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Supplemental Information

Tubastatin A maintains adult skeletal muscle stem cells in a quiescent state *ex vivo* and improves their engraftment ability *in vivo*

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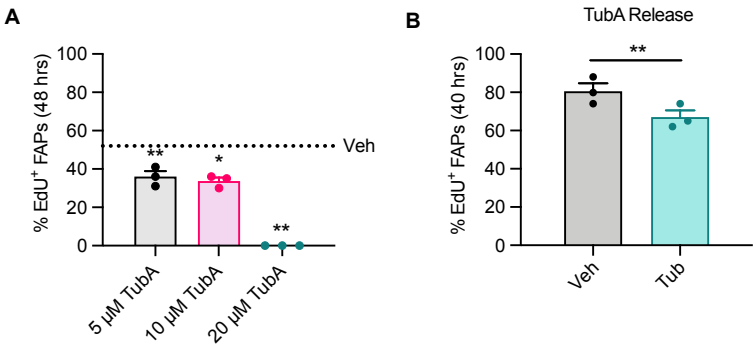
A
% EdU+ FAPs (48 hrs)
100
80
60
40
20
0
Veh
**
*
**
5 μM TubA
10 μM TubA
20 μM TubA
B
TubA Release
% EdU+ FAPs (40 hrs)
100
80
60
40
20
0
**
Veh
Tub

Figure S1

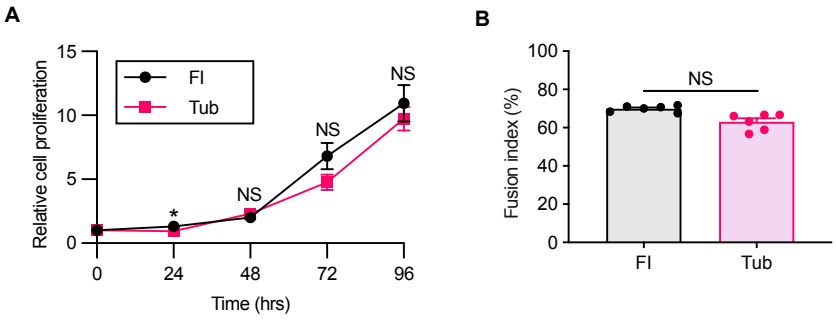
A
B
Relative cell proliferation
Time (hrs)
FI
Tub
NS
NS
NS
*
Fusion index (%)
NS
FI
Tub

Figure S2

Supplemental Figure Legends

Figure S1. TubA prevents FAP entry into the cell cycle. A, FACS-isolated FAPs were treated with different doses of TubA for 48 hrs while being cultured continuously in EdU to assess S-phase progression. The percentage of EdU+ FAPs was quantified (*n*= 3 mice). **B**, FAPs were cultured in the absence or presence of 20 μM of TubA for 48 hrs. Cells were then cultured for 40 hrs in the absence of TubA but the presence of EdU, after which the number of EdU+ FAPs was quantified (*n*=3 mice). Error bars represent ± s.e.m. *P <0.05, **P<0.01; two-tailed paired t-test.

Figure S2. *In vitro* proliferation and differentiation characteristics of TubA-released MuSCs are comparable to those of MuSCs^{FI}. A, FACS-isolated MuSCs were cultured in the presence of TubA for 72 hrs, then released from TubA and maintained in culture for 96 hrs. Cells were fixed at the moment of TubA release (0 hrs) or 24, 48, 72, and 96 hrs after TubA release. As a control, FACS-isolated MuSCs were grown in culture for 96 hrs. The number of MuSCs at each time point was quantified (*n*=12 mice). **B**, FACS-isolated MuSCs were cultured in the presence of TubA for 72 hrs, then released from TubA and cultured in wash media for 48 hrs. Cells were then cultured in differentiation media for another 48 hrs. As a control, FACS-isolated MuSCs were grown in wash media for 48 hrs and then in differentiation media for another 48 hrs. Myonuclear fusion index was measured as the percentage of DAPI+ nuclei from each well that were within MHC+ myotubes that contained more than one nucleus (*n*= 6 mice). Error bars represent ± s.e.m. *P <0.05; NS: not significant; two-tailed t-test.

Supplemental Experimental Procedures

EdU assay. To analyze S-phase entry of MuSCs *ex vivo*, FACS-isolated MuSCs were cultured in the presence of 10 μM EdU (Thermo Fisher) for different periods of time (indicated in each experiment). Cells were fixed with 4% PFA for ten minutes, permeabilized using 0.5% Triton in PBS, and blocked using 3% BSA in PBS. Cells were then stained with the Click-iT EdU Cell Proliferation Kit (Thermo Fisher) and DAPI. The percentage of EdU^+ cells was quantified automatically using the Volocity software.

