure of the X-ray film. Films were imaged with Gel Doc XR System (Bio-Rad Laboratories Inc., Hercules, CA). The optical density of each band was measured by ImageJ.

**Optical cryo-imaging**

Hearts were collected for the 3D cryo-imaging protocol (n = 7 WT, n = 7 TG). The snap-frozen hearts, stored at −80°C until the day of study, were imaged in our custom-made cryo-imager at the Biophotonics Lab, University of Wisconsin-Milwaukee. We have reported extensively on the cryo-imaging system in previous studies [23–25]. In brief, a microtome blade sequentially slices the embedded tissue. For each slice, the autofluorescence images from reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) were captured to measure the redox state of the mitochondrial metabolism in the tissue. Major NADH and FAD signals captured originate from mitochondria with negligible contributions from cytoplasmic sources [26,27]. The 3D rendered images using z-stacks of all the image slices for both NADH and FAD signals, and the NADH to FAD redox ratio of each heart was calculated voxel by voxel as previously explained [23–25]. The intensity histogram distribution of redox ratio (NADH/FAD) for each heart was determined, and the mean value (RR) calculated as a quantitative marker for the oxidative state of the tissue.

**Amino acids analysis**

The heart tissue (20 mg) was mixed with 150μL of 10% (w/w) trichloroacetic acid, and centrifuged immediately (4°C, 30 min, 10000 g) to remove precipitated protein. All samples were kept on ice to minimize chemical reactions of thiol metabolites. The amino acid concentrations were measured by an automatic amino acid analyzer (L-8800; Hitachi, Tokyo, Japan). Briefly, amino acids, separated by cation exchange chromatography, were detected spectro-photometrically after post-column reaction with the ninhydrin reagent.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Significance of the difference between two groups was calculated by unpaired Student’s two-tailed t-test. Variance in the heart weight to body weight ratio was compared by F-test. Comparison of survival curves was performed by Mantel-Cox log-rank test as well as Gehan-Breslow-Wilcoxon test. The size distribution of mitochondria was compared by the Kolmogorov-Smirnov test.

**Results**

In order to assess the effect of Tmem135 overexpression on the heart, we compared the heart from adult transgenic mice over-expressing wild-type Tmem135 (Tmem135 TG) under the control of the β-actin promoter [11] to that of age-matched, littermate non-transgenic control (WT) mice. Comparison of the mean heart weight to body weight ratio (n = 7 WT, n = 9 TG) at 14 months of age indicated 27% increase in the heart size relative to the body size in Tmem135 TG adult mice compared to WT mice although the difference did not reach statistical significance by unpaired t-test with Welch’s correction (p = 0.085) (Fig 1A). We observed
Fig 1. *Tmem135* TG mice display disease phenotypes in the heart. A) Heart to body weight ratios of 14 month old mice show a trend of increase in *Tmem135* TG mice indicative of mild cardiac hypertrophy (n = 7 WT, n = 9 TG; p = 0.085 by t-test). Representative Masson's trichrome-stained hearts at 14 months of age are shown. B) Masson's trichrome-stained 6-month-old hearts show collagen in blue (left). The percentage of blue-stained area (% Blue) is significantly increased in *Tmem135* TG hearts compared to that in WT hearts (n = 4 WT, n = 4 TG; p < 0.05 by t-test) (center). Immunohistochemistry of 6-month-old heart tissue indicates increase in the collagen III protein (right). C) WGA staining (green, far left) of transverse sections was used to quantify cardiomyocyte size (outlined by WGA staining) and extra-cellular space. While the cardiomyocyte size was comparable between *Tmem135* TG and WT mice (n = 6 WT, n = 6 TG, >200 cells per sample examined, p = 0.0642 by t-test), extracellular space is significantly increased in *Tmem135* TG hearts (n = 6 WT, n = 6 TG; p < 0.05 by t-test). D) Survival curves show that *Tmem135* TG mice die earlier compared to WT littermates (n = 11 WT, n = 21 TG, P < 0.0001 by Log-rank test and Gehan-Breslow-Wilcoxon test).
significantly greater variance in the heart weight to body weight ratio among the *Tmem135* TG group compared to the WT group (*p*<0.01 by F-test), possibly reflecting the variability in the genetic background of mice which is the mixture of FVB/NJ and C57BL/6J. Gross histological examination did not show marked morphological changes in the *Tmem135* TG heart (Fig 1A). However, closer examination revealed pathological changes. We examined the amount of collagen accumulation by measuring the percentage of blue-stained area in the Masson’s tri-chrome-stained heart sections. We observed that the *Tmem135* TG heart had significantly increased collagen compared to the WT heart at 6 months of age (Fig 1B; *n* = 4 WT, *n* = 4 TG, *p*<0.05 by t-test) indicating increased fibrosis, which is often associated with cardiac hypertrophy [28–30]. We also conducted immunohistochemistry using a collagen III antibody, which is a marker for extracellular collagen and found that it was also increased in the *Tmem135* TG heart (Fig 1B). Since cardiac hypertrophy could be due to cardiomyocyte hypertrophy, we examined the size of cardiomyocytes outlined by WGA staining in transverse sections of the heart at 14 months of age (Fig 1C). The analysis did not indicate a change in the size of cardiomyocytes in the *Tmem135* TG heart compared to the WT heart (Fig 1C; *n* = 6 WT, *n* = 6 TG, *p* = 0.0642 by t-test). These results suggest that mild cardiac hypertrophy in *Tmem135* TG mice is mainly due to increased fibrosis/collagen accumulation. We, then, conducted electron microscopy to assess changes in the ultrastructure of individual cardiomyocytes. Electron micrographs of the left ventricle muscle tissue showed large vacuoles co-occupying the space between myofibrils with mitochondria at varying severity (Fig 2A). We also observed that

