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Supplementary Materials for

Targeting RalGAPα1 in skeletal muscle to simultaneously improve postprandial glucose and lipid control

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Table S1. The list of antibodies used in this study.

Antibody Name	Company	Cat No.
Rabbit anti-AS160	Merck Millipore	07-741
Rabbit anti-pT642-AS160	Life Technologies	441071G
Rabbit anti-pS2448-mTOR	Cell Signaling Technology	#5536
Rabbit anti-mTOR	Cell Signaling Technology	#2983
Rabbit anti-PKB	Cell Signaling Technology	#9272
Rabbit anti-pS473-PKB	Cell Signaling Technology	#9271
Rabbit anti-pT308-PKB	Cell Signaling Technology	#3038
Rabbit anti-pT389-S6K	Cell Signaling Technology	#9205
Rabbit anti-S6K	Cell Signaling Technology	#9202
Rabbit anti-GSK3	Cell Signaling Technology	#5676
Rabbit anti-pS21/9-GSK3	Cell Signaling Technology	#9331
Rabbit anti-RalA	Cell Signaling Technology	#4799
Rabbit anti-HA	Cell Signaling Technology	#3724
Rabbit anti-CD36	Abcam	ab133625
Rabbit anti-GLUT4	Abcam	ab654
Rabbit anti-pS307-IRS1	ABclonal	AP0371
Rabbit anti-IRS1	ABclonal	A0245
Rabbit anti-RalB	ABclonal	A4069
Mouse anti-Flag	Sigma	F9291
Mouse anti-CD36	Santa Cruz Biotechnology	sc-7309
Rabbit anti-GFP	Santa Cruz Biotechnology	sc-8334
Mouse anti-GAPDH	Santa Cruz Biotechnology	sc-32233
Mouse anti-Tubulin	Santa Cruz Biotechnology	sc-8035

Table S2. Primer information for quantitative real-time fluorescence PCR analysis of expression of target genes.

Gene name	Forward primer	Reverse primer
Cd36	5′-CACAGATGCAGCCTCCTTTC-3′	5′-AGCACACCATACGACGTACA-3′
Fatp1	5′-CTACCACTCTGCAGGGAACA-3′	5′-CAGGTAGCGGCAGATTTCAC-3′
Fabp3	5′-CCCCTCAGCTCAGCACCAT-3′	5′-CAGAAAAATCCCAACCCAAGAAT-3′
Acc1	5′-GCCTCTTCCTGACAAACGAG-3′	5′-TGACTGCCGAAACATCTCTG-3′
Fasn	5′-GCTTCGCCAACTCTACCATG-3′	5′-CCATCGCTTCCAGGACAATG-3′
Acl	5′-ACCGGCAAAGAACTCCTGTA-3′	5′-ATGTCCCAGGCGAGGTTTTA-3′
AceCS1	5′-CTCCATTGTGTTTGCAGGCT-3′	5′-GTCATTCATGCCCAGCTCTG-3′
Pnpla2	5′-CAACGCCACTCACATCTACG-3′	5′-ACCAGGTTGAAGGAGGGATG-3′
Srebp1a	5′-GATGTGCGAACTGGACACAG-3′	5′-CATAGGGGGCGTCAAACAG-3′
Srebp1c	5′-TGTTGGCATCCTGCTATCTG-3′	5′-AGGGAAAGCTTTGGGGTCTA-3′
Hmgcs1	5′-GCCGTGAACTGGGTCGAA-3′	5′-GCATATATAGCAATGTCTCCTGCAA-3′
Hmgcr	5′-CTTGTGGAATGCCTTGTGATTG-3′	5′-AGCCGAAGCAGCACATGAT-3′
PPARα	5′-CCACGAAGCCTACCTGAAGA-3′	5′-TTCTCGGCCATACACAAGGT-3′
Cpt1	5′-ACTCCTGGAAGAAGAAGTTCA-3′	5′-AGTATCTTTGACAGCTGGGAC-3′
Acox1	5′-ATCACGGGCACTTATGC-3′	5′-TCTCACGGATAGGGACA-3′
PPARγ	5′-GAGAGGTCCACAGAGCTGATT-3′	5′-GATGTGCGAACTGGACACAG-3′
Cebpα	5′-CAAGAACAGCAACGAGTACCG-3′	5′-GTCACTGGTCAACTCCAGCAC-3′
RalGAPα1	5′-ACAGAAAGGTCACAAGTCTCAA-3′	5′-TCCTGAAGAGCTTGCAACCA-3′
RalGAPα2	5′-CAGGAGTGGAGAAGGCAAGA-3′	5′-TGGGGCTGTAACCTTGAGAG-3′
RalGAP β	5′-AAATCCAAGGAGCCACTGGA-3′	5′-GGCTCCAACTGCTTATTCCG-3′
RalA	5′-ACAGGATGGCTGCAAACAAG-3′	5′-TGAACTGCAGAGTCAGAGCA-3′
RalB	5′-CTTTCCCTCCTCAACACCCT-3′	5′-AGCCTTCCCTTCATCTGCTT-3′
Cre	5′-GCCTGCATTACCGGTCGATGC-3′	5′-CAGGGTGTTATAAGCAATCCC-3′
actin	5′-GAGAGGGAAATCGTGCGTGACA-3′	5′-GTTTCATGGATGCCACAGGAT-3′

