Activation of the Hippo signalling pathway mediates mitochondria dysfunction and dilated cardiomyopathy in mice

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RNA sequencing data were uploaded to the Gene Expression Omnibus (GSE106201)
Supplementary Materials

Expanded Methods

Transmission electron microscopy (EM) and quantification
Mitochondrial ultrastructure was studied in freshly collected LV specimens by EM (3 LV samples per age per genotype). Samples were dissected into 1 mm³ blocks, immediately fixed with 2.5% glutaraldehyde buffer (2 h at 37°C), and then post-fixed in 1% OsO₄ (2 h at 4°C). The samples were dehydrated in a graded series of alcohol and then exposed to propylene oxide for infiltration of the embedding medium, Epon 812 resin in 0.1 mol/L sodium phosphate buffer. Using LKB-V/Leica UC7 ultramicrotome (LKB-V/NOVA, Sweden and Leica, Germany), the embedded blocks were cut into sections (50-70 nm). Sections were stained with acidified uranyl acetate, followed by a modification of Sato's triple lead stain, and viewed with a transmission electron microscope (TEM; H-7650; Hitachi, Tokyo, Japan). For each sample, images from randomly chosen fields were obtained at magnifications of ×4,000, ×10,000 and ×30,000, respectively (6-10 images at each magnification per heart sample). The images (×10,000 magnification) were analysed, in a blinded fashion, using ImageJ software on at least 8 images per heart. Area of individual mitochondria was manually measured and average used. The sum of all measurements of mitochondria size per image represented the total area by mitochondria per image field. Mitochondria with complete or incomplete outer membrane were individually observed and counted to obtain percentage of mitochondria with incomplete membrane. Sarcomere length was determined in 8-10 images (×10,000 magnification) by drawing a line parallel to myofibril orientation across over 5-10 sarcomeres. Approximately 30 sarcomeres were measured from one image and the averages from all images calculated for comparison.

Mitochondrial lipid and amino acid profiling

Tissue homogenization and lipid extraction. Tissue samples were homogenized using a sonicator probe in ice cold PBS. Lipids were extracted from homogenized tissue as previously described. In brief, tissue or plasma was mixed with 10× volume of butanol:methanol (1:1) with 10 mM ammonium formate containing a mixture of internal standards. Samples were vortexed thoroughly and set in a sonicator bath for 1 hour at room temperature. Samples were then centrifuged (14,000 g, 10 min, 20 °C) and the supernatant was transferred into sample vials with glass inserts for mass spectrometry analysis.

Liquid chromatography mass spectrometry (LC/MS/MS). The detailed lipidomics and assay conditions have been reported previously. Briefly, lipidomic analysis was performed by LC ESI-MS/MS using an Agilent 1290 liquid chromatography system and an Agilent 6490 QQQ mass spectrometer. We employed source conditions identical to those previously described. Liquid chromatography was performed on a Zorbx C18, 1.8 μm, 50× 2.1 mm column (Agilent Technologies). Solvents A and B consisted of water, acetonitrile and isopropanol in the ratio (50:30:20) and (90:9:1) respectively, both containing 10 mM ammonium formate with solvents B also containing 5 μM medronic acid. Gradient conditions were as previously described [1, 2]. Columns were heated to 45 °C and the auto-sampler regulated to 25 °C.
**Analysis of mitochondria-rich lipid species.** Quantification of lipid species was determined by comparison to the relevant internal standards. In the present study that was focused on mitochondria, only 3 lipids that are largely or entirely localized in mitochondria were analysed, i.e. acyl-carnitine (AC), cardiolipin (CL) and ubiquinone (or coenzyme Q10, CoQ10). Lipid characterization and quantification were conducted as we previously described in detail [1, 2]. Alterations in the species of the three lipids were expressed as relative change to that of nTG control.

