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

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The MEF2 factors regulate transcription during cardiac and skeletal myogenesis. MEF2 factors establish skeletal muscle commitment by amplifying and synergizing with MyoD. While phosphorylation is known to regulate MEF2 function, lineage-specific regulation is unknown. Here, we show that phosphorylation of MEF2C on T^{80} by skeletal myosin light chain kinase (skMLCK) enhances skeletal and not cardiac myogenesis. A phosphorylation-deficient MEF2C mutant (MEFT80A) enhanced cardiac, but not skeletal myogenesis in P19 stem cells. Further, MEFT80A was deficient in recruitment of p300 to skeletal but not cardiac muscle promoters. In gain-of-function studies, skMLCK upregulated myogenic regulatory factor (MRF) expression, leading to enhanced skeletal myogenesis in P19 cells and more efficient myogenic conversion. In loss-of-function studies, MLCK was essential for efficient MRF expression and subsequent myogenesis in embryonic stem (ES) and P19 cells as well as for proper activation of quiescent satellite cells. Thus, skMLCK regulates MRF expression by controlling the MEF2C-dependent recruitment of histone acetyltransferases to skeletal muscle promoters. This work identifies the first kinase that regulates MyoD and Myf5 expression in ES or satellite cells.

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Introduction

Cellular differentiation is controlled by cascades of regulatory genes comprising combinations of widely expressed and cell type-restricted transcription factors. The MEF2 proteins (MEF2A-D) consist of a family of transcription factors that have a central role during the development of several tissues, including cardiac and skeletal muscle (Potthoff and Olson, 2007). While MEF2 can synergize with tissue-specific transcription factors in the various lineages (Molkentin *et al*, 1995; Naidu *et al*, 1995; Black *et al*, 1996; Morin *et al*, 2000), the signalling pathways that regulate MEF2 function in a lineage-specific manner remain to be determined.

P19 embryonal carcinoma (EC) cells are a well-established pluripotent embryonic stem (ES) cell model that has shed light on unique aspects of molecular mechanisms regulating cardiac and skeletal muscle development (Skerjanc, 1999; van der Heyden and Defize, 2003). Results in P19 cells have been confirmed in animal models and/or ES cells (Pandur *et al*, 2002; Karamboulas *et al*, 2006a; Kennedy *et al*, 2009). Following 4 days of cellular aggregation in the presence of dimethylsulfoxide (DMSO) to induce differentiation, P19-derived cardiomyocytes first appear on day 6, while skeletal muscle first appears on day 9. Similarly, mouse ES cells differentiate into cardiac muscle by day 6 and skeletal muscle by day 15, with a profile of gene expression analogous to P19 cells (van der Heyden and Defize, 2003; Kennedy *et al*, 2009; Gessert and Kuhl, 2010). Previously, we have shown that MEF2C can induce skeletal and cardiac myogenesis as well as neurogenesis in aggregated P19 stem cells (Skerjanc *et al*, 1998; Ridgeway *et al*, 2000; Skerjanc and Wilton, 2000), providing a unique tissue culture system in which to examine the cell type-specific regulation of MEF2C.

The activity and stability of MEF2 transcription factors are controlled by phosphorylation. The transcriptional activity of MEF2 family members can be enhanced upon phosphorylation by several kinases, including p38 MAPK, ERK5/BMK1, protein kinase C, and casein kinase-II (Molkentin *et al*, 1996b; Han *et al*, 1997; Kato *et al*, 1997; Yang *et al*, 1998; Ornatsky *et al*, 1999; Zhao *et al*, 1999; Cox *et al*, 2003; Barsyte-Lovejoy *et al*, 2004). Phosphorylation of MEF2A by ERK family members can target it for degradation, suggesting that ERK kinases have a dual function during myogenesis (Cox *et al*, 2003). In contrast, MEF2D transactivation properties are potently abolished upon phosphorylation by PKA at Ser-121 and Ser-190 (Du *et al*, 2008). Thus, it is clear that the MEF2 family is regulated by posttranslational modification, although the regulation of MEF2 by kinases in P19 EC or ES cells is not understood.

MLCK is important in regulating muscle contraction, cell motility, membrane events, and cell morphology (Gallagher and Stull, 1997; Kamm and Stull, 2001). In vertebrates, three genes code for MLCK, including smooth muscle (sm), skeletal muscle (sk), and cardiac muscle (c) MLCK (Kamm and Stull, 1986; Kennelly *et al*, 1987; Seguchi *et al*, 2007; Chan *et al*, 2008). Unlike, skeletal myosin light chain kinase (skMLCK) and cMLCK, which are specifically expressed in skeletal and cardiac muscles, respectively, smMLCK is expressed ubiquitously in a wide range of tissues. MLCK has a serine/threonine-kinase catalytic core and a regulatory segment containing autoinhibitory and calmodulin-binding domains (Herring *et al*, 1990; Takashima, 2009). Myosin II

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regulatory light chain is the only known substrate for MLCK (Kamm and Stull, 2001; Takashima, 2009).

To discover proteins that may regulate MEF2C function in a tissue-specific manner, we used the tandem affinity purification strategy (Cox *et al*, 2003) and identified skMLCK as a MEF2C-interacting protein during P19 cell differentiation. We set out to determine if skMLCK regulates MEF2C and if this regulation was lineage specific. We identified a novel role for skMLCK in regulating skeletal muscle commitment by controlling MEF2C-mediated recruitment of p300 to specific promoters.

Results

skMLCK binds and phosphorylates MEF2C

In order to identify proteins that regulate MEF2C, P19 stem cells expressing a TAP-tagged MEF2C were generated and characterized, confirming that MEF2C-TAP is functional and can enhance both cardiomyogenesis and skeletal myogenesis (Supplementary Figure S1A–C), as shown previously for untagged MEF2C (Skerjanc *et al*, 1998; Ridgeway *et al*, 2000). Purification and mass spectrometric analysis of proteins interacting with tagged MEF2C on day 5 of differentiation led to the identification of skMLCK as a novel MEF2C-interacting protein (Supplementary Figure S1D). Western blot analysis with anti-skMLCK antibodies validated the mass spectrometric findings (Supplementary Figure S1D) and demonstrated that skMLCK is present at low levels in C2C12 myoblasts and upregulated during differentiation (Supplementary Figure S1F). SkMLCK can be localized to the nucleus and the cytoplasm (Pujol *et al*, 1993) (Supplementary Figure S1E), indicating that it can be located in the same subcellular compartment as the transcription factor MEF2C.

Identification of the interaction of MEF2C and skMLCK led us to study the expression profile of skMLCK during P19 EC myogenesis. Quantitative real-time PCR (Q–PCR) analysis showed that the expression of skMLCK transcripts increased during differentiation, almost 10-fold by day 9, compared with day 0 (Figure 1A). The transcripts of MEF2C increased on days 2–5 and 9, representing cardiac and skeletal muscle induction, respectively. GATA-4, found in cardiac muscle precursors (Grepin *et al*, 1997), showed increased levels starting at day 2 and peaking at days 5–6 (Figure 1A). Cardiomyocytes were observed by day 6 of differentiation (Figure 1A; Supplementary Figure S3). The expression of the skeletal premyogenic mesoderm gene, Pax3, peaked from days 4 to 6 and the myogenic regulatory factors (MRFs) increased starting on day 7, indicating commitment into the skeletal muscle lineage (Figure 1A). Skeletal myocytes were observed by day 9 of differentiation (Figure 1; Supplementary Figure S3). Expression of structural proteins such as myosin

