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Supplementary Materials for

Mitophagy controls beige adipocyte maintenance through a Parkin-dependent and UCP1-independent mechanism

Xiaodan Lu, Svetlana Altshuler-Keylin, Qiang Wang, Yong Chen, Carlos Henrique Sponton, Kenji Ikeda, Pema Maretich, Takeshi Yoneshiro, Shingo Kajimura*

*Corresponding author. Email: shingo.kajimura@ucsf.edu

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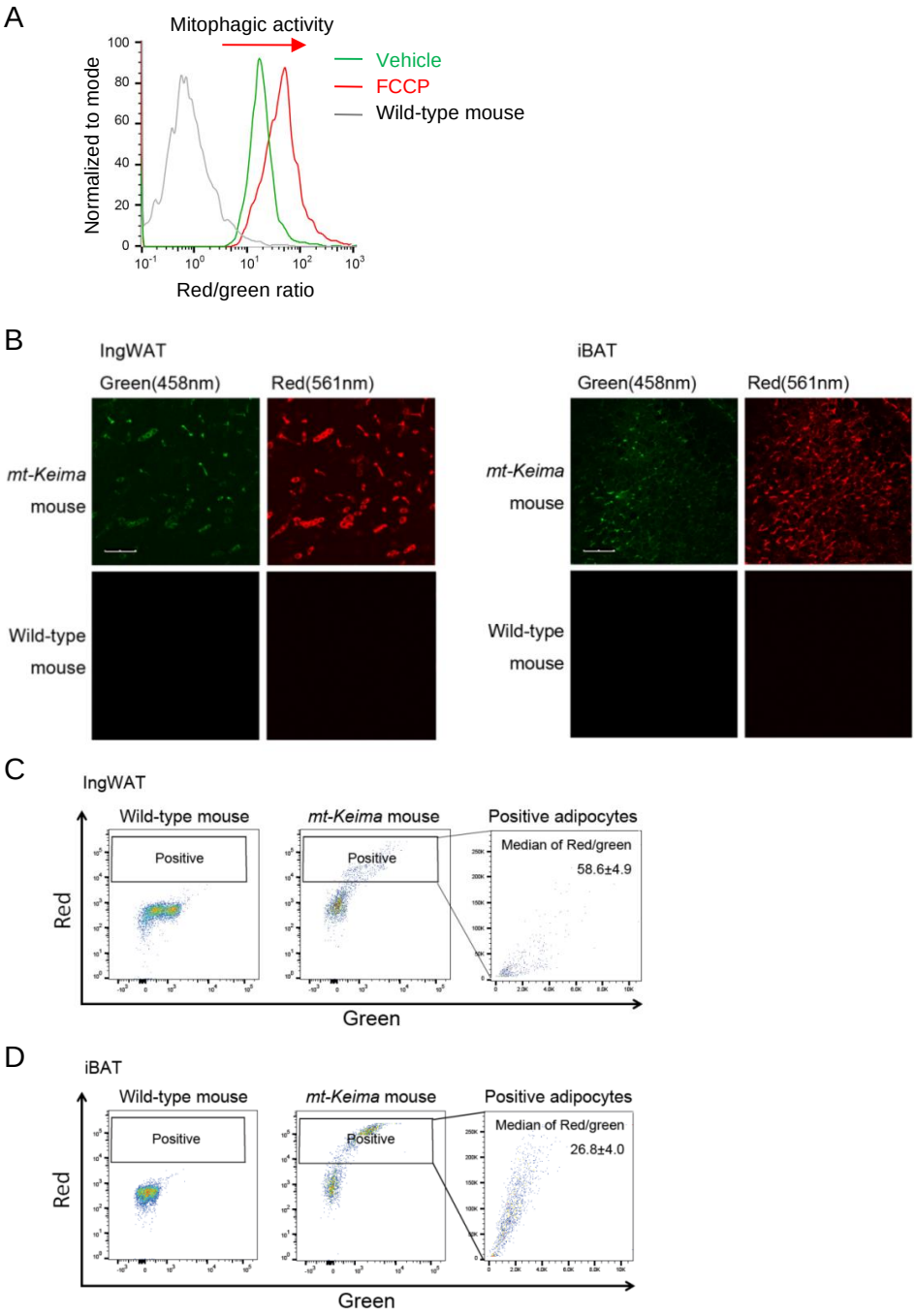


