

## Enhanced MyoD-Induced Transdifferentiation to a Myogenic Lineage by Fusion to a Potent Transactivation Domain

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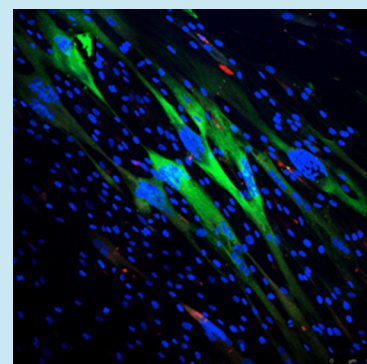
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### S Supporting Information

**ABSTRACT:** Genetic reprogramming holds great potential for disease modeling, drug screening, and regenerative medicine. Genetic reprogramming of mammalian cells is typically achieved by forced expression of natural transcription factors that control master gene networks and cell lineage specification. However, in many instances, the natural transcription factors do not induce a sufficiently robust response to completely reprogram cell phenotype. In this study, we demonstrate that protein engineering of the master transcription factor MyoD can enhance the conversion of human dermal fibroblasts and adult stem cells to a skeletal myocyte phenotype. Fusion of potent transcriptional activation domains to MyoD led to increased myogenic gene expression, myofiber formation, cell fusion, and global reprogramming of the myogenic gene network. This work supports a general strategy for synthetically enhancing the direct conversion between cell types that can be applied in both synthetic biology and regenerative medicine.



**KEYWORDS:** genetic reprogramming, myogenesis, MyoD, muscle cell therapy, protein engineering, transcription factor, regenerative medicine, adipose stem cell, mesenchymal stem cell

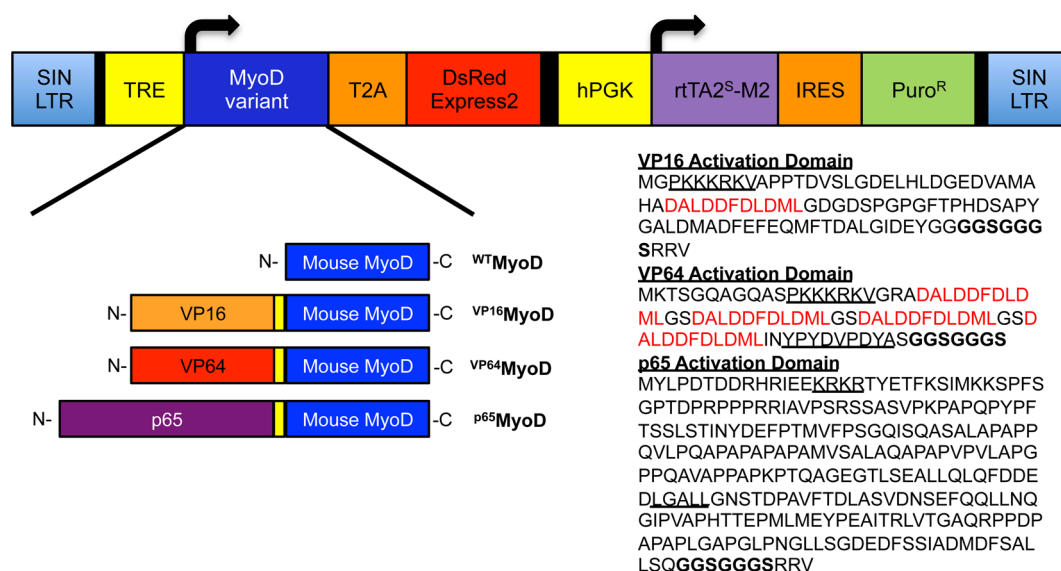
Genetic reprogramming is the conversion of one cell type to another via the activation of gene networks that control a particular cell phenotype.<sup>1</sup> Conventional methods of genetic reprogramming involve the overexpression of master regulatory transcription factors that control the gene networks corresponding to the desired cell phenotypes. This strategy has been used to induce somatic cells to pluripotency<sup>2</sup> and to directly convert one somatic cell type to another, such as the conversion of fibroblasts to myoblasts,<sup>3,4</sup> cardiomyocytes,<sup>5</sup> neurons,<sup>6–8</sup> or hepatocytes.<sup>9,10</sup> These new cell types can then be used for patient-specific disease modeling, drug screening, or regenerative medicine.<sup>11</sup> One significant challenge to genetic reprogramming is achieving sufficiently robust activation of gene networks to induce the new cell phenotype. For example, the generation of induced pluripotent stem cells (iPSCs) often produces many incompletely reprogrammed cells, with residual epigenetic memory of the starting cell type.<sup>12,13</sup> The rate of reprogramming is slow, often taking several weeks or months.<sup>14,15</sup> The overall efficiencies of genetic reprogramming are low and can be highly variable depending on the methods, cell types, and reprogramming factors used. The efficiency of reprogramming is known to correspond to the magnitude and duration of expression of the exogenous transcription factors.<sup>14,16</sup> Although these issues are most well-characterized for iPSCs, these challenges are general across all types of

reprogramming.<sup>1–10</sup> We hypothesized that genetic reprogramming may be improved by enhancing the transactivation potential of reprogramming factors through protein engineering.

The engineering of transcription factors to control mammalian cell processes has a wide variety of applications in biotechnology, including synthetic biology<sup>17</sup> and gene and cell therapy.<sup>18</sup> The most common methods for engineering synthetic transcription factors have been based on the programmable DNA-binding domains of zinc finger proteins,<sup>19</sup> Transcription activator-like effectors (TALEs),<sup>20–24</sup> and most recently the CRISPR/Cas9 system.<sup>25–27</sup> These transcription factor platforms consist of the DNA-binding domain fused to potent transcriptional activation domains, most commonly the tetramer of the minimal transactivation domain of the VP16 protein from herpes simplex virus,<sup>28</sup> referred to as VP64.<sup>29,30</sup> In some cases, the activation domain of the human NF- $\kappa$ B RelA transcription factor p65<sup>31</sup> is used, particularly for therapeutic applications in which it is desirable to avoid nonhuman protein components, although it is generally not as potent as VP64.<sup>23</sup> These modular transcription factors have been useful for targeted activation of specific genes for genetic screens,<sup>32–34</sup>

