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Supplemental Information

A CRISPR/Cas9-Based System for Reprogramming Cell Lineage Specification

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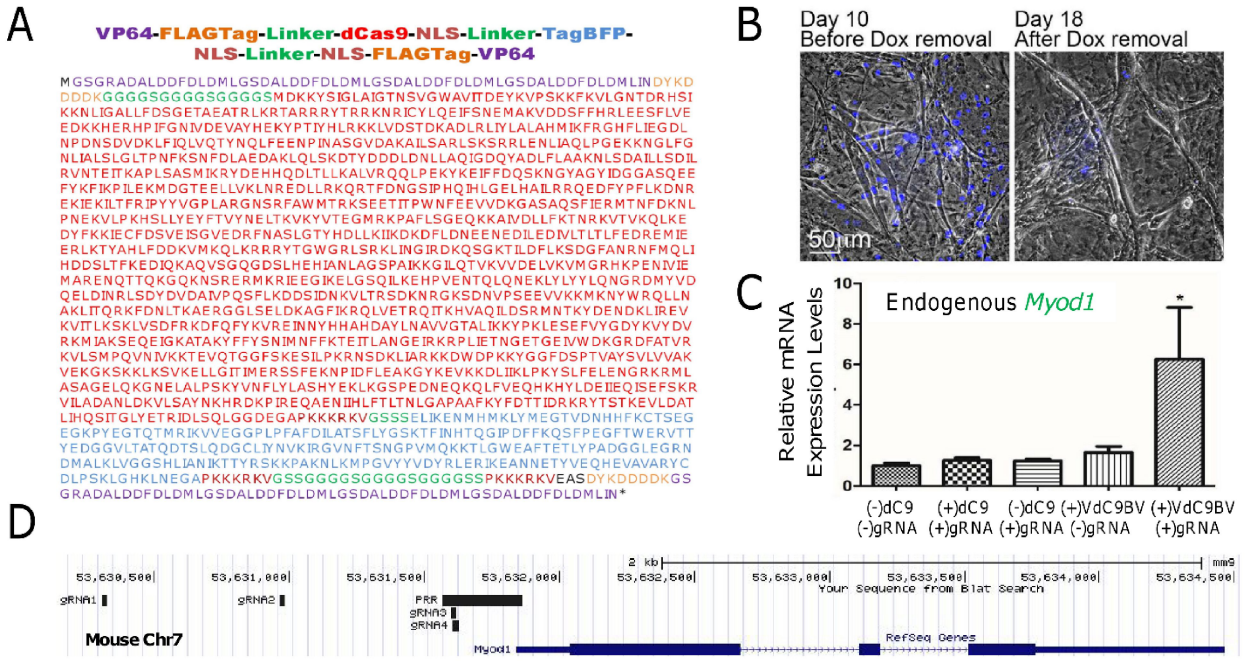

Figure S1: Supplemental Figure 1, related to Figure 1. Transactivation of the endogenous *Myod1* gene by a RNA-guided, nuclease-inactive, doxycycline-inducible ^{VP64}dCas9-BFP^{VP64} (VdC9BV) fusion protein.

(A) Amino acid sequence of the ^{VP64}dCas9-BFP^{VP64} fusion protein. **(B)** Doxycycline-inducible ^{VP64}dCas9-BFP^{VP64} expression. Following doxycycline addition to the culture medium, BFP is detected in the nuclei of multinucleated myotubes formed from reprogramming C3H10T1/2 cells. On removal of doxycycline most of the nuclei lose BFP expression. **(C)** ^{VP64}dCas9-BFP^{VP64} (VdC9BV) needs a gRNA to activate the endogenous *Myod1* gene. VdC9BV or gRNA alone cannot activate the gene. The VP64 fusion is needed for activation as gRNA+dCas9 cannot activate the gene. The studies were done by transfecting murine cell line C3H10T1/2. Control group = Mock transfection, (-) dC9 and (-) gRNA. (n=3 biological replicates, One Way ANOVA with Dunnett's Multiple Comparison Test with all groups compared to the Control, P<.05). Fold change in

expression is relative to the control group. **(D)** Mouse *Myod1* locus-specific gRNAs #1 - #4 represented in relation to the *Myod1* gene on Chromosome 7 and its proximal regulatory region (PRR, -275 to +20 relative to transcription start site). The representation has been done using UCSC genome browser BLAT search tool.

