DOI:
10.1002/stem.2757

Document Version
Peer reviewed version

Delta-like 4 on nascent myotubes activates Notch3 to regulate self-renewal in skeletal muscle stem cells

SiewHui Low[1,4], Josephine L. Barnes[2], Peter S. Zammit[3] and Jonathan R. Beauchamp[4]

[1]Carnegie Institution for Science, Baltimore MD 21201, USA.

[2]University College London, Centre for Inflammation and Tissue Repair, London WC1E 6JF, UK.

[3]King's College London, Randall Centre for Cellular and Molecular Biophysics, London SE1 1UL, UK.

[4]Centre for Biomedical Sciences, School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, TW20 0EX, UK.

Correspondence: SiewHui Low, Ph.D., Carnegie Institution for Science, Baltimore MD 21201, USA.

Telephone: 410-246-3039 ; Fax: 410-243-6311 ; Email: siewhuilow@gmail.com

Author contributions:

S.L, J.L.B and J.R.B. conceptualized the study; S.L and J.L.B performed experiments, all authors analysed and interpreted results, J.R.B. initiated and supervised the project. All four authors wrote, discussed and edited the manuscript.

ABSTRACT

Notch signalling is essential to maintain skeletal muscle stem cells in quiescence. The precise roles of the different Notch receptors are, however, incompletely defined. Here we demonstrate a role for Notch3 in the self-renewal of muscle stem cells. We found that Notch3 is active in quiescent C2C12 reserve cells, and Notch3 over-expression and knockdown studies in C2C12 and primary satellite cells reveal a role in self-renewal. The Notch ligand Delta-like 4 (Dll4) is expressed by newly formed myotubes and interaction with this ligand is sufficient to maintain Notch3 activity in quiescent C2C12 reserve cells to prevent activation and progression into the cell cycle. Thus our data suggest a model whereby during regeneration, expression of Dll4 by nascent muscle fibres triggers Notch3 signalling in associated muscle stem cells to recruit them to quiescence, thereby renewing the stem cell pool.

INTRODUCTION

Satellite cells (SCs) are resident skeletal muscle stem cells for postnatal muscle growth, maintenance, and repair/regeneration [1-3]. Notch signalling has been implicated in maintaining SC quiescence [4,5] as well as promoting SC proliferation during regeneration [6,7]. Notch receptor activation is initiated by interaction with a DSL (for Delta-Serrate- Lag) ligand that promotes a series of conformational and proteolytic events that release the active Notch intracellular domain (ICD) which translocates to the nucleus and associates with the DNA-binding protein CSL (for CBF1/Su(H)/Lag-1: also known as RBP-Jκ or Rpbsuh). In the absence of Notch ICD, CSL functions as a transcriptional repressor: binding of Notch ICD displaces associated co-repressor molecules and recruits a complex of co-activators, thereby acting as a switch for the expression of Notch-responsive genes, primarily those of the *Hes* and *Hey* families [8]. Although the core pathway is highly conserved, Notch signalling has a diverse (often conflicting) range of context-dependent biological functions probably due to the selective activation of a functionally heterogeneous group of downstream effectors by different combinations of the four Notch receptors (Notch1-4) and five DSL ligands (Delta-like (Dll) 1, 3 and 4 and Jagged 1 and 2) identified in mammals. For example, Notch1 has been reported to mediate SC transition into quiescence *in vivo* [9], and also myoblast proliferation *in vitro* [6]. *Notch3* is expressed by quiescent SCs [10, 11] and *Notch3* mutant mice regenerate more muscle and SCs after repeated injuries [12]. Although, these studies implicate Notch3 in SC homeostasis, the underlying cellular mechanism(s) are unclear.

MATERIALS AND METHODS

Primary Cell Culture

Mice were bred and experimental procedures passed by the Ethical Review Process Committee of King's College London were carried out under the provisions of the Animals (Scientific Procedures) Act 1986. Freshly isolated myofibers [13, 14] were mildly digested with 0.125% trypsin–EDTA for 10 min at 37 °C, and the released satellite cells were plated on Matrigel in 75 cm^2 flasks or 6-well plates and maintained at low density for several days in DMEM supplemented with 20% FBS, 1% chicken embryo extract, 4 mM L-glutamine and 100 IU/ml penicillin/100 μg/ml streptomycin. Cells were then trypsinised and seeded in Lab-Tek 8-well chamber slides for transfection.