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**Figure 1.** MyoD lentiviral delivery system.  $^{WT}$ MyoD,  $^{VP64}$ MyoD,  $^{VP16}$ MyoD, and  $^{p65}$ MyoD were cloned into a Tet-ON lentiviral vector. This vector constitutively expresses the reverse tetracycline transactivator (rtTA $^{2S}$ -M2) and the puromycin resistance gene (Puro $^R$ ) from the human phosphoglycerate kinase (hPGK) promoter. The rtTA $^{2S}$ -M2 and Puro $^R$  are coexpressed from the same mRNA via an internal ribosomal entry site (IRES). The MyoD-T2A-dsRed Express2 expression cassette is downstream of the tetracycline response element (TRE) promoter. The rtTA $^{2S}$ -M2 binds to the TRE and activates expression of the downstream genes in the presence of doxycycline. The T2A ribosomal skipping peptide results in the expression of two separate proteins from a single mRNA. The peptide sequence of each transcriptional activation domain is shown. The minimal activation domain of VP16 is shown in red. PKKKRKRK is the SV40 nuclear localization signal and p65 contains the native nuclear localization signals KRKR and LGALL (underlined). VP64 also contains an HA epitope tag (YPYDVPDYA) that was not utilized in this study (underlined). All fusion proteins contain a flexible serine/glycine linker GGGSGGS (bold) between the activation domain and MyoD.

gene therapy,<sup>35–40</sup> and creating gene circuits and synthetic gene regulation systems.<sup>41–43</sup> Although these applications are well-suited for the targeted activation of a single gene, genetic reprogramming requires the coordinated regulation of many nodes of natural gene networks as is typically performed by naturally occurring reprogramming factors.<sup>44</sup> Thus, we sought to combine principles from each of these approaches by attaching potent transcriptional activation domains to a natural reprogramming factor to increase the efficiency and/or rate of cell fate conversion.

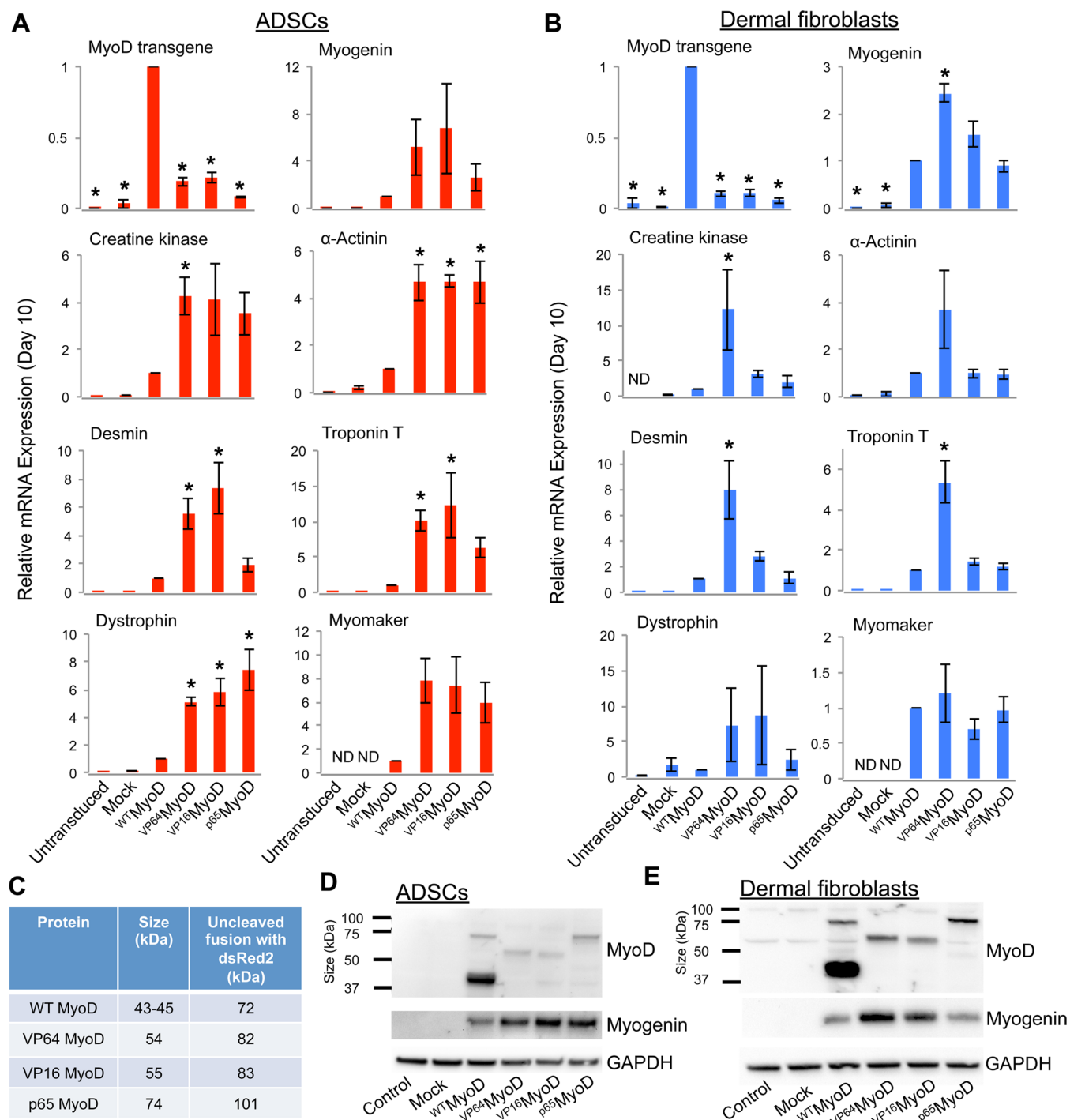
Fusion to the VP16 activation domain or its oligomers to enhance the activity of natural transcription factors has been useful for a variety of applications. In the most well-known example, VP16 was fused to the tetracycline-dependent repressor to create the widely used tetracycline-regulated gene expression system.<sup>45</sup> VP64 has also been used to direct chromatin remodeling.<sup>46</sup> HIF-1 $\alpha$  is a transcription factor that responds to hypoxia to coordinate a network of genes involved in angiogenesis and wound healing. To remove its oxygen-dependence for gene therapy applications, the oxygen-sensing domain was removed and the truncated protein was fused to the VP16 transactivation domain.<sup>47</sup> Several variants of this chimeric transcription factor potently induced angiogenesis in multiple animal models<sup>48</sup> and one variant was later used in gene therapy clinical trials to treat ischemia.<sup>49</sup> In the context of genetic reprogramming, VP16 has been fused to transcription factors to enhance the efficiency of reprogramming mouse and human fibroblasts to iPSCs<sup>50</sup> and also to convert liver to pancreas in a *Xenopus* model.<sup>51</sup> In addition to the viral VP16 domain, the transfer of transactivation domains between mammalian transcription factors has also increased their potency for some reprogramming applications.<sup>52,53</sup> Collectively, these successes suggest that there is considerable opportunity

to enhance natural transcription factors through protein engineering to guide the coordination of gene networks.

