Expanded View Figures

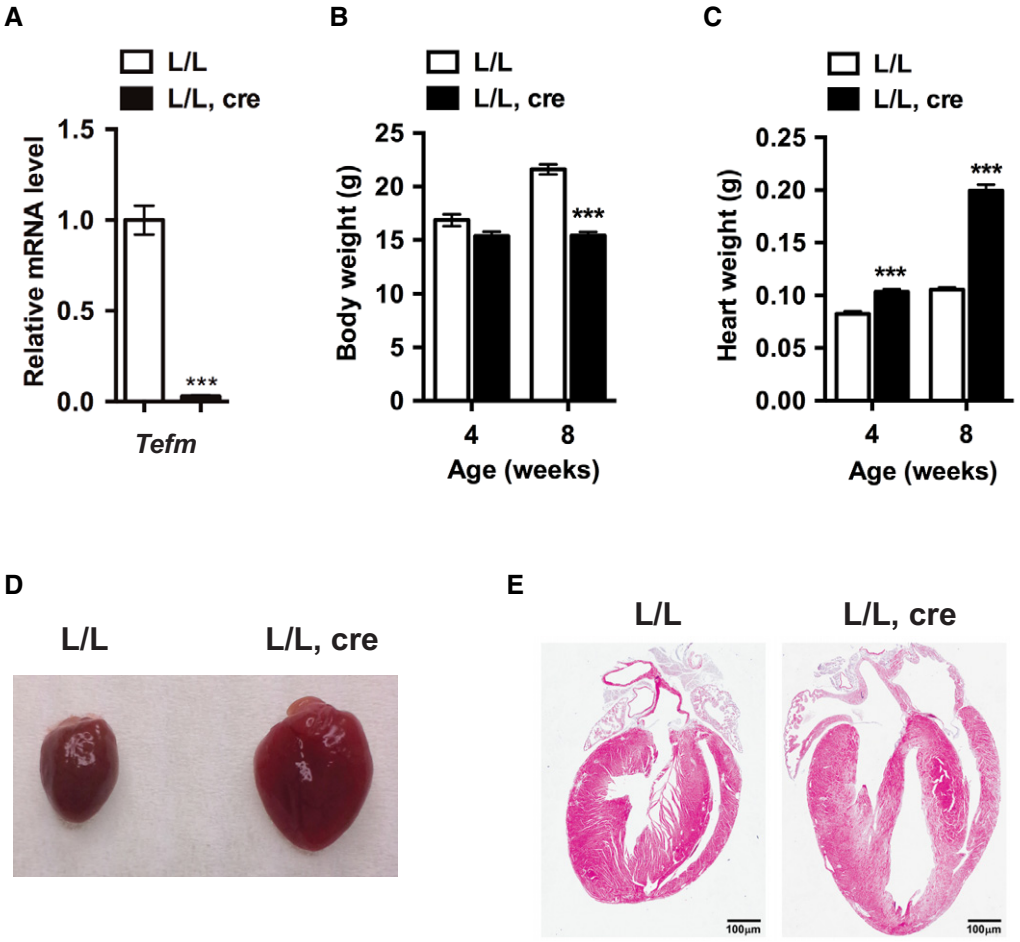

Figure EV1. Disruption of *Tefm* in the germline and heart.

A RT–qPCR analyses of the RNA level of *Tefm* in 8-week-old control (L/L) and *Tefm* knockout (L/L, cre) mice (n = 10 mice for each group).

B Body weight of 4- and 8-week-old control and *Tefm* knockout mice. At 4 weeks: L/L n = 22, L/L, cre n = 15; 8 weeks: L/L n = 41, L/L, cre n = 39.

C Heart weight of 4- and 8-week-old control and *Tefm* knockout mice. At 4 weeks: L/L n = 22, L/L, cre n = 15; 8 weeks: L/L n = 41, L/L, cre n = 39.

D Representative images of hearts of 8-week-old control and *Tefm* knockout mice (n = 5 mice for each group).

E Representative images of hematoxylin and eosin staining showing heart structure and morphology in 8-week-old control and *Tefm* knockout mice (n = 5 mice for each group). Scale bar, 100 μm.

Data information: In (A–C), data are presented as mean ± SEM. ***P < 0.001; Student's t-test. (A) P = 3,666241e-010; (B) 4 weeks P = 0.0541737, 8 weeks P = 1.126271e-016; (C) 4 weeks P = 1.207998e-006, 8 weeks P = 1.305101e-026.