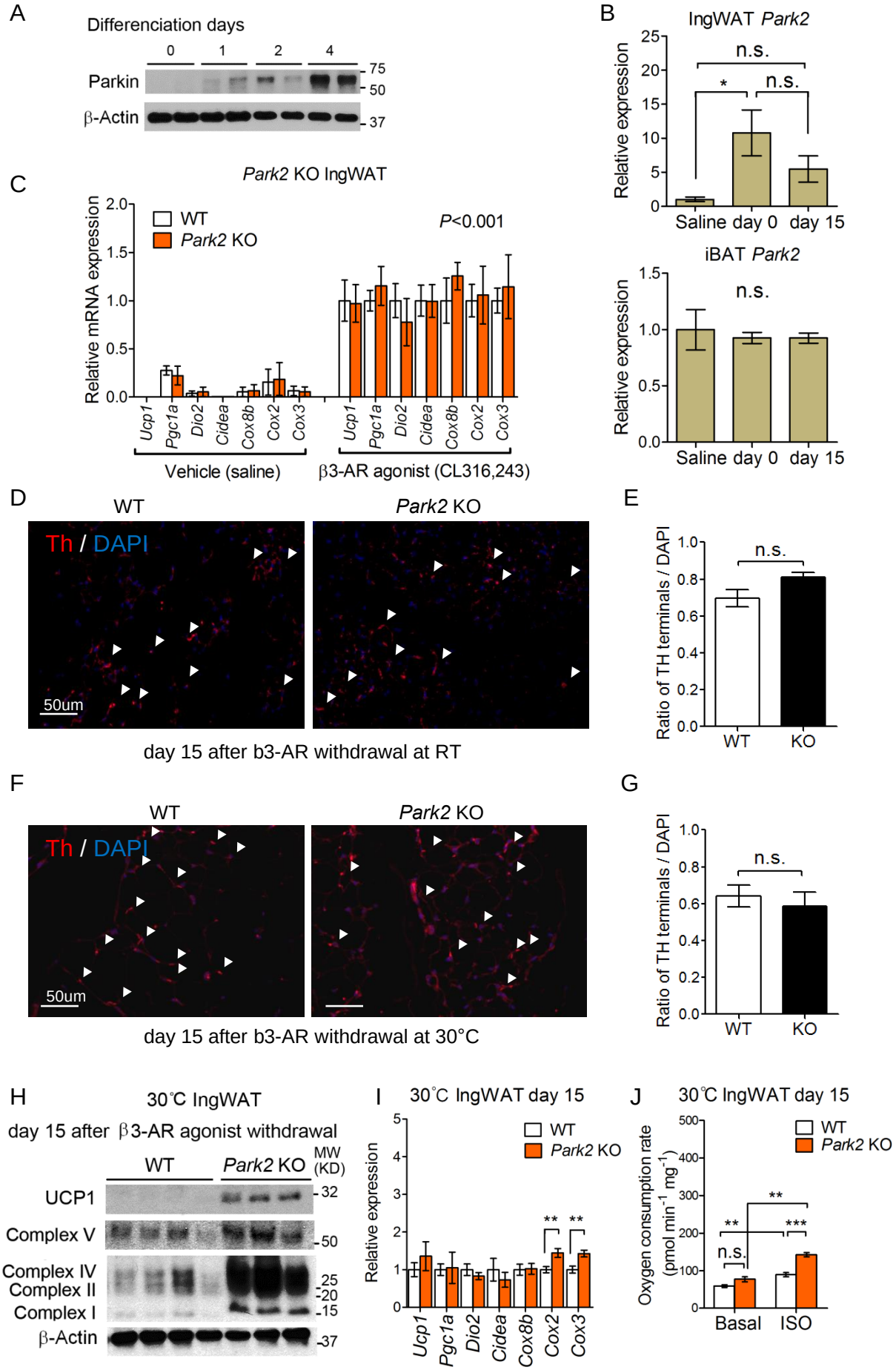
Supplementary Figure 1. Validation of mitophagy monitoring in *mt-Keima* mice.

(A) Mature adipocytes were isolated from the inguinal white adipose tissue of *mt-Keima* mice or wild-type mice (gray) as a negative control. Vehicle (green) or FCCP at 10 μM (red) were added to the isolated adipocytes. Signals from green (458nm) and red (561nm) channels were detected by flow cytometry. n=3 mice per group.