Supple. Fig. 1

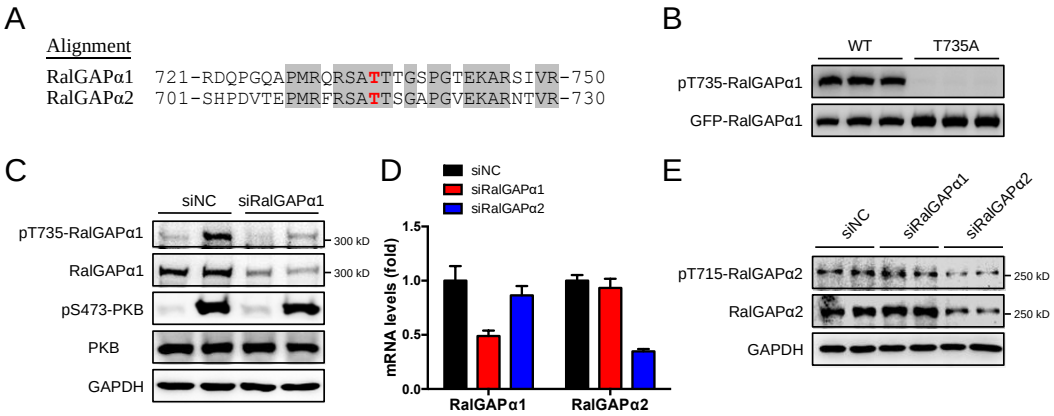

Fig. S1. Specificity of the pT735-RalGAPα1 antibody. (**A**) Alignment of sequences surrounding RalGAPα1-Thr^{735} and RalGAPα2-Thr^{715}. RalGAPα1-Thr^{735} and RalGAPα2-Thr^{715} are highlighted in red. The pT715-RalGAPα2 peptide (EPMRFRSApTTSGAPGVEK, Thr^{715} highlighted in red) was used to raise phospho-specific antibody. (**B**) Phosphorylation of the GFP tagged RalGAPα1$^{E590\text{-}D1232}$ WT and Thr735Ala mutant proteins were detected with the pT735-RalGAPα1 antibody. (**C**) RalGAPα1-Thr^{735} phosphorylation in L6 myotubes upon knockdown of RalGAPα1. Endogenous RalGAPα1 runs at ~ 300 kD in cell lysates of L6 myotubes. (**D**) mRNA levels of RalGAPα1 and RalGAPα2 in primary mouse hepatocytes upon knockdown of these two genes, respectively. (**E**) RalGAPα2-Thr^{715} phosphorylation in primary mouse hepatocytes upon knockdown of RalGAPα1 or RalGAPα2. Endogenous RalGAPα2 runs at ~ 260 kD in cell lysates of primary mouse hepatocytes.

Supple. Fig. 2

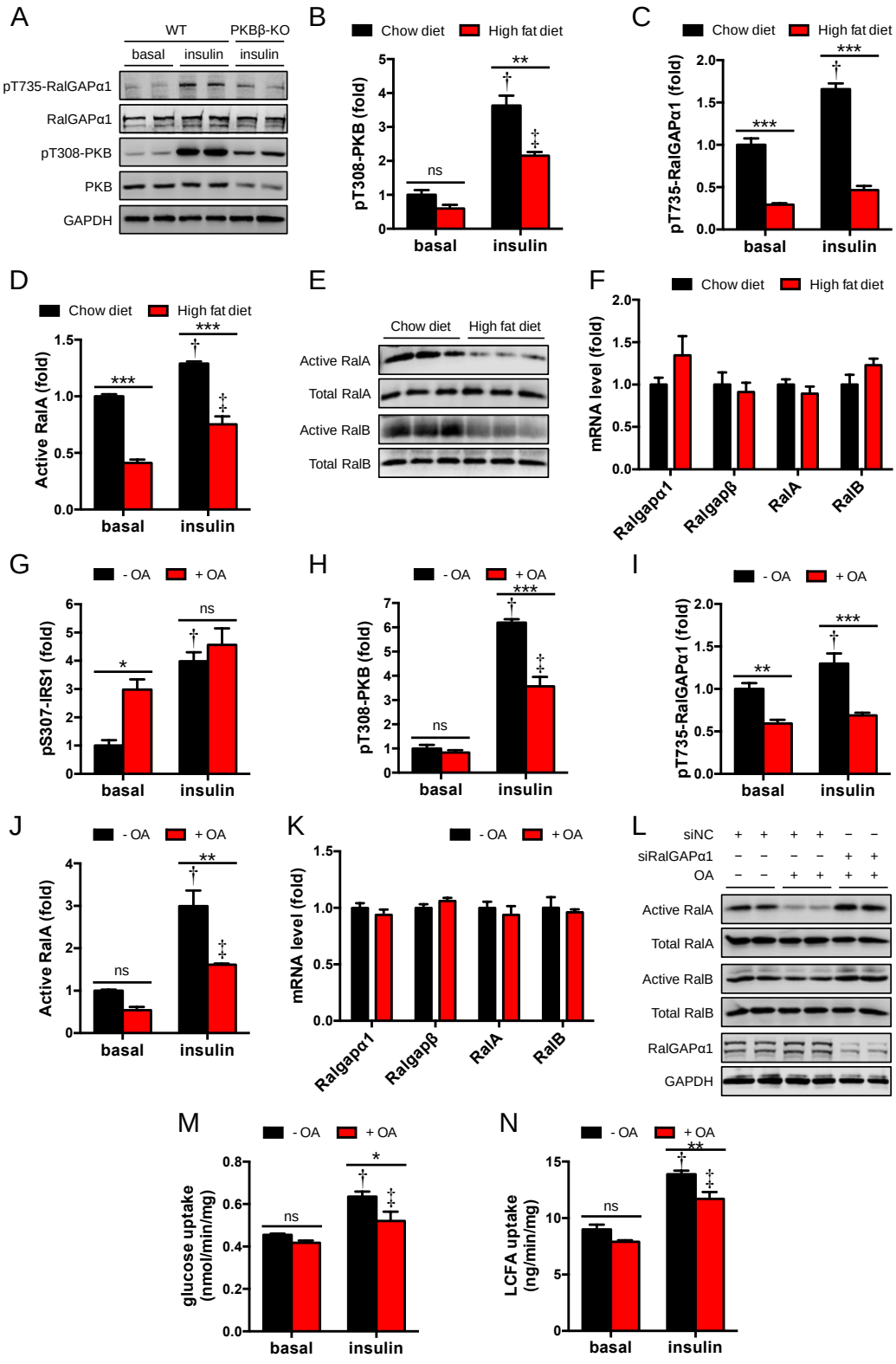
A
WT
PKBβ-KO
basal
insulin
insulin
pT735-RalGAPα1
RalGAPα1
pT308-PKB
PKB
GAPDH
B
Chow diet
High fat diet
pT308-PKB (fold)
ns
basal
insulin
C
pT735-RalGAPα1 (fold)
D
Active RalA (fold)
E
Active RalA
Total RalA
Active RalB
Total RalB
F
mRNA level (fold)
Ralgapα1
Ralgapβ
RalA
RalB
G
- OA
+ OA
pS307-IRS1 (fold)
H
pT308-PKB (fold)
I
pT735-RalGAPα1 (fold)
J
Active RalA (fold)
K
mRNA level (fold)
L
siNC
siRalGAPα1
OA
Active RalA
Total RalA
Active RalB
Total RalB
RalGAPα1
GAPDH
M
glucose uptake (nmol/min/mg)
N
LCFA uptake (ng/min/mg)