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Figure EV2. Depletion of TEFM in heart causes mitochondrial dysfunction.

A Quantification of the relative mitochondrial mass by electron microscopy analysis of the heart of 8-week-old control and *Tefm* knockout mice (n = 5 mice for each group).

B Quantitative PCR analyses of the relative mtDNA levels in 4- and 8-week-old control and *Tefm* knockout mice (n = 8 mice at each time-point for each group).

C, D Oxygen consumption rates measured using an Oroboros oxygen electrode in heart mitochondria isolated from 8-week-old control and *Tefm* knockout mice (n = 5 mice for each group). Phosphorylating, non-phosphorylating, and uncoupled respiration under carbonyl cyanide 3-chlorophenylhydrazone states were measured using pyruvate, glutamate, and malate or succinate and rotenone as substrates.

E Western blot analyses of subunits of OXPHOS at the age of 8 weeks (n = 15 mice for each group). Nucleus-encoded subunits of complex I (NDUFB8), complex II (SDHB), complex III (UQCRC2), complex IV (MTCOX1), and complex V (ATP5A) were analyzed. VDAC was used as loading control.

F Analysis of the assembly of the OXPHOS complexes by BN-PAGE in heart mitochondria of 8-week-old control *Tefm* knockout mice (n = 6 mice for each group). OXPHOS complexes were detected by western blot following the BN-PAGE, including complex I (NDUFV2), complex II (SDHA), complex III (UQCRC1), complex IV (COXIV), and complex V (ATP5A).

Data information: In (A–D), data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student's t-test. A: $P = 0.0003$; B: 4 weeks: *Nd1/18S* $P = 0.0135973$, *Nd6/18S* $P = 0.00955924$, *Cyb/18S* $P = 0.0114725$, 8 weeks: *Nd1/18S* $P = 1.449815e\text{-}005$, *Nd6/18S* $P = 6.995544e\text{-}005$, *Cyb/18S* $P = 4.002903e\text{-}006$. C: P = Complex I Phosphorylating P=1.355075e-008, Non-phosphorylating $P = 0.00279867$, Uncoupled $P = 0.000431079$; D: Complex II Phosphorylating P=1.399847e-007, Non-phosphorylating $P = 4.396380e\text{-}008$, Uncoupled $P = 1.662621e\text{-}006$.

