ScienceAdvances

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Supplementary Materials for

Human myotube formation is determined by MyoD–Myomixer/Myomaker axis

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Table S1. Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell Lines		
10T1/2	ATCC	Cat#CCL-226
Lenti-X 293T	Clontech	Cat#632180
Platinum-A 293	Cell Biolabs	Cat#RV-102
MYMX$^{-/-}$ human myoblasts	This study	N/A
MYMK$^{-/-}$ human myoblasts	This study	N/A
MYMX/K double KO human myoblasts	This study	N/A
MYOD$^{-/-}$ human myoblasts	This study	N/A
MYOG$^{-/-}$ human myoblasts	This study	N/A
Cell culture reagents		
Skeletal Muscle Cell Basal Medium	PromoCell	Cat#C-23260
Dulbecco's Modified Eagle's Medium-high glucose	Sigma	Cat#D5796
GemCell™ U.S. Origin Fetal Bovine Serum	GemCell™ U.S.	Cat#100-500
Horse Serum, heat inactivated	ThermoFisher Scientific	Cat#26050070
Growth Medium Supplement Mix	PromoCell	Cat#C-39365
Penicillin-Streptomycin	Gibco™	Cat#15140122
TrypLE	Gibco™	Cat#12605-028
Trypsin EDTA	Sigma-Aldrich	Cat#T4049
GlutaMAX™	Gibco™	Cat#35050-061
Gentamicin Sulfate	BioWhittaker	Cat#17-518Z
Polybrene	Millipore	Cat#TR-1003-G
Chemicals		
(Z)-4-hydroxytamoxifen	CAYMAN CHEMICAL	Cat#14854
Cycloheximide	CAYMAN CHEMICAL	Cat#14126
FuGENE6	Promega	Cat#E2692
Lenti-X Concentrator	Clontech	Cat#PT4421-2
PFA	Electron Microscopy Sciences	Cat#15710
Triton X-100		
2-Propanol	Fisher Scientific	Cat#A416-4
Methanol	Fisher Scientific	Cat#A412-4
Ethanol	Decon Laboratories	Cat#2716
Oligonucleotides for detection of human genes		
Primers for MYMK genotyping-F: CTTCCTTCCCAGCCATCCAG	This study	N/A
Primers for MYMK genotyping-R: GGGCTAGTGAGCAGGGACTA	This study	N/A
Primers for MYMX genotyping-F: AACTGAAGGGAGGGGGAACT	This study	N/A
Primers for MYMX genotyping-R: TGGAGGACAGAGGGGCAATA	This study	N/A
Primers for MYOD genotyping-F: TTTGCTATCTACAGCCGGGG	This study	N/A
Primers for MYOD genotyping-R: GATATAGCGGATGGCGTTGC	This study	N/A
Primers for MYOG genotyping-F:	This study	N/A

GCGGGAGAAAGAAGGGGAAT		
Primers for MYOG genotyping-R: CTATGTTCCCCACCCCAACC	This study	N/A
Primers for MYMK qPCR-F: TGTGCGGATCTACCATGACC	This study	N/A
Primers for MYMK qPCR-R: GACGCTCTTGTCTGGGTACAG	This study	N/A
Primers for MYMX qPCR-F: CTGATTCTGAGCAGCAGTTCT	This study	N/A
Primers for MYMX qPCR-R: AATGAACAGCAGACAGCCCA	This study	N/A
Primers for MYOD1 qPCR-F: CGACGGCATGATGGACTACA	This study	N/A
Primers for MYOD1 qPCR-R: TATATCGGGTTGGGGTTCGC	This study	N/A
Primers for MYOG qPCR-F1: GGGGAAAACTACCTGCCTGTC	This study	N/A
Primers for MYOG qPCR-R1: AGGCGCTCGATGTACTGGAT	This study	N/A
Primers for MYOG qPCR-F2: GCCAACCCAGGGGATCAT	This study	N/A
Primers for MYOG qPCR-R2: CCCGGCTTGGAAGACAATCT	This study	N/A
Primers for MYF5 qPCR-F: CGCCTGAAGAAGGTCAACCA	This study	N/A
Primers for MYF5 qPCR-R: ACATTCGGGCATGCCATCAG	This study	N/A
Primers for MYF6 qPCR-F: CTTCAGCTACAGACCCAAACA	This study	N/A
Primers for MYF6 qPCR-R: CCCTGGAATGATCGGAAACA	This study	N/A
Primers for MEF2C qPCR-F: GCAACAGCAACACCTACATAAC	This study	N/A
Primers for MEF2C qPCR-R: GTAGAAGGCAGGGAGAGATTTG	This study	N/A
Primers for MYH1 qPCR-F: CCCTACAAGTGGTTGCCAGTG	This study	N/A
Primers for MYH1 qPCR-R: CTTCCCTGCGCCAGATTCTC	This study	N/A
Primers for MYH3 qPCR-F: ATTGCTTCGTGGTGGACTCAA	This study	N/A
Primers for MYH3 qPCR-R: GGCCATGTCTTCGATCCTGTC	This study	N/A
Primers for MYH8 qPCR-F: CCAAAACAAGCCGTTTGATGC	This study	N/A
Primers for MYH8 qPCR-R: AGCACTCCAGGCTCGTGTA	This study	N/A
Primers for 18S qPCR-F: GTAACCCGTTGAACCCCATT	This study	N/A
Primers for 18S qPCR-R:	This study	N/A