C2C12 maintenance, differentiation and reserve cell isolation

C2C12 myoblasts (European Collection of Cell Cultures) were maintained in growth medium supplemented with 20% foetal calf serum (PAA Laboratories) and induced to differentiate in medium containing 5% horse serum (PAA Laboratories) as described previously [13]. After 5 days of differentiation, cultures were separated into myotube and reserve fractions by partial trypsinisation. Briefly, cultures were rinsed with PBS and then incubated in 0.01% trypsin (Sigma) in PBS until the myotubes were seen to detach from the substrate. The released myotubes were retained and the adherent cells rinsed again with PBS and then subjected to a series of 3, 1 min incubations in 0.01% trypsin (with intervening PBS washes) to remove residual myoblasts and myocytes. The remaining reserve cells were either processed to obtain RNA or protein, or re-plated for

activation in growth medium or in co-culture. The successful enrichment of the myotube and reserve cell fractions was confirmed by RT-PCR (Fig. S1).

Western Blotting

15μg of total protein (extracted by cell lysis in RIPA buffer containing protease inhibitor cocktail (Pierce)) were separated on 4-12% polyacrylamide gradient gels (In Vitrogen) and transferred onto nitrocellulose membrane (Hybond-ECL: GE Healthcare). Membranes were probed with goat polyclonal anti-Notch 1 (M20, 1:200), anti-Notch3 (M20, 1:200), anti-Jagged 1 (C20, 1:200), anti-Dll1 (T-20, 1:100), anti-Dll4 (C20, 1:100) (all from Santa Cruz Biotechnology), rat monoclonal anti-activated Notch1 (bTAN 20, 1:50, DSHB), mouse monoclonal anti-cyclin A (C4710, 1:1000) and anti-α-tubulin (T9026, 1:2500) (both from Sigma). Primary antibodies were detected using appropriate species-specific, HRP-conjugated secondary antibodies (DakoCytomation) in conjunction with an ECL chemiluminescent detection kit (Pierce). An intermediate biotinylated secondary antibody followed by streptavidin-HRP (1:4000, Invitrogen) was used to detect Notch3.

Reverse Transcription and PCR (RT-PCR)

Total RNA was extracted using Trizol (Invitrogen) according to manufacturer's instructions. RT-PCRs were carried out using a One-Step kit (Qiagen) with the following murine gene-specific primer pairs (all 5'→3'): Notch1 forward TGGACGCCGCTGTGAGTCA, Notch1 reverse TGGGCCCGAGATGCATGTA (202bp product, 28 cycles); Notch3 forward CCTGGATGCTGGGGCGGACAC, Notch3 reverse

CGGCATGGCTGGCGATGAGCT (218bp product, 28 cycles); MyoD forward CGGCGGCAGAATGGCTACGA, MyoD reverse GAGGGGCGGCGTCGGGAGAC (253bp product, 25 cycles); HPRT forward GCTGGTGAAAAGGACCTCTC, HPRT Reverse CACAGGACTAGAACACCTGC (249bp product, 25 cycles). Additional RT-PCRs presented in Fig. S1 were carried out using: CD34 forward TGGAATCCGAGAAGTGAGGT, CD34 reverse ACCCAGCCTTTCTCCTGTAG (442bp product, 28 cycles); myogenin forward TTGCTCAGCTCCCTCAACCA, myogenin reverse TGGGCTGGGTGTTAGCCTTA (445bp product, 28 cycles).

Retroviruses

An eGFP-mouse Notch1 ICD fusion cDNA (EGFP-NIC [15]) was cloned into pMSCV-puro (Clontech); cDNA encoding the murine Notch3 intracellular domain fused to a carboxy-terminal HA tag (N3IC-HA [16]) was cloned into pMSCV-IRES-GFP, a modified version of pMSCV-puro [19]. Retroviruses were packaged in HEK 293T cells using standard methods.