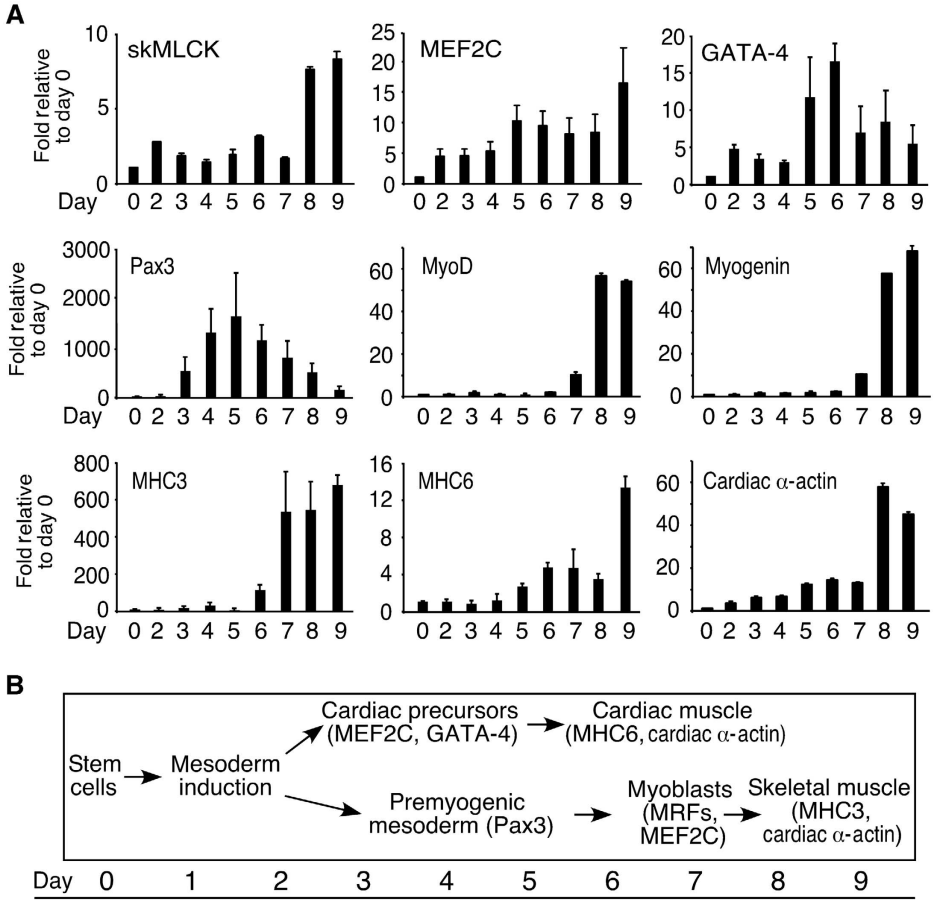

Figure 1 skMLCK is upregulated during skeletal myogenesis in P19 cells. (**A**) Q–PCR was performed for the indicated genes in a time course of P19 cell differentiation with DMSO. Results were normalized to β-actin, and expressed relative to day 0. Data are shown as mean ± s.e.m. ($n = 3$). (**B**) A schematic outline of the cardiac and skeletal muscle differentiation programmes that occur simultaneously during P19 cell differentiation.

heavy chain 3 (MHC3), MHC6, and cardiac α-actin showed increased levels from days 5–6 and 8–9, indicating the waves of cardiac and skeletal muscle formation, respectively (Figure 1A). The difficulty of discerning skeletal versus cardiac muscle-specific structural protein markers is evident, although MHC3 appears to be predominantly skeletal muscle specific. The time line for the various stages of skeletal and cardiac muscle, along with the genes expressed, is outlined in Figure 1B.

The novel interaction between TAP-tagged MEF2C and skMLCK was validated using co-immunoprecipitation assays. Endogenous MEF2C was co-immunoprecipitated with endogenous skMLCK in extracts from C2C12 myocytes using skMLCK-specific antibodies (Figure 2A). Further, co-transfection of HEK-293 cells with Flag-MEF2C with constructs containing wild-type skMLCK, revealed that the full-length skMLCK protein physically interacted with MEF2C (Figure 2B, lane 2). To determine which domain of skMLCK interacted with MEF2C, several skMCLK mutants were examined. MEF2C-interacting mutants included His-skMLCK-ATP and His-skMLCK-Calm, defective in the putative ATP and calmodulin-binding domains, respectively (Figure 2B, lanes 5 and 6). In contrast, a C-terminally truncated skMLCK mutant (skMLCK420) was inefficiently co-immunoprecipitated with

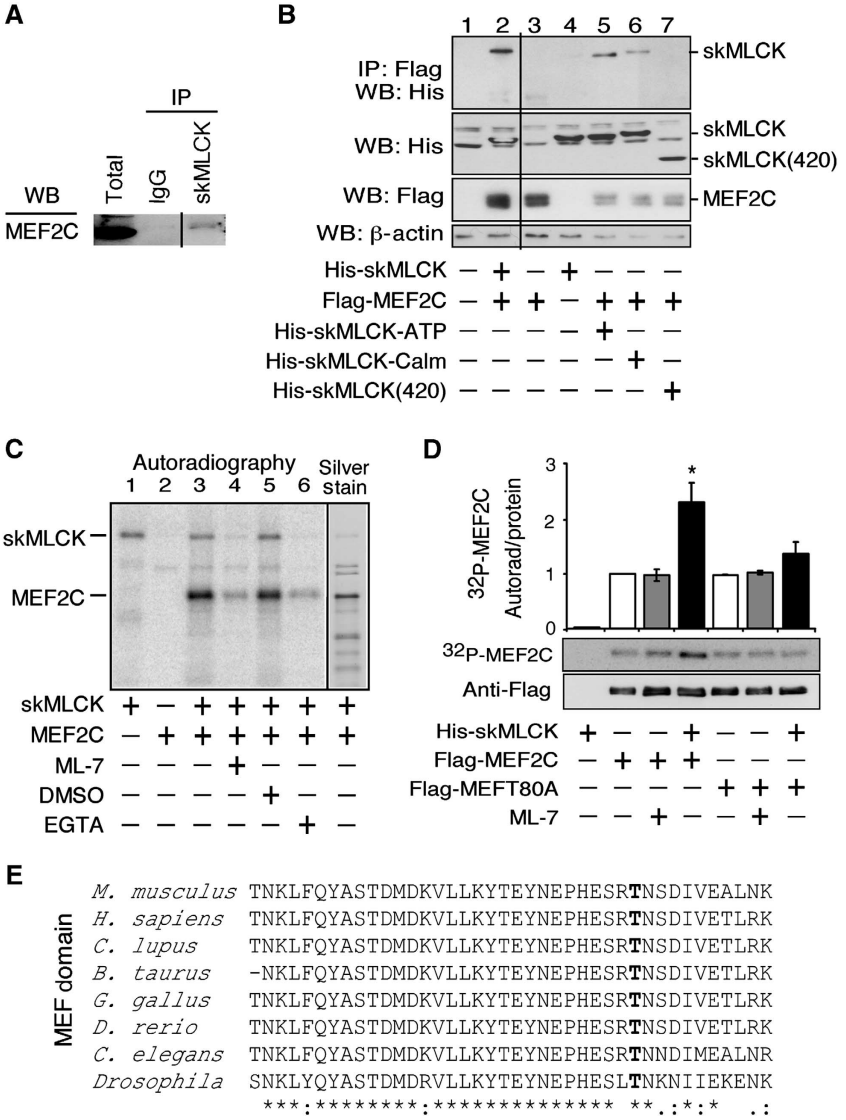

Figure 2 skMLCK physically interacts with and phosphorylates MEF2C *in vivo* and *in vitro*. (**A**) The *in vivo* interaction between MEF2C and skMLCK was observed in C2C12 myoblasts that were differentiated under serum starvation conditions. Co-immunoprecipitation (IP) was performed using anti-skMLCK antibodies conjugated to magnetic beads followed by western blot with antibodies against MEF2C. Anti-IgG antibodies were used in a control IP. Intervening lanes have been removed for clarity, marked by a black line. (**B**) Flag-tagged MEF2C and His-tagged skMLCK or its mutants were co-transfected into HEK-293 cells. Co-immunoprecipitation (IP) using anti-Flag-agarose resin was followed by western blot analysis (WB) with anti-His antibodies. WB for His-, Flag-, and β-actin show expression prior to IP. Intervening lanes have been removed for clarity, marked by a black line. (**C**) *In vitro* kinase assays were performed with recombinant His-MEF2C incubated with purified skMLCK as indicated and visualized by silver stain or autoradiography. (**D**) *In vivo* kinase assays were performed in HEK-293 cells co-transfected as indicated. After immunopurification with an anti-Flag resin, western blot analysis and autoradiography were performed. An Image J program was used to measure band intensities and the intensity of each ^{32}P-radiolabelled band was normalized to the corresponding level of MEF2C protein. The data are shown as the normalized average ^{32}P intensity ± s.e.m. ($n=3$). (**E**) The MEF-domain protein sequence alignment from different species, showing conservation of T^{80}. $^{*}P<0.05$.

MEF2C, indicating that the interaction required the C-terminal domain of skMLCK (Figure 2B, lane 7).

To determine if skMLCK could phosphorylate MEF2C, an *in vitro* kinase assay was performed. Purified His-tagged MEF2C protein was phosphorylated by a commercially available skMLCK protein preparation in the presence of Ca^{2+}, Mg^{2+}, and [^{32}P]-γ-ATP as a phosphate donor and the absence of calmodulin (Figure 2C, lane 3). Phosphorylation of MEF2C was lost in the absence of skMLCK and reduced in the presence of EGTA or the skMLCK inhibitor ML-7 (Figure 2C, lanes 4 and 6). Phosphorylation of MEF2C also occurred in the presence of calmodulin, which is required for optimal myosin light chain phosphorylation, but the autophosphorylation of skMLCK was more intense (Gao *et al*, 1992) (Supplementary Figure S2). Mass spectrometric analysis of the skMLCK preparation did not reveal any contaminating kinases. Silver staining showed an equal loading of the blot (Supplementary Figure S2). Thus, skMLCK phosphorylates MEF2C *in vitro*.