CCATCCAATCGGTAGTAGCG		
Oligonucleotides for detection of mouse genes		
Primers for MymX qPCR-F: CTGAGCTCCCAAGACATGAG	This study	N/A
Primers for MymX qPCR-R: TGGAGGCCTCTCCAGAAT	This study	N/A
Primers for MymK qPCR-F: GCCTTTACCACCTTCTCCCC	This study	N/A
Primers for MymK qPCR-R: GCACAGCACAGACAAACCAG	This study	N/A
Primers for MyoD1 qPCR-F: CCACTCCGGGACATAGACTTG	This study	N/A
Primers for MyoD1 qPCR-R: AAAAGCGCAGGTCTGGTGAG	This study	N/A
Primers for 18s qPCR-F: ACCGCAGCTAGGAATAATGGA	This study	N/A
Primers for 18s qPCR-R: GCCTCAGTTCCGAAAACCA	This study	N/A
Oligonucleotides for human cDNA cloning		
Primers for MYMK-C Amplification-F: CGCGGATCCGCCACCATGGGCACTC	This study	N/A
Primers for MYMK-C Amplification-R: GCTCGAGTCATGCTTCTGGTTCCACG CACGCGCAGCAAAG	This study	N/A
Primers for MYF5 ORF Amplification-F: ATGGACGTGATGGATGGC	This study	N/A
Primers for MYF5 ORF Amplification-R: TCATAGCACATGATAGATAAGCCTGG	This study	N/A
Primers for MYF6 ORF Amplification-F: ATGATGATGGACCTTTTTGAAACT	This study	N/A
Primers for MYF6 ORF Amplification-R: TTACTTCTCCACCACTTCCTC	This study	N/A
Primers for MEF2C ORF Amplification-F: ATGGGGAGAAAAAAGATTCAGATTAC	This study	N/A
Primers for MEF2C ORF Amplification-R: TCATGTTGCCCATCCTTCAG	This study	N/A
Western Blot Reagents		
RIPA buffer	Sigma	Cat#R0278
4x Laemmli sample buffer	BIO-RAD	Cat#161-0747
PVDF membrane	Millipore	Cat#ISEQ00010
Protease inhibitor	Roche	Cat#04693159001
SuperSignal West Dura Substrate	ThermoFisher Scientific	Cat#34075
Membrane fractionation kit	ThermoFisher Scientific	Cat#89842
cDNA Preparation Reagents		
TRIzol	Invitrogen	Cat#15-596-018
Chloroform	Alfa Aesar	Cat#J67241
Superscripts™ III First-Strand Synthesis System	Invitrogen	Cat#18080051
Immunostaining Reagents		

Bovine Serum Albumin	GEMINI	Cat#700-107P
PBS	Sigma	Cat#P5368-10PAK
Antibodies		
GAPDH	Santa Cruz Biotechnology	Cat#sc-32233
α-Tubulin	Santa Cruz Biotechnology	Cat#sc-8035
Insulin Receptor β	Cell Signaling Technology	Cat#3020S
Myomixer	ThermoFisher Scientific	Cat#PA5-47639
Myomaker	This study	
Myosin	DSHB	Cat#MF20
MyoD	Santa Cruz Biotechnology	Cat# SC-304
MyoG	DSHB	Cat#F5D
Biotin Anti-C-tag Conjugate	ThermoFisher Scientific	Cat#7103252100
HRP Streptavidin	Vector Laboratories	Cat#SA-5004
Donkey anti-sheep IgG-HRP Conjugate	Santa Cruz Biotechnology	Cat#SC-2473
Goat Anti-Mouse IgG (H+L)-HRP Conjugate	Invitrogen	Cat#A28177
Goat Anti-Rabbit IgG (H+L)-HRP Conjugate	Invitrogen	Cat# A27036
Goat anti-Mouse IgG (H+L), Superclonal™ Recombinant Secondary Antibody, Alexa Fluor 555	Invitrogen	Cat#A28180
Goat anti-Mouse IgG (H+L), Superclonal™ Recombinant Secondary Antibody, Alexa Fluor 488	Invitrogen	Cat#A28175
Goat anti-Rabbit IgG (H+L), Superclonal™ Recombinant Secondary Antibody, Alexa Fluor 555	Invitrogen	Cat#A27039
Goat anti-Rabbit IgG (H+L), Superclonal™ Recombinant Secondary Antibody, Alexa Fluor 488	Invitrogen	Cat#A27034
Plasmids		
pLenti-V2	Addgene	Cat#52961
psPAX2	Addgene	Cat#12260
pMD2.G	Addgene	Cat#12259
pLentiSAM v2	Addgene	Cat#92062
pLentiMPH v2	Addgene	Cat #89308
pMXs-Puro Retroviral Vector	Cell Biolabs	Cat#RTV-012
pLOVE-GFP	Addgene	Cat#15949
MyoD-pCLBabe	Addgene	Cat#20917
pLv-CMV-MyoD-ER(T)	Addgene	Cat#26809
Software and Algorithms		
ImageJ v1.52a	NIH	RRID:SCR_003070
Microsoft Excel	Microsoft	RRID:SCR_016137
GraphPad Prism 8.3.0	Graphpad	RRID:SCR_002798
Adobe Photoshop (CS6)	Adobe	RRID:SCR_014199

