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Supplemental Information
FOXO3 Promotes Quiescence in Adult Muscle Stem Cells during the Process of Self-Renewal

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Inventory of Supplemental information

Figure S1 relates to Figure 1.

Figures S2 relates to Figure 2.

Figure S3 relates to Figure 2.

Figure S4 relates to Figure 4.

Figure S5 relates to Figures 5 and 6.

Supplementary Materials and Methods

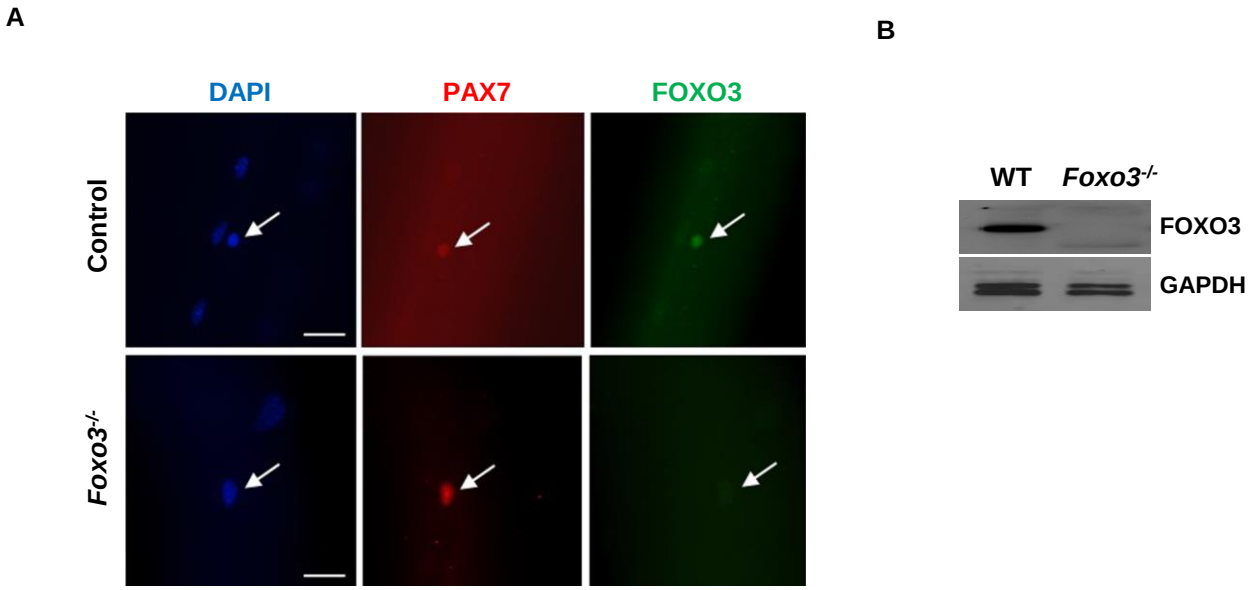

Figure S1. Specificity of the FOXO3 antibody.

(**A**) Single myofibers from wild type and *Foxo3*$^{-/-}$ mice were fixed immediately after isolation and immunostained for PAX7 and FOXO3. Arrows point to PAX7^{+ve} SCs. Such cells are positive for FOXO3 staining in the control and negative in *Foxo3*$^{-/-}$ SCs, thus displaying specificity of the FOXO3 antibody. (**B**) A Western blot analysis of lysates from proliferating SC progeny isolated from wild type and *Foxo3*$^{-/-}$ muscles was performed also to show that the FOXO3 antibody is specific. GAPDH serves as loading control. Scale bars represent 50 μm in (A).