Fig. S2. Effects of HFD/fatty acids on the RalGAPα1/β complex and RalA/B activities in skeletal muscle and L6 myotubes. (A) Thr^{735} phosphorylation of RalGAPα1 in gastrocnemius muscle from the wild-type and PKBβ-KO mice treated with insulin. (**B**) Quantitation of PKB-pT308 phosphorylation in soleus muscle of male DIO mice (5-month-old) in response to insulin. Representative blots were shown in Fig. 1A. n = 3. † indicates $p < 0.001$ (Chow diet insulin vs Chow diet basal), and ‡ indicates $p < 0.01$ (High fat diet insulin vs High fat diet basal). (**C**) Quantitation of RalGAPα1-pT735 phosphorylation in soleus muscle of male DIO mice (5-month-old) in response to insulin. Representative blots were shown in Fig. 1A. n = 3. † indicates $p < 0.001$ (Chow diet insulin vs Chow diet basal). (**D**) Quantitation of GTP-bound active RalA in soleus muscle of male DIO mice (5-month-old) in response to insulin. Representative blots were shown in Fig. 1A. n = 3. † indicates $p < 0.01$ (Chow diet insulin vs Chow diet basal), and ‡ indicates $p < 0.01$ (High fat diet insulin vs High fat diet basal). (**E**) Levels of GTP-bound active RalA and RalB in gastrocnemius muscle of *ad libitum* DIO male mice at the age of 5 months. (**F**) mRNA levels of RalGAPα1/β and RalA/B in gastrocnemius muscle of *ad libitum* DIO male mice at the age of 5 months. n = 7-8. (**G**) Quantitation of IRS1-pS307 phosphorylation in OA-treated L6 myotubes in response to insulin. Representative blots were shown in Fig. 1B. n = 3. † indicates $p < 0.01$ (− OA insulin vs − OA basal). (**H**) Quantitation of PKB-pT308 phosphorylation in OA-treated L6 myotubes in response to insulin. Representative blots were shown in Fig. 1B. n = 3. † and ‡ indicate $p < 0.001$ (insulin vs basal). (**I**) Quantitation of RalGAPα1-pT735 phosphorylation in OA-treated L6 myotubes in response to insulin. Representative blots were shown in Fig. 1B. n = 3. † indicates $p < 0.05$ (− OA insulin vs − OA basal). (**J**) Quantitation of GTP-bound active RalA in OA-treated L6 myotubes in response to insulin. Representative blots were shown in Fig. 1B. n = 3. † indicates $p < 0.001$ (− OA insulin vs − OA basal), and ‡ indicates $p < 0.05$ (+ OA insulin vs + OA basal). (**K**) mRNA levels of RalGAPα1/β and RalA/B in OA-treated L6 myotubes. n = 4. (**L**)Levels of GTP-bound active RalA and RalB in L6 myotubes treated with or without oleic acid (OA) in the presence or absence of RalGAPα1 down-regulation via siRNA. (**M**-**N**) Uptake of glucose (M) or

LCFA (N) in OA-treated L6 myotubes in response to insulin. n = 6. † indicates $p < 0.001$ (− OA insulin vs − OA basal), and ‡ indicates $p < 0.05$ for glucose uptake (+ OA insulin vs + OA basal) or $p < 0.001$ for LCFA uptake (+ OA insulin vs + OA basal). The data are given as the mean ± SEM. * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. ns, not significant.