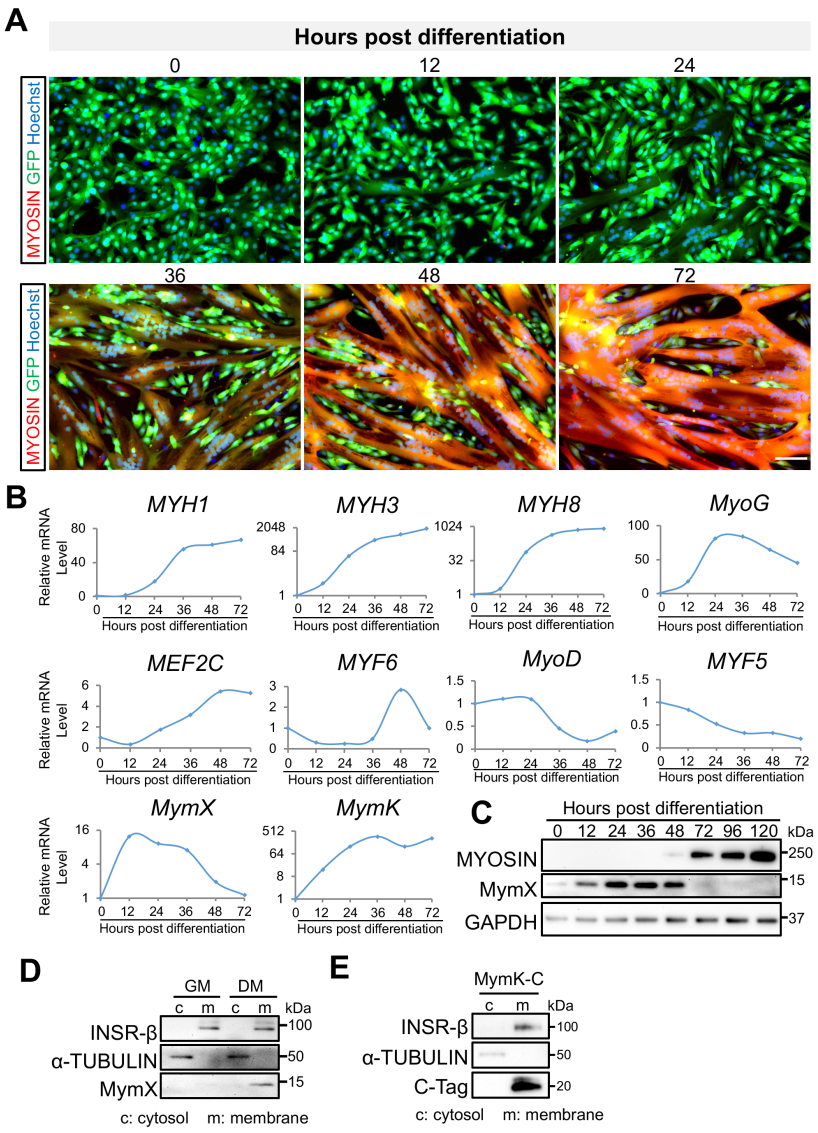

fig. S1 (relating to Figure 1). Characterizations of human myoblast differentiation.
(**A**) Characterizations of the fusion and differentiation potentials of human myoblasts. Cells were labelled by GFP to visualize the syncytium at early stages of differentiation. Scale bar, 100 μm. (**B**) qPCR results that measured expression for a panel of muscle-specific genes. (**C**) Protein levels of myosin heavy chain (MF20) and Myomixer in human myoblasts at various stages of differentiation. (**D** and **E**) Western blots of proteins from cytosolic (c) and total membrane (m) fractions of human myoblasts. GM: growth medium; DM: differentiation medium for 36 hours. INSULIN RECEPTOR-β (INSR-β) was used as a positive control of membrane protein isolation. α-TUBULIN was used as a positive control of cytosolic protein isolation. For panel (E), due to the lack of human MymK antibody, human myoblasts were transfected with a C-terminus tagged version of human MymK: MymK-C, and recognized by a C-tag antibody.

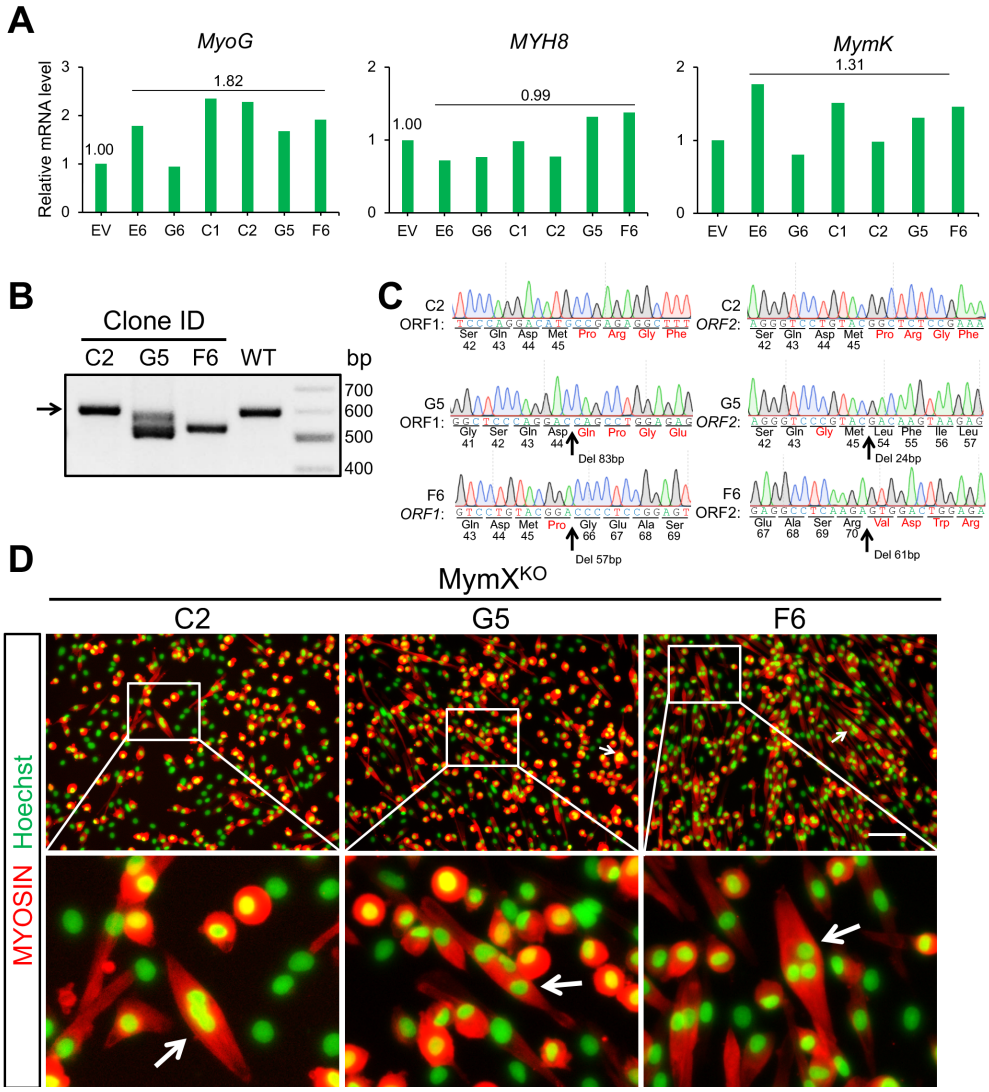

fig. S2 (relating to Figure 1). Analyses of another three human MymX^{KO} myoblast clones. (**A**) Relative mRNA levels of *MyoG, MYH8* and *MymK* in WT or a group of MymX^{KO} human myoblast clones. Average fold changes of gene expression were labelled. (**B**) MymX genotyping results for human MymX^{KO} myoblast clones. Arrow points to the position of WT-size amplicon. (**C**) Sanger sequencing results of MymX genotyping PCR products as shown in (B). The frame-shifted codons were highlighted in red. Arrow indicates the position of big deletion. (**D**) Myosin immunostaining results of human MymX^{KO} myoblasts. Cells were differentiated for three days. Arrows point to the multinucleated myosin+ myotubes. Scale bar, 100 μm.