To identify the sites in MEF2C that are phosphorylated by skMLCK, *in vitro* kinase assays were performed followed by LC–MS/MS analysis (Abu-Farha *et al*, 2008). Phosphorylation of MEF2C was observed on a tryptic peptide corresponding to amino acids 80–89 of MEF2C, identifying a single phosphorylation in this peptide at Threonine-80 (T^{80}). Notably, the peptide was mapped to the MEF2 domain, which is essential for homodimerization and heterodimerization of MEF2 family members, binding to DNA, and interaction with the inhibitor HDAC4 (Lu *et al*, 2000). Alignment of the MEF2 domain shows that T^{80} is conserved in MEF2C proteins from different species (Figure 2E).

To investigate whether skMLCK phosphorylates MEF2C in cells, *in vivo* phosphorylation assays were conducted. After incubation of transfected HEK-293 cells with [^{32}P]-orthophosphate, Flag-MEF2C was purified on an anti-Flag-agarose resin and examined by western blot analysis to determine Flag-MEF2C expression and by autoradiography to identify the level of phosphorylation. Quantification revealed a statistically significant 2.5-fold increase in the intensity of Flag-MEF2C phosphorylation in the presence of skMLCK (Figure 2D). Mutation of T^{80} to Alanine, creating MEFT80A, resulted in a lack of enhancement of phosphorylation in the presence of skMLCK, suggesting that T^{80} is the major site of MEF2C phosphorylation by skMLCK (Figure 2D). HEK-293 cells did not contain endogenous MLCK activity, since treatment with ML-7 did not change the baseline MEF2C phosphorylation levels (Figure 2D) and skMLCK protein was not detected by western blotting (Supplementary Figure S1F). Immunoblotting with anti-flag antibodies indicated an equal loading of the purified Flag-MEF2C protein (Figure 2D). Therefore, skMLCK binds and phosphorylates MEF2C on T^{80}.

Phosphorylation-deficient MEF2C cannot enhance skeletal myogenesis

To explore the functional relevance of MEF2C phosphorylation at T^{80} on endogenous promoters during myogenesis, stable P19 cells expressing wild-type Flag-MEF2C, Flag-MEFT80A, or Flag-MEFT80D mutants were isolated and aggregated to induce differentiation. While MEFT80A is phosphorylation deficient, MEFT80D should be a phosphomimetic mutant. On day 9 of differentiation, immunofluorescence using antibodies against the muscle-specific marker MHC showed that both P19[MEF2C] and P19[MEFT80D] cultures, but not P19[MEFT80A], enhanced skeletal muscle generation three-fold compared with P19[Control] cultures (Figure 3A and B). Notably, all three cell lines induced comparable numbers of cardiomyocytes expressing MHC (MHC^{+ve}), indicated by their rounded morphology (data not shown), and contained comparable levels of Flag-tagged wild-type or mutant MEF2C protein (Figure 3C). Taken together, these data indicate that phosphorylation of T^{80} in MEF2C is required for MEF2C-enhanced skeletal myogenesis.

Q–PCR analyses supported the results obtained by immunofluorescence. While P19[MEF2C] and P19[MEFT80D] cultures showed a statistically significant enhancement of the skeletal myoblast/myocyte-specific markers MyoD, myogenin and Myf5, compared with P19[Control] cells, P19[MEFT80A] cultures did not (Figure 3D). A similar pattern was observed for mRNA transcripts of the muscle structural proteins cardiac α-actin and MHC3 on day 9, indicating a lack of upregulation of skeletal myogenesis in P19[MEFT80A] cultures. Note that under the conditions used, MEF2C enhanced skeletal myogenesis more efficiently than cardiomyogenesis (Figure 3D). Interestingly, the cardiac muscle markers GATA-4 and Nkx2-5 had similar increases in transcript levels in the wild-type MEF2C and its mutant cell cultures (Figure 3D). Furthermore, cardiac α-actin and MHC6 transcript levels on day 6 of differentiation, representative of cardiac muscle, were similar in all cell lines examined (Figure 3D), indicating a similar extent of cardiomyogenesis. The reduced ability to generate skeletal muscle by the MEFT80A mutant was not due to lower levels of MEF2C or skMLCK expression, since the transcript levels of wild-type and mutant MEF2C and skMLCK on day 9 were similar in all three cell lines (Figure 3D). Taken together, our results show that the phosphorylation of T^{80} in the MADS/MEF2 domain has a critical effect on the ability of MEF2C to enhance skeletal but not cardiac myogenesis.

skMLCK is necessary and sufficient for enhanced skeletal myogenesis

The importance of the T^{80} phosphorylation and the interaction of MEF2C with skMLCK prompted us to study the effect of skMLCK stable overexpression during P19 cell myogenesis. Cells were examined by immunofluorescence with MF20 on day 9 of differentiation and the number of MHC^{+ve} skeletal myocytes was counted and found to be increased four-fold in P19[skMLCK] cultures compared with P19[Control] cells (Figure 4A and B). Q–PCR analysis of MRF expression revealed a 10–15-fold induction over control of MyoD, Myogenin, and Myf5 transcripts, indicating enhanced commitment into skeletal muscle (Figure 4C). Further, a five-fold increase was observed in mRNA levels of the structural genes cardiac α-actin and MHC in P19[skMLCK] compared with P19[Control] cultures (Figure 4C). In contrast, the cardiac muscle marker, GATA-4, and the skeletal muscle progenitor markers Pax3 and Meox1, were not significantly upregulated on day 6. These results suggest that skMLCK exerts its effect at the commitment stage of P19 skeletal myogenesis, and not cardiac myogenesis, by amplifying the expression of the MRFs and not the premyogenic mesoderm markers.

To investigate the effect of skMLCK inhibition on skeletal myogenesis in mES and P19 cells, ML-7, a cell-permeable, potent, and selective competitive inhibitor for the ATP-bind-