Figure S2

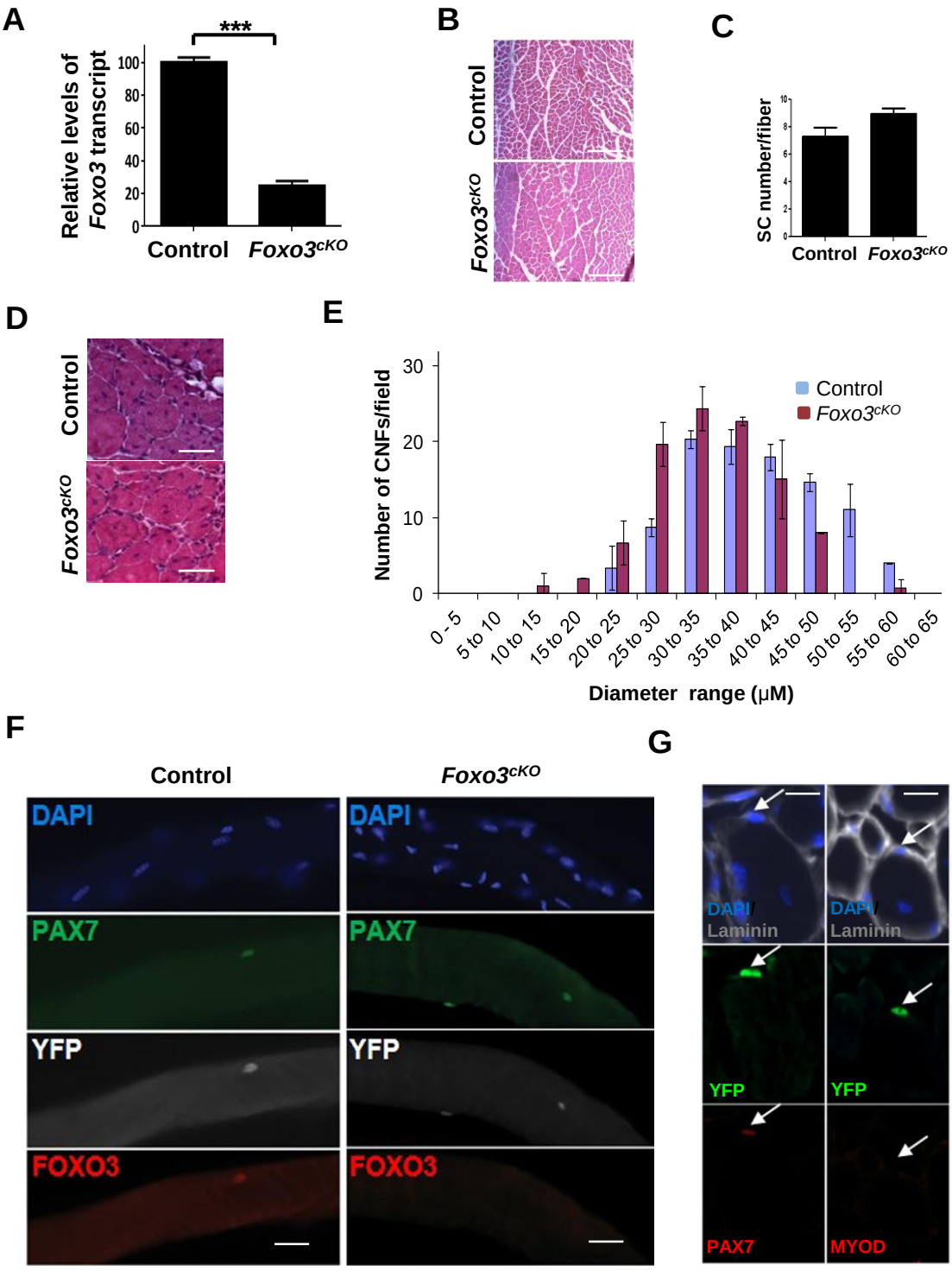
A
***
Relative levels of Foxo3 transcript
100
80
60
40
20
0
Control
Foxo3cKO
B
Control
Foxo3cKO
C
SC number/fiber
10
8
6
4
2
0
Control
Foxo3cKO
D
Control
Foxo3cKO
E
Number of CNFs/field
30
20
10
0
Control
Foxo3cKO
0 - 5
5 to 10
10 to 15
15 to 20
20 to 25
25 to 30
30 to 35
35 to 40
40 to 45
45 to 50
50 to 55
55 to 60
60 to 65
Diameter range (µM)
F
Control
Foxo3cKO
DAPI
PAX7
YFP
FOXO3
G
DAPI/ Laminin
YFP
PAX7
MYOD

Figure S2. Characterization of FOXO3 expression and function during quiescence and upon activation following injury.