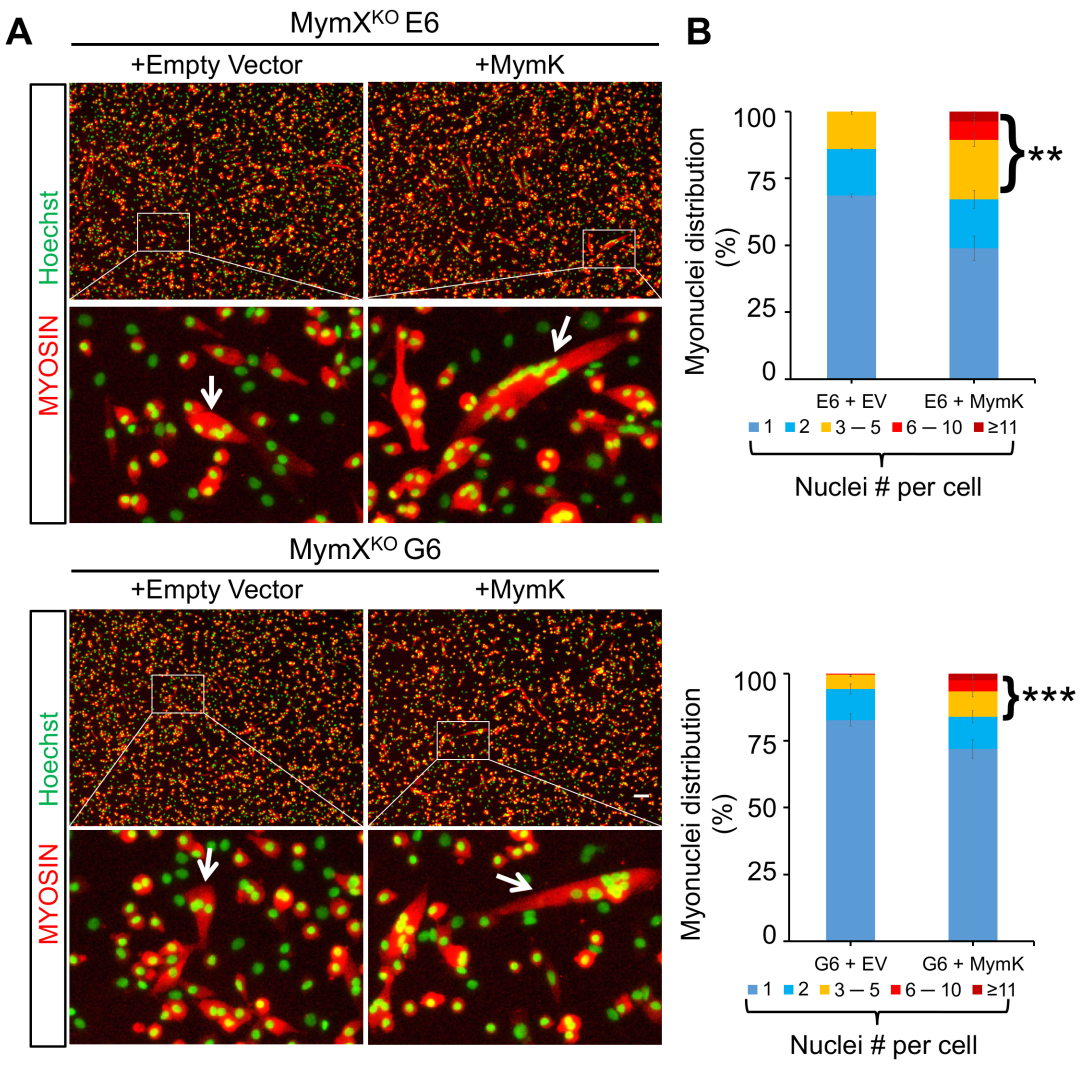

fig. S3 (relating to Figure 2). Overexpression of MymK promotes fusion of MymX^{KO} myoblasts.

(**A**) Myosin immunostaining results of human MymX^{KO} myoblasts to show retroviral expression of human Myomaker (MymK) can increase fusion and myotube size of MymX^{KO} myoblasts. Cells were differentiated for three days. Arrows point to the multinucleated myosin+ myotubes. Scale bar, 100 μm. (**B**) Quantification results for experiments as shown in (A). $^{**}P < 0.01$, $^{***}P < 0.001$. Data are mean ± SEM.

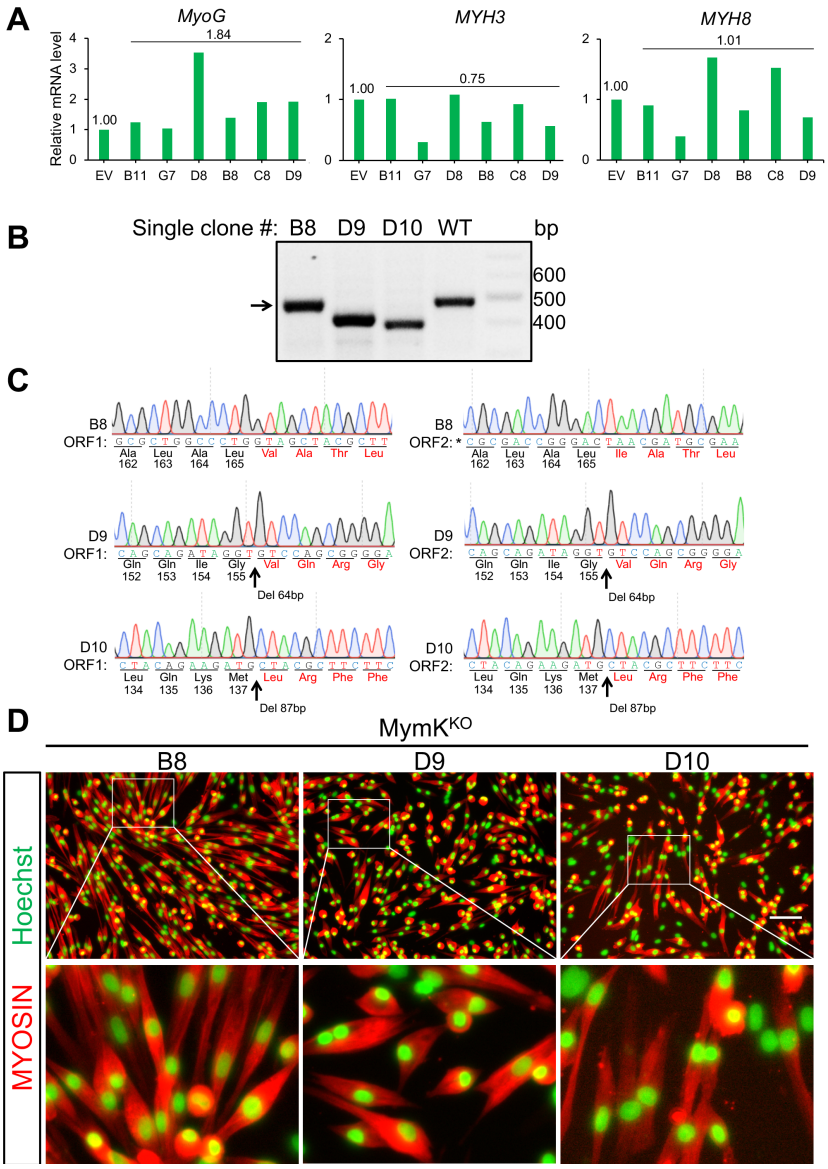

fig. S4 (relating to Figure 2). Analyses of another three clones of human MymK^{KO} myoblasts.

