Supplementary Materials: CaMKK2 Suppresses Muscle Regeneration through the Inhibition of Myoblast Proliferation and Differentiation

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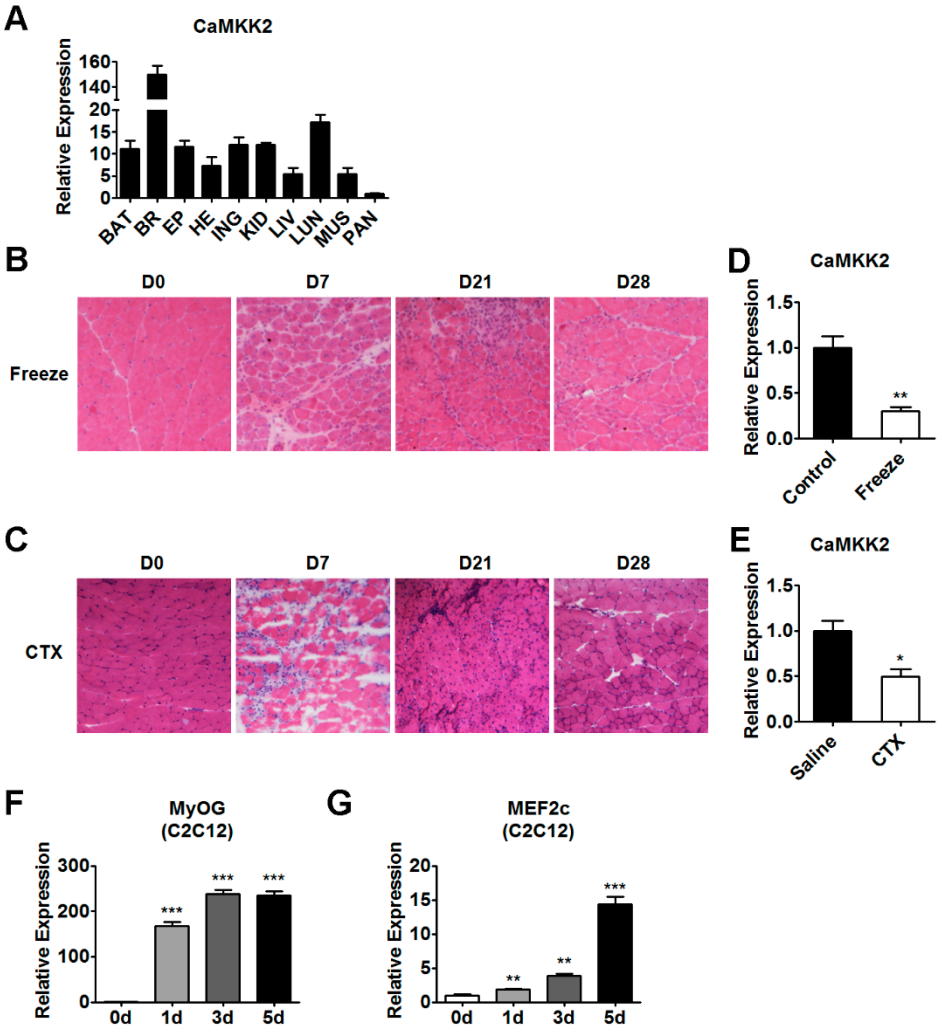

Figure S1. The expression of CaMKK2 in different tissues and myogenic markers during C2C12 myoblasts differentiation. (**A**) Quantitative RT-PCR analysis of CaMKK2 mRNA levels in different tissues as indicated (n = 3) (BAT: brown adipose tissue; BR: brain; EP: epididymal fat; HE: heart; ING: inguinal fat; KID: kidney; LIV: liver; LUN: lung; MUS: muscle; PAN: pancreas); (**B**,**C**) H&E staining of regenerating gastrocnemius muscles from mice at 7, 21 and 28 days following freeze injury (**B**) or CTX injury (**C**); (**D**,**E**) Quantitative RT-PCR analysis of CaMKK2 mRNA levels in freeze injury-induced regeneration (n = 4) or in CTX injury-induced regeneration (n = 4); (**F**,**G**) Quantitative RT-PCR analysis of myogenin (MyOG) (**F**) and MEF2c (**G**) mRNA levels during C2C12 myoblasts differentiation (n = 3). Means ± SEM (error bars) are shown. *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$.

