HDAC4 promotes Pax7-dependent satellite cell activation and muscle regeneration

Moon-Chang Choi[1], Soyoung Ryu[1], Rui Hao[1], Bin Wang[1], Meghan Kapur[1], Chen-Ming Fan[2], and Tso-Pang Yao[1,*]

[1]Department of Pharmacology and Cancer Biology, Duke University, Durham, NC 27710

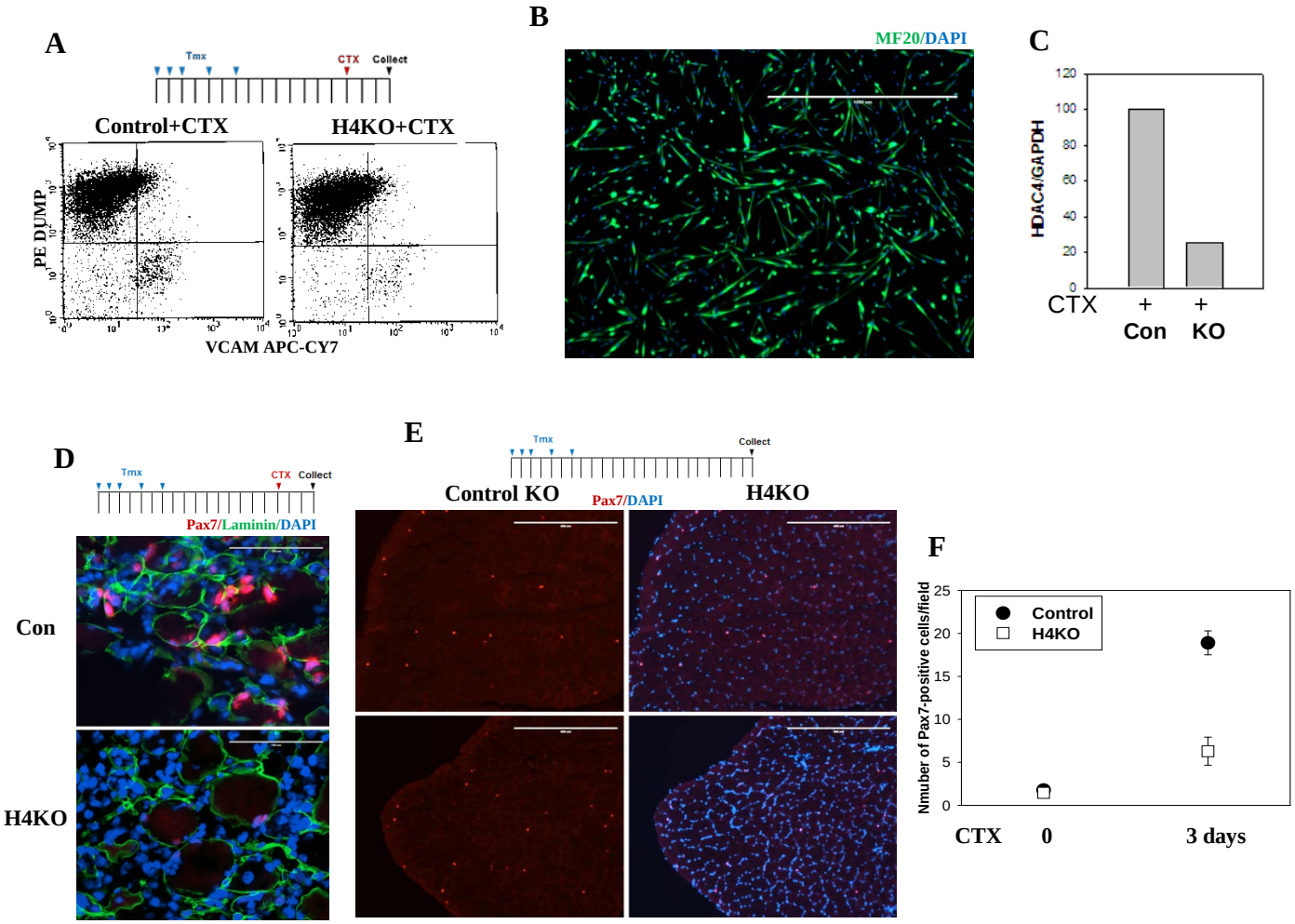
[2]Department of Embryology, Carnegie Institution of Washington, Baltimore, MD 21218