(B) Confocal images of green (458nm) and red (561nm) fluorescence in inguinal WAT (left) and interscapular BAT (right) from *mt-Keima* mice. Adipose tissues from wild-type mice were included as a negative control. Scale bars, 100 μm.

(C) Mature adipocytes were isolated from the inguinal WAT from *mt-Keima* mice or wild-type control mice.

(D) Mature adipocytes were isolated from the interscapular BAT from *mt-Keima* mice or wild-type control mice.


A
Differenciation days
0
1
2
4
Parkin
β-Actin
75
50
37
B
IngWAT Park2
Relative expression
n.s.
*
n.s.
Saline day 0
day 15
iBAT Park2
n.s.
C
Park2 KO IngWAT
WT
Park2 KO
P<0.001
Relative mRNA expression
Ucp1
Pgc1a
Dio2
Cidea
Cox8b
Cox2
Cox3
Vehicle (saline)
β3-AR agonist (CL316,243)
D
WT
Park2 KO
Th / DAPI
50um
day 15 after b3-AR withdrawal at RT
E
Ratio of TH terminals / DAPI
n.s.
WT
KO
F
WT
Park2 KO
Th / DAPI
50um
day 15 after b3-AR withdrawal at 30°C
G
Ratio of TH terminals / DAPI
n.s.
WT
KO
H
30℃ IngWAT
day 15 after β3-AR agonist withdrawal
WT
Park2 KO
MW (KD)
UCP1
Complex V
Complex IV
Complex II
Complex I
β-Actin
32
50
25
20
15
37
I
30℃ IngWAT day 15
WT
Park2 KO
Relative expression
**
**
Ucp1
Pgc1a
Dio2
Cidea
Cox8b
Cox2
Cox3
J
30℃ IngWAT day 15
WT
Park2 KO
Oxygen consumption rate (pmol min-1 mg-1)
**
**
***
n.s.
Basal
ISO

Supplementary Figure 2. Genetic requirement of Parkin for beige adipocyte maintenance in vivo.

(A) Wild-type mouse pre-adipocytes were differentiated in culture. Parkin protein expression was detected by immunoblotting in total cell lysates collected at day 0, day 1, day 2, and day 4 of differentiation. ß-actin was used as a loading control. The molecular weight (MW) is shown on the right. n=2 wells of cell samples per group. Data are representative of three independent experiments.

(B) Relative mRNA level of *Park2* in the inguinal WAT and the interscapular BAT from control and *Park2* KO mice at day 0 and day 15 following ß3-AR agonist withdrawal. **P*<0.05 by one-way ANOVA with post hoc test by Tukey's method. n.s., not significant. n=3-4 mice per group. Data are presented as mean ± s.e.m.

(C) Relative mRNA levels of thermogenic genes (as indicated) in the inguinal WAT of control and *Park2* KO mice. ****P*<0.001 by two-way ANOVA with post hoc test by Tukey's method between ß3-AR agonist-treated group and vehicle-treated group. n=3-4 mice per group. Data are presented as mean ± s.e.m.

(D) Immunohistochemistry staining of tyrosine hydroxylase (TH) in the inguinal WAT of WT and *Park2* KO mice at day 15 following ß3-AR agonist withdrawal under ambient temperature (RT). DAPI staining was performed to visualize the nucleus. Scale bar, 50 µm.

(E) Quantification of TH-positive cells in (D). n.s., not significant by unpaired Student's *t*-test. n=4 mice per group. Data are presented as mean ± s.e.m.