**Fig 2. Cardiomyocyte and mitochondrial morphology are affected in *Tmem135* TG hearts.** A) Electron micrographs of WT and *Tmem135* TG heart tissues at 7 months of age show varying severity of vacuolization in the cardiomyocytes of *Tmem135* TG mice (7100X). B) Electron micrographs indicate decreased mitochondrial size in *Tmem135* TG cardiomyocytes (7100X). C) Quantification of the cardiomyocyte size (*n* = 4 WT [8655 mitochondria examined], *n* = 4 TG [8950 mitochondria examined]) shows significant decrease in the average size (inset, *p*<0.01 by t-test), and size distribution of mitochondria in *Tmem135* TG hearts (*p*<0.0001 by Kolmogorov-Smirnov test).

https://doi.org/10.1371/journal.pone.0201986.g002
when present, the vacuoles often disrupted the contacts between neighboring mitochondria and increased the space between myofibrils (Fig 2A, intermediate and severe). Variability in the severity of this phenotype may be also due to the mixed genetic background of these mice. We examined the survival of Tmem135 TG mice and found that they died earlier than WT controls (Log-rank test, \( P < 0.0001 \); Gehan-Breslow-Wilcoxon test, \( P < 0.0001 \)) beginning around 4 months, and the majority of Tmem135 TG mice succumb before 1.5 years (Fig 1D). The heart abnormalities may contribute to the earlier deaths observed in Tmem135 TG mice.

A previous study showed that TMEM135 is a novel regulator of mitochondrial dynamics [11]. Specifically, in vitro overexpression of Tmem135 resulted in a mitochondrial dynamic shift towards increased fission, which resulted in smaller mitochondria in Tmem135 TG fibroblast cells [11]. Therefore, we tested whether mitochondrial morphology is affected in the myocardium of Tmem135 TG mice (Fig 2B). We measured the surface area of mitochondria in electron micrographs of cardiomyocytes in 7-month-old Tmem135 TG (\( n = 4, 8950 \) mitochondria examined) and WT (\( n = 4, 8655 \) mitochondria examined) hearts (Fig 2C). The average mitochondria size was decreased in the Tmem135 TG heart compared to the WT heart (Fig 2C, inset, \( p < 0.01 \) by t-test). Additionally, the size distribution of the mitochondria was shifted towards smaller sizes in the Tmem135 heart (Fig 2C, \( p < 0.0001 \) by Kolmogorov-Smirnov test). These data suggest that mitochondrial dynamics are shifted towards increased fission in the Tmem135 TG cardiomyocytes in vivo.