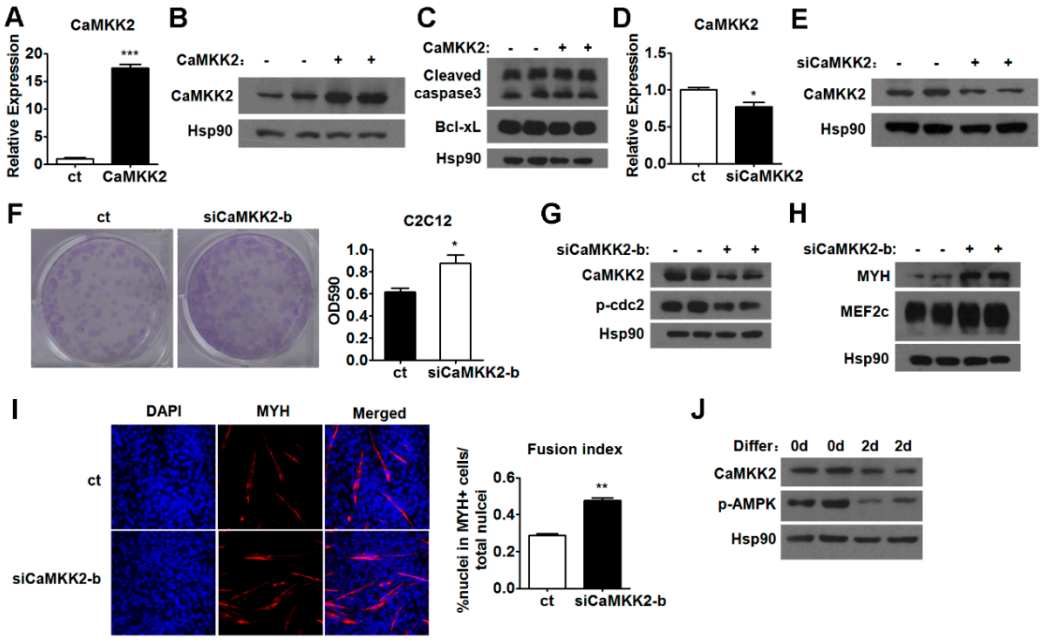

Figure S2. CaMKK2 levels in C2C12 myoblasts and p-AMPK protein levels in undifferentiated and differentiated C2C12 cells. (**A**) CaMKK2 mRNA levels in C2C12 cells transfected with CaMKK2 plasmids (n = 3); (**B**) Representative western blot showing the CaMKK2 protein levels in C2C12 cells transfected with CaMKK2 plasmids; (**C**) Representative western blot showing the cleaved caspase3 and Bcl-xL protein levels in C2C12 cells transfected with CaMKK2 plasmids; (**D**) CaMKK2 mRNA levels in C2C12 cells transfected with siCaMKK2 (n = 3); (**E**) Representative western blot showing the CaMKK2 protein levels in C2C12 cells transfected with siCaMKK2; (**F**) Representative micrographs of crystal violet-stained C2C12 cell colonies, which were transfected with siCaMKK2-b as indicated (**left**), absorbance of each well at 590 nm (**right**) (n = 3); (**G**) Representative western blot showing the CaMKK2 and p-Cdc2 Tyr15 protein levels in C2C12 cells transfected with siCaMKK2-b; (**H**) Representative western blot showing the MYH, MEF2c protein levels in C2C12 myotubes transfected with siCaMKK2-b; (**I**) Immunofluorescence analysis of MYH was performed in C2C12 myotubes 48 h after siCaMKK2-b transfection (**left**), quantification of the fusion index of indicated cells (**right**) (n = 3); (**J**) Representative western blot showing the p-AMPK and CaMKK2 protein levels in undifferentiated (Day 0, 0d) and differentiated (Day 2, 2d) C2C12 cells as indicated. Experiments were repeated at least twice. Means ± SEM (error bars) are shown. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$.