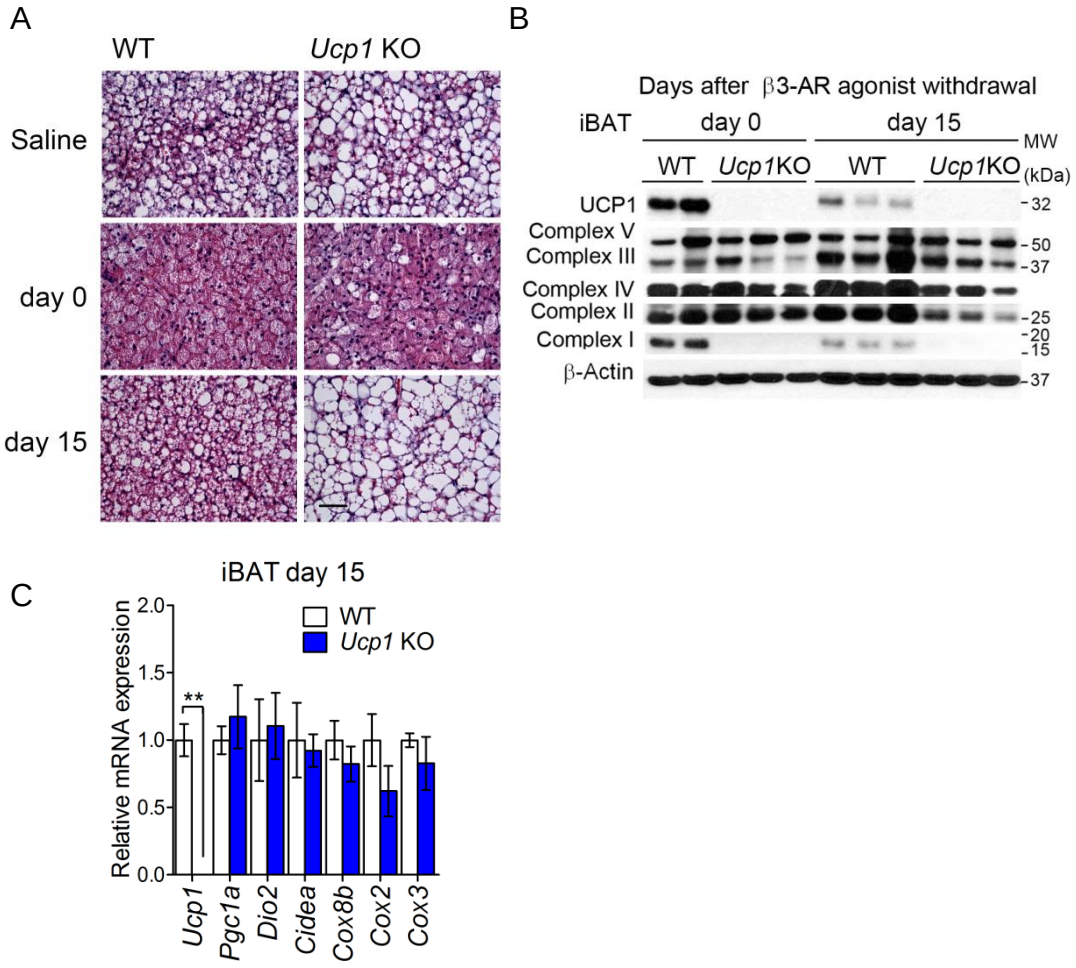
(F) Immunohistochemistry staining of TH in the inguinal WAT of WT and *Park2* KO mice at day 15 following ß3-AR agonist withdrawal at thermoneutrality (30˚C). DAPI staining was performed to visualize the nucleus. Scale bar, 50 µm.

(G) Quantification of TH-positive cells in (D). n.s., not significant by unpaired Student's *t*-test. n=4 mice per group. Data are presented as mean ± s.e.m.

(H) Representative immunoblotting for UCP1 and mitochondrial complexes (as indicated) in the inguinal WAT of control and *Park2* KO mice treated with the ß3-AR agonist CL316,243 at day 15 at thermoneutrality (30˚C). ß-actin was used as a loading control. The molecular weight (MW) is shown on the right. n=3-4 mice per group.

(I) Relative mRNA expression of thermogenic genes (as indicated) in the inguinal WAT of control and *Park2* KO mice treated with CL316,243 at day 15 under thermoneutrality (30˚C). ***P*<0.01 by unpaired Student's *t*-test. n=7-8 mice per group. n.s., not significant. Data are presented as mean ± s.e.m.

(J) OCR in the inguinal WAT of control and *Park2* KO mice at day 15 following ß3-AR agonist withdrawal under thermoneutrality (30˚C). OCR data were shown per 1 mg of tissue. Tissues were treated with isoproterenol (ISO) or vehicle (basal). ***P*<0.01, ****P*<0.001 by two-way ANOVA with post hoc test by paired or unpaired Student's *t*-test. n=4 mice per group. n.s., not significant. Data are presented as mean ± s.e.m.

