Supplementary data

Title: Inhibitors of tyrosine phosphatases and apoptosis reprogram lineage marked differentiated muscle to myogenic progenitor cells

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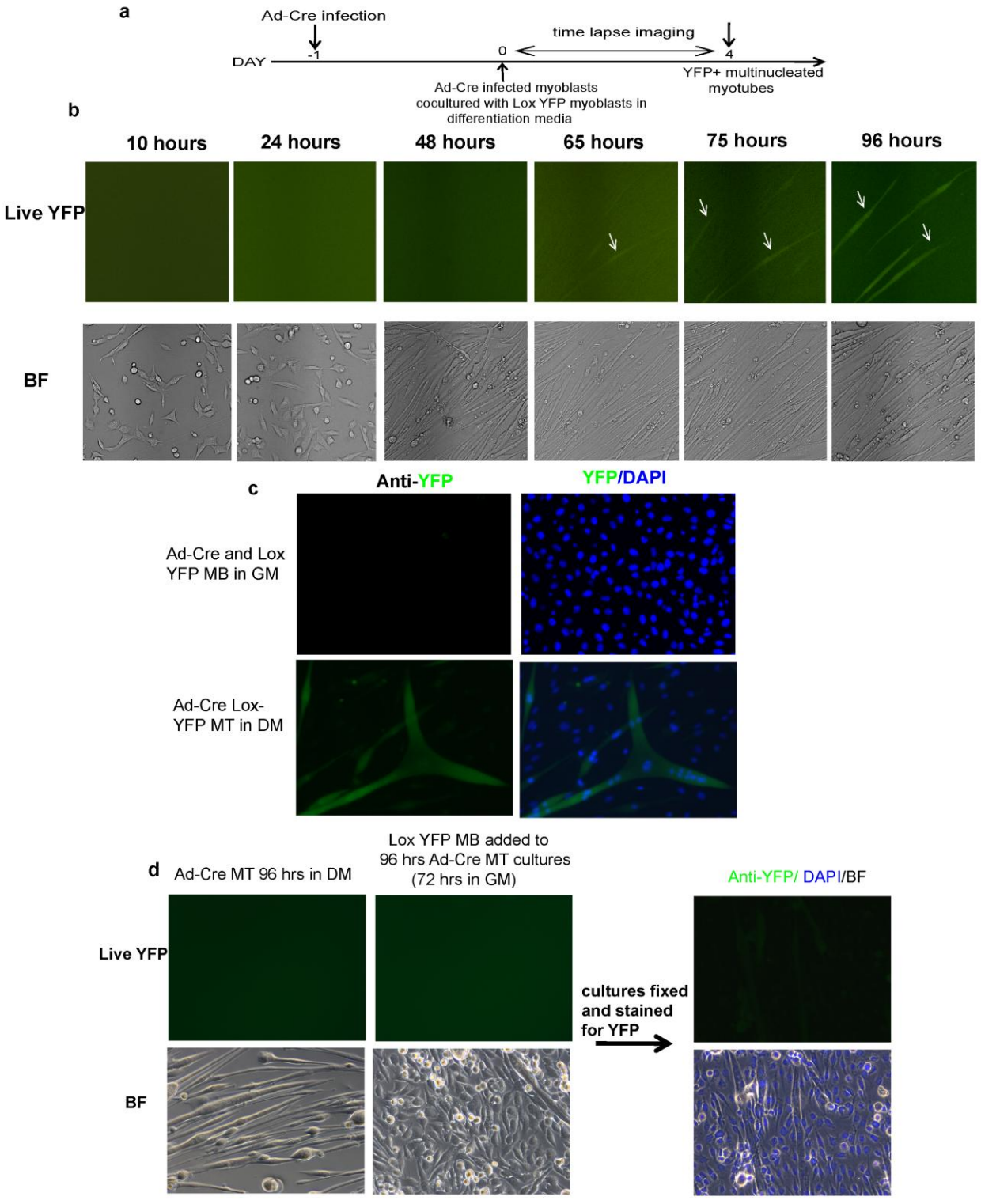
Supplementary information includes

