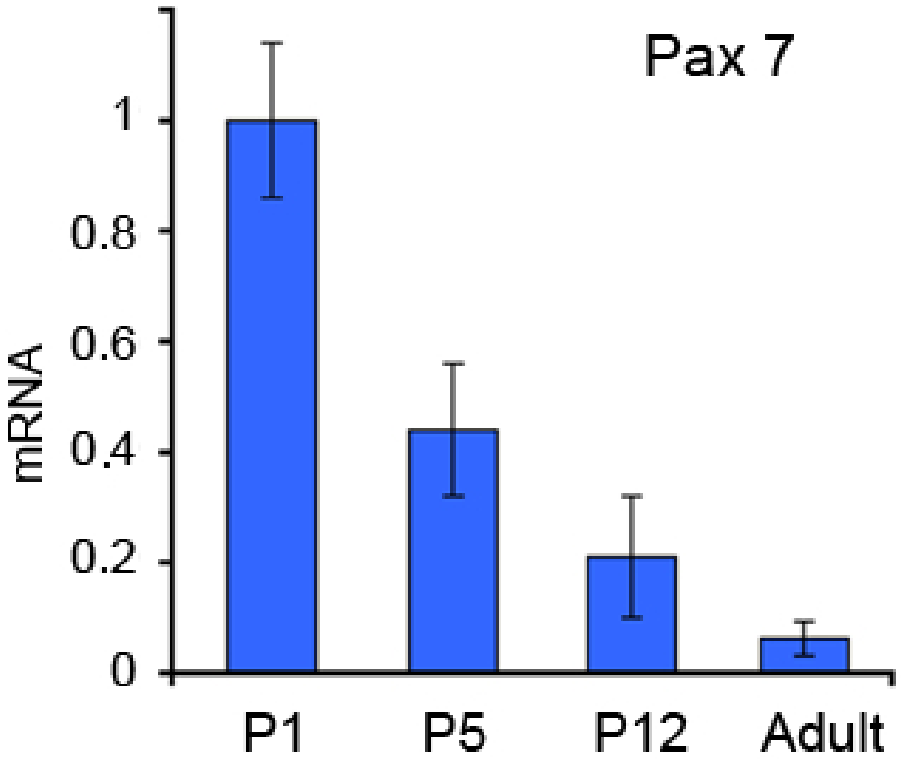
a


Pax 7
mRNA
1
0.8
0.6
0.4
0.2
0
P1
P5
P12
Adult

b

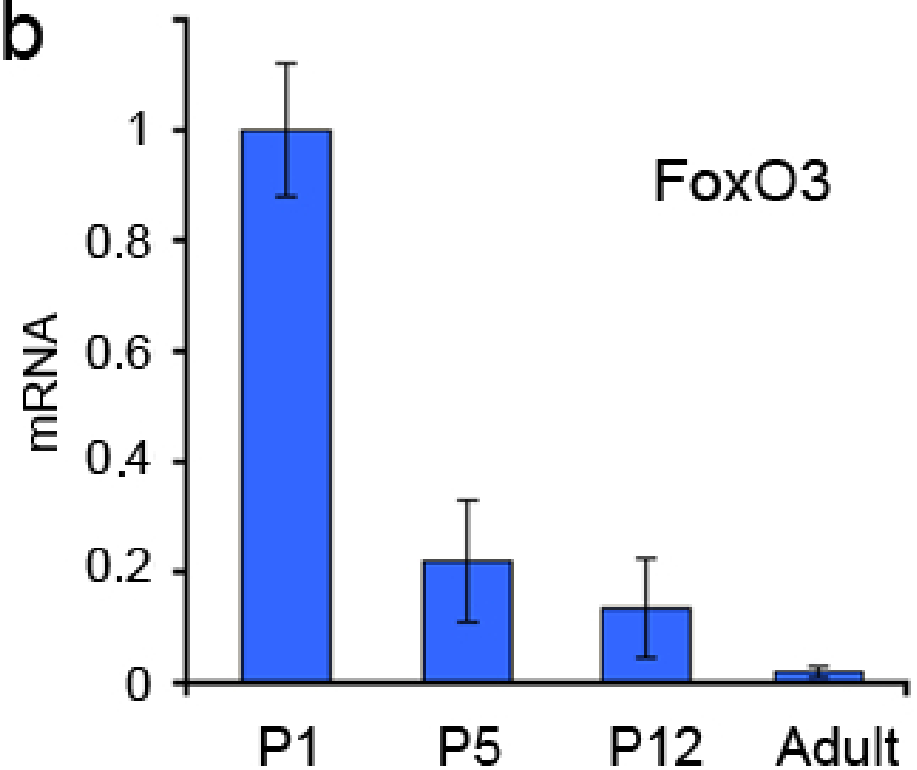

FoxO3
mRNA
1
0.8
0.6