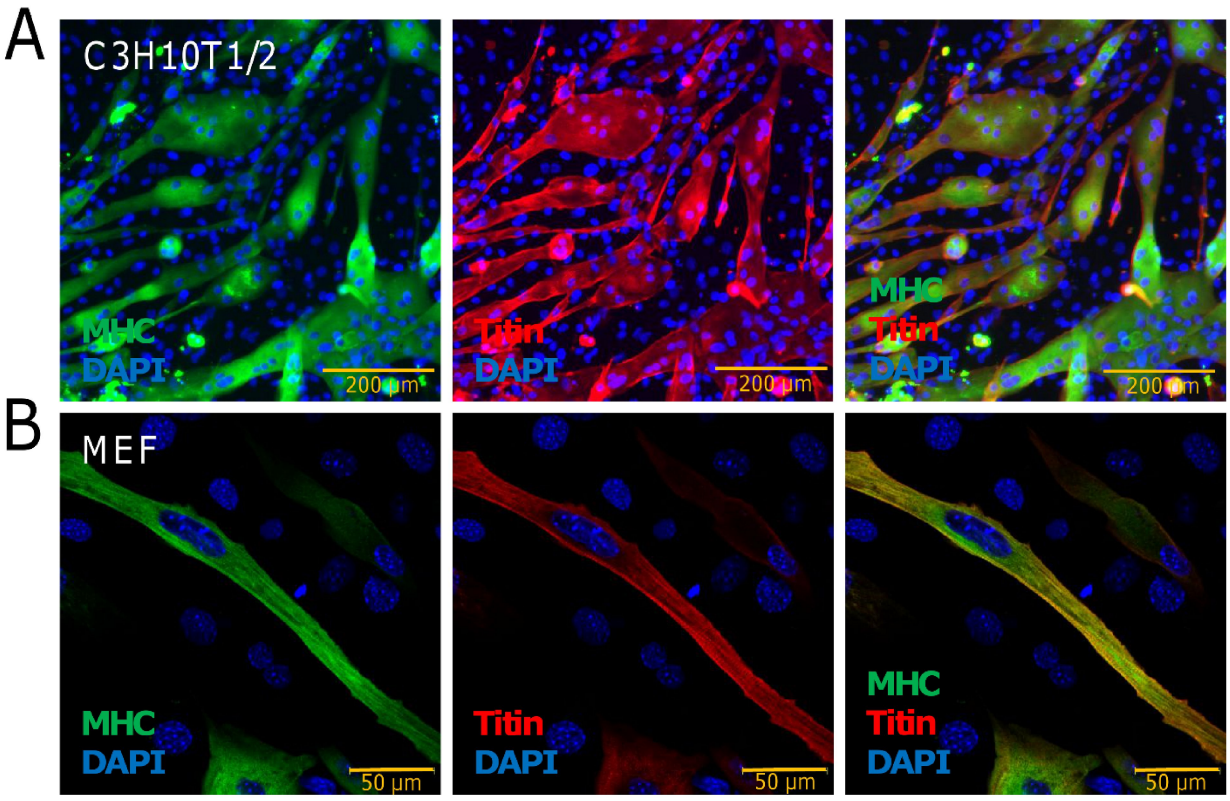

Figure S2: Supplemental Figure 2, related to Figure 2. Immunocytochemistry (ICC) staining of reprogrammed C3H10T1/2 cells and mouse embryonic fibroblasts (MEFs) for additional myogenic markers.

(A) Immunofluorescence microscopic imaging of reprogrammed C3H10T1/2 cells showing multinucleated myotubes characterized by the presence of sarcomeric proteins Myosin heavy chain (MHC) and Titin. **(B)** Confocal imaging of reprogrammed MEFs showing the emergence of cross-striated organization of sarcomeric Myosin heavy chain and Titin.