To identify molecular pathways affected in the Tmem135 TG heart, we conducted RNA sequencing (RNAseq) of 6-month-old Tmem135 TG and WT hearts to compare gene expression profiles. We detected 1,144 differentially expressed (DE) genes between TG and WT, where 505 genes were decreased, and 639 genes were increased in the Tmem135 TG heart compared with WT controls (Fig 3). Using the Database for Annotation, Visualization, and Integrated Discovery Functional Annotation Tool (DAVID) for gene ontology (GO) term analysis, we categorized DE genes in the Tmem135 TG heart (Table 1). We observed over-representation of the mitochondria-associated GO terms in the down-regulated gene set. Down-regulation of mitochondria-associated genes is consistent with mitochondrial dysfunction [11] along with fragmentation observed in vitro in Tmem135 TG fibroblasts [11] as well as
in vivo in the Tmem135 TG heart (Fig 2B and 2C). GO terms associated with the endoplasmic reticulum (ER) and stress response as well as those associated with the extracellular matrix are enriched in the up-regulated genes for Tmem135 TG hearts (Table 2).

The GO term analysis suggested that the ER stress may play a role in the development of Tmem135 TG heart phenotypes. Genes in all three branches of the unfolded protein response (UPR) activated by ER stress [31] were increased in Tmem135 TG hearts (Fig 4A). We conducted western blot analysis to test the protein expression of key ER stress markers including protein kinase RNA-like endoplasmic reticulum kinase (PERK) [32], phosphorylated PERK, inositol-requiring protein-1 (IRE1α) [33], 78-kilodalton Glucose Regulated Protein (GRP78)/binding immunoglobulin protein (Bip) [34], protein disulfide isomerase (Pdi) [35,36], eukaryotic translation initiation factor (eIF2α) [37], and phosphorylated eIF2α as well as key UPR transcription factors activating transcription factor (ATF)6 [38], and ATF4 [39]. We performed this analysis at 2 months of age, in order to also test whether increase in the ER stress

Table 1. Top 10 GO terms enriched in the down-regulated gene list of Tmem135 TG mice.

| GO root category | rank | GO term                                      | Genes (n) | EASE score |
|------------------|------|----------------------------------------------|-----------|------------|
| Biological Process | 1    | generation of precursor metabolites and energy | 68        | 3.36E-48   |
|                   | 2    | electron transport chain                      | 45        | 7.62E-41   |
|                   | 3    | oxidation reduction                           | 83        | 2.45E-33   |
|                   | 4    | cellular respiration                           | 18        | 7.83E-14   |
|                   | 5    | energy derivation by oxidation of organic compounds | 21    | 6.21E-13   |
|                   | 6    | establishment of localization                  | 112       | 5.08E-11   |
|                   | 7    | transport                                      | 111       | 7.46E-11   |
|                   | 8    | oxidative phosphorylation                      | 15        | 1.06E-10   |
|                   | 9    | coenzyme metabolic process                     | 22        | 1.20E-10   |
|                   | 10   | metabolic process                              | 242       | 2.91E-10   |

Cellular Component

| rank | GO term                                      | Genes (n) | EASE score |
|------|----------------------------------------------|-----------|------------|
| 1    | mitochondrion                                | 189       | 3.30E-93   |
| 2    | mitochondrial part                           | 119       | 3.00E-78   |
| 3    | organelle inner membrane                     | 90        | 6.87E-68   |
| 4    | mitochondrial inner membrane                 | 88        | 1.46E-67   |
| 5    | mitochondrial membrane                        | 92        | 2.26E-63   |
| 6    | mitochondrial envelope                        | 93        | 5.31E-62   |
| 7    | organelle envelope                           | 98        | 5.73E-54   |
| 8    | envelope                                     | 98        | 8.15E-54   |
| 9    | cytoplasmic part                             | 248       | 2.50E-46   |
| 10   | respiratory chain                            | 38        | 7.57E-42   |

