Cell reports

Supplemental information

Lethal cardiomyopathy in mice lacking transferrin receptor in the heart

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SUPPLEMENTAL FIGURES:

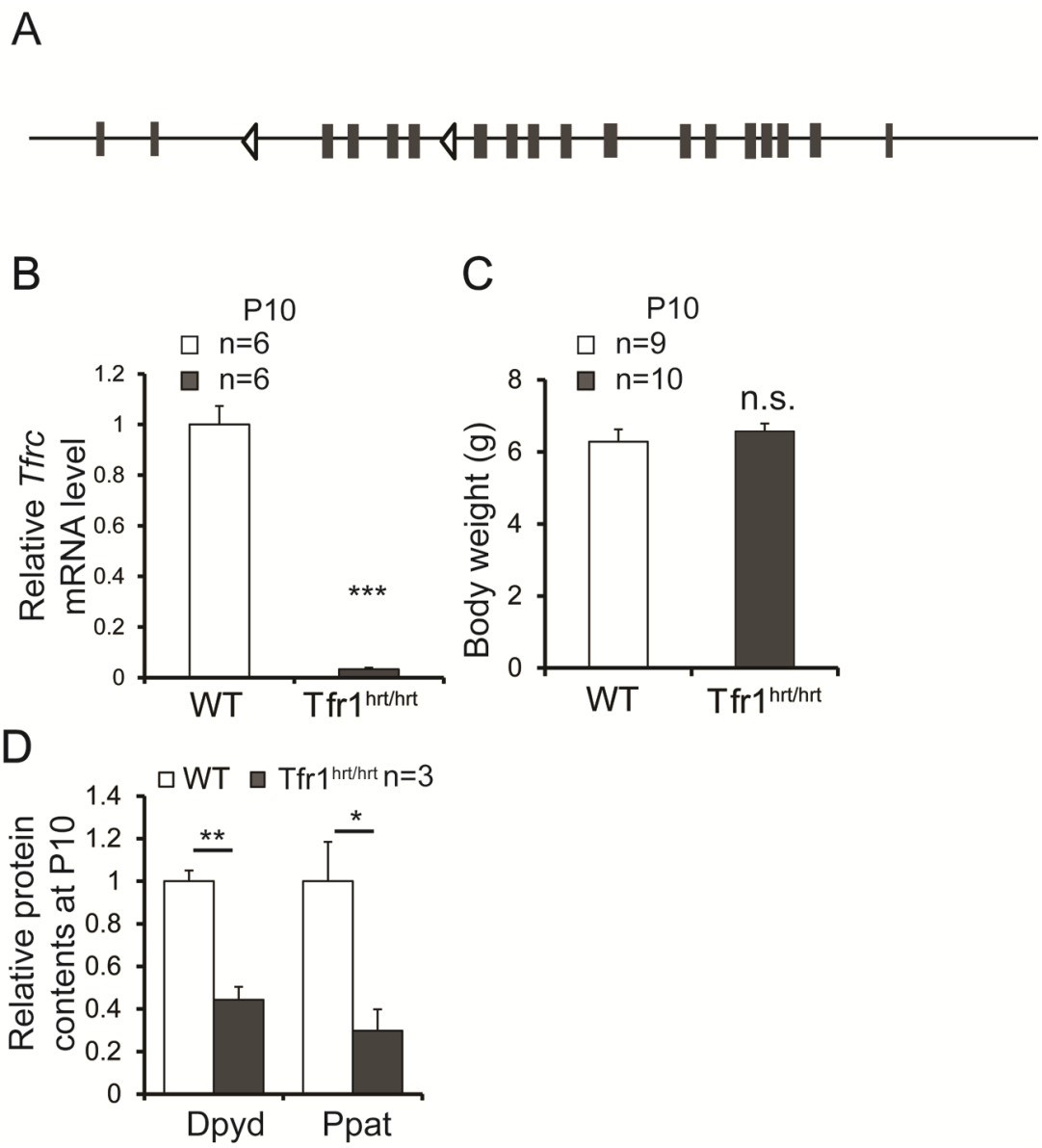

Figure S1

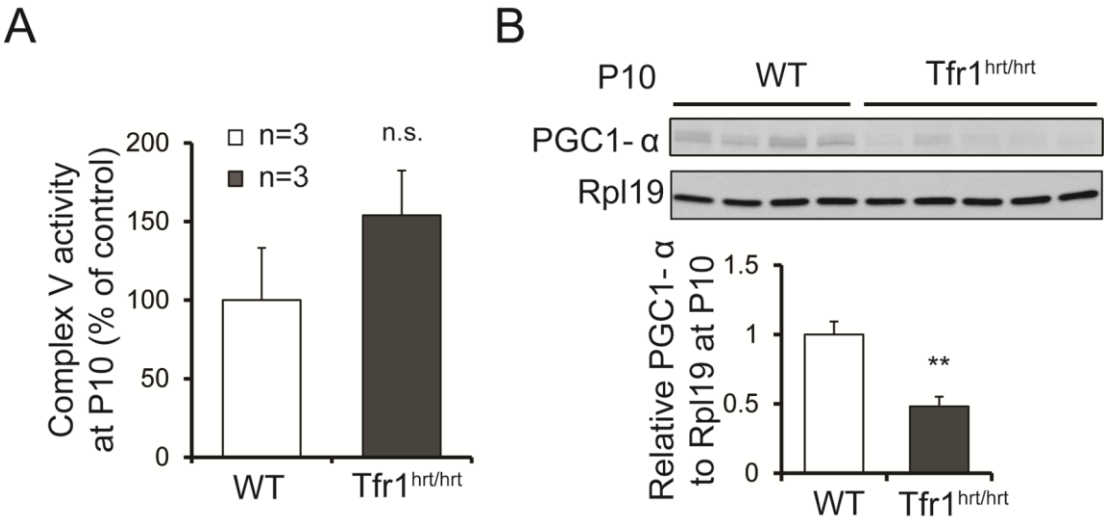
A
B
n=3
n=3
n.s.
Complex V activity at P10 (% of control)
200
150
100
50
0
WT
Tfr1hrt/hrt
P10
WT
Tfr1hrt/hrt
PGC1- α
Rpl19
Relative PGC1- α to Rpl19 at P10
1.5
1
0.5
0
**
WT
Tfr1hrt/hrt