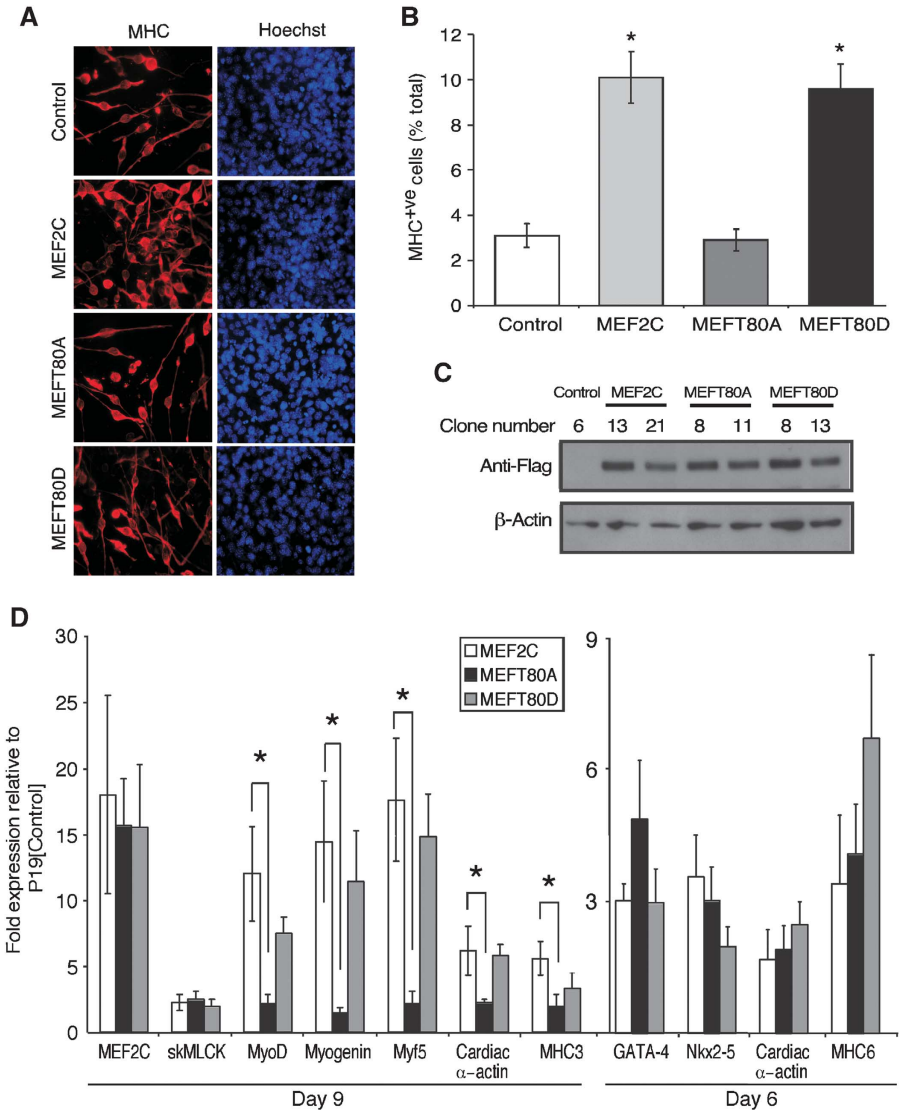

Figure 3 MEFT80D, but not MEFT80A enhances skeletal myogenesis. (**A**) P19[Control], P19[MEF2C], P19[MEFT80A], and P19[MEFT80D] stable cell lines were differentiated and examined by immunofluorescence with an anti-MHC antibody, MF20, to detect skeletal muscle, and counter stained with Hoechst dye to visualize the nuclei ($\times 400$). (**B**) Skeletal myocytes and total nuclei for 10 random fields from different clones were counted and shown as a percentage of total cells, $\pm$s.e.m. ($n=3$). (**C**) Western blot analysis of total protein extracts with anti-flag antibodies, showed similar levels of exogenous wild-type and mutant MEF2C protein. (**D**) Q–PCR analysis from RNA harvested on day 6 or 9 was performed with the genes indicated. Results were normalized to β-actin, and expressed as normalized fold-change relative to P19[Control] cells ($n=15$). *$P<0.05$.

ing site of MLCK was used (Kuhlmann *et al*, 2007). mES cells were differentiated in the presence of ML-7 (0, 300, 600 nM) and examined by immunofluorescence for MHC on day 15. ML-7 treatment resulted in a decrease of skeletal, but not cardiac, muscle formation (Figure 4D). The inhibition of skeletal myogenesis was due to a significant reduction (35–55%) in MyoD, myogenin, Myf5, and MHC3 mRNA transcript levels (Figure 4D). In contrast, GATA-4 transcripts were unaffected (Figure 4D), indicating no loss of cardiomyogenesis. Furthermore, the skeletal premyogenic mesoderm markers, Meox1 and Pax3 showed no significant changes in their mRNA levels (Figure 4D). Inhibition of skeletal, but not cardiac, myogenesis at the stage of muscle commitment was also observed in P19 cells treated with ML-7 (Supplementary Figure S3). Therefore, gain-and-loss-of-function studies identify skMLCK as a key regulatory kinase during the commitment stage of skeletal, but not cardiac, muscle formation in differentiating embryonic and P19 stem cells.

Inhibition of skMLCK reduced the activation and subsequent differentiation of quiescent satellite cells

Since previous studies have shown that MEF2C is highly expressed in quiescent satellite cells (Pallafacchina *et al*, 2010), we isolated adult mouse satellite cells and examined their activation and differentiation status when freshly isolated (day 0) and after 3 days culture, with or without ML-7. Q–PCR analysis of satellite cells cultured for 3 days showed upregulated levels of Pax7, MyoD, and Myf5, indicative of satellite cell activation, and upregulated levels of myogenin and MHC3, indicative of early myoblast differentiation (Figure 5). In the presence of ML-7, MyoD, Myf5, myogenin, and MHC3 were significantly downregulated but Pax7 levels

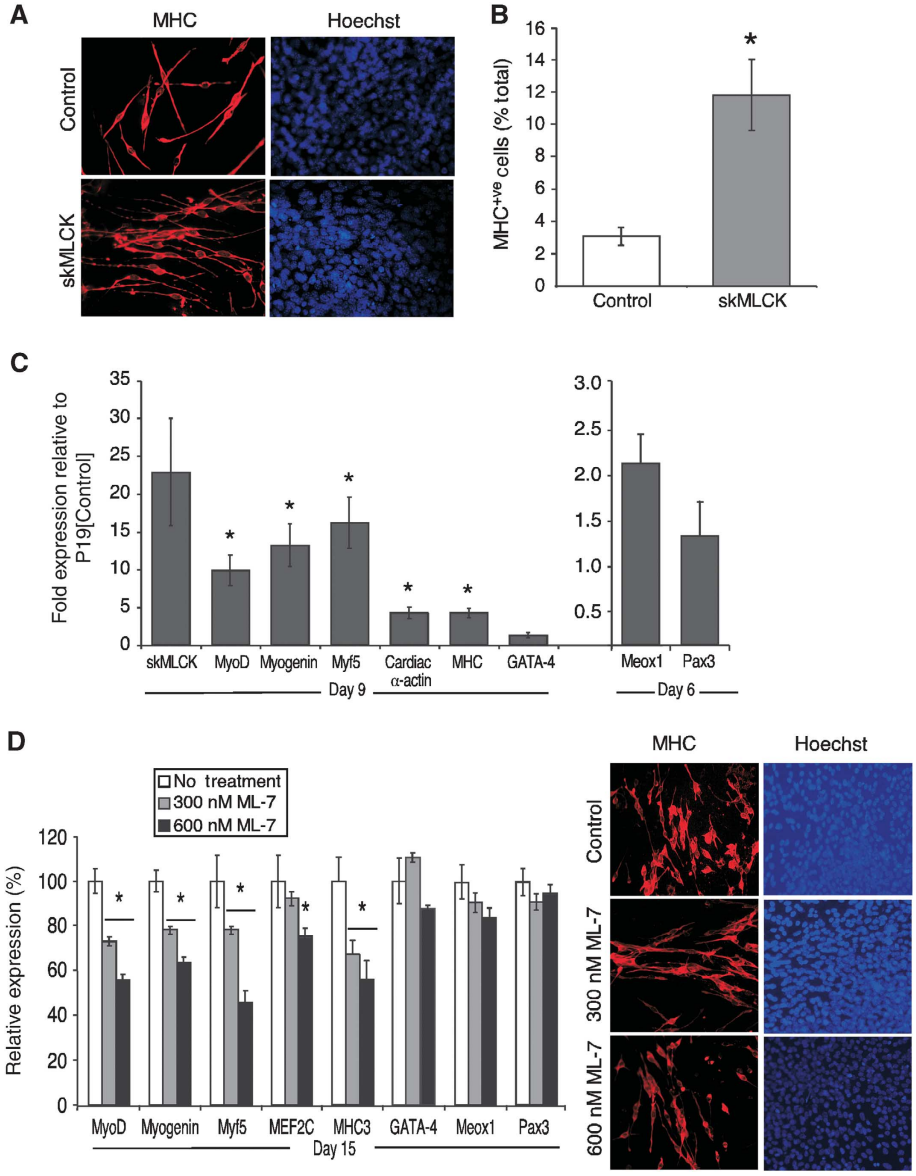

Figure 4 skMLCK is necessary and sufficient for efficient myogenesis. (**A**) Immunofluorescence with MF20 antibodies, showing enhanced myogenesis in day 9 differentiated P19[skMLCK] cells compared with P19[Control] cells. Cells were counter stained with Hoechst dye ($\times 400$). (**B**) MHC^{+ve} skeletal myocytes and total nuclei were counted and the percentage of total cells was calculated ($n=3$). (**C**) Q–PCR analysis of RNA harvested on days 6 and 9 of differentiation from P19[Control] and P19[skMLCK] cells ($n=10$). (**D**) Skeletal myogenesis was inhibited in mouse ES cells differentiated in the presence of ML-7 for 15 days. Q–PCR analysis was performed and the results were normalized to GAPDH, with levels expressed as normalized fold-change over undifferentiated cells and as a percentage of the control untreated cells ($n=3$). Immunofluorescence was performed with MF20 antibodies and stained with Hoechst dye ($\times 400$). $^*P<0.05$.

remained relatively unchanged (Figure 5). MEF2C was expressed at high levels in the freshly isolated satellite cells, in agreement with previous findings (Pallafacchina *et al*, 2010), and was still present but at reduced levels after 3 days of culture. Thus, MEF2C appears to be expressed at all stages of satellite cell myogenesis. Notably, treatment with ML-7 reduced MEF2C expression significantly by day 3. Taken together, our data suggest that skMLCK is important for the activation of satellite cells and their subsequent differentiation.

Recent work has shown that Pax7 expression in satellite cells is regulated by the p38α kinase-dependent repression by PRC2 (Palacios *et al*, 2010). We compared inhibition of skMLCK, by ML-7, with inhibition of p38 kinase by SB203580 (SB). In agreement with the previous study (Palacios *et al*, 2010), SB markedly enhanced Pax7 expression and efficiently inhibited expression of MEF2C, myogenin, and MHC (Figure 5). Notably, both MyoD and Myf5 were upregulated by SB, although MyoD was upregulated only at the lowest concentration. Thus, in accordance with what has been previously reported (Palacios *et al*, 2010), SB expanded the population of activated satellite cells and inhibited their subsequent differentiation.