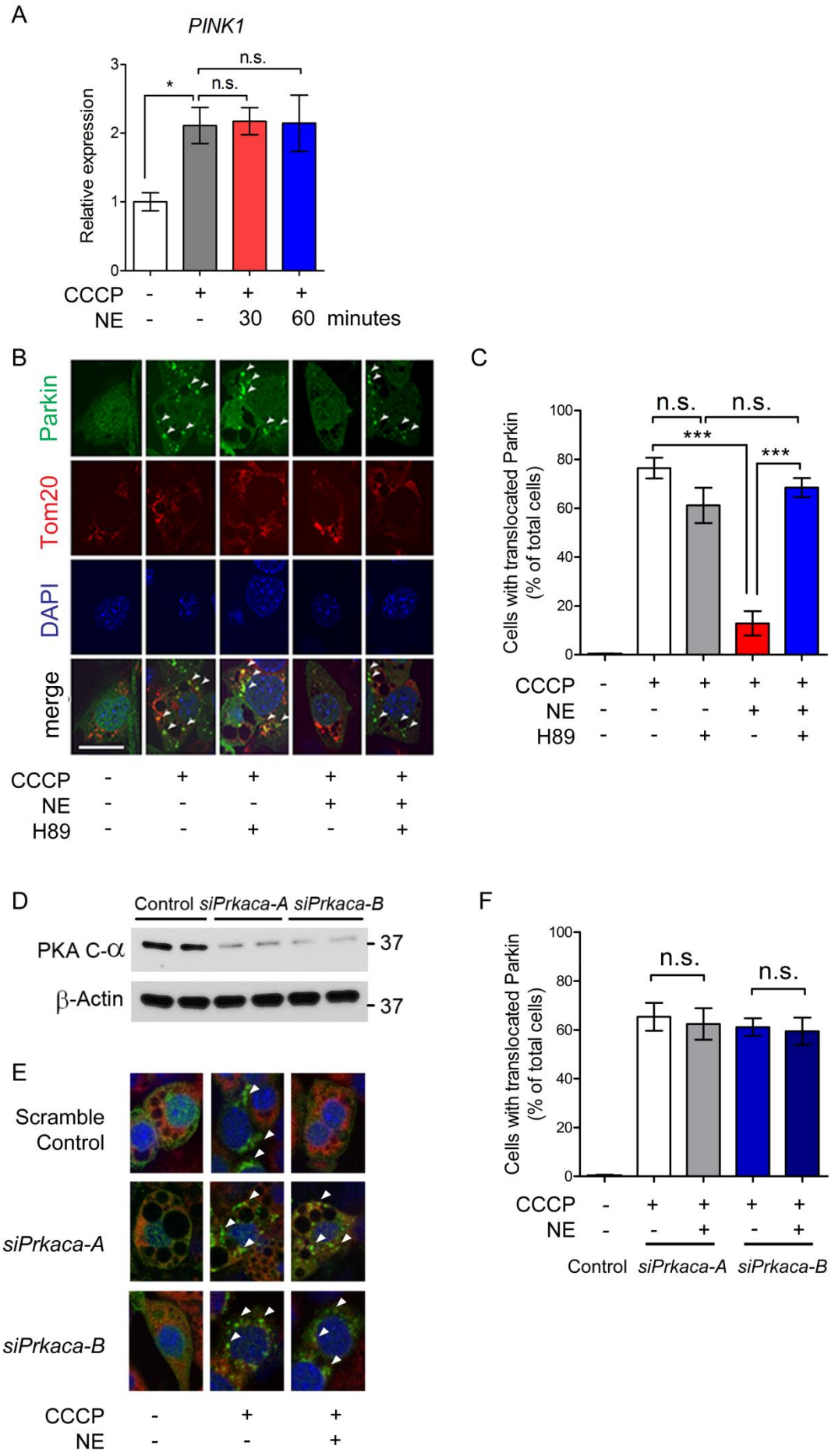


Supplementary Figure 3. Analyses of the iBAT of *Ucp1* KO mice after β_3-AR agonist withdrawal.

(A) Hematoxylin and eosin (H & E) staining of the interscapular BAT of control and *Ucp1* KO mice treated with vehicle (saline) or CL316,243 at day 0 and day 15 following ß3-AR agonist withdrawal. n=3 mice per group. Scale bars, 100μm.

(B) Representative immunoblotting for UCP1 and mitochondrial complexes (as indicated) in the interscapular BAT of control and *Ucp1* KO mice treated with CL316,243 at day 0 and day 15 following ß3-AR agonist withdrawal. ß-actin was used as a loading control. The molecular weight (MW) is shown on the right. Data are representative of three independent experiments.