Retroviral Infection and Immunostaining of Satellite Cells on Isolated Myofibres

Myofibres were isolated from extensor digitorum longus muscles of 12 week-old C57Bl/10 mice as described by Rosenblatt *et al.* [20]. Freshly isolated myofibre preparations were retrovirally infected and then maintained in suspension for 72 h in DMEM supplemented with 10% horse serum and 0.5% chick embryo extract (PAA Laboratories) as described previously [19].

Fibres were fixed for 20 min in 4% paraformaldehyde in PBS, permeabilised with 0.1% Triton X-100 for 10 min and then incubated for at least 30 minutes in 20% (v/v) normal goat serum in PBS to block non-specific antibody binding, all at room temperature. Rabbit polyclonal anti-GFP (1:400, Molecular Probes) and either mouse monoclonal anti-Pax7 (1:50; DSHB), anti-MyoD (clone 5.8a, 1:50; DakoCytomation) or anti-Dll4 (C20, 1:50; Santa Cruz Biotechnology) were applied for 16 h at 4°C. Fibres were incubated for 1 h at room temperature in flurochrome-conjugated species-specific secondary antibodies (Molecular Probes) to visualise primary antibody binding. All antibodies were diluted in 6% horse serum in PBS and the fibres were mounted in DakoCytomation Faramount fluorescent mounting medium containing 100ng/ml 4,6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain.

Retroviral Infection and Immunostaining of C2C12 Myoblasts

Proliferating C2C12 myoblasts were infected with retroviruses as described previously [19]. After 48 hours, infected GFP+ve cells were sorted using a FACS DiVa flow cytometer and cell sorter (Becton Dickinson) and expression of the relevant Notch ICD confirmed by Western blotting. Infected myoblasts were seeded into LAB-TEK® Permanox™ 8-well chamber slides (Nunc) in growth medium and 24 h later, pulsed for 1 hour with 40μM BrdU. Cultures were fixed, permeabilised and immunostained with either mouse monoclonal anti-MyoD antibody (see above) or rat monoclonal anti-BrdU antibody (clone BU1/75, 1:500, Abcam) as described previously [13]. Primary antibody binding was detected using species-specific flurochrome-conjugated seconday antibodies

(Molecular Probes) before mounting in DakoCytomation Faramount fluorescent mounting medium containing 100ng/ml (DAPI).

shRNA knockdown of Notch3 and Dll4

Knockdown of Notch3 and Dll4 expression was achieved using a SureSilencing™ shRNA/GFP plasmid (SABiosciences) encoding the shRNA TCCCAGCGAGCATCCTTATTT and AACAGTGGGCCAAAGGGTTAT, respectively. Effective knockdown was confirmed by Western blot analysis of Notch3 and Dll4 expression in transiently transfected C2C12 cultures (Fig. S2 A and B). Proliferating myoblasts were co-transfected with pCI-neo and either the knockdown plasmid or the supplied control plasmid consisting of an encoding an irrelevant, scrambled shRNA sequence, using Lipofectamine 2000 (Invitrogen) and selected with G-418. GFP expression was confirmed by flow cytometry before seeding in LAB-TEK® Permanox™ 8-well chamber slides for differentiation. After 5 days in differentiation medium, cultures were fixed, permeabilised and immunostained with mouse monoclonal anti-skeletal muscle myosin (clone MF20, 1:50, DSHB) and rabbit polyclonal anti-GFP antibodies (A11622, 1:400, Molecular Probes), followed by appropriate species-specific fluorochrome-conjugated secondary antibodies, as above.

Co-culture of Reserve Cells With DSL Ligand-Expressing 3T3 Cells

Full-length murine Dll4 and Dll1 [21] and Jagged 1 [22] cDNAs were each cloned into pCI-neo (Promega) and transfected into 3T3 fibroblasts using Lipofectamine 2000. Transfected cells were selected by using G-418 and expression of the relevant ligand

confirmed by RT-PCR and Western blotting (no endogenous expression of Dll1, Dll4 or Jagged 1 was detected).