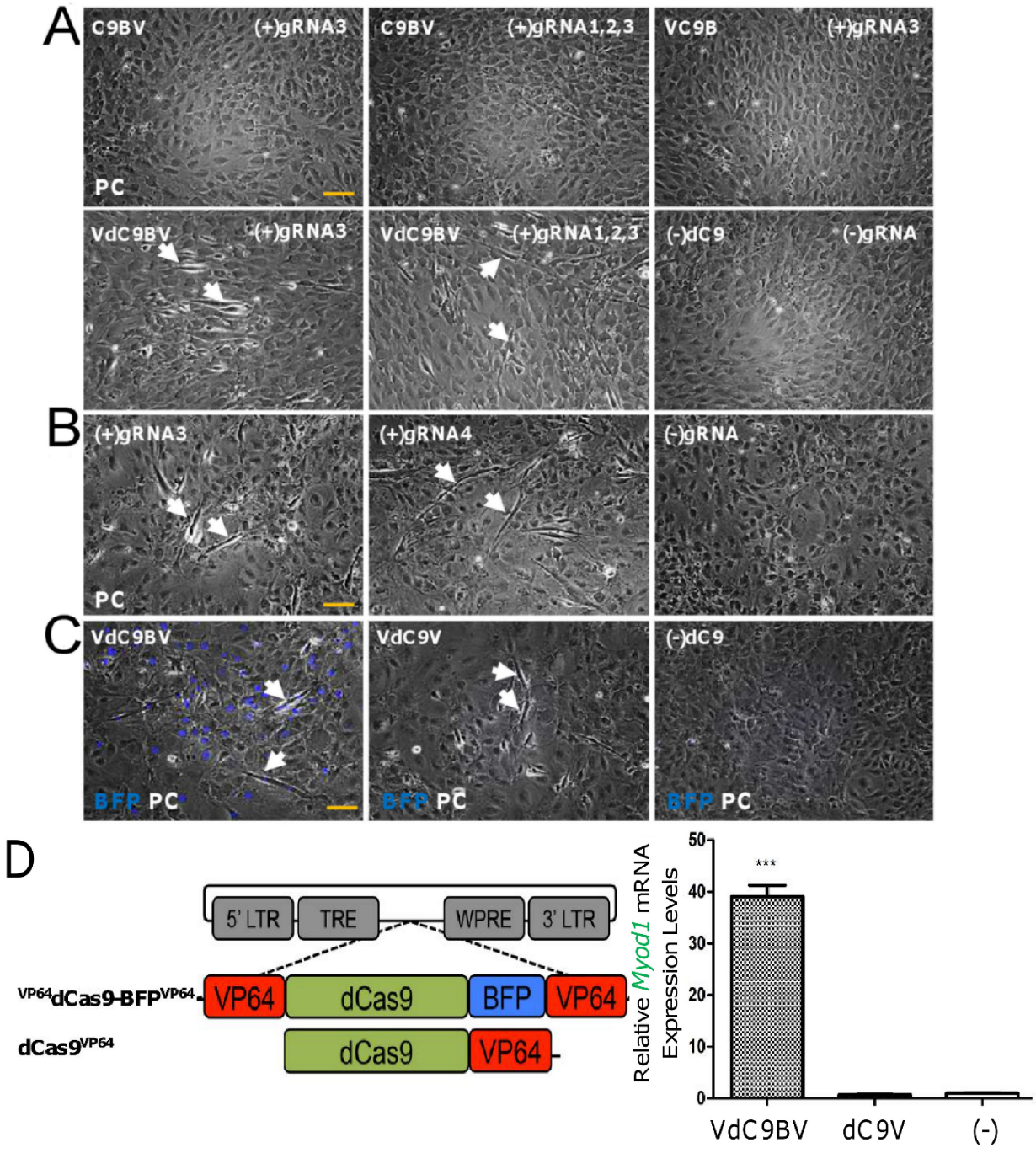

Figure S3: Supplemental Figure 3, related to Figure 3. Characteristics of gRNA-guided ^{VP64}dCas9-BFP^{VP64} (VdC9BV)-mediated activation of the endogenous *Myod1* gene locus.