[*]Corresponding author

SUPPLEMENTARY INFORMATION

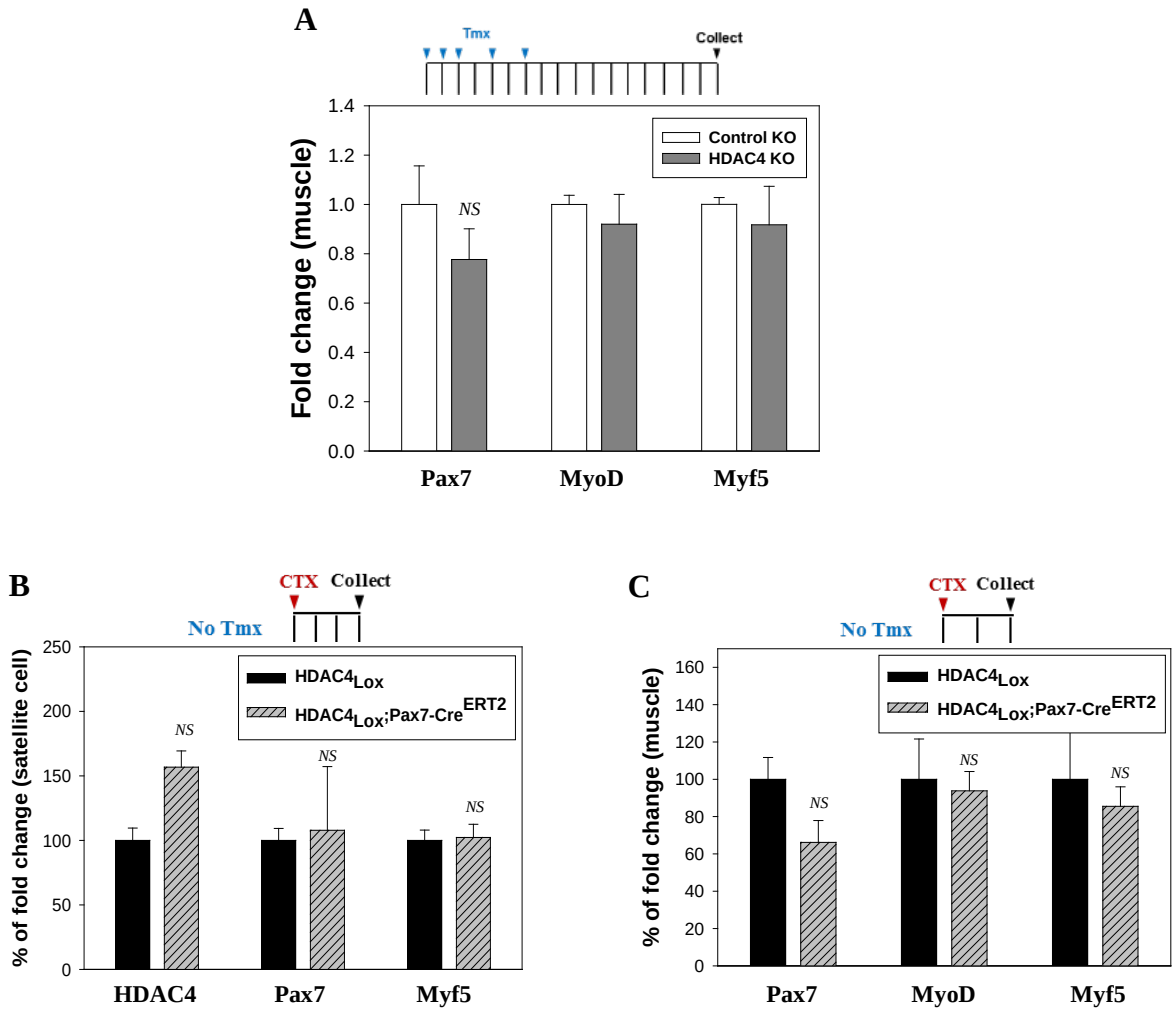
Inventory of Supplementary Information:

Figure S1



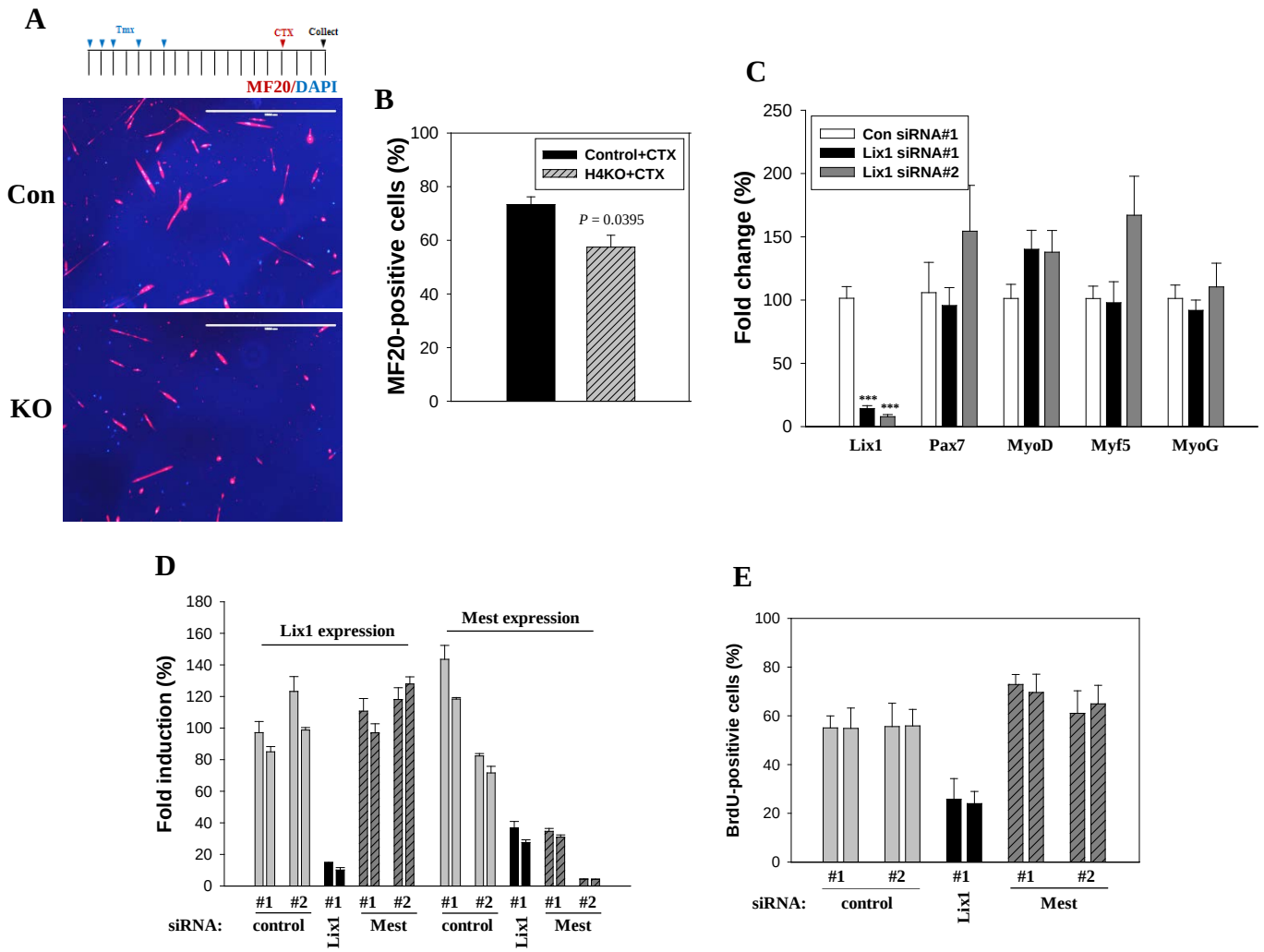
Supplementary Figure S1. HDAC4 inactivation inhibits SC expansion induced by acute muscle injury. (**A**) Isolation of SCs. 8-week-old control and HDAC4 SC-KO male mice were treated with tamoxifen (Tmx) and then CTX as indicated. Muscles from 3 control and 3 KO mice were combined into each group and SCs (PE-/APC-Cy7+ population) were isolated by FACS sorting. Note relatively low population of SCs isolated from KO compared to control. (**B**) Myotube formation of purified SCs. Isolated SCs from muscles treated with CTX for 2 days in 12-week-old C57BL/6 male mice were cultured in GM for 2 days and then differentiated in DM for 2 days. Myotubes were stained by MF20 antibody. DNA was visualized by DAPI staining. Scale bar: 1,000 μm. (**C**) KO efficiency of HDAC4. Sorted SCs in (**A**) were cultured for 4 days in DMEM containing 10% horse serum (HS) and subjected to RNA analysis. Columns, mean of PCR triplicate. (**D**) Pax7-positive SC staining in control and HDAC4 KO damaged muscles. Larger images were taken from samples used in Fig 1D. Scale bar: 100 μm. (**E**) Effects of HDAC4 KO on numbers of quiescent SCs expressing Pax7 in undamaged muscle. TA muscles from 7-week-old control or SC-KO male mice were stained with Pax7 antibody to mark quiescent SCs. Scale bar: 400 μm. (**F**) Inhibition of SC expansion by loss of HDAC4. Combined graph made from Supplementary Fig S1E and Fig 1E. For quantification of SC numbers in undamaged muscle (day 0), approximately, 50 grids (each 250 μm x 250 μm) per muscle were counted. Columns, mean; bars, SEM. n = 4 for control and n = 3 for HDAC4 KO.