(**A**) FACS-purified QSCs were isolated from *Foxo3*^{cKO} muscles and control muscles and analyzed for levels of *Foxo3* transcript. Although the *Foxo3* locus is not recombined in all SCs due to the limited efficiency of Cre recombinase, we observed that the normalized *Foxo3* expression levels in *Foxo3*^{cKO} muscles were significantly reduced compared to controls. Control and *Foxo3*^{cKO} samples represent triplicate experiments of pooled RNA from 2 mice for each experiment (***, $p < 0.005$). (**B**) One month after the initiation of tamoxifen treatment, muscles were harvested from *Foxo3*^{cKO} and control mice and cryosections were stained with Hematoxylin and Eosin. *Foxo3*^{cKO} muscles appeared grossly normal and were indistinguishable from control muscles. (**C**) Single myofibers were isolated from tamoxifen-treated control and *Foxo3*^{cKO} muscles and assessed for the number of SCs per fiber. There was no statistically significant difference in the number of SCs in muscles of the two strains. Fibers were obtained from 3 mice per genotype. (**D**) One month after the initiation of tamoxifen treatment, muscles of *Foxo3*^{cKO} and control mice were injured and harvested one week later. Cryosections were stained with Hematoxylin and Eosin to reveal the presence of regenerating fibers with central nuclei. (**E**) The histogram shows that there were no significant differences in the diameters of CNFs in control and *Foxo3*^{cKO} muscles (n=300 fibers per genotype). (**F**) Single fibers from tamoxifen-treated *Foxo3*^{cKO} and control muscles were fixed immediately after isolation and immunostained for PAX7, YFP and FOXO3. Arrows point to PAX7^{+ve}, YFP^{+ve} SCs that stain positive for FOXO3 in control muscles but are negative for FOXO3 expression after Cre-mediated recombination in *Foxo3*^{cKO} muscles. (**G**) Injured muscles from tamoxifen-treated control mice were harvested 1month later and stained for Laminin, YFP, and either PAX7 or MYOD. Arrows point to sublaminar YFP^{+ve} SCs, demonstrating that they are PAX7^{+ve} and MYOD^{-ve}, indicative of a quiescent, self-renewed population. Scale bars represent 200 μm in (B), 50 μm in (D) and (G) and 100 μm in (F).