Molecular Function

| rank | GO term                                      | Genes (n) | EASE score |
|------|----------------------------------------------|-----------|------------|
| 1    | oxidoreductase activity                       | 72        | 4.71E-23   |
| 2    | catalytic activity                            | 203       | 2.50E-13   |
| 3    | monovalent inorganic cation transmembrane transporter activity | 20 | 5.94E-13 |
| 4    | hydrogen ion transmembrane transporter activity | 19 | 2.22E-12 |
| 5    | inorganic cation transmembrane activity       | 21        | 8.39E-11   |
| 6    | coenzyme binding                             | 23        | 1.45E-10   |
| 7    | NADH dehydrogenase (ubiquinone) activity      | 11        | 1.64E-10   |
| 8    | NADH dehydrogenase activity                   | 11        | 1.64E-10   |
| 9    | NADH dehydrogenase (quinone) activity         | 11        | 1.64E-10   |
| 10   | cofactor binding                              | 27        | 1.74E-10   |

Down-regulated GO terms ranked by biological process, cellular component, and molecular function. Bold terms are those associated with mitochondrial energy production.

https://doi.org/10.1371/journal.pone.0201986.t001
response is observed at an earlier timepoint. We found that a majority of these ER stress markers are increased in the Tmem135 TG heart compared to the WT heart at 2 months of age (Fig 4B and 4C, S1 Fig). These results indicate that the UPR is activated by increased ER stress in the Tmem135 TG mouse heart at an early stage of phenotype progression, further suggesting its involvement in the development of the heart phenotypes. In addition, we examined whether expression of target genes downstream of primary UPR is affected in the Tmem135 TG heart using RNAseq data. The analysis identified known targets of UPR transcription factors in the DE gene set, most of which are upregulated in the Tmem135 TG heart (Fig 4A and 4D). A majority of these target genes (88 genes) are direct targets of ATF4 (Fig 4D), suggesting that ATF4 is the dominant UPR transcription factor activated by ER stress in the Tmem135 TG heart.

Oxidative stress is considered as one of the factors that could trigger ER stress [40]. Our previous study showed increased reactive oxygen species (ROS) in cultured Tmem135 TG fibroblasts [11], which may be also increased in the Tmem135 TG heart. Using optical cryo-

| GO root category         | rank | GO term                      | Genes (n) | EASE score |
|-------------------------|------|------------------------------|-----------|------------|
| Biological Process      | 1    | cellular adhesion            | 51        | 8.49E-10   |
|                         | 2    | biological adhesion          | 51        | 9.09E-10   |
|                         | 3    | oxoacid metabolic process    | 44        | 1.14E-08   |
|                         | 4    | carboxylic acid metabolic process | 44      | 1.14E-08   |
|                         | 5    | organic acid metabolic process | 44       | 1.20E-08   |
|                         | 6    | inflammatory response        | 28        | 1.95E-08   |
|                         | 7    | response to stress           | 79        | 2.24E-08   |
|                         | 8    | cellular ketone metabolic process | 44     | 2.35E-08   |
|                         | 9    | response to unfolded protein | 14        | 2.76E-08   |
|                         | 10   | response to wounding         | 35        | 5.05E-08   |
| Cellular Component      | 1    | extracellular region         | 150       | 3.71E-29   |
|                         | 2    | extracellular region part    | 91        | 3.23E-25   |
|                         | 3    | extracellular matrix         | 57        | 3.60E-25   |
|                         | 4    | proteinaceous extracellular matrix | 54      | 1.54E-23   |
|                         | 5    | extracellular matrix part    | 24        | 4.03E-14   |
|                         | 6    | endoplasmic reticulum        | 74        | 1.44E-13   |
|                         | 7    | endoplasmic reticulum part   | 29        | 6.66E-09   |
|                         | 8    | cytoplasmic part             | 200       | 6.89E-08   |
|                         | 9    | collagen                     | 9         | 9.88E-08   |
|                         | 10   | basement membrane            | 15        | 1.52E-07   |
| Molecular Function      | 1    | glycosaminoglycan binding    | 24        | 2.78E-12   |
|                         | 2    | pattern binding              | 25        | 4.90E-12   |
|                         | 3    | polysaccharide binding       | 25        | 4.90E-12   |
|                         | 4    | heparin binding              | 20        | 2.15E-11   |
|                         | 5    | carbohydrate binding         | 37        | 1.22E-10   |
|                         | 6    | protein binding              | 259       | 5.40E-09   |
|                         | 7    | growth factor binding        | 16        | 1.13E-08   |
|                         | 8    | binding                      | 430       | 1.84E-07   |
|                         | 9    | extracellular matrix structural constituent | 10      | 3.60E-07   |
|                         | 10   | platelet-derived growth factor binding | 6    | 4.53E-06   |

