Inhibition of cardiac PERK signaling promotes peripartum cardiac dysfunction

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Peripartum cardiomyopathy (PPCM) is a life-threatening heart failure occurring in the peripartum period. Although mal-angiogenesis, induced by the 16-kDa N-terminal prolactin fragment (16 K PRL), is involved in the pathogenesis, the effect of full-length prolactin (23 K PRL) is poorly understood. We transfected neonate rat cardiomyocytes with plasmids containing 23 K PRL or 16 K PRL in vitro and found that 23 K PRL, but not 16 K PRL, upregulated protein kinase RNA-like endoplasmic reticulum kinase (PERK) signaling, and hypoxia promoted this effect. During the perinatal period, cardiomyocyte-specific PERK homogenous knockout (CM-KO) mice showed PPCM phenotypes after consecutive deliveries. Downregulation of PERK or JAK/STAT signaling and upregulation of apoptosis were observed in CM-KO mouse hearts. Moreover, in bromocriptine-treated CM-KO mice, cardiac function did not improve and cardiomyocyte apoptosis was not suppressed during the peripartum period. These results demonstrate that interaction between 23 K PRL and PERK signaling is cardioprotective during the peripartum term.
Results

PERK signaling is upregulated by 23 k PRL, but not 16 k PRL, in neonatal rat cardiomyocytes (NRCMs). To investigate the potential roles of the 23 K PRL-PERK axis in PPCM, we treated NRCMs with recombinant 23 K PRL (50 nM) under normoxia or hypoxia (4% O₂, 24 h) (Fig. 1A). The phosphorylation of PERK occurred to a greater extent under hypoxia, but not under normoxia. The cleavage of 23 K PRL into 16 K PRL was also induced under hypoxia (Fig. 1A–C). These effects were reversed by treatment with a PERK inhibitor (PERKi, 1 μM). The expression of cleaved caspase 3, which is related to apoptosis, was increased in hypoxic NRCMs with 23 k PRL treatment compared to normoxic cells with or without the treatment. PERKi treatment did not affect the expression of 23 k PRL in hypoxic NRCMs.

Next, to discriminate the effect of 23 K PRL from that of 16 K PRL, we transfected NRCMs with plasmids containing LacZ, 23 k PRL, or 16 k PRL. The expression of Atf4, one of PERK target genes, was increased...
involved in the regulation of cardiac angiogenic and hypertrophic responses. Between the transcriptomic analysis and pathway analysis, we depicted representative genes on heatmaps (CTRL NP, CTRL PP, CM-KO NP, and CM-KO PP).

Genes involved in pathways of translation and oxidative stress were enhanced during the postpartum term. RNA-Seq was performed to investigate changes in the transcriptome during the postpartum period in CTRL and CM-KO mice. Each analysis was performed with whole myocardial tissues obtained at the study endpoint (after four consecutive deliveries). All RNA-Seq results are provided in Table S2. A total of 8865 genes exhibited more than fivefold increase in expression in at least one of the replicates in all groups (CTRL NP, CTRL PP, CM-KO NP, and CM-KO PP).

First, we compared gene expression between CTRL NP and CTRL PP (Fig. 3A). Upregulated genes (FC > 1 and P < 0.05) were defined as “PP upregulated genes” (Fig. 3A).

Gene enrichment and functional annotation analyses were performed on these genes, using the DAVID tool (Fig. 3B). In Gene Ontology analysis, genes involved in translation, oxidation–reduction process, metabolic process, response to oxidative stress, and cellular response to interferon-beta were the most enriched.

PERK deletion strongly modulates gene expression in PP mice. To investigate the relationship between the transcriptomic analysis and pathway analysis, we depicted representative genes on heatmaps (Fig. 3C). The expression of Dna1c,31 and Rbm312 (PERK inhibitors) was inhibited in CTRL or CM-KO PP compared to CTRL NP, resulting in enhanced PERK signaling (related to translation) in the peripartum period. The PERK target genes, Atf4, Atf5, Ddit3, Eprs, Herpud1, and Stat3, found to be upregulated in PP groups.

