SUPPLEMENTAL MATERIAL

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Chromatin immunoprecipitation (ChIP)- ChIP was performed following the protocol from Upstate (Temecula, CA). Briefly, cells were treated with 1% formaldehyde at 37°C for 10 min to cross-link chromatin and lysed with SDS lysis buffer containing protease inhibitors. Then the cell extracts were sonicated to shear DNA and immunoprecipitated using anti-MyoD (C-20, Santa Cruz Biotechnology), or anti-pMEK1 (Cell Signaling Technology) antibody. Immunoprecipitates containing protein-DNA complexes were washed according to the instruction. In order to reverse cross-links 20 μl of 5 M NaCl was added to the eluates (500 μl) and heated at 65°C for 4 h. The eluates were then treated with 10 μl of proteinase K (20 μg/ml) in a buffer containing 20 μl of 1M Tris-HCl (pH 6.5) and 10 μl of 0.5 M EDTA, and incubated at 45°C for 1 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. To analyze ChIP products, PCR primers (forward primer: 5'-ACCCGAGATGCCTGGTTATA-3'; backward primer: 5'-TCCATGGTGTACAGAGCCTA-3') were designed to flank the MyoD consensus DNA sequence in the muscle creatine kinase (MCK) promoter. PCR was performed with samples of genomic input and ChIP DNAs for 35 cycles of denaturation at 97°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min.

siRNA experiments- C2C12 cells were transfected with a Smart Pool of siRNA (SI) targeting MEK1 (Dharmacon Inc., Chicago, IL; 5'-GCCAGCAUCUGAGCCUUUA-3', 5'-GAUGGUGGGUCCUUGGAUC-3', 5'-GGUACAUGCUUUCAUCAAA-3' and 5'-CGGGAGAAGCACAAGAUUA-3') or scramble siRNA (SC; 5'-CCUACGCCACCAAUUUCGUdTdT-3') as a control. Cells were plated and transfected with Lipofectamine 2000 (Invitrogen) on the next day according to the manufacture's protocol.

Differentiation assay- 10T1/2 cells were grown to approximately 80-90% confluence in the growth medium (GM: 10% fetal bovine serum prepared in DMEM) and transfected with wild type MyoD or mutant MyoD-Y156F together with pEGFP-N1 plasmid using Lipofectamine 2000. On the next day the GM was switched to the differentiation medium (DM: 2% horse serum prepared in DMEM). After 2 days, cells were photographed using fluorescent microscope (Carl Zeiss AX10) and the number of myotubes containing more than 2 nuclei was counted.

LEGENDS TO SUPPLEMENTAL FIGURES

Fig. S1. Active MEK1 and MyoD co-localized in the nuclei of differentiating myotube. 10T1/2 cells were transfected with mCherry-MyoD using Lipofectamine 2000 (Invitrogen), and growth medium (GM) was switched to diffrentiation medium (DM) on the next day. The cells kept in DM for 1 day were transfected with EGFP-MEKEE (ΔN) and incubated for 1 day. The subcellular localization of active MEK1 (A) and MyoD (B) was observed with fluorescence microscope (Carl Zeiss AX10, x400 magnifictions). Nuclei were stained with DAPI (C). (D) is a merged image of A, B and C.

Fig. S2. Activated MEK1 interacts with free MyoD, but not with MyoD bound to the MyoD consensus sequence (E box) of chromatin. C2C12 cells grown to approximately 70-80% confluence in GM were switched to DM to induce differentiation and kept in DM for 3 days. The cell extracts were used for ChIP analysis using anti-MyoD (C-20) or anti-pMEK1 antibody as described in Supplemental Experimental Procedures. An amplified DNA band (119 bps) was seen from samples immunoprecipitated with anti-MyoD antibody and the sonicated cell extract (Input). No band was detected from samples immunoprecipitated with anti-MEK1 antibody or without antibody as a control.