Supple. Fig. 3

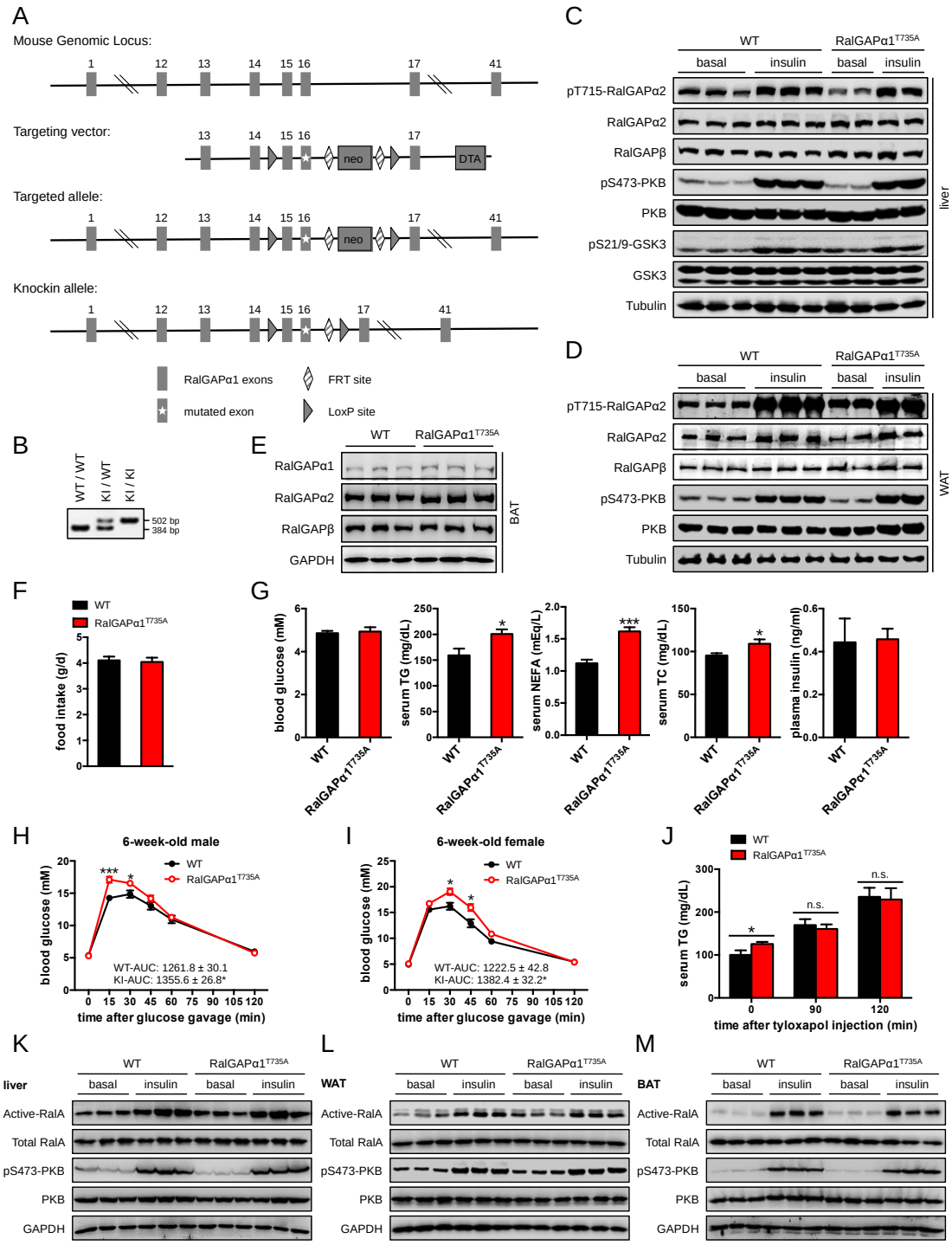

Fig. S3. Generation and characterization of the RalGAPα1^{Thr735Ala} knock-in mice. (**A**) Diagrammatic illustration of the strategy for generation of the RalGAPα1^{Thr735Ala} knockin mice. (**B**) PCR-based genotyping of the RalGAPα1^{Thr735Ala} knockin mice. (**C-D**) Phosphorylation and expression of RalGAPα2 and other key components of insulin-PKB pathway in the liver (C) and WAT (D) of the wild-type and RalGAPα1^{Thr735Ala} knockin mice (2-month-old) in response to insulin. (**E**) Expression of RalGAPα1, RalGAPα2 and RalGAPβ in the BAT of wild-type and

RalGAPα1^{Thr735Ala} knockin mice (2-month-old). (**F**) Food intake of the male wild-type and RalGAPα1^{Thr735Ala} knockin mice (3-month-old). n = 7-8. (**G**) Blood glucose, plasma insulin, TG, NEFA and TC in the overnight-fasted male WT and RalGAPα1^{Thr735Ala} knockin mice at the age of 7-8 weeks. n = 8-9. (**H**-**I**) Oral glucose tolerance test in the male (H) or female (I) wild-type and RalGAPα1^{Thr735Ala} knockin mice at age of 6 weeks. The values show the glucose area under the curve during glucose tolerance test. n = 5-7. (**J**) Serum TG levels in the overnight-fasted WT and RalGAPα1^{Thr735Ala} knockin male mice (5-month-old) upon administration of tyloxapol via intraperitoneal injection. n = 7-8. (**K**-**M**) Levels of GTP-bound active RalA in the liver (K), WAT (L) and BAT (M) of the wild-type and RalGAPα1^{Thr735Ala} knockin mice (2-month-old) in response to insulin. The data are given as the mean ± SEM. * indicates $p < 0.05$, and *** $p < 0.001$. ns, not significant.