Figure S2

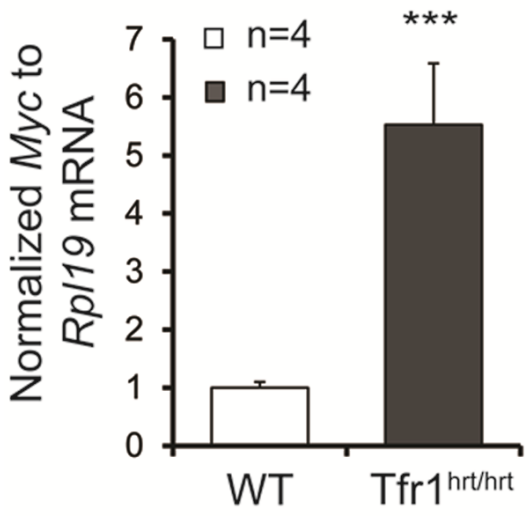

Figure S3

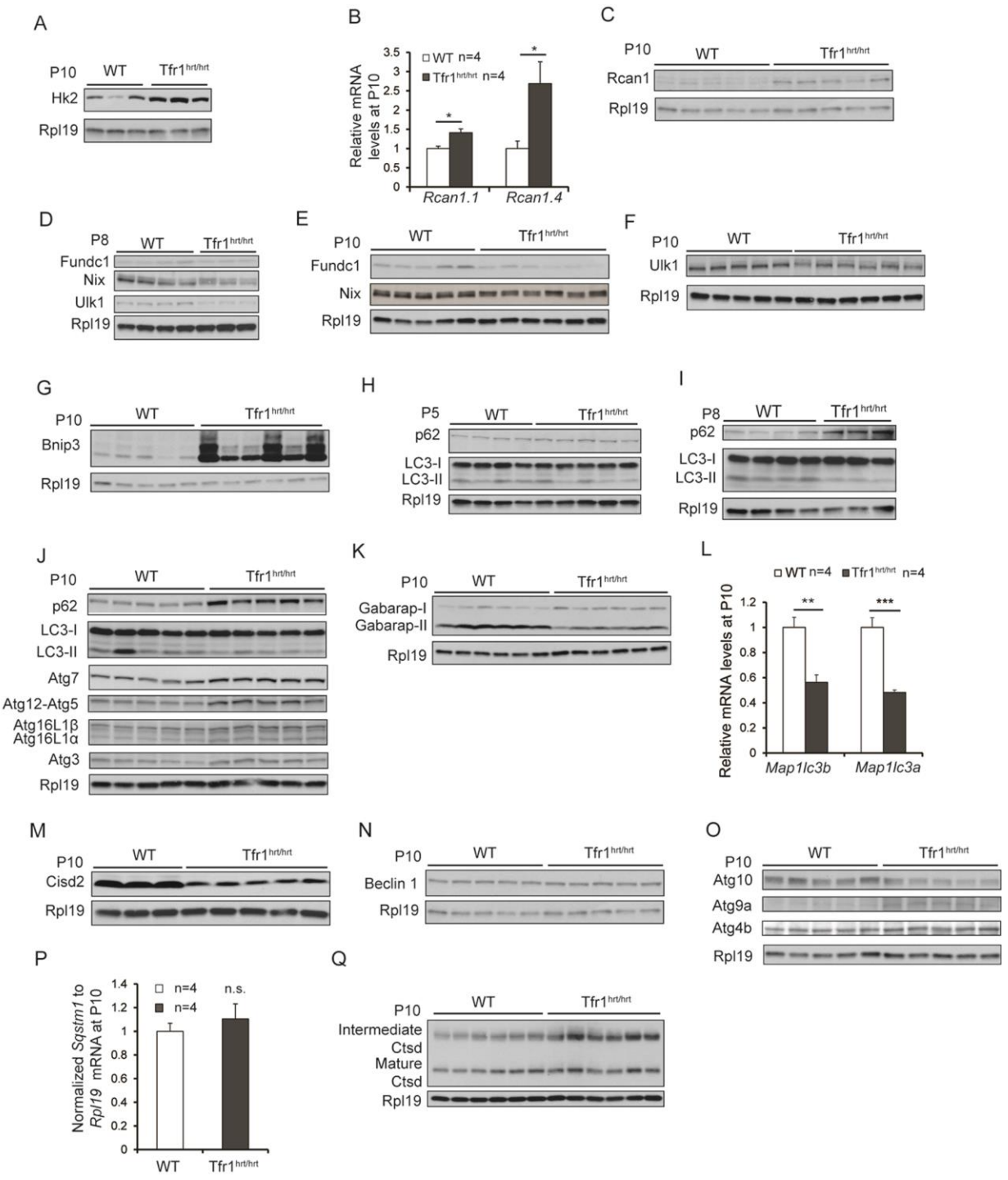
A
P10
WT
Tfr1hrt/hrt
Hk2
Rpl19
B
WT n=4
Tfr1hrt/hrt n=4
Relative mRNA levels at P10
Rcan1.1
Rcan1.4
C
P10
WT
Tfr1hrt/hrt
Rcan1
Rpl19
D
P8
WT
Tfr1hrt/hrt
Fundc1
Nix
Ulk1
Rpl19
E
P10
WT
Tfr1hrt/hrt
Fundc1
Nix
Rpl19
F
P10
WT
Tfr1hrt/hrt
Ulk1
Rpl19
G
P10
WT
Tfr1hrt/hrt
Bnip3
Rpl19
H
P5
WT
Tfr1hrt/hrt
p62
LC3-I
LC3-II
Rpl19
I
P8
WT
Tfr1hrt/hrt
p62
LC3-I
LC3-II
Rpl19
J
P10
WT
Tfr1hrt/hrt
p62
LC3-I
LC3-II
Atg7
Atg12-Atg5
Atg16L1β
Atg16L1α
Atg3
Rpl19
K
P10
WT
Tfr1hrt/hrt
Gabarap-I
Gabarap-II
Rpl19
L
WT n=4
Tfr1hrt/hrt n=4
Relative mRNA levels at P10
Map1lc3b
Map1lc3a
M
P10
WT
Tfr1hrt/hrt
Cisd2
Rpl19
N
P10
WT
Tfr1hrt/hrt
Beclin 1
Rpl19
O
P10
WT
Tfr1hrt/hrt
Atg10
Atg9a
Atg4b
Rpl19
P
n=4
n=4
n.s.
Normalized Sqstm1 to Rpl19 mRNA at P10
WT
Tfr1hrt/hrt
Q
P10
WT
Tfr1hrt/hrt
Intermediate Ctsd
Mature Ctsd
Rpl19