In this study, we evaluated the effects of fusing potent activation domains to the transcription factor MyoD, the master regulator of the skeletal myoblast lineage.<sup>54</sup> In certain nonmyogenic lineages, MyoD overexpression causes upregulation of the myogenic gene network and conversion to a myoblast phenotype including cell fusion into multinucleated myotubes.<sup>3,4</sup> Myogenic reprogramming induced by MyoD overexpression has been widely explored as a means for cell-based gene therapy for skeletal muscle disorders such as muscular dystrophy.<sup>55–60</sup> Compared to wild-type MyoD, the enhanced MyoD variants induced greater levels of specific myogenic markers, greater levels of cell fusion, and greater overall reprogramming of global gene expression in both primary human dermal fibroblasts and adipose-derived stem cells. Collectively, these results support the paradigm of enhancing natural transcription factors through protein engineering and specifically provide a potential mechanism for autologous cell therapies for musculoskeletal disorders.

## RESULTS AND DISCUSSION

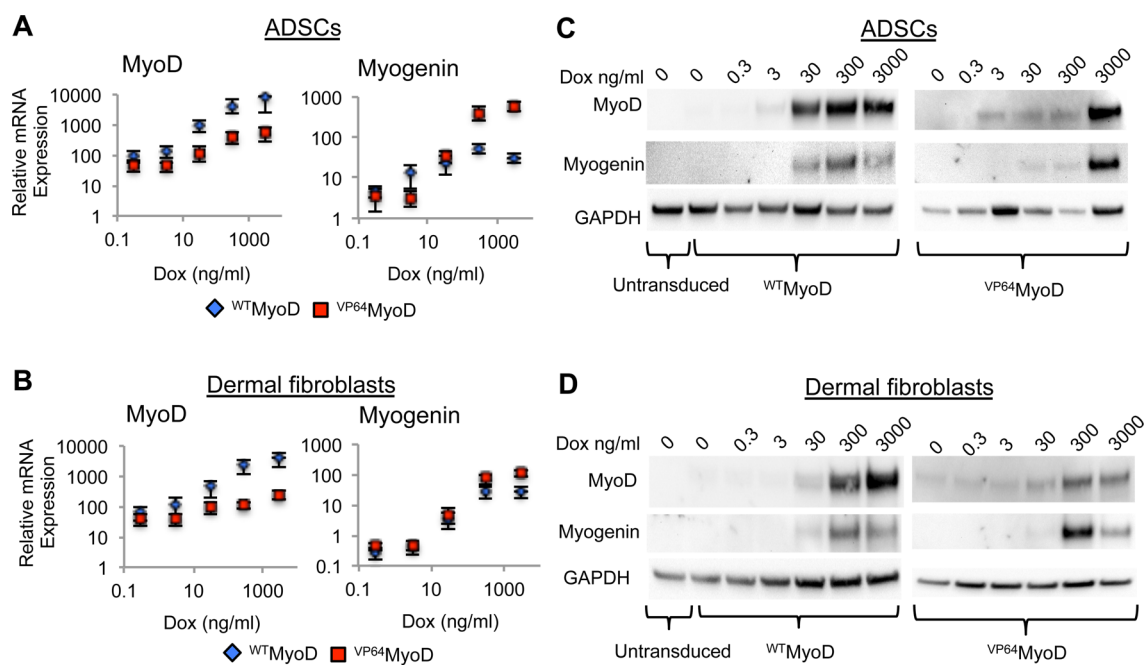
To construct enhanced MyoD variants, potent activation domains were genetically fused to the N-terminus of wild-type (WT) mouse MyoD ( $^{WT}$ MyoD) to construct  $^{VP16}$ MyoD,  $^{VP64}$ MyoD, and  $^{p65}$ MyoD. VP16, VP64, and p65 are modular transcriptional activation domains (Figure 1). The mouse MyoD cDNA was used to distinguish exogenous MyoD expression from endogenous human MyoD expression. VP16 is the activation domain from a natural transcription factor originally isolated from the herpes simplex virus and is widely used in transcription factor engineering.<sup>28</sup> VP64 is a tetramer of the minimal VP16 domain required for transcriptional



**Figure 2.** Upregulation of myogenic markers in ADSCs and dermal fibroblasts. Cells were transduced with lentivirus that expresses each MyoD variant or a mock control consisting of the lentiviral vector without a transgene. After 10 days of expression induced by 3  $\mu\text{g}/\text{mL}$  doxycycline, ADSCs (A) and dermal fibroblasts (B) were assessed for the relative expression levels of a variety of myogenic genes by qRT-PCR. Expression levels were normalized to <sup>WT</sup>MyoD and the  $\beta$ -actin house-keeping gene.  $n = 3$  independent experiments. All  $p$ -values are compared to <sup>WT</sup>MyoD ( $*p < 0.05$ ). In both the ADSCs (D) and fibroblasts (E), expression of each MyoD fusion was determined by Western blot, which demonstrated efficient translational skipping by the T2A peptide. Expected protein sizes are provided in part C.

activation. p65 is the activation domain of the human NF- $\kappa$ B RelA transcription factor.<sup>31</sup> Since overexpression of MyoD causes cells to exit the cell cycle and terminally differentiate, an inducible tetracycline-regulated (Tet-On) lentiviral delivery vector<sup>61,62</sup> was used to control the timing and magnitude of expression of the engineered transcription factors.

We tested the reprogramming capacity of our engineered MyoD variants in two human cell types: primary adipose-derived stem cells (ADSCs) and primary dermal fibroblasts. ADSCs are adult stem cells isolated from adipose tissue.<sup>63</sup> Human dermal fibroblasts are a terminally differentiated cell type that can be obtained from a minimally invasive skin biopsy. Both of these cell types are attractive starting material for



**Figure 3.** Dose-dependence of myogenic gene expression on levels of  $^{WT}MyoD$  and  $^{VP64}MyoD$ . ADSCs and dermal fibroblasts were transduced with lentivirus that expresses either  $^{WT}MyoD$  or  $^{VP64}MyoD$ , selected with puromycin, and then treated with the indicated dose of doxycycline. After 10 days of induction, ADSCs (A) and fibroblasts (B) were assessed for MyoD and myogenin expression by qRT-PCR. Expression levels were normalized to untransduced fibroblasts and the  $\beta$ -actin house-keeping gene.  $n = 3$  independent experiments. The transduced ADSCs (C) and fibroblasts (D) were also assayed for MyoD and myogenin protein expression by Western blot.

disease modeling, drug screening, and patient-specific cellular therapies, as they are relatively easy to obtain and can be expanded in culture. Both the ADSCs and fibroblasts were transduced with lentivirus containing each tetracycline-inducible MyoD expression cassette, selected with puromycin to obtain a pure cell population, and subsequently treated with doxycycline for 10 days to induce transgene expression.