Supple. Fig. 4

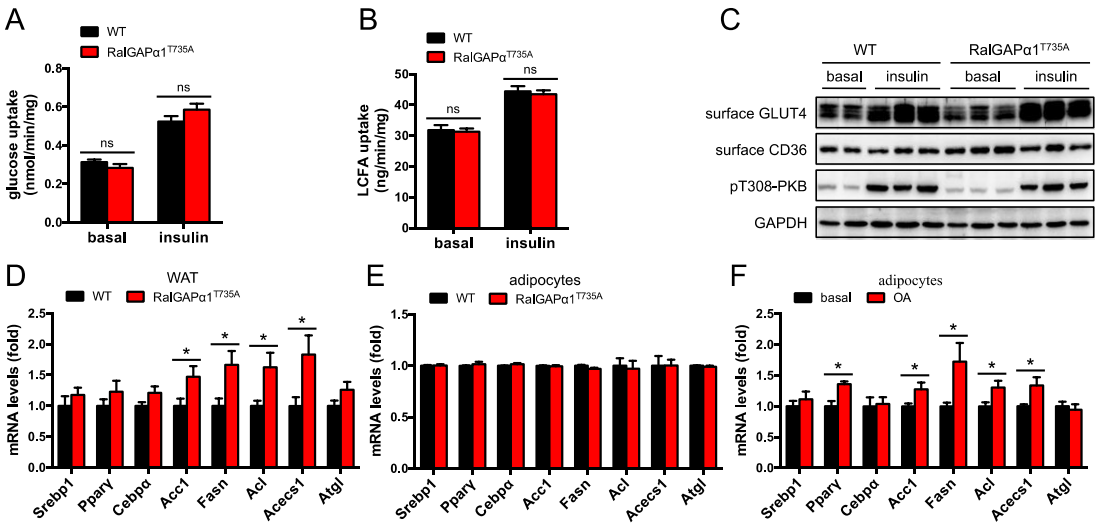

Fig. S4. Uptake of glucose and LCFAs and expression of genes for lipid metabolism in adipose tissues and primary adipocytes. (**A-B**) Uptake of glucose (A) or LCFA (B) in primary brown adipocytes in response to insulin. n = 6. (**C**) Cell surface GLUT4 and CD36 in the BAT in response to insulin. Subcellular fractionation was performed in the BAT, and GLUT4 and CD36 on the plasma membrane were detected via western blot. (**D**) mRNA levels of genes for lipid metabolism in the WAT of WT and RalGAPα1^{Thr735Ala} knockin male mice at the age of 2 months. n = 6. (**E**) mRNA levels of genes for lipid metabolism in primary white adipocytes from WT and RalGAPα1^{Thr735Ala} knockin male mice. n = 4-6. (**F**) mRNA levels of genes for lipid metabolism in wild-type primary white adipocytes treated with or without OA. n = 5. The data are given as the mean ± SEM. * indicates $p < 0.05$. ns, not significant.