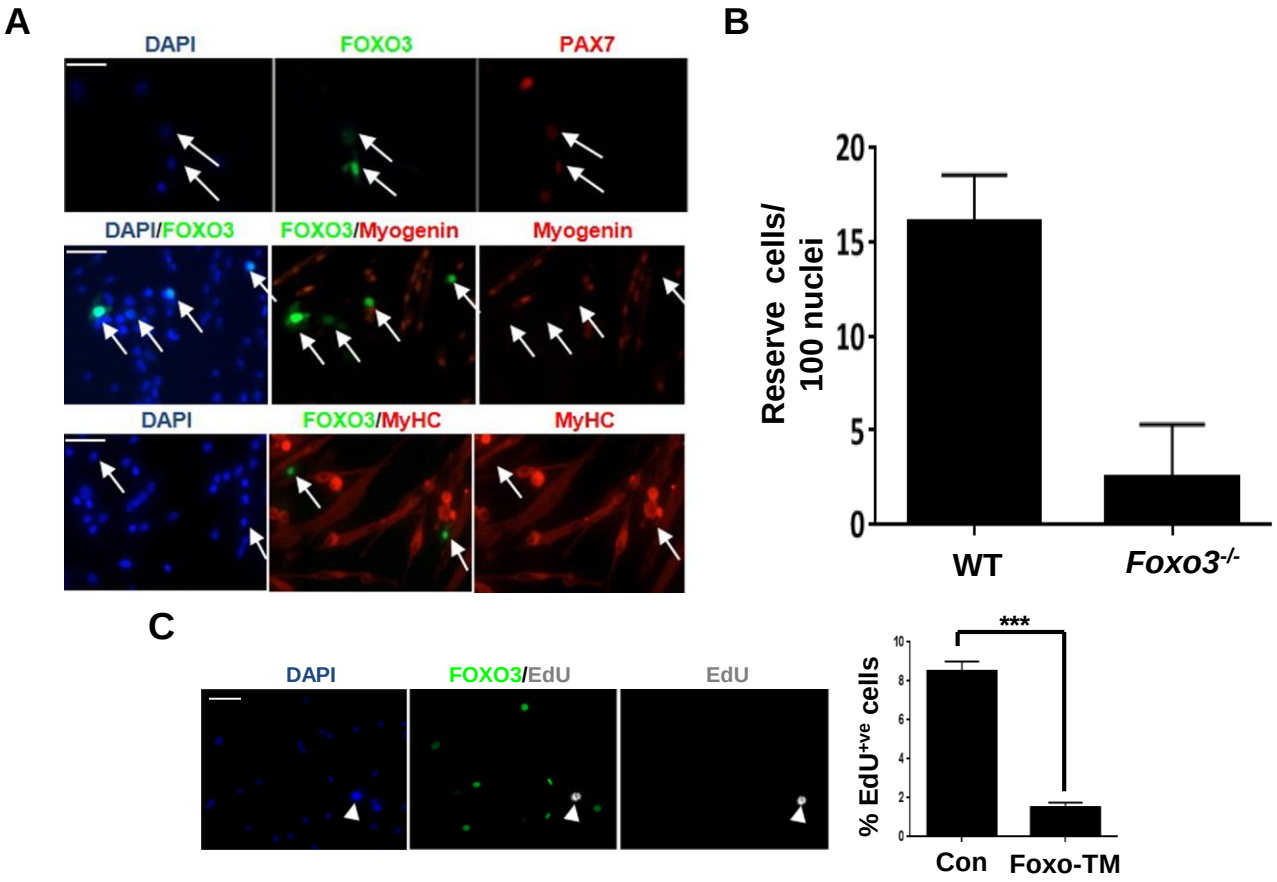

Figure S3. FOXO3 promotes the quiescent state in proliferating SC progeny.

(**A**) Proliferating SC progeny from *Foxo3*$^{-/-}$ cultures were transfected with GFP-FOXO3-TM, switched to low mitogen medium, and then stained for markers of reserved cells, PAX7 (top row) or markers of terminal differentiation Myogenin (middle row) and MyHC (bottom row). Arrows point to cells expressing GFP-FOXO3-TM, that were nearly all PAX7^{+ve} and uniformly Myogenin^{-ve}/ MyHC^{-ve}.
(**B**) Proliferating progeny from *Foxo3*$^{-/-}$ SC cultures were induced to differentiate in mitogen-poor medium for three days and stained for markers of reserve cells and of terminally differentiated cells as in panel (H) markers. The graph shows that there are vastly fewer reserve cells in *Foxo3*$^{-/-}$ cultures than in wild type cultures and represent values from the same culture for each genotype that were plated in different wells (n=3 wells) (***, $p < 0.005$). (**C**) Proliferating SC progeny from *Foxo3*$^{-/-}$ cultures maintained in growth-promoting medium were tested for proliferative activity as determined by EdU incorporation following transfection with a plasmid expressing GFP-FOXO3-TM or a control plasmid. Except for the rare GFP-FOXO3-TM^{+ve} cell that was EdU^{+ve} (closed arrowhead), nearly all of the GFP-FOXO3-TM^{+ve} cells were EdU^{-ve}. The graph shows reduced EdU incorporation in GFP-FOXO3-TM transfected cells compared to control cells and represent values from 3 independent transfections for each sample (n=3 wells) (***, $p < 0.005$). Scale bars represent 200 μm in (A) and (C).