**Amino acid analysis of heart and plasma.** The extracted myocardial samples were further analysed for specific amino acids using a separate targeted HILIC-MS/MS method. An Acquity UPLC BEH Amide 1.7 µm 2.1 × 100 mm column (Waters) was used. In this assay Solvent A comprises of 50% acetonitrile in water, whereas Solvent B comprises of 95% acetonitrile in water, both with 10 mM ammonium formate. Chromatography was used to separate out analytes prior to mass spectrometry analysis. Starting at 100% B, this was held for 1 minute before a linear gradient to 0% B over 5 minutes. 1 minute was then spent holding 0% B before switching to 100% B over 0.1 minute, then held at 100% B for an additional 2.9 minutes for equilibration. Total run time was 10 minutes per sample. Results were normalised to a spike internal standard (L-Leucine-5,5,5-d3, Sigma Aldrich).

**High performance liquid chromatography (HPLC) assay of ubiquinone:** Tissue content of ubiquinone in LVs of 3-wk-old mice was determined using HPLC. LV tissues (20 mg) were homogenized in 2 ml extract (N-hexane: ethanol = 8:1), centrifuged (3,000 rpm for 5 min), and supernatant was harvested. This process was repeated 3 times and the final supernatant was evaporated to dryness in a vacuum centrifuged concentrator (1500 rpm, 37°C, 50 min). Sample was redissolved in pure ethanol (100 µL) and assayed using SHIMADZU HPLC system (LC-2030C 3D, mobile phase: 3:7 methanol:ethanol 1 ml/min). Ubiquinone was identified by an internal standard and the amount quantified using ubidecarenone (Sigma, Lot# LRAC3727) derived standard curve.

**References**
1. Tham YK, Bernardo BC, Huynh K, Ooi JYY, Gao XM, Kiriazis H, et al. Lipidomic Profiles of the Heart and Circulation in Response to Exercise versus Cardiac Pathology: A Resource of Potential Biomarkers and Drug Targets. Cell Rep. 2018;24:2757-2772.
2. Huynh K, Barlow CK, Jayawardana KS, Weir JM, Mellett NA, Cinel M, et al. High-Throughput Plasma Lipidomics: Detailed Mapping of the Associations with Cardiometabolic Risk Factors. Cell Chem Biol. 2019;26:71-84 e4.

**Quantitative real-time PCR**
RNA was extracted from LV tissue using RNAiso PLUS (Takara Bio, Japan, Code No. 9108/9109). RNA (1 µg) was used for cDNA synthesis. Quantitative PCR was performed by SYBR-based RT-PCR assays using PrimeScript™ reagent kit with gDNA Eraser (Perfect Real-Time) (Takara, Code No: RR047A), and TB Gree™ Premix Ex Tag™ II Tli RNAaseH Plus (Takara, Code No. RR820A). Table S1 lists target genes examined and sequences of primers (Sangon Biotech, Shanghai, China). Quantitative real-time PCR reaction was performed using Bio-Rad CFX96 Real-time PCR Detection System with the following two step procedures: Step-1: 95 ºC for 30 sec, and Step-2 (PCR reaction): 40 cycles of amplification at 95 ºC for 5 sec and 60 ºC for 30 sec. The expression of target genes was
normalized to that of GAPDH using the method of $2^{-\Delta\Delta C_t}$, and presented relative to that of the nTG value.

**YAP silencing by small interfering RNA in H9c2 cells.**

YAP gene knockdown was performed in rat cardiomyoblasts (H9c2) using small interfering RNA (siRNA, GenePharma, Shanghai), according to manufacturer's instructions. Cells were cultured in 6-well plates with standard antibacterial-free medium. To make siRNA delivery medium, 2.5 μL lipofectamine 2000 (Invitrogen 11668019) was added in 250 μL Opti-MEM (Gibco, 11058021) and mixed for 5 min, followed by addition of specific rat YAP-siRNA (F: 5-GGA GAA GUU UAC AUA ATT-3; R: 5-UUA UGU AGU AAA CUU CUC CTT-3) or non-targeting control siRNA (10 nM each) with 20 min allowed for full mixing. After reaching 40-50% confluence, cells were incubated with the siRNA delivery medium (0.5 mL per well containing 1.5 mL standard medium). Six hours afterward, the delivery medium was replaced. After incubation for another 48 h, cells were harvested and protein was extracted for immunoblotting assay.