Supple. Fig. 5

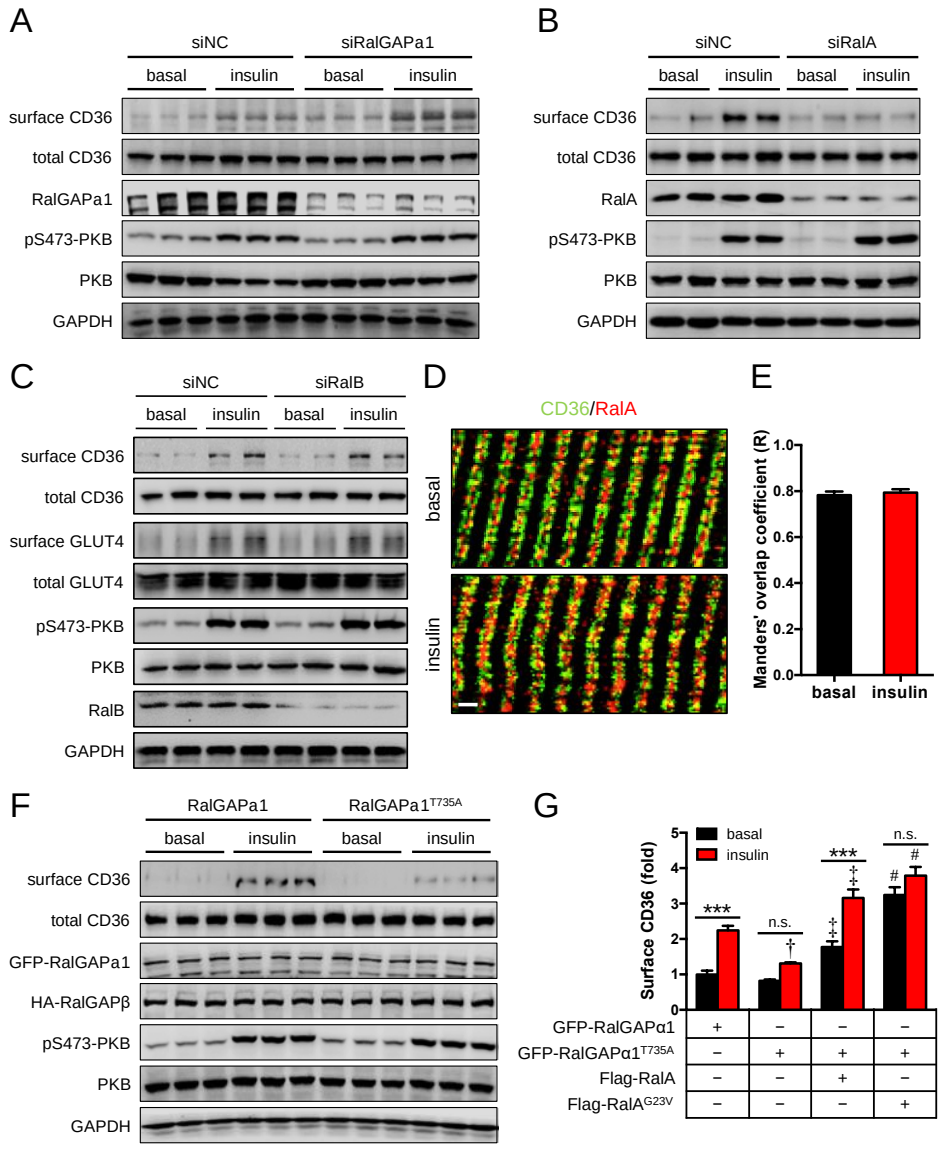

Fig. S5. Cell surface and total CD36 in L6 myotubes. (**A**) Cell surface and total CD36 in L6 myotubes upon knockdown of RalGAPα1 via siRNA. Cells were treated with or without insulin. (**B**) Cell surface and total CD36 in L6 myotubes upon knockdown of RalA via siRNA. Cells were treated with or without insulin. (**C**) Cell surface and total CD36 in L6 myotubes upon knockdown of RalB via siRNA. Cells were treated with or without insulin. (**D**-**E**) Immunostaining of CD36 and RalA in mouse soleus muscle in response to insulin. Mouse anti-CD36 and Rabbit anti-RalA antibodies were used in the immunostaining. Colocalization of CD36 and RalA (Manders' overlap coefficient) was determined using Image-Pro Plus, and shown in

E. n = 16. (**F**) Cell surface and total CD36 in L6 myotubes overexpressing RalGAPα1 WT or Thr735Ala mutant proteins. Cells were treated with or without insulin. (**G**) Cell surface and total CD36 levels in L6 myotubes overexpressing wild-type or mutant GFP-RalGAPα1 proteins in the presence or absence of overexpression of wild-type or mutant Flag-RalA proteins. Cells were stimulated with or without insulin. Representative blots were shown in Fig. 3J. Quantitative data of cell surface CD36 that were normalized to total CD36 were shown here. n = 4. † indicates $p < 0.05$ (RalGAPa1^{T735A} alone insulin vs RalGAPa1 alone insulin). ‡ indicates $p < 0.01$ (RalA/RalGAPa1^{T735A} basal vs RalGAPa1^{T735A} alone basal) or $p < 0.001$ (RalA/RalGAPa1^{T735A} insulin vs RalGAPa1^{T735A} alone insulin). # indicates $p < 0.001$ (RalA^{G23V}/RalGAPa1^{T735A} basal vs RalGAPa1^{T735A} alone basal) or $p < 0.001$ (RalA^{G23V}/RalGAPa1^{T735A} insulin vs RalGAPa1^{T735A} alone insulin). The data are given as the mean ± SEM. *** indicates $p < 0.001$. ns, not significant.