For co-culture, 3T3 fibroblasts were grown to confluence in LAB-TEK® Permanox™ 8-well chamber slides (for analysis by immunofluorescent staining) or 10cm-diameter dishes (for Western blot analysis) prior to the addition of reserve cells. C2C12 myoblasts were transfected with pmaxFP-Green-N (Amaxa) using Lipofectamine 2000 and transfectants selected with G-418. Cultures of GFP+ve myoblasts were allowed to differentiate for 5 days, the reserve cells were isolated and then applied to the fibroblast monolayers (4000 reserve cells/well; 2 x 10^5 reserve cells/dish) an approximate ratio of 1:3 (reserve cell: fibroblast). Co-cultures were maintained for 24 h in growth medium prior to analysis. Co-cultures maintained in chamber slides were pulsed with BrdU for 1 h and analysed by immunostaining; co-cultures in dishes were used to produce protein and RNA for Western blotting and RT-PCR.

RESULTS

We initially investigated the role of Notch3 by use of the C2C12 reserve cell model [18] to model cell fates adopted during muscle regeneration. In this model, when stimulated to differentiate, most cells form multinucleated myotubes, but a fraction of cells become quiescent reserve cells (Fig. 1A). Reserve cells resemble quiescent SCs in that they express Pax7 and CD34 but not MyoD, bind Lysenin, and can be reactivated to again proliferate [17-19]. Evaluating the levels of active Notch1 and Notch3 during the differentiation time course by Western blot (Fig. 1B), we found that active Notch1 is present throughout, whilst Notch3 is up-regulated 1 day after differentiation is induced,

reaching maximum levels after 3 days. Notch3 up-regulation was coincident with the appearance of myotubes. After 5 days, myotube and reserve cell fractions were separated for Western blot analysis, which revealed that active Notch1 was mainly in myotubes, whereas active Notch3 was exclusively in reserve cells, consistent with *Notch3* expression in quiescent SCs [10,11].

To examine the role of Notch3, we activated Notch signalling through Notch3 and Notch1 by retroviral-mediated expression of a constitutively active Notch3-intracellular domain (Notch3-ICD) or a Notch1-ICD (for comparison) in SCs on isolated murine muscle fibres (Fig. 2A-C); GFP co-expression was used for identifying transduced cells. 72 hours after infection we found that, whereas expression of Notch1 ICD had no significant effect on proliferation, SC expressing active Notch3 ICD gave rise to significantly fewer progeny compared with controls infected with parental retrovirus, whereas expression of Notch1 ICD had no significant effect on proliferation. In the control preparations, ~50% of GFP+ve cells were Pax7+ve (stem cell fate) and ~75% expressed MyoD (progenitor fate). In contrast, ~ 90% of cells expressing Notch1-ICD or Notch3-ICD were Pax7+ve. Notch1-ICD reduced the MyoD+ve fraction, whilst Notch3-ICD nearly abolished this fraction. Similar results were found using C2C12 myoblasts (Fig. 2D): both Notch1-ICD and Notch3-ICDs reduced the MyoD+ve fraction, whilst only Notch3-ICD reduced their proliferation rate, as assessed measuring by BrdU incorporation. There are conflicting reports on the effect of Notch1 activity on muscle stem cell behaviour: whilst overexpression of exogenous Notch1-ICD has been shown to promote proliferation [6,7], SC-specific genetic gain-of-function studies suggest that

Notch1 activity directly inhibits cell division [9]. We found that whilst transfection with Notch1-ICD significantly inhibited myogenic progression in terms of MyoD expression, there was no effect on proliferation rate, possibly because of a maximal response to endogenous Notch1 activity. In contrast, Notch3 induced a MyoD-ve, non-proliferative 'quiescent' state in SC, C2C12 and primary myoblasts.

Conversely, we asked whether reducing Notch3 would reduce the number of myoblasts entering quiescence? We used shRNA (with GFP co-expression) to knockdown *Notch3*, just prior to initiating differentiation in C2C12 myoblasts (Fig. 2E) and primary myoblasts (Fig. S2C-D); efficient reduction of Notch3 expression was achieved (Fig. S2A). *Notch3* knockdown resulted in more myotubes (expressing skeletal muscle myosin, SkMM) (Fig. 2E, Fig. S2C), more MyoD+ve and fewer Pax7+ve cells (Fig. S2D) compared with the scrambled shRNA control. The proportion of GFP+ve SkMM-ve cells (i.e. reserve cells) was significantly lowered by *Notch3* knockdown, relative to controls (Fig 2E, Fig. S2C), whereas N3-ICD overexpressing primary cultures contained less myotubes compared to controls (Fig. S2C). Significantly, *Dll4* knockdown shRNA also resulted in more myotubes (Fig. 2F) and fewer undifferentiated cells (Fig. 2F) compared with the scrambled shRNA control.

