Supporting Online Material for:

Skeletal myosin light chain kinase regulates skeletal myogenesis by phosphorylation of MEF2C

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This PDF file includes supplementary:

Methods:

Tandem Affinity Purification

To identify MEF2C associated proteins at early stages of skeletal muscle development, P19[MEF2C-TAP] and P19[TAP] cells were aggregated in the presence of Me_2SO and cellular protein extracts were prepared from the differentiated cells on day 5. To ensure sufficient material, 50 plates (150 mm) were used for each cell line per purification. Protein complexes of the tagged MEF2C were purified from P19[MEF2C-TAP] lysates using streptavidin and calmodulin columns using the tandem affinity purification procedure described by InterPlay mammalian expression TAP purification kit (Stratagene, USA, http://www.stratagene.com/manuals/240101.pdf). Co-eluted proteins were visualized by silver staining after fractionation on SDS/PAGE.

Protein Processing and LC-MS/MS

Following SDS-PAGE, protein bands were excised and subjected to in-gel tryptic digestion as previously described (Abu-Farha et al, 2008). Digestions were carried out overnight using sequencing grade trypsin obtained from Promega (Madison, WI). Peptides from gel bands were extracted, hydrated in 20μL of 5% formic acid, and subjected to analysis on LC-MS/MS. Peptide and MS/MS mass tolerances are set at ± 2 Da and 0.8 Da, respectively. MS/MS data were then analyzed and matched to mouse and/or human protein sequences in the NCBI database (nrdb) using the Mascot database search engine (Matrix Science, UK) with Ser/Thr phosphorylation as a fixed modification and oxidation as a variable modification. LC-MS/MS analysis of samples identified several peptides of skMLCK

that were present with high confidence in complex with MEF2C but not in the TAP control samples.

References:

Abu-Farha M, Lambert JP, Al-Madhoun AS, Elisma F, Skerjanc IS, Figeys D (2008) The tale of two domains: proteomics and genomics analysis of SMYD2, a new histone methyltransferase. *Mol Cell Proteomics* **7**(3)**:** 560-572.

Figure Legends:

Figure S1. Identification of skMLCK as a Novel MEF2C Associated Protein

(A) MEF2C-TAP activated a MEF2-responsive promoter. P19 cells were transiently transfected with plasmids encoding the muscle reporter 4X MEF2-luciferace, Renilla luciferase and Flag tagged MEF2C, TAP tagged MEF2C or un-tagged MEF2C. After 24 hr, cells were lysed and luciferase activity was measured. Data are means ± SEM (n=3).

(B) MEF2C-TAP enhanced skeletal myogenesis in P19 cells. Control P19[TAP] and P19[MEF2C-TAP] cells were differentiated in the presence of DMSO to induce skeletal myogenesis. On day 9, cells were fixed and reacted with Hoechst dye to detect nuclei (blue) and anti-MHC antibody (red).

(C) MEF2C-TAP protein was present during P19 cell differentiation. Total protein extracts from P19, P19[TAP] and P19[MEF2C-TAP] on days 0, 6, or 9 of differentiation, were examined by western blot with antibodies against calmodulin binding peptide (CBP-TAP) or MEF2C.

(D) P19[TAP] and P19[MEF2C-TAP] cells were differentiated in the presence of DMSO to induce skeletal myogenesis. On day 5, MEF2C-TAP was purified using the TAP purification protocol. Western blotting using an anti-MEF2C antibody identified MEF2C-TAP in the P19[MEF2C-TAP] purification. Silver staining showed protein bands associated with MEF2C-TAP compared to control TAP cells. Proteins were identified by LC-MS/MS. This gel is representative of two purification experiments. Western blot analysis using an anti-skMLCK antibody confirmed that skMLCK is co-purified with MEF2C.

(E) skMLCK can shuttle between the nucleus and the cytoplasm. HEK-293 cells were transfected with YFP-skMLCK. After 24hrs, the cells were treated with or without 10 μM leptomycin for 4 hours and examined by fluorescence microscopy.

(F) Western Bolt Analysis for the endogenous expression of skMLCK in HEK-293 and in differentiated versus non differentiated C2C12 cells. Protein extracts

were examined by Western blot with antibodies against skMLCK. Results show that skMCK is not detected in HEK-293 cells and is upregulated during C2C12 differentiation.