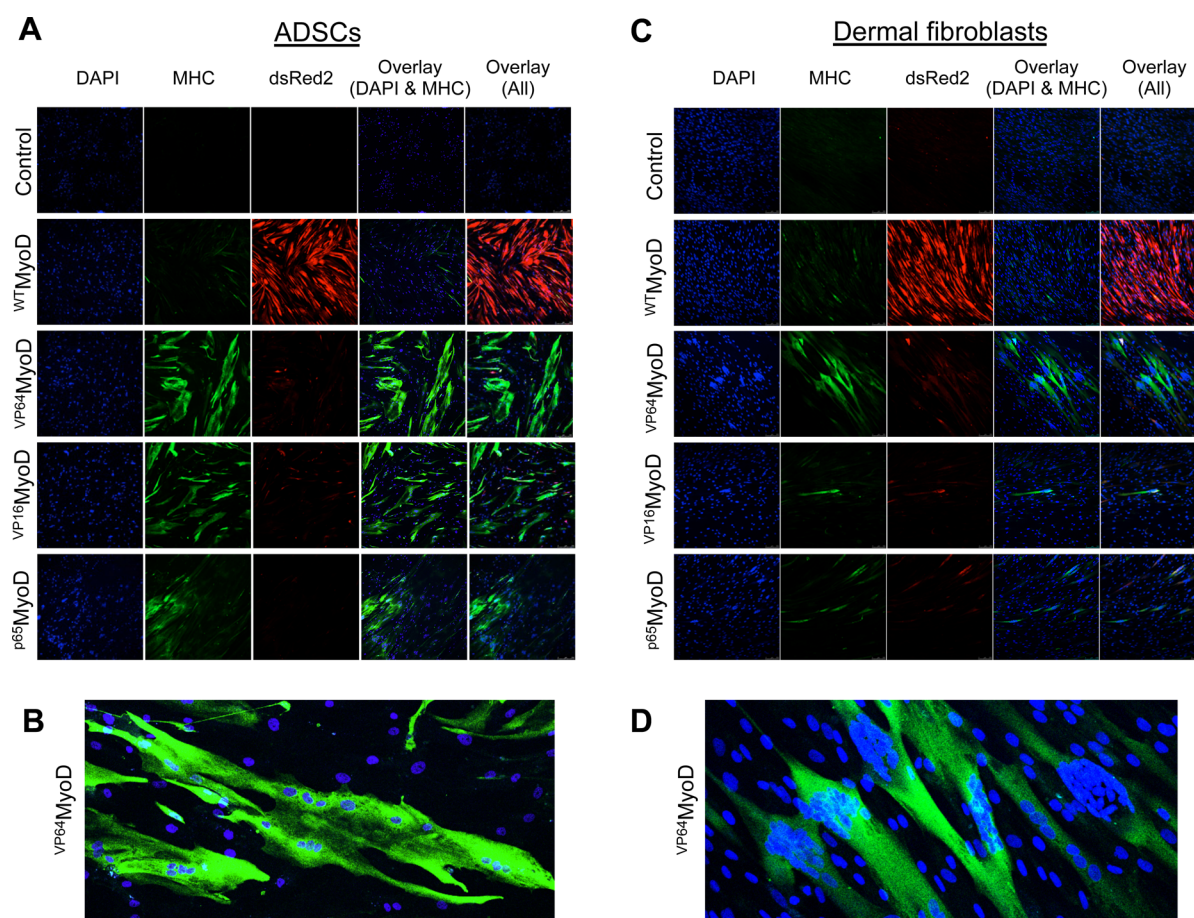
After 10 days of induction, the engineered variants upregulated myogenic markers in ADSCs and fibroblasts to a greater degree than  $^{WT}MyoD$  (Figure 2A, B). The three engineered variants behaved similarly in ADSCs, whereas  $^{VP64}MyoD$  was significantly more robust in the dermal fibroblasts, perhaps because of greater plasticity of the multipotent ADSCs. Notably,  $^{WT}MyoD$  was expressed at much higher levels than the engineered transcription factors at both the mRNA (Figure 2A, B) and protein levels (Figure 2C–E). To assess the dose-dependence of MyoD and its engineered variants on myogenic gene induction, we measured marker expression at various doses of MyoD by manipulating the concentration of doxycycline (Figure 3). As expected,  $^{WT}MyoD$  and  $^{VP64}MyoD$  both upregulate the myogenic gene network in a dose-dependent manner, but interestingly,  $^{VP64}MyoD$  is more potent than  $^{WT}MyoD$  despite its lower expression level. To ensure that the enhanced transcription factor activity was not specific to mouse MyoD, we constructed human  $^{VP64}MyoD$ . Of the eight myogenesis-associated genes analyzed, there were no significant differences in gene expression induced by human versus mouse  $^{VP64}MyoD$  (Supporting Information Figure 1).

Myogenic differentiation is characterized by a phenotypic change from mononuclear cells to fusion into multinucleated muscle fibers. Through immunofluorescence staining we observed that the engineered MyoD variants promote increased cell fusion and elevated levels of myosin heavy chain expression

*in vitro* when compared with  $^{WT}MyoD$  after 10 days of expression (Figure 4). Similar to the effects on gene expression (Figure 2), the multipotent ADSCs responded more robustly to the reprogramming factors than the dermal fibroblasts. Similar effects were also observed when overexpressing human  $^{WT}MyoD$  or  $^{VP64}MyoD$  (Supporting Information Figure 2).