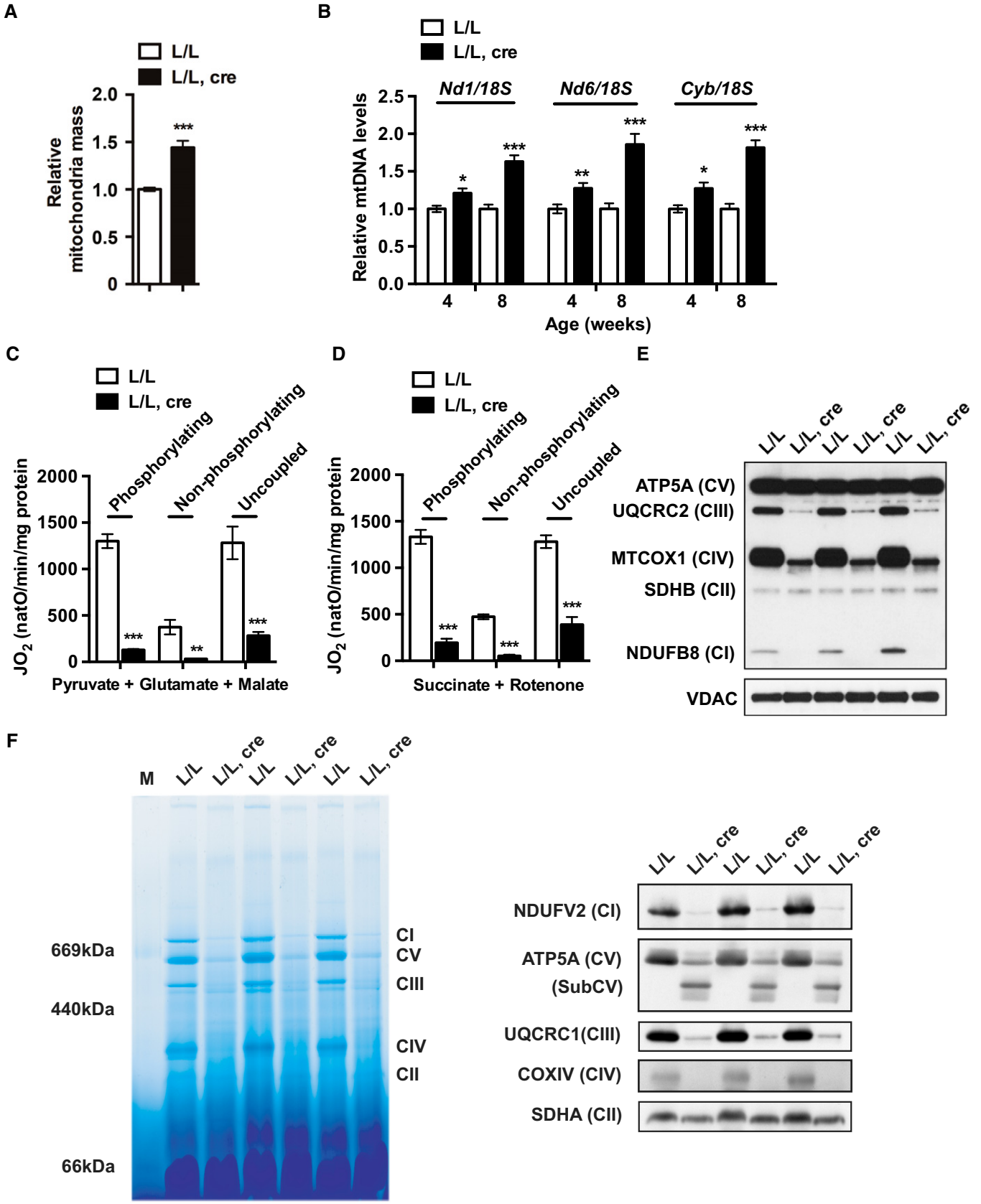

A
L/L
L/L, cre
Relative mitochondria mass
2.0
1.5
1.0
0.5
0
***
B
L/L
L/L, cre
Nd1/18S
Nd6/18S
Cyb/18S
Relative mtDNA levels
2.5
2.0
1.5
1.0
0.5
0.0
*
***
**
***
*
***
4
8
4
8
4
8
Age (weeks)
C
L/L
L/L, cre
Phosphorylating
Non-phosphorylating
Uncoupled
JO2 (natO/min/mg protein
2000
1500
1000
500
0
***
**
***
Pyruvate + Glutamate + Malate
D
L/L
L/L, cre
Phosphorylating
Non-phosphorylating
Uncoupled
JO2 (natO/min/mg protein
2000
1500
1000
500
0
***
***
***
Succinate + Rotenone
E
L/L
L/L, cre
L/L
L/L, cre
L/L
L/L, cre
ATP5A (CV)
UQCRC2 (CIII)
MTCOX1 (CIV)
SDHB (CII)
NDUFB8 (CI)
VDAC
F
M
L/L
L/L, cre
L/L
L/L, cre
L/L
L/L, cre
669kDa
440kDa
66kDa
CI
CV
CIII
CIV
CII
L/L
L/L, cre
L/L
L/L, cre
L/L
L/L, cre
NDUFV2 (CI)
ATP5A (CV)
(SubCV)
UQCRC1(CIII)
COXIV (CIV)
SDHA (CII)

Figure EV2.

Figure EV3. Proteome of *Tefm* knockout heart mitochondria.

A Beeswarm plot presenting the distribution of differentially expressed proteins (Benjamini–Hochberg adj. $P < 0.05$) in mitochondrial pathways after knockout of *Tefm* at 8 weeks of age ($n = 5$ mice for each group).

B Heatmap showing differentially expressed respiratory chain complexes assembly factors (upper panel) and RNA metabolism-related proteins (lower panel) in *Tefm* knockout mice in comparison to controls [L/L, cre/L/L].

C Volcano scatterplots depicting the expression levels of the subunits of the small (28S) and large (39S) ribosomes in green and blue, respectively. Red dotted line, Benjamini–Hochberg adj. $P = 0.05$.