Figure S2



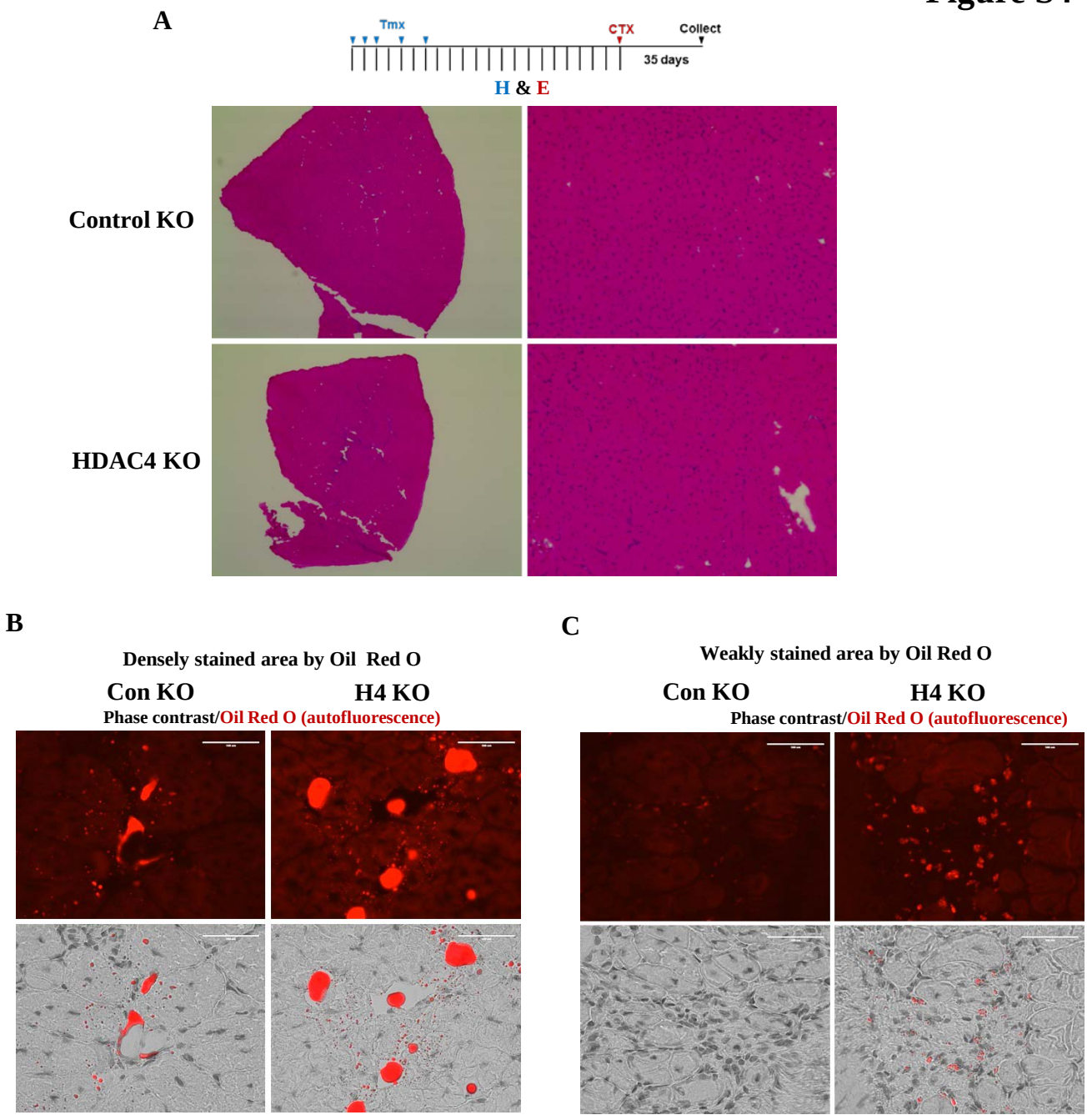
Supplementary Figure S2. mRNA expression of Pax7 and myogenic factors is not changed either in HDAC4 KO undamaged muscle or in damaged muscle from Tmx-untreated mice. (**A**) mRNA expression of genes in undamaged muscle. Experimental design is shown. mRNA expression for indicated genes was examined in intact TA muscles from 10-week-old control or HDAC4 SC-KO male mice. Columns, mean; bars, SEM (n = 3 for each group). *NS*, not significant, $P > 0.05$. (**B**) mRNA expression of genes in activated SCs isolated from Tmx-untreated mice. CTX-activated SCs isolated from 8-week-old male mice were cultured for 3 days in GM. Medium was changed at 2 days after plating. Bars, SEM (n = 3 for each group). (**C**) mRNA expression of genes in TA muscles from Tmx-untreated mice. mRNA expression of indicated genes was examined in damaged TA muscles from 10-week-old control or HDAC4 SC-KO female mice. Columns, mean; bars, SEM (n = 3 for each group). *NS*, not significant, $P > 0.05$.