The expression of Atf4, Herpud1, and Stat3 was decreased in CM-KO PP compared to CTRL PP; however, the expression of other genes did not differ between them. Ern1, Xhp1, and Atf6 belonged to the other UPR branches.

The expression of Ern1 was the highest in CTRL NP. The expression of Xhp1 was suppressed in CM-KO PP compared to CTRL NP or PP compared to CTRL NP or PP, respectively. The expression of Atf6 was inhibited in CM-KO PP compared to CTRL PP. The expression of PP upregulated genes such as Mmp2, Mmp3, Mmp9, Sod1, Stat1, and Stat2 was the highest in CTRL PP. Mmp2, Mmp3, Mmp9, and Sod1 were involved in the oxidative–reduction process and response to oxidative stress. Stat1 and Stat2 were involved in the cellular response to interferon-beta. The expression of Nppb, Serpine1, Cysc, and Tnfrsf1a was the highest in CM-KO PP. Nppb is a biomarker for HF. Nppa-1 (Serpine1) is required as a co-factor for 16 K PRL signaling. Cysc belongs to the cytochrome c family of proteins and plays a major role in cell apoptosis. TNFRSF1A binds to TNF, leading to apoptosis. The expression of TNF was increased in CM-KO PP mice compared to CTRL PP mice (Fig. 21). Overall, the expression of genes related to translation, oxidative stress, and inflammation was decreased in CM-KO PP mice, while those related to HF and apoptosis were upregulated.
Figure 2. Cardiac-specific PERK homogenous knockout (CM-KO) mice develop peri-partum cardiomyopathy (PPCM). (A) Kaplan–Meier survival curve in CM-KO (n = 32) female mice versus control (CTRL, PERK lox/lox, n = 39) female mice. Log-rank test was performed between them. (B) Representative echocardiographic images in mice of each group. (C) (D) %FS (C) and Left ventricular end-diastolic dimension (Dd, D) in mice of each group: nulliparous (NP) CTRL (n = 22 mice), postpartum (PP) CTRL (n = 37 mice), NP CM-KO (n = 24 mice), and PP CM-KO (n = 24 mice). (E) Heart weight:tibial length (HW/TL) ratios of mice in each group. (F) CD31 staining of hearts in each group. Cardiac sections stained with isolectin B4 (blood vessels, green), WGA (cell membranes, red), and nuclei (Hoechst, blue). Scale bars: 50 μm. (G) Capillary density was quantified in HPF (high power field). n = 6 mice per group. (H) Quantification of cardiomyocyte cross-sectional area. n = 50 sets of cells per group. (I) The expressions of TNF, ErbB4 and Vegf in hearts of each group; all normalized to GAPDH (n = 5 mice per group). (J) Using the aggresome-specific ProteoStat dye, which showed an enhanced fluorescence signal of aggregated protein accumulation. Scale bars: 20 μm. (K) Protein aggregomes levels fluorometry intensity (%). n = 10 sets of cells per group.
Figure 3. Comparison of hearts after consecutive deliveries with or without PERK deletion, using RNA sequencing. LV myocardium from NP or PP CTRL and CM-KO mice were subjected to RNA sequencing and subsequent differential expression analysis (n = 3 mice per group). (A) Volcano plot of RNA sequencing data, depicting mRNA data P values, calculated using the unpaired t test, versus fold change (FC) in comparisons between CTRL NP and CTRL PP mice. Upregulated (FC >1) and different (P<0.05) genes were defined as “PP upregulated genes (red)”. (B) DAVID functional Gene Ontology analysis of biological processes for PP upregulated genes. Top5 enrichments were shown. (C) Representative genes related to (B) enrichments, were depicted. Statistical tests used were one-way ANOVA with Bonferroni correction. (D) Western blot analysis of phosphorylated PERK (p-PERK), PERK, phosphorylated eIF2a (p-eIF2a), eIF2a, phosphorylated Stat3 (p-Stat3), Stat3, phosphorylated Akt (p-Akt), Akt, and prolactin (23 k PRL, 16 k PRL) in hearts of each group. n = 4 mice per group. (E,F) Densitometric quantification of the p-PERK (E) or 16 k PRL (F) to GAPDH protein ratio (n = 4 mice per group). Data represent mean ± SD; P values were measured by two-way ANOVA with Bonferroni correction. (G) Cathepsin D activity in hearts. n = 10 mice per groups. Data represent mean ± SD; P values were measured by two-way ANOVA with Bonferroni correction.
Cleavage of 23 K PRL was not observed in the hearts or sera of CM-KO PP mice. To further study PERK signaling activity, we performed western blotting analysis and observed increased phosphorylation of PERK, eIF2a, STAT3, and AKT in heart tissues of CTRL PP mice compared to CTRL NP mice (Fig. 3D). However, the levels of p-PERK were suppressed in CM-KO PP mice compared to CTRL PP mice (Fig. 3E). Previous studies have suggested that many enzymes, including cathepsin D and MMP5, lead to cleavage of 23 K PRL into 16 K PRL. We found an upregulation of 16 K PRL expression in the hearts (Fig. 3D,F) and sera (Fig. S1E) of PP CTRL mice, but not in CM-KO PP mice, whereas cathepsin D activity was not different between the hearts of CTRL PP mice and CM-KO PP mice (Fig. 3G). However, the expression of MMP-2, -3, and -9 in CTRL PP mice was the most upregulated among all groups (Fig. 3C). Thus, MMP5-mediated cleavage of 23 K PRL into 16 K PRL was suppressed in CM-KO PP mice. It was shown that 16 K PRL signaling inhibited the expression of ERBB4, whereas STAT3 promoted it. Using qRT-PCR, we confirmed that the expression of ERBB4 was suppressed in CM-KO mice compared to CTRL mice under both NP and PP conditions (Fig. 2I). As a result, 16 K PRL signaling was not advanced in CM-KO PP mice.