Upregulated GO terms ranked by biological process, cellular component, and molecular function

https://doi.org/10.1371/journal.pone.0201986.t002
imaging [23–25], we examined the redox state of the Tmem135 TG heart (Fig 5). This method detects changes in the oxidation state of mitochondrial metabolic coenzymes NADH (NAD in the reduced form) and FAD (FADH\textsubscript{2} in its oxidized form), and provides a quantitative marker for oxidative stress (the ratio of NADH/FAD; the redox ratio) in tissues [23–25]. The 3D cryo-imaging revealed lower NADH and higher FAD fluorescence signals in Tmem135 TG hearts compared with WT hearts (Fig 5A) resulting in lower redox ratio (Fig 5B and 5C) (n = 7 WT, n = 7 TG, p < 0.001 by t-test). These results indicate higher levels of oxidative stress in Tmem135 TG hearts compared with WT hearts, and suggest the possibility that increased oxidative stress may trigger ER stress in the Tmem135 TG heart.
The GO term analysis of the RNAseq data also showed a significant enrichment of genes associated with the extracellular matrix (Table 2) in the \textit{Tmem135} TG heart, which is consistent with the histological data showing increased collagen (Fig 1C). In order to examine the collagen synthesis pathway, we conducted qPCR analysis on genes associated with the procollagen pathway. The RNA sequencing results showed that mRNA for enzymes involved in the procollagen production (arginosuccinate lyase [ASL], pyrroline-5-carboxylate reductase 1 [PYCR1], and prolyl 4-hydroxylase [P4H]) were up-regulated, while an enzyme that suppresses collagen production, proline dehydrogenase (PRODH), was down-regulated in the \textit{Tmem135} TG heart. Furthermore, some of the secondary metabolites within the urea cycle pathway and the procollagen production pathway, ornithine (Orn) and proline (Pro), were increased in the \textit{Tmem135} TG heart (Fig 6A). These data indicate that the molecular pathway leading to collagen accumulation is activated in the \textit{Tmem135} TG heart (Fig 6B).

Pathologies in the \textit{Tmem135} TG heart including hypertrophic cardiomyopathy and collagen accumulation (Fig 1A and 1B) indicative of fibrosis are also observed in the aging heart \cite{41}. Moreover, TMEM135 was found to be involved in the regulation of the aging process in the retina \cite{11}. Therefore, we compared the gene expression profile of the \textit{Tmem135} TG heart with publicly available datasets for the aged heart. We obtained gene expression data of the...
Heart abnormalities caused by Tmem135 overexpression

Fig 6. Collagen synthesis pathways are affected in Tmem135 TG mice. A) Quantification of small molecules in the heart lysate of 2-month-old WT and Tmem135 TG mice shows significant increase of molecules in the collagen synthesis pathway (Orn and Pro) in the Tmem135 TG heart (n = 4 WT, n = 4 TG; \(*) p<0.05, **** p<0.0001 \) by t-test). Orn: ornithine, Pro: proline, Cit: citrulline, Hypro: hydroxyproline. B) Schematic of the collagen synthesis pathway leading to fibrogenesis depicting significantly affected molecules and enzymes in Tmem135 TG hearts. (green = increased, red = decreased, black = unchanged).