Table S1. Oligonucleotide sequences of mouse primers for gene expression by RT-PCR

| Gene name | Primer Sequence (5'―3')               |
|-----------|--------------------------------------|
| Ctgf      | F: GACCCAACCTATGATGCGAGCC            |
|           | R: ACTTGGCCCTGTATGTCTTCACA           |
| Lgals3    | F: AACACGAAGCAGGACAATAACTGG          |
|           | R: GCAGTAGGTGAGCAGCTCGGTGAGC        |
| IL6       | F: GGTACATCCCTCGAAGGCGATCT          |
|           | R: GTGCCCTTTTGCACCTGTTTCAC           |
| Ax1       | F: ATGCCAGTCAAGTGGATTGCT             |
|           | R: CACACATCGCTCTTGCTGGT              |
| Ankrd1    | F: GAGACACCCCCACTGCATGAT             |
|           | R: TTCCGGCAAGCTTCTGGACC              |
| Ndufa12   | F: ACCCTCCGAGCAGTACATCCA             |
|           | R: GTGTCGAAGTGGGAAACCCAT             |
| Ndufab1   | F: CTTAGGGGAGTACGAGGAGGA             |
|           | R: GTCTAAGTCAAGTGGGAGTG              |
| Ndufs6    | F: CCTGAGAGCGATCTGGGATACTTT          |
|           | R: TGGGCTTCGAGCTAAAGTTG             |
| Atp5e     | F: GAGGCTACTCTGAAAGGACC             |
|           | R: ACCGAGATGTAGCTAGTCTGA             |
| Txn2      | F: GCTAGAGAAGATGGTGCGCAAGCA          |
|           | R: TCCTGCCTGATACCCACAAACTTG          |
| Ndufa3    | F: TGCTGGGCGCTCTGCATTTAA            |
|           | R: CAGGGCATGTTCCCTGGCATTCT           |
| Gapdh     | F: TGAAGCAGGAGCATCTGAGGG            |
|           | R: CGAAGTGGGAAGAGTGAGGG             |

Abbreviations: please refer to online supplemental Table 3.
Table S2. Source and characteristics of antibodies used for immunoblotting