Bromocriptine (BCR) does not ameliorate cardiac function during the postpartum term in CM-KO mice. Previous studies have shown that anti-PRL agents, such as BCR, a dopamine D2 receptor agonist, not only improved cardiac function by reducing the plasma 16 K PRL levels but also suppressed cardiac hypertrophy by suppressing the plasma aldosterone and 23 K PRL levels during the lactation term. We examined the effect of BCR in two consecutive deliveries in CTRL or CM-KO mice. BCR treatment did not affect cardiac function in these mice during the first or second postpartum term (Fig. 4A). BCR treatment did not change systolic blood pressure (sBP) in any group (Fig. 4B). We then examined the cardiac TNF levels using qRT-PCR. BCR treatment was found to inhibit TNF expression in CTRL PP mice but not in CM-KO PP mice (Fig. 4C). BCR treatment did not affect the expression of VEGFA neither in CTRL nor CM-KO PP mice. Next, we isolated cardiomyocytes from these mice after the second delivery to examine the presence of necrotic or apoptotic cells. We measured the populations of necrotic (DAPI+/Annexin V−) or apoptotic (DAPI−/Annexin V+) cardiomyocytes by flow cytometry (Fig. 4D). The populations of necrotic cells were suppressed by BCR treatment in both CTRL and CM-KO PP mice (Fig. 4E). However, apoptotic cell subpopulations were suppressed by BCR treatment in CTRL but not in CM-KO PP mice (Fig. 4F). These results suggest that PERK deletion reversed the cardioprotective effect of BCR during the peripartum term, reducing the levels of TNF and apoptotic cardiomyocytes.

Effects of 23 K PRL in NP mice. To identify the factors through which BCR suppresses the expression of TNF in CTRL mice during the peripartum period, we treated CTRL or CM-KO NP mice with 23 K PRL for three weeks. Neither of the treatments affected cardiac function (Fig. S2A) or sBP (Fig. S2D). PRL treatment induced cardiac dilatation and cardiac hypertrophy in CM-KO but not in CTRL mice (Fig. S2B,C). The cardiac expression of TNF was advanced by PRL treatment in CM-KO mice and not in CTRL mice (Fig. S2E). These results suggest that 23 K PRL treatment induced cardiac mal-remodeling and the upregulation of TNF in CM-KO but not in CTRL mice.