0.4
0.2
0
P1
P5
P12
Adult

Fig. S1

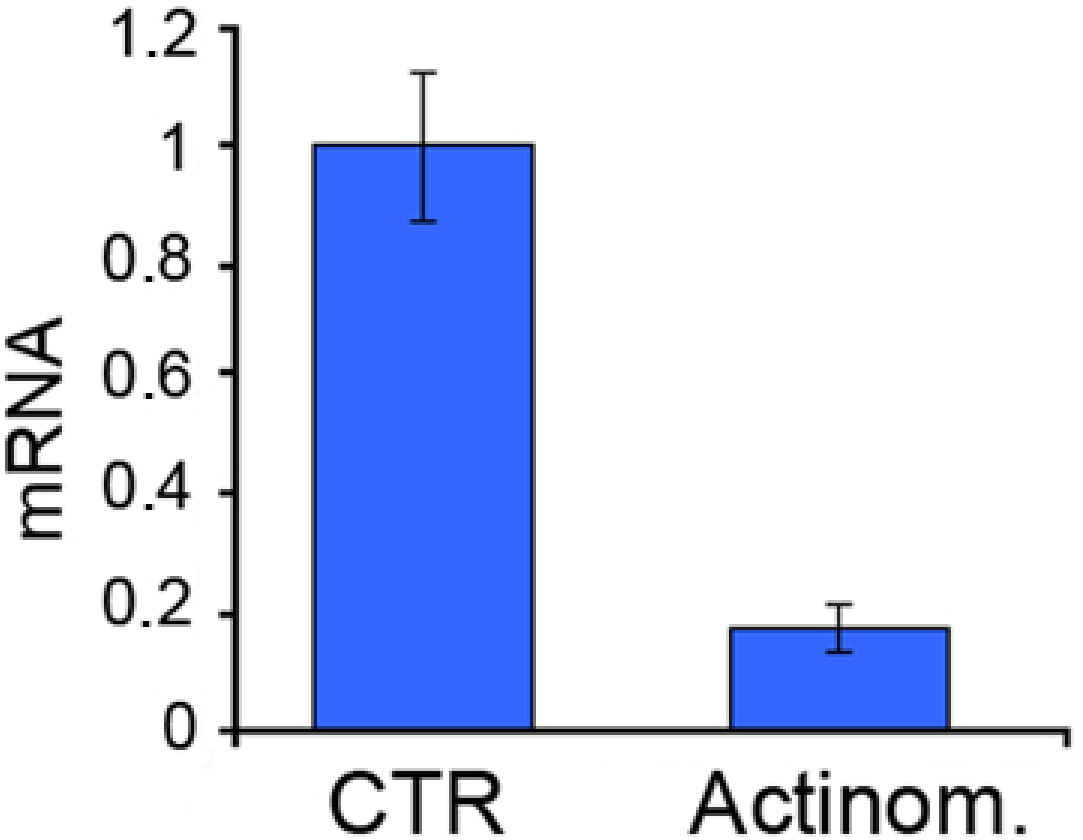
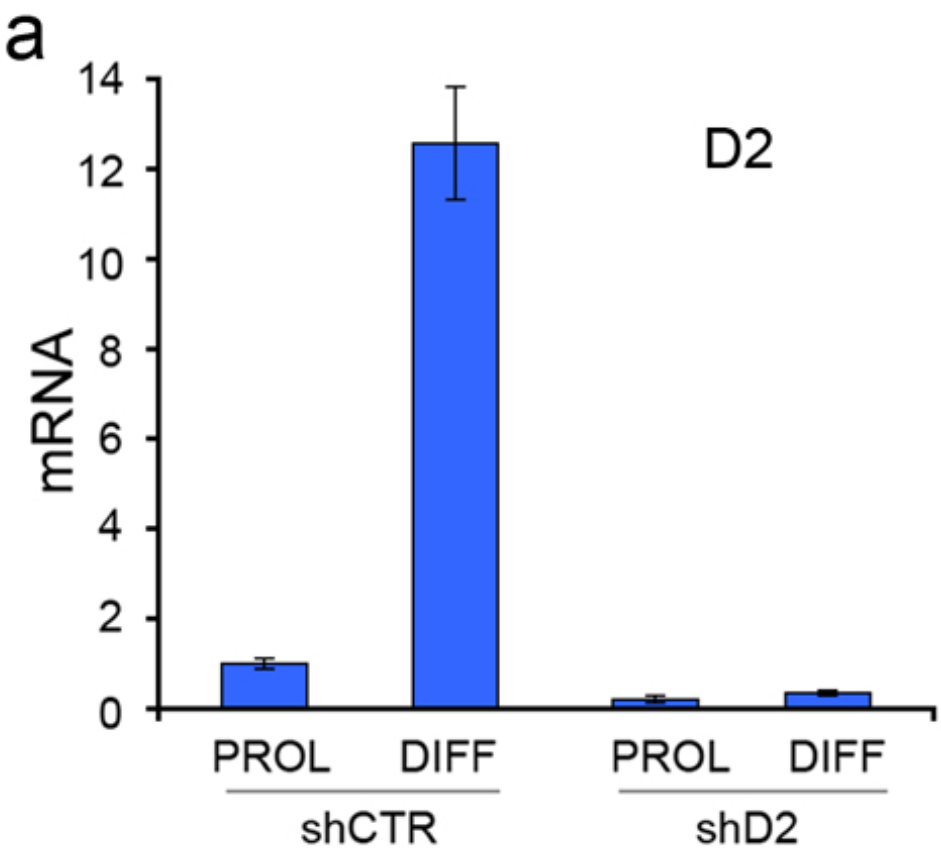
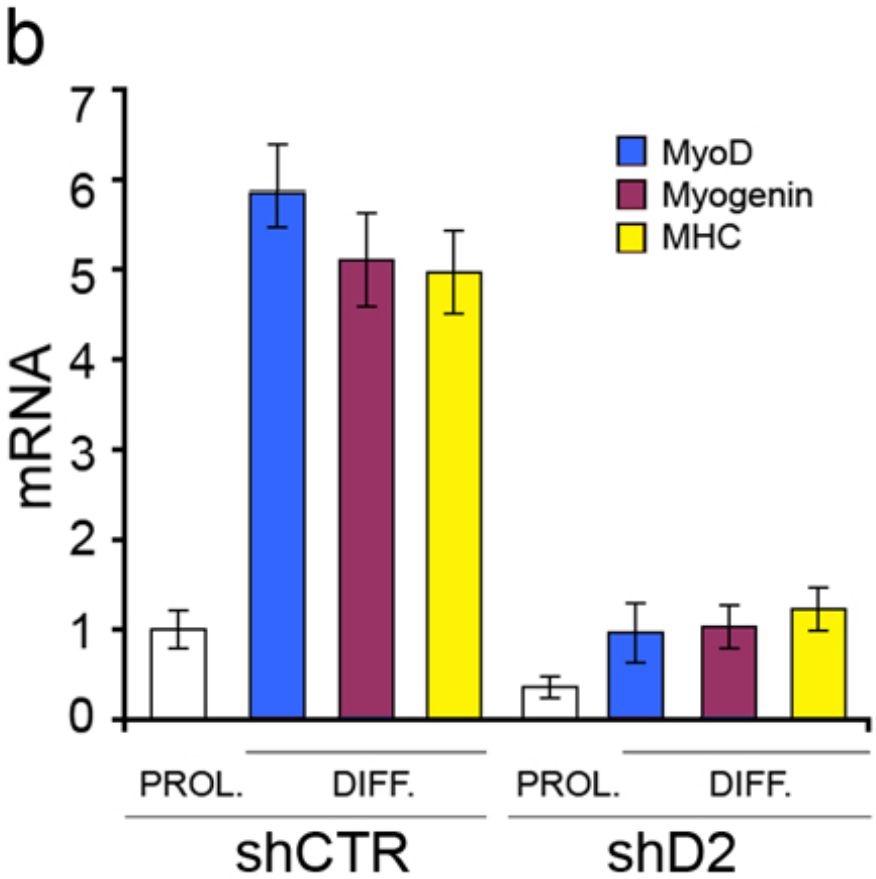