(A) C-terminal-only and N-terminal-only VP64 fusion with dCas9-BFP cannot reprogram C3H10T1/2 cells into skeletal myocytes. Only VP64 fused to both N- and C-terminus of dCas9 can activate the endogenous *Myod1* gene sufficiently for myotube formation. The cells were imaged for the presence of developing myotubes (indicated by white arrows) which indicates initiation of reprogramming. Scale bar = 100μm. **(B)** gRNA4, designed with target sequence on the antisense strand, is able to trigger reprogramming in conjunction with VdC9BV similar to gRNA3 that targets the sense strand. The cells were imaged for the presence of developing myotubes (indicated by white arrows) which indicates initiation of reprogramming. Scale bar = 100μm. **(C)** Both BFP-fused and -unfused forms of VdC9BV transgene can activate the endogenous *Myod1* gene for skeletal reprogramming. The cells were imaged for the presence of developing myotubes (indicated by white arrows) which indicates initiation of reprogramming. Scale bar = 100μm. **(D)** Illustration depicting dCas9-based activators $^{\mathbf{VP64}}$dCas9-BFP$^{\mathbf{VP64}}$ (VdC9BV) and dCas9$^{\mathbf{VP64}}$ (dC9V) and qRT.PCR analysis showing failure of the C-terminal VP64 fusion to dCas9 (dCas9$^{\mathbf{VP64}}$, a BFP-less form) to activate the *Myod1* locus of C3H10T1/2 cells ($P \leq .001$, One-way ANOVA, Dunnett's post hoc test, n=3 biological replicates). All the experiments **(A-D)** were done in C3H10T1/2 cells. Images were acquired at six days post-transduction.