| Protein       | supplier       | Lot number | concentration | MW (kDa) |
|---------------|----------------|------------|---------------|----------|
| Mst1          | CST            | #3682      | 1:1000        | 59       |
| p-Yap         | CST            | #4911      | 1:1000        | 69       |
| Yap           | CST            | #12395     | 1:1000        | 69       |
| PGC-1α        | proteintech    | 66369      | 1:1000        | 100      |
| NRF1          | CST            | #46743S    | 1:1000        | 68       |
| AMPKα         | CST            | #2532      | 1:1000        | 62       |
| OPA1          | CST            | #80471S    | 1:3000        | 80-100   |
| OPA3          | proteintech    | 15638-1-AP | 1:1000        | 20       |
| Mfn1          | abcam          | ab104274   | 1:1000        | 84       |
| DRP1          | CST            | #8570S     | 1:1000        | 82       |
| Fis1          | abcam          | ab71498    | 1:1000        | 17       |
| p53           | abcam          | ab26       | 1:1000        | 53       |
| LC3A          | CST            | #4599S     | 1:1000        | 14,16    |
| Pink1         | abcam          | ab23707    | 1:500         | 66       |
| Bnip3         | abcam          | ab10433    | 1:1000        | 30       |
| MT-ND1        | abcam          | ab181848   | 1:5000        | 36       |
| SDHA          | CST            | #11998     | 1:3000        | 70       |
| COXIV         | proteintech    | 11242-1-AP | 1:4000        | 17       |
| OGDH          | abcam          | ab137773   | 1:5000        | 116      |
| PDH           | CST            | #3205      | 1:3000        | 43       |
| Bax           | CST            | #2772S     | 1:1000        | 20       |
| Bcl-2         | CST            | #3498S     | 1:1000        | 26       |
| Hif-1α        | CST            | #36169     | 1:1000        | 120      |
| VDAC1         | proteintech    | 10866-1-AP | 1:4000        | 31       |
| TFAM          | abcam          | ab131607   | 1:2000        | 28       |
| Anti-MYH6     | abcam          | ab207926   | 1:3000        | 223      |
| Anti-MYH7B    | abcam          | ab172967   | 1:3000        | 221      |
| GAPDH         | proteintech    | 10494-1-AP | 1:4000        | 36       |
| CTGF          | proteintech    | 23936-1    | 1:500         | 38       |
| Yap1          | santa          | sc-376830  | 1:500         | 70       |
| Taz           | CST            | #84185     | 1:1000        | 55       |
| Tead1         | santa          | sc-393976  | 1:500         | 50       |
| Tead1         | CST            | #12292     | 1:1000        | 50       |
| Lamin B1      | Proteintech    | 66095-1-1g | 1:3000        | 66       |
| Galectin-3    | abcam          | ab2785     | 1:1000        | 30       |
| IL-6          | CST            | 12912T     | 1:1000        | 24       |
| NDUFA12       | abcam          | Ab192617   | 1:4000        | 17       |
| NDUFA1B       | abcam          | Ab181021   | 1:1000        | 12       |
| NDUFS6        | abcam          | Ab195808   | 1:1000        | 14       |
| Txn2          | abcam          | Ab185544   | 1:1000        | 12       |
| POLRmt        | Invitrogen     | PA5-28129  | 1:1000        | 130      |
| IgG           | Beyotime       | A7028      |               |          |
Table S3. Full list of abbreviations used in Figures

| Fig 1 Abbreviations | Full term                                      |
|---------------------|-----------------------------------------------|
| TG, nTG             | Transgenic, non-transgenic                    |
| Mst1                | mammalian sterile-20 like kinase 1            |
| MHC                 | myosin heavy chain                            |
| YAP                 | yes-associated protein                        |
| GAPHD               | glyceraldehyde-3-phosphate dehydrogenase      |
| LV                  | left ventricle or left ventricular            |
| LA                  | left atrium                                   |
| Ao                  | aorta                                         |

| Fig 4 Abbreviations | Full term                                                                                            |
|---------------------|--------------------------------------------------------------------------------------------------------|
| PGC-1α              | peroxisome proliferator-activated receptor γ coactivator 1-α                                         |
| NRF1                | nuclear respiratory factor 1                                                                         |
| AMPKα               | AMP-activated protein kinase α                                                                        |
| Mfn1                | mitofusin 1                                                                                            |
| OPA1/3              | optic atrophy 1/3, mitochondrial dynamin-related protein                                              |
| Drp1                | dynamin-related protein 1                                                                            |
| Fis1                | fission, mitochondrial 1                                                                             |
| Bnip3               | BCL2 interacting protein 3                                                                            |
| Pink1               | PTEN induced kinase 1                                                                                  |
| LC3II/I             | microtubule associated protein 1 light chain 3 alpha                                                   |
| MT-ND1              | NADH dehydrogenase, subunit 1 (complex I)                                                             |
| SDHA                | succinate dehydrogenase complex flavoprotein subunit A                                               |
| COX IV              | cytochrome c oxidase subunit 4                                                                       |
| OGDH                | oxoglutarate dehydrogenase                                                                          |
| PDH                 | pyruvate dehydrogenase                                                                               |
| Bcl-2               | BCL2 apoptosis regulator                                                                              |
| Bax                 | BCL2 associated X, apoptosis regulator                                                                |
| VDAC1               | voltage-dependent anion channel 1                                                                    |
| GAPDH               | glyceraldehyde-3-phosphate dehydrogenase                                                             |