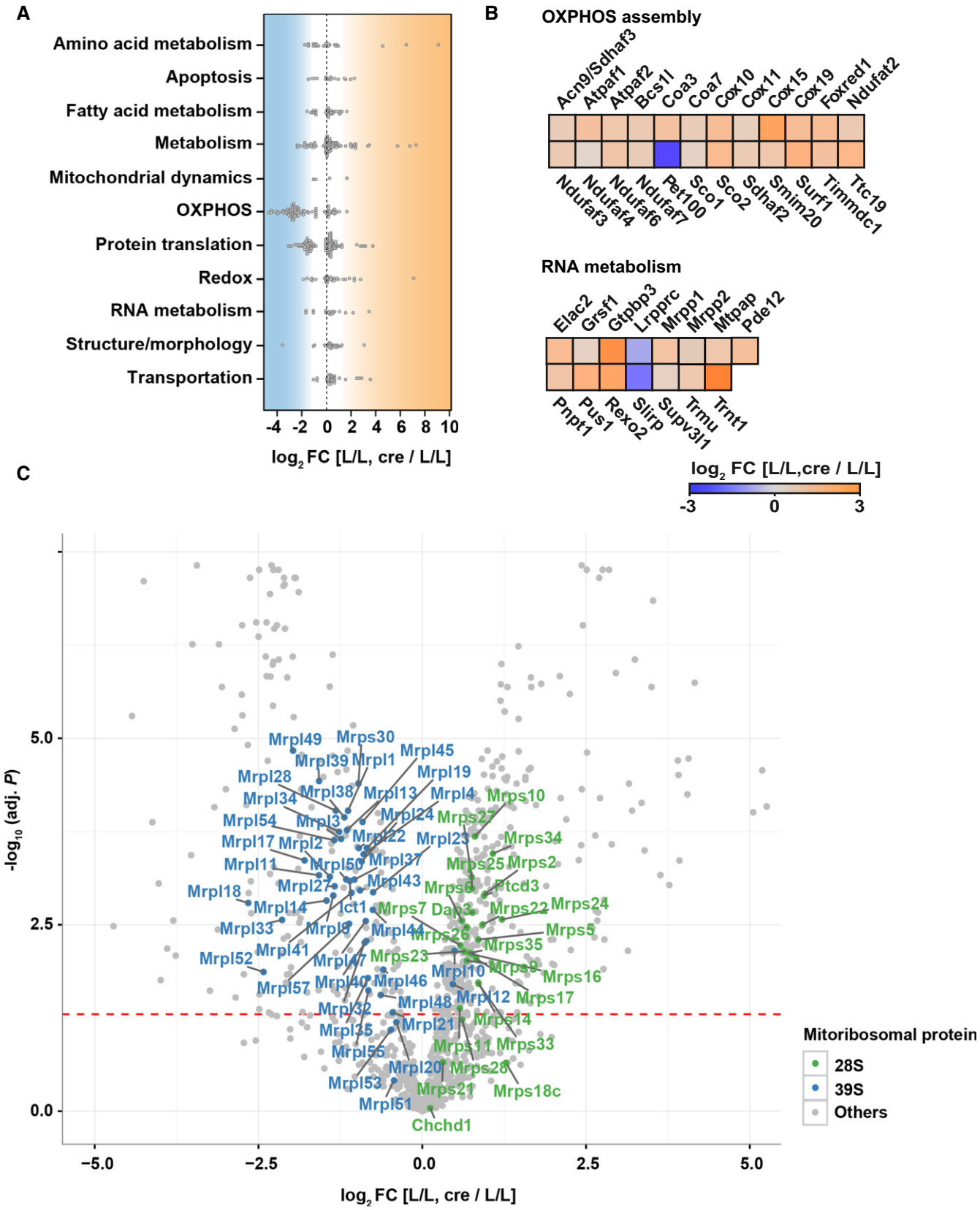

A
Amino acid metabolism
Apoptosis
Fatty acid metabolism
Metabolism
Mitochondrial dynamics
OXPHOS
Protein translation
Redox
RNA metabolism
Structure/morphology
Transportation
-4 -2 0 2 4 6 8 10
log2 FC [L/L, cre / L/L]
B
OXPHOS assembly
Acn9/Sdhaf3
Atpaf1
Atpaf2
Bcs1l
Coa3
Coa7
Cox10
Cox11
Cox15
Cox19
Foxred1
Ndufat2
Ndufaf3
Ndufaf4
Ndufaf6
Ndufaf7
Pet100
Sco1
Sco2
Sdhaf2
Smim20
Surf1
Timmdc1
Ttc19
RNA metabolism
Elac2
Grsf1
Gtpbp3
Lrpprc
Mrpp1
Mrpp2
Mtpap
Pde12
Pnpt1
Pus1
Rexo2
Slirp
Supv3l1
Trmu
Trnt1
C
log2 FC [L/L,cre / L/L]
-3 0 3
-log10 (adj. P)
5.0
2.5
0.0
−5.0 −2.5 0.0 2.5 5.0
log2 FC [L/L, cre / L/L]
Mrpl49 Mrps30 Mrpl45 Mrpl39 Mrpl1 Mrpl19 Mrpl28 Mrpl38 Mrpl13 Mrpl4 Mrps10 Mrpl34 Mrpl24 Mrps27 Mrpl54 Mrpl3 Mrpl22 Mrpl23 Mrps34 Mrpl17 Mrpl2 Mrps25 Mrps2 Mrpl11 Mrpl50 Mrpl37 Mrpl43 Mrps6 Ptcd3 Mrpl18 Mrpl27 Mrps7 Dap3 Mrps22 Mrps24 Mrpl14 Ict1 Mrpl8 Mrpl44 Mrps26 Mrps5 Mrpl33 Mrps35 Mrpl41 Mrpl47 Mrps23 Mrps9 Mrps16 Mrpl52 Mrpl10 Mrpl57 Mrpl40 Mrpl46 Mrpl12 Mrps17 Mrpl48 Mrpl32 Mrpl21 Mrps14 Mrpl35 Mrps11 Mrps33 Mrpl55 Mrpl20 Mrps28 Mrpl53 Mrps21 Mrps18c Mrpl51 Chchd1
Mitoribosomal protein
28S
39S
Others

Figure EV3.

Figure EV4. Characterization of whole-body *Tefm* and *Polrmt* double-heterozygous knockout mice and the effects of TEFM depletion on mitochondrial transcripts and the mtDNA replication machinery.

A Western blot analyses of POLRMT and TEFM protein levels in heart mitochondrial extracts from 16-week-old *Tefm* and *Polrmt* double-heterozygous knockout mice (n = 6 mice for each group). VDAC was used as loading control.

B Quantitative PCR analyses of the relative mtDNA levels in 16-week-old *Tefm* and *Polrmt* double-heterozygous knockout mice (n = 6 mice for each group). *18S* was used to normalize for nuclear input.

