SUPPLEMENTARY FIGURE LEGENDS

Fig. S1: Validation of the partial trypsination protocol. Expression of CD34, myogenin and MyoD were used as markers of reserve cells, differentiated cells and myoblasts/early myotubes, respectively. RT-PCR of total RNA from unseparated cultures (U), myotube (MT), reserve cell (RC) fractions and two intermediate washes showed that the MT fraction contained MyoD and myogenin transcripts, whilst the RC fraction expressed only CD34, consistent with the expected phenotypes. Cells detached by intermediate washes expressed MyoD and/or myogenin, presumably myoblasts and differentiated mononucleated myocytes.

Fig. S2: (**A** and **B)** Confirmation of knockdown efficiency of Notch3 (**A**) and Dll4 (**B**) protein expression by shRNA.

Proliferating C2C12 myoblasts were transfected with SureSilencing™ shRNA/GFP plasmids containing either the shRNA sequence (knockdown) or an irrelevant, scrambled sequence. After 24 hours, the cultures were transferred to differentiation medium for 48 hours and expression of Notch3 and Dll4 analysed by Western blotting. Transfection with the relevant shRNA resulted in highly effective knockdown of Notch3 (**A**) and Dll4 (**B**) compared with untransfected (C2C12) and control cultures transfected with the scrambled sequence (Cont.). Expression of Notch1 was unaffected in Notch3 knockdown cultures (**A**); Notch3 expression was decreased in Dll4 knockdown cultures (**B**). α-tubulin expression was used as a loading control (α-Tub).

(C and **D**) The effects of Notch3 knockdown and expression of constitutively active Notch3 on the differentiation of mouse primary myoblasts.

To investigate the effects of Notch3 overexpression, satellite cells isolated from mouse hind limb muscles were infected with retrovirus encoding the Notch3 ICD (N3ICD) or empty vector (Control), both with an *IRES-eGFP* to mark infected cells: to investigate the effects of Notch3 inhibition, satellite cells were transfected with shRNA (N3 knockdown) or an irrelevant, scrambled sequence (Cont shRNA).

(**C**) Cultures were allowed to differentiate for 5 days and immunostained for the expression of skeletal muscle myosin (SkMM, red) as a marker of differentiation and counterstained with DAPI (blue) to reveal all nuclei. Representative images of infected/transfected cultures are shown. The proportion of nuclei in differentiated, SkMM+ve cells were counted and the data shown in the adjacent bar graph. Expression of constitutively active Notch3 (ICD) resulted in a significant decrease in the percentage of nuclei in differentiated cells compared with cultures infected with empty virus (Control). Conversely, inhibition of Notch 3 activity (Knockdown) caused a significant increase in the proportion of nuclei in differentiated cells compared with cultures transfected with the control, scrambled shRNA (Control). These results show that Notch3 expression inhibits the differentiation of primary myoblasts. The SkMM-ve mononucleated cells remaining in the cultures were then analysed for the expression of GFP to determine the proportion that had been infected/transfected. The data are presented as bar graphs and show that in cultures infected with Notch3 ICD (ICD), significantly more undifferentiated cells were GFP+ve compared with cultures infected with empty retrovirus (Control); whereas in cultures where Notch3 activity had been inhibited by shRNA knockdown (Knockdown), significantly fewer undifferentiated cells were GFP+ve than in cultures transfected with the scrambled shRNA (Control). This

suggests that the effects of modulating Notch3 activity on differentiation determined by the percentage of nuclei in myotubes are underestimations as they do not account for transfection/infection efficiencies. Thus, whilst ≈30% of cells remained undifferentiated in Notch3 knockdown cultures, the majority of these (≈90%) were untransfected, GFP-ve and presumably expressing Notch3. Similarly, the inhibitory effects of Notch3 ICD are likely to have been underestimated as a proportion of the cells were uninfected, GFP-ve and therefore Notch3 ICD-ve.

(**D**)The status of the remaining undifferentiated cells was further investigated by immunostaining for the expression of either Pax7 or MyoD. Representative images are shown stained for Pax7 (red), GFP (green) and counterstained with DAPI (blue) (Pax7/GFP/DAPI), or MyoD (red), GFP (green), also counterstained with DAPI (blue) (MyoD7/GFP/DAPI). Quantitation of these experiments are presented in the adjacent bar graphs. In control cultures infected with empty vector (Notch3 ICD control) or transfected with control shRNA (Notch3 shRNA Knockdown control), ≈5% of the transduced, GFP+ve cells were Pax7+ve. However, in cultures expressing Notch3 ICD (which contained significantly more undifferentiated cells (**B**)), the proportion of Pax7+ve/GFP+ve cells was 4-5 fold greater (N3ICD: representative Pax7+ve/GFP+ve cells are indicated by arrows; Pax7-ve/GFP+ve by arrowheads). In contrast, almost all remaining GFP+ve mononucleated cells in the Notch3 shRNA knockdown cultures were Pax7-ve (N3 knockdown: representative Pax7-ve/GFP+ve cells are indicated by arrowheads).

In replicate control cultures (empty vector or scrambled shRNA) the majority (≈70-80%) of GFP+ve mononucleated cells were MyoD+ve. MyoD expression was significantly

reduced to ≈5% in cultures expressing constitutively active Notch3 (N3ICD: representative MyoD-ve/GFP+ve are indicated by arrowheads), but was increased to greater than 90% of the few remaining GFP+ve mononucleated cells in shRNA knockdown cultures (N3knockdown: representative MyoD+ve/GFP+ve are indicated by arrows).