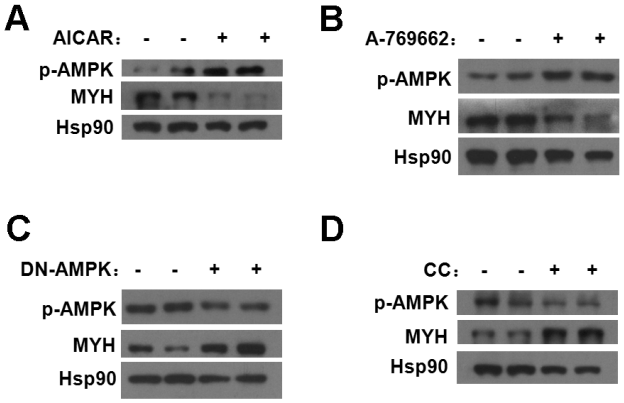

Figure S3. AMPK activation or inhibition affectsC2C12 myoblasts differentiation. (**A**,**B**) Representative western blot showing the MYH and p-AMPK protein levels in C2C12 myotubes treated with 0.25 mM AICAR or 80 μM A-769662 (**B**) for 48 h; (**C**,**D**) Representative western blot showing the MYH and p-AMPK protein levels in C2C12 myotubes treated with DN-AMPK (**C**) or10 μM Compound **C** (D, CC) for 48 h. Experiments were repeated at least twice.