Figure S2. SkMLCK phosphorylates MEF2C *in vitro*. Recombinant His-MEF2C was incubated with purified skMLCK at 30^{o}C in a reaction mixture containing [^{32}P]-γ-ATP in the presence or absence of Mg^{2+}, Ca^{2+} or increasing concentrations of calmodulin. After SDS-PAGE, the gel was Silver stained and subjected to autoradiography. Bands corresponding to MEF2C and skMLCK proteins are indicated by the black and white arrowheads, respectively.

Figure S3. Inhibition of MLCK resulted in a loss of skeletal myocytes in P19 cells. P19 cells were aggregated in the presence of 1% DMSO for 4 days with either 0, 300 or 600 nM ML-7 for 9 days. (A) Cells were fixed on day 9 of differentiation. Immunofluorescence was performed using an Anti-Myosin Heavy Chain antibody, MF20, to detect skeletal muscle (B,D,F,H) or cardiac muscle (J,L,N,P) , and counter stained with Hoechst dye (A,C,E,G; I ,K,M,O) to visualize the nuclei. Magnification is 200X. Skeletal and cardiac myocytes and total nuclei for 10-12 fields were counted, at a magnification of 200X, for each treatment, for two independent experiments (n=2), and illustrated as the percentage of differentiated skeletal myocytes, +/-SEM.

(B) RNA was harvested on day 9 and examined by Q-PCR. Levels were expressed as fold change over untreated cells and as a percentage of the DMSO - treated cells. Results were normalized to β-actin and are averages ± s.e.m. (n=3;* $p < 0.05$).

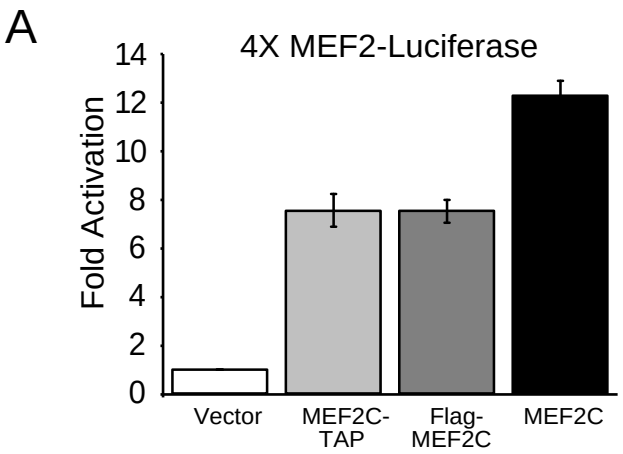
Table 1. Reverse and Forward primers used for Cloning and Mutagenesis

MEFT80D-F	GAGCCGCATGAGAGCCGG**GAC**AACTCAGA**T**ATCGTGGAGACGTTGAGAAAG
MEFT80D-R	CTTTCTCAACGTCTCCACGAT**A**TCTGAGTT**GTC**CCGGCTCTCATGCGGCTC
MEFT80A-R	GAGCCGCATGAGAGCCGG**G**CAAACTCAGA**T**ATCGTGGAGACGTTGAGAA
MEFT80A-F	TTCTCAACGTCTCCACGATATCTGAGTTTGCCCGGCTCTCATGCGGCTC
skMLCK-ATP mutant-F	AAGGAGGCGCTCG**C**AGGTG**C**CAAGT**C**TG**C**GGCAGTCTGTAC**C**TGC
skMLCK-ATP mutant-R	GCA**G**GTACAGACTGCC**G**CA**G**ACTTG**G**CACC**T**GCGAGCGCCTCCTT
skMLCK-cal mutant-F	AAGAAATACCTCATGAA**C**AG**CT**GCTGGAA**C**AAAAACTTCATTGCTG
skMLCK-cal mutant-R	CAGCAATGAAGTTTTT**G**TTCCAGC**AGC**T**G**TTCATGAGGTATTTCTT
	Bold nucleotides represent the mutant sites.
skMLCK clone-F	CTAGAAGCTTACATGGCGACAGAAAATGGAGC
skMLCK clone-R	TTATACTCGAGGACCCCCAGAGCCA