(**A**) Relative mRNA levels of *MyoG*, *MYH3* and *MYH8* in WT or human MymK^{KO} myoblast clones. Average fold changes of gene expression were labelled. (**B**) MymK genotyping results. Arrow points to the position of WT-size amplicon. (**C**) Sanger sequencing results of MymK genotyping PCR products as shown in (B). The frame-shifted codons were highlighted in red. Arrow indicates the position of big truncation. (**D**) Myosin immunostaining results to show the absence of fusion in MymK^{KO} myoblast clones. Cells are differentiated for three days. Nuclei were counterstained with Hoechst and pseudo colored in green. Scale bar, 100 μm.

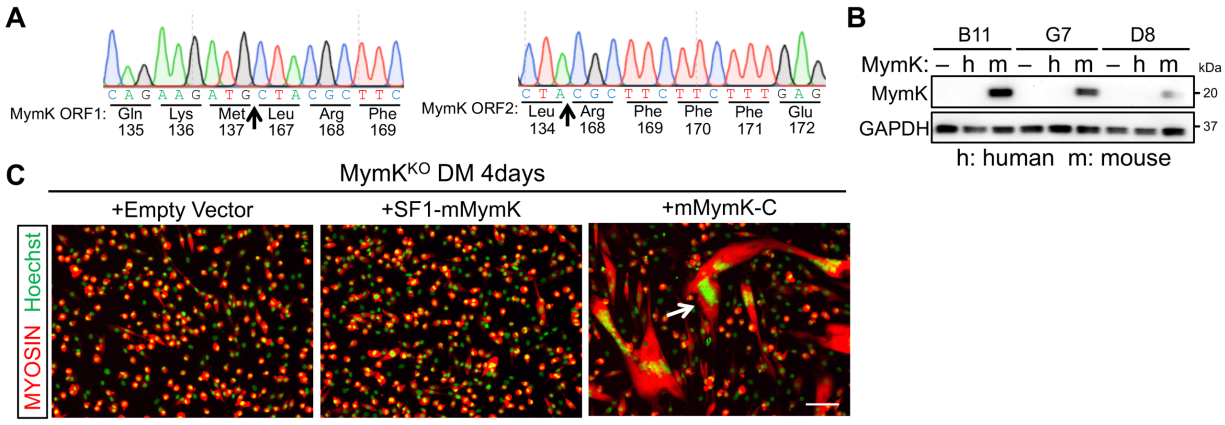

fig. S5 (relating to Figure 2).
(**A**) Generation of human MymX and MymK double KO (dKO) myoblasts by CRISPR deletions of MymK gene in MymX^{KO} myoblasts (clone # G6). Arrow indicates the position of big deletion. (**B**) Western blot analysis of MymK orthologs that were overexpressed in MymK^{KO} clones. Note that mouse MymK antibody does not recognize human MymK protein. (**C**) Myosin immunostaining results to show that C-tagged MymK (mMymK-C) but not flag-tagged MymK (SF1-mMymK) can rescue fusion of human MymK^{KO} myoblasts. Scale bar, 100 μm.

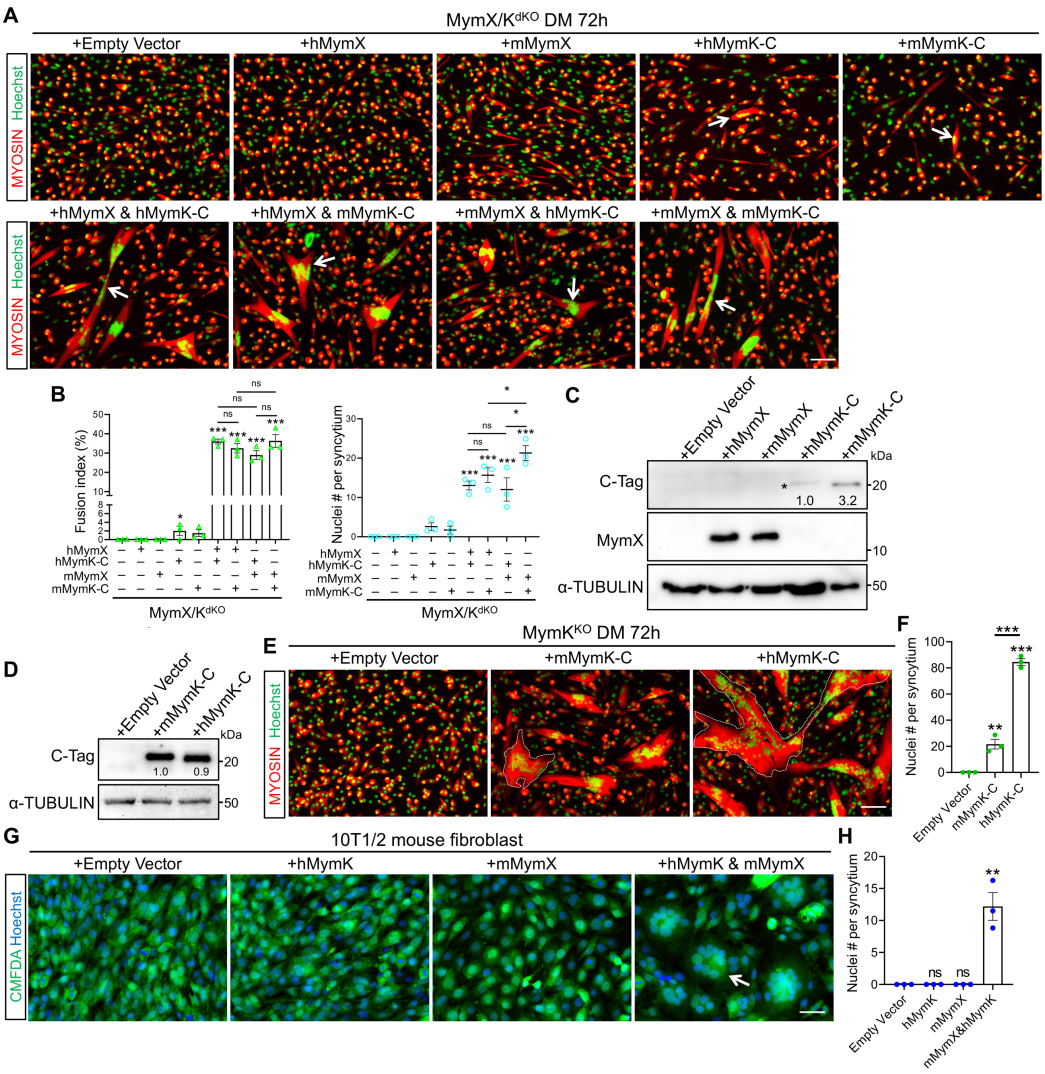

fig. S6 (relating to Figure 2). Comparisons of fusogenic activities for human and mouse MymX/MymK.