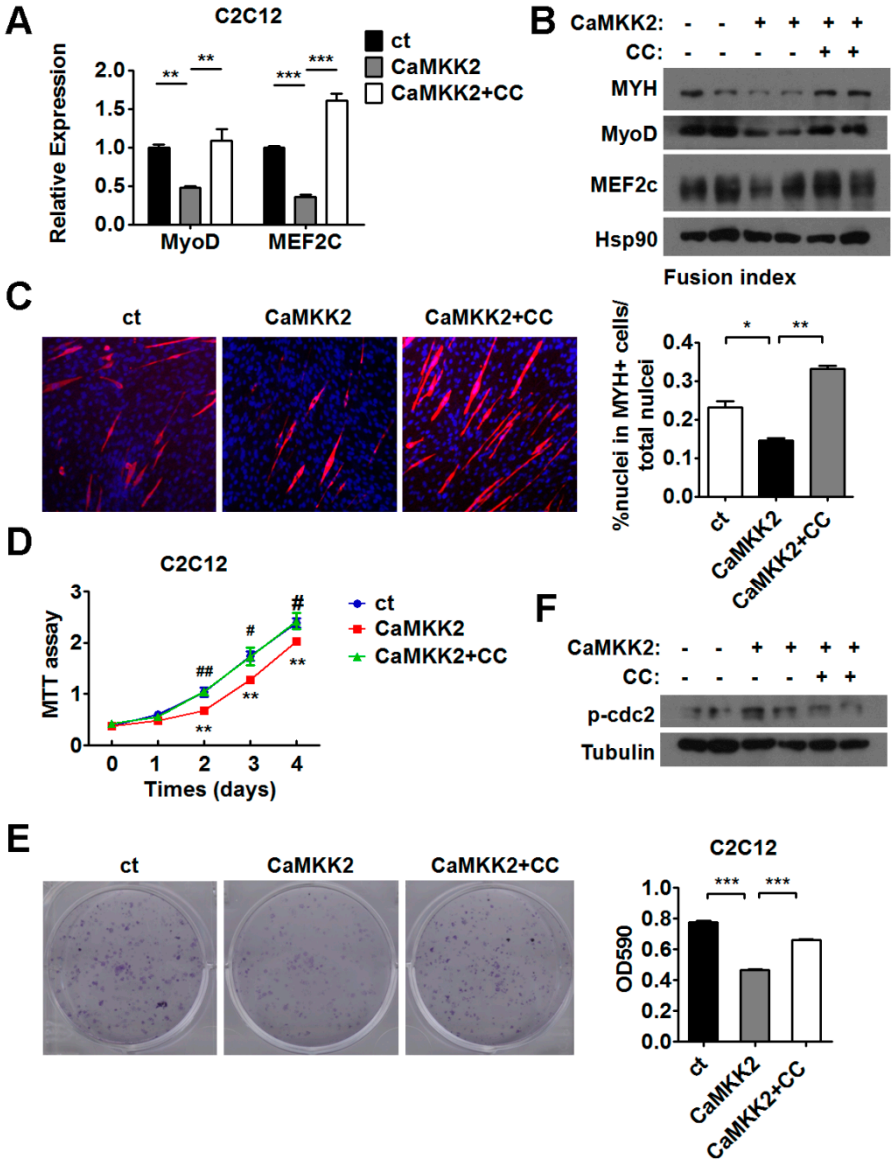

Figure S4. CaMKK2 inhibits C2C12 myoblasts proliferation and differentiation through AMPK activation. (**A**) Quantitative RT-PCR analysis of MyoD and MEF2c mRNA levels in C2C12 myotubes transfected with CaMKK2 plasmids and then treated with 10 μM Compound C (CC) for 48 h (n = 3); (**B**) Representative western blot showing the MYH, MyoD and MEF2c protein levels in C2C12 myotubes transfected with CaMKK2 plasmids and then treated with 10 μM Compound C for 48 h; (**C**) Immunofluorescence analysis of MYH was performed in C2C12 myotubes transfected with CaMKK2 plasmids and then treated with 10 μM Compound C for 48 h (**left**), quantification of the fusion index of indicated cells (right) (n = 3). Means ± SEM (error bars) are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; (**D**) MTT assay of the proliferation ability of C2C12 myoblasts transfected with CaMKK2 or control plasmids, and then incubated with 10 μM Compound C and maintained over a period of 5 days. Means ± SEM (error bars) are shown. **, $p < 0.01$ CaMKK2-treated group vs. control group; #, p <0.05; ##, $p < 0.01$ CaMKK2 and CC treated group vs. CaMKK2-treated group; (**E**) Representative micrographs of crystal violet-stained C2C12 cell colonies, which were transfected with CaMKK2 or control plasmids, and then incubated with 10 μM Compound C (**left**), absorbance of each well at 590 nm (**right**) (n = 3); (**F**) Representative western blot showing the p-cdc2 protein levels in C2C12 myotubes transfected with CaMKK2 plasmids and then treated with 10 μM Compound C for 48 h. Experiments were repeated at least twice. Means ± SEM (error bars) are shown. ***, $p < 0.001$.