Table 2. Reverse and Forward primers used for Q-PCR analysis and CHIP analysis

GENES	FORWARD PRIMER	REVERSE PRIMER
ß-actin	AAATCGTGCGTGACATCAAA	AAGGAAGGCTGGAAAAGAGC
MyoD	CCCCGGCGGCAGAATGGCTACG	GGTCTGGGTTCCCTGTTCTGTT
Myf5	CCTGTCTGGTCCCGAAAGAAC	GACGTGATCCGATCCACAATG
Myogenin	GCAATGCACTGGAGTTCG	ACGATGGACGTAAGGGAGTG
Pax3	TTTCACCTCAGGTAATGGGACT	GAACGTCCAAGGCTTACTTTGT
Meox1	TGGCCTATGCAGAATCCATTCC	RTGGATCTGAGCTGCGCA TGTG
Gata4	AAAACGGAAGCCCAAGAACCT	TGCTAGTGGCATTGCTGGAGT
alpha-actin	GTGGTATTGCCGATCGTATG	CTTGCTGATCCACATTTGCT
MEF2C	TCTGTCTGGCTTCAACACTG	TGGTGGTACGGTCTCTAGGAT
skMLCK	AGACTACCATCTGACGGACCT	AGTCGATTATCTTCACCAAGTC
cMLCK	AAGCTGACGCCTCTAGGAC	CTCCTGCCTGGGTTAGGTC
MHC	ACAACCCCTACGATTATGCGT	ACGTCAAAGGCACTATCCGTG
Nkx2.5	AAGCAACAGCGGTACCTGTC	GCTGTCGCTTGCACTTGTAG
MyoG promoter	AAAGGAGAGGGAAGGGGAAT	AACTGCTGGGTGCCATTTA
GATA4 Promoter	AAGCGCTCTTTTCTCCTTCC	GTGAGGGCTACAGGGAGTGA

Supp. Fig. S1


A
4X MEF2-Luciferase
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10
8
6
4
2
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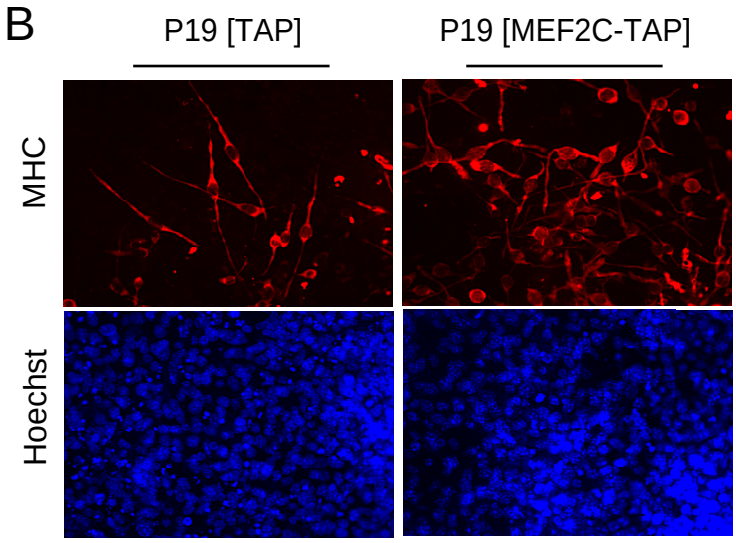
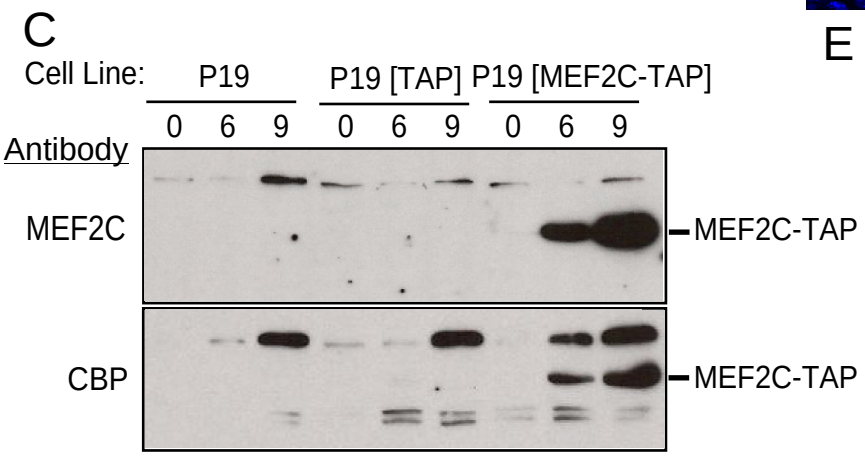
Vector
MEF2C-TAP
Flag-MEF2C
MEF2C