In comparing the two pathways, SB was a better inhibitor of differentiation than ML-7 and the latter was a superior inhibitor of satellite cell activation than SB. To determine how the two pathways would interact, we treated satellite cells with both SB and ML-7. The observed outcome was more

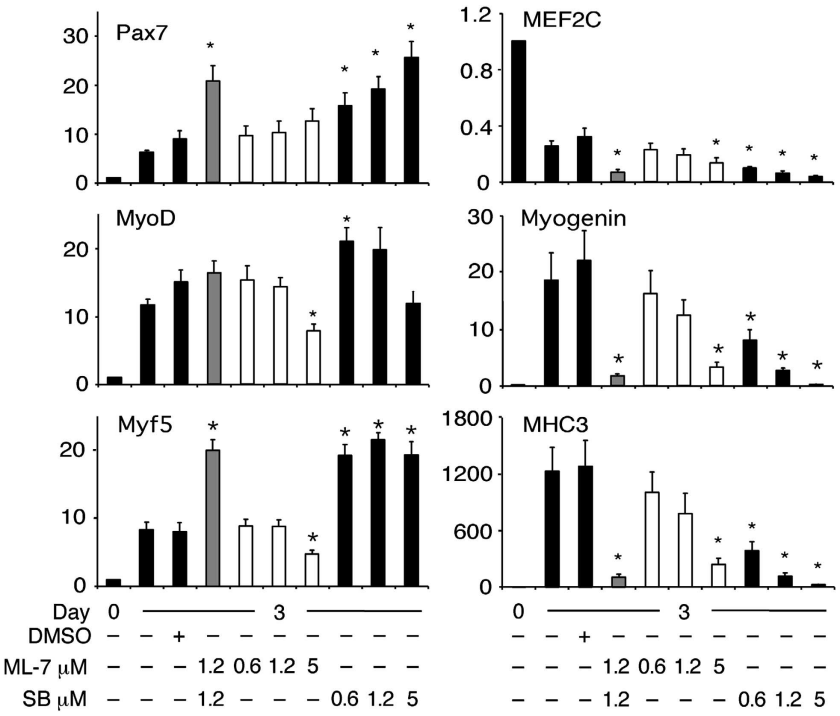

Figure 5 Inhibition of skMLCK reduced the activation of adult satellite cells. Isolated mouse satellite cells were cultured in the presence or absence of increasing concentrations of ML-7, SB203580, separately or together, as indicated. On day 3, RNA was harvested and Q–PCR analysis was performed. Results were normalized to β-actin, and expressed as fold-change relative to non-treated cells ($n=5$; $^*P<0.05$).

similar to that of SB inhibition, with high Pax7 and Myf5 expression and greatly reduced MEF2C, myogenin, and MHC3 (Figure 5). Thus, the two pathways did not synergize but the SB treatment overrode the inhibition of MyoD and Myf5 by ML-7. In summary, skMLCK regulates the expression of MyoD and Myf5 during satellite cell activation.

skMLCK phosphorylation of MEF2C regulates MyoD function

In light of the ability of skMLCK to phosphorylate MEF2C and enhance skeletal muscle commitment in P19 cells, the effect of mutant MEF2C and skMLCK was examined on the myogenic conversion of C3H10T1/2 embryonic fibroblasts by MyoD. Myogenic conversion was evaluated by Q–PCR analysis of cardiac α-actin and myogenin transcripts, in cells transiently transfected with MyoD and/or skMLCK, MEF2C or its T^{80} mutants. Co-transfection of MEF2C or MEFT80D with MyoD significantly increased the transcript levels of cardiac α-actin and myogenin (Figure 6A). In contrast, MEFT80A did not increase myogenin or cardiac α-actin transcript levels over MyoD alone (Figure 6A). Co-transfection with skMLCK significantly enhanced MyoD-directed myogenic conversion and this enhancement was reduced by the skMLCK inhibitor, ML-7. Finally, ML-7 reduced the observed synergy between MyoD and MEF2C (Figure 6A). Similar levels of wild-type and mutant MEF2C proteins were detected in these experiments, as shown by a western blot analysis with an anti-Flag antibody (Figure 6B). SkMLCK transcripts were present at low levels in 10T1/2 fibroblasts and were upregulated by MyoD and MEF2C expression (data not shown), similar to the upregulation of skMLCK in myogenesis of C2C12 (Supplementary Figure S1F) and P19 cells (Figure 1). These results indicate that the synergy between MyoD and MEF2C activity on endogenous promoters is regulated, at least in part, by the phosphorylation of MEF2C-T^{80} by skMLCK.

Promoter analysis was performed to determine if the phosphorylation-deficient (MEFT80A) or phosphomimetic (MEFT80D) mutants would modulate the transcriptional activity of MEF2C. Transient expression of wild-type or mutant MEF2C resulted in similar levels of enhancement of luciferase activity, driven by a MyoD- and MEF2-responsive promoter, in the presence or absence of MyoD (Figure 6C). Thus, in contrast to the above findings analysing endogenous gene expression, the phosphorylation of T^{80} is not essential for the transcriptional activity of MEF2C, or its synergistic activation with MyoD, on non-chromatinized, exogenous promoters.

skMLCK phosphorylation of MEF2C regulates recruitment of histone acetyltransferases to skeletal muscle promoters

Since MEFT80A could activate exogenous (Figure 6C) but not endogenous (Figures 3 and 6A) promoters, we examined the ability of MEFT80A to bind to endogenous MEF2 DNA-binding sites, using chromatin immunoprecipitation (ChIP) followed by Q–PCR. Both MEF2C and MEFT80A bound to the myogenin and GATA-4 promoters (Figure 7A), specifically in the region of a conserved MEF2 DNA-binding site. Thus, the MEFT80A mutant appeared to bind MEF2 sites in chromatin as efficiently as wild-type MEF2C protein.

To determine if histone methylation/acetylation levels were changed, ChIP was performed with antibodies against trimethylated-K4 on Histone 3 ($H3K4^{me3}$) and against acetylated H3K14 ($H3K14^{ac}$), indicative of actively transcribed chromatin. The levels of $H3K4^{me3}$ on the myogenin promoter were comparable in P19[MEF2C] and P19[MEFT80A] cell

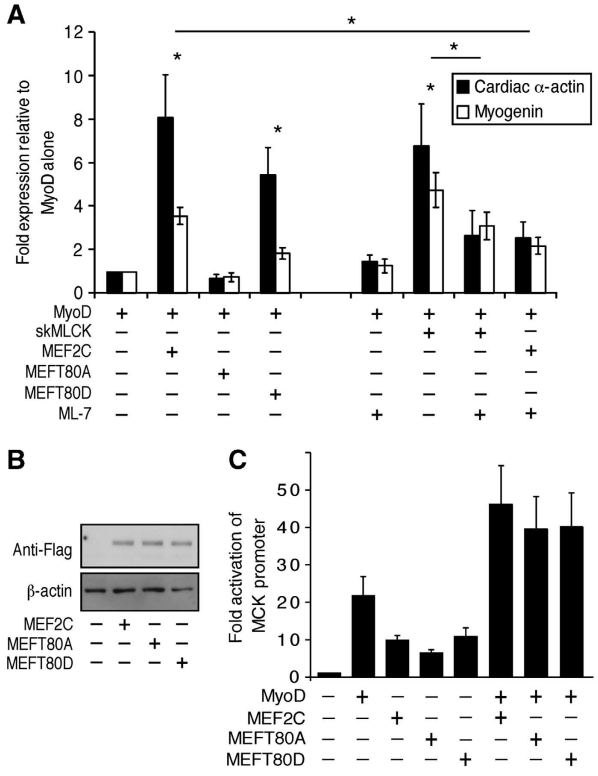

Figure 6 MEFT80A cannot synergize with MyoD on endogenous skeletal muscle-specific promoters. (**A**) Myogenic conversion assays were performed in C3H10T1/2 fibroblasts, transiently transfected with plasmids as indicated, with or without ML-7 treatment. Q–PCR analysis of cardiac α-actin and myogenin transcript levels were normalized to transfected GFP transcripts and expressed relative to cells transfected with MyoD alone ($n=3$). (**B**) Western blot analysis with anti-flag antibodies, showing similar protein expression of wild-type and mutant MEF2C in transfected C3H10T1/2 cells. (**C**) A reporter assay with the muscle reporter MCK-luciferase shows similar levels of synergy for MEF2C or its mutants with MyoD on an exogenous promoter. Luciferase activity was measured and normalized against Renilla ($n=4$). $^*P<0.05$.