We next examined the activities of Notch1 and Notch3 as reserve cells re-entered the cell cycle. Isolated reserve cells were stimulated with growth medium and pulse-labelled with BrdU at selected time points. Almost all cells entered S-phase within 12-24 hours (Fig. 3A). Re-entering the cell cycle was preceded by down-regulation of *Notch3* mRNA and active Notch3 protein. In contrast, *Notch1* mRNA and active Notch1 protein displayed concomitant up-regulation with *MyoD* mRNA and CyclinA (Fig. 3B,C).

Proliferating myoblasts expressed Notch ligands Dll1, Jagged1 and barely detectable levels of Dll4. Expression of all three increased during differentiation but with different distributions: Dll1 expression was higher in myotubes than in reserve cells; Dll4 was restricted to myotubes, and Jagged 1 to reserve cells. Dll3 was not detected and Jagged2 showed the same pattern as Jagged1.The findings suggested that the presence of Dll1 or Dll4 on new myotubes, or Dll1 or Jagged1 on reserve cells, could be responsible for activating Notch3 in adjacent cells.

To test their roles individually, reserve cells from differentiated GFP+ve C2C12 cultures were isolated and co-cultured in growth medium with wildtype NIH3T3 cells (control) or NIH3T3 cells expressing Dll1, Dll4 or Jagged1, to activate Notch signalling. When cultured alone or with control NIH3T3 cells, approximately 90% of the reserve cells had entered the cell cycle and incorporated BrdU after 24 hours. Co-culture with NIH3T3 cells expressing Dll1 or Jagged1 had no effect on reserve cell activation and proliferation (Fig 4A). In marked contrast, co-culture with Dll4-expressing cells significantly inhibited reserve cell activation and proliferation. After 24 hours in growth medium, only~20% had entered the cell cycle and incorporated BrdU. The remaining~ 80% had not incorporated BrDU and were still quiescent, as determined by lysenin binding (Fig. 4B). Importantly, reserve cells co-cultured with Dll4-expressing NIH3T3 cells contained the active form of Notch3, but the inactive form of Notch1 (Fig. 4C), as opposed to the presence of the active forms of both Notch3 and Notch1 under co-culture with control NIH3T3 or those expressing Dll1 or Jagged1 (Fig. 4C). RT-qPCR data further confirmed that exposure of reserve cells to Dll4 maintained *Notch3* expression and prevented up-regulation of *Notch1* and *MyoD* (Fig 4D). In freshly isolated murine

myofibres, immunolabelling revealed that quiescent satellite cells were exposed to Dll4 until at least 24 hours of culture, but that Dll4 expression was lost by 48 hours (Fig. 4E).

DISCUSSION

Different combinations of Notch receptor and ligand are known to result in different outcomes, even within the same biological system [21, 23]. Here we defined the paired actions of Notch3 in stem/progenitor cells and Dll4 in muscle fibres to establish and maintain a quiescent stem cell phenotype. It is interesting to note that Bi *et al*. [24] reported that Notch1 activation in myofibres results in the upregulation of Dll4 expression which could contribute to the sequestration and maintenance of the satellite cell population during regeneration. Our results help explain the *Notch3* mutant muscle regeneration phenotype, in which SCs do not adopt self-renew effectively and excessive muscle regeneration occurs [12]. Notch1 was recently been shown to instruct proliferative SCs to transition into quiescence in juvenile mice [9]. It is possible that Notch1 has a unique property during the juvenile stage, or that inactivation of Notch1 also inadvertently reduce or inhibit Notch3 activity. However, these data are consistent with the hypothesis that myotubes provide the signal to impose SC quiescence. Such a feedback mechanism by which differentiated cells are responsible for the recruitment and maintenance of a stem cell compartment may explain how, following widespread damage muscle regeneration restores both functional tissue and an equivalent stem cell compartment.