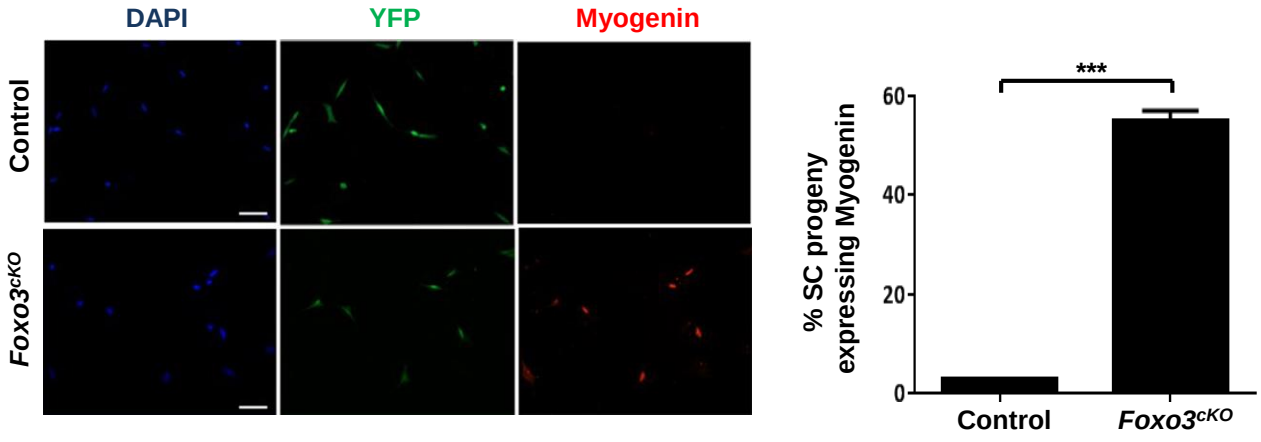

Figure S4. *Foxo3*^{cKO} SCs display an increased tendency to differentiate in vitro.

One month after tamoxifen treatment, FACS-sorted purified SCs from *Foxo3*^{cKO} and control mice were assessed for their ability to differentiate in vitro by plating them in low-mitogen media for 18 hours. The graph shows that a significantly greater fraction of *Foxo3*^{cKO} SC progeny express Myogenin compared to control SCs (n=3 mice per genotype) (***, $p < 0.005$). Scale bar represents 100 μm.