Fig. S2


a
D2
mRNA
14
12
10
8
6
4
2
0
PROL
DIFF
PROL
DIFF
shCTR
shD2


b
mRNA
7
6
5
4
3
2
1
0
MyoD
Myogenin
MHC
PROL.
DIFF.
PROL.
DIFF.
shCTR
shD2

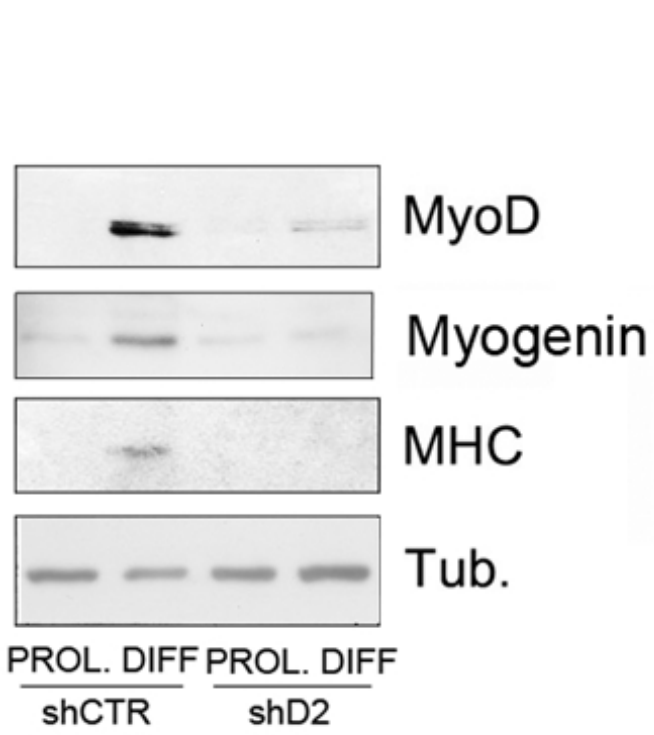

c
MyoD
Myogenin
MHC
Tub.
PROL.
DIFF
PROL.
DIFF
shCTR
shD2

Fig. S3

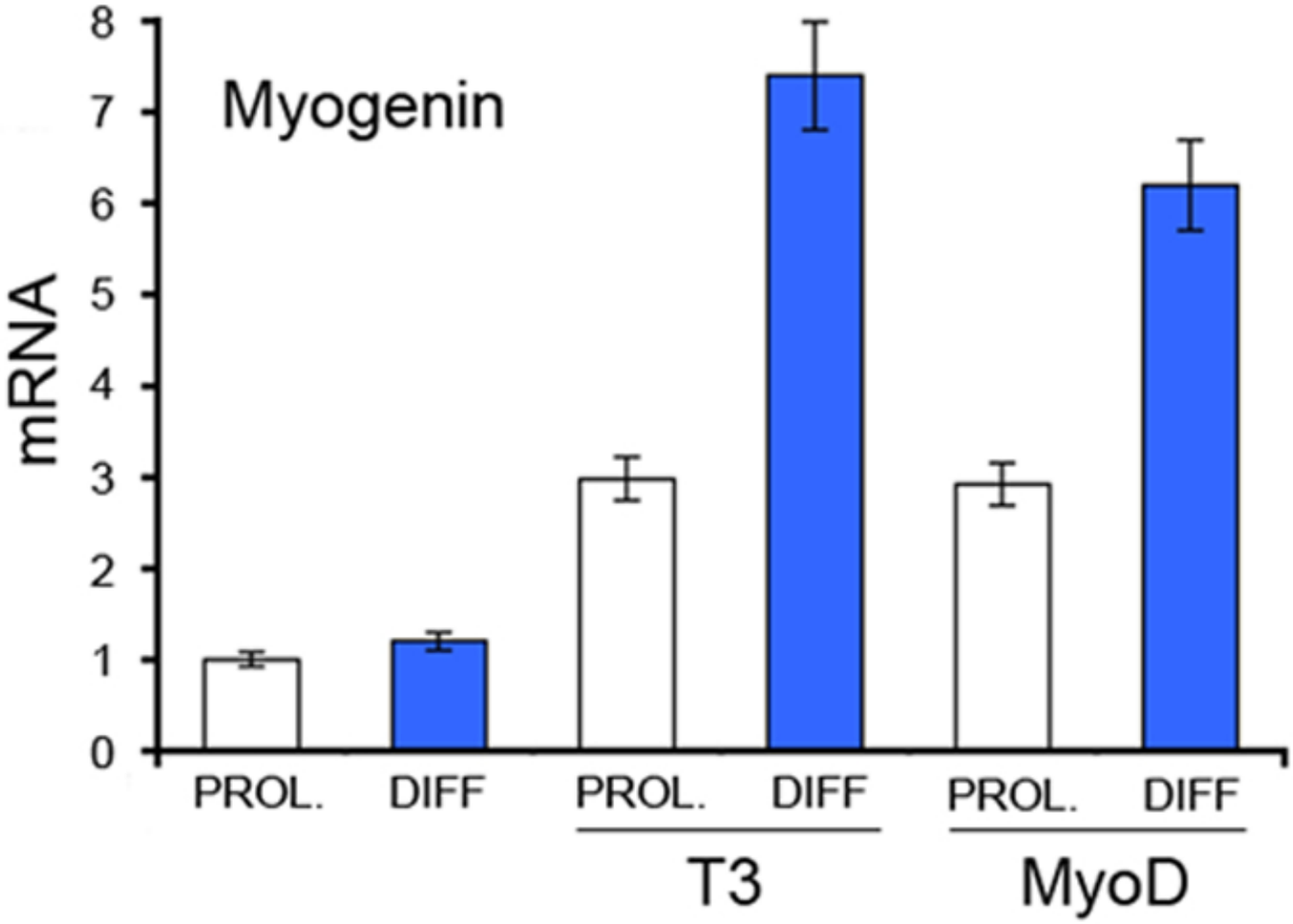
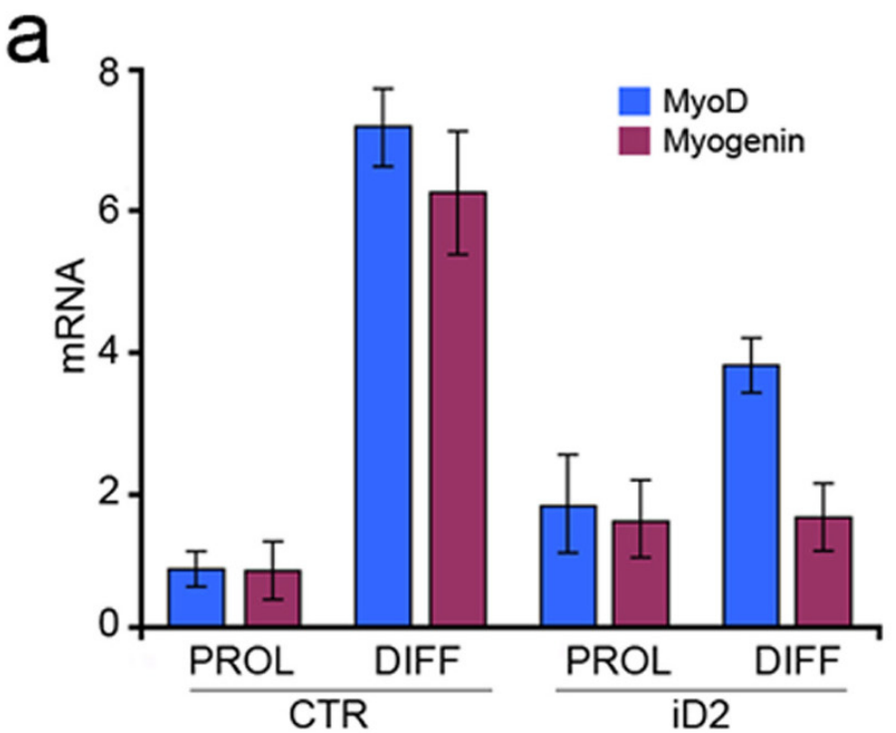
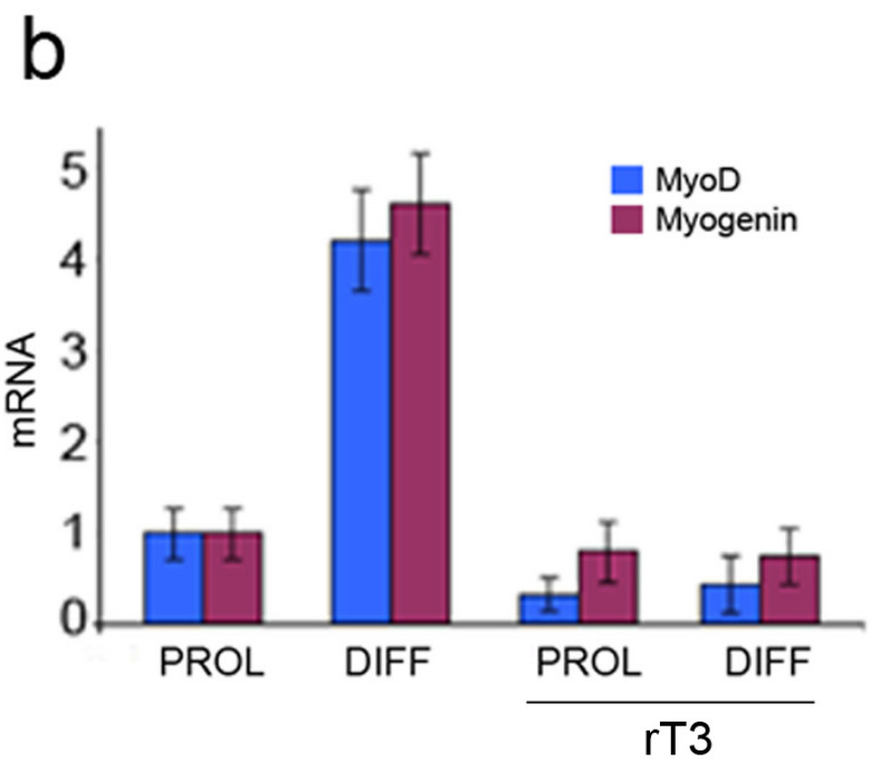