CONCLUSION

Our work shows that the expression of Dll4 in myotubes activates Notch3 in adjacent myoblasts to signal them to stop proliferating and return to quiescence. By thus controlling muscle stem cell self-renewal, the Dll4/Notch3 axis restores the stem cell pool after muscle repair/regeneration to be able to respond to further needs of homeostasis, hypertrophy or repair.

ACKNOWLEDGEMENTS

We would like to thank K. Tezuka (Gifu University, Japan) and I. Screpanti (University "La Sapienza", Italy) for the EGFP-NIC (Notch1 ICD) and Notch3IC-HA (Notch3 ICD) plasmids, respectively. Jagged 1 cDNA was provided by M. Bevan (University of Washington, USA), Dll1 and Dll4 cDNAs were a gift from A. Freitas (Pasteur Institute, France). And we also thank Dr. Chen-Ming Fan for critical reading of the manuscript. This work was supported by the MYORES Network of Excellence, contract 511978, from the European Commission 6th Framework Programme. The Zammit laboratory is generously supported by the Association Francaise contre les Myopathies (20082, 16050 and 17865), the Medical Research Council (MR/P023215/1), Muscular Dystrophy UK (RA3/3052/1) and the FSH Society (FSHS-82013-06 and FSHS-82016-03) and the King's Health Partner's Research and Development Challenge Fund (R151006).

The authors declare no conflict of interest and have approved the final article.

REFERENCES

1. Hawke TJ, Garry DJ. Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol*. 2001; 91(6):2414.
2. Collins CA, Olsen I, Zammit PS et al. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell.* 2005;122(2):289-301.
3. Montarras D, Morgan J, Collins CD et al. Direct isolation of satellite cells for skeletal muscle regeneration. *Science*. 2005;309(5743):2064-7.
4. Mourikis P, Sambasivan R, Castel D et al. A critical requirement for notch signaling in maintenance of the quiescent skeletal muscle stem cell state. *Stem Cells*. 2012, 30 (2): 243-252.
5. Bjornson CR, Cheung TH, Liu L et al. Notch Signaling Is Necessary to Maintain Quiescence in Adult Muscle Stem Cells. *Stem Cells*. 2012; 30(2): 232–242.
6. Conboy IM, Rando TA: The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev Cell*. 2002, 3 (3): 397-409.
7. Brack AS, Conboy IM, Conboy MJ et al. A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell Stem Cell*. 2008;2(1):50-9.
8. Fiúza UM, Arias AM. Cell and molecular biology of Notch. *J Endocrinol*. 2007;194(3):459-74.

9. Wen Y, Bi P, Liu W et al. Constitutive Notch Activation Upregulates Pax7 and Promotes the Self-Renewal of Skeletal Muscle Satellite Cells. *Mol Cell Biol*. 2012:32(12) 2300-2311.
10. Fukada S, Uezumi A, Ikemoto M et al. Molecular signature of quiescent satellite cells in adult skeletal muscle. *Stem Cells*. 2007;25(10):2448-59.
11. Kuang S, Kuroda K, Le Grand F et al. Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell*. 2007;129(5):999-1010.
12. Kitamoto T and Hanaoka K. Notch3 null mutation in mice causes muscle hyperplasia by repetitive muscle regeneration. *Stem Cells.* 2010;28(12):2205-16.
13. Beauchamp JR, Heslop L, Yu DS et al. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *The Journal of Cell Biology*. 2000;151, 1221-1234.
14. Zammit PS, Golding JP, Nagata Y et al. Muscle satellite cells adopt divergent fates: a mechanism for self- renewal? *The Journal of Cell Biology*. 2004;166, 347-357.
15. Tezuka K, Yasuda M, Watanabe N et al., J Bone Miner Res 17, 231 (2002). J Bone Stimulation of osteoblastic cell differentiation by Notch. *Miner Res*. 2002 Feb;17(2):231-9.
16. Felli MP, Vacca A, Calce A et al. PKCθ mediates pre-TCR signaling and contributes to Notch3-induced T-cell leukemia. *Oncogene* 2005;**24**, 992–1000.
17. Nagata Y, Kobayashi H, Umeda M et al. Sphingomyelin levels in the plasma membrane correlate with the activation state of muscle satellite cells. *J Histochem Cytochem*. 2006;54(4):375-84.