Fig. S3. MEK1 phosphorylates MyoD. 10T1/2 cells were transfected with HA-MEKEE (ΔN), an active form or HA-MEKAA (ΔN), an inactive form. After 36 h, cells were extracted and the immunoprecipitates prepared using anti-HA antibody were incubated with 2 μg of either GST or GST-MyoD protein in a kinase buffer including 5 μCi [γ-^{32}P] ATP at 37°C for 30 min. The mixtures were separated by 10% SDS-PAGE, and the gel was dried and exposed to X-ray film (right panel). The positions of GST and GST-MyoD (~65 kDa) were identified by immunoblotting using anti-GST antibody (left panel). MyoD was phosphorylated by HA-MEKEE (ΔN) but not HA-MEKAA (ΔN).

Fig. S4. Knockdown of MEK1 reduced the MyoD protein level. C2C12 cells grown in the GM were transfected with siRNA (SI) targeting MEK1 or scramble siRNA (SC) as a control using Lipofectamine 2000 (Invitrogen). On the next day, the cells were switched to DM and kept for 2 days. The protein levels were analyzed by immunoblotting using anti-MEK1, anti-MyoD (C-20), and anti-pMyoD-Y156 antibodies (A). The intensity of MEK1 dramatically reduced in cells transfected with siRNA (SI) targeting MEK1. The intensity of MyoD band (especially the upper band which could be modified forms) reduced with disappearance of pMyoD-Y156. (B) The quantitative ratio of MyoD to α-tubulin is shown. The results show the mean ± standard deviation of triplicate experiments. (**P<0.01; ***P<0.001)

LEGENDS TO SUPPLEMENTAL FIGURES

Fig. S5. Mutant MyoD-Y156F showed low myogenic activity compared to wild type MyoD. 10T1/2 cells were transiently transfected with wild type MyoD (MyoD-WT) or mutant MyoD-Y156F together with pEGFP-N1 plasmid as described in Supplemental Experimental Procedures. Compared with wild type MyoD, the number of myotubes in the cells transfected with mutant MyoD was reduced (Carl Zeiss AX10, x200 magnifications). Myotube with above two nuclei was indicated with a arrow. Nuclei were stained with DAPI.