Cell size, Pyronin Y, and Annexin V assays. To assess cell size, RNA content, and cell survival, MuSCs cells were cultured in growth media (Ham's F-10 media containing 20% fetal bovine serum, 2.5 ng/mL bFGF (PeproTech), 100 U/mL penicillin, and 100 μg/mL streptomycin). At various time-points after plating (indicated in each experiment), cells were trypsinized using TrypLE Select (Thermo Fisher). To test cellular diameter, cells were assayed with a Moxi Flow combined Coulter counter and flow cytometer (Orflo Technologies). For RNA content, MuSCs were blocked for forty-five minutes at 37°C with wash media, and 10 μM Hoechst 33342 (Thermo Fisher), and then stained with 0.1 mg/mL Pyronin Y (Santa Cruz sc-203755) for fifteen minutes at 37°C in the same medium. To assess for cell survival, detached cells were collected and pooled with attached cells after gentle detachment with TrypLE Select. Cells were then stained with an Annexin V antibody (BioLegend) and with Propidium Iodide (BioLegend) for ten minutes at 4°C. Each analysis was done with 1000 cells using an Aria II or Aria III machine. Cell size was recorded in the FSC-A channel, pyronin Y intensity in the PE-Cy5 channel, and Annexin V signal in the FITC channel.

Differentiation assay. To assess differentiation, freshly-isolated MuSCs were grown in wash media for 48 hrs and then in differentiation media (DMEM media containing 5% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin) for another 48 hrs. To examine the differentiation capacity of TubA-released MuSCs, freshly-isolated MuSCs were cultured in the presence of TubA for 72 hrs. TubA was then washed out and cells were cultured in wash media for 48 hrs. Next, cells were grown in differentiation media for another 48 hrs. Myonuclear fusion index was measured as the percentage of DAPI+ nuclei from each well that were within MHC+ myotubes that contained more than one nucleus. Myonuclear fusion index was quantified from immunofluorescent DAPI and MHC images using the thresholding and contour functions (adaptiveThreshold, findContours, contourArea, and arcLength) of the open source Python package, OpenCV (https://pypi.org/project/opencv-python/), and the Image.getpixel function of the open source Python Imaging Library (https://pypi.org/project/Pillow/).

Immunofluorescence assay. To examine MyoD, Pax7, Ift20, Arl13b, or detyrosinated tubulin (detyr-tub) expression, MuSCs were fixed with 4% formaldehyde for ten minutes, permeabilized with 0.2% Triton X-100 in PBS for another ten minutes, blocked in PBS containing 10% FBS, 1% BSA, 0.1% Tx100 and 0.01% NaN_3 for 60 minutes, and then stained with antibodies against MyoD (1:100, BD 554130), Pax7 (1:50, DSHB AB_528428), Ift20 (1:1000, Sigma-Aldrich HPA021376), Arl13b (1:1000, NeuroMab clone N295B/66) or detyr-tub (1:500, Abcam ab48389) overnight at 4°C. Nuclei were stained with DAPI. The fraction of $MyoD^+$ cells, $Pax7^+$ cells, $Ift20^+$ cells, $Arl13b^+$ cells, and detyr-tub+ cells was quantified using Volocity software. To analyze Pax7 expression levels, $DAPI^+$ nuclear regions were identified automatically using the contour functions (findContours, contourArea, and arcLength) of the OpenCV2 Python library,

and mean fluorescence intensity of the Pax7 channel was calculated using the Image.getpixel() command from the Python Imaging Library and the np.mean() command from the NumPy Library.

Muscle injury. For transplantation experiments, lower hindlimbs of NSG mice were anesthetized with isoflurane and tibialis anterior (TA) muscles were injured with 40 μL of 1.2% barium chloride (Sigma) injected over fifteen intramuscular pokes. TA muscles from recipient mice were injured 72 hrs before transplantation.

MuSC transplantation and tamoxifen treatment. In order to label MuSCs with RFP, $Pax7^{CreER}$; $Rosa26R^{RFP}$ mice received tamoxifen (Sigma) at 80 mg/kg in 100% corn oil. Tamoxifen was delivered via intraperitoneal injection for seven consecutive days. After tamoxifen injections, a chase period of two weeks was given to the mice before any experiment was performed. MuSCs were then isolated based on RFP expression via FACS. RFP+ MuSCs were maintained in culture in the presence of either TubA or DMSO for 72 hrs before being used as donors. RFP+ MuSCs were trypsinized using TrypLE Select (Thermo Fisher) and resuspended in PBS before transplantation. Twenty-thousand cells were suspended in 40 μL of PBS and injected into injured TA muscles of recipient NSG mice. Either ten days or four weeks after transplantation, the mice were sacrificed, and their muscles were analyzed.

In the experiments where TubA was added to cycling MuSCs, freshly isolated RFP+ MuSCs were expanded for 72 hrs in growth media and then treated with either TubA or DMSO for 48 hrs in wash media before transplantation. RFP+ MuSCs were trypsinized using TrypLE Select and resuspended in PBS before transplantation. Twenty-thousand cells were suspended in 40 μL

of PBS and injected into injured TA muscles of recipient NSG mice. Ten days after transplantation, the mice were sacrificed and their muscles were analyzed.