(C) Relative mRNA expression of indicated genes in the interscapular BAT of control and *Ucp1* KO mice treated with CL316,243 at day 15 following ß3-AR agonist withdrawal. n=3 mice per group. $^{**}P<0.01$ by unpaired Student's *t*-test. n.s., not significant. Data are expressed as mean ± s.e.m.


A
PINK1
Relative expression
n.s.
n.s.
*
CCCP - + + +
NE - - 30 60 minutes
B
Parkin
Tom20
DAPI
merge
CCCP - + + + +
NE - - - + +
H89 - - + - +
C
Cells with translocated Parkin (% of total cells)
n.s.
n.s.
***
***
CCCP - + + + +
NE - - - + +
H89 - - + - +
D
Control siPrkaca-A siPrkaca-B
PKA C-α
β-Actin
37
37
E
Scramble Control
siPrkaca-A
siPrkaca-B
CCCP - + +
NE - - +
F
Cells with translocated Parkin (% of total cells)
n.s.
n.s.
CCCP - + + + +
NE - - + - +
Control siPrkaca-A siPrkaca-B

Supplementary Figure 4. Regulation of Parkin recruitment to the mitochondria by the PKA signaling pathway.

(A) Relative mRNA expression of *Pink1* in beige adipocytes. Cells were treated with vehicle or norepinephrine (NE) for 30 or 60 minutes followed by CCCP treatment. **P*<0.05 by one-way ANOVA with post hoc test by unpaired Student's *t*-test. n=3 wells of cell samples per group. Data are presented as mean ± s.e.m.

(B) YFP-Parkin was expressed in mouse beige adipocytes. Differentiated adipocytes were pretreated with a PKA inhibitor (H89) or vehicle for 1 hour. Subsequently, cells were treated with CCCP and/or NE. Mitochondria were stained with the Tom20 antibody (red) and nuclei with DAPI (blue). YFP-Parkin localization to the mitochondria was analyzed by confocal microscopy. Scale bars, 20 μm. Arrowheads indicate the punctae of YFP-Parkin recruited to the mitochondria.

(C) Quantification of Parkin localization in the mitochondria in (B). ****P*<0.001 by one-way ANOVA with post hoc test by Tukey's method. n.s., not significant. 412 cells were analyzed for each experimental group. Data are represented as mean ± s.e.m. The study was repeated in three independent experiments.

(D) siRNA of *Prkaca* (*siPrkaca-A* and *siPrkaca-B*) or scramble control siRNA (Control) were transfected to the beige cells expressing YFP-Parkin. PKA C-α protein expression was detected by immunoblotting. ß-actin was used as a loading control. Molecular weight (MW) is shown on the right. The study was repeated in three independent experiments.

(E) Cells in (D) were treated with CCCP and/or NE. YFP-Parkin localization to the mitochondria was analyzed by confocal microscopy. Scale bars, 20 μm. Arrowheads indicate the punctae of YFP-Parkin localization to mitochondria.

(F) Quantification of Parkin localization in the mitochondria in (E). n.s., not significant, by one-way ANOVA with post hoc test by Tukey's method. 476 cells were analyzed for each experimental group. Data are presented as mean ± s.e.m. The study was repeated in three independent experiments.

Supplementary Table 1. Oligonucleotide sequences of quantitative RT-PCR primers.

Gene Name	Forward	Reverse
Park2	GGGATTCAGAAGCAGCCAGA	CCACACTGAACTCGGAGCTT
Ucp1	CACCTTCCCGCTGGACACT	CCCTAGGACACCTTTATACCTAATGG
Pgc1α	AGCCGTGACCACTGACAACGAG	GCTGCATGGTTCTGAGTGCTAAG
Dio2	CAGTGTGGTGCACGTCTCCAATC	TGAACCAAAGTTGACCACCAG
Cidea	ATCACAACTGGCCTGGTTACG	TACTACCCGGTGTCCATTTCT
Cox8b	GAACCATGAAGCCAACGACT	GCGAAGTTCACAGTGGTTCC
Cox2	ACCTGGTGAACTACGACTGCT	CCTAGGGAGGGGACTGCTCA
Cox3	CTTCACCATCCTCCAAGCTTCA	AGTCCATGGAATCCAGTAGCCAT
Prkaca	CCACTACGCCATGAAGATCT	ACCTGTACATGGTCATGGAG
Pink1	GTCAGGAGATCCAGGCAATT	CATTGGCAAGGGTTGCAATG