Figure S4

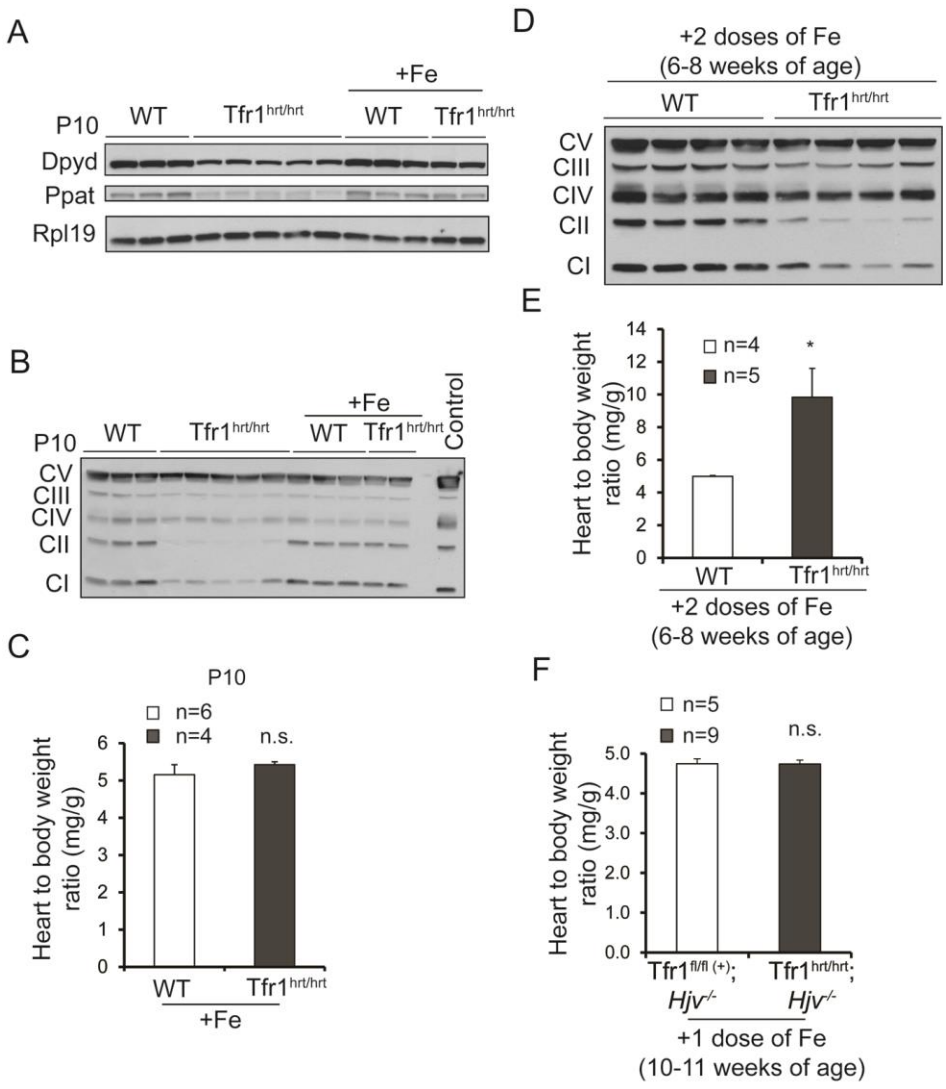
A
+Fe
P10
WT
Tfr1hrt/hrt
WT
Tfr1hrt/hrt
Dpyd
Ppat
Rpl19
B
+Fe
P10
WT
Tfr1hrt/hrt
WT
Tfr1hrt/hrt
Control
CV
CIII
CIV
CII
CI
C
P10
n=6
n=4
n.s.
Heart to body weight ratio (mg/g)
WT
Tfr1hrt/hrt
+Fe
D
+2 doses of Fe
(6-8 weeks of age)
WT
Tfr1hrt/hrt
CV
CIII
CIV
CII
CI
E
n=4
n=5
*
Heart to body weight ratio (mg/g)
WT
Tfr1hrt/hrt
+2 doses of Fe
(6-8 weeks of age)
F
n=5
n=9
n.s.
Heart to body weight ratio (mg/g)
Tfr1fl/fl (+); Hjv-/-
Tfr1hrt/hrt; Hjv-/-
+1 dose of Fe
(10-11 weeks of age)

Figure S5

SUPPLEMENTAL FIGURE LEGENDS:

Figure S1. Floxed *Tfrc* allele configuration and preliminary characterization of Tfr1$^{hrt/hrt}$ mice (related to Figures 1, 2).