| Fig 5 Abbreviations | Full term                                                                                            |
|---------------------|--------------------------------------------------------------------------------------------------------|
| AC                  | acylcarnitine                                                                                    |
| CL                  | cardiolipin                                                                                       |
| Coq2-10             | hydroxybenzoate polyprenyltransferases 2-10, mitochondrial                                       |
| Pdss1/2             | Decaprenyl-diphosphate synthase subunit 1/2                                                         |
| CPT1/2              | Carnitine palmitoyltransferases                                                                     |
| CACT                | Carnitine/acylcarnitine translocase                                                                 |
| TAZ                 | transcriptional coactivator with PDZ-binding motif                                                  |
| HADHa/b             | hydroxacyl-CoA dehydrogenase trifunctional multi-enzyme complex                                    |
| LCLAT1              | lysocardiolipin acyltransferase 1                                                                   |
| CRLS1               | cardiolipin synthase 1                                                                             |
| PLA2G6              | phospholipase A2 group VI                                                                           |
| TAMM41              | TAM41 mitochondrial translocator assembly and maintenance homolog                                   |
| PGS1                | phosphatidyglycerophosphate synthase 1                                                              |
| HSD10               | fibrous sheath interacting protein 1                                                                 |
| PLD6                | phospholipase D family member 6                                                                      |
| **Fig 6 Abbreviations** | **Full term** |
|-------------------------|--------------|
| Glut1                   | glucose transporter1 |
| HIF-1α                  | hypoxia inducible factor-1α |
| BCAA                   | Branched-chain amino acid |
| BCAT2                  | branched-chain-amino-acid aminotransferase |
| BCKDH                 | Branched-chain keto acid dehydrogenase E1 |
| BCKDK                 | Branched-chain keto acid dehydrogenase kinase |
| PPM1K                | protein phosphatase 1K |
| Mecc1/2               | methylcrotonoyl-CoA carboxylase |
| IVD                | isovaleryl-CoA dehydrogenase |
| ACAT1               | acetyl-CoA acetyltransferase 1 |
| Acad              | acetyl-CoA dehydrogenase short chain |
| Pcca            | propionyl-CoA carboxylase subunit alpha |
| Mtr            | 5-methyltetrahydrofolate-homocysteine methyltransferase |
| NOX2/4       | NADPH oxidases2/4 |

| **Fig 7 Abbreviations** | **Full term** |
|-------------------------|--------------|
| IP                      | immunoprecipitation |
| TAZ                     | Tafazzin |
| CTGF                    | Connective tissue growth factor |
| Lgala3 or Gal3          | Galectin-3 |
| Ankrd1                  | Ankyrin repeat domain 1 |
| Ax1                     | AXL receptor tyrosine kinase |
| Hsf1/2                  | Heat shock transcription factor 1/2 |
| Txn2                    | Thioredoxin, mitochondrial |
| Ndufs                  | NADH:ubiquinone oxidoreductase subunits |
| Apt5e                  | ATP synthase 5E |
| IL-6                    | Interleukin-6 |

| **Fig 8 Abbreviations** | **Full term** |
|-------------------------|--------------|
| mtDNA                   | Mitochondrial DNA |
| Tfam                    | transcription factor A, mitochondrial |
| Polrmt                  | RNA polymerase, mitochondrial |
| Tfb2m                   | transcription factor B2, mitochondrial |
| Mterf1a                 | mitochondrial transcription termination factor 1a |
SUPPLEMENTAL FIGURES

Figure S1.