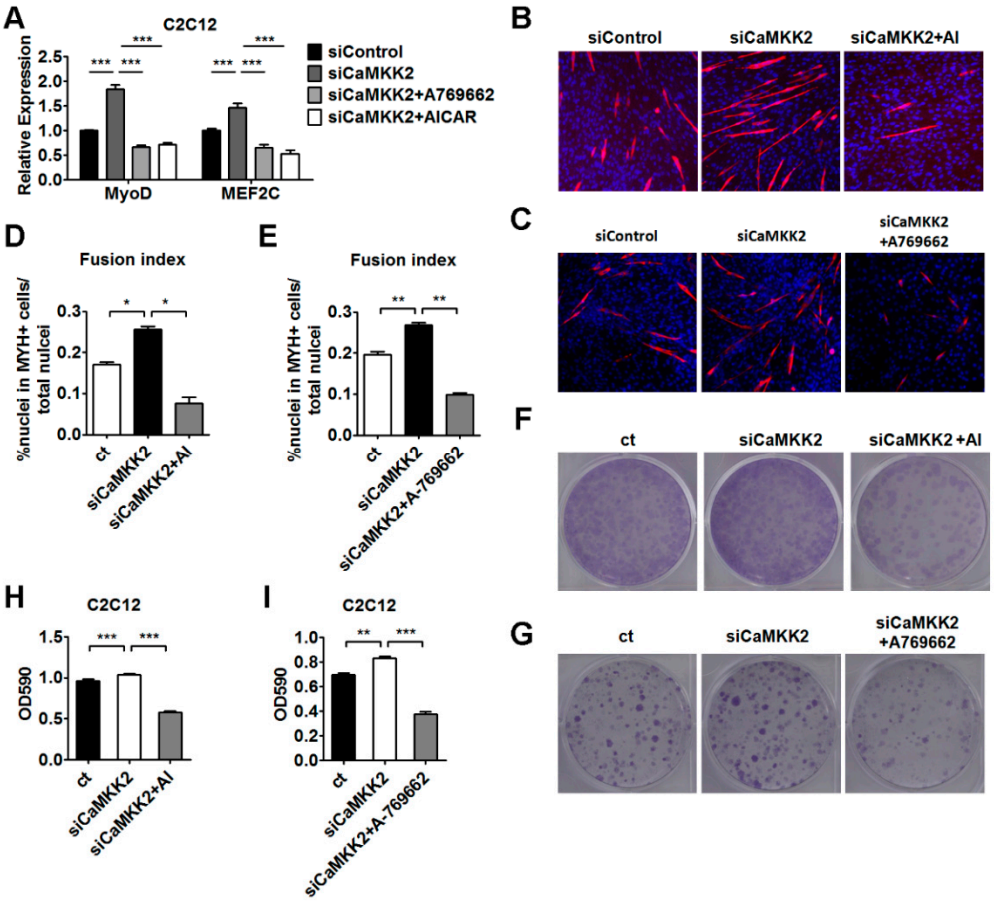

Figure S5. A-769662 and AICAR attenuates the effect of CaMKK2 knockdown in C2C12 myoblasts proliferation and differentiation. (**A**) Quantitative RT-PCR analysis of MyoD, and MEF2c mRNA levels in C2C12 myotubes transfected with siCaMKK2 and then treated with 0.25 mM AICAR or 80 μM A-769662 for 48 h (n = 3); (**B**,**C**) Immunofluorescence analysis of MYH was performed in C2C12 myotubes transfected with siCaMKK2 and then treated with 0.25 mM AICAR (**B**) or 80 μM A-769662 (**C**) for 48 h; (**D**,**E**) Quantification of the fusion index of C2C12 myotubes transfected with siCaMKK2 and then treated with 0.25 mM AICAR (**D**) or 80 μM A-769662 (**E**) for 48 h (n = 3); (**F**,**G**) Representative micrographs of crystal violet-stained C2C12 cell colonies, which were transfected with siCaMKK2 and then treated with 0.25 mM AICAR (**F**) or 80 μM A-769662 (**G**) for 48 h; (**H**,**I**) Absorbance at 590 nm of crystal violet-stained C2C12 cell colonies, which were transfected with siCaMKK2 and then treated with 0.25 mM AICAR (**H**) or 80 μM A-769662 (**I**) for 48 h (n = 3). Means ± SEM (error bars) are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

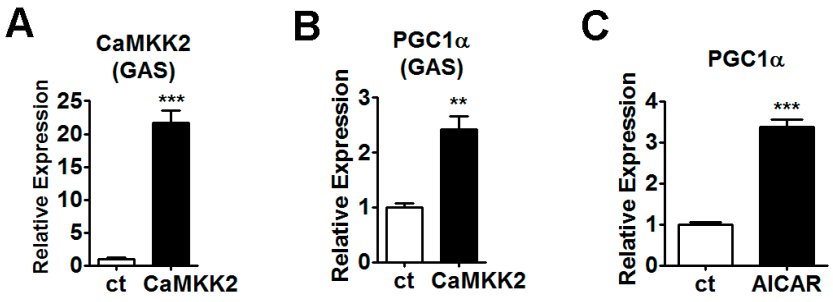

Figure S6. CaMKK2 expression in mice after electroporation with CaMKK2 plasmids and PGC1α expression in C2C12 cells treated with AICAR. (**A**,**B**) Quantitative RT-PCR analysis of CaMKK2 (**A**) and PGC1α (**B**) mRNA levels in gastrocnemius muscles (GAS) after electroporation with CaMKK2 and control plasmids (n = 3); (**C**) Quantitative RT-PCR analysis of PGC1α mRNA levels in C2C12 cells treated with 0.25 mM AICAR for 48 h (n = 3). Means ± SEM (error bars) are shown. **, $p < 0.01$; ***, $p < 0.001$.

