Supplemental data

Supplemental data figure legend

Figure S1

The pS711 antibody specifically detects S711 only when it is phosphorylated A) AICAR-stimulated (2 mM; 40 min) soleus muscles were homogenized in a buffer ±phosphatase inhibitors (i.e., NaF, sodium pyrophosphate, β-glycero-phosphate, and Na_3VO_4). Subsequently, samples were incubated ± 2U μg^{-1} λ-phosphatase for 30 min at 30°C. The reaction was stopped by adding Laemmli buffer to the samples and heating them at 96°C for 5 min. Samples were then subjected to SDS-PAGE. The single band detected using the pS711 antibody disappears when lysates are prepared without phosphatase inhibitors and treated with λ-phosphatase. Thus, the pS711 antibody detects a phospho-protein running at ~160 kDa. B) To verify that the single band visualized with the pS711 antibody in A) was indeed TBC1D4, TBC1D4 from basal, AICAR- and insulin-stimulated samples was immunoprecipitated from 200 µg of protein over-night at 4°C with anti-TBC1D4 antibody bound to protein-G beads from pools of basal, AICAR- (2 mM; 40 min), and insulin- (60 nM; 40 min) stimulated soleus muscles. TBC1D4 immunoprecipitants, the supernatants, and lysate samples cleared with beads were subsequently subjected to SDS-PAGE probing the membranes with antibodies recognizing total TBC1D4 and pS711. TBC1D4 was depleted from the supernatants and enriched in the immunoprecipitant (B, top panel). Moreover, the band detected with the pS711 antibody was also removed from the supernatant and recovered in the immunoprecipitant (B, lower panel). Thus, the pS711 antibody specifically detects TBC1D4. C) To confirm that the pS711 antibody did not cross-react with any other phosphorylation sites on TBC1D4, TBC1D4-WT and TBC1D4-S711A DNA constructs were injected and expressed in tibialis anterior muscles of ICR mice (n=4). Following recovery (7 days) expression of total TBC1D4 and phosphorylated S711 were evaluated to confirm site-specificity of the S711 antibody. In muscles ectopically expressing TBC1D4-WT a strong band running slightly higher than 160 kDa was detected using the pS711 antibody. In muscles expressing the TBC1D4-S711A mutant, however, no signal could be obtained at a similar molecular weight. Thus, the pS711 antibody specifically detects S711 of TBC1D4 only when phosphorylated.

Figure S2

Down-stream signaling from Akt is diminished in Akt2 KO mice in response to insulin Soleus muscles (n=6) from WT and Akt2 KO mice were incubated *in vitro* in the absence (black bars) or presence (white bars) of insulin (60 nM; 30 min). Muscle lysates were prepared followed by Western blot analyses using either A) a phospho-Akt substrate (PAS) antibody and quantifying the 160 kDa band or B) a phospho-GSK-3 antibody. ** Indicates treatment effect ($p<0.01$) and †† indicates genotype effect ($p<0.01$).