Myogenin
mRNA
8
7
6
5
4
3
2
1
0
PROL.
DIFF
PROL.
DIFF
PROL.
DIFF
T3
MyoD

Fig. S4


a
8
6
4
2
0
mRNA
MyoD
Myogenin
PROL
DIFF
PROL
DIFF
CTR
iD2


b
5
4
3
2
1
0
mRNA
MyoD
Myogenin
PROL
DIFF
PROL
DIFF
rT3

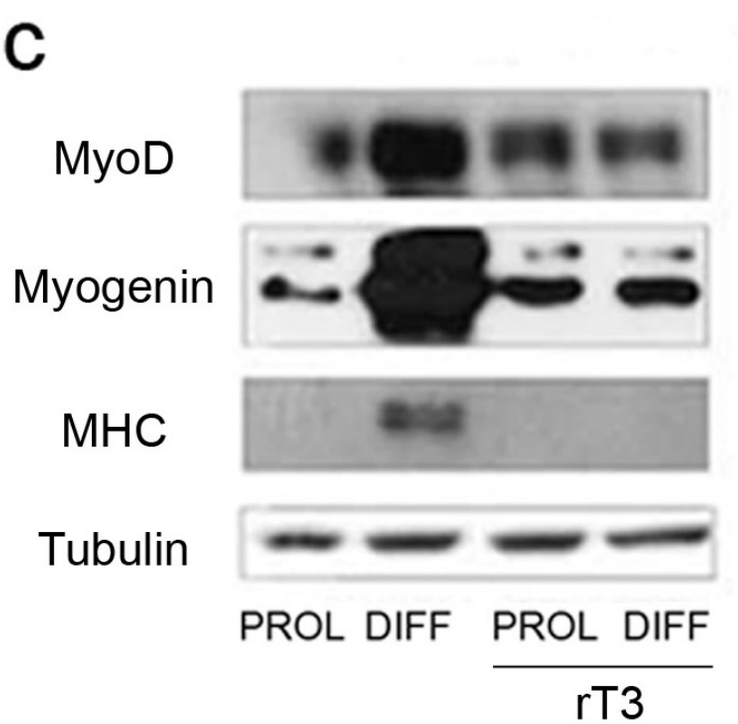
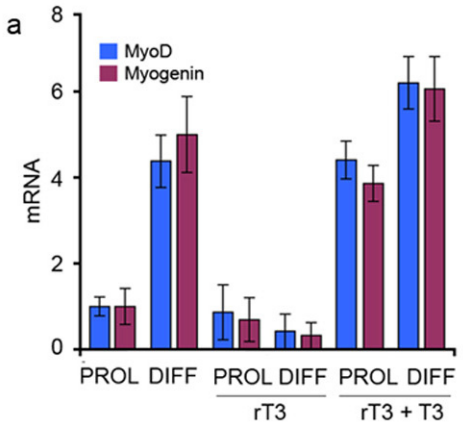

c
MyoD
Myogenin
MHC
Tubulin
PROL
DIFF
PROL
DIFF
rT3

Fig. S5


a
MyoD
Myogenin
mRNA
0
2
4
6
8
PROL DIFF PROL DIFF PROL DIFF
rT3
rT3 + T3

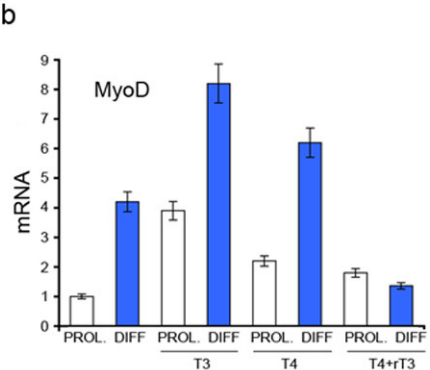
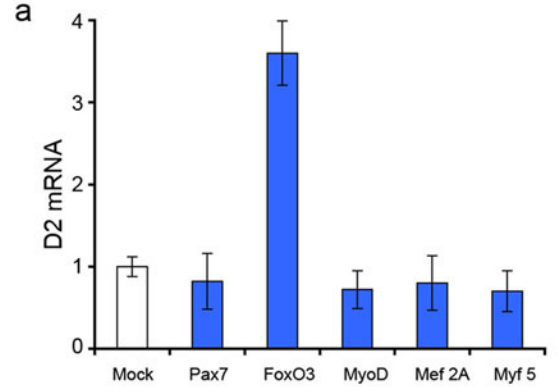
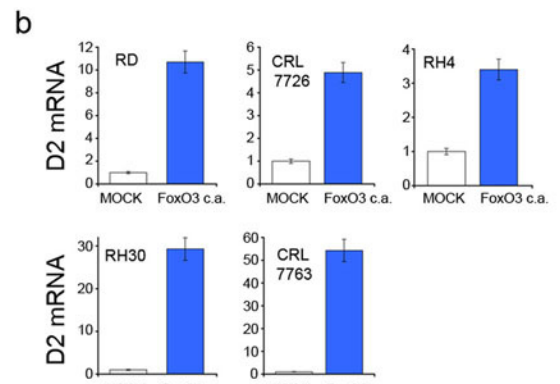