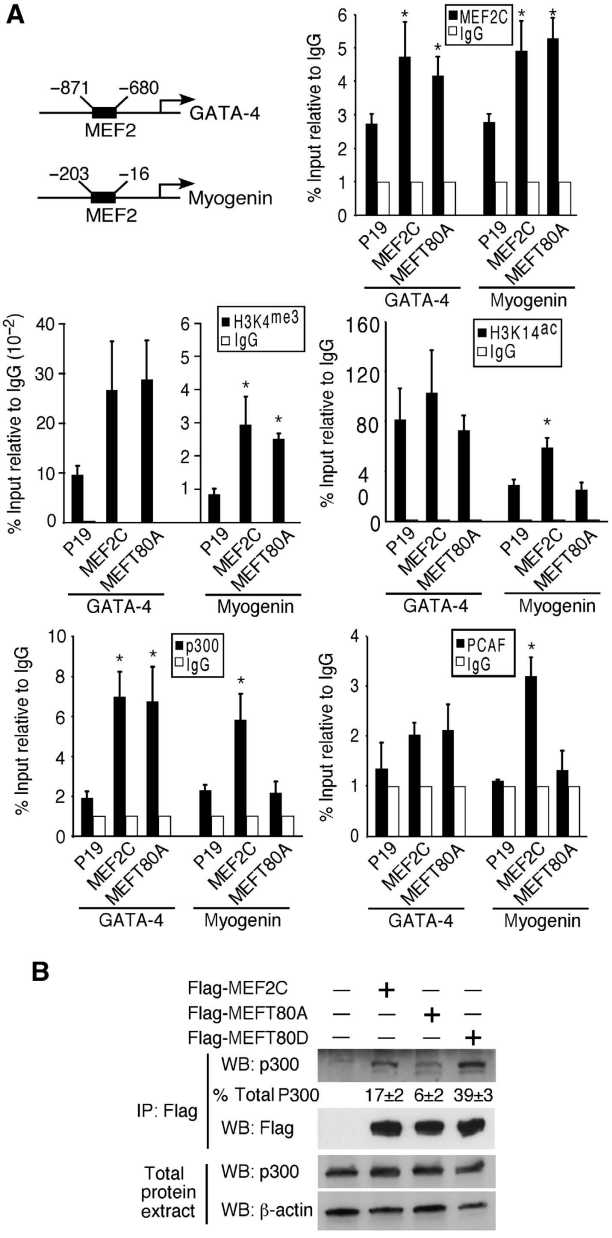

Figure 7 MEFT80A cannot recruit p300/PCAF to endogenous skeletal muscle-specific promoters. (**A**) ChIP was performed using the indicated antibodies and analysed by Q–PCR with primers flanking the MEF2C site in the myogenin or GATA-4 promoters. Graphs represent Q–PCR analysis from day 7 of differentiation for P19, P19[MEF2C], or P19[MEFT80A] cultures. Relative enrichment was calculated as the percent chromatin input normalized to IgG ($n=4$). (**B**) HEK-293 cells were transfected with Flag-MEF2C, -MEFT80A, or -MEFT80D. Co-immunoprecipitation (IP) using anti-Flag-agarose resin was followed by western blot analysis (WB) with antibodies against endogenous p300. Western blots were prepared from total extracts, reacted with antibodies as indicated, and quantified using the Image J program. The p300 Co-IP band intensities were normalized to the intensity of their corresponding control β-actin bands and then to total p300 for each sample ($n=3$). $^*P<0.05$.

lines, indicating similar levels of H3K4 trimethylation in these cell lines (Figure 7A). However, ChIP performed with antibodies against acetylated H3K14 indicated that the levels of $H3K14^{ac}$ were significantly lower at the myogenin promoter, but not the GATA-4 promoter, in P19[MEFT80A] cells as compared with P19[MEF2C] cells (Figure 7A).

To determine if the loss of acetylation was due to deficient recruitment of a histone acetyltransferase (HAT), we performed ChIP with antibodies against p300 and PCAF, which have been shown to bind MEF2C (Sartorelli *et al*, 1997). While wild-type MEF2C could recruit both p300 and PCAF to the myogenin promoter, the MEFT80A mutant could not (Figure 7A). In contrast, p300 was recruited efficiently by both MEF2C and MEFT80A to the GATA-4 promoter.

To determine if the decrease of p300 recruitment was due to inefficient binding to MEFT80A, co-immunoprecipitation studies were performed in HEK-293 cells transfected with wild-type MEF2C, MEFT80A, or MEFT80D. A decrease in the interaction of p300 with MEFT80A was observed, compared with the interaction with MEF2C and MEFT80D (Figure 7B). Therefore, the MEFT80A mutant appears defective in enhancing H3K14 acetylation on skeletal, but not cardiac, promoters, likely due to a loss of recruitment of p300 and/or PCAF.

Discussion

Our data support a model whereby the ability of MEF2C to establish commitment to the skeletal muscle lineage is regulated, at least in part, by skMLCK phosphorylation of MEF2C on T^{80}. In the absence of phosphorylation, MEF2C can bind

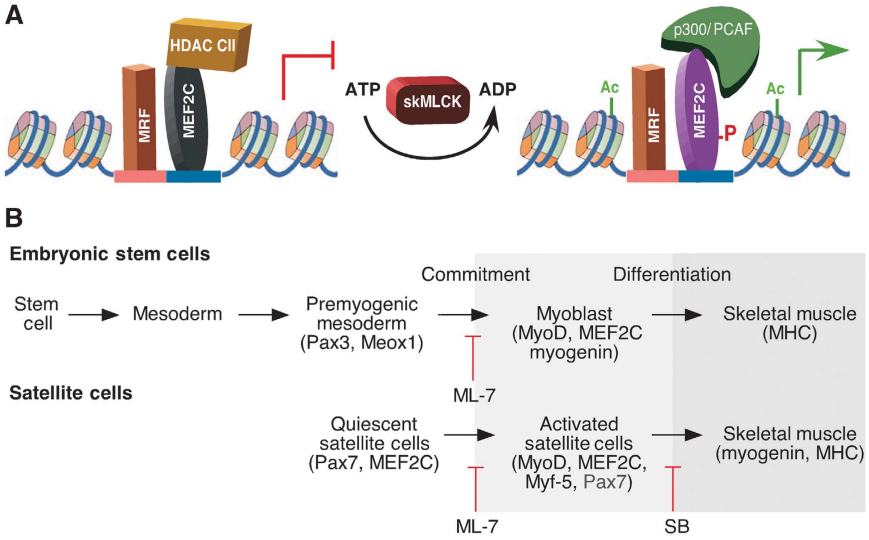

Figure 8 Working model for the mechanism by which skMLCK can enhance myogenesis. (**A**) Previous studies have shown that MEF2C is inhibited by class II HDACs (HDAC CII), synergizes with MRFs, and recruits p300 to promoters (Sartorelli *et al*, 1997; Lu *et al*, 2000; Potthoff and Olson, 2007). Here, we show that skMLCK directly phosphorylates MEF2C, leading to p300/PCAF recruitment, increased acetylation of skeletal muscle-specific genes, and enhanced skeletal myogenesis. (**B**) Comparison of the stages of myogenesis in embryonic stem cells and satellite cells. ML-7 reduced the efficient upregulation of MRFs in the ES-derived premyogenic mesoderm or during the activation of quiescent satellite cells. In agreement with other studies (Palacios *et al*, 2010), SB inhibited the loss of Pax7, required for myoblast formation and subsequent differentiation.

to endogenous skeletal muscle promoters but cannot recruit p300/PCAF, leading to a lack of histone acetylation (Figure 8A). As a consequence, the synergy between MRFs and MEF2C is disrupted, resulting in minimal MRF upregulation and a deficit in the formation of committed skeletal myoblasts (Figures 3, 4, and 6) or in the activation of quiescent satellite cells (Figure 5). Thus, skMLCK and, by implication, regulators of skMLCK (Kamm and Stull, 2001), control the transition from skeletal muscle progenitors or quiescent satellite cells, which can repopulate the satellite cell niche (Montarras *et al*, 2005; Kuang *et al*, 2008), to skeletal myoblasts or activated satellite cells, respectively (Figure 8B).

SkMLCK belongs to a family of Ca^{2+}-dependent protein kinases and the phosphorylation of MEF2C by skMLCK was found to require Ca^{2+} (Figure 2). Sufficient intracellular Ca^{2+} levels are vital for C2C12 differentiation into skeletal muscle (Porter *et al*, 2002). Further, Ca^{2+}-activated signalling has a crucial role in regulating myogenesis by a variety of mechanisms, including the promotion of E protein-MyoD heterodimerization (Hauser *et al*, 2008), the loss of HDAC4/5 repression of MEF2 by Ca^{2+}/CaM-dependent kinase (Lu *et al*, 2000; McKinsey *et al*, 2000), and the activation of calcineurin (Delling *et al*, 2000; Friday *et al*, 2000). Thus, our results are consistent with the importance of Ca^{2+} in regulating skeletal muscle development and reveal a novel mechanism of transcriptional regulation by Ca^{2+}.