To measure cell fusion, we developed a modified version of a previously reported quantitative assay that is based on a conditional luciferase expression scheme.<sup>53</sup> In this system, two lentiviral vectors were constructed: one vector that constitutively expresses Cre recombinase (LV-Cre) and a second vector encoding a luciferase expression cassette containing a 1 kb stuffer sequence flanked by loxP sites (LV-Floxed Luc). The stuffer sequence interrupts the luciferase coding sequence after the start codon such that luciferase expression is reconstituted only in response to Cre-mediated recombination (Figure 5A). One cell population was transduced with LV-Cre and the other cell population with LV-Floxed Luc (Figure 5B). When the cells from the two populations fuse to form a multinucleated myotube, Cre recombinase expressed from one nucleus enters neighboring nuclei harboring the lentivirally integrated Floxed Luc cassette and removes the stuffer sequence, thereby creating a functional luciferase gene (Figure 5C). To conduct this assay, cells were first transduced with the tet-responsive vector carrying the MyoD variant of interest and selected in puromycin to obtain a pure population. This cell population was divided and transduced with either LV-Cre or LV-Floxed Luc. The cells were remixed, plated, and induced with doxycycline. After 10 days of induction, cells were assayed for the relative levels of luciferase activity (Figure 5B). Further corroborating the immunofluorescence staining, the engineered MyoD variants induce enhanced cell fusion compared to  $^{WT}MyoD$  in both cell types (Figure 5D–E). Once again, the effects were similar for each variant in the ADSCs, whereas





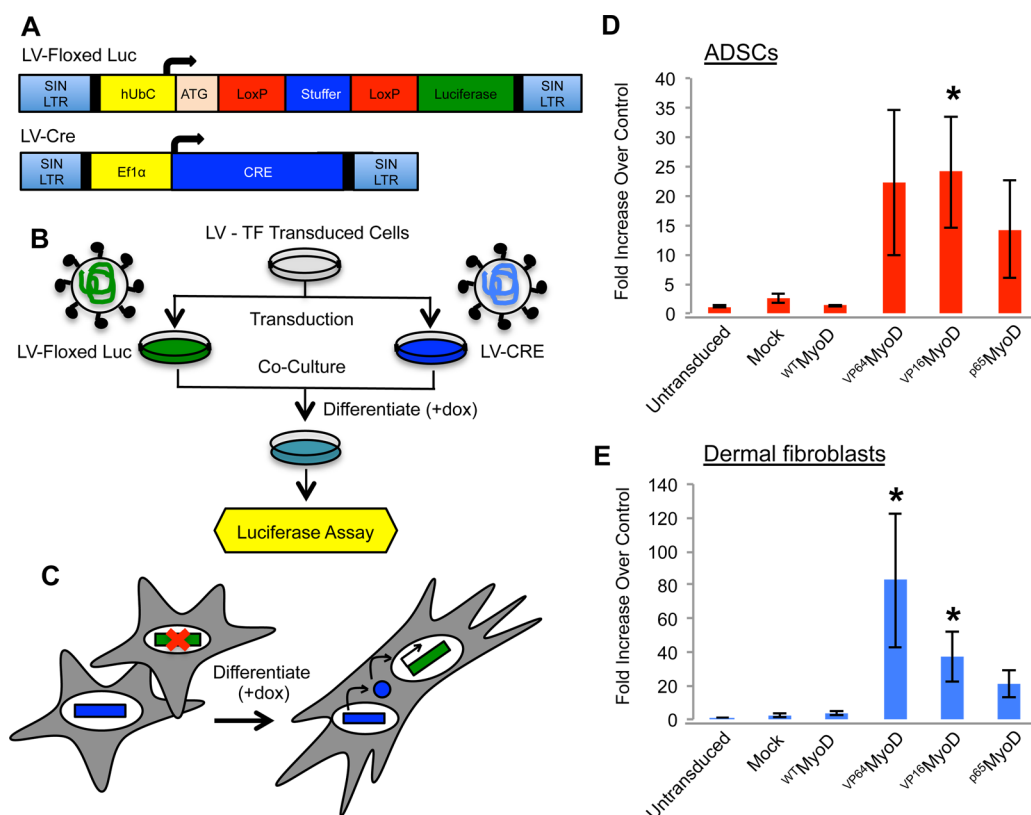
**Figure 4.** Cell differentiation and fusion induced by MyoD-mediated cell reprogramming. Following 10 days of transgene expression, cell fusion and expression of skeletal myosin heavy chain (MHC, green) was assessed by immunofluorescence staining in ADSCs (A and B) and dermal fibroblasts (C and D). Nuclei are shown in blue (DAPI) and dsRed2-Express indicates the relative levels of MyoD transgene expression.

$VP^{64}MyoD$  had the greatest effect in the dermal fibroblasts. Additionally, human MyoD showed similar enhancements with the addition of VP64 (Supporting Information Figure 3).

Next we evaluated the relative effects of  $WTMyoD$  and  $VP^{64}MyoD$  on gene expression across the transcriptome. We performed RNA-seq analysis on human dermal fibroblasts in which  $WTMyoD$  or  $VP^{64}MyoD$  was induced for 10 days. Genome-wide gene expression levels were compared to untreated control cells. Of the 30 053 transcripts included in the analysis, 4763 were significantly altered by  $WTMyoD$  overexpression (false discovery rate (FDR)  $< 10^{-6}$ ). Of these, 3135 (~66%) were also significantly changed by  $VP^{64}MyoD$  (FDR  $< 10^{-6}$ ), although  $VP^{64}MyoD$  overexpression led to changes in an additional 3559 genes that were not significantly affected by  $WTMyoD$  (FDR  $< 10^{-6}$ ), further demonstrating the enhanced activity of the engineered transcription factor in global reprogramming of cellular gene networks (Figure 6A). The majority of differentially expressed genes were upregulated, consistent with the role of MyoD as a transcriptional activator (Figure 6B). The downregulated genes are likely secondary effects of global reprogramming and transdifferentiation from the fibroblastic lineage. Of the genes that were differentially expressed in both  $WTMyoD$ - and  $VP^{64}MyoD$ -treated samples compared to untreated controls, we observed that  $VP^{64}MyoD$  generally induced a greater effect as indicated by a best fit line with a slope greater than one (Figure 6B).  $VP^{64}MyoD$  had about a 2-fold greater effect than  $WTMyoD$  (Figure 6C). The RNA-

seq analysis also showed that specific myogenic markers were induced to a greater extent by  $VP^{64}MyoD$  compared to  $WTMyoD$  (Figure 6D), confirming the qRT-PCR results (Figure 2).

