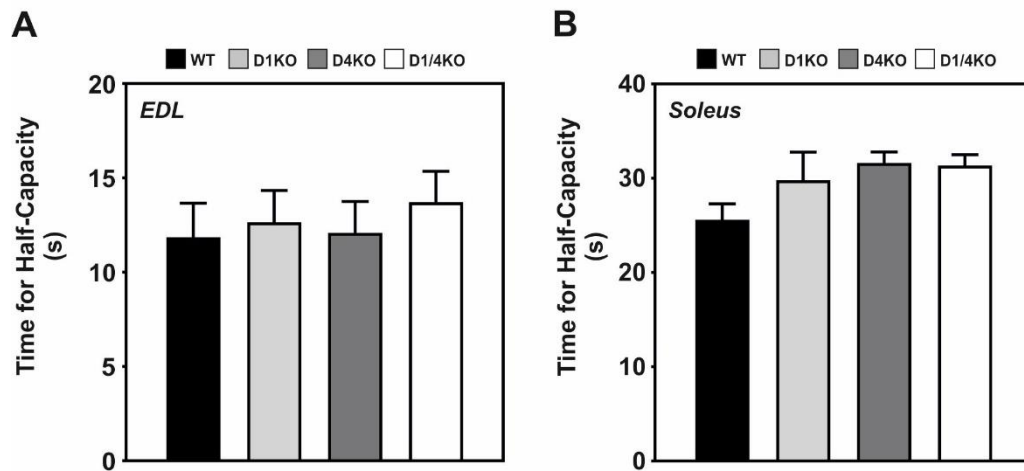
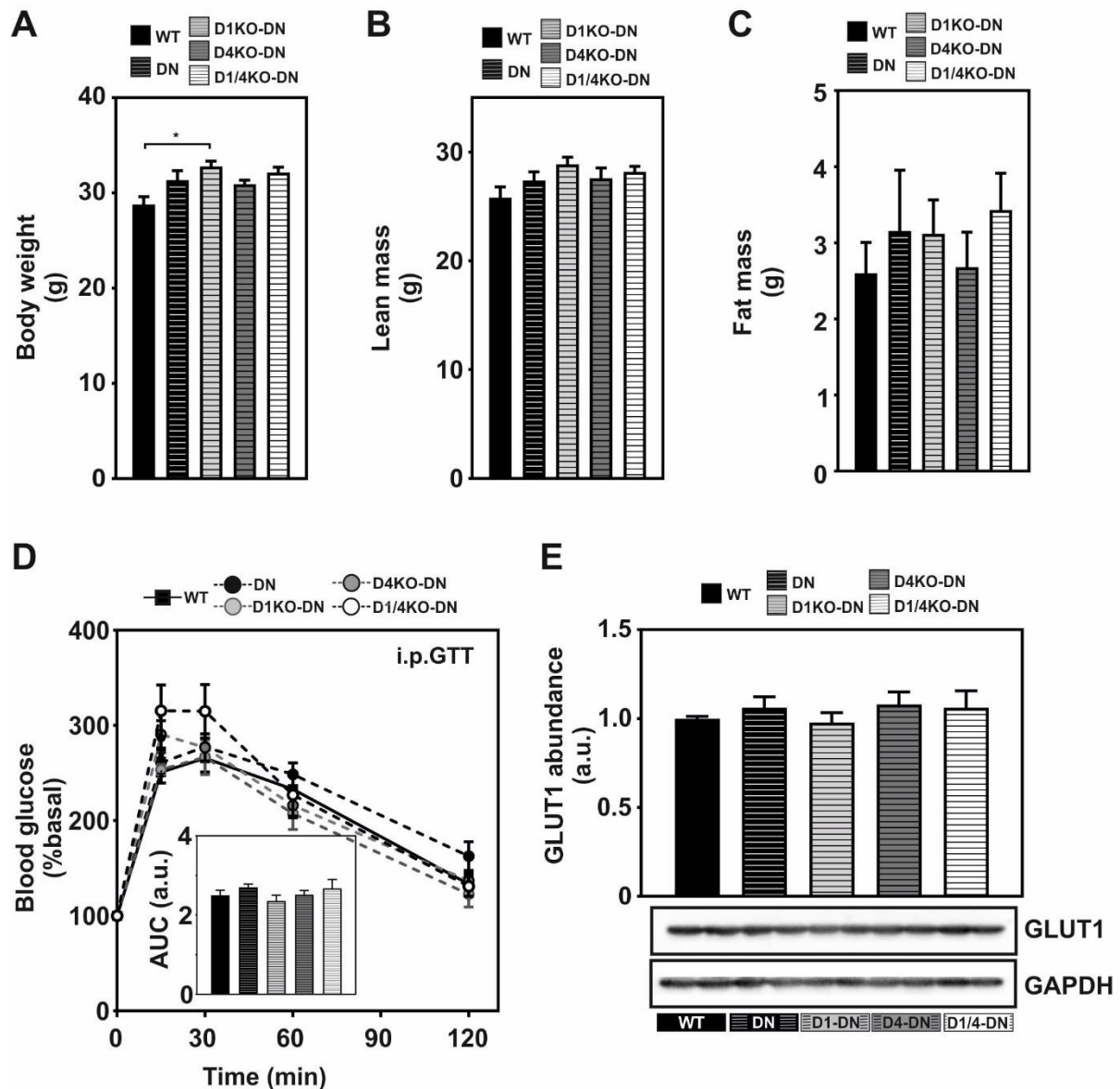