Together, these findings show that expression of constitutively active Notch3 inhibits the differentiation of primary skeletal muscle cultures, resulting in the persistence of an increased number mononucleated cells with increased Pax7 and decreased MyoD expression, consistent with a shift towards the quiescent reserve cell phenotype at the expense of terminal differentiation. The requirement for Notch3 activity is further suggested by the observations that in shRNA knockdown cultures, the number of undifferentiated cells is significantly reduced and those that do remain are Pax7-ve/MyoD+ve, indicative of unfused myoblasts rather than quiescent reserve cells.

In **C** and **D**, bar graphs show the mean ± SEM from 3 cultures (100-200 cells counted per culture), statistically compared using Student's t-test. Scale bar equals 50μM.

FIGURE S1

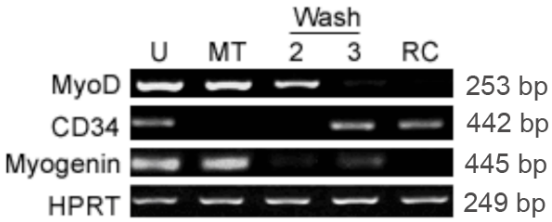
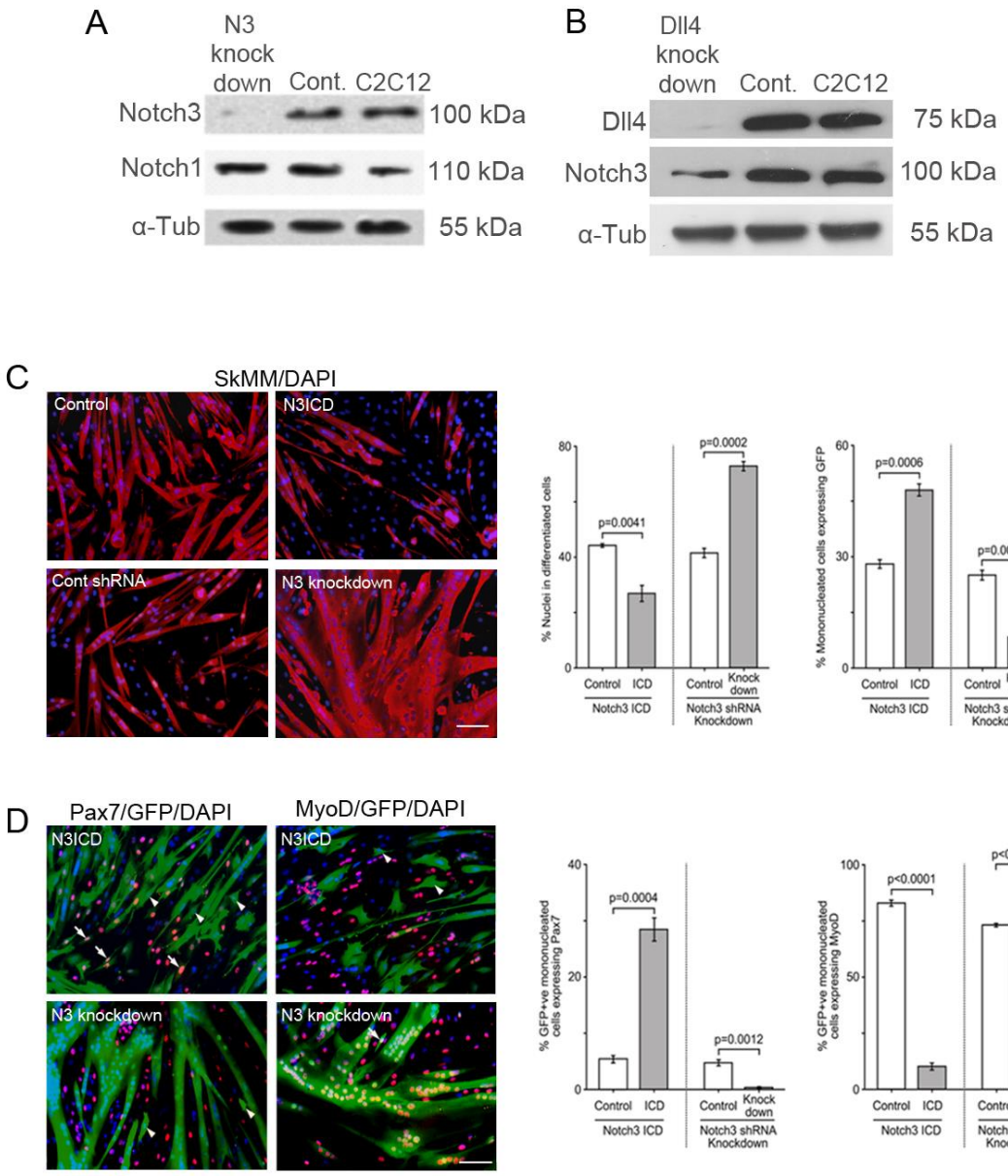

Wash
U
MT
2
3
RC
MyoD
CD34
Myogenin
HPRT
253 bp
442 bp
445 bp
249 bp

FIGURE S2


A
N3 knock down
Cont.
C2C12
Notch3
100 kDa
Notch1
110 kDa
α-Tub
55 kDa
B
Dll4 knock down
Cont.
C2C12
Dll4
75 kDa
Notch3
100 kDa
α-Tub
55 kDa
C
SkMM/DAPI
Control
N3ICD
Cont shRNA
N3 knockdown
% Nuclei in differentiated cells
p=0.0041
p=0.0002
Control
ICD
Knock down
Notch3 ICD
Notch3 shRNA Knockdown
% Mononucleated cells expressing GFP
p=0.0006
p=0.0007
D
Pax7/GFP/DAPI
MyoD/GFP/DAPI
N3ICD
N3 knockdown
% GFP+ve mononucleated cells expressing Pax7
p=0.0004
p=0.0012
% GFP+ve mononucleated cells expressing MyoD
p<0.0001
p<0.0001