Discussion

Multiple signaling pathways and various cytokines have been individually shown to be involved in the pathogenesis of PPCM; however, systematic evaluation of the interactions among them has not been extensively reported. In this study, we comprehensively examined how 23 K PRL-PERK axis modulated PPCM-related signaling pathways, using transcriptomic analysis.

It is known that 23 K PRL protects against hypoxia or inflammation-induced cell death through AKT and STAT3 signaling. However, the involvement of UPR or PERK signaling with PRL signaling has not been reported. Our data suggest that 23 K PRL, but not 16 K PRL, activated the PERK signaling both in normoxic and hypoxic NRCMs (Fig. 1A). In CTRL PP mice, the expression of PERK inhibitors, such as Dnaaj3 and Rbm3, was decreased, resulting in the activation of PERK downstream signaling. Additionally, pathway analysis showed that the expression of translation-related genes, such as PERK target genes, was the highest in postpartum hearts (Fig. 3B). PERK signaling involves interaction with the other signaling molecules of UPR, namely IRE1α-XBP1 and ATF6. PERK deletion induced PPCM-like phenotype during the postpartum period, suppressing the expression of Xbp1, Atf6, and oxidative stress response genes, such as Stat3 and Sod1 (Fig. 3C). However, acute inhibition of PERK activity reduced processing of 23 k PRL to 16 k PRL (Fig. 1A,C). Thus, consecutive PERK inhibition induces PPCM-like phenotype, but temporary PERK inhibition during the peripartum period may improve the cardiac mal-angiogenesis. If selective PERK inhibitors are developed in future, these could be candidates for new drugs of PPCM.

Furthermore, 16 K PRL is a metabolite of 23 K PRL produced by cathepsin D- and MMP5-mediated cleavage. The expression of Mmps was induced by JAK-STAT signaling. The expression of Mmp2, Mmp3, Mmp9, Stat1, Stat2, and Stat3 was increased in CTRL PP mice, but not in CM-KO PP mice (Fig. 3C). As a result, 16 K PRL was produced in CTRL PP mice, but not in CM-KO PP mice (Fig. 3D). Moreover, 16 K PRL has an antiangiogenic effect; however, cardiac angiogenesis in CTRL PP mice was the most advanced among all groups (Fig. 2F,G). The expression of a pro-angiogenic gene, Vegfa (Fig. 2I), was the highest in CTRL PP mice. In PPCM patients, circulating and cardiac PAI-1 expression are up-regulated. Circulating PAI-1 (Serpine1) seems to induce vascular impairment via the uPAR/NFkB/miR-146a pathway. The expression of Serpine1, inhibited by Stat3, was suppressed in CTRL PP mice compared to CM-KO PP mice (Fig. 3C). Additionally, 16 K PRL has a proinflammatory effect in endothelial cells and activates NFκB signaling. We showed that 16 K PRL upregulated the expression of Nfkb2 in NRCMs (Fig. 1E). Interferon-beta target genes were also upregulated in CTRL PP
Figure 4. Bromocriptine (BCR) suppresses apoptosis in CTRL PP mice, but not CM-KO PP mice during. (A) Serial changes in %FS in CTRL and CM-KO mice with vehicle or bromocriptine (BCR) during the second delivery (n = 5 mice in each group). (B,C) systolic blood pressure (sBP, B) and the cardiac TNF and VEGFA levels (C) in CTRL and CM-KO mice after second delivery (n = 5 mice in each group). (D) Representative flow cytometric images in isolated cardiomyocytes of each mouse group (n = 5 mice in each group). (E,F) Quantification of necroptotic (DAPI+/Annexin V+, E), apoptotic (DAPI+/Annexin V+, F) cardiomyocytes of each mouse group (n = 5 mice in each group). Data represent mean ± SD; ※P<0.05 two-way ANOVA with Bonferroni correction.
mice (Fig. 2B). Therefore, vascular impairment induced by 16 K PRL and PAI-1 was not observed in CTRL PP mice, but cardiac inflammation occurred.