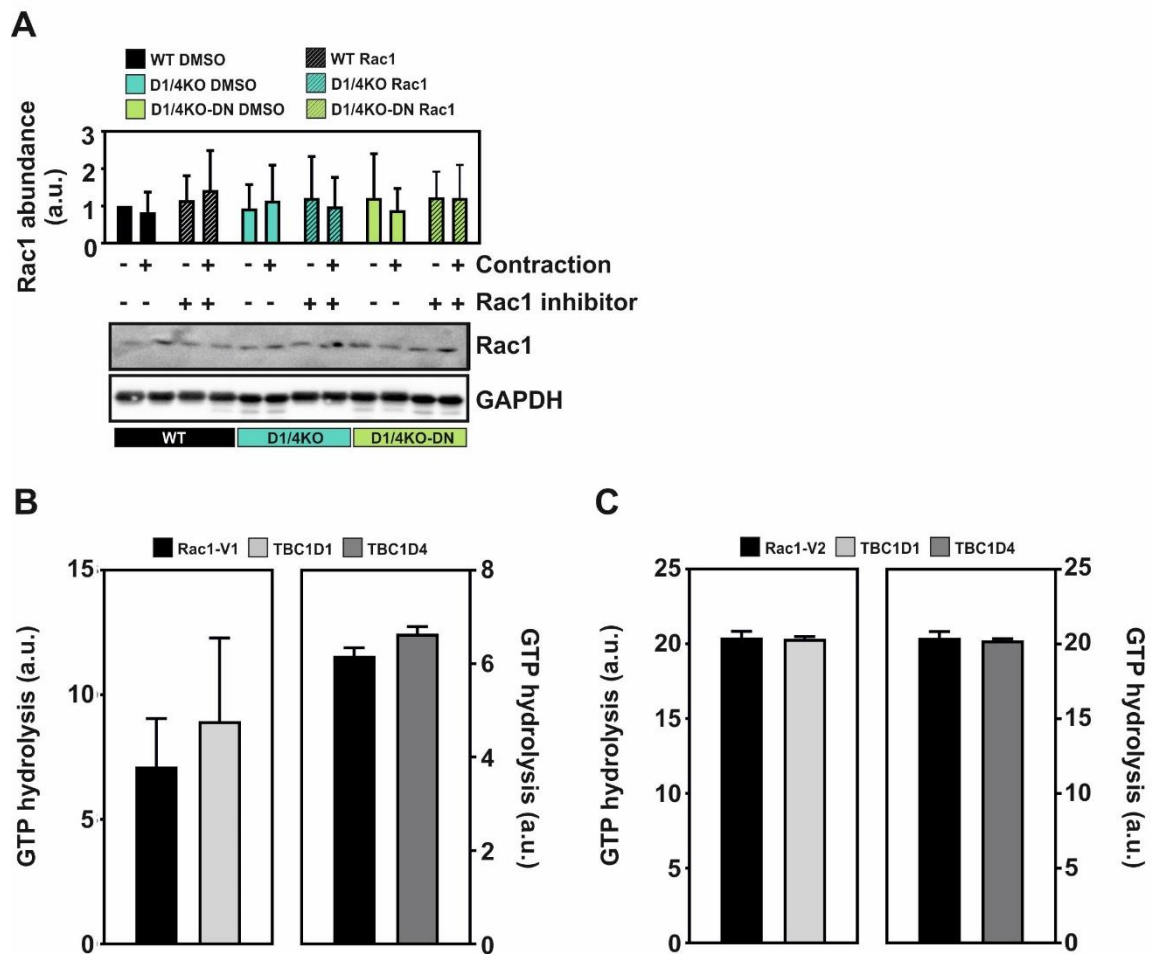
Supplemental material



Supplemental Figure 1: Time for Half-Capacity of *EDL* (A) and *Soleus* (B) muscle from RabGAP-deficient mice. Skeletal muscles from *Tbc1d1*- (D1KO), *Tbc1d4*-deficient (D4KO) and double-deficient *Tbc1d1/Tbc1d4*-deficient (D1/4KO) mice were isolated and assayed for Time for Half-Capacity of *EDL* (n = 8-9) and *Soleus* muscle (n = 3-7). Data are presented as mean \pm SEM. WT vs. D1KO vs. D4KO vs. D1/4KO (one-way ANOVA with Dunnett correction).



Supplemental Figure 2: Body weight (A), lean (B) and fat (C) mass measured via nuclear magnetic resonance (NMR) from WT, DN, D1KO-DN, D4KO-DN and D1/4KO-DN mice. Data are presented as mean \pm SEM (n = 3-7). * P < 0.05, (one-way ANOVA with Tukey correction). Glucose tolerance (D) was measured after intraperitoneal injection of glucose (2 g/kg body weight, 20% solution) and the area under the curve (AUC) was determined for quantification. Data are presented as mean \pm SEM (n = 7-11). WT vs. DN vs. D1KO-DN vs. D4KO-DN vs. D1/4KO-DN (one-way ANOVA with Tukey correction). Representative Western Blot of GLUT1 (E) in *Gastrocnemius* muscle from WT, DN, D1KO-DN, D4KO-DN and D1/4KO-DN mice normalized on GAPDH. Data are presented as mean \pm SEM (n = 8). WT vs. DN vs. D1KO-DN vs. D4KO-DN vs. D1/4KO-DN (one-way ANOVA with Tukey correction).