b
MyoD
mRNA
0
1
2
3
4
5
6
7
8
9
PROL. DIFF PROL. DIFF PROL. DIFF PROL. DIFF
T3
T4
T4+rT3

Fig. S6


a
D2 mRNA
0
1
2
3
4
Mock
Pax7
FoxO3
MyoD
Mef 2A
Myf 5


b
D2 mRNA
RD
MOCK
FoxO3 c.a.
CRL 7726
RH4
RH30
CRL 7763

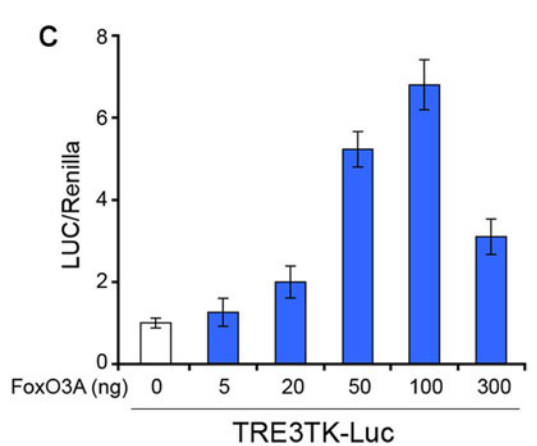
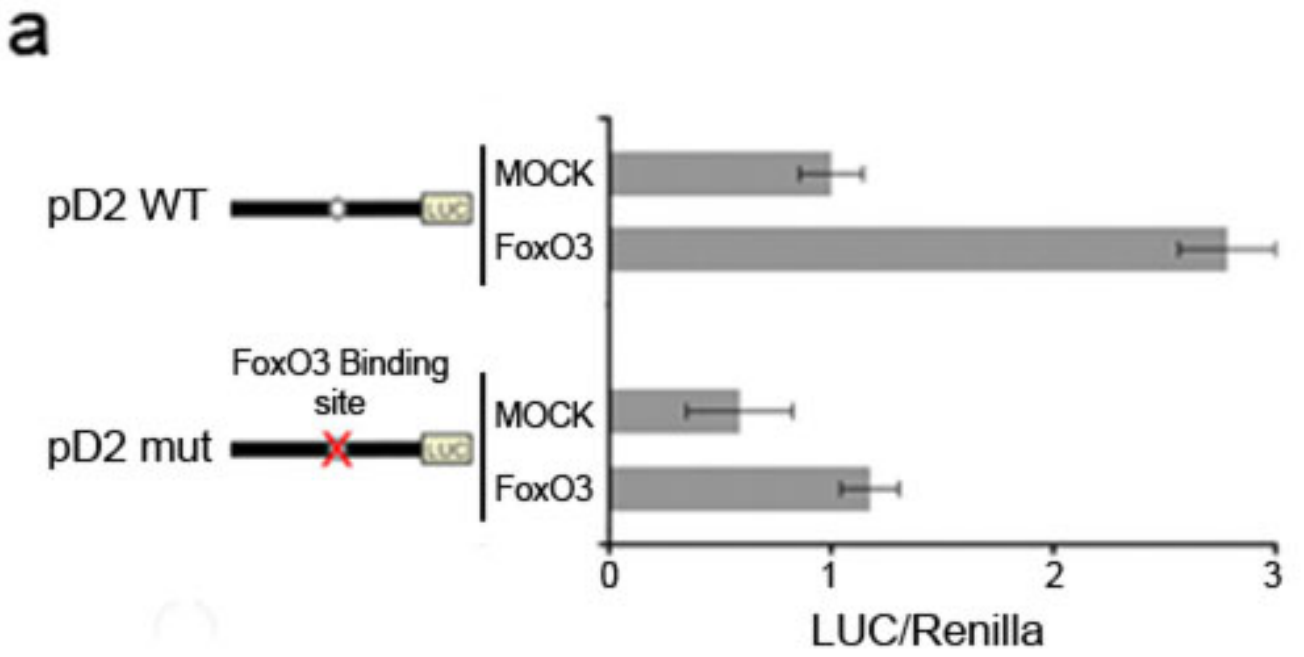

c
LUC/Renilla
0
2
4
6
8
FoxO3A (ng)
0
5
20
50
100
300
TRE3TK-Luc

Fig. S7


a
pD2 WT
MOCK
FoxO3
FoxO3 Binding site
pD2 mut
MOCK
FoxO3
0
1
2
3
LUC/Renilla

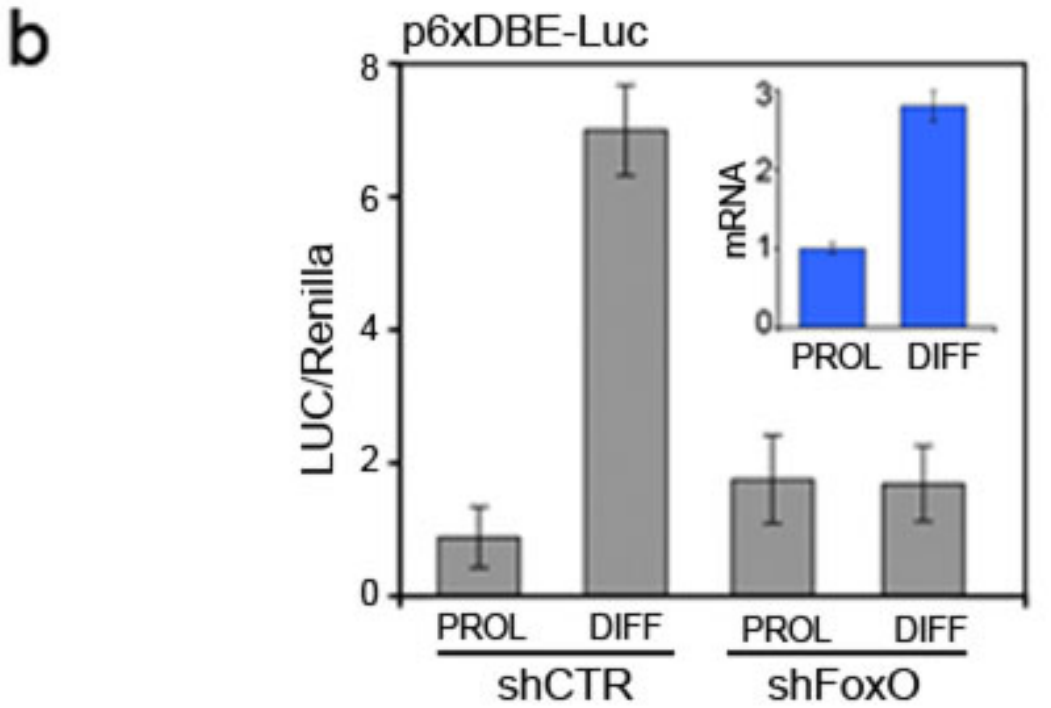

b
p6xDBE-Luc
LUC/Renilla
0
2
4
6
8
mRNA
0
1
2
3
PROL
DIFF
PROL
DIFF
PROL
DIFF
shCTR
shFoxO