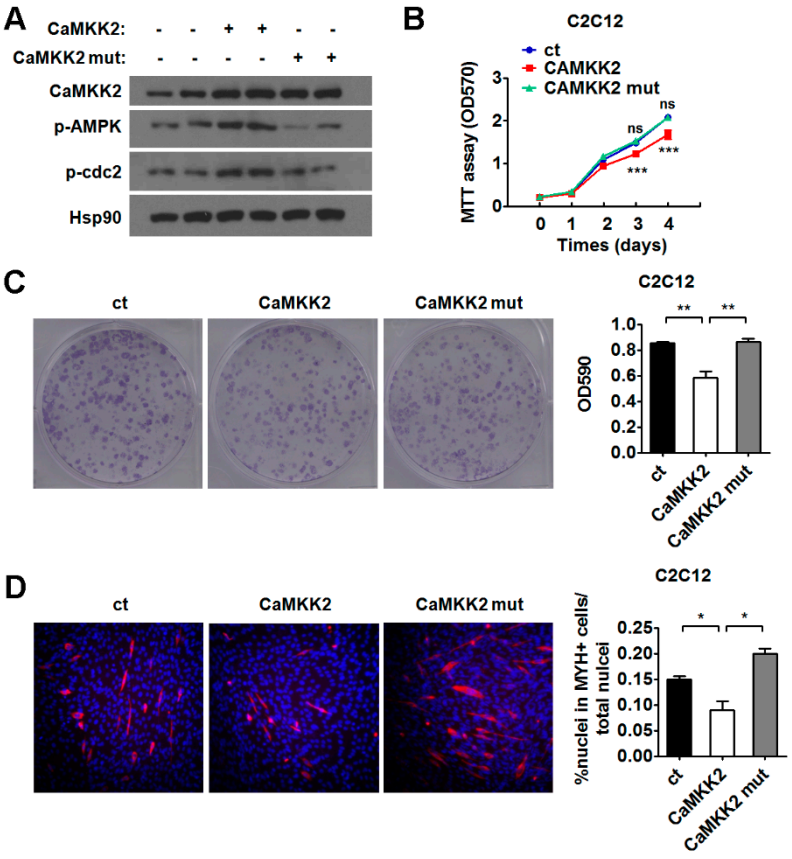

Figure S7. CaMKK2 K193A mutation could not lead to cell cycle retardation or inhibit myoblast differentiation in vitro. (**A**) Representative western blot showing the p-AMPK and p-cdc2 protein levels in the C2C12 cells transfected with CaMKK2 or CaMKK2 K193A plasmids; (**B**) MTT assay of the proliferation of C2C12 myoblasts transfected with CaMKK2 or CaMKK2 K193A plasmids and maintained over a period of 5 days. Means ± SEM (error bars) are shown. ***, $p < 0.001$, CaMKK2-treated group vs. control group; ns, $p \geq 0.05$ CaMKK2-mut treated group vs. CaMKK2-treated group; (**C**) Representative micrographs of crystal violet-stained C2C12 cell colonies, which were transfected with CaMKK2 or CaMKK2 K193A plasmids as indicated (**left**), absorbance of each well at 590 nm (**right**) ($n = 3$); (**D**) Immunofluorescence analysis of MYH was performed in C2C12 myotubes 48 h after CaMKK2 plasmids transfection (**left**), quantification of the fusion index of indicated cells (**right**) ($n = 3$). Experiments were repeated at least twice. Means ± SEM (error bars) are shown. *, $p < 0.05$; **, $p < 0.01$.