Supplemental Figure 3: (A) Western Blot analysis of Rac1 in *EDL* muscle of WT, DN, D1KO-DN, D4KO-DN and D1/4KO-DN mice, normalized on GAPDH, to determine whether preincubation with Rac1 inhibitor or contraction stimulus has an impact on Rac1 protein abundance. ~15 pmol affinity-purified GST-Rac1 variant 1 (B) and 2 (C) loaded with [γ - 32 P]GTP was incubated with 3 pmol of purified full-length TBC1D1 or TBC1D4. Briefly, GST-Rac1 variants bound to glutathione-sepharose was loaded with [γ - 32 P]GTP (Hartmann Analytic, Braunschweig, Germany) in a buffer containing 50 mM Tris-HCl, pH 8, 2.5 mM DTT and, 5 mM MgCl₂. The matrix was washed stepwise with the same buffer to remove unbound GTP. [γ - 32 P]GTP bound Rac1 was eluted using 10 mM reduced glutathione and then incubated with full-length TBC1D1 and TBC1D4, respectively, at room temperature (RT) for 30 minutes. The reaction was stopped with 0.5 M EDTA, radioactive [32 P]phosphate was separated by filtration through activated charcoal and measured by scintillation counting as described (Mafakheri *et al.*, J Biol Chem, 2018. 293(46): p. 17853-17862). [32 P]phosphate produced was normalized to the amount of radioactivity of [γ - 32 P]GTP bound Rac1 proteins.