C Western blot analyses of subunits from individual OXPHOS complexes at the age of 16 weeks (n = 6 mice for each group). Nucleus-encoded subunits of complex I (NDUFB8), complex II (SDHB), complex III (UQCRC2), complex IV (MTCOX1), and complex V (ATP5A) were analyzed. VDAC was used as loading control.

D Northern blot analyses of mitochondrial transcripts of mt-rRNAs and mt-mRNAs in 16-week-old *Tefm* and *Polrmt* double-heterozygous knockout mouse hearts (n = 8 mice for each group). *18S rRNA* was used as loading control.

E Northern blot analyses of mitochondrial mt-tRNA transcripts in 16-week-old *Tefm* and *Polrmt* double-heterozygous knockout mouse hearts (n = 8 mice for each group). *5.8S rRNA* was used as loading control.

F RT–qPCR analyses of the mitochondrial transcripts in 8-week-old control and *Tefm* knockout mice (n = 12 mice for each group).

G Western blot analyses of TFAM and TFB2M in the 8-week-old control and *Tefm* knockout mice (n = 12 mice for each group). SDHA was used as loading control.

H Western blot analyses of the subunit A of the DNA polymerase γ (POLγA), DNA helicase TWINKLE, and ssDNA-binding protein (SSBP1) in 8-week-old control and *Tefm* knockout mice (n = 12 mice for each group). VDAC was used as loading control.

Data information: In (B), data are presented as mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA). There is no difference between groups. In (F), data are presented as mean ± SEM. ***$P < 0.001$; Student's t-test. (F) HSP transcripts: *12S rRNA* P = 0.000728399; *16S rRNA* P = 1.10763E-06; *Nd1* P = 2.63285E-06; *Nd2* P = 0.00152084; *Cox1* P = 1.29269E-06; *Cox2* P = 1.9267E-05; *Atp8* P = 3.05955E-07; *Atp6* P = 0.000849436; *Cox3* P = 4.98506E-05; *Nd3* P = 4.07442E-05; *Nd4* P = 1.43029E-06; *Nd5* P = 6.04136E-05; *Cyb* P = 0.00882284; LSP transcript: *Nd6* P = 0.00139773.