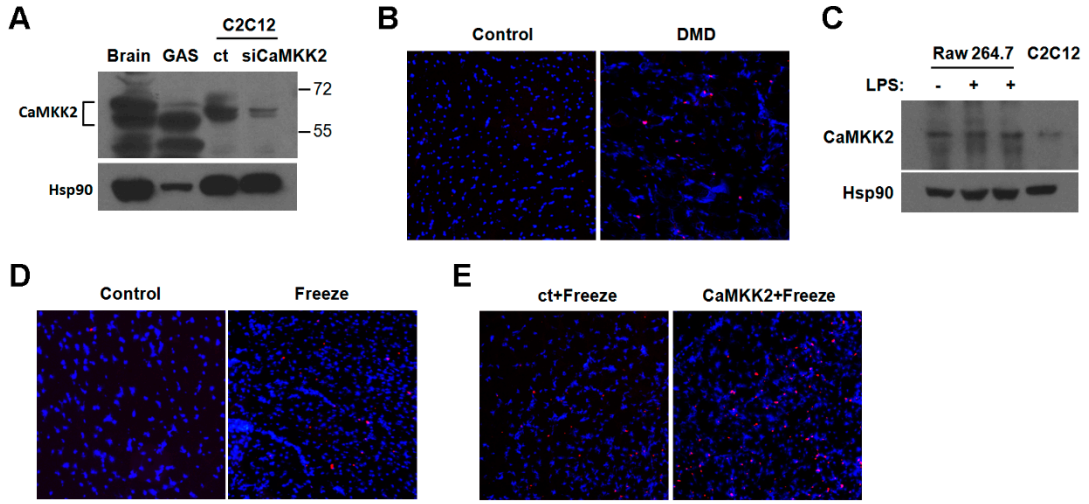

Figure S8. CD68 staining in DMD patient and mice gastrocnemius muscles. (**A**) Representative western blot showing the CaMKK2 protein levels in brain, gastrocnemius muscle and C2C12 cell transfected with siCaMKK2 or ct; (**B**) CD68 staining of muscle from DMD patient (Blue: DAPI, Red: CD68); (**C**) Representative western blot showing the CaMKK2 protein levels in Raw 264.7 and C2C12 cells; (**D**) CD68 staining of regenerating gastrocnemius muscle (**Blue**: DAPI, **Red**: CD68); (**E**) CD68 staining of regenerating gastrocnemius muscles from mice after electroporation with CaMKK2 and control plasmids at 14 days following freeze injury (**Blue**: DAPI, **Red**: CD68). Experiments were repeated at least twice.

Table S1. Primers for quantitative real-time PCR.

Gene	Accession No.	Primer Sequence	Product Length
CaMKK2	NM_001199676	Forward: AGACCAGGCCCGCTTCTACT Reverse: GAAGATCTTGCGGGTCTCTG	241
MyoD	NM_010866.2	Forward: CGCCACTCCGGGACATAG Reverse: GAAGTCGTCTGCTGTCTCAAAGG	71
MEF2C	NM_001170537.1	Forward: GTCAGTTGGGAGCTTGCACTA Reverse: CGGTCTCTAGGAGGAGAAACA	112
Myogenin	NM_031189.2	Forward: AGTGAATGCAACTCCCACAG Reverse: ACGATGGACGTAAGGGAGTG	137
cyclin A1	NM_001305221.1	Forward: TGATGCTTGTCAAATGCTCAGC Reverse: AGGTCCTCCTGTACTGCTCAT	101
cyclin D1	NM_007631.2	Forward: GCGTACCCTGACACCAATCTC Reverse: CTCCTCTTCGCACTTCTGCTC	183
cyclin E1	NM_007633.2	Forward: GTGGCTCCGACCTTTCAGTC Reverse: CACAGTCTTGTCAATCTTGGCA	101
p27	NM_009875.4	Forward: TCAAACGTGAGAGTGTCTAACG Reverse: CCGGGCCGAAGAGATTTCTG	103
Pax7	NM_011039.2	Forward: TCTCCAAGATTCTGTGCCGAT Reverse: CGGGGTTCTCTCTCTTATACTCC	132
PGC1α	NM_008904.2	Forward: CAATGAATGCAGCGGTCTTA Reverse: GTGTGAGGAGGGTCATCGTT	198
GAPDH	NM_001289726.1	Forward: ACATCATCCCTGCATCCACT Reverse: GTCCTCAGTGTAGCCCAAG	224

Table S2. Densitometric analysis for Western blots.