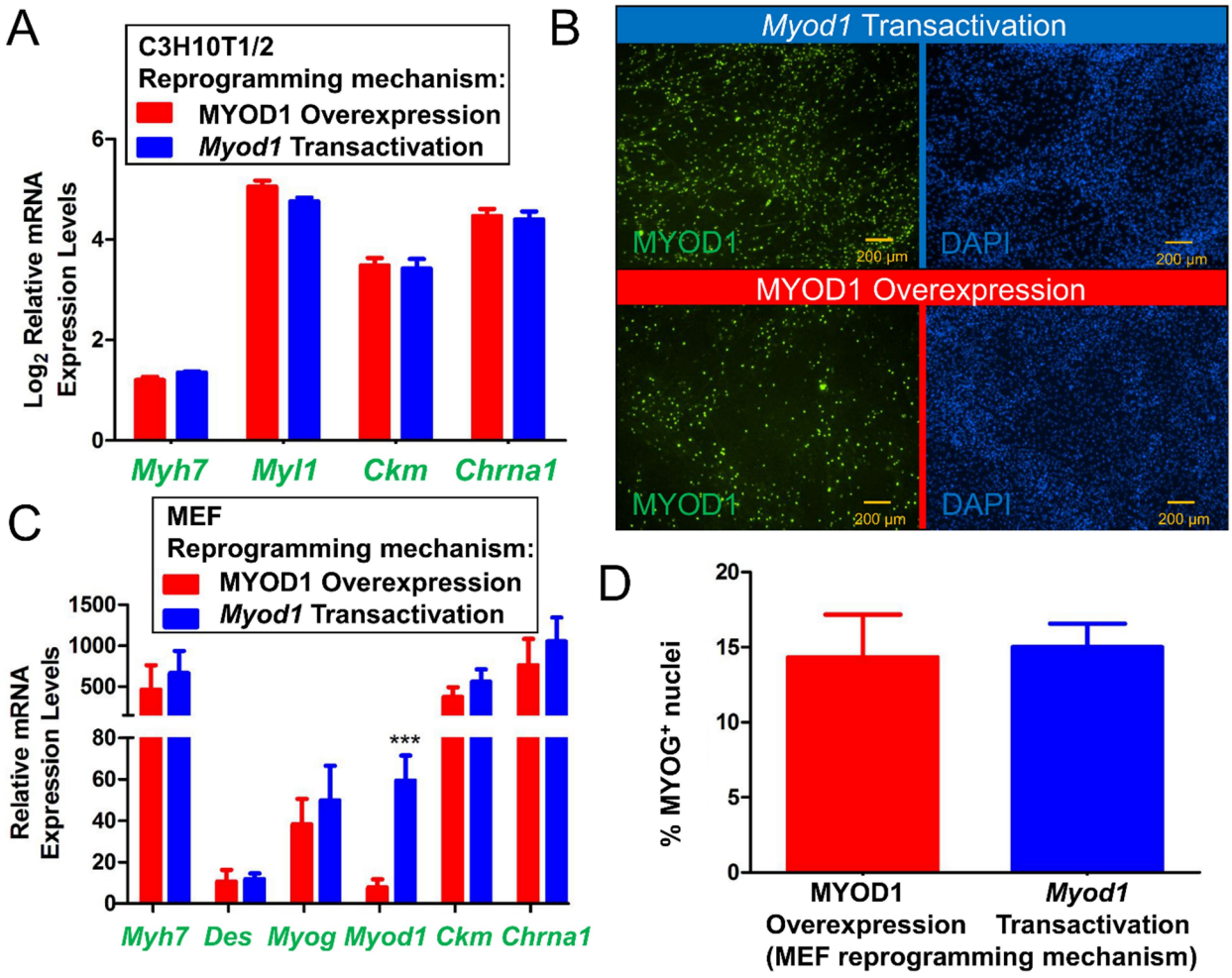

Figure S4: Supplemental Figure 4, related to Figure 4. Comparison of skeletal reprogramming by gRNA-guided ^{VP64}dCas9-BFP^{VP64} (VdC9BV)-mediated transactivation of the endogenous *Myod1* gene locus and by transgenic MYOD1 overexpression.

(A) Expression of myogenic markers in C3H10T1/2s (detected by qRT.PCR) on day 18 post-transduction by VdC9BV-mediated transactivation of the endogenous *Myod1* gene is similar to that activated by transgenic MYOD1 overexpression (comparison of individual markers was done by one-way ANOVA with Bonferroni post tests, n=3 biological replicates, error bars=SEM). Fold change in expression is relative to the (-) gRNA (-)

MYOD1 transgene control group. Doxycycline induction of VdC9BV and MYOD1 transgenes was carried out for 8 days till day 10 post-transduction. **(B)** Percentage of DAPI-stained nuclei that also express MYOD1 protein in C3H10T1/2s (detected by ICC) on day 18 post-transduction by transactivation of the endogenous *Myod1* gene is higher than that mediated by transgenic MYOD1 overexpression. **(C)** Expression of myogenic markers in mouse embryonic fibroblasts (detected by qRT.PCR) on day 22 post-transduction by transactivation of the endogenous *Myod1* gene is similar ($P>.05$) to that mediated by transgenic MYOD1 overexpression except for the expression of *Myod1* mRNA ($P\leq.001$) (comparison of individual markers was done by one-way ANOVA with Bonferroni post tests, n=3 biological replicates, Error bars=SEM). Fold change in expression is relative to the (-) gRNA (-) MYOD1 transgene control group. The doxycycline induction of VdC9BV and MYOD1 transgenes was carried out for 10 days till day 12 post-transduction. **(D)** Percentage of DAPI-stained nuclei that also express MYOG in mouse embryonic fibroblasts (detected by ImageJ-aided counting from ICC images) on day 22 post-transduction in the *Myod1* gene transactivation group (mean=~15%) is statistically similar to that mediated by transgenic MYOD1 overexpression (mean=~14.5%) (Two-tailed unpaired t test, n=3 biological replicates, Error bars=SEM).

Movie S1: Supplemental Movie S1, related to Figure 1. Video showing spontaneous twitching of skeletal myocytes reprogrammed from mouse embryonic fibroblast on post transduction day 22.

Supplemental Experimental Procedures

Guide RNA plasmid construction. Ubiquitin promoter-driven Green Fluorescent Protein cassette was removed from FUGW plasmid (Addgene plasmid 14883) by digesting with PacI and XhoI. For expressing a chimeric gRNA from the human U6 promoter a gBlock fragment (Integrated DNA Technologies, Inc., USA) was synthesized (Mali et al., 2013). It incorporated a BsmBI site, distal to the U6 promoter, for easy incorporation of new gRNA spacer sequences. The gBlock was then cloned into the restriction-digested FUGW backbone (Figure 1C). For targeting 23bp sequences in the genome of the form $NNNNNNNNNNNNNNNNNNNN_{(N=20)}$-$NGG_{(PAM)}$, plus and minus strand DNA oligos were designed to contain the protospacer sequence. An overhang was produced after annealing of the oligos that was complementary to the overhangs created in the vector after digestion with BsmBI. After ligation, the gRNA expression plasmids were subsequently packaged in lentiviral vectors.