Fig. S1

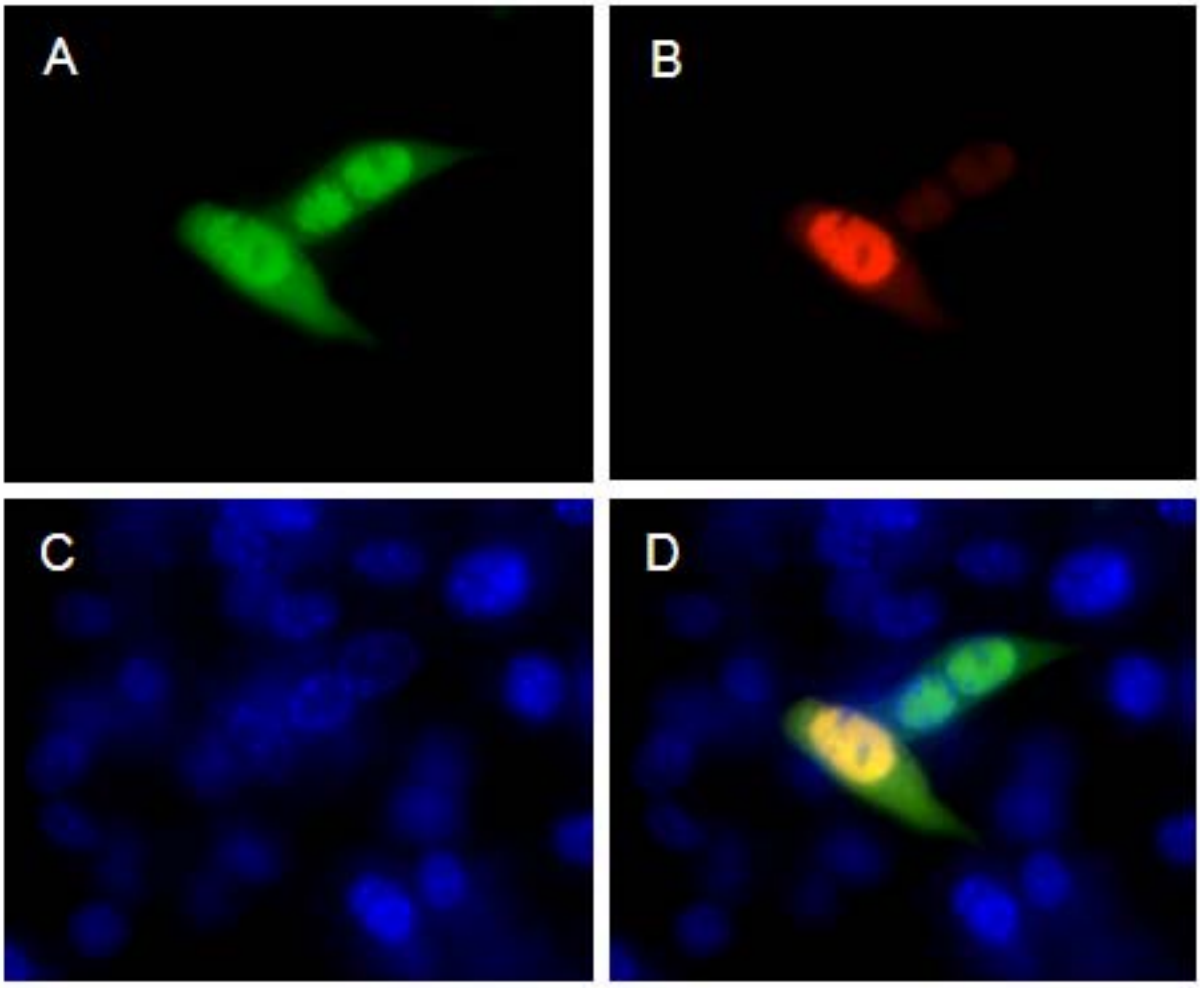
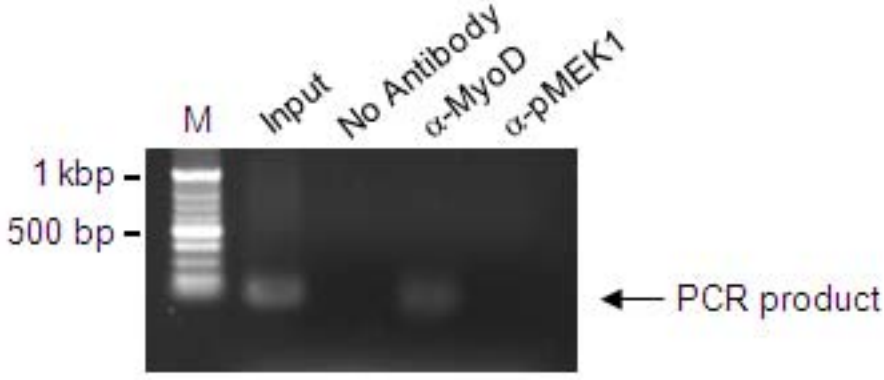

A
B
C
D

Fig. S2


M
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α-pMEK1
1 kbp
500 bp
PCR product