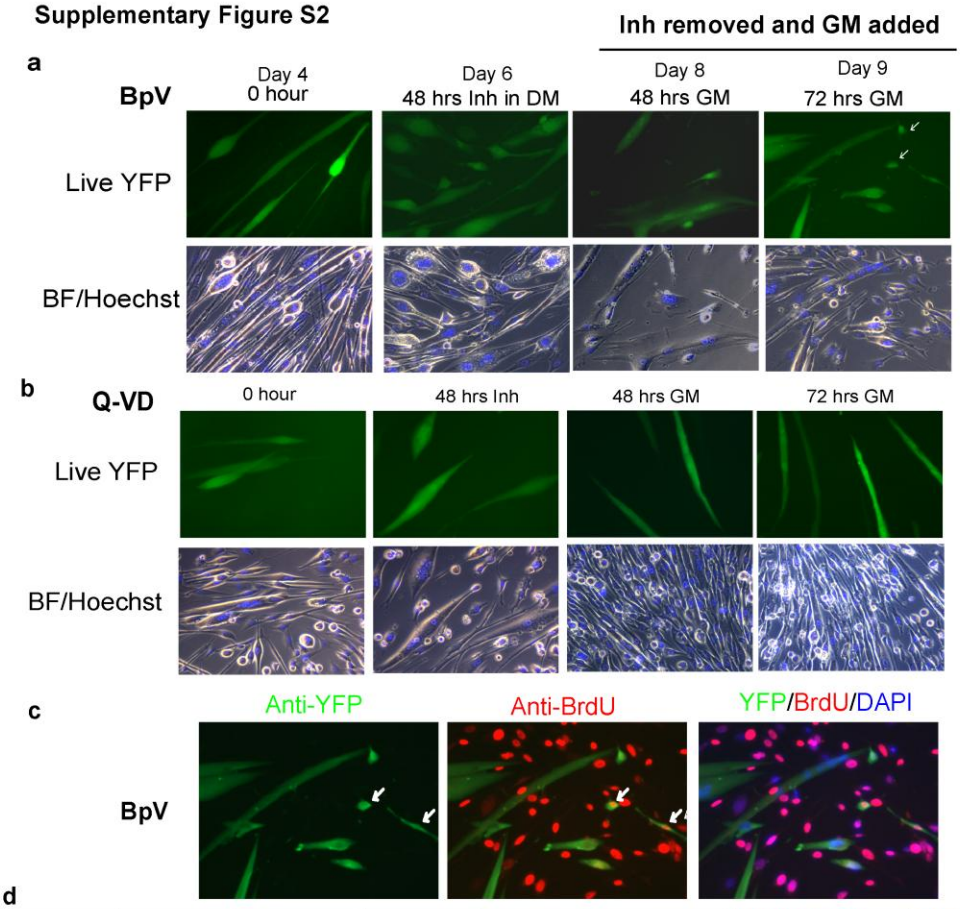
1. Supplementary Figures S1-S6
2. Supplementary legends and methods