Table 1: Summary AMPK-DN-RabGAP-deficient mouse phenotypes (versus WT)

	DN	D1KO-DN	D4KO-DN	D1/4KO-DN
Whole-body glycemia				
Blood glucose 6 h fasted	↔	↔	↓	↓
HOMA-IR	↑	↑*	↔	↔
Glucose tolerance (i.p.)	↔	↔	↔	↔
AICAR tolerance (i.p.)	↑	↔	↔	↔
Blood glucose 16 h fasted	↔	↔	↔	↔
Blood glucose 1 h refed	↔	↔	↑****	↑****
Plasma levels				
Plasma insulin 6 h fasted	↑	↑	↔	↔
Plasma insulin 16 h fasted	↔	↔	↔	↔
Plasma insulin 1 h refed	↔	↓	↔	↔
Plasma TGs 16 h fasted	↑*	↑	↔	↔
Plasma TGs 1 h refed	↑	↑	↔	↑
Plasma FFAs 16 h fasted	↑**	↔	↑	↔
Plasma FFAs 1 h refed	↔	↔	↔	↔
Liver phenotype				
Liver TGs	↔	↑*	↑	↑**
Liver glycogen	↔	↔	↓	↓
Liver PCK1	↔	↔	↔	↑
Liver PCK2	↔	↔	↔	↔
Liver GS abundance	↔	↔	↔	↑**
Liver pGS/GS	↔	↔	↔	↓
Acute exercise phenotype				
Time to exhaustion	↓****	↓****	↓**	↓****
VO _{2max}	↓*	↓**	↔	↔
RER average	↔	↔	↔	↔
Skeletal muscle TGs	↔	↔	↔	↔
Skeletal muscle glycogen	↓	↓	↔	↔
Contraction-stimulated glucose uptake				
Glucose uptake EDL basal	↓	↓	↓	↓*
Glucose uptake EDL contracted	↓****	↓****	↓**	↓****
Glucose uptake Soleus basal	↓	↓	↓*	↓
Glucose uptake Soleus contracted	↓**	↓	↓**	↓****
Gene expression analysis				
<i>Nuak1</i>	↑	↑	↑	↑
<i>Nuak2</i>	↔	↑*	↑	↑
<i>Sik1</i>	↑	↑	↔	↑
<i>Sik2</i>	↔	↔	↔	↔
<i>Sik3</i>	↔	↔	↔	↔
Protein abundance analysis				
ARK5 (NUAK1)	↑*	↑*	↑*	↑
GLUT4 abundance EDL	↔	↓*	↔	↓
GLUT4 abundance Soleus	↔	↔	↓*	↓**
SNARK (NUAK2)	↔	↔	↔	↔

AMPK-dependent trait
RabGAP-dependet trait
Combined trait
Not affected by any of the afore mentioned

Supplemental Figure 4: Summary of AMPK-DN-RabGAP-deficient mouse phenotypes. * P < 0.05,