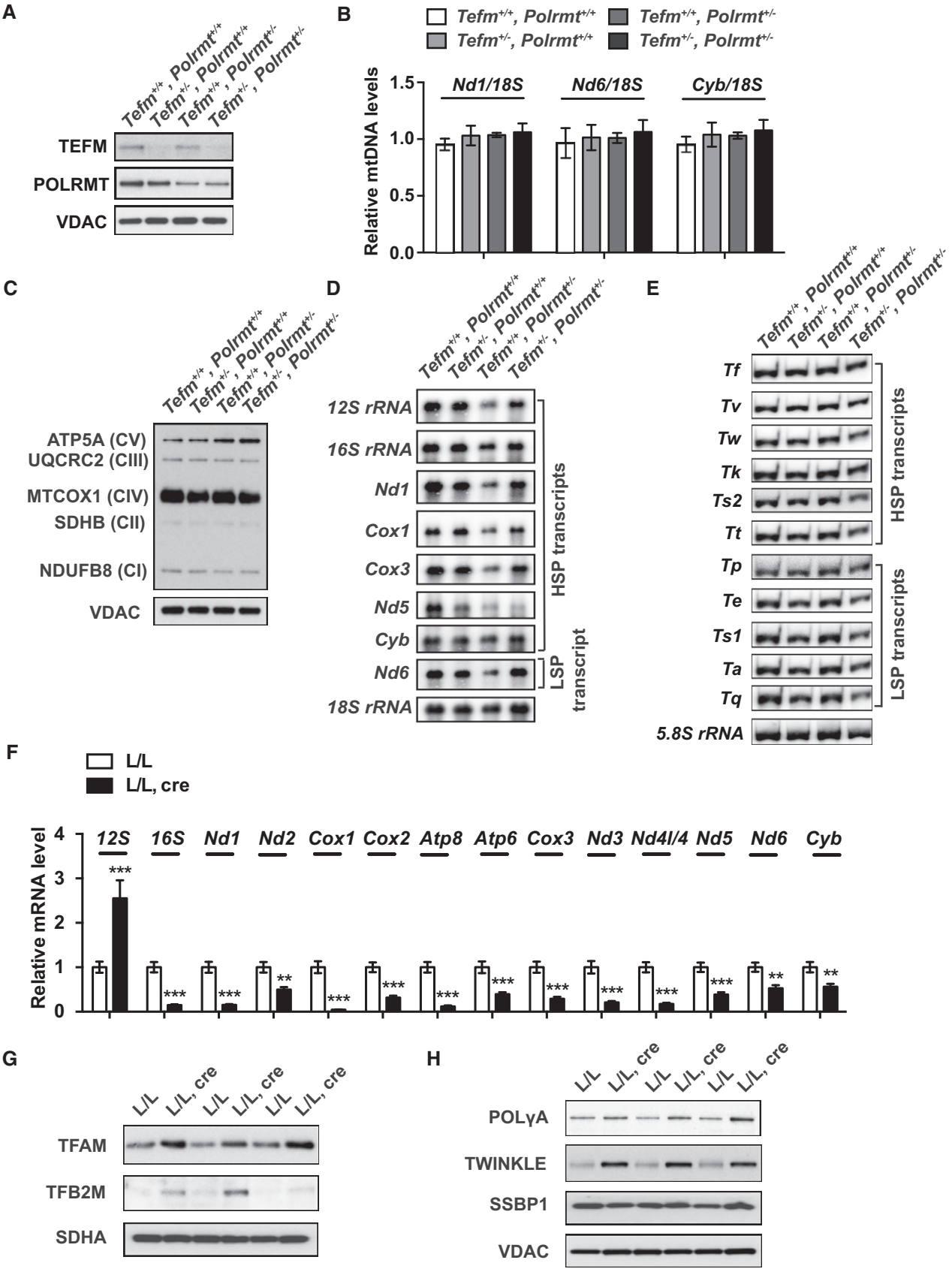
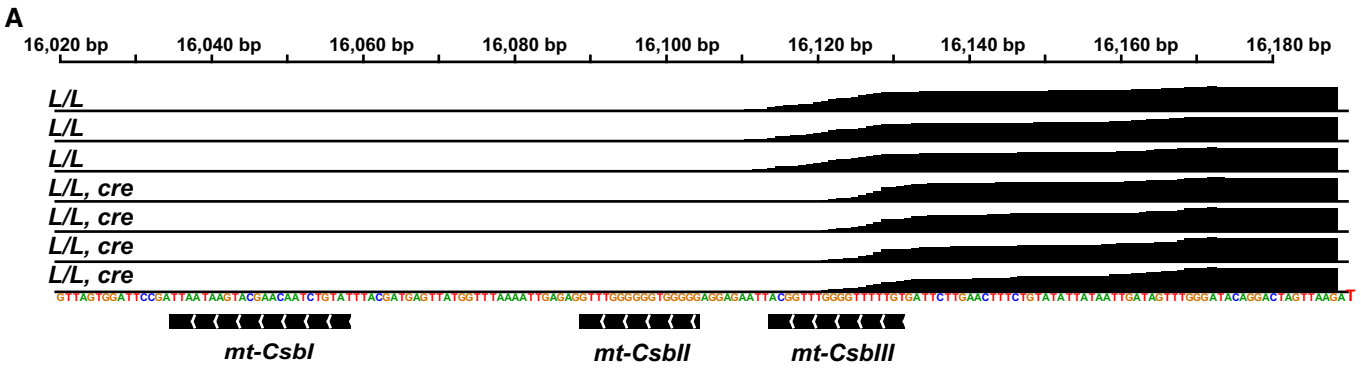