To characterize the roles of each group of differentially expressed genes, we performed functional annotation clustering using the Database for Annotation, Visualization, and Integrated Discovery tool (DAVID).<sup>64,65</sup> We found that the top four gene clusters differentially expressed by both  $WTMyoD$  and  $VP^{64}MyoD$  were involved in key processes important for myogenesis including cell cycle regulation, extracellular matrix proteins, contractile proteins, and cytoskeletal proteins (Supporting Information Table 1). The top functional clusters found differentially expressed only in  $WTMyoD$ -treated cells were genes involved in translation, vascularization, cell adhesion, and extracellular matrix production (Supporting Information Table 2). The top functional clusters found differentially expressed only in  $VP^{64}MyoD$  treated cells were nucleotide binding proteins, pleckstrin proteins, and metabolic proteins involved in degradation processes (Supporting Information Table 3). Additional studies are necessary to elucidate the differential regulation of these gene groups. Proteins containing pleckstrin homology domains associate with transmembrane proteins such as G proteins and phosphatidylinositol. These proteins recruit factors to the cellular membrane and facilitate signal transduction pathways.<sup>66</sup> Therefore, this cluster of proteins may be involved with cell



**Figure 5.** Quantitative analysis of cell fusion induced by MyoD-mediated cell reprogramming. (A) Two lentiviral vectors were constructed that contain a constitutive Cre recombinase expression cassette (LV-Cre) or a luciferase cassette that is dependent on Cre recombinase activity for expression (LV-Floxed Luc). (B) Cells transduced with each dox-inducible MyoD variant were separately transduced with either LV-Floxed Luc or LV-Cre. These two cell populations were mixed at a ratio of 1:10 LV-Cre/LV-Floxed Luc. (C) As the two cell populations fuse to form a multinucleated myotubes, Cre recombinase expressed from one nucleus enters neighboring nuclei harboring the Floxed Luc cassette. Cre removes the stuffer sequence and induces expression of a functional luciferase gene. (D) ADSCs and (E) dermal fibroblasts were induced to express the MyoD variant and were assayed for relative luciferase activity on Day 10.  $n = 3$  or 4 independent experiments. All  $p$  values are compared to WT MyoD ( $*p < 0.05$ ).

signaling pathways important for cell fusion and differentiation.<sup>67</sup> Ubiquitination of the VP16 monomer of VP64 is necessary for transcriptional activation<sup>68</sup> and also serves as a mechanism to prime transcription factors and promote transcriptional elongation.<sup>69</sup> This is consistent with our observations that VP64 MyoD stimulates expression of gene clusters associated with proteolysis and is expressed at lower levels compared to WT MyoD.

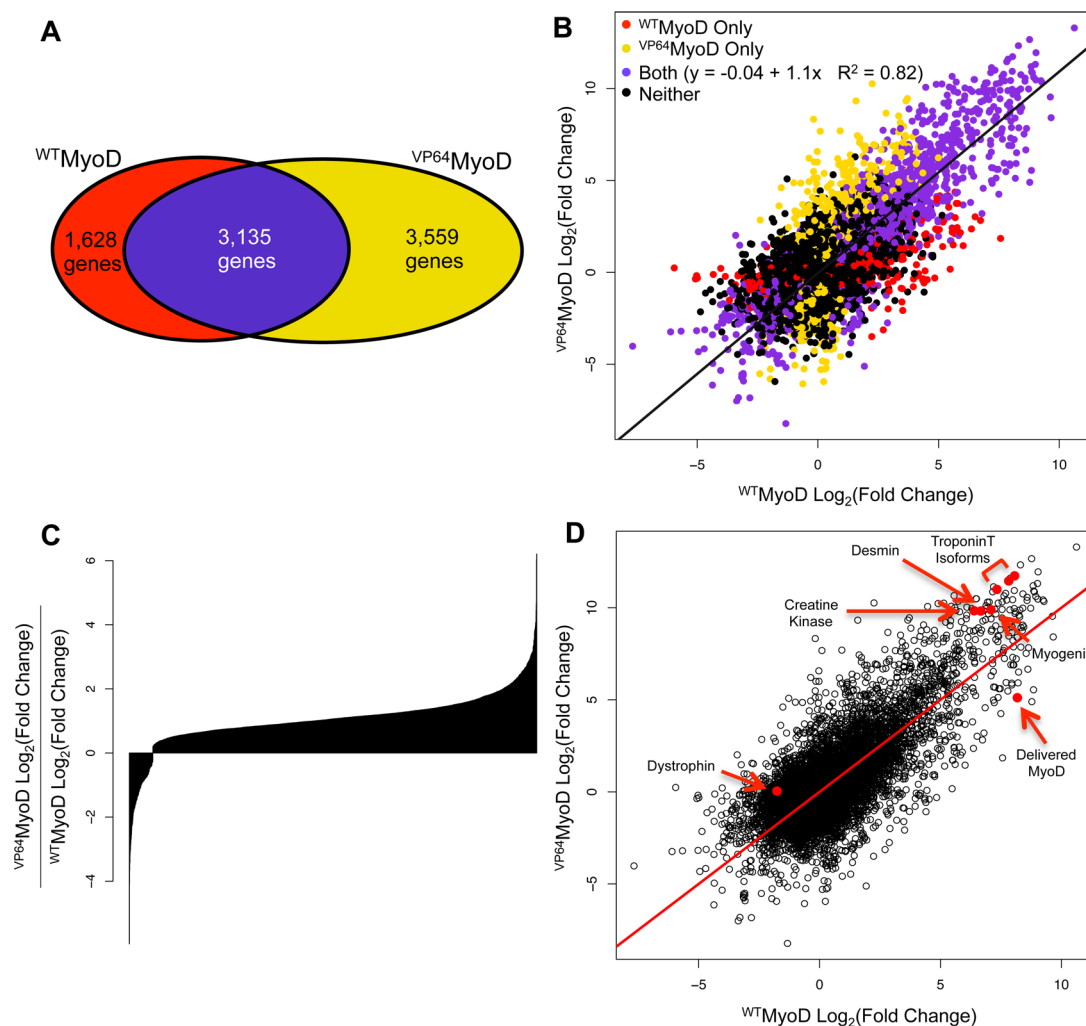
Our results demonstrate that VP64 MyoD orchestrates myogenic differentiation *in vitro* more effectively than the wild-type protein. Time course experiments suggest that the elevated levels of myogenic gene expression induced by VP64 MyoD promotes a faster rate of myogenic reprogramming compared to cells treated with WT MyoD (Supporting Information Figures 4 and 5). This is an important finding for drug screening,<sup>70</sup> disease modeling,<sup>71</sup> muscle tissue engineering,<sup>72</sup> and regenerative medicine<sup>73</sup> as genetic reprogramming with VP64 MyoD may provide new avenues for generating sources of myogenic cells. This study complements previous work in engineering Oct4 and MyoD variants with potent transcriptional activation domains to enhance cell reprogramming efficiencies.<sup>50,52,53</sup> Therefore, the addition of potent activation domains to wild-type transcription factors is a generalizable approach for increasing transcriptional activity.