(**A**) Myosin immunostaining results to show the rescue of fusion defects of MymX/K dKO myoblasts by MymK-C expression or co-expression of MymX and MymK-C. Arrows point to multinucleated myosin+ cells. Cells were differentiated for three days. Scale bar, 100 μm. (**B**) Quantification results of fusion outcomes in human MymX/K^{dKO} myoblasts in various co-expression combinations of human or mouse MymX or MymK. n = 3. *P < 0.05, ***P < 0.001, ns: not significant. Data are mean ± SEM. (**C**) Representative Western blot analyses to compare protein overexpression levels in MymX/K^{dKO} myoblasts. Star indicates the MymK band of correct size. The relative ratios of target band intensity normalized to that of loading control (α-TUBULIN) were labeled. (**D**) Representative Western blot measurements of human or mouse MymK-C for experiments in (E, F). The relative ratios of target band intensity normalized to that of loading control (α-TUBULIN) were labeled. (**E**) Myosin immunostaining results to show the higher fusogenic activity of human MymK-C compared with mouse MymK-C. Scale bar, 100 μm. (**F**) Quantification results of fusion for experiments as shown in (E). (**G**) Fluorescence images of cell cytosol dye CMFDA to show that human MymK alone cannot induce fibroblast fusion. Scale bar, 50 μm. (**H**) Quantification results of fusion for experiments as shown in (G). n = 3. **P < 0.01, ns: not significant. Data are mean ± SEM.

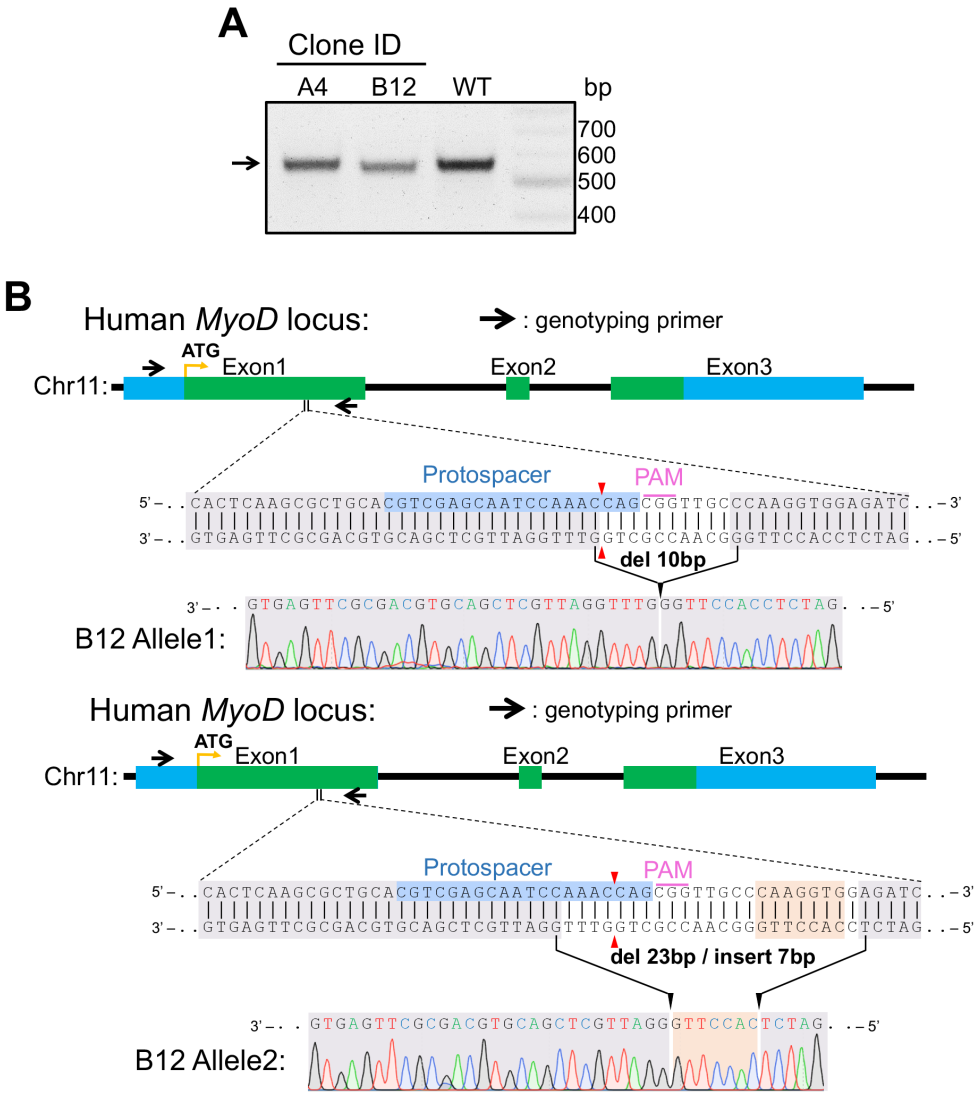

fig. S7 (relating to Figure 4). Genotyping analyses of human MyoD^{KO} myoblast. (**A**) MyoD genotyping results. Arrow points to the position of WT-size amplicon. (**B**) Human MyoD gene structure and Sanger sequencing results that confirmed biallelic deletions of MyoD gene in the second KO clone (B12). Del: deleted.