** P < 0.01, *** P < 0.001, **** P < 0.0001, WT vs. DN, D1KO-DN, D4KO-DN, D1/4KO-DN.

Supplemental Table 1: Chemicals and buffer ingredients

Compound	Supplier
[γ - ³² P]GTP	Hartmann Analytic, Brunswick, Germany
5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR)	Toronto Research Chemicals, North York ON, Canada
Bovine serum albumin (BSA)	Sigma Aldrich, Steinheim, Germany
Bromophenol blue	Merck, Darmstadt, Germany
Complete protease inhibitor cocktail	Roche Diagnostics, Mannheim, Germany
Deoxy-D-Glucose 2-(1,2-3-H(N))	Hartmann Analytic, Brunswick, Germany
Dithiothreitol (DTT)	Sigma Aldrich, Steinheim, Germany
D-Mannitol	AppliChem, Darmstadt, Germany
Ethylendiamintetra acetic acid (EDTA)	Serva, Heidelberg, Germany
Ethylenglycol-bis(aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA)	Serva, Heidelberg, Germany
Glycine	Appllichem, Darmstadt, Germany
Hyaluronidase	Sigma Aldrich, Steinheim, Germany
Isoflurane	Piramal Enterprises, Mumbai, India
Magnesium chloride (MgCl ₂)	Roche Diagnostics, Mannheim, Germany
Mannitol, D-(1-14C)	PerkinElmer, Seer Green, UK
Methanol	Carl Roth, Karlsruhe, Germany
Milk powder	Carl Roth, Karlsruhe, Germany
PhosSTOP phosphatase inhibitor cocktail	Roche Diagnostics, Mannheim, Germany
Rac1 Inhibitor (NSC23766)	Sigma Aldrich, Steinheim, Germany
Sodium dodecyl sulfate	Appllichem, Darmstadt, Germany
Sodium chloride	Carl Roth, Karlsruhe, Germany
Tris-HCl	Appllichem, Darmstadt, Germany
Triton X-100	Roche Diagnostics, Mannheim, Germany
Tween-20	MP Biomedicals, Solon, USA

Supplemental table 2: Genotyping primers

Mouse DNA was isolated from tail tips using the Invisorb® Genomic DNA Kit II (STRATEC Molecular GmbH, Berlin, Germany) according to manufacturer's instructions.

Primer	Sequence (5' → 3')
<i>Ampk</i> kinase dead	Fwd: CGA GGT CGA CGG TAT CGA TAA GCT TGA TAT C Rev: GAA GGA ACC CGT TGG AGG ACT GGA GGC GAG G
<i>Tbc1d1</i> -WT	Fwd: GGA CAA GCA GCT TTC TTG TTT Rev: TCC TGG TCC AGA AGC GAG
<i>Tbc1d1</i> -KO	Fwd: CAA CAT TCT GAA GGC CTT CTG Rev: TCC CTG GCT ACA AGC TGA GT
<i>Tbc1d4</i> <i>Tbc1d4</i> -WT <i>Tbc1d4</i> -KO	Fwd: AGT AGA CTC AGA GTG GTC TTG G Rev: GTC TTC CGA CTC CAT ATT TGC Rev: GCA GCG CAT CGC CTT CTA TC

Supplemental Table 3: Buffers

Description	Ingredients
Binding buffer (for <i>in vitro</i> GAP assay)	50 mM Tris-HCl, pH 8, 2.5 mM DTT and, 5 mM MgCl ₂ , pH 8
Elution buffer (for <i>in vitro</i> GAP assay)	20 mM Tris-HCl, pH 7.5, 1 mM DTT, 10 mM reduced GSH, 2.5 mM MgCl ₂ , pH 7.5
Krebs-Henseleit buffer (KHB)	Stock I: 118.5 mM NaCl, 0.047 mM KCl, 0.012 mM KH ₂ PO ₄ , 0.25 mM NaHCO ₃ Stock II: 0.025 mM CaCl ₂ , 0.012 mM MgSO ₄ , 0.05 mM HEPES
Laemmli sample buffer	20 % (v/v) Glycol; 8 % (w/v) Bromophenol blue + 400 mM DTT
Lysis buffer (for tissue protein isolation)	20 mmol/L Tris-HCl, 150 mmol/L sodium chloride, 1 mmol/L EGTA, 1 mmol/L EDTA, 1%(v/v) Triton-X-100 and a proteinase inhibitor and a phosphatase inhibitor cocktail
SDS-PAGE 1x electrophoresis buffer	25 mM Tris-HCl, 192 mM Glycine, 0.1 % (w/v) SDS
SDS-PAGE 1x separation gel buffer (pH 8.8)	500 mM Tris-HCl, 0.4 % SDS
SDS-PAGE 1x stacking gel buffer (pH 6.8)	0.5 M Tris, 0.4 % SDS
Transfer buffer (Tank blotting)	25 mM Tris-HCl, 192 mM Glycine, 20 % Methanol
Tris-buffered Saline with Tween (TBS-T)	10 mM Tris; 0,15 M NaCl; pH 8,0; 0,05 % TWEEN-20