(A) Schematic diagram of floxed *Tfrc* allele allowing for deletion of exons 3-6. LoxP sites are shown as white triangles.

(B) Levels of *Tfrc* mRNA in hearts from littermate wild type (WT) and Tfr1$^{hrt/hrt}$ mice at P10.

(C) Body weights of littermate WT and Tfr1$^{hrt/hrt}$ mice at P10.

(D) Quantitation of cytoplasmic Fe-S cluster proteins Dpyd and Ppat at P10 (Fig 2E).

Data are presented as means ± SEM. P-values were determined by one-way ANOVA. The sample size (n) is indicated; *p<0.05; **p<0.01; ***p<0.001; n.s., not significant.

Figure S2. Complex V activity and PGC1-α expression (related to Figure 3).

(A) Complex V enzymatic activity at P10.

(B) PGC1-α protein level by immunoblotting.

Data are presented as means ± SEM. P-values were determined by one-way ANOVA. The sample size (n) is indicated; **p<0.01; n.s., not significant.

Figure S3. *Myc* mRNA (related to Figure 4).

Data are presented as means ± SEM. P-values were determined by one-way ANOVA. The sample size (n) is indicated; ***p<0.001.

Figure S4. Immunoblot detection of proteins and real-time PCR measurement of RNAs of genes involved in autophagy and mitophagy in hearts from WT and Tfr1$^{hrt/hrt}$ mice (related to Figure 5).

(A-K,M,N,O) Multiple autophagy- and mitophagy-related proteins were examined by immunoblotting 10-, 8- and 5-day old littermate heart samples as indicated in each panel.

(L) mRNA levels for *Map1lc3b* and *Map1lc3a*.

(P) Normalized levels of p62 (Sqstm1) mRNA were similar in hearts from WT and $Tfr1^{hrt/hrt}$ littermates at 10 days of age.

(Q) Immunoblot showing increased Ctsd in hearts from $Tfr1^{hrt/hrt}$ mice.

Data are presented as means ± SEM. P-values were determined by one-way ANOVA. The sample size (n) is indicated; *p<0.05; **p<0.01; ***p<0.001; n.s., not significant.

Figure S5. Ongoing iron loading was necessary to rescue $Tfr1^{hrt/hrt}$ mice (related to Figure 6).

(A) Amounts of Fe-S cluster proteins Dpyd and Ppat in hearts from 10-day old WT and $Tfr1^{hrt/hrt}$ mice untreated or treated with iron dextran.

(B) Representative ETC complex proteins in hearts from 10-day old WT and $Tfr1^{hrt/hrt}$ mice untreated or treated with iron dextran.

(C) Heart weight to body weight ratios at P10 for WT and $Tfr1^{hrt/hrt}$ littermates treated with 1 dose of iron dextran treated on P3.

(D) Representative ETC complex proteins in hearts from 6-8 week old WT and $Tfr1^{hrt/hrt}$ mice treated with 2 doses of iron dextran (at P3 and P7).

(E) Heart weight to body weight ratios at 6-8 weeks for WT and $Tfr1^{hrt/hrt}$ mice treated with 2 doses of iron dextran (at P3 and P7).

(F) Heart to body weight ratios at 10-11 weeks for $Tfr1^{fl/fl(+)}$; $Hjv^{-/-}$ and $Tfr1^{hrt/hrt}$; $Hjv^{-/-}$ mice treated with iron dextran only at P3.

Data are presented as means ± SEM. P-values were determined by one-way ANOVA. The sample size (n) is indicated; *p<0.05; n.s., not significant.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Primers for Genotyping mice by PCR

Gene name	**Forward primer sequence**	**Reverse primer sequence**
Tfrc	5'-CAGTAATCCCAGAGGAATCATTAG-3'	5'-CTAAACCGGGTGTATGACAATG-3'
Cre	5'-CCATATTGGCAGAACGAAAAC-3'	5'-GCTTCAAAAATCCCTTCCAGG-3'
Hjv WT	5′-GAATGGCTTCCTTCCATCAA-3′	5′-ATCTTCAAAGGCTGCAGGAA-3′
Hjv$^{-/-}$	5′-CGGATGGTTTTTGGCAGTTAG-3′	5′-GCCTTTACGATATCTCAGTCC-3′