18. Yoshida N, Yoshida S, Koishi K et al. Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *J Cell Sci*. 1998;111 (Pt 6):769-79.
19. Zammit PS, Relaix F, Nagata Y et al. Pax7 and myogenic progression in skeletal muscle satellite cells. *J Cell Sci*. 2006;119: 1824-1832.
20. Rosenblatt JD, Lunt AI, Parry DJ, Partridge TA. Culturing satellite cells from living single muscle fiber explants. In Vitro Cell Dev Biol Anim. 1995 Nov;31(10):773-9.
21. de La Coste, A., and A. A. Freitas. Notch signaling: distinct ligands induce specific signals during lymphocyte development and maturation. *Immunol. Lett.* 2006;102: 1–9.
22. Lehar SM, Dooley J, Farr AG et al. Notch ligands Delta 1 and Jagged1 transmit distinct signals to T-cell precursors. *Blood.* 2005 Feb 15;105(4):1440-7.
23. Rutz, S, Mordmuller B, Sakano S et al. Notch ligands Delta-like1, Delta-like4 and Jagged1 differentially regulate activation of peripheral T helper cells. *Eur. J. Immunol*. 2005;35: 2443–2451.
24. Bi P, Yue F, Sato Y et al. Stage-specific effects of Notch activation during skeletal myogenesis. *eLife*. 2016; 5: e17355.

FIGURE LEGENDS

Fig. 1: Active Notch 3 is expressed by quiescent myogenic cells. (**A**) Schematic depicting the alternate cell fates adopted during myogenic differentiation in vitro. When cultures of proliferating myoblasts (Mb) are induced to differentiate, most fuse together to form differentiated, multinucleated myotubes (Mt). In contrast, a minority become reserve cells (RC), adopting a phenotype equivalent to that of a quiescent satellite cell. After 5 days, Mt and RC fractions can be separated by partial trypsination. (**B**) Western blot analysis of Notch receptor and ligand expression in differentiating C2C12 cultures and in separated Mt and RC fractions, prepared after 5 days of differentiation (U = unseparated cultures). The two bands on blots probed with antibodies raised against the Notch receptor intracellular domains correspond to the membrane-bound inactive form (upper band) and to the active form (lower band) released by proteolytic cleavage [8]. α-Tubulin was used as a loading control.

Fig. 2: Notch 3 ICD promotes exit from the cell cycle. (**A**, **B** and **C**) Satellite cells on freshly isolated muscle fibres were transduced with retrovirus encoding Notch1 ICD (N1 ICD) or Notch3 ICD (N3 ICD) or an empty control vector (Cont), with an *IRES-eGFP* marking transduced cells. Immunostaining for Pax7 and GFP 24 h after transduction showed that approximately 50% of satellite cells had been infected. (**A**) The number of GFP+ve cells per fibre (n = 10 fibres per group) and the proportion of GFP+ve cells expressing either Pax7 or MyoD (n = 150-250 GFP+ve cells) were determined after 72 h. Data are mean ± SEM, compared using Student's t-test. (**B**) Representative clusters of satellite cell progeny, immunolabelled for Pax7 (red) and GFP (green). Arrowheads

indicate Pax7-ve/GFP+ve cells. (**C**) Representative clusters immunolabelled for MyoD (red) and GFP (green), arrowheads indicate MyoD-ve/GFP+ve cells. GFP produced as a fusion with Notch1 ICD localised to the nucleus; GFP in control and Notch3 ICD-expressing cells was produced from an IRES and therefore is cytoplasmic. Preparations were counterstained with DAPI to reveal all nuclei: scale bar represents 10μM. (**D**) Effects of Notch3 and Notch 1 ICD on C2C12 myoblasts. Proliferating myoblasts transduced with the same retroviruses were pulsed for 1 h with BrdU and immunolabelled for MyoD and BrdU. Representative untransfected (C212) and Notch3 ICD-expressing cultures are shown, positive cells show red nuclear staining. Data are the mean ± SEM from 3 cultures (100-200 cells per culture), statistically compared using Student's t-test. Scale bar equals 50μM. (**E** and **F**) shRNA knockdown of Notch3 (E) or Dll4 (**F**). C2C12 myoblasts transfected with an shRNA (N3 or Dll4 knockdown) or an irrelevant, scrambled sequence (Cont) were allowed to differentiate for 5 days. Cultures were stained for skeletal muscle myosin (SkMM, red), a marker of differentiation and counterstained with DAPI to reveal all nuclei (blue) to determine the proportion of nuclei in differentiated cells. Immunolabelling of replicate cultures for GFP was used to determine the proportion of undifferentiated cells expressing the shRNA plasmids. Data are the mean ± SEM of 3 cultures (200 nuclei per culture), compared by Student's t-test. Scale bar equals 100μM.