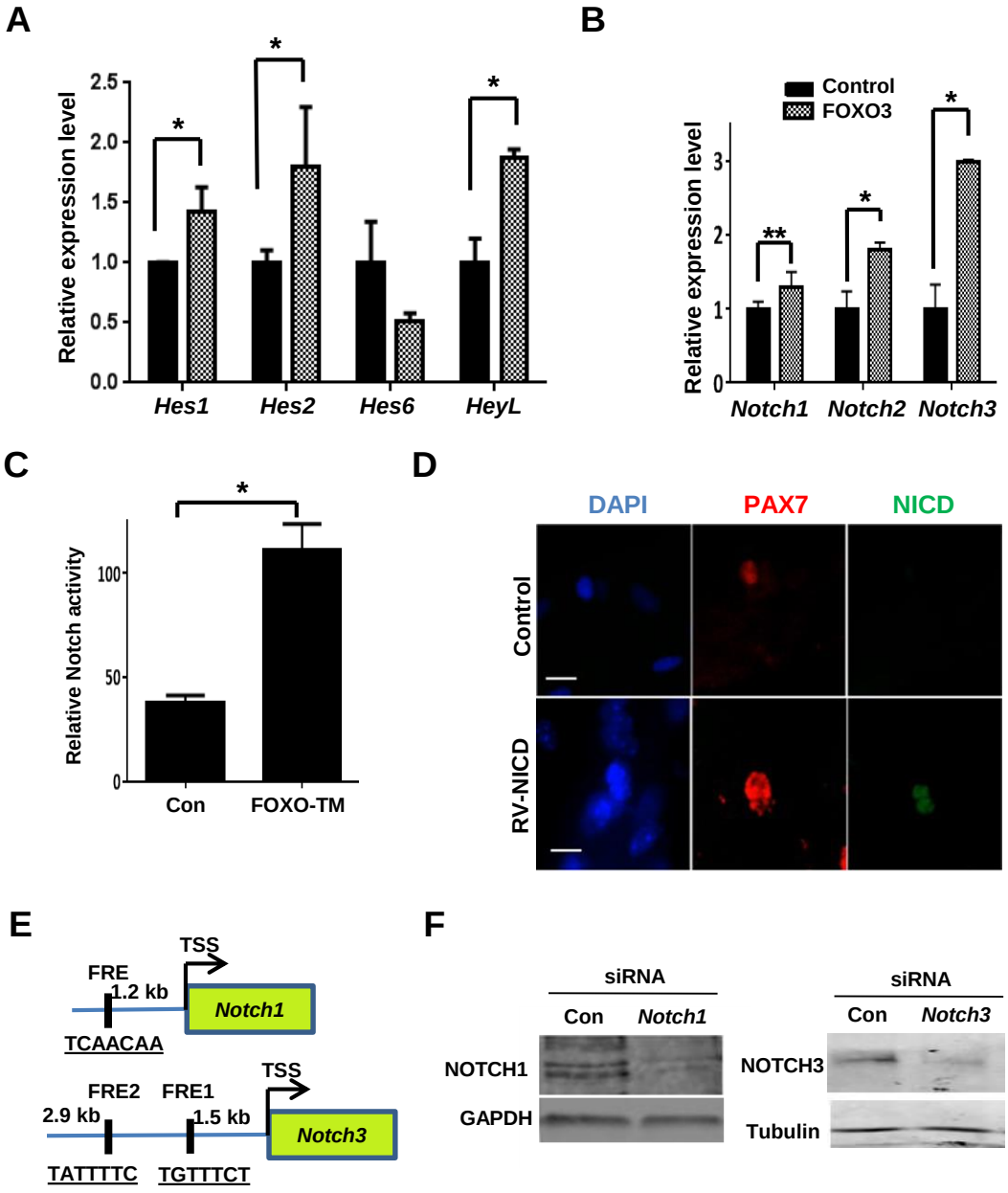
A
Relative expression level
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
Hes1
Hes2
Hes6
HeyL
B
Control
FOXO3
Relative expression level
3
2
1
0
**
*
*
Notch1
Notch2
Notch3
C
Relative Notch activity
100
50
0
*
Con
FOXO-TM
D
DAPI
PAX7
NICD
Control
RV-NICD
E
TSS
FRE
1.2 kb
Notch1
TCAACAA
TSS
FRE2
FRE1
2.9 kb
1.5 kb
Notch3
TATTTTC
TGTTTCT
F
siRNA
Con
Notch1
NOTCH1
GAPDH
siRNA
Con
Notch3
NOTCH3
Tubulin

Figure S5. FOXO3 regulates the expression of *Notch1* and *Notch3* and increases Notch signaling.