Currently, most reprogramming protocols require extended transcription factor expression. The most effective method for

extended transgene expression is lentiviral transduction, but there are concerns regarding the stable integration of expression factor cassettes in the cellular genome. For this reason, reprogramming cells with nonintegrating transient expression methods such as plasmid DNA transfection,<sup>74–77</sup> mRNA transfection,<sup>78</sup> or the delivery of cell-permeable proteins<sup>79</sup> are being widely explored. However, the resulting reprogramming efficiencies are much lower and these methods are technically challenging requiring repeated dosing over extended time periods. As our results demonstrate upregulation of the myogenic gene network in only 10 days to levels requiring 30 days of WT MyoD expression (Supporting Information Figures 4 and 5), the use of engineered transcription factors in conjunction with transient delivery methods such as DNA and RNA transfection may prove advantageous. Therefore, synthetic transcription factors that induce fast and efficient remodeling of gene networks may enable widespread applications of genetic reprogramming in many areas of biotechnology.

## METHODS

**Cells.** HEK293T cells were obtained from the American Tissue Collection Center (ATCC, Manassas, VA) through the Duke University Cell Culture Facility and were maintained in DMEM supplemented with 10% FBS and 1% penicillin streptomycin. Primary human dermal fibroblasts (Catalog ID:



**Figure 6.** Genome-wide gene expression following reprogramming of dermal fibroblasts by  $^{WT}$ MyoD or  $^{VP64}$ MyoD. RNA-seq was performed on untreated control fibroblasts and fibroblasts expressing either  $^{WT}$ MyoD or  $^{VP64}$ MyoD for 10 days ( $n = 2$ ). Fold changes in expression of each gene were calculated relative to the untreated control samples. The genes shown are those different than control with a false discovery rate (FDR)  $< 10^{-6}$ . (A and B) Many genes were differentially regulated by both  $^{WT}$ MyoD and  $^{VP64}$ MyoD compared to control (purple), while other genes were exclusively regulated by only  $^{WT}$ MyoD (red) or only  $^{VP64}$ MyoD (yellow). Of the genes that were differentially expressed in samples treated with  $^{WT}$ MyoD or  $^{VP64}$ MyoD,  $^{VP64}$ MyoD generally induced a greater overall effect as indicated by a best-fit line with a slope greater than one. (C) For the transcripts differentially expressed by both  $^{VP64}$ MyoD and  $^{WT}$ MyoD compared to control (purple data points in B), the effect of  $^{VP64}$ MyoD was generally greater. (D) The RNA-seq analysis also confirms the qRT-PCR results (Figure 2B). Specific myogenic genes assayed by qRT-PCR are indicated in red and show greater activation by  $^{VP64}$ MyoD than  $^{WT}$ MyoD (line indicates  $y = x$ ). This analysis also confirmed lower levels of MyoD transgene in the  $^{VP64}$ MyoD samples compared to  $^{WT}$ MyoD.

GM03348) were obtained from Coriell Institute (Camden, New Jersey) and were maintained in DMEM supplemented with 10% FBS and 1% penicillin streptomycin. Primary human adipose derived stem cells (ADSCs) were isolated as described previously<sup>80</sup> and were cultured in growth media of DMEM/F12 supplemented with 10% FBS, 1% penicillin streptomycin, 5 ng/mL human epidermal growth factor, 1 ng/mL human basic fibroblast growth factor, and 0.25 ng/mL human transforming growth factor- $\beta$ . All cells were cultured at 37 °C with 5% CO<sub>2</sub>.

**Viral Production and Transduction.** All lentiviral vectors used in this study are second generation. A lentivirus production protocol was adapted from methods previously described.<sup>81</sup> Briefly, 3.5 million HEK293T cells were plated per 10 cm dish. The following day, cells were transfected with 20  $\mu$ g of transfer vector, 6  $\mu$ g of pMD2G, and 10  $\mu$ g psPAX2 using a calcium phosphate transfection. The media was changed 12–14 h post-transfection. The viral supernatant was collected 24

and 48 h after this media change, pooled, and passed through a 0.45  $\mu$ m filter. For transduction, the cell medium was replaced with viral supernatant supplemented with 4  $\mu$ g/mL Polybrene. The viral supernatant was changed 24–48 h later.

**MyoD-Directed Genetic Reprogramming.** The transcriptional activation domains VP16, VP64, and p65 were genetically fused to the N-terminus of either the mouse or human wild-type (WT) MyoD via a short flexible serine glycine linker. The VP16 sequence was isolated from Addgene plasmid 11351. VP64 is a fusion of four copies of the minimal VP16 domain. p65 was isolated from Addgene plasmid 21966. Amino acid sequences of all the activation domains are in Figure 1. The MyoD variants were subcloned into a Tet-ON lentiviral vector.<sup>61,62</sup> In this vector, each MyoD variant is coexpressed with dsRed-Express2, via a T2A ribosomal skipping peptide.<sup>82</sup> All the Tet inducible lentiviral vectors are available on Addgene (Addgene plasmids 60623–60629).



Human dermal fibroblasts and human ADSCs were transduced with the Tet-ON LV carrying each MyoD variant. Cells were selected in 1  $\mu\text{g}/\text{mL}$  puromycin to obtain a pure population of transduced cells. Cells were expanded in standard growth medium supplemented with puromycin. Selected cells were grown to confluence and MyoD transgene expression was induced by supplementing the medium with 3  $\mu\text{g}/\text{mL}$ , doxycycline unless indicated otherwise. Cells were given fresh media supplemented with doxycycline every 2 days. All differentiation studies for the fibroblasts were conducted in standard growth medium (DMEM supplemented with 10% FBS and 1% penicillin streptomycin). For the ADSCs, cells were seeded in plates coated with 1:10 poly-L-lysine and all differentiation studies were conducted in standard growth media without the growth factors (DMEM/F12 supplemented with 10% FBS and 1% penicillin streptomycin). Control ADSC samples were grown in standard growth media because withdrawing the growth factors without inducing differentiation causes cell death.

**Quantitative Reverse Transcription PCR.** RNA was isolated using the RNeasy Plus RNA isolation kit (Qiagen). cDNA synthesis was performed using the SuperScript VIL0 cDNA Synthesis Kit (Invitrogen). Real-time PCR using SsoFast EvaGreen Supermix (Bio-Rad) was performed with the CFX96 Real-Time PCR Detection System (Bio-Rad). Oligonucleotide primers and PCR conditions are reported in Supporting Information Table 4. Primer specificity was confirmed by agarose gel electrophoresis and melting curve analysis. Reaction efficiencies over the appropriate dynamic range were calculated to ensure linearity of the standard curve (Supporting Information Figure 6). The results are expressed as fold-increase mRNA expression normalized to  $\beta$ -actin expression using the  $\Delta\Delta\text{Ct}$  method. Reported values are the mean and SEM from two or three independent experiments performed on different days ( $n = 2$  or  $3$ ) where technical replicates were averaged within each experiment.