Fig. S8

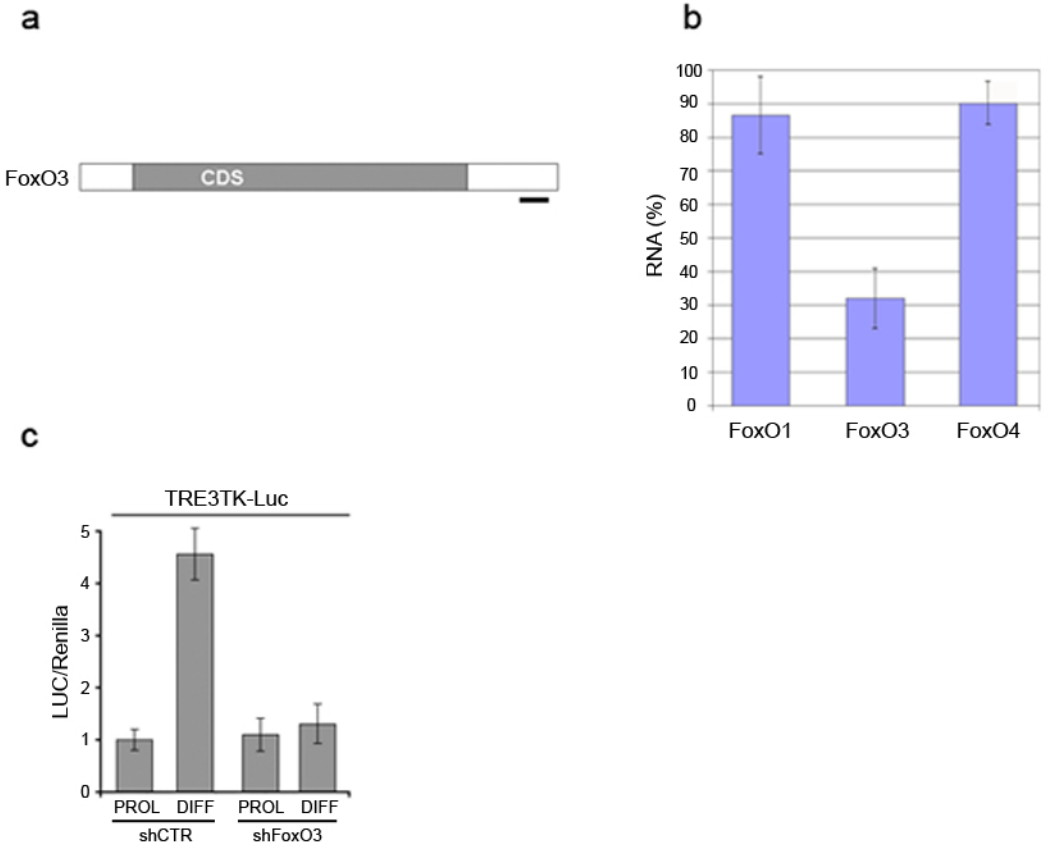

a
FoxO3
CDS
b
RNA (%)
100
90
80
70
60
50
40
30
20
10
0
FoxO1
FoxO3
FoxO4
c
TRE3TK-Luc
LUC/Renilla
5
4
3
2
1
0
PROL
DIFF
PROL
DIFF
shCTR
shFoxO3

Fig. S9

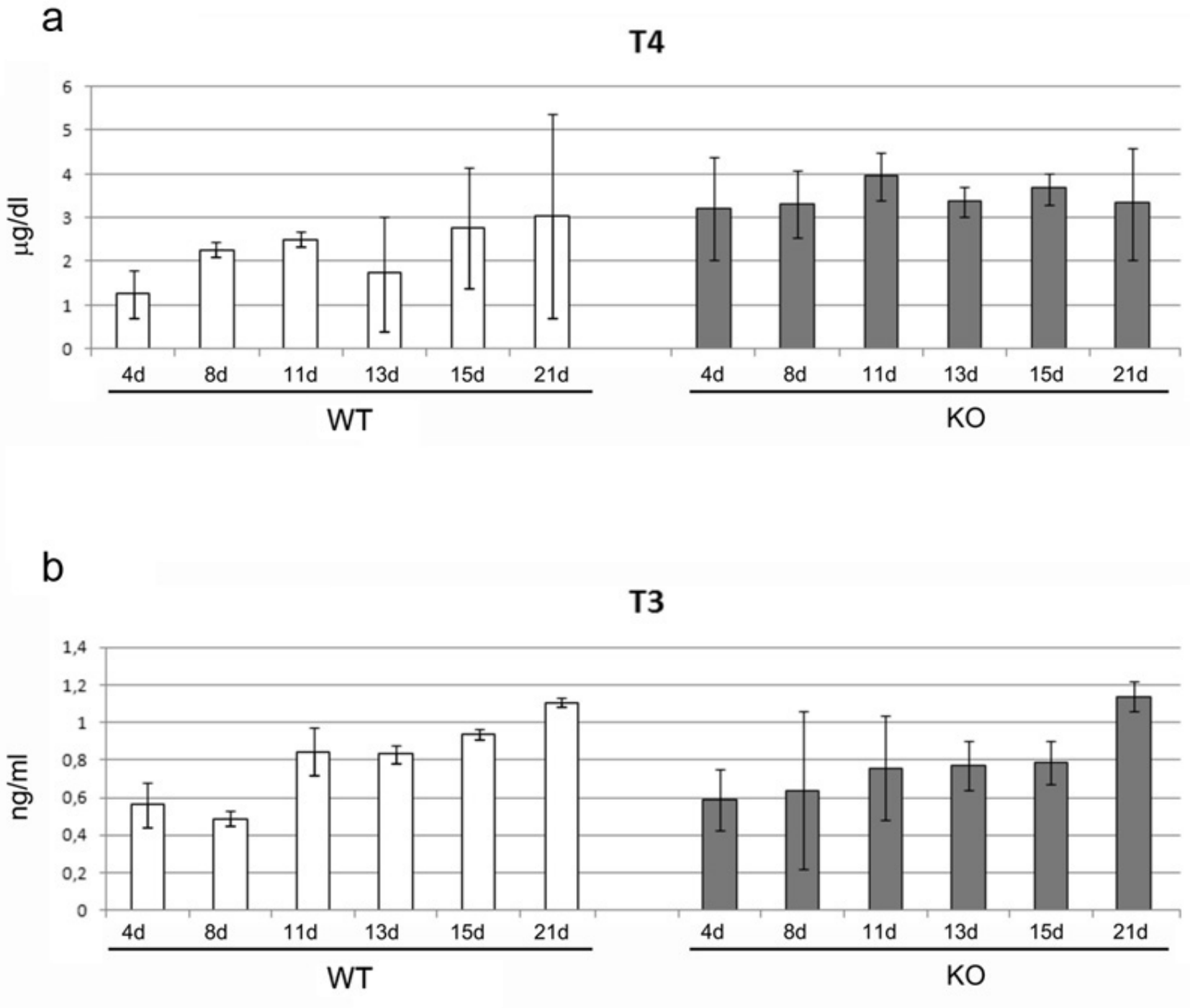

a
T4
µg/dl
6
5
4
3
2
1
0
4d
8d
11d
13d
15d
21d
WT
KO
b
T3
ng/ml
1,4
1,2
1
0,8
0,6
0,4
0,2
0