(**A-B**) Proliferating SC progeny were infected with lentiviral vectors expressing wild type FOXO3 or control virus (the transfection efficiency was > 90%). RNA was isolated and quantitative RT-PCR was performed using primers specific to Notch targets and receptors analyzed in Panel A and B, respectively. All Notch targets analyzed in Panel A (except for *Hes6*) were up-regulated in FOXO3-expressing cultures. Among the Notch receptor transcripts, there was a modest increase in *Notch1*, while *Notch2* and *Notch3* were significantly up-regulated in FOXO3-overexpressing cultures. RNA expression levels are normalized to levels in control transfected cultures and represent values from three independent wells (n=3) (**, $p = 0.089$; *, $p < 0.05$) (**C**) SC progeny were co-transfected with *pHes1-Luc* and with either a plasmid expressing FOXO-TM or a control plasmid. Cells expressing FOXO-TM displayed more than a 3-fold increase in reporter activity compared to cells expressing the control plasmid (n= 3 wells representing 3 independent transfections for each sample) (*, $p < 0.01$) (**D**) SCs associated with single fibers were infected with retroviral vectors expressing NICD (RV-NICD) or control virus and stained for PAX7 and NICD after 72 hours in culture. Greater than 90% of $PAX7^{+ve}$ cells were $NICD^{+ve}$. Scale bar represents 50 μm. (**E**) Bioinformatic analysis identified an FRE in the *Notch1* promoter and 2 FREs in the *Notch3* promoter. The *Notch1* promoter containing the FRE with the consensus sequence, TCAACAA, is located 1.2 kb upstream of the *Notch1* TSS. FRE1 in *Notch3* containing the consensus sequence, TGTTTCT, is located 1.5 kb upstream of the *Notch3* TSS, while FRE2 with the consensus sequence, TATTTTC, is located 2.9 kb upstream of the *Notch3* TSS. (**F**) FACS-sorted SCs were incubated with siRNAs to *Notch1*, *Notch3*, or cyclophilin (control). After 24 hours, cells were lysed and a Western blot analysis performed with antibodies to NOTCH1 (left panel) and NOTCH3 (right panel) revealed that both receptors were undetectable in the knockdown cells. GAPDH and tubulin serve as loading controls respectively.

Supplemental Material and Methods

Animals

Foxo$^{-/-}$ and *Foxo3*$^{fl/fl}$ mice were generated in the lab of Dr. Ronald DePinho (Dana Farber Cancer, Boston). Wild type FVB/N mice were purchased from Charles River. *Foxo3*$^{fl/fl}$ mice were crossed with *Pax7CreER*^{tm} (referred in the text to as *Pax7*$^{CreER/+}$) mice (Nishijo et al, 2009) to generate *Pax7*$^{CreER/+}$*; Foxo*$^{fl/+}$ mice.

Immunofluorescence and histology

TA muscles were harvested, fixed for 4 hours by rocking in 0.5% electron-microscopy grade PFA (Electron microscopy Sciences, Hatfield, PA), frozen in Optimal Cutting Temperature mounting media (Sakura Finetek, Torrance, CA), and collection of 8 μm cryosections. Histological analysis was performed using Hematoxylin and Eosin (Sigma-Aldrich) staining. For immunofluorescence, cryosections were postfixed in 2% PFA for 10 minutes and permeabilized using 0.3% TritonX-100 in PBS followed by incubation in blocking buffer containing 10% donkey serum. For FOXO3 immunostaining, antigen retrieval method was used on 7 μM cryosections collected on Superfrost slides (Fisher) and boiled in 0.5% TritonX-100/Citrate buffer (10 mM, pH 6) for 10 minutes prior to incubation in blocking buffer. Primary antibodies used include rabbit anti-GFP (1:500, Invitrogen), rabbit anti-FOXO3 (described previously

in Brunet et al., 1999;Greer et al., 2007), mouse anti-Myogenin (1:200, Becton/Dickson), mouse anti-PAX7 (1:200, DSHB), rat anti-Laminin (1:1000, Sigma-Aldrich). Species-specific secondary antibodies (donkey) were conjugated to Alexa 488, Alexa 594, or Alexa 647 and used at a concentration of 1:1000 (Invitrogen), and 4',6-diamidino-2-phenylindole (DAPI, 1: 5000) was used to visualize nuclei.
For FOXO3 detection, goat anti-FITC-Alexa 488 (Invitrogen, catalogue no. A11096) was used as a tertiary fluorochrome to enhance the fluorescence signal from SCs in vivo.

Drug treatments

Tamoxifen (Sigma-Aldrich, St. Louis, MO) was dissolved in corn oil at a concentration of 50 mg/ml, and control and experimental mice were injected with 100 µl (5 mg) twice a week for 3 weeks to induce Cre-mediated excision. Mice were allowed to recover for 1 week before any experimental procedure. For assessment of proliferation, EdU (Invitrogen, Carlsbad, CA) dissolved in sterile phosphate buffered saline (PBS) was added to the media of SCs in culture to a final concentration of 10 µM. To activate endogenous FOXO3, LY (Calbiochem) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at a concentration of 20 mg/ml. LY stocks were stored at -20^{o}C protected from light.