**Western Blot.** Cells were lysed in RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma). Protein concentration was measured using BCA protein assay reagent (Thermo Scientific) and BioTek Synergy 2 Multi-Mode Microplate Reader. Lysates were mixed with loading buffer and boiled for 5 min; equal amounts of protein were run in NuPage 10% Bis-Tris Gel polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose membranes. Nonspecific antibody binding was blocked with TBST (50 mM Tris, 150 mM NaCl and 0.1% Tween-20) with 5% nonfat milk for 1 h at room temperature. The membranes were incubated with the following primary antibodies: anti-MyoD (1:250 dilution, Santa Cruz, Sc-32758) in 5% BSA in TBST overnight at 4  $^{\circ}\text{C}$ ; anti-Myogenin (1:250 dilution, Santa Cruz, Sc-12732) in 5% BSA in TBST, overnight at 4  $^{\circ}\text{C}$ ; anti-GAPDH (1:5,000 dilution, Cell Signaling, clone 14C10) in 5% milk in TBST 30 min at room temperature. The membranes were washed with TBST for 15 min and incubated for 30 min with anti-rabbit HRP-conjugated antibody (1:5000 dilution, Sigma, A6154) or anti-mouse HRP-conjugated antibody (1:5000 dilution, Santa Cruz, SC-2005) in 5% milk in TBST and subsequently washed with TBST for 15 min. Membranes were visualized using the ImmunoStar WesternC Chemiluminescence Kit (Bio-Rad) and images were captured using a ChemiDoc XRS+ System and processed using ImageLab software (Bio-Rad).

**Immunofluorescence Staining.** Cells transduced with Tet-On LV expressing each MyoD variant were plated on

autoclaved glass coverslips (1 mm, Thermo Scientific). Fibroblasts were plated directly on coverslips while the ADSCs were seeded on coverslips coated with 1:10 poly-L-lysine. Following 0, 10, 20, or 30 days of transgene expression, cells were fixed in 4% PFA and prepared for immunofluorescence staining. Samples were permeabilized in blocking buffer (PBS supplemented with 5% BSA, 0.2% Triton X-100, and 2% goat serum) for 1 h at room temperature. Samples were incubated with MF20 supernatant primary antibody (1:200 dilution, Hybridoma Bank) in blocking buffer overnight at 4  $^{\circ}\text{C}$ , and rinsed for 15 min in PBS. Samples were incubated with anti-mouse fluorescein-conjugated antibody (1:200, Invitrogen A10683) for 1 h at room temperature. Cells were incubated with DAPI diluted 1:5000 in PBS for 5 min and washed with PBS for 15 min. Coverslips were mounted with ProLong Gold Antifade Reagent (Invitrogen) and imaged using a Leica SP5 inverted confocal microscope.

**Quantitative Cell Fusion Assay.** To conduct the fusion assay, either the fibroblasts or ADSCs were transduced with the respective Tet-ON MyoD lentivirus and selected using puromycin. Each pure cell population was subsequently divided and transduced with either LV-Cre (Addgene plasmid 30205)<sup>83</sup> or LV-Floxed Luc (Addgene plasmid 60622). Following transduction, these cell populations were mixed and plated in a ratio of one part LV-Cre transduced cells to ten parts LV-Floxed Luc cells in 24 well plates. Once grown to confluence, the cells were induced to express the MyoD variant with 3  $\mu\text{g}/\text{mL}$  doxycycline. Medium containing fresh doxycycline was replenished every 2 days. Cells were harvested after 10 days of MyoD expression and assayed for luciferase expression. Cells were pelleted and washed with PBS. Pellets were resuspended in 100  $\mu\text{L}$  of lysis buffer (100 mM  $\text{KH}_2\text{PO}_4$  + 0.2% Triton-X, pH 7.8) and incubated at room temperature for 10 min. The cell debris was pelleted and 30  $\mu\text{L}$  of the supernatant from each sample was transferred to an opaque 96-well plate. Each sample was mixed with 30  $\mu\text{L}$  of Bright-Glo reagent (Bright-Glo Luciferase Assay System, Promega). Luminescence was measured by a BioTek Synergy 2 Multi-Mode Microplate Reader with 1-s scan time. Each MyoD variant had a matched set of samples that only received the LV-Floxed Luc vector to serve as control. All luciferase data is presented as a fold increase over background from the matched Luc only samples. Reported values are the mean and SEM from three or four independent experiments performed on different days ( $n = 3$  or  $4$ ) where technical replicates were averaged within each experiment.

**RNA-seq.** RNA-seq libraries were constructed as previously described.<sup>84</sup> Briefly, first-strand cDNA was synthesized from oligo(dT) Dynabead-captured mRNA using SuperScript VIL0 cDNA Synthesis Kit (Invitrogen). Second-strand cDNA was synthesized using DNA polymerase I (New England Biolabs). cDNA was purified using Agencourt AMPure XP beads (Beckman Coulter). Nextera transposase (Illumina; 5 min at 55  $^{\circ}\text{C}$ ) was used to simultaneously fragment and insert sequencing primers into the double-stranded cDNA. Transposition reactions were halted using QG buffer (Qiagen) and fragmented cDNA was purified on AMPure XP beads. Indexed sequencing libraries were generated by six cycles of PCR. Libraries were sequenced using 50-bp paired-end reads on one lane of an Illumina HiSeq 2000 instrument at the Duke Genome Sequencing and Analysis Core Resource. Reads were aligned to human RefSeq transcripts using Bowtie.<sup>85</sup> The significance of differential expression of <sup>WT</sup>MyoD and



<sup>VP64</sup>MyoD treated samples compared to untreated control samples, including correction for multiple hypothesis testing, was calculated using DESeq.<sup>86</sup> Sequencing data has been deposited to the Gene Expression Omnibus, Accession code: GSE62448.

**Statistical Analysis.** At least two independent experiments were compiled as means and standard errors of the mean.

Effects were evaluated with multivariate ANOVA and Dunnett's post hoc test using JMP 10 Pro.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Supporting Figures S1–S6 and Tables S1–S4. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Author Contributions

A.M.K. conducted all experiments. A.M.K., P.I.T., C.M.V., T.M.G., D.G.O., F.G., T.E.R., and C.A.G. contributed to experimental design and data analysis. A.M.K. and C.A.G. wrote the manuscript with editing and approval by all authors.

### Notes

The authors declare no competing financial interest.

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