The following gRNA-targeted sites were used in the study:

Mouse *Myod1* (*Myod1* Transcript Position: chr7:53,631,844-53,634,462) gRNA-targeted sequence:

gRNA1(+strand chr7:53630309-53630328): GTATCAGAGACAAAAACCGT;

gRNA2(-strand chr7:53630967-53630986): TTCTTAAGGATCGTGATGGC;

gRNA3(+strand chr7:53631602-53631621): TAGCCAAGTGCTACCGCGTA;

gRNA4(-strand chr7:53631608-53631627): CAGCCATACGCGGTAGCACT.

Human *MYOD1* (*MYOD1* Transcript Position: chr11:17,741,110-17,743,678) gRNA-targeted sequence:

(+strand chr11:17740919-17740938): ACCTAGCGCGCACGCCAGTG.

^{VP64}dCas9-BFP^{VP64} plasmid construction. The dCas9-BFP fusion cassette was digested from the pdCas9::BFP-humanized plasmid (Addgene plasmid 44247). Two gBlock DNA fragments were synthesized: one having the N-terminal VP64, FLAG tag, and linker sequence and the other having the C-terminal VP64 along with a FLAG tag, an additional nuclear localization signal, and a linker sequence. The lentiviral backbone along with the doxycycline inducible promoter was derived from Tet-O-FUW-Myt1l (Addgene plasmid 27152) (Vierbuchen et al., 2010). All four fragments were digested to generate compatible sticky ends. All the digested fragments were then joined together by a four-way ligation reaction to form TetO-FUW-^{VP64}dCas9-BFP^{VP64} (Figure 1C). The final amino acid sequence of the construct is provided in Supplemental Figure 1A. This plasmid was used to produce all the other forms including N-terminal-only VP64, C-terminal-only VP64, and the BFP-deleted fusion construct. Q5 High Fidelity DNA polymerase (New England Biolabs. MA, USA) along with corresponding primers were used to PCR amplify the required fragments to be ligated back into the doxycycline inducible viral backbone to generate all the other forms mentioned above. A doxycycline inducible form of MYOD1 was also generated by inserting a human *MYOD1* element (LIFESEQ3292930, Open Biosystems) into the same lentiviral plasmid backbone.

Cell culture, transfection and viral transduction. Primary mouse embryonic fibroblasts (PMEF) (Lonza), human embryonic kidney 293T cells (HEK 293T) (ATCC) and C3H10T1/2 cells (ATCC) were utilized in this study. All the cell types were initially cultured with 10% fetal bovine serum (FBS) (Atlanta Biologicals) in DMEM-HG (GIBCO-11960) supplemented with L-glutamine, pyruvate and MEM-NEAA (GIBCO). The HEK293Ts were utilized for lentivirus generation and for verifying the efficacy of ^{VP64}dCas9-BFP^{VP64}