Figure S3



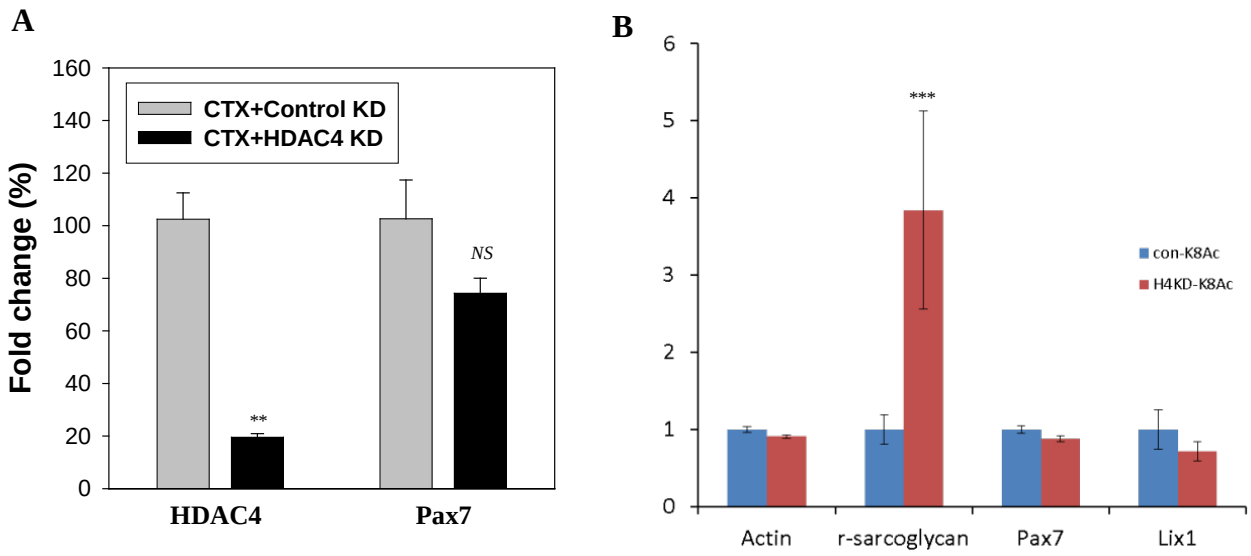
Supplementary Figure S3. HDAC4 KO slightly affects SC differentiation, whereas Lix1 is a critical factor for SC proliferation. (**A,B**) Effect of HDAC4 KO on SC differentiation. Isolated SCs from TA and GA muscles treated with CTX for 3 days in 7-week-old control and KO male mice were cultured for 2 days in DMEM containing 10% HS and then differentiated for 2 days in DM containing 2% HS. Myotubes were monitored by staining cells with MF20 antibody. Representative images (**A**) and quantification (**B**) are shown. Approximately, 1,000 DAPI-positive nuclei were counted per muscle. Columns, mean; bars, SEM. $n = 3$ for each group. Scale bar: 1,000 μm. (**C**) mRNA expression of Lix1, Pax7 and myogenic factors after Lix1 KD in activated SCs. Experiment was paired with Fig 3D. Columns, mean; bars, SEM. $n = 3$ for triplicate wells. ***$P < 0.001$ versus control siRNA#1. (**D**) mRNA expression of Lix1 and Mest after depletion of Lix1 or Mest in SCs. Pooled SCs isolated from TA and GA muscles treated with CTX for 2 days in 11-week-old C57BL/6 male mice (7 mice) were transfected with two different sets of control, Mest siRNAs, or a Lix1 siRNA for 2 days. Relative fold change was calculated compared to control KD. Error bars were generated from real-time PCR triplicates and represent SD ($n = 2$ per group). (**E**) Effects of Lix1 or Mest KD on SC proliferation. Experiment was performed with (**D**). BrdU was added 4 h before fixation. Values are means from 10 to 25 counting fields (10X); error bars indicate SD. Approximately, 4,000 nuclei were counted per well. $n = 2$ per group.