Bioluminescence (BLI) imaging. For BLI experiments, mice were anaesthetized using isoflurane and intraperitoneally injected with d-Luciferin (50 mg/ml, Biosynth International Inc.). Immediately after the injection, mice were imaged using the Xenogen IVIS-Spectrum System (Caliper Life Sciences). Images were taken every minute using medium binning, maximum sensitivity (f-stop 1) and a 1 min exposure time. Imaging was performed until the bioluminescent signal reached saturation. Living Image Software (Caliper Life Sciences) was then used to analyze the images obtained. For the analysis, a small region of interest (ROI) was generated and used in every transplanted TA muscle.

Histology. To test for MuSC engraftment ability in transplantation experiments, muscles were fixed with 0.5% formaldehyde for six hours, cryoprotected with 20% sucrose overnight, and then fresh-frozen in liquid nitrogen cooled isopentane. Transverse 10 μm cryosections were collected and fixed with 2% formaldehyde for ten minutes. The muscles were then stained for laminin using a rat anti-laminin α2 antibody (1.5 μg/mL, Abcam ab11576) for 2 hrs. DNA was stained with DAPI. Images from the entire section were taken and the number of RFP+ myofibers was manually quantified using Volocity software.

RNA-Seq. Freshly isolated MuSCs or MuSCs grown *ex vivo* for 24 hrs in the presence of either DMSO or TubA were snap-frozen, and total RNA was extracted using the RNeasy Micro Plus Kit (Qiagen) according to manufacturer's instructions. Reverse transcription was performed with

5 ng RNA using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara). The cDNA was then sheared with a Covaris S2 ultrasonicator and library constructions were performed using the Ovation Ultralow Multiplex system (NuGEN). Libraries underwent paired-end 150-bp sequencing at Novogene with an Illumina HiSeq 2000 to a depth of 20-40 million reads.

RNAseq reads were processed with trim_galore for quality and adapter removal (www.bioinformatics.babraham.ac.uk/projects/trim_galore). Reads were then mapped to the mouse genome (mm10) with Ensembl transcript annotations using (Dobin et al., 2013). Uniquely mapping exonic reads were summarized over genes with the featureCounts module of Subread (Liao et al., 2014). Counts were analyzed using edgeR (Robinson et al., 2010), normalizing with the Trimmed Mean of M-values method and performing differential expression testing with Cox-Reid estimations of tagwise dispersions and the negative binomial GLM likelihood ratio test. The Benjamini-Hochberg method was used to generate false discovery rates.

Gene expression heatmaps were generated in R using the pheatmap package. Mean group FPKM values were log2-transformed for color mapping, and genes were clustered by Manhattan distance and single linkage.

Genes were analyzed with GSEA (Subramanian et al., 2005) using the MSigDB Hallmark gene sets (Liberzon et al., 2015) (enrichment statistic p 1, ranking metric Signal2Noise, expressed gene set size range 15-500). For overrepresentation analysis (ORA), fold-change <2 and Benjamini-Hochberg-corrected p-value >0.05 were used to classify genes as "similar" between $MuSCs^{FI}$ and $MuSCs^{Tub}$. FDR 1% was used to identify genes that were significantly different between $MuSCs^{FI}$ and $MuSCs^{Veh}$ as well as between $MuSCs^{Tub}$ and $MuSCs^{Veh}$. The REACTOME and KEGG pathway databases were used to annotate genes.

RT-qPCR. Total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen) and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed with the LightCycler 480 I SYBR Green PCR Master Mix (Roche). Each measurement was performed in triplicate and the values were averaged and normalized to *Gapdh*. Relative expression was calculated using the comparative CT method (Pfaffl, 2001). RT-qPCR primer sequences were, from 5' to 3':

Cyclin D1: AGACCATTCCCTTGACTGC, AAGCAGTTCCATTTGCAGC

Cyclin-dependent kinase 1: GTCCGTCGTAACCTGTTGAG,

TGACTATATTTGGATGTCGAAG

Polo like kinase 4: AGGAGAAACTAATGAGCACCACA, TGGCTCTCGTGTCAGTCCAA

GLI-Kruppel family member GLI3: AAGCGGTCCAAGATCAAGC,

TGTTCCTTCCGGCTGTTC

Smoothened: AGAGCAAGATGATCGCCAAG, CCATCATGGGAGACAGTGTG

Gapdh: GACTTCAACAGCAACTCCCAC, TCCACCACCCTGTTGCTGTA

Statistics. When data are summarized using a column with individual data points, each data point represents a biological replicate and error bars represent s.e.m. In time series graphs each data point represents the mean and the error bars represent s.e.m. Significance was calculated using two-tailed *t*-tests, except in the RT-qPCR experiments in which one tailed *t*-tests were performed. Differences were considered to be statistically significant at the $P < 0.05$ level.

Supplemental References

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Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics *30*.

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Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics *26*.