to transactivate the endogenous human *MYOD1* locus. The other two cell types were utilized in reprogramming studies. For reprogramming the cells were seeded at a density of 25,000/well of a 12-well plate. Transfection of C3H10T1/2 was done by using Lipofectamine LTX with plus reagent (Life Technologies). Cells were transfected at a confluency of 70%. 2 µg of plasmid DNA was delivered to each well of a 12-well plate at a ratio of 4:1:1 (^{VP64}dCas9-BFP^{VP64}:M2rtTA:U6-gRNA). For transduction, the lentiviruses were added at a total MOI (multiplicity of infection) of 60: ^{VP64}dCas9-BFP^{VP64}=MOI of 40, M2rtTA=MOI of 10, U6-gRNA=MOI of 10. For the experimental controls, empty FUW lentiviral backbone (Ubiquitin C promoter removed from the FUW lentiviral construct) was transduced to maintain the total MOI (Figure 1C). To improve transduction, Sequebrene was added to the medium at a concentration of 8 µg/ml. Doxycycline at a concentration of 3 µg/ml was added to cultures on day 2 post transduction. In the case of C3H10T1/2 cells, doxycycline induction was continued till day 10 in a low serum differentiation media composed of 2% Horse Serum (Sigma Aldrich) in DMEM-HG (GIBCO-11960) supplemented with L-glutamine, pyruvate and MEM-NEAA (GIBCO). Subsequently the media was switched to a doxycycline-free low serum media. On day 18 post-transduction the cells were fixed and stained and RNA was extracted for RT.PCR. For the PMEFs, doxycycline was continued till day 12 and subsequently switched to a no-doxycycline medium till day 22. For PMEFs the differentiation media had 10% FBS till day 12 and subsequently changed to 2% FBS (Figure 2E).

Lentiviral production. Lentiviral particles were produced by transfecting HEK 293T cells with the transfer plasmid (TetO-FUW-^{VP64}dCas9-BFP^{VP64}, TetO-FUW-dCas9-BFP^{VP64}, TetO-FUW-^{VP64}dCas9-BFP, TetO-FUW-^{VP64}dCas9^{VP64}, U6-gRNA, FUW-M2rtTA, Empty

FUW) along with 2nd generation packaging plasmids (Addgene plasmid 12260: psPAX2 and Addgene plasmid 12259: pMD2.G). The procedure for lentivirus generation and extraction has been described elsewhere (Chakraborty et al., 2013). For determining the titer of the lentivirus, a qRT.PCR-based lentiviral titration kit was used according to the manufacturer's instructions (Applied Biological Materials Inc., Richmond, Canada).

Quantitative real time reverse-transcription polymerase chain reaction (qRT.PCR) and reverse-transcription polymerase chain reaction (RT.PCR). The primers were purchased from IDT (Coralville, Iowa). Total RNA was extracted with a Clontech RNA clean-up kit. cDNA was generated by iScript cDNA Synthesis kit (Bio-Rad). PCR was done by utilizing *Taq* DNA Polymerase with ThermoPol® Buffer (NEB). The cycling conditions were: Activation: 95°C for 30 sec; Denaturation: 95°C for 20 sec, Annealing: 58°C for 30 sec, Extension: 68°C for 60 sec, for varying number of cycles; Final Extension: 72°C for 5 min. For qRT.PCR, SsoAdvanced™ Universal SYBR® Green Supermix from Bio-Rad was used according to the manufacturer's instruction. The expression levels are presented as fold change relative to a control group (*delta-delta Ct method).* GAPDH was used as the housekeeping endogenous control gene to normalize target gene expression.

Primer sequences for quantitative real time reverse-transcription polymerase chain reaction (qRT.PCR) and reverse-transcription polymerase chain reaction (RT.PCR). The following primers were utilized in this study:

dCas9: AGGGATTAAGGAGCTCGGGT, AGGAAGCTCTGAGGGACGAT;

Mu-*Myod1*: AGCGACACAGAACAGGGAAC, TCGAAAGGACAGTTGGGAAG;

Mu-*Myog*: GAGACATCCCCCTATTTCTACCA, GCTCAGTCCGCTCATAGCC;

Mu-*Desmin*: GTGGATGCAGCCACTCTAGC, TTAGCCGCGATGGTCTCATAC;

Mu-*Myl1*: AAGATCGAGTTCTCTAAGGAGCA, TCATGGGCAGAAACTGTTCAAA;

Mu *Ckm*: CTGACCCCTGACCTCTACAAT, CATGGCGGTCCTGGATGAT;

Mu-*Chrna1*: GCACCTGGACCTATGACGGC, TAAGACAGAGATGCTCAGCG;

Mu-*Myh7*: CTCAAGCTGCTCAGCAATCTATTT, GGAGCGCAAGTTTGTCATAAGT;

Mu-*Gapdh*: TGCGACTTCAACAGCAACTC, CTTGCTCAGTGTCCTTGCTG;

Hu-*MYOD1*: CTTTGCTATCTACAGCCGGG, GAGTGCTCTTCGGGTTTCAG;

Transgenic Hu-MYOD1: CGGCATGATGGACTACAGCG,

CAGGCAGTCTAGGCTCGAC;

Hu-*GAPDH*: GGAGCGAGATCCCTCCAAAAT, GGCTGTTGTCATACTTCTCATGG.