Electron Microscopy

The heart was removed and rinsed quickly in cold Krebs-Henseleit buffer (Krebs and Henseleit, 1932), and then immersed in 5% glutaraldehyde buffer. Tissue for electron microscopic examination were post-fixed for 1 hr in 2% osmium tetroxide, dehydrated through successive acetones, infiltrated with Epon (EM bed 812; Electron Microscopy Sciences) embedded, and polymerized in a 70°C oven overnight. Epon blocks were trimmed and ultrathin sections (70nm) were cut on a Reichert-Jung Ultracut E microtome (Leica Microsystems, Wetzlar, Germany) and collected on copper mesh grids. Sections were counterstained with uranyl acetate and lead citrate and examined in Philips CM-12 electron microscope (FEI, Hillsboro, OR). Samples were prepared and imaged by Duke University Medical Center Research Electron Microscopy Services.

Western Blot Analysis

Mouse hearts were homogenized in 15 volumes of ice-cold RIPA buffer in the presence of protease inhibitors and phosphatase inhibitors (Roche) using glass homogenizers. Protein lysates were collected as the supernatant after centrifugation at 14,000 rpm for 15 min at 4 °C. Protein concentrations were determined using the DC protein assay (Bio-Rad). Antibodies were used recognizing Dpyd, Cathepsin D, Tom20, PGC1-α (Santa Cruz Biotechnology), LC3, Gabarap, p62, Beclin-1, Ulk1, Atg3, Atg4B, Atg7, Atg5, Atg12, Atg16L1, Bnip3, Nix, H-Ferritin (Cell Signaling Technology), Atg10 (ThermoFisher Scientific), Fundc1 (Aviva Systems Biology), Rcan1 (Abgent), Rpl19 (Sigma-Aldrich), Tfr1 (Invitrogen), Ppat and Atg9A (Novus Biologicals). Total OXPHOS Rodent WB Antibody Cocktail was purchased from Abcam.

RNA Extraction, Microarray Gene Expression Analysis and PCR

Total heart RNA was isolated from flash-frozen tissue using the RNeasy Fibrous Tissue Mini Kit (Qiagen) and reverse transcribed as previously described (Schmidt et al., 2008). For the microarray analysis, we used the Affymetrix GeneChip® Mouse Genome 430 2.0 Array. A minimum of 200 ng RNA was used for each sample. RNA samples were processed by the Duke Microarray Shared Resource Services. Results have been deposited online (Xu and Andrews, 2015).Transcript abundance was quantified by 2-step real-time PCR, using amplification conditions as previously described (Schmidt et al., 2008). Primers were summarized below and Rpl19 was used as an internal control.

Gene name	**Forward primer sequence**	**Reverse primer sequence**
Tfrc	5’-TCAAGCCAGATCAGCATTCTC-3’	5’-AGCCAGTTTCATCTCCACATG-3’