Supplementary Figure S1



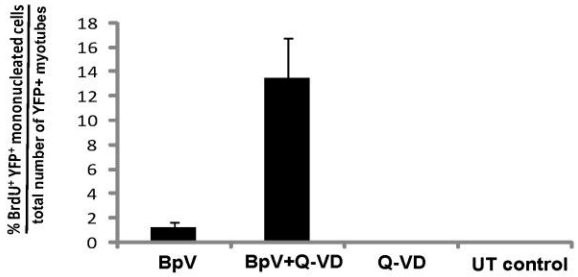
Supplementary Figure S1, related to Figure 1: Cre-Lox myotube formation. **a**. Schematic of time lapse imaging experiment. **b.** Representative images (10X) of time lapse experiments performed upon co-culture of Ad-Cre infected MB with Lox YFP MB in the ratio of 1:2 in DM. Cre-Lox myotube formation was imaged every one hour for the total period of 96 hours**.** White arrows show YFP+ myotubes**. YFP expression is observed upon physiological fusion of Ad-Cre and Lox YFP myoblasts in differentiation medium**. **c.** Ad-Cre infected MB were co-cultured with Lox-YFP MB in mitogenic growth medium (GM) and parallel in differentiation medium (DM) for 96 hours and subsequently immunostained with anti-YFP antibody. **d.** Ad-Cre infected myoblasts were differentiated to form myotubes in low serum conditions. Lox YFP myoblasts were added after 96 hours to Ad-Cre myotube cultures in mitogenic rich medium for another 72 hours and subsequently immunostained with anti-YFP antibody. The myotubes obtained from the physiological fusion of Ad-Cre-Lox-YFP MB in DM were YFP^{+} whereas co-culture of mononucleated cells expressing Ad-Cre and Lox-YFP MB in GM and addition of Lox YFP myoblasts to Ad-Cre myotubes in GM did not show YFP expression.