Cell fusion assay. C3H10T1/2 cells were sequentially transduced with TetO-FUW-^{VP64}dCas9-BFP^{VP64} and M2rtTA virus. This cell population was subsequently divided and transduced with either LV-CRE or LV-Floxed Luc. These cell populations were mixed and plated in a 1:1 ratio of LV-CRE transduced cells to LV- Floxed Luc cells and transduced with the *Myod1* gRNA virus. The cells were induced to express ^{VP64}dCas9-BFP^{VP64} by adding 3 µg/ml doxycycline to the medium on day 6 post-transduction with ^{VP64}dCas9-BFP^{VP64}. Medium containing fresh doxycycline was replenished every two days for the next eight days. Subsequently doxycycline was withdrawn on day 14 post-transduction with ^{VP64}dCas9-BFP^{VP64}. Fresh medium without doxycycline was then replenished every two days. Samples were harvested on day 14 and day 22 and assayed for luciferase expression. Briefly, cells were pelleted and washed with PBS. Pellets were re-suspended in 100 µL of lysis buffer (100mM KH2PO4 + 0.2% Triton-X, pH 7.8) and incubated at room temperature for 10 min. The cell debris was pelleted and 30 µl of the supernatant from each sample was transferred to an opaque 96-well plate. Each sample was mixed with

30 µL of Bright-Glo reagent (Bright-Glo Luciferase Assay System, Promega). Luminescence was measured by a BioTek Synergy 2 Multi-Mode Microplate Reader with 1-second scan time. All luciferase data is presented as a fold increase over background of samples transduced with VP64dCas9-BFPVP64, M2rtTA, and LV-Floxed Luc (Figure 2D).

Cell staining, imaging, analysis and illustrations. The cells were then stained with primary antibodies against: Desmin (Monoclonal Anti-Pig Desmin, mouse IgG1 isotype, Sigma Aldrich, D1033), MYOD1 (Monoclonal Anti-Mouse MYOD1, mouse IgG1 isotype, Pierce, MA141017), Myogenin (Monoclonal Anti-Rat Myogenin, mouse IgG1 isotype, Developmental Studies Hybridoma Bank, F5D), FLAG™ Epitope Tag (Mouse monoclonal IgG2b, Pierce, MA1-91878), F-Actin (Alexa Fluor 594 Phalloidin, Life Technologies, A12381), α-Actinin (Monoclonal Anti-Rabbit Skeletal α-Actinin, mouse IgG1 isotype, Sigma Aldrich, A7811), Myosin heavy chain (Monoclonal Anti-Chicken Sacrcomeric Myosin, mouse IgG2b isotype, Developmental Studies Hybridoma Bank, MF 20), Titin (Monoclonal Anti-Bovine Titin, mouse IgM isotype, Developmental Studies Hybridoma Bank, 9 D10). DAPI was used to stain the nucleus. Alexa Fluor 488 Goat Anti-mouse IgG, Alexa Fluor 568 Goat Anti-rabbit IgG and Alexa Fluor 568 Goat Anti-mouse IgM (Life technologies) were used as the secondary antibody for ICC. Fluorescent images were captured using Nikon Eclipse TE2000-U with a Roper Scientific CoolSnap HQ camera or Zeiss 510 inverted confocal microscope. Acquired images were displayed by using Nikon NIS Elements or Zeiss AIM LSM Image browser for the confocal images. ImageJ (http://rsb.info.nih.gov/ij) was used for counting the marker^{+} nuclei on the ICC images. Three fields were randomly chosen for each replicate. ImageJ was also used to manually

define the nuclear and the cytoplasmic regions on the images and to calculate the average fluorescence intensity. Illustrations were created by adapting templates from Servier Medical Arts (http://www.servier.com/Powerpoint-image-bank, licensed under a Creative Commons Attribution 3.0 Unported License).

Supplemental References

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