Figure S1

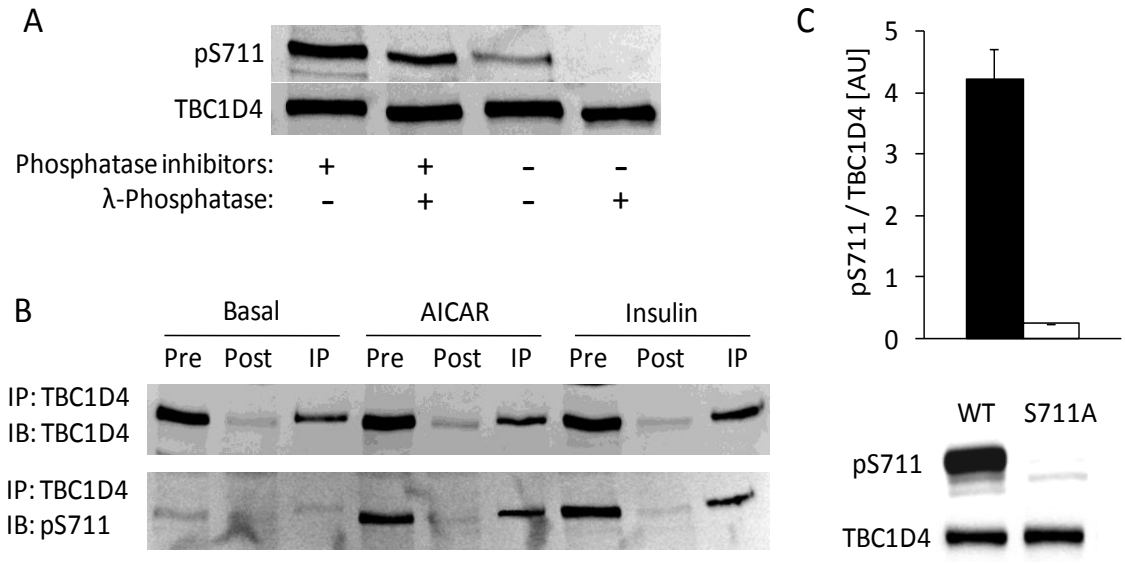

Figure S2

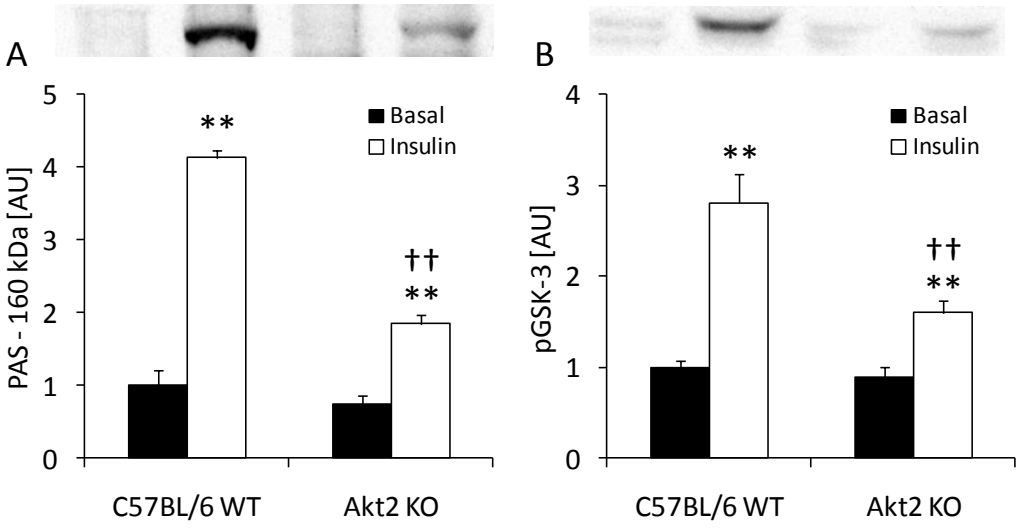

Table S1

Phosphorylation of proteins in AICAR- or contraction-signaling pathways

		pT172 AMPK		pS227 ACC	
Genotype	Condition	Basal	Stimulated	Basal	Stimulated
α2AMPK WT	AICAR	100±9	116±8*	100±2	170±11**
α2AMPK KD	AICAR	58±4††	77±9*/††	40±6††	88±11**/††
α2AMPK WT	Contract.	100±12	332±34**	100±9	232±21**
α2AMPK KD	Contract.	113±7	226±21**/†	47±4††	155±12**/††

Of note, the dominant negative AMPKα2 construct expressed in the transgenic animals retains the ability of being phosphorylated on T172. However, activity of the AMPK construct is severely blunted (26). The increased phosphorylation of ACC in response to both contraction and AICAR in the AMPKα2 KD mice has been reported previously (39). All values are arbitrary and related to the basal value in WT muscles. */** indicates different from basal within genotype; $P<0.05/0.01$. †/†† indicates different between genotypes within treatment condition; $P<0.05/0.01$.