B
P19 [TAP]
P19 [MEF2C-TAP]
MHC
Hoechst


C
Cell Line:
P19
P19 [TAP]
P19 [MEF2C-TAP]
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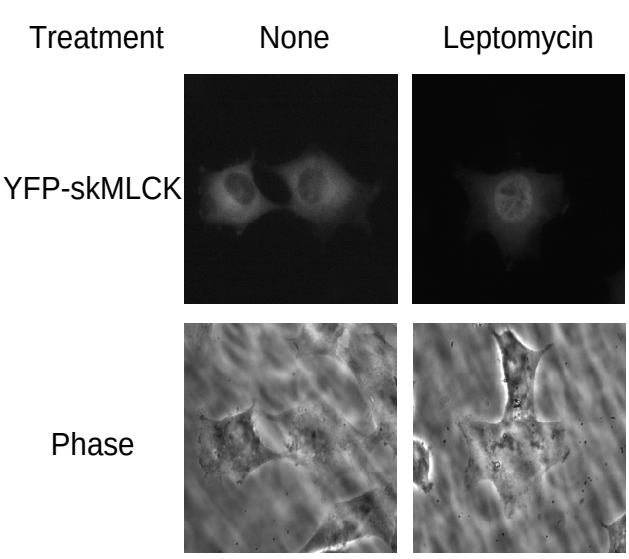
MEF2C-TAP
CBP
MEF2C-TAP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Treatment
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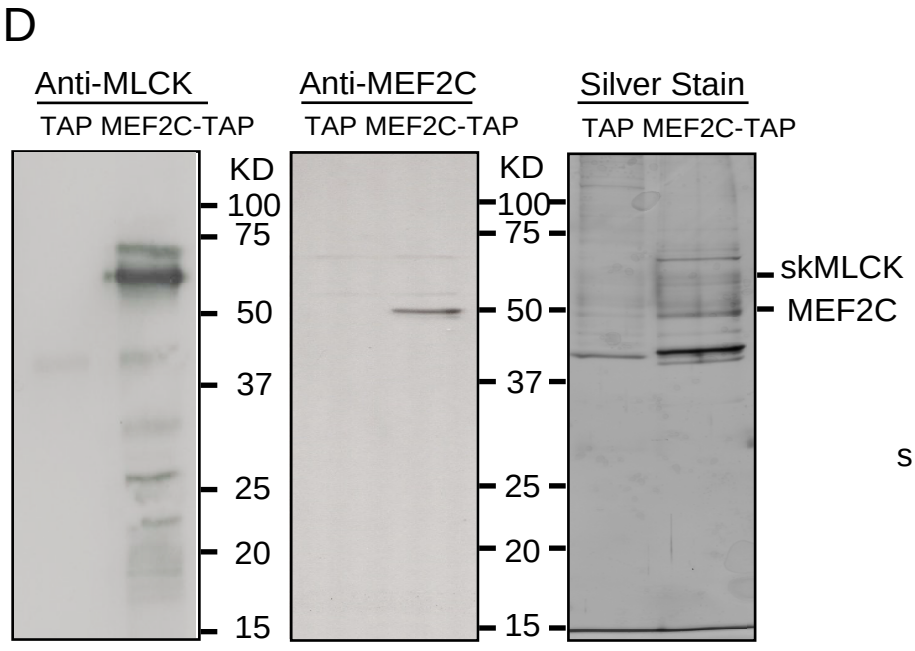
YFP-skMLCK