Chromatin Immunoprecipitation

ChIP experiments were performed as described (Lee et al., 2006). 10^7 proliferating SC progeny were cultured in Ham's F10 media (Invitrogen) containing 20% Fetal Bovine Serum (Invitrogen). To activate endogenous FOXO3, cells were incubated in 20 μm LY in low serum containing media for 2 hours. Chromatin was cross-linked with 1% formaldehyde for 10 minutes, followed by quenching with 0.125 M Glycine for 5 minutes. Cells were washed in PBS and scraped in Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0 and protease inhibitors), and chromatin was sheared with a Sonicator (Misonix) seven times for 30 seconds at an amplitude setting of 15. Lysate was diluted in ChIP dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl) and incubated with 10 μg of FOXO3 antibody (Santa Cruz Biotechnology) or rabbit IgG (Santa Cruz Biotechnology), bound to Protein G coupled to Dynabeads (Invitrogen), by rocking overnight at 4^{o}C. Beads were washed in RIPA buffer (50 mM Hepes-KOH, pH 7.6, 500 mM LiCl, 1 mM EDTA, 1% NP40, 0.7% sodium deoxycholate) and bound complexes were eluted at 65^{o}C for 1 hour in buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0. ChIP products were analyzed by semi-quantitative PCR using specific primers for the following genes,

p27^{KIP1} (forward): 5'-CAGAGTTTGCCTGAGACCCAA-3'

p27^{KIP1} (reverse): 5'-GCAGGAGAGCCAGGATGTCA-3'

FRE in *Notch1* (forward): 5'-TGGAATCTGGAACGAACAAC-3'

FRE in *Notch1* (reverse): 5'-CTGGCCCTTTAGTGCCTTTC-3'

FRE1 in *Notch3* (forward): 5'-AAAGCCAGCCTGGTCTACCT-3'

FRE1 in *Notch3* (reverse): 5'-TCTTCGGTCCACAAAGAAGC-3'

FRE2 in *Notch3* (forward): 5'-TCCTGGAAAAGGGATCTTGTT-3'

FRE2 in *Notch3* (reverse): 5'-GACTCCTACAAGTGGTCCTCTGA-3'

Viral Production and Transduction

Lentiviral particles were generated in 293T cells by transfecting 1 μg of pVSV-G, 6 μg of Delta 8.9 and 6 μg of FUW-*rfp* or FUW-*rfp-Foxo3* in 100 mm dishes using FuGENE6 (Roche) according to the manufacturer's protocol. For generating NICD1-expressing retroviral vectors, Phoenix cells were transfected with pLPCX-NICD1 (gift from Dr. Ernesto Canalis). Supernatant containing viral particles was collected between 48 and 72 hours post-transfection, filtered through 0.45 μm polyethanesulfone (PES) filters, concentrated using a PEG virus precipitation kit (BioVision Inc.), and stored at -80°C. Proliferating SC progeny were plated at a density of 10^5 cells in a 30 mm dish. Viral supernatant along with 8 μg/ml polybrene was added to the medium and incubated for 24 hours at 37°C. Fresh medium was added after 24 hours and cultures were maintained for 72 hours before analysis.

Transfections

Sorted SCs were plated at a density of 10,000 cells per well and were transfected with

control siRNA to Cyclophilin B (Thermo Scientific) or siRNAs to *Notch1* (SIGMA, catalogue # SASI_Mm01_00104902) and *Notch3* (Santa Cruz Biotechnology, catalogue # sc-37136) using X-tremeGene siRNA Transfection Reagent (Roche).

Western Blotting

Protein extracts from FACS-sorted QSCs and ASCs were obtained by lysing approximately 2 x 10^5 SCs in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). The proteins were resolved by SDS-PAGE (8%) and transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies followed by incubation with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies and visualized using Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA).

Supplementary References

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