Supplementary Figure S2



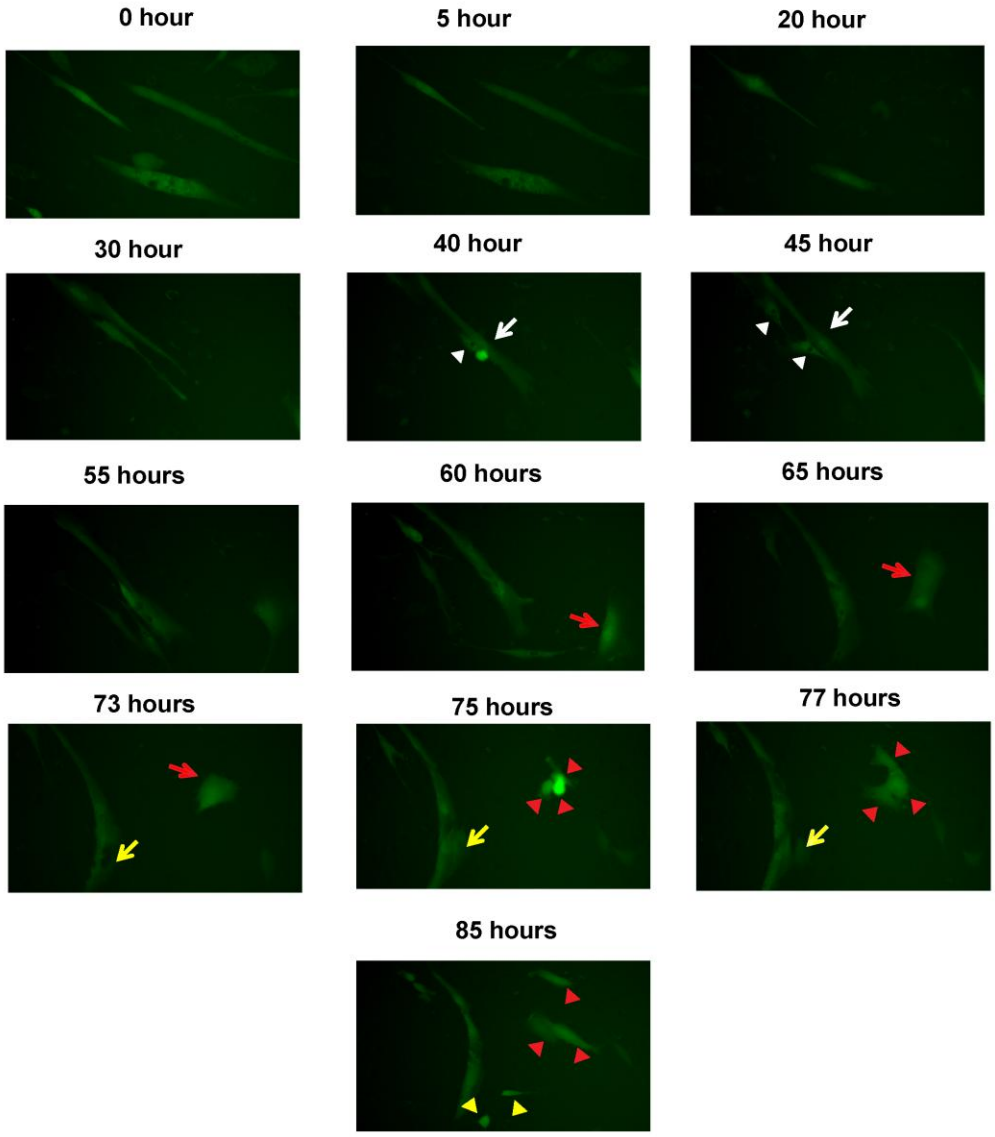
d

	Total number of YFP$^+$ MT per well (before inhibitor addition) [n=4]	Total number of myonuclei in YFP+ MT (based on an average of 4 myonuclei per MT)	Estimated total number of Lox YFP myonuclei in YFP$^+$ MT (based on Cre:Lox MB ratio of 1:2)	Number of YFP$^+$ myotubes after inhibitor treatment per well (n=4)	Number of de-differentiated YFP$^+$ cells per well	**Reprogramming Efficiency Method 1** (total number.of de-differentiated mononucleated. YFP$^+$ cells / total number of YFP$^+$ MT before treatment) X100	**Reprogramming Efficiency Method 2** (total number of de-differentiated mononucleated YFP$^+$ cells/ estimated total number of Lox YFP myonuclei in YFP$^+$ MT) X 100
BpV	2691± 128	10764	7176	1340	32± 11	~1.18%	~ 0.44%
BpV+ Q-VD	2600± 128	10410	6940	1532	350±108	~13.46%	~ 5.04%
Q-VD	2511± 240	10044	6696	2447	0	0%	0%
Untreated control	2635± 334	10540	7026	2543	0	0%	0%