Figure 1								
	Figure 1B	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	1	1.00	1.52	1.17	1.20	0.39	0.60	0.22	0.38
	2	1.00	0.93	1.46	1.37	1.18	1.18	1.25	1.76
	Figure 1C	**1**	**2**	**3**	**4**	**5**	**6**		
	1	1.00	0.2	0.82	0.49	0.80	0.49		
	2	1.00	1.19	0.91	1.02	1.14	1.40		
	Figure 1D	**1**	**2**	**3**	**4**	**5**	**6**		
	1	1.00	0.12	0.84	0.86	1.47	0.67		
	2	1.00	1.15	1.05	1.43	1.07	1.03		
	Figure 1G	**1**	**2**	**3**	**4**	**5**	**6**		
	1	1.00	2.22	5.85	11.43	16.90	20.05		
	2	1.00	2.55	4.15	4.58	5.24	4.31		
	3	1.0	0.90	0.79	0.80	0.64	0.59		
	4	1.00	1.23	0.83	0.66	0.45	0.22		
	5	1.00	0.99	0.96	0.93	0.94	1.06		
Figure 2								
	Figure 2F	**1**	**2**	**3**	**4**				
	1	1.00	1.33	2.43	2.76				
	2	1.00	1.06	1.04	1.14				
	Figure 2H	**1**	**2**	**3**	**4**				
	1	1.00	1.89	2.92	3.45				
	2	1.00	0.86	0.52	0.29				
	3	1.00	0.97	0.44	0.40				
	4	1.00	0.74	0.84	0.80				
Figure 3								
	Figure 3B	**1**	**2**	**3**	**4**				
	1	1.00	1.13	1.81	1.42				
	2	1.00	0.89	0.64	0.36				
	3	1.00	0.90	0.58	0.56				
	4	1.00	0.83	0.37	0.55				
	5	1.00	1.07	1.10	1.00				
Figure 4								
	Figure 4E	**1**	**2**	**3**	**4**				
	1	1.00	0.79	0.55	0.39				
	2	1.00	0.94	0.60	0.71				
	3	1.00	1.21	0.68	0.67				
	4	1.00	0.89	0.97	0.94				
	Figure 4H	**1**	**2**	**3**	**4**				
	1	1.00	1.07	0.70	0.57				
	2	1.00	1.19	1.92	1.82				
	3	1.00	1.34	2.49	2.26				
	4	1.00	1.02	0.85	0.82				

Table S2. *Cont.*

Figure 5							
	Figure 5A	**1**	**2**	**3**	**4**		
	1	1.00	1.08	1.68	1.83		
	2	1.00	1.20	1.81	1.77		
	3	1.00	0.80	0.83	0.80		
	4	1.00	1.11	1.04	0.92		
	Figure 5C	**1**	**2**	**3**	**4**	**5**	**6**
	1	1.00	0.82	0.35	0.63	1.37	1.28
	2	1.00	1.05	0.83	0.64	1.05	1.12
	3	1.00	1.03	0.70	0.47	0.74	0.89
	4	1.00	0.87	0.87	0.96	0.79	0.85
	5	1.00	1.03	1.73	1.89	0.97	0.58
	6	1.00	0.76	1.05	1.07	1.82	1.74
	Figure 5G	**1**	**2**	**3**	**4**	**5**	**6**
	1	1.00	1.13	1.57	1.31	1.05	1.10
	2	1.00	1.26	1.19	0.95	1.01	0.93
Figure 6							
	Figure 6B	**1**	**2**	**3**	**4**		
	1	1.00	1.07	1.89	1.51		
	2	1.00	1.14	1.12	1.01		
	Figure 6D	**1**	**2**	**3**	**4**		
	1	1.00	0.76	0.25	0.47		
	2	1.00	1.20	1.13	1.23		
	Figure 6G	**1**	**2**	**3**	**4**		
	1	1.00	1.04	3.59	3.61		
	2	1.00	1.10	0.86	0.97		
	Figure 6H	**1**	**2**	**3**	**4**		
	1	1.00	0.96	1.72	1.71		
	2	1.00	1.02	0.85	0.87		