Nppa	5’-TCGTCTTGGCCTTTTGGCT-3’	5’-TCCAGGTGGTCTAGCAGGTTCT-3’
Nppb	5’- AAGTCCTAGCCAGTCTCCAGA-3’	5’- GAGCTGTCTCTGGGCCATTTC-3’
Myh7	5'-GTGCCAAGGGCCTGAATGAG-3'	5'-GCAAAGGCTCCAGGTCTGA-3'
Acta1	5'-ATGTGCGACGAAGACGAGACCA-3'	5'-AGGGTCAGGATACCTCGCTTG-3'
Polrmt	5'-CTCCTCCCACATGATGCTGAC-3'	5'-AATTGCTCGCGGCATACCT-3'
Nd4	5'-CATCACTCCTATTCTGCCTAGCAA-3'	5'-TCCTCGGGCCATGATTATAGTAC-3'
Cytb	5'-GCCACCTTGACCCGATTCT -3'	5'-TTGCTAGGGCCGCGATAAT-3'
Cox3	5'-CGGAAGTATTTTTCTTTGCAGGAT-3'	5'-CAGCAGCCTCCTAGATCATGTG-3'
Ppargc1a	5'-AGAAGTCCCATACACAACCGCAGTCGCAA -3'	5'-CTTGGAGCTGTTTTCTGGTGCTGCAAGGA-3'
Ppargc1b	5'-GCTCTGGTAGGGGCAGTGA-3'	5'-TCCTGTAAAAGCCCGGAGTAT-3'
Itgb1bp3	5'-TACAGCCAACGCTACTTCCT-3'	5'-GGGACTTCATGCCATCTAAA-3'
Slc3a2	5'-TGATGAATGCACCCTTGTACTTG-3'	5'-TCCCCAGTGAAAGTGGA-3'
Slc7a5	5'-TGGTCTTCGCCACCTACTTG-3'	5'-AAGCCGAGCAAAATGATGAG-3'
Art1	5'-CAGGGGCTACTCCTTTTTCC-3'	5'-CCCAGACCTGCACTTCTTTT-3'
Art4	5'-AAGAAAAGAAGTGCAGGTCTGG-3'	5'-AGAGCAGGAAGCAGAAATGG-3'
Art5	5'-TGTGTCCTCAAGAGCAGTCG-3'	5'-CAACTCTGGTTGGACAGGT-3'
Ppara	5'-GCCTGTCTGTCGGGATGT-3'	5'-GGCTTCGTGGATTCTCTTG-3'
Rxrg	5′-CCGCTGCCAGTACTGTCG-3′	5′-ACCTGGTCCTCCAAGGTGAG-3′
Fatp1	5′-CGCTTTCTGCGTATCGTCTG-3′	5′-GATGCACGGGATCGTGTCT-3′
Rcan1.1	5'-TCGACTGCGTAGATGGAGG-3'	5'-TGGTGTCCTTGTCATATGTTCTG-3'
Rcan1.4	5'-CTTGTGTGGCAAACGATGATG-3'	5'-TGGTGTCCTTGTCATATGTTCTG-3'
Map1lc3b	5'-CGTCCTGGACAAGACCAAGT-3'	5'-ATTGCTGTCCCGAATGTCTC-3'
Map1lc3	5'-TGTCCTGGATAAGACCAAGT-3'	5'-TTCATCCTTCTCCTGTTCAT-3'

a		