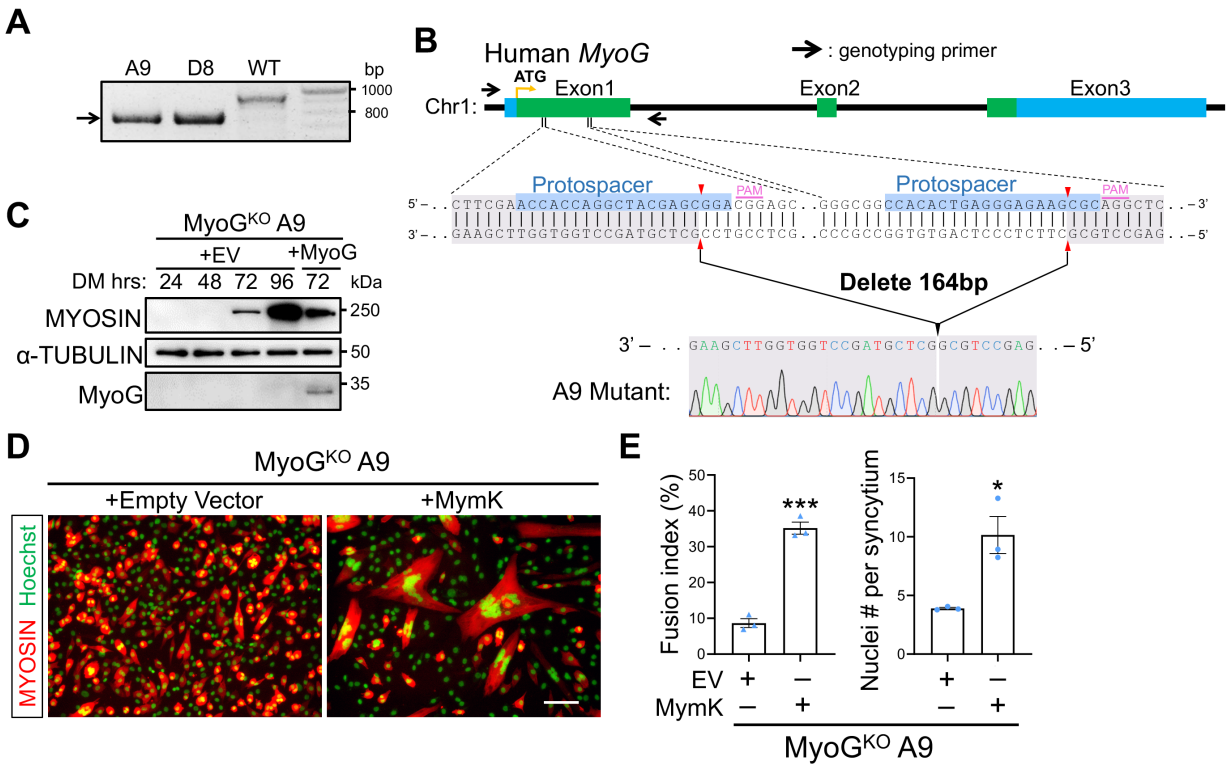

fig. S8 (relating to Figure 5).
(**A**) Genotyping results for two human MyoG^{KO} myoblast clones. Arrow points to the position of WT-size amplicon. (**B**) Human MyoG gene structure and Sanger sequencing results that confirmed biallelic deletions of MyoG in the clone # A9. (**C**) Western blot results of human myoblasts at various time points post differentiation. DM: differentiation medium. (**D**) Myosin immunostaining results to show the rescue of fusion defect of human MyoG^{KO} myoblasts (clone # A9) by retroviral expression of MymK. Cells were differentiated for three days. Scale bar, 100 μm. (**E**) Quantification results of myoblast fusion for the experiments as shown in (D). *n* = 3. **P* < 0.05, ****P* < 0.001. Data are mean ± SEM.

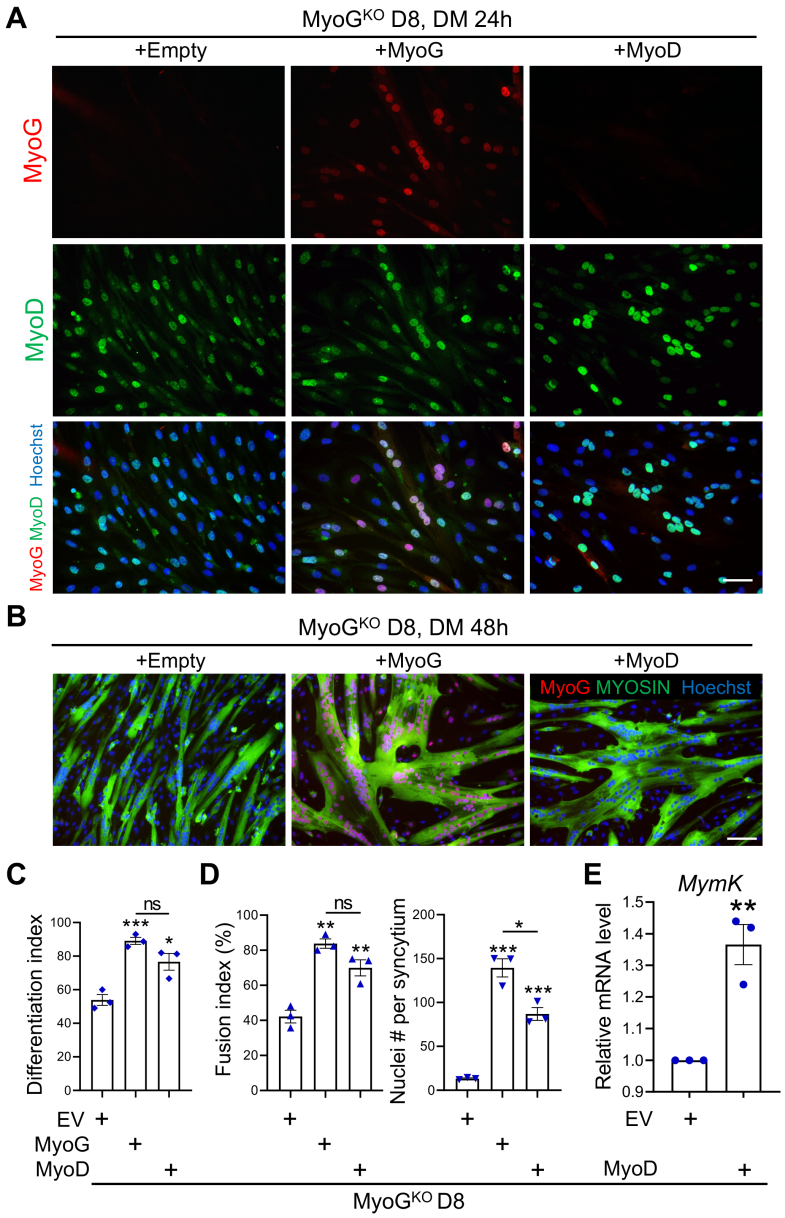

fig. S9 (relating to Figure 5).
(**A**) Immunostaining results of human MyoG^{KO} myoblasts with retroviral expression of MyoG, MyoD or empty control vector. Staining of MyoD confirmed its normal expression in MyoG^{KO} cells. Individual fluorescence channels for MyoD and MyoG staining were shown. Cells were differentiated for one day. Scale bar, 50 μm. (**B**) Immunostaining results of myosin and myogenin to show the rescue of fusion defects of MyoG^{KO} myoblasts by retroviral expression of either MyoG or MyoD. Cells were differentiated for two days. Scale bar, 100 μm. (**C** and **D**) Quantification results of differentiation index (C) and fusion index (D) of another human MyoG^{KO} myoblast clone with retroviral expression of MyoG, MyoD or empty control (EV). (**E**) Relative mRNA level of *MymK* in MyoG^{KO} cells. $n = 3$. ns, no significant difference, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are mean ± SEM.