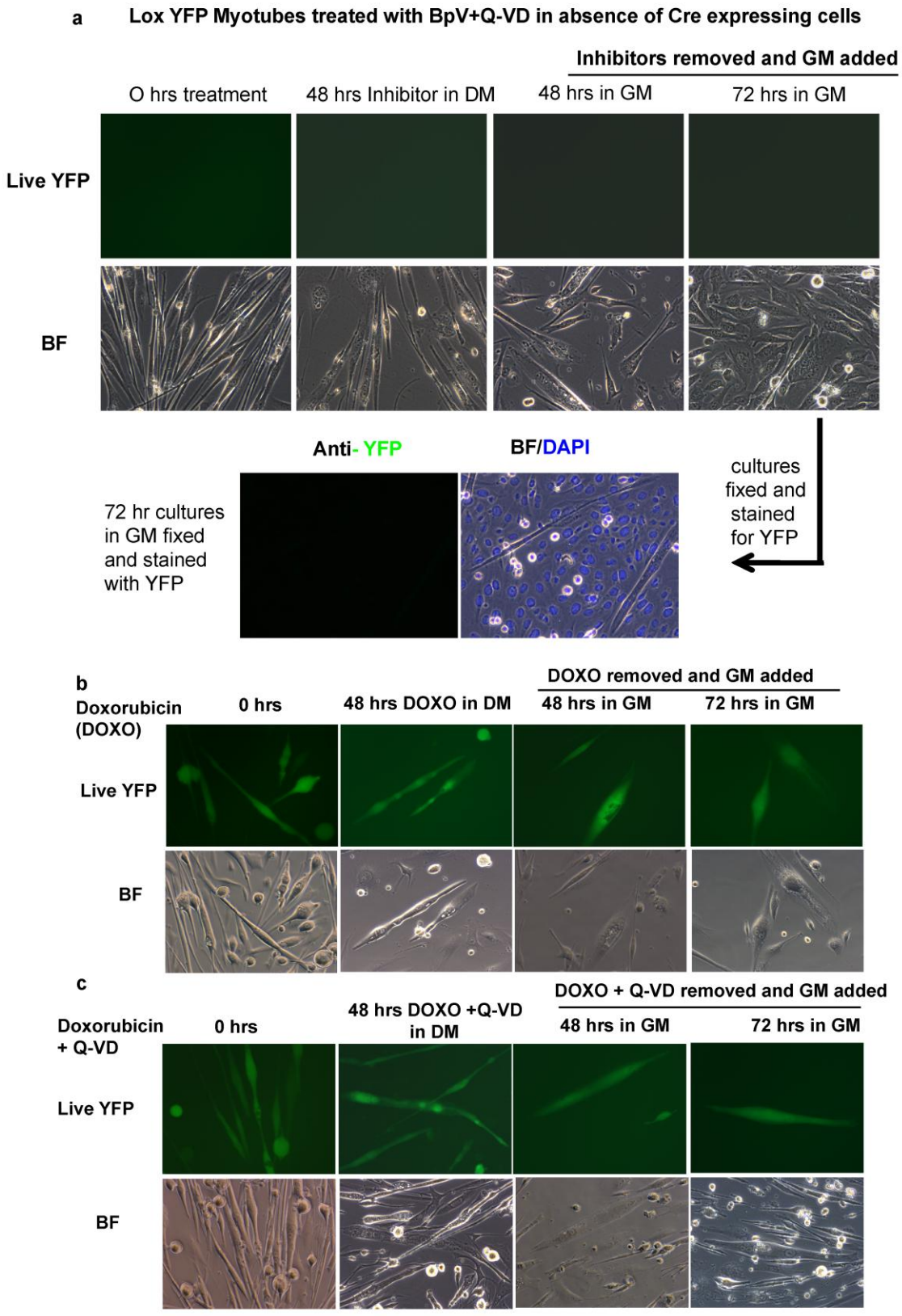
Supplementary Figure S2, related to Figure 3: a. BpV treatment induces de-differentiation in small fraction of Cre-Lox myotubes. Ad-Cre-Lox YFP myotubes were treated with BpV alone for total of 48 hrs in DM. Later inhibitor was removed and cultures switched to GM for 72 hrs. Rare YFP+ mononucleated cells were observed (white arrow**). b. Addition of apoptosis inhibitor does not induce dedifferentiation of Ad-Cre-Lox YFP myotubes**. Q-VD was added to Cre-Lox myotube cultures for 48 hrs in DM and then switched to GM for 72 hrs after inhibitor removal. No mononucleated YFP+ cells were seen. **c. YFP+ mononucleated cells obtained after BpV treatment incorporate BrdU**. Ad-Cre-Lox YFP myotube cultures after BpV treatment and subsequent switch to GM were pulsed with BrdU for 12 hrs and immunostained with BrdU and YFP antibody (white arrows). **d. Calculation of reprogramming efficiency.** Table represents the detailed quantitative analysis of different experimental conditions used in de-differentiation experiments. Histogram represents de-differentiation frequency of different experimental conditions (See also main text methods section).