A
Tefm+/+, Polrmt+/+
Tefm+/-, Polrmt+/+
Tefm+/+, Polrmt+/-
Tefm+/-, Polrmt+/-
TEFM
POLRMT
VDAC
B
Tefm+/+, Polrmt+/+
Tefm+/-, Polrmt+/+
Tefm+/+, Polrmt+/-
Tefm+/-, Polrmt+/-
Relative mtDNA levels
1.5
1.0
0.5
0.0
Nd1/18S
Nd6/18S
Cyb/18S
C
ATP5A (CV)
UQCRC2 (CIII)
MTCOX1 (CIV)
SDHB (CII)
NDUFB8 (CI)
VDAC
D
12S rRNA
16S rRNA
Nd1
Cox1
Cox3
Nd5
Cyb
Nd6
18S rRNA
HSP transcripts
LSP transcript
E
Tf
Tv
Tw
Tk
Ts2
Tt
Tp
Te
Ts1
Ta
Tq
5.8S rRNA
HSP transcripts
LSP transcripts
F
L/L
L/L, cre
Relative mRNA level
4
3
2
1
0
12S
16S
Nd1
Nd2
Cox1
Cox2
Atp8
Atp6
Cox3
Nd3
Nd4l/4
Nd5
Nd6
Cyb
***
**
G
L/L
L/L, cre
TFAM
TFB2M
SDHA
H
POLγA
TWINKLE
SSBP1
VDAC

Figure EV4.



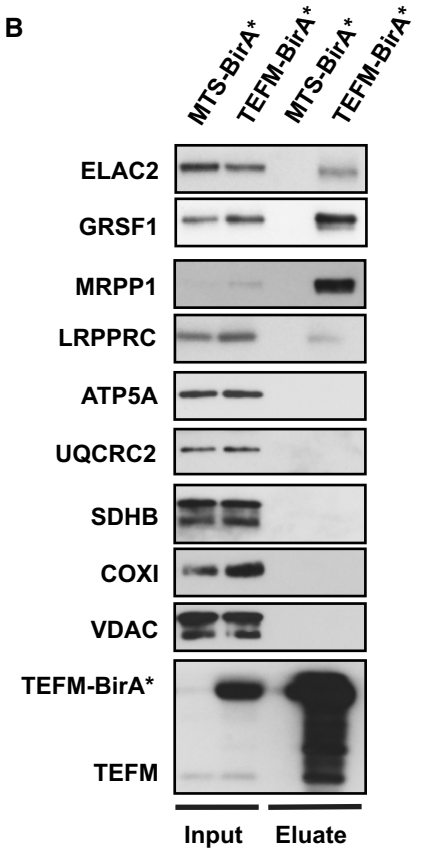


Figure EV5. Transcriptome-wide analysis of *Tefm* knockout hearts and TEFM interacts with mtRNA processing factors.

A Normalized read counts corresponding to the light-promoter region from small RNA sequencing of mitochondrial transcripts showing the *7S RNA* read counts (L/L, $n = 4$; L/L, cre $n = 3$).

B Western blot analyses of RNA processing-related proteins in isolated mitochondria (input) and eluates when TEFM-BirA* was used as a bait. Eluate (100%), input (1%).