PERK-DDIT3 signaling is known to induce apoptosis via ER-associated degradation (ERAD). However, a previous study has shown that inhibition of PERK during ER stress induces ERAD-independent apoptosis. In this study, PERKi did not inhibit the expression of cleaved caspase 3, a marker for apoptosis, in hypoxic NRCMs (Fig. 1A). The expression of Cycs was the highest in CM-KO PP mice, whereas that of Ddit3 was not increased (Fig. 3C).

TNF-TNFRSF1A signaling is a pivotal pathway for apoptosis in cardiomyocytes. Furthermore, the expression of Tnf (Fig. 2I) and Tnfrsf1a (Fig. 3C) was advanced in CM-KO PP mice compared to CTRL PP mice. A previous study has revealed that 23 k PRL treatment upregulated the expression of Tnf, while BCR downregulated it. To examine their effects, we treated PP mice with BCR and NP mice with 23 K PRL. These treatments did not affect the cardiac function in these mice (Fig. 4A, Fig. S2A). BCR inhibited the population of apoptotic cardiomyocytes in CTRL mice, but not in CM-KO mice (Fig. 4F). BCR did not suppress the cardiac TNF level in CM-KO mice (Fig. 4C), while 23 K PRL promoted it (Fig. S2E). Moreover, 23 K PRL induced cardiac malremodeling (Fig. S2B,C).

Furthermore, we evaluated the expression of genes involved in PPCM, such as titin (Ttn) and cardiac troponin T (Tnnt2). RNA-seq revealed that the expression levels of Ttn and Tnnt2 was the highest in CTRL PP mice compared to the others. Moreover, the expression of Rbm20, which controls titin (Ttn) splicing, and the expression of Ttn-associated genes was the highest in CM-KO PP mice. TTN and TNNT2 are involved in passive stiffness and contraction of muscle, respectively. Therefore, it is difficult to induce a cardiac muscle stress response during pressure overload without the upregulation of these genes in CM-KO mice.

The expression of Stat1, Stat2, and Stat3 was also increased in the hearts of CTRL PP mice compared to CM-KO PP mice (Fig. 3C). STAT3 is cardioprotective, suppressing the production of ROS by upregulating the expression of antioxidative genes, such as SOD. However, the roles of STAT1 and STAT2 in postpartum hearts remain to be uncovered and need to be investigated in the future.

In conclusion, our findings suggest that peripartum 23 K PRL-PERK interaction is cardioprotective, involving the downregulation of apoptosis and 16 K PRL production and the upregulation of STAT3 signaling (Fig. 5A,B).

**Materials and methods**

**Ethics statement.** All experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments (P18-097) and strictly adhered to the guidelines for animal experimentation of the University of Tokyo. This study was carried out in compliance with the ARRIVE guidelines.
Cells and reagents. Isolation and culture of primary rat neonate cardiomyocytes (NRCMs) was performed as described16. NRCMs placed in a humidified incubator set at 37 °C in either normoxic conditions (21% O2) or hypoxic conditions (4% O2) with or without rat recombinant PRL (50 nM ProSpec) or PERK inhibitor (gsk2606414, 1 μM, Medkoo) for 24 h (h). NRCMs were also transfected with 23 k PRL plasmid (addgene #73123) or 16 k plasmid, which is mutated from 23 k PRL plasmid, using KOD plus mutagenesis kit (Toyobo). siRNAs and plasmids were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer’s protocol.

Mice. To generate mice with cardiomyocyte-specific deletion of PERK mice homzygous for the floxed PERK (The Jackson Laboratory, Bar Harbor, ME) were crossed with αMHC-Cre mice. Mice were maintained on a standard rodent chow diet with 12-h light and dark cycles. Mice were bred starting at the age of 8 weeks. Administration of bromocriptine (4 mg/kg/day, Cayman Chemical) was added in drinking water. For chronic (3 week) administration, osmotic minipumps (Alzet; LEP or PRL 400 iU/kg/day) were implanted in sedated nulliparous mice.