Supplementary Figure S3



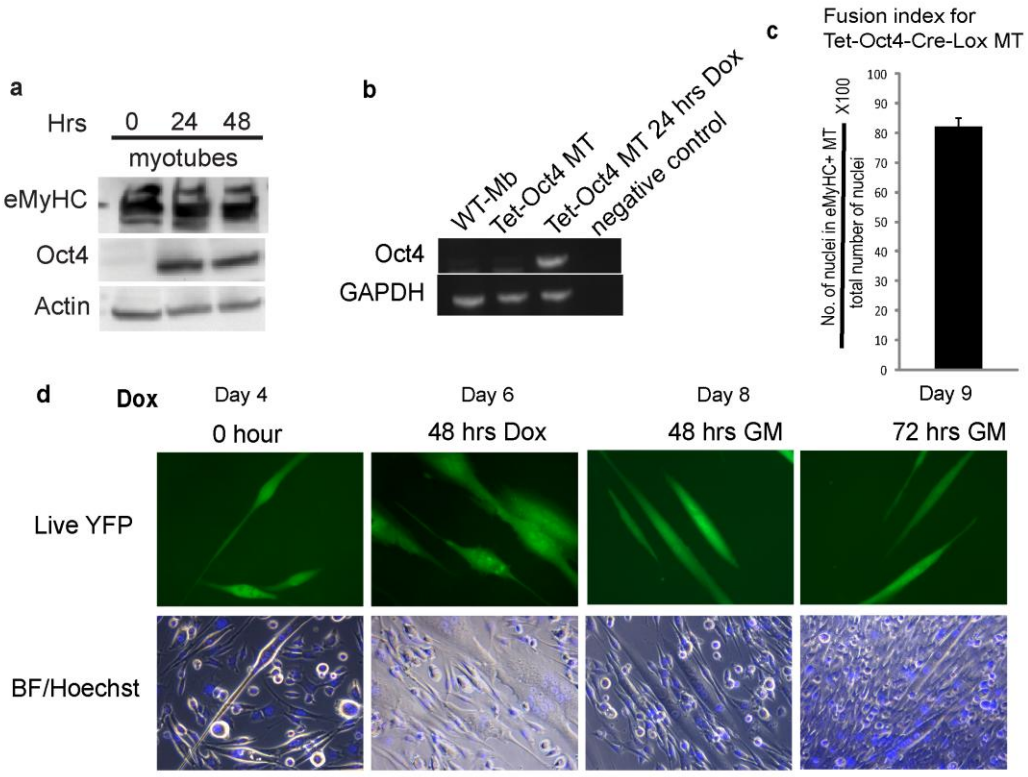
Supplementary Figure S3, related to Figure 3: Time lapse snapshots of myotube de-differentiation. Ad-Cre MB were co cultured with Lox YFP MB for 4 days and inhibitor mix added for 48 hours which was then switched to GM. Time lapse images (10X) of Cre-Lox YFP+ myotubes were taken everyone hour for period of 4 days. Shown are time lapse still images of YFP+ myotubes at different time points. Bold White, red and yellow arrows shows the myotube which de-differentiated to give rise to mononucleated YFP+ reprogrammed cells (shown in small white, red and yellow arrow heads).