Lpin1	5'-CCTTCTATGCTGCTTTTGGGAACC-3'	5'-GTGATCGACCACTTCGCAGAGC-3'
Epas1	5'-TAAAGCGGCAGCTGGAGTAT-3'	5'-ACTGGGAGGCATAGCACTGT-3'
Slc2a1	5'-GCTTATGGGCTTCTCCAAACT-3'	5'-GGTGACACCTCTCCCACATAC-3'
Slc16a3	5'-TCACGGGTTTCTCCTACGC-3'	5'-GCCAAAGCGGTTCACACAC-3'
Pdk1	5'-GCTACTCAACCAGCACTCCT-3'	5'-CCTGGTGATTTCGCATTT-3'
Lonp1	5'-ATGGTGGAGGTTGAGAATGTAG-3'	5'-TGGTCTCTTCCAGAACATCTTG-3'
Bnip3	5'-GGCGTCTGACAACTTCCACT-3'	5'-AACACCCAAGGACCATGCTA-3'
Ldha	5'-AGGCTCCCCAGAACAAGATT-3'	5'-TCTCGCCCTTGAGTTTGTCT-3'
Vegfa	5'-CCTGGTGGACATCTTCCAGGAGTACC-3'	5'-GAAGCTCATCTCTCCTATGTGCTGGC-3'
Ndrg1	5'-CATTTTGCTGTCTGCCATG-3'	5'-CCATGCCAATGACACTCTTG-3'
Hk2	5'-TGATCGCCTGCTTATTCACGG-3'	5'-AACCGCCTAGAAATCTCCAGA-3'
Gpi1	5'-GCCAAAGTGAAAGAGTTTGGA-3'	5'-ATGGAAAGTCCAATGGCTGA-3'
Pfkl	5'-CTGCTGGTGATTGGTGGCTTTG-3'	5'-TTGCTGATGGTGGCTGGGATG-3'
Aldoa	5'-GTGGGAAGAAGGAGAACCTG-3'	5'-CTGGAGTGTTGATGGAGCAG-3'
Tpi	5'-CCAGGAAGTTCTTCGTTGGGG-3'	5'-CAAAGTCGATGTAAGCGGTGG-3'
Gapdh	5'-CATGGCCTTCCGTGTTCCTA-3'	5'-GCGGCACGTCAGATCCA-3'
Pgk1	5'-CTCCGCTTTCATGTAGAGGAAG-3'	5'-GACATCTCCTAGTTTGGACAGTG-3'
Pgam1	5'-CTGTGCAGAAGAGAGCAATCC-3'	5'-CTGTCAGACCGCCATAGTGT-3'
Eno1	5'-TGCGTCCACTGGCATCTAC-3'	5'-CAGAGCAGGCGCAATAGTTTTA-3'
Pkm2	5'-TGTCTGGAGAAACAGCCAAG-3'	5'-TCCTCGAATAGCTGCAAGTG-3'
Sqstm1	5'-GAAGAATGTGGGGGAGAGTGTGG-3'	5'-TGCCTGTGCTGGAACTTTCTGG-3'
Rpl19	5'-AGGCATATGGGCATAGGGAAGAG-3'	5'-TTG ACC TTC AGG TAC AGG CTG TG-3'
c-Myc	5'-CCTAGTGCTGCATGAGGAGA-3'	5'-TCTTCCTCATCTTCTTGCTCTTC-3'
Optn	5'-CAAGTGACCTCTCTGTTTAAGG-3'	5'-GCCTGCTCCATCTTGATTT-3'