Transthoracic echocardiography was performed with a VEVO2100 (Visualsonics, Toronto, Canada) system in nonanesthetized mice. Parasternal short-axis projections were visualized and M-mode recordings at the mid-ventricular level were recorded. Heart rate, left ventricular (LV) end-systolic and end-diastolic dimensions were averaged from 5 beats. LV percentage of fractional shortening (FS) was then calculated as 100 × (LVEDD–LVESD)/LVEDD. Systolic blood pressure (sBP), diastolic blood pressure (dBP), and mean blood pressure (mBP) were measured by tail-cuff plethysmography from Muromachi Kikai Co., Ltd. (Tokyo, Japan) before mice were killed. Rectal body temperature was measured by thermometer.

Isolation of cardiomyocytes from adult mice. The isolation and purification of cardiomyocytes from the adult mice were performed as described before1. Briefly, the cardiomyocytes were isolated from mouse hearts via coronary perfusion with collagenase type 2 (Worthington).

mRNA isolation, cDNA synthesis and qRT-PCR. mRNA from tissue or cells was isolated with the Trizol or miRNeasy Mini Kit (Qiagen). cDNA synthesis was made with High Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific) and quantitative PCR was performed with THUNDERBIRD SYBR qPCR Mix (TOYOBO, #QPS-201), using LightCycler480 (Roche Applied Science) or QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific). GAPDH was used as a loading control. PCR primers are these; rat ATF4 (Forward 5′-CAT CAC TTT GAT GTG TGA ATT TCC-3′, Reverse 5′-AAA AAC GAA AGC GCA AGA AA-3′), mouse ErbB4 (Forward 5′-CTA CTA GGT ACC GCC AGA AG-3′, Reverse 5′-GCC TTA CGG ACC TCT TCT AT-3′), mouse VEGFA (Forward 5′-CAT GTC TAT GGT CAA GAG AAG-3′, Reverse 5′-CAT CAC TTT GAT GTG TGA ATT TCC-3′), mouse GAPDH (Forward 5′-GTG CCG GCT AGC CAA GAT-3′, Reverse 5′-GCC TTA CGG ACC TCT TCT AT-3′).

RNA-seq library preparation. Total RNAs from NRCMs and hearts were isolated as described above. The RNA integrity score was calculated with the RNA 6000 Nano reagent (Agilent Technologies) in a 2100 Bioanalyzer (Agilent Technologies). RNA-Seq libraries were prepared with a TruSeq RNA Library Prep Kit (Illumina). The libraries were sequenced on a HiSeq 2500 system (Illumina) as single read 100 (NRCMs) or 150 (hearts) base reads.

RNA-seq data analysis. Sequence reads were aligned to the rat or mouse reference genome (RGSC6.0/rn6 or GRcm38/mm10) with HISAT2 (version: 2.1.0) with the default parameters. After enumerating the mapped reads on the gene positions deposited in the geocode database (https://www.gencodegenes.org/mouse/), the FPKMs (fragments per kilobase of exon per million reads) of all of the deposited genes were calculated by Cufflinks with the default parameters. RNA-seq signals were visualized with the Integrated Genome Viewer (Version 2.4.8) (http://software.broadinstitute.org/software/igv/). The RNA-seq signal of each locus was normalized by the following basis:

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Signal \text{ of each locus} = \frac{\text{Number of mapped reads on each locus} \times 1,000,000}{\text{Total number of mapped reads}}
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Reproducibility between RNA-seq experiments. The reproducibility of the genome-wide RNA-seq signals in the biological replicates was examined under all conditions. The FPKM values were used as the RNA-seq signals. Subsequently, the correlation coefficients between three biological replicates were calculated based on the FPKMs of each reference gene.

Volcano plot. To visualize the dispersion of mRNAs, the log2 fold changes (FCs) of expression levels (horizontal axis) and the –log10 P values (vertical axis) were plotted.