Dilated cardiomyopathy phenotype of 3-wk Mst1-TG mice. Images of lungs and the liver from nTG and Mst1-TG mice at 3 weeks (A) in comparison to that of 6-mo-old counterparts (B). C, body weight-normalized organ weights of 3-wk-old male and female mice. Pulmonary and hepatic congestion in TG mice indicated presence whole heart failure. *P < 0.05, †P<0.01, #P < 0.001 vs. nTG group.
Figure S2.

Cardiac transcriptome by RNA sequencing.

RNA sequencing data were collected from LV tissues of mice aged at 3-wk (n = 4 genotype) and 15-wk (n = 6 for nTG and n = 7 for TG). We generated 25 M (3-wk) or 38.6 M (15-wk) reads/sample, of which 83.3% of reads were uniquely mapped (SD 2.1%). An average of 24.0 M reads was assigned to genes (SD 3.2 M). A, Venn diagrams showing number of genes detected in 3-wk and 15-wk TG hearts (left), and number of differentially expressed genes (DEGs, FDR<0.05) due to Mst1-overexpression in both age groups (right). B, Volcano plots of DEGs of 3-wk and 15-wk TG mice relative to respective nTGs. Red dots denote genes with FDR < 0.05). For clarity reason, only DEGs are presented. C, Venn diagram of number of genes showing significant changes due to Mst1 overexpression in both age groups. D, Filled contour plot of DEGs in TG hearts showing a positive correlation between 3-wk and 15-wk TG groups.
Figure S3.
Downregulation of gene sets of mitochondrial biosynthesis, assembly and turnover in TG hearts.
Filled contour plots showing that 3-wk and 15-wk TG hearts exhibited similar changes in the down-regulation of gene sets related to mitochondria biosynthesis, assembly, turnover, cristae formation, protein import and metabolism of cofactors.
Figure S4.

**Downregulation of gene-sets of mitochondrial metabolism.** Filled contour plots showing that 3-wk and 15-wk TG mouse hearts exhibited consistent down-regulation of gene-sets related to mitochondria metabolism.
**Figure S5.**

**Downregulation of mitochondrial protein import genes in Mst1-TG hearts by RNAsequencing.**

Heatmap for mitochondrial protein import genes (set size n = 57) in 3-wk and 15-wk Mst1-TG hearts. Among the gene-sets were numerous members of translocases of outer-membrane (TOMs) or translocases of inner-membrane (TIM) that mediate inward transportation of cytosolic proteins into mitochondria. Group size: n = 4/group for 3-wk and n = 6-7/group for 15-wk group.
Figure S6.

Upregulation in fibrotic pathway gene-sets in 3-wk and 15-wk Mst1-TG mouse hearts and myocardial fibrosis in adult TG hearts.

A, Filled contour plots showing similarity in 3-wk (n = 4/group) and 15-wk (n = 6-7/group) TG relative to nTG hearts in upregulated expression of gene-sets related to extracellular matrix formation/turover and fibrogenesis. B, Representative histology of nTG and TG hearts (left ventricle, LV) with Masson trichrome staining for collagen in blue colour. Note that significant interstitial fibrosis (in blue) of LV myocardium was evident in 6-month-old but not in 3-wk-old TG mice.
Figure S7.
Lack of changes in certain pathways by transcriptome of 3-wk and 15-wk Mst1-TG mouse hearts.

Heatmap for selected pathways (cellular responses to stress, hemostasis, apoptosis, signal transduction, cardiac conduction, muscle contraction) in Mst1-TG hearts. Note the lack of differences between nTG and TG groups in expression pattern of these gene sets.
Figure S8.

Changes in YAP-target genes in TG heart by transcriptome.

A, Volcano plots showing up- and down-regulated YAP-target genes in 3-wk and 15-wk TG relative to respective nTG hearts. Red dots denote genes with FDR < 0.05. B, Venn diagram of downregulated genes in both TG groups. Bar graph depicting the number of downregulated mitochondrial genes as percentages of total downregulated YAP-target genes.