Supplementary Figure S4



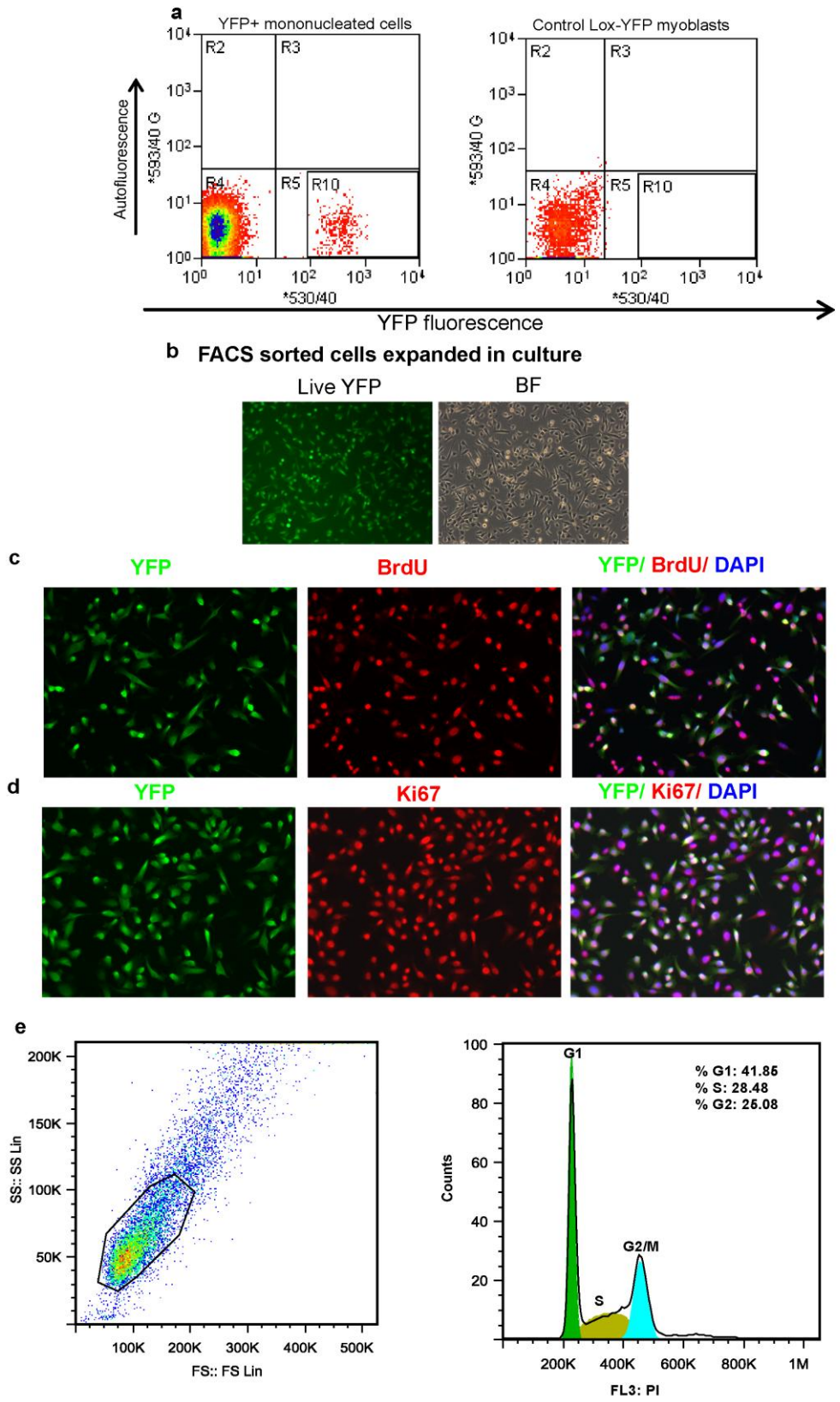
Supplementary Figure S4, related to Figure 3: a. Lox YFP myotubes do not express YFP upon inhibitor mix treatment. Lox YFP myoblasts in the absence of Cre expressing cells were differentiated in low serum conditions. Epifluorescent images of 4 day old Lox YFP myotube cultures treated with inhibitor mix for 48 hrs and then switched to GM at different time points do not show YFP expression. Subsequent immunostaining with anti-YFP antibody confirm the absence of YFP expression upon inhibitor treatment in Lox YFP myotubes. **b and c. Apoptosis inducing agent, doxorubicin does not induce de-differentiation of YFP+ myotubes.** Ad-Cre Lox YFP myotube cultures were treated with doxorubicin (0.2uM) with and without apoptosis inhibitor (10uM) for 48 hrs and then switched to mitogenic rich growth medium. Epifluorescent images at different time points show the absence of YFP+ mononucleated cells.

Supplementary Figure S5



Supplementary Figure S5, related to Figure 3: Tet-Oct4 myotubes express Oct4 upon doxycycline addition. Primary myoblasts derived from Tet-Oct4 mice were allowed to fuse into myotubes for 96 hours in DM followed by Dox administration for 24 and 48 hours. Cells were harvested and analyzed for Oct4 protein and mRNA levels. Tet-Oct4 myotubes treated for 24 and 48 hours with Dox induced Oct4 protein and mRNA level whereas untreated myotubes (0 hour) did not express Oct4. eMyHC served as myotube marker and actin as a loading control. RT-PCR analysis of these cells confirmed the tight regulation of Oct4 by Dox at mRNA level. GAPDH was used as a control **c. Fusion index of Cre-Lox-YFP myotubes.** The histogram quantifies the percent of number of nuclei in $eMyHC^{+}$ to the total number of nuclei in cultures of Ad-Cre-Lox-YFP myotubes expressed as mean and standard deviation n=3**. d . Induction of Oct4 alone does not cause de-differentiation of YFP+ myotubes.** Tet-Oct4 Cre Lox-YFP^{+} MT were induced for oct4 by Dox addition for 2 days in differentiation medium and switched to growth medium. No de-differentiation was observed in this set of experiment