The regulation of myogenesis by skMLCK was both lineage and stage specific, having little effect on the formation of either cardiac muscle or skeletal muscle progenitors. Cardiac muscle genes, such as GATA-4 and Nkx2-5, were still upregulated by the MEFT80A mutant or after treatment with ML-7, and were not upregulated by skMLCK overexpression. Similarly, skeletal muscle progenitor genes, such as Pax3/7 and Meox1, were unaffected by changes in skMLCK activity. In contrast, expression of the MRFs along with muscle structural genes was dependent on skMLCK activity. Finally, although MEF2C binds and recruits p300 to muscle promoters (Sartorelli *et al*, 1997), the mutant MEFT80A appeared deficient in p300 recruitment. It is likely that other transcription factors present in MEF2C complexes in the cardiac muscle lineage can compensate for the loss of p300 recruitment by MEFT80A. Thus, specificity of regulation by skMLCK appears to be mediated by the ability of MEF2C to recruit p300 to skeletal versus cardiac muscle promoters at the stage of muscle commitment.

Interestingly, the phosphorylation-deficient MEF2C mutant could still enhance H3K4 trimethylation on skeletal muscle promoters, indicating a specific role for skMLCK in regulating histone acetylation, as opposed to p38, which can regulate histone trimethylation via MEF2D, but not p300 recruitment in myoblasts (Rampalli *et al*, 2007; Serra *et al*, 2007; Guasconi and Puri, 2009). However, other effects of skMLCK on chromatin cannot be ruled out. Overall, our data suggest that, in contrast to the cardiac muscle lineage, the complex of transcription factors bound to MEF2C in the skeletal muscle pathway requires MEF2C-T^{80} phosphorylation for efficient p300 recruitment.

Previous studies have analysed T^{80} in the context of mutating $ESRT^{77-80}$ to VNQA and found that this mutant could still homodimerize and heterodimerize and bind to HDAC4 and DNA (Molkentin *et al*, 1996a; Lu *et al*, 2000). These studies agree with our finding that MEFT80A could bind and activate exogenous promoters as efficiently as wild-type MEF2C.

The finding that MEF2C is highly expressed in quiescent satellite cells (Pallafacchina *et al*, 2010) (Figure 6) suggests that the recruitment of class II HDACs by MEF2C (Lu *et al*, 2000; McKinsey *et al*, 2000) may have an important role in satellite cell formation or maintenance. From gene profiling

analysis, HDAC2, 4, and 11 transcripts are present at high levels in quiescent satellite cells (Pallafacchina *et al*, 2010). Release of HDAC repression by CamK or PKD1 signalling (McKinsey *et al*, 2000; Kim *et al*, 2008) may then allow for the phosphorylation of MEF2C by skMLCK and the subsequent upregulation of MyoD and Myf5, creating an activated satellite cell (Figure 8). This transition appears crucial in that MRF expression in activated satellite cells is detrimental towards their ability to reconstitute the satellite cell niche after transplantation (Montarras *et al*, 2005; Kuang *et al*, 2007).

The recent finding that p38α signalling regulates Pax7 expression and expansion of satellite cells (Palacios *et al*, 2010) led us to compare the inhibition of skMLCK with that of p38. As expected, SB treatment blocked the downregulation of Pax7, and appeared to enhance activated satellite cell proliferation by upregulating Myf5 expression. Treatment with both drugs resulted in a blockade of ML-7 inhibition, consistent with SB functioning downstream to prevent a reduction in Pax7 expression. Higher concentrations of ML-7 may provide a more extensive downregulation of MyoD and Myf5. The efficient inhibition of differentiation by SB may be due in part to a reduction in MEF2 activity, since p38 kinase phosphorylation of MEF2 is important for its activity during muscle differentiation (Wu *et al*, 2000; Penn *et al*, 2004). Future experiments will determine whether ML-7 treatment is beneficial towards the use of satellite cells for muscle therapy.

Mice lacking skMLCK showed no obvious phenotype, including no change in body mass or viability, although the efficiency of satellite cell formation was not examined in these mice (Zhi *et al*, 2005). Since smMLCK is also expressed in skeletal muscle, it is possible that it could compensate for the loss of skMLCK during development, similar to the previously shown compensation of MyoD by MRF4 and Myf5 (Kassar-Duchossoy *et al*, 2004). Similarly, mice lacking MEF2C in skeletal or cardiac muscle lineages do not display early differentiation defects (Lin *et al*, 1997; Potthoff *et al*, 2007). This is likely due to compensation by other MEF2 family members, since disruption of MEF2 function with dominant negative approaches results in a loss of differentiation into cardiac or skeletal muscle in transgenic mice or C2C12 cells, respectively (Ornatsky *et al*, 1997; Karamboulas *et al*, 2006a). ML-7, which inhibited MyoD and Myf5 upregulation in mouse ES, P19, and satellite cells, inhibits all forms of MLCK. Thus, skMLCK may not be the only MLCK that can regulate skeletal muscle commitment.

In addition to muscle contraction, MLCK regulates a variety of other processes, including cell morphology, cell motility, and membrane events (Kamm and Stull, 2001). These events may be mediated in non-muscle cells by MLCK phosphorylation of myosin II. Since the MEF2 family is expressed in a wide variety of tissues and regulates a broad range of proteins (Sandmann *et al*, 2006; Potthoff *et al*, 2007), our results implicate the regulation of MEF2 as a mechanism for MLCK function in both muscle and non-muscle cells. Thus, it is possible that MEF2 factors may be regulated by MLCK in a variety of biological processes.

In summary, we have identified a novel phosphorylation of MEF2C by skMLCK that regulates the commitment of cells to the skeletal muscle lineage at least in part by regulating the ability of MEF2C to recruit p300 to skeletal muscle-specific promoters. Inhibition of MLCK activity reduced the upregulation of MRFs that occurs during the commitment of mES and P19 cells to myoblasts and during the activation of quiescent satellite cells. Our work supports the use of P19 cells as a model system to identify novel molecular pathways regulating myogenesis and our findings may lead to innovative approaches to muscle replacement therapies.

Materials and methods

P19 cell culture and DNA transfections

P19 EC cells were cultured as described (Wilton and Skerjanc, 1999; Karamboulas *et al*, 2006a) and stable cell lines were generated following the published procedures (Ridgeway and Skerjanc, 2001; Karamboulas *et al*, 2006b). Briefly, P19 cells were transfected using the FuGENE-6 transfection system (Roche Diagnostics, USA) with 0.7 μg of PGK-Puro, 1 μg of PGK-LacZ, and 2.5 μg of B17 in the presence of 2.04 μg MEF2C-TAP, Flag-MEF2C, Flag-MEFT80A, Flag-MEFT80D, or His-skMLCK constructs. P19 control cells were transfected with the appropriate vector control. Stable clonal populations were selected in a media containing 2 μg/ml puromycin. Clones with relatively similar expression of the exogenous genes were selected for further studies.

The differentiation protocol was performed as described previously (Karamboulas *et al*, 2006a, b) with minor modifications. Cells were aggregated at a density of 5×10^5 in Petri dishes in serum-supplemented medium containing 1% DMSO. Four days later, the aggregates were plated in tissue culture plates. At days 6 or 9 following differentiation, cultures were fixed or RNA/protein were harvested, as described (Skerjanc *et al*, 1998; Skerjanc and Wilton, 2000). Suboptimal serum conditions were used to minimize the extent of skeletal muscle development in the control cultures (Skerjanc, 1999; Wilton and Skerjanc, 1999).

Mouse ES differentiation and treatment with ML-7

D3 mouse ES cells were grown and differentiated as described previously (Kennedy *et al*, 2009). Briefly, differentiation was induced by aggregating the cells at a density of 4×10^4 cells/ml, for 2 days in hanging drops and 5 days in NTC plates. On day 7, the embryoid bodies were transferred to TC plates or 0.1% gelatin-coated glass coverslips. On day 10, the cells were cultured in low serum medium containing N2 supplement and allowed to grow for a further 5 days, at which time skeletal myocytes were observed. The cells were treated with ML-7 (0, 300, or 600 nM) from days 3 to 15 of differentiation. These concentrations are far below the concentrations at which ML-7 inhibits other kinases. According to the manufacturer (http://www.emdchemicals.com/life-science-research/ml-7-hydrochloride/EMD_BIO-475880/p_uuid?ProductID=PwGb.s1OzZcAAAEiLTxCeVC_), the primary target for ML-7 is MLCK with $K_i = 300$ nM, the secondary targets are PKA ($K_i = 21$ μM) and PKC ($K_i = 42$ μM).