Fig. S10

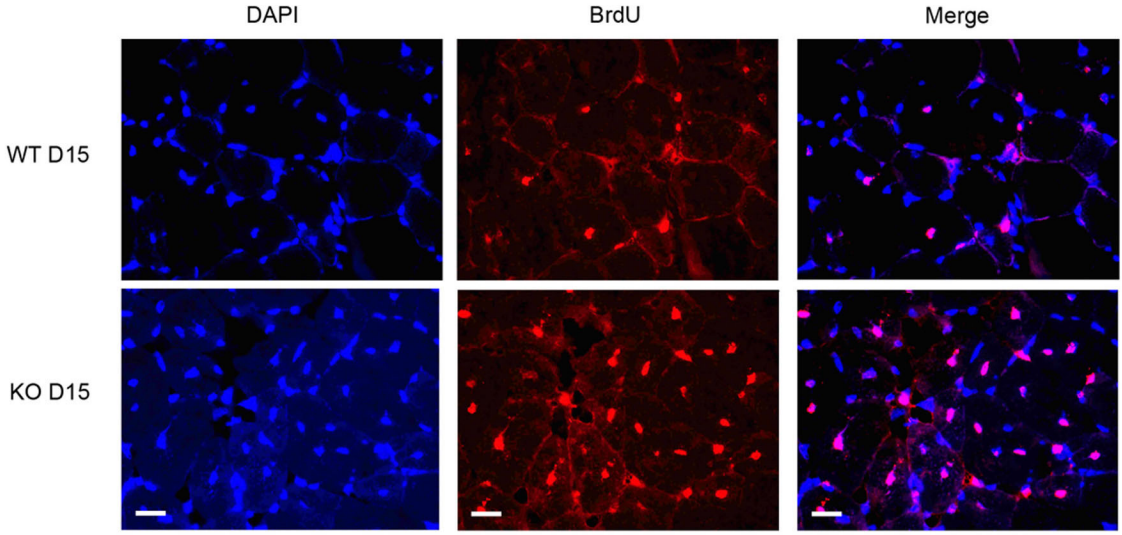


Fig. S11

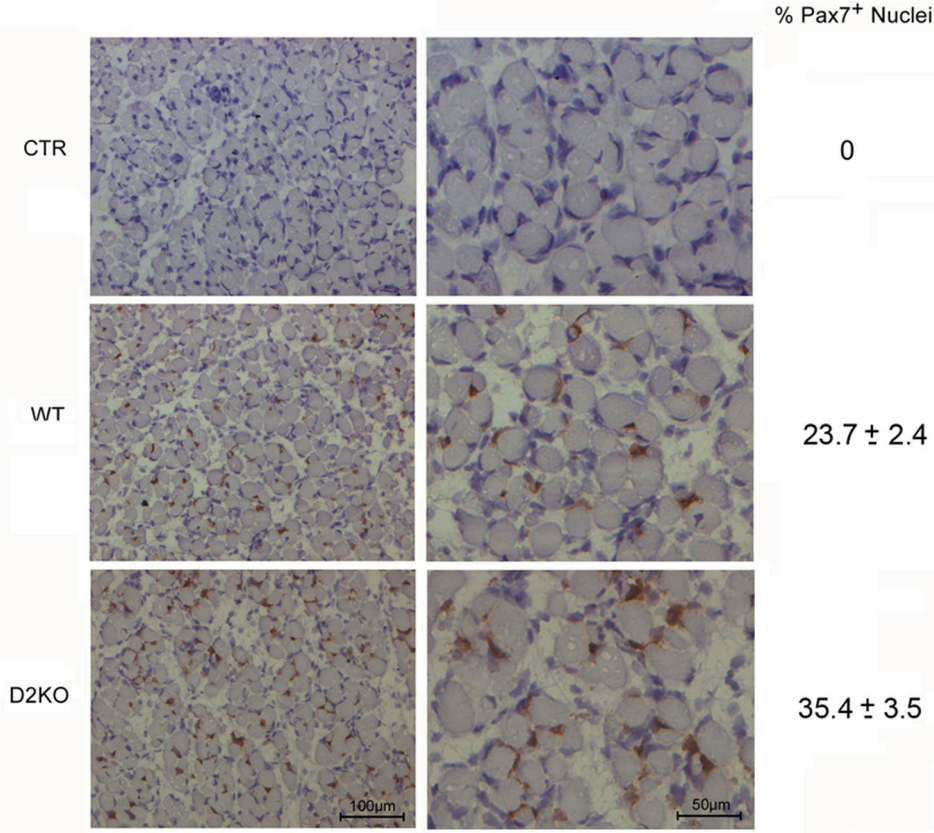


Fig. S12

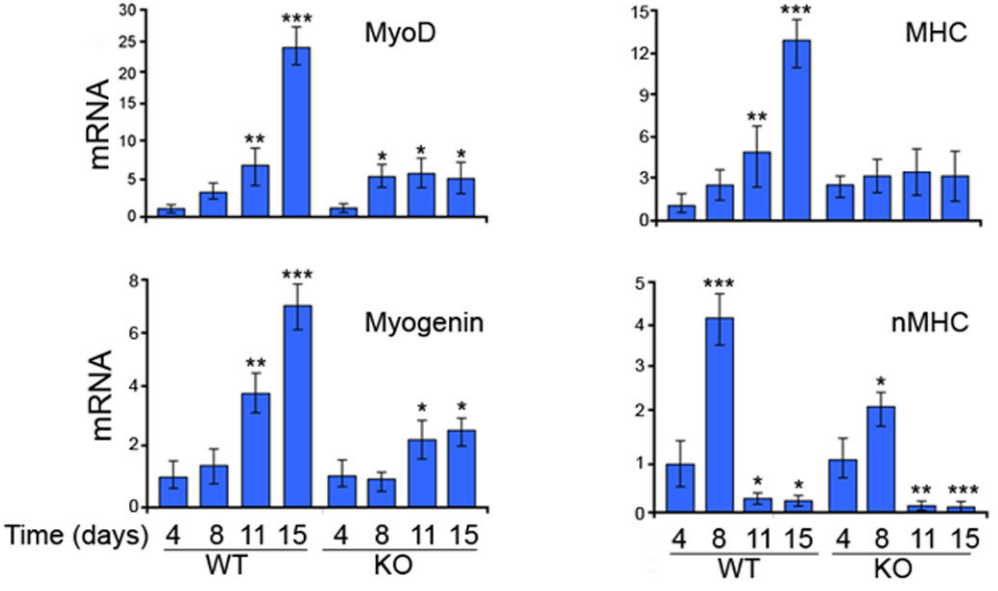

MyoD
MHC
Myogenin
nMHC
mRNA
mRNA
Time (days) 4 8 11 15 4 8 11 15
WT
KO
4 8 11 15 4 8 11 15
WT
KO

Fig. S13

Table I.

Primers used for RT-PCR analysis		
Gene	**Forward primer (5' - 3')**	**Reverse primer (5' - 3')**
Cyclophilin A	CGCCACTGTCGCTTTTCG	AACTTTGTCTGCAAACAGCTC
dio2	CTTCCTCCTAGATGCCTACAAAC	GGCATAATTGTTACCTGATTCAGG
dio3	CCGCTCTCTGCTGCTTCAC	CGGATGCACAAGAAATCTAAAAGC
FoxO3	TTGTCCCAGATCTACGAGTGGA	CGTGCCTTCATTCTGAACGCGCA
MHC2	CCATTCAGAGCAAAGATGCAGG	GCATAACGCTCTTTGAGGTTG
myoD	GACCTGCGCTTTTTTGAGGACC	CAGGCCCACAGCAAGCAGCGAC
myogenin	TTGCTCAGCTCCCTCAACCAGGA	TGCAGATTGTGGGCGTCTGTAGG
dio2 5′ flanking	CGCTCCTGGAGAACCTGGAGAA	AAGACCAGGTTCAGGCTCTGCA
Neonatal MHC	CACCTTCGCCTGTAATTTGTC	GAACTTGAAGGAGAGGTCGA
pax7	ACCCTGATGCATGGTTGATGG	GTCTGGTTCAGTAACCGGCGTG
Primers used for cloning and EMSA		
dio2 5′ flanking	GCCGGAGCTCTATTCCCTCTGATAAGATCATTAC	GCCGCTCGAGCTTCTCTGCCTCCTCGGTCAGT
dio2 FoxO binding site WT	GAAAGTATGTTTACAACGGAGTAAAGACAGAGCG	CGCTCTGTCTTTACTCCGTTGTAAACATACTTTC
dio2 FoxO binding site mutant	GAAAGTATGCCCACAACGGAGTCCCGACAGAGCG	CGCTCTGTCGGGACTCCGTTGTGGGCATACTTTC
Primers used for shRNA plasmids generation		
CTR forward	AATTCAAGCAGCAGAAGTACCTGTCGCAAGAGACGACAGGTACTTCTGCTGCTTTTTTTC	
CTR reverse	TCGAGAAAAAAAGCAGCAGAAGTACCTGTCGTCTCTTGCGACAGGTACTTCTGCTGCTTG	
dio2 forward	AATTCGCATGGACAATAATGCCAACGCAAGAGACGTTGGCATTATTGTCCATGCTTTTTC	
dio2 reverse	TCGAGAAAAAGCATGGACAATAATGCCAACGTCTCTTGCGTTGGCATTATTGTCCATGCG	
FoxO3 forward	AATTCGTCCTTTCCCTAGCACACTTACAAGAGATAAGTGTGCTAGGGAAAGGACTTTTTC	
FoxO3 reverse	TCGAGAAAAAGTCCTTTCCCTAGCACACTTATCTCTTGTAAGTGTGCTAGGGAAAGGACG	