Phase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Anti-MEF2C
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Silver Stain
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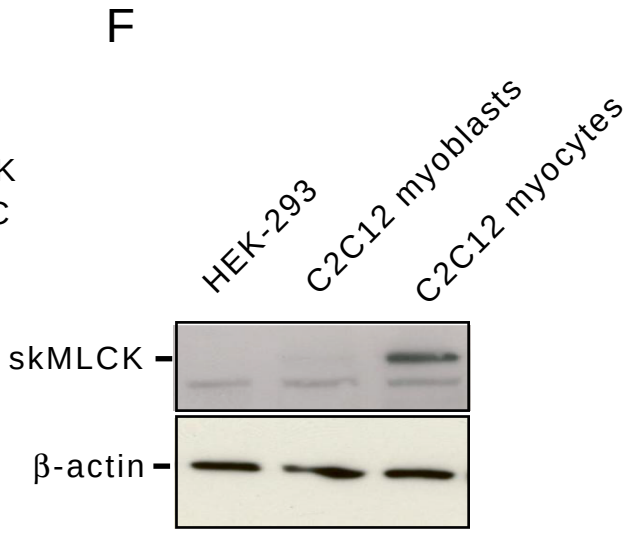
50
37
25
20
15
skMLCK
MEF2C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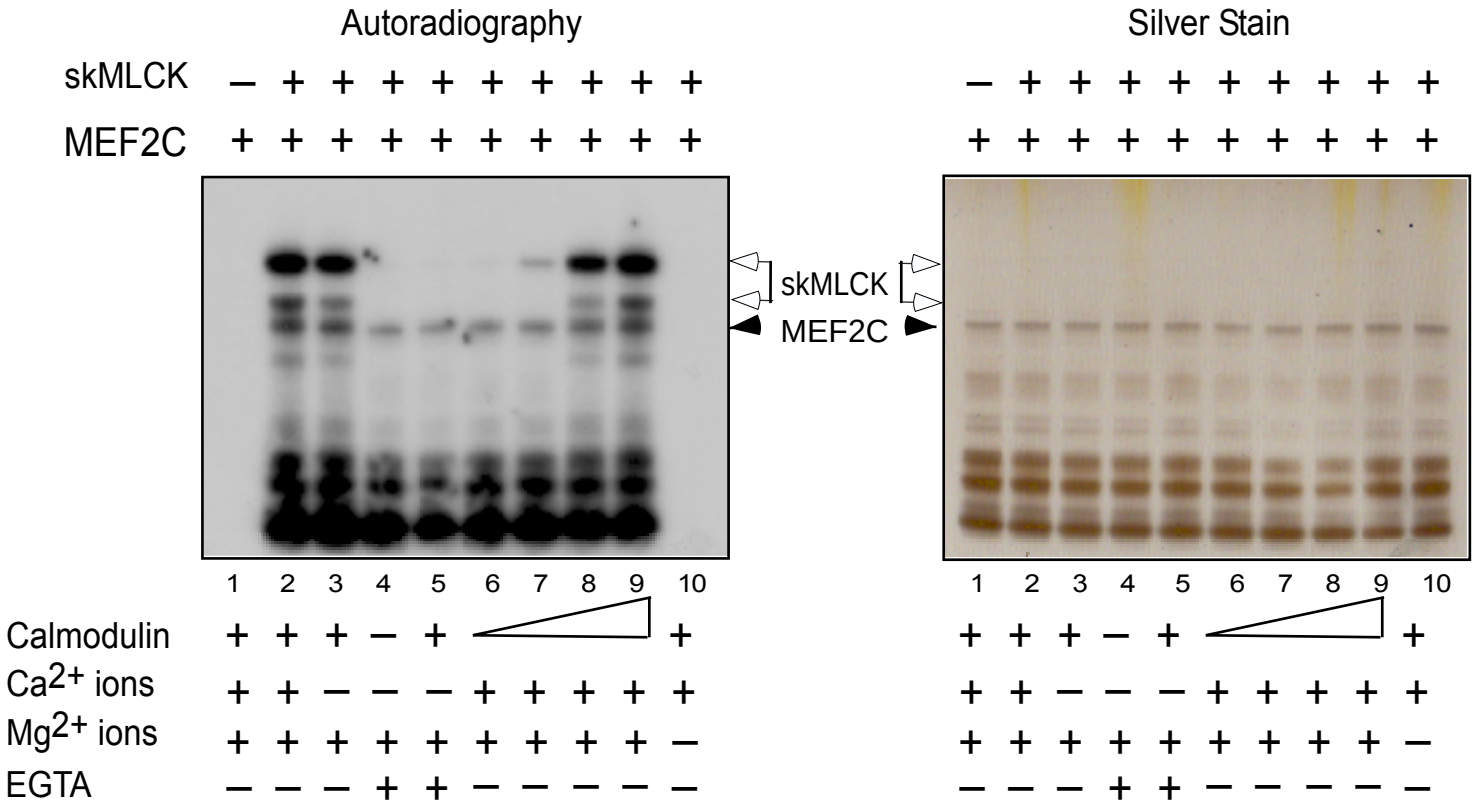
C2C12 myoblasts
C2C12 myocytes
skMLCK
β-actin

Supp. Figure S2



Supp. Figure S3