Fig. S3

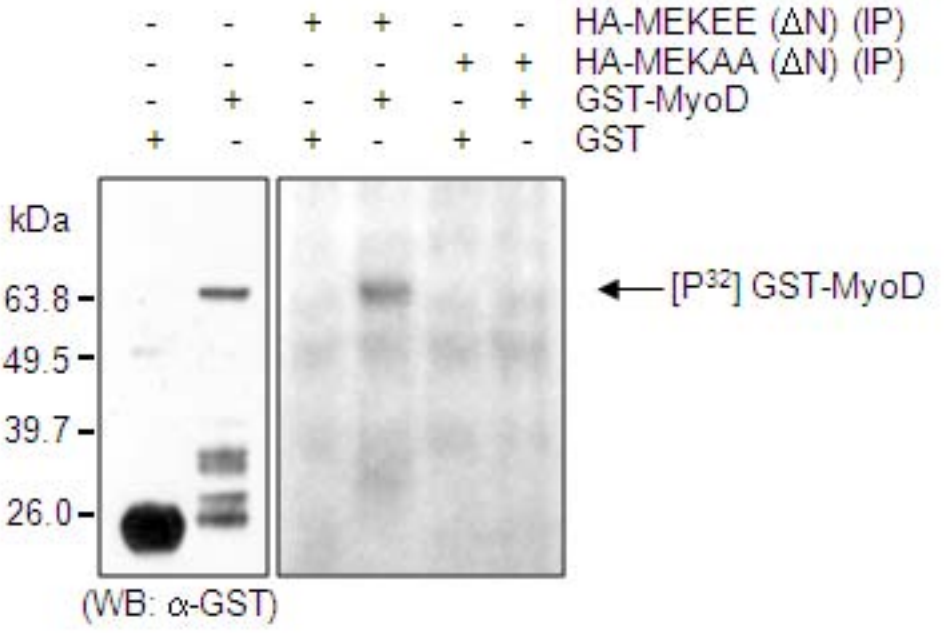
- - + + - - HA-MEKEE (ΔN) (IP)
- - - - + + HA-MEKAA (ΔN) (IP)
- + - + - + GST-MyoD
+ - + - + - GST
kDa
63.8
49.5
39.7
26.0
(WB: α-GST)
[P32] GST-MyoD

Fig. S4

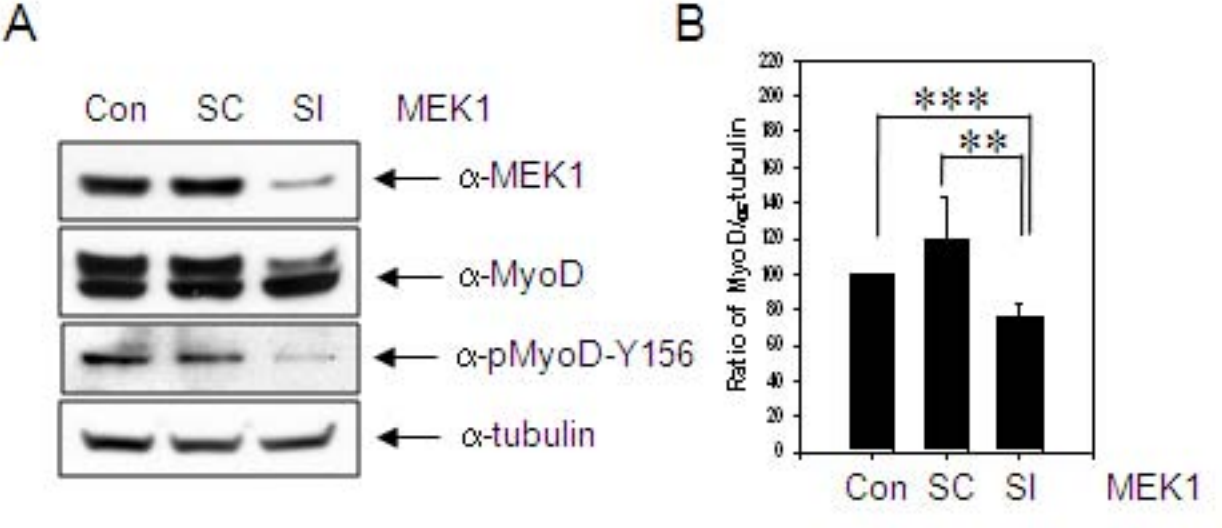
A
Con SC SI MEK1
α-MEK1
α-MyoD
α-pMyoD-Y156
α-tubulin
B
Ratio of MyoD/α-tubulin
***
**
Con SC SI MEK1

Fig. S5