Supplemental data

Fig. S1.

(a-b) Anterior tibialis muscles from mice at 1, 5, 12 and 21 postnatal days (P1 to adult) were homogenized and cDNAs were prepared for real time PCR analysis of Pax7 and FoxO3 expression. CyclophilinA was used as internal control. Normalized copies of the target gene in tibialis muscle at P1 were set as 1. Data are shown as average of 3 separate assays analyzing 4 mice for each group, ± standard deviation.

Fig. S2.

C2C12 cells, cultured in proliferative or differentiating medium for 48h, were treated with Actinomycin D for 12 hours, and cDNAs were prepared to perform real time PCR using specific D2 and cyclophilinA (as internal control) oligonucleotides. Normalized copies of D2 gene in differentiated cells without Actinomycin treatment were set as 1. Error bars represent s.d.

Fig. S3.

(a) C2C12 cells were transfected with shRNA plasmid specific for D2 knockdown (shD2) or scrambled control oligonucleotides (shCTR) and cultured in proliferating or differentiative conditions. Total RNA was extract to measure D2 mRNA levels by real time PCR. CyclophilinA mRNA was measured as internal control. Normalized copies of D2 in proliferating cells transfected with shCTR were set as 1. **(b)** MyoD, myogenin and MHC mRNA levels were measured by real time PCR from shD2 and shCTR cells, cultured in proliferating or differentiative medium. **(c)** Western blot analysis of myoD, myogenin, MHC and tubulin of total lysates from shD2 and shCTR cells. Error bars represent s.d.

Fig. S4.

C2C12 cells were cultured in thyroid hormone deprived medium and treated with 30 nM T3 or transfected with myoD expression plasmid as indicated. Myogenin mRNA was measured by real time PCR as marker of differentiation. Error bars represent s.d.

Fig. S5.

(a) C2C12 cells were transiently transfected with RNAi oligonucleotides specific for D2 knockdown (iD2) or scramble control oligonucleotides (CTR) and cultured in proliferating or differentiative conditions. Total RNA was extract to measure myoD and myogenin mRNA levels by real time PCR. CyclophilinA mRNA was measured as internal control. Normalized copies of each gene in proliferating cells transfected with CTR were set as 1. **(b)** cDNAs were prepared from C2C12 cells cultured in the presence of rT3 or vehicle. cDNAs were analyzed for MyoD, myogenin and cyclophilinA expression by RT-PCR. For each gene, normalized copies of the target gene in proliferating cells were set as 1. **(c)** Western blot analysis of total lysates from C2C12 cells cultured as in b. Tubulin was used as loading control.

Fig. S6.

(a) C2C12 cells, cultured in proliferating or differentiating medium, were treated for 48h with vehicle, rT3 or rT3 plus T3. MyoD and myogenin mRNA levels were measured by real time PCR. **(b)** MyoD mRNA levels in C2C12 cells grown in proliferative or differentiating medium and treated with T3, T4 or T4 plus rT3 for 48 hours.

Fig. S7.

(a) C2C12 cells were transiently transfected with the indicated transcription factors and total mRNAs were analyzed for D2 expression by real time PCR. Normalized copies of D2 gene in Mock transfected cells were arbitrarily set as 1. **(b)** D2 mRNA was measured in different rhabdomyosarcoma cell lines transfected with FoxO3 or an empty vector. Normalized copies of the *Dio2* gene in Mock-transfected cells were set as 1. **(c)** C2C12 cells were transiently co-transfected with TRE3TK-Luc plasmid, different amount of FoxO3 expressing vector and CMV-Renilla plasmid as internal control. The LUC/Renilla value from mock-transfected plasmid was arbitrarily set as 1. Error bars represent s.d.

Fig. S8.

(a) C2C12 cells were co-transfected with a 1.4kb mouse *Dio2* promoter (pD2 WT) or its mutant in the putative FoxO3 binding site (pD2mut) and CMV-Flag (Mock) or FoxO3 plasmids. **(b)** C2C12 cells were stably transfected with a control (shCTR) or a FoxO3 shRNA (shFoxO3) constructs. FoxO3 activity was analyzed by measuring the activity of the FoxO3 responding promoter (p6xDBE-Luc). Inside panel: endogenous FoxO3 mRNA level was measured in proliferating and differentiated C2C12 cells.

Fig. S9.

(a) C2C12 cells were transfected with shRNA plasmid specific for FoxO3 knockdown (shFoxO3) targeting the indicated region within the FoxO3 3'UTR. **(b)** Specificity of shFoxO3 silencing was analyzed by measuring mRNA levels of FoxO1, FoxO3 and FoxO4 in shFoxO3 cells versus shCTR cells. CyclophilinA mRNA was measured as internal control. **(c)** shCTR or shFoxO3 cells were transiently transfected with a T3-responsive (TRE3TKLUC) and CMV-Renilla plasmids, and differentiation was induced for 48h.

Fig. S10.

(a-b) Serum thyroid hormone levels during mouse muscle regeneration experiment from wild type (wt) and D2 null mice (KO). Each time point represents the mean from three different animals.

Fig. S11.

Immunofluorescent staining for BrDU, DAPI or merged images in sections from tibialis anterior muscle fromD2KO and WT mice at 15 days following CTX injection. Scale bar = 50 μm.

Fig. S12.

Immunohistochemical analysis for Pax7 positive cells in sections from tibialis anterior muscle from D2KO and WT mice (post-natal day 4). The percentage of Pax7 positive cells / total cells is indicated. CTR indicates the absence of primary antibody.

Fig. S13.

MyoD, MHC, Myogenin, and neonatal MHC mRNAs were measured by real time PCR from WT and D2KO mice at the indicated times following cardiotoxin injection. For each gene, normalized copies of the target gene in WT tissue at 4 days after CTX injection were set as 1. Error bars represent s.d.* P<0.05, **P<0.01, ***P<0.001 compared with D2 activity at 2 days following injection, n=5.

Table I

Primers used in the study