Supplemental table 4: Antibodies

Name	Host Species	Supplier	Ordering #	Dilution used in TBS-T
AMPK α	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	#2532	1:1000 + 5 % BSA
ARK5	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	#4458	1:1000 + 5 % BSA
GAPDH (14C10)	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	#2118	1:1000 + 5 % milk powder
GLUT1	Rabbit	Prof. Schürmann (DIfE, Potsdam, Germany)		1:1000 + 5 % milk powder
GLUT4	Rabbit	Prof. Al-Hasani (DDZ, Düsseldorf, Germany)		1:1000 + 5 % milk powder

Glycogen Synthase	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	#3893	1:1000 + 5 % milk powder
p-Glycogen Synthase (Ser ⁶⁴¹)	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	#3891	1:1000 + 5 % BSA
PCK1	Rabbit	Abcam (Cambridge, UK)	#ab87340	1:1000+ 5 % BSA
PCK2	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	#6924	1:1000 + 5 % BSA
Rac1	Mouse	Cytoskeleton (Denver, CO, USA)	Arc03	1:1000 + 5 % milk powder
SNARK	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	#4100	1:1000 + 5 % BSA
TBC1D1 (V796)	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	#4629	1:1000 + 5 % milk powder
pTBC1D1 Ser ²³¹	Sheep	Dr. Hastie (MRC-PPU, Dundee, Scotland)		1:1000 + 5 % BSA
pTBC1D1 Ser ⁶⁶⁰	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	#6928	1:1000 + 5 % BSA
pTBC1D1 Ser ⁷⁰⁰	Rabbit	Cell Signaling Technology (Danvers, MA, USA)	#6929	1:1000 + 5 % BSA
TBC1D4	Rabbit	Merck Millipore (Burlington, MA, USA)	#07-741	1:1000 + 5 % milk powder
Anti-Rabbit-HRP	Goat	Dianova (Hamburg, Germany)	315-035-008	1:20,000

Supplemental Table 5: List of SYBR-Green primers for qPCR

Target gene	Primer sequences (5 → 3')
<i>Gapdh</i>	Fwd: CCA GGT TGT CTC CTG CGA CT Rev: ATA CCA GGA AAT GAG CTT GAC AAA GT
<i>Nuak1-fwd</i>	Fwd: GGC TCT CCA ACC TGT ACC AG Rev: TAA AGC AAG ACA CCC AGG GC
<i>Nuak2-fwd</i>	Fwd: AGG ATC TGC TGC ACA TAC GGA Rev: TGG CCG CTC ACT GAT GTA ATC
<i>Rps29-fwd</i>	Fwd: TGA AGG CAA GAT GGG TCA C Rev: GCA CAT GTT CAG CCC GTA TT
<i>Sik1-fwd</i>	Fwd: GGC ACC GAG TCA CCA AAA CG Rev: AAG TTT CAT GAG CTG GAC CTC C
<i>Sik2-fwd</i>	Fwd: CGT CGG CCT AGC ACC ATT G Rev: GTG GGA GGA GGG TAG ATC GC
<i>Sik3-fwd</i>	Fwd: AGC TGC TGA AGA CGT GGT GT Rev: GTG CTC CCA TCA AAC GGC AG