https://doi.org/10.1371/journal.pone.0201986.g006

heart of 25–28 month old C57BL/6J WT mouse heart (GSE12480) and those of a diet-induced obesity model (GSE47022) [21] as well as those from the respective control mice (young [4–6 month old] C57BL/6J WT mice and non-obese mice on control diet, respectively) from public database. We determined the DE gene sets of each pathology model compared to controls, and then determined the similarity of the DE genes between the Tmem135 TG heart and the other 2 models using GeneOverlap in R programming language. Through this analysis, we found that DE genes in the 6-month old Tmem135 TG mouse heart significantly overlapped with DE genes in aged (25–28 months old) WT mouse heart. Among up-regulated DE genes, 301 genes were found to overlap between the Tmem135 TG heart and aged heart while 86 genes overlap between the obese model and the aged heart and 28 genes overlap between the Tmem135 TG heart and the obese model (Fig 7A). Similarly, among down-regulated DE genes in each condition, 114 overlapped genes were found between the Tmem135 TG and aged heart while 35 genes overlapped between the obese model and the aged heart and 32 genes overlapped between the Tmem135 TG heart and the obese model (Fig 7B). Using GeneOverlap simulation
in the R package, we confirmed significant overlap between DE gene sets of hearts from Tmem135 TG mice, aged (23–25 month-old) mice, and a diet-induced obesity mouse model. 

**Fig 7.** The profile of DE genes in Tmem135 TG hearts is similar to that of aged hearts. Comparison of DE gene sets of hearts from Tmem135 TG mice, aged (23–25 month-old) mice, and a diet-induced obesity mouse model. GeneOverlap simulation in R confirmed significant overlap in the up-regulated (A) and down-regulated (B) DE gene sets of Tmem135 TG and aged mouse hearts. p = 7.6e-167 and p = 1.3e-65, respectively, by Fisher's Test.

https://doi.org/10.1371/journal.pone.0201986.g007

in the R package, we confirmed significant overlap between DE gene sets of Tmem135 TG heart and the aged heart (p = 7.6e-167 and p = 1.3e-65 for upregulated and downregulated genes, respectively). In addition, we performed function-based comparison of DE genes in the Tmem135 TG and aged heart using over-represented gene ontology (GO) terms. Using the DAVID analysis, we found that 65% and 29% of GO terms enriched in up-regulated and down-regulated DE (respectively) in the Tmem135 TG heart overlap with those enriched in the aged heart. This further suggested functional correlation between the Tmem135 TG mouse heart and aged heart. Thus, the Tmem135 TG mouse heart shares common features with the aged heart not only in pathological phenotypes but also in the gene expression profiles.

**Discussion**

In this study, we investigated the effect of overexpression of the Tmem135 gene, which encodes a novel regulator of mitochondrial dynamics [11], in the heart. Overexpression of Tmem135 affects the size of mitochondria in cardiomyocytes in vivo and leads to pathological phenotypes in the heart including collagen accumulation and hypertrophy. Furthermore, the gene expression analysis showed that genes associated with the ER stress pathway are upregulated in the Tmem135 TG heart. It also showed that gene expression changes in the heart of Tmem135 TG mice significantly overlap with those of aged mice, suggesting that mitochondrial dynamics may be involved in the normal aging process of the heart. Thus, our study revealed the pathological consequence of dysregulated mitochondrial dynamics due to overexpression of Tmem135, and suggested downstream molecular changes that may underlie those disease pathologies.