Supple. Fig. 6

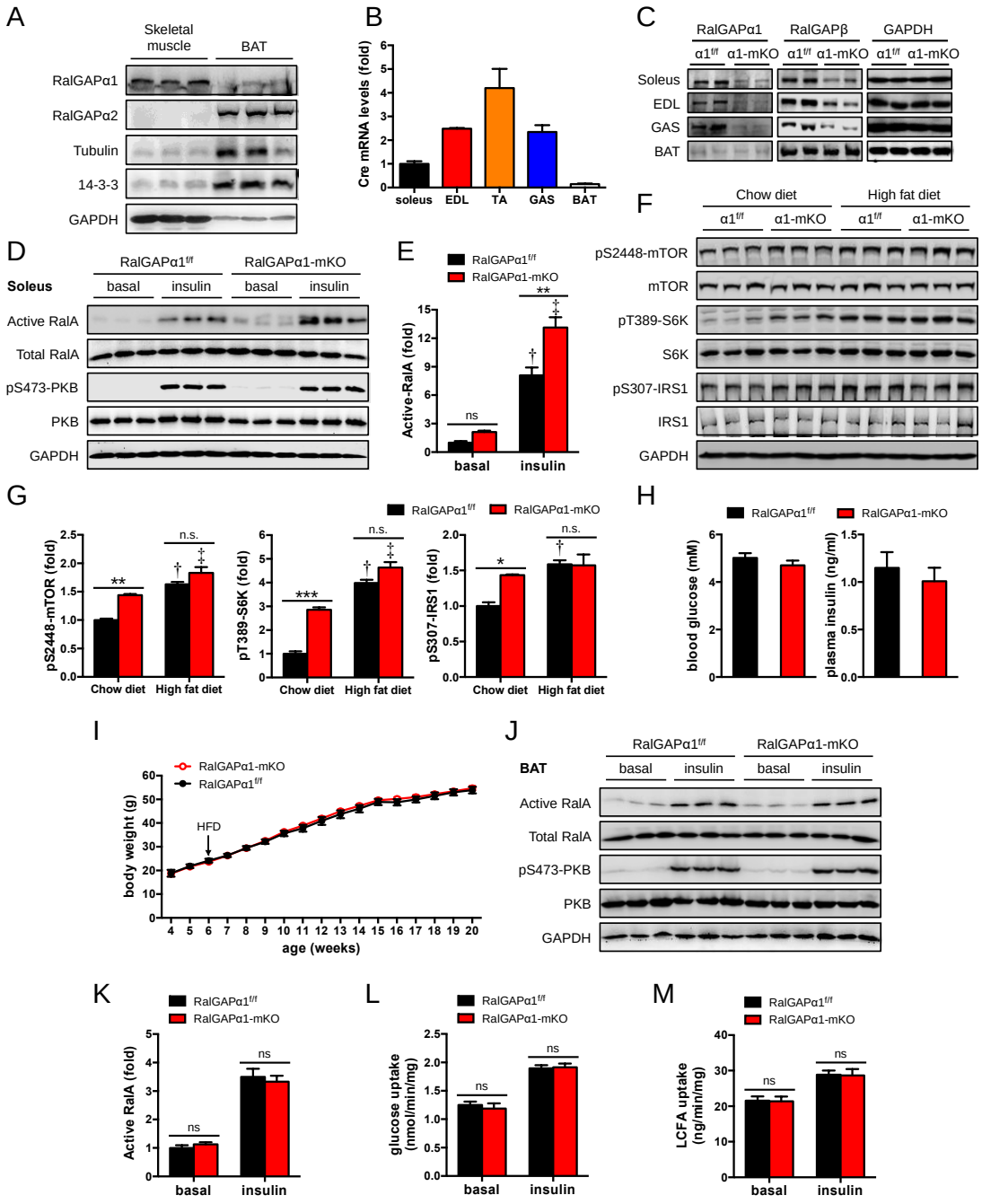

Fig. S6. Characterization of RalGAPα1-mKO mice. (**A**) Protein expression of RalGAPα1 and RalGAPα2 in mouse gastrocnemius muscle and BAT. 40 μg of tissue lysates were loaded for each lane. (**B**) mRNA levels of Cre recombinase in different skeletal muscle and BAT of the Mlc1f-Cre mice. n = 3-4. (**C**) Expression of RalGAPα1 and RalGAPβ in different skeletal muscle and BAT from the male RalGAPα1-mKO mice (3-month-old). (**D-E**) GTP-bound active RalA in soleus

muscle of the male RalGAPα1-mKO mice (3-month-old) in response to insulin. Active RalA in the blots shown in D were quantified in E. n = 3. † and ‡ indicate $p < 0.001$ (insulin vs basal). (**F-G**) Phosphorylation and expression of mTOR, S6K and IRS1 in skeletal muscle of the RalGAPα1-mKO male mice fed on chow or high fat diet. Blots in F were quantified in G. For pS2448-mTOR, † indicates $p < 0.001$ ($RalGAP\alpha1^{f/f}$ High fat diet vs $RalGAP\alpha1^{f/f}$ Chow diet), and ‡ indicates $p < 0.01$ (RalGAPα1-mKO High fat diet vs RalGAPα1-mKO Chow diet). For pT389-S6K, † and ‡ indicate $p < 0.001$ (High fat diet vs Chow diet). For pS307-IRS1, † indicates $p < 0.01$ ($RalGAP\alpha1^{f/f}$ High fat diet vs $RalGAP\alpha1^{f/f}$ Chow diet). (**H**) Blood glucose in the overnight fasted $RalGAP\alpha1^{f/f}$ and RalGAPα1-mKO male mice at the age of 8 weeks. Plasma insulin in the $RalGAP\alpha1^{f/f}$ and RalGAPα1-mKO male mice fed *ad libitum* at the age of 5 months. n = 7-8. (**I**) Body weight of the $RalGAP\alpha1^{f/f}$ and RalGAPα1-mKO male mice fed on high fat diet. n = 7-10. (**J-K**) Levels of GTP-bound active RalA in the BAT of male RalGAPα1-mKO mice (3-month-old) in response to insulin. Blots shown in J were quantified in K. n = 3. (**L-M**) Uptake of glucose (L) or LCFA (M) in primary brown adipocytes in response to insulin. n = 5-6. The data are given as the mean ± SEM. * indicates $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. ns, not significant.