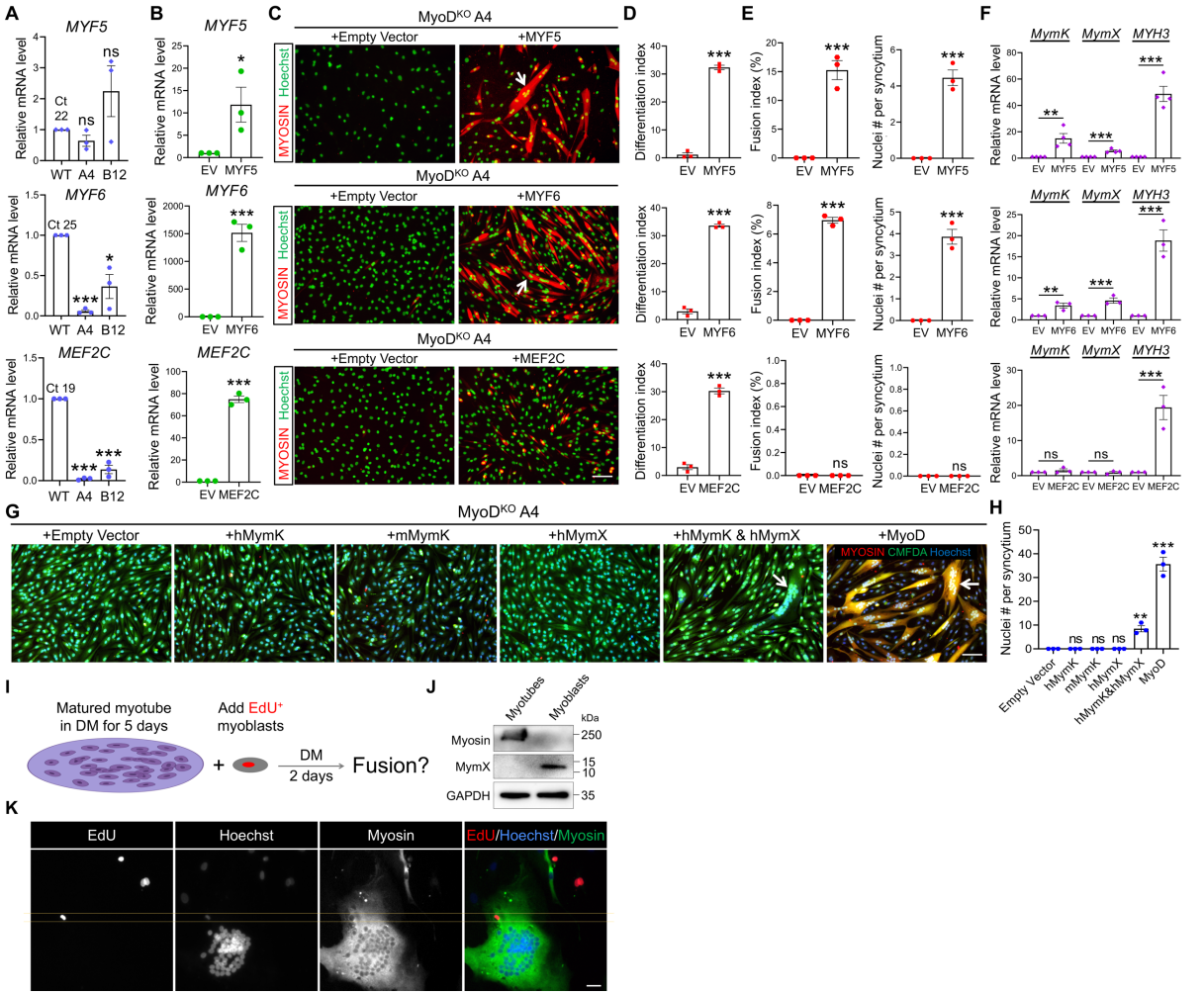

fig. S10 (relating to Figure 5). MYF5 and MYF6 can only weakly rescue the fusion defects of MyoD^{KO} myoblasts.

(**A** and **B**) qPCR results that measured the expression changes of *MYF5*, *MYF6* and *MEF2C* in MyoD^{KO} myoblasts (A). Threshold cycle value (Ct) for each of these genes in WT control group was provided. Overexpression of *MYF5*, *MYF6* and *MEF2C* in MyoD^{KO} clone # A4 was confirmed in (B). (**C**) Myosin immunostaining results. MyoD^{KO} myoblasts were differentiated for three days for MYF5 and five days for MYF6 and MEF2C groups. Note that myosin+ cells can be detected in *MYF5*, *MYF6* and *MEF2C* overexpression conditions. However, small myotubes (pointed by arrows) were only found in MYF5 and MYF6 overexpression groups. Scale bar, 100 μm. (**D** and **E**) Quantification results of differentiation index (D) and fusion index (E) for experiments as shown in (C). (**F**) qPCR results of *MymK*, *MymX* and *MYH3* in human MyoD^{KO} myoblasts. Note that MEF2C overexpression did not induce MymX and MymK expression. (**G**) Immunostaining results of myosin, cytosol dye CMFDA and Hoechst to show that fusion of human MyoD^{KO} myoblasts can be induced by expression of MymK & MymX or MyoD, but not by MymK alone. Cells were differentiated for 48 hours. Scale bar, 100 μm. (**H**) Quantification results of fusion for experiments as shown in (G). Statistic comparisons were all made with empty vector group. ns, no significant difference. n = 3. *P < 0.05, **P < 0.01, ***P < 0.001. Data are mean ± SEM. (**I**) Schematic of experiment design to examine whether matured myotubes can fuse with myoblasts. Myoblasts were EdU pre-labelled and washed three times before mixing with post-differentiation myotubes. DM: differentiation medium. (**J**) Western blotting analyses of Myosin, MymX and Gapdh in human myoblasts (DM 1 day) and myotubes (DM 5 days). Note that MymX is expressed in nascent myocytes but not in matured myotubes. (**K**) Immunofluorescent images of EdU and Myosin (MF20) after cell mixing as illustrated in I. Note the EdU+ nucleus highlighted in-between the yellow gridlines was incorporated by the giant Myosin+ myotube at its peripheral. Scale bar: 50 μm.