**TMEM135 as a mitochondrial fission factor in the heart**

TMEM135 was recently identified as a novel factor that regulates mitochondrial dynamics [11]. Overexpression of Tmem135 induces increased fragmentation of mitochondria in fibroblast cells (in vitro) [11] and in the retinal tissue (in vivo) (Ikeda A: unpublished data). Our
study in the heart showed that the mitochondrial size in cardiomyocytes of Tmem135 TG mice is decreased compared to WT mice (Fig 2B and 2C), indicating that TMEM135 has the same function in cardiomyocytes to induce mitochondrial fission. Thus, Tmem135 TG mice provide a new model to study the role of mitochondrial dynamics in the heart. The consequence of dysregulated mitochondrial dynamics in the heart has been investigated by targeting genes involved in mitochondrial dynamics in mice. Using Drp1 and Mfn1/Mfn2 conditional KO mice, Song et al. showed that mitochondrial dynamics defects lead to heart disease phenotypes [10]. When Drp1 is ablated in the adult mouse heart causing hyper-fused mitochondria, the mice show dilated cardiomyopathy, while ablation of both Mfn1 and Mfn2 in the adult mouse heart causes a decrease in mitochondrial fusion leading to hypertrophy of the heart [10]. These results suggest the importance of balanced mitochondrial dynamics in maintaining normal structures and functions of the heart tissue. In another study, imbalanced processing of mitochondrial dynamin-like GTPase OPA1 causes fragmentation of mitochondria and leads to dilated cardiomyopathy [42]. Although cardiac Mfn1/Mfn2 deficiency, induced processing of OPA1, and Tmem135 overexpression all result in fragmentation of mitochondria in cardiomyocytes, the phenotypes of the heart in these models are distinct. Mfn1/Mfn2 deficient mice [10] and Tmem135 TG mice (Fig 1A) show cardiac hypertrophy while mice with induced processing of OPA1 develop dilated cardiomyopathy [42]. Collagen accumulation indicative of cardiac fibrosis is observed in Tmem135 TG mice (Fig 1B) and mice with induced OPA1 processing [42], but not in Mfn1/Mfn2 deficient mice [10]. Most recently, it was also reported that mitochondrial fragmentation due to cardiac overexpression of Drp1 did not result in any cardiac pathologies [43]. These differences could be possibly due to multiple molecular pathways affecting mitochondrial fusion and fission in the heart, and thus producing different pathological consequences depending on which pathway is affected to cause fragmentation of mitochondria. Additionally, the genetic background of mice could be another factor that potentially affects phenotypic differences considering that these mouse models were on different genetic backgrounds. Mfn1/Mfn2 deficient mice [10] were on a C57BL/6J background, while mice with induced OPA1 processing [42] had a mixture of C57BL/6 and FVB/N backgrounds. Mice with cardiac overexpression of Drp1 [43] were generated by crossing Drp1 transgenic mice on a FVB/N background with myh6 promoter-driven doxycycline-suppressible line whose genetic background was not described [44]. Tmem135 TG mice used in the current study were on a mixed background of FVB/N and C57BL/6. We observed variability in the severity of phenotypes among these mice (Figs 1A and 2A), further suggesting the contribution of genetic factors on the severity of cardiac abnormalities caused by defective mitochondrial dynamics. Investigation into the downstream molecular changes as well as genetic factors that affect phenotype manifestations in these mouse models could further reveal how mitochondrial dynamics affect the structure and function of the heart.

**ER stress is upregulated in the Tmem135 TG heart**

The GO Term analysis of RNAseq data indicated that ER stress-related genes constitute a large proportion of upregulated genes in the Tmem135 TG heart. In addition, we observed upregulation of ER stress associated factors at the protein level (Fig 4B and 4C). These results indicate that ER stress is upregulated in the Tmem135 TG heart. The relation between mitochondrial dynamics and ER stress has been reported in different tissues. Disruption of Mfn2 has been shown to increase ER stress in fibroblasts and cultured cardiomyocytes [45] while inhibition of DRP1 alleviates stress in cells [46]. Filippi et. al also showed that increased mitochondrial fission by constitutively activating DRP1 is sufficient to induce ER stress in the brain [47]. These findings suggest that an imbalance toward mitochondrial fission induces ER stress. In case of
the Tmem135 TG heart, it may be also true that mitochondrial fragmentation induced by overexpression of Tmem135 initiates the ER stress response. It is possible that increased oxidative stress due to dysfunctional mitochondria mediate this process. In addition to our earlier observation of dysfunctional mitochondria and increased ROS in cultured Tmem135 TG fibroblasts [11], our optical cryo-imaging indicated significantly increased oxidative stress in Tmem135 TG hearts (Fig 5). Since there is accumulating evidence that ER stress signaling is elicited in response to oxidative triggers [40], our results suggest increased oxidative stress due to mitochondrial abnormalities may trigger ER stress in the Tmem135 heart. On the other hand, a number of studies indicated communication between ER and mitochondria [48]. ER stress has been shown to increase mitochondrial ROS generation [40,49,50], and can cause functional defects in mitochondria as observed in diseases such as neurodegeneration [51] and multiple sclerosis [52]. Therefore, it is possible that mitochondrial defects and ER stress enhance each other to make the phenotypes more severe in this fashion. The Tmem135 TG mouse model can be used to investigate the molecular pathways affected by increased mitochondrial fission and ER stress, and how they may interact with each other to cause heart pathologies in vivo.