Supple. Fig. 7

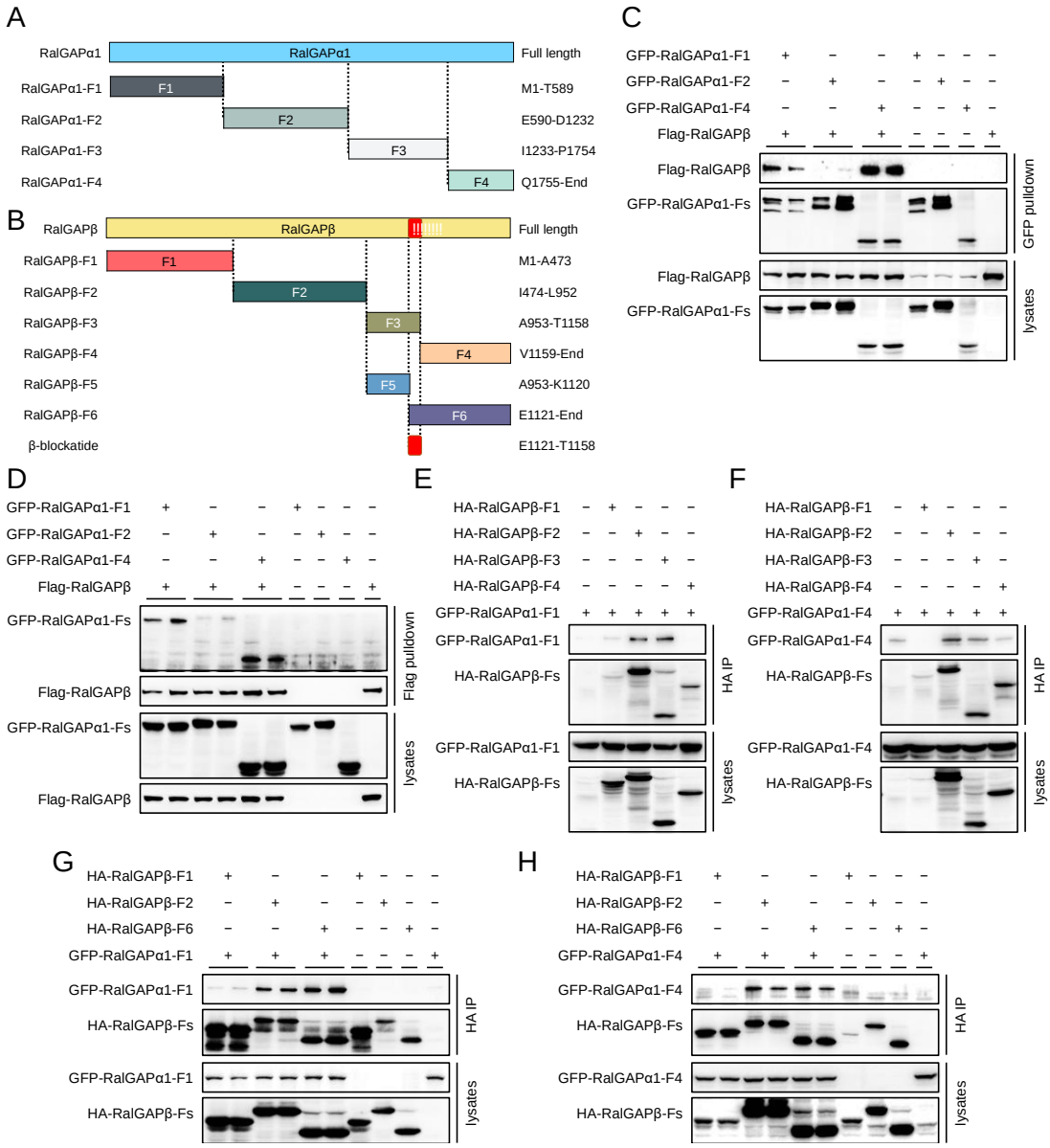

Fig. S7. Mapping of interaction domains on RalGAPα1 and RalGAPβ. (**A-B**) Diagrammatic illustration of fragments of RalGAPα1 (A) and RalGAPβ (B) for mapping of interaction domains between these two proteins. (**C-D**) Mapping of regions on RalGAPα1 that interact with RalGAPβ. GFP-tagged RalGAPα1 fragments were co-expressed with Flag-tagged RalGAPβ. C, Flag-RalGAPβ was detected via immunoblotting in the immunoprecipitates of GFP-tagged RalGAPα1 fragments. D, GFP-tagged RalGAPα1 fragments were detected via immunoblotting in the immunoprecipitates of Flag-RalGAPβ. (**E-H**) Mapping of regions on RalGAPβ that

interact with RalGAPα1. HA-tagged RalGAPβ fragments were co-expressed with GFP-tagged RalGAPα1-F1 (E and G) or RalGAPα1-F4 (F and H). GFP-tagged RalGAPα1 fragments were detected via immunoblotting in the immunoprecipitates of HA-tagged RalGAPβ fragments.

Supple. Fig. 8

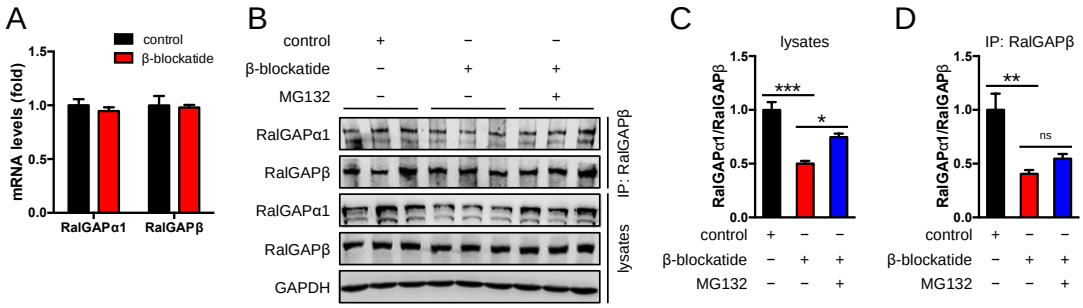

Fig. S8. Expression, degradation, and interaction of RalGAPα1 and RalGAPβ in L6 myotubes expressing β-blockatide. (**A**) mRNA levels of endogenous RalGAPα1 and RalGAPβ in L6 myotubes expressing the β-blockatide. Cells transfected with an empty vector were used as control. n = 6. (**B-D**) Protein expression and interaction of RalGAPα1 and RalGAPβ in L6 myotubes expressing the β-blockatide. Myotubes were treated with or without MG132 for 4 h before lysis. Cells transfected with an empty vector were used as control. Endogenous RalGAPβ was immunoprecipiated using the anti-RalGAPβ antibody, and RalGAPα1 was detected in the immunoprecipitates via immunoblotting. C, quantitation of RalGAPα1 in cell lysates. D, quantitation of RalGAPα1 in RalGAPβ immunoprecipitates. RalGAPα1 signals were normalized with RalGAPβ in cell lysates or immunoprecipitates. n = 3.
The data are given as the mean ± SEM. ns, not significant.