Atf4	GAGCTTCCTGAACAGCGAAGTG	TGGCCACCTCCAGATAGTCATC
Ddit3	GACTCAGCTGCCATGACTG	GCGACAGAGCCAGAATAACAG
Fgf21	TCCAAATCCTGGGTGTCAAA	CAGCAGCAGTTCTCTGAAGC

Spectrophotometric Analysis of Activities of Single Respiratory Chain Complexes I–IV

All reactions were performed using a computer-tunable spectrophotometer (Spectramax Plus Microplate Reader, Molecular Devices, Sunnyvale, CA) at 20-s intervals for 5 minutes at room temperature. The reaction conditions for assaying activity of each complex were described previously (Janssen et al., 2007; Spinazzi et al., 2011). The total volume of each reaction was 200 μl. Each sample had three replicates for total activity for and inhibitor-insensitive activity. For complexes I and II, we measured 2,6-dichloroindophenol (DCIP) reduction by electrons accepted from decylubiquinol. The reduction of DCIP was followed by spectrophotometer at 600 nm. 20 μg heart homogenates were used in each reaction for both complex I and complex II activity assays. Complex III activity was measured by following the reduction of cytochrome c at 550 nm. Complex IV activity was measured by following the oxidation of reduced cytochrome c at 550 nm. 3.6 μg and 1 μg of heart homogenate was used in each reaction for complex III and complex IV activity assays, respectively. The addition of standard respiratory chain inhibitors for each complex was used to ascertain the specificity of the enzymatic assays. The same amount of protein was used for each sample.

Statistical Analysis

One-way ANOVA was performed when the comparison was between the means of WT and Tfr1$^{hrt/hrt}$. Two-way ANOVA followed by Bonferroni *post-hoc* was performed for multiple comparisons. Survival analysis (**Fig 7E**) was performed using Logrank in Graphpad software. $P < 0.05$ was considered statistically significant.

An independent statistical analysis for survival analysis was also performed by Dr. Kingshuk Roy Choudhury as follows:

We modelled the survival time as follows:

$$T_{ij} = \mu + \beta_j + \varepsilon_{ij}$$

Where T_{ij} is the survival time (in days) for the *i*-th mouse in the *j*-th group. The model explains this in terms of a baseline mean μ, an effect β_j for the *j*-th group (relative to baseline) and inter-mouse variation ε_{ij}. We fit the model using ordinary least squares (1-way ANOVA).

For the NR rescue experiment, the baseline is Tfr1$^{hrt/hrt}$ with PBS (Table A). There is no significant improvement in mean survival when using just Tfr1$^{hrt/hrt}$ (0.625 days, p-value = 0.26, Table B). There is, however a significant improvement in the Tfr1$^{hrt/hrt}$ with NR group (2.29 days, p-value <0.001). In WT, the improvement is over 19.6 days (all observations were censored in this group).

Analysis of survival:

	Effect	**Std. Error**	**t value**	**P-value**
Baseline	10.375	0.402	25.787	
Tfr1$^{hrt/hrt}$	0.625	0.540	1.158	0.258

$Tfr1^{hrt/hrt}$ with NR	2.292	0.484	4.739	<0.001
WT with NR	19.625	0.493	39.827	<0.001
WT with PBS	19.625	0.569	34.491	<0.001

SUPPLEMENTAL REFERENCES:

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