The ATF4 pathway is specifically activated in the Tmem135 TG heart

In Tmem135 TG mice, the expression of genes and proteins involved in three major UPR pathways are up-regulated. Among those pathways, the ATF4 pathway especially shows major changes. First, we observed very little expression of the ATF4 protein and its activator, phosphorylated eIF2α [53] in the WT heart, while their expression is largely upregulated in the Tmem135 TG heart (Fig 4B and 4C). Secondly, known ATF4 targets also show the most significant changes among targets of the UPR pathways (Fig 4D). Thus, in the Tmem135 TG model, ATF4 may play a major role in mediating ER stress in the heart. Recent studies have linked mitochondrial abnormalities to induction of ER stress [48] and activation of ATF4 [54,55]. In addition to ER stress, ECM-related genes are highly represented in the DE gene set of the Tmem135 TG heart. This is consistent with the histological data showing increased collagen in the Tmem135 TG heart (Fig 1B), indicative of ECM remodeling. Upon further investigation of the procollagen pathway, we observed increases in metabolites and expression of enzymes that facilitate the production of procollagen (Fig 6). ER stress and activation of the UPR have been suggested as a pro-fibrotic stimulus in the heart as well as other internal organs [56]. Increased ATF4 activity in the Tmem135 TG heart may possibly be the cause of fibrosis in the Tmem135 TG heart, since pyrroline-5-carboxylate reductase 1 (PYCR1), which is upregulated in the Tmem135 TG heart, is a key enzyme for synthesis of collagen, and its expression has been shown to depend on ATF4 [57]. Further investigation using conditional knockout mice for Atf4 combined with Tmem135 TG mice may reveal the role of ATF4 in pathogenesis in the Tmem135 TG heart.

The Tmem135 TG heart shows similar gene expression profiles and pathologies to aging hearts

Alterations in the balance of mitochondrial dynamics have been linked to aging and age-dependent disease phenotypes. For instance, in mouse skeletal muscle, elongation of the mitochondria has been linked to aging-associated phenotypes [58]. In the kidney of diabetic mice, mitochondrial fragmentation precedes the development of histological damages observed in kidneys [59]. Both cancer and neurodegenerative disease are linked to dysregulated mitochondrial dynamics [60,61]. In this study, we found that the Tmem135 TG heart displays some of the aging and age-related disease phenotypes in the heart including hypertrophy and collagen accumulation indicative of fibrosis (Fig 1A and 1B). In addition, we compared the gene
expression profile of the Tmem135 TG heart to that of the aged mouse heart, which showed a significant overlap of genes between Tmem135 TG and aged hearts (Fig 7). Consistent with the phenotypic overlap in collagen accumulation/fibrosis, ECM-related genes were found in the overlap. Mitochondria-related genes were also prominent within the overlap, suggesting that changes that occur in mitochondrial functions during the process of aging may be involved in the development of aging-associated heart pathologies. Thus, Tmem135 TG may provide a mouse model in which certain aspects of heart aging is accelerated.

Supporting information

S1 Fig. Phosphorylated PERK in Tmem135 TG hearts. A) Western blot analysis for phosphorylated PERK in WT and Tmem135 TG hearts at 2 months of age. B) Quantification of phosphorylated PERK shows a trend of increase in Tmem135 TG hearts compared to WT hearts although it did not reach statistical significance (n = 5 TG, n = 5 WT, p = 0.056 by t-test).

(TIF)

Acknowledgments

The authors thank Satoshi Kinoshita for generation of frozen sections and the University of Wisconsin Medical School Electron Microscopy Facility and Ben August for technical support and use of the facility equipment. This work was supported by a grant from the National Institutes of Health (NIH R01 EY022086), a research agreement with Ajinomoto Co., Inc., a professorship from the Retina Research Foundation (Walter H. Helmerich Research Chair) and Timothy William Trout Professorship in Eye Research to AI, Core Grant for Vision Research (P30 EY016665), the National Science Foundation Graduate Research Fellowship under Grant No. 403–4030513 to SL, and UWM RGI 101x290 to MR. The work in NS laboratory is supported by an unrestricted award from Research to Prevent Blindness to the Department of Ophthalmology and Visual Sciences, Retina Research Foundation, EY026078, EPA 83573701, and Stein RPB Innovation Award.

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