Supplementary Figure S6



Supplementary Figure S6, related to Figure 4. FACS sorting of YFP^{+} mononucleated progeny of de-differentiated myotubes. a. Quadrant plot showing FACS sorting profiles of YFP^{+} mononucleated cells and Lox-YFP MB that served as control. **b.** Epifluorescent image of sorted YFP cells (95% of the cells were positive for YFP). BF represents bright field image of the same population. **c and d**. **FACS sorted dedifferentiated YFP+ cells divide in culture conditions.** De-differentated YFP+ cells were expanded in culture and immunostained with BrdU and Ki67 antibodies. The YFP+ cells stained positive for these two markers**. e**. **Cell cycle analysis for de-differentiated YFP+ cells**. YFP+ proliferating mononucleated cells were stained with propidium iodide and analyzed by flow cytometry. Scatter plot depicts the gated cell population used for cell cycle analysis. Histogram represents the different phases of de-differentiated YFP+ cells in cell cycle.

Supplementary legend

Supplementary Movie S1 related to Figure 1: Time lapse imaging for Cre-Lox YFP+ myotube formation. Ad-Cre recombinase infected myoblasts were co-cultured with Lox YFP myoblast in differentiation medium on and staged for live cell imaging in a humidified chamber at 37^0C and 5% CO_2. 10X Images were captured every one hour and movie made both for Bright Field (BF) and YFP expression for the total of 96 hours.

Supplementary Movie S2 related to Figure 3: Time lapse imaging for de-differentiation of Cre-Lox YFP+ myotubes to mononucleated YFP+ proliferating cells. Ad-Cre Lox YFP+ myotube cultures were treated with BpV+Q-VD for the total of 48 hours in differentiation medium and then switched to serum rich growth medium. The cultures were staged for 10X live cell imaging every one hour for the total of 96 hours for YFP expression and movie was captured.

Supplementary experimental procedure

Time lapse imaging microscopy: Ad-Cre expressing myoblasts were co-cultured with Lox YFP myoblasts on ECM coated 12 well plates and within 4 hours of co-culturing, these cells were staged for live cell imaging in a humidified chamber at 37^0C at 5% CO2 connected to Nikon_ TE 2000 imaging microscope station. The 10X images were captured every one hour for total of 96 hours for BF and YFP and movies were made using NIS elements advanced software. For dedifferentiation assays, 96 hour Cre-Lox myotubes were treated with 10uM BpV and 10uM Q-VD for 48 hours in differentiation medium and switched to growth medium where inhibitors were removed. In growth

medium, YFP+ cells were captured live every one hour for the total of 96 hours and analyzed using NIS elements advanced software.

RNA isolation and semi-quantitative PCR: : RNA isolation was performed by RNAeasy Kit (Qiagen) according to manufacture recommendations For semi-quantitative, 0.5ug RNA was reverse transcribed by superscript III first strand synthesis (Invitrogen) according to manufacture recommendations. For semi-quantitative PCR, reaction was carried out for 34 cycles using BioRad icycler. PCR products were run on 1.5% agaorse gel containing ethidium bromide and bands visualized using BioRad Gel Doc imaging system. Mouse Oct4 primer sequences were obtained as described elsewhere (Chen et al., 2006).

Reference

Chen, S., Do, J.T., Zhang, Q., Yao, S., Yan, F., Peters, E.C., Scholer, H.R., Schultz, P.G., and Ding, S. (2006). Self-renewal of embryonic stem cells by a small molecule. Proc Natl Acad Sci U S A *103*, 17266-17271.