Isolation of mouse satellite cells

The hind limb muscles were isolated from 3-week-old C57BL/6 mice (five animals treated individually). The muscles were digested with 10 mg/ml collagenase in DMEM for 1 h at 37°C. Satellite cells were isolated by three cycles (1 h each) of incubation in tissue culture plates to remove fibroblasts. The satellite cells were placed on Matrigel-coated (Invitrogen) plates in growth media containing 10% fetal-calf serum, 100 U/ml penicillin/streptomycin, and 292 ng/ml L-glutamine in DMEM for 3 days. Under these conditions, both the activation of satellite cells, as shown by MyoD and Myf5 upregulation, and their early differentiation, as shown by myogenin and MHC3 upregulation, were observed. However, the cultures appeared to be just beginning to differentiate and multinucleated myotubes were not yet observed. On a daily basis, cells were treated with ML-7 or SB203580 (Calbiochem) at final concentrations of 0.6, 1.2, or 5 μM, or both drugs at 1.2 μM.

DNA constructs and mutagenesis

Human MEF2C cDNA was subcloned into the appropriate restriction sites of the eukaryotic expression plasmids pCMV-Taq2 and pCTAP (Stratagene, USA). MEF2C mutants, MEFT80A and MEFT80D, were generated by a Site-Directed Mutagenesis Kit (Stratagene, USA) using the primers in Supplementary Table S1 and following the protocol recommended by the manufacturer. Human skMLCK was cloned from cDNA generated from human skeletal

RNA (Clontech, USA) by PCR using Advanced-GC cDNA PCR kit (Clontech, USA) and skMLCK-specific primers (Supplementary Table S1). The skMLCK mutants resulting in the loss of the ATP-binding site, or the calmodulin-binding site were generated using a Site-Directed Mutagenesis Kit (Stratagene, USA; Supplementary Table S1). The skMLCK C-terminal deletion mutant was generated by an insertion of a stop codon after amino-acid 420, resulting in a deletion of the C-terminal 300 aa containing the putative kinase active site and the calmodulin domains.

Western blot, immunoprecipitation, and silver stain

Total cellular proteins were prepared by lysing cells in modified RIPA buffer and western blots were performed as described (Al-Madhoun *et al*, 2004; Abu-Farha *et al*, 2008). Western blots were probed with antibodies against the calmodulin-binding peptide, MEF2C, skMLCK (Santa Cruz Biotechnology, USA), anti-His tag (Invitrogen, USA), monoclonal anti-FLAG M2-peroxidase (HRP) or β-actin (Sigma-Aldrich, USA). Co-immunoprecipitation and silver staining was performed as described previously (Al-Madhoun *et al*, 2007; Abu-Farha *et al*, 2008).

Immunofluorescence and luciferase activity

For immunofluorescent labelling, cells were fixed in cold methanol and incubated first with mouse anti-MHC monoclonal antibody supernatant MF20 (Bader *et al*, 1982), followed by goat anti-mouse IgG(H + L) Cy3-linked antibody (Jackson ImmunoResearch Laboratories), as described previously (Gianakopoulos and Skerjanc, 2005). Luciferase activity was measured using the Dual luciferase assay system (Promega, USA) as previously described (Al-Madhoun *et al*, 2004).

RNA extraction, cDNA synthesis, and quantitative Q–PCR reactions

Total RNA was extracted from cells using the RNeasy Kit (Qiagen Inc., Canada) following the protocol described by the manufacturers. First-strand cDNA was synthesized from 1 μg RNA by reverse transcription using QuantiTect Reverse Transcription Kit (Qiagen Inc., Canada). Q–PCR reactions were performed as described (Savage *et al*, 2009). Primer pairs (Supplementary Table S2) were selected from the PrimerBank (Wang and Seed, 2003) at website (http://pga.mgh.harvard.edu/primerbank). The reactions and data analysis were performed on the ABI 7300 system (Applied Biosystems, Canada) using SDS software. Relative gene expression was calculated using the comparative Ct method as previously described (Savage *et al*, 2009). Unless indicated otherwise, results were normalized to β-actin, and averages ± s.e.m. are shown expressed as fold-change relative to P19[Control] cells.

Expression and purification of recombinant proteins

MEF2C cDNA was constructed into PET30a bacterial expression vector and was expressed in *Escherichia coli*, BL 21 (DH3) pLys host cells, which were induced by 0.5 mM IPTG (Al-Madhoun *et al*, 2002). The protein was purified on Ni-NTA His-binding resins column (Invitrogen, USA) (Al-Madhoun *et al*, 2002). Purified human skMLCK was obtained from a commercially available source (ProQinase Tools and Tests, USA).

Statistical analysis and sequence alignment

Statistical significance was estimated with a one-tailed Student's *t*-test assuming equal variance and error is s.e.m. (* indicates $P<0.05$). Sequences were extracted from PubMed website and alignments were performed using Clustal W program (http://www.ebi.ac.uk/Tools/clustalw2). The accession numbers for MEF2C were *H. sapiens* NP_002388; *M. musculus* NP_079558.1; *C. lupus* XP_536298.1; *B. taurus* NP_001039578.1; *G. gallus* XP_001231662.1; *D. rerio* AAC05226.1; *C. elegans* NM_060040.4; *Drosophila* NP_477018.1.

In vitro and in vivo kinase assays

The skMLCK phosphorylation assay was carried out as described (Kennelly *et al*, 1990) with minor modifications. Purified His-MEF2C and skMLCK were incubated at 30°C for 30 min in a buffer system containing 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM NaCl, 5 mM DTT, 1 μM ATP, 0.3 μM [γ-^{32}P]-ATP (NEN Life Science Products, USA), with and without 0.5 mM $CaCl_2$, 25 mM EGTA, or 5 μM ML-7. The enzyme activity was terminated by heat inactivation in the presence of 1 × SDS-loading buffer.

The *in vivo* kinase assay was performed as described previously (Huang and Chen, 2005) with some modifications. HEK-293 were co-transfected with Flag-MEF2C or Flag-MEFT80A constructs with/without His-skMLCK in the presence or absence of ML-7. Eighteen hours later, the cells were incubated in phosphate-free Dulbecco's modified Eagle's medium for 5 h. Fresh phosphate-free DMEM media containing 0.05 mCi/ml [^{32}P]-orthophosphate (NEN Life Science Products, USA) was then added to the cells for another 4 h. The labelled cells were washed with Tris-buffered saline and lysed in 1 ml of lysis buffer containing 50 mM Tris–HCl, pH 7.5, 300 mM NaCl, and 1 × Mini Complete protease-inhibitor cocktail and 1 × phosphatase-inhibitor cocktail, PhosSTOP (Roche, Laval, Canada). Labelled Flag-MEF2C was purified from cell extracts on anti-Flag-agarose resin as described in the manufacturer's protocol (Sigma-Aldrich, USA), and examined by western blot using monoclonal anti-FLAG M2-peroxidase (HRP; Sigma-Aldrich, USA) antibodies. Labelled proteins were visualized using a phosphor-imager (Typhoon TRIO, variable mode imager, GE Healthcare Bio-science).

Myogenic conversion of C3H10T1/2 fibroblasts

10T1/2 fibroblasts were cultured in 10% 1:1 cosmic calf-fetal bovine serum in α-minimum Eagle's medium. One day before transfection, cells were seeded in a six-well plate at a density of 1×10^5 cells/well. The cells were then transfected using the FuGENE-6 transfection reagent following the procedure recommended by the manufacturer (Roche Diagnostics, USA). The myogenic conversion was driven by 1 μg Flag-MyoD in the absence or presence of 1 μg His-skMLCK, Flag-MEF2C, Flag-MEFT80A, or Flag-MEF2T80D constructs and 0.2 μg pEGFP-N1 vector (Clontech Laboratories, Inc., Palo Alto, CA). After 48 h, cells were transferred to differentiation medium, containing 0.5% horse serum, for 4 days with or without daily administration of 600 nM ML-7 (Calbiochem, USA). Transfection efficiency was scored by analysing GFP transcripts and myogenic conversion was quantified by real-time Q–PCR analysis.

Chromatin immunoprecipitation

ChIP assays were performed as described (Savage *et al*, 2009) with minor modifications. P19, P19[MEF2C], or P19[MEFT80A] clones were aggregated for 4 days in the presence of DMSO, and cells were harvested for ChIP analysis on day 7. Relative enrichment of binding sites compared with the IgG negative control immunoprecipitation was analysed from 50 μg of chromatin using Q–PCR, as described above. Primers sequences for myogenin and GATA-4 are listed in (Supplementary Table S2).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: ASM and ISS designed the study, collected and analysed the data. ASM performed the experiments except for Supplementary Figure S3, which was carried out by VM. GL isolated satellite cells under the supervision of NWB, who also helped interpret the data for the satellite cells analysis. DF interpreted the data for the mass spectroscopy analysis. ASM and ISS wrote and edited the manuscript. All of the authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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