Signaling pathway analysis. Gene annotation enrichment analysis was performed for KEGG pathway analysis, using the functional annotation tool in DAVID Bioinformatics Resources 6.8 (http://david.abcc.ncifcrf.org).
Flow cytometry. Dead cells and debris were excluded using forward scatter/side scatter (FSC/SSC). For the analysis of dying cells, FITC labeled Annexin V (1:100 dilution, BioLegend) and 4′,6-diamidino-2-phenylindole (DAPI, 1:1000 dilution, Sigma-Aldrich) can also be used. Necrotic or apoptotic cardiomyocytes were defined as DAPI+/Annexin V+, or DAPI-/Annexin V+ cells, separately.

Protein aggregation and ROS assays. Protein aggregation was assessed in isolated cardiomyocytes from adult mice, using Proteostat protein aggregation assay kits (Enzo Life Sciences). Proteostat emits fluorescence when it binds to the tertiary structure of aggregated proteins. To double-label protein aggregates and aggresome-like inclusion bodies in cells and cell lysates, we incubated with anti-α-actinin antibody (Sigma-Aldrich)/secondary antibody-fluorescein (488 nm) in PBS and Proteostat dye in Enzyme assay buffer for 30 min at room temperature, followed by observation under a fluorescent microscope and image acquisition.

Capillary density and cardiomyocyte cross-sectional area. Capillary density was determined as the ratio of capillaries to 100 cardiomyocytes in transversely sectioned LV tissue immunostained with isolectin (Figs. 1A,F, 3D) did not include full length membranes, with membrane edges visible. To determine the expression of ROS in isolated adult cardiomyocytes, we used ROS Fluorometric Assay Kit (Elabscience).

CD activity assay. Supernatant from freshly isolated LV tissue was generated by mincing LV tissue. Cathepsin D (CD) activity was determined in LV supernatant by using the Cathepsin D Activity Assay Kit (Raybio).

Western blotting. Mouse tissue or cultured NRCMs were homogenized in lysis buffer (cell signaling technology, #9803) with 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysates were separated on 5–20% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk, the membranes were probed with one of the following primary antibodies overnight at 4 °C: anti PERK antibody (1:200, Santa cruz), anti p-PERK (Thr980) antibody (1:100, cell signaling technology), anti eIF2α antibody (1:1000, cell signaling technology), anti PRL (N-terminal) antibody (1:1000, Acris Antibodies GmbH), anti phosphorylated STAT3 antibody (1:2000, cell signaling technology), anti STAT3 antibody (1:1000, cell signaling technology), anti cleaved caspase3 antibody (1:1000, cell signaling technology), anti GAPDH antibody (1:1000, cell signaling technology), and anti transferin antibody (1:1000, Santa cruz).

This was followed by a 1-h incubation with secondary antibodies conjugated with horseradish peroxidase (HRP) at room temperature. Bound antibodies were detected by chemiluminescence with the ECL detection system. Relative protein levels were quantified using the Image J program (NIH, Bethesda, MD). Some of images (Figs. 1A,F, 3D) did not include full length membranes, with membrane edges visible.

Statistical analysis. The data are described as mean ± SD. Parametric tests were used after verification to ensure the variables in each group were normally distributed. Student’s un-paired t tests, as well as one-way or two-way analysis of variance (ANOVA) with Bonferroni correction were performed using the Graph Pad Prism8.4.2. The clustering displayed in the heatmap was also performed using this software.

Data availability The RNA sequence data can be accessed through the Gene Expression Omnibus (GEO) under the NCBI accession number GSE168536 and GSE169063. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.
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Author contributions
T.S., conceived the project, designed the study, and interpreted the results; A.T. performed RNA-seq library preparation; T.S. performed computational analyses; Y.H., T.K., Y.W. provided support for computational analyses; Y.K. and R.N. provided support for his Statistical analysis; T.S. generated PERK KO mice, conducted the functional analysis; T.S. performed biochemical experiments, immunohistochemistry experiments and analyzed the data; Y.U. provided experimental and analytical support; T.S. wrote the manuscript with feedback from all authors.

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Competing interests
The authors declare no competing interests.

Additional information
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