Figure S4



Supplementary Figure S4. Delayed muscle regeneration and increased fat infiltration in HDAC4 KO muscle. (**A**) HDAC4 KO delays muscle regeneration. Experimental design is shown. TA muscles in 9-week-old male mice were treated with CTX for 5 weeks, collected TA muscles were stained with H & E for histology. (**B,C**) Effect of HDAC4 KO on lipid accumulation in regenerating muscle. Fluorescence images were taken from samples in Fig 4D to enhance signal intensity of weakly-stained lipid. Regenerating control and HDAC4 KO TA muscles were stained with Oil Red O and hematoxylin to mark lipid and DNA, respectively. Densely-(**A**) or weakly-(**B**) stained areas by Oil Red O are shown.

Figure S5



Supplementary Figure S5. Regulation of Pax7 by HDAC4 is indirect. (**A**) mRNA expression of HDAC4 and Pax7 after *in vitro* knockdown of HDAC4 in activated SCs. Activated SCs from CTX-damaged muscles in 10-week-old-C57BL/6 male mice were isolated and transfected with control or HDAC4 siRNA and cultured for 2 days in GM. Columns, mean; bars, SEM. $n = 3$ for triplicate wells. $^{**}P < 0.01$; *NS*, not significant, $P > 0.05$. (**B**) ChIP analysis using acetyl-histone H4 (K8) antibody was performed on control or HDAC4 KD C2C12 myoblasts. Note that HDAC4 deficiency had little effect on acetyl-histone H4 (K8) level in either Pax7 or Lix1 promoter region, whereas γ-sarcoglycan, a known HDAC4 target, was hyper-acetylated in HDAC4 KD cells. Columns, mean; bars, SD. $n = 3$. $^{***}P < 0.001$ versus control KD-K8Ac.