Fig. 3: During reserve cell activation, Notch3 signalling is suppressed while Notch1 signalling is activated. (**A**) Cultures of C2C12 reserve cells were transferred to growth medium and analysed at the shown time points following a 1 h pulse with BrdU.

Representative cultures immunolabelled to reveal BrdU (red) and counterstained with DAPI (blue) to identify all nuclei are shown. Data are the mean ± SEM of 3 cultures (100-200 cells per culture). Scale bar equals 50μM. (**B**) RT-PCR analysis of *Notch3*, *Notch1* and *MyoD* expression during reserve cell reactivation, with *HPRT* as a control (**C**) Western blot analysis of cultures harvested at the same time points. Active corresponds to the active form of Notch3, generated by cleavage at the membrane to generate N3-ICD; Inactive indicates the higher molecular mass membrane-bound form; and Unprocessed refers the receptor prior to S1 cleavage [8]. Antibody specific for the active form of Notch1 was used.

Fig. 4: Dll4 signals through Notch3 to maintain myogenic quiescence. (A-D) GFP+ve reserve cells were maintained in growth medium for 24 h either alone (RC), or in co-culture with wildtype NIH3T3 fibroblasts (Cont) or NIH3T3 cells expressing Dll1, Dll4 or Jagged 1 (Jag1). (**A**) Cultures pulsed with BrdU for 1 h (23-24 h) were immunolabelled for GFP (green) to identify reserve cells and BrdU incorporation (red), and counterstained with DAPI to reveal all nuclei, and the proportion of GFP+ve/BrdU+ve cells determined. Representative images of reserve cells co-cultured with wildtype NIH3T3 cells (Cont) and NIH3T3 cells expressing Dll4 only are shown. Arrows indicate BrdU-ve reserve cells; arrowheads indicate BrdU+ve reserve cells. (**B**) Replicate co-cultures were incubated with lysenin, a marker of quiescence [17] and the proportion of GFP+ve cells labelled with lysenin determined. Representative images of reserve cells co-cultured with control (Cont) and Dll4+ve NIH3T3 cells only are shown, immunolabelled for GFP (green) and lysenin binding (red). The arrow indicates a

lysenin+ve reserve cell in co-culture with control NIH3T3 cells: arrowheads indicate lysenin-ve reserve cells in co-culture with Dll4+ve NIH3T3 cells. All data are the mean of 3 cultures ± SEM (200 GFP+ve cells per culture), compared using Student's t-test. Scale bar equals 20μM. (**C**) Western blot and (**D**) RT-qPCR analysis of *Notch1* and *Notch3* expression in NIH3T3 cells alone (3T3), reserve cells cultured alone (RC) and in reserve cells co-cultured with wildtype NIH3T3 cells (3T3) or with NIH3T3 fibroblasts expressing either Dll1, Dll4 or Jagged1 (Jag1). (**E**) Single muscle fibres were isolated and grown in culture for 48 h. At least 30 fibres were analysed at 0 h (T0), 24 h (T24) or 48 h (T48) in culture. Cells labelled with Pax7 (green, arrows), Dll4 (red, arrows) and DAPI (blue) can be observed on isolated muscle fibres at 0 h (T0) and 24 h (T24) however Dll4 (red, arrowhead) was not detectable in fibres cultured for 48 h (T48). Scale bar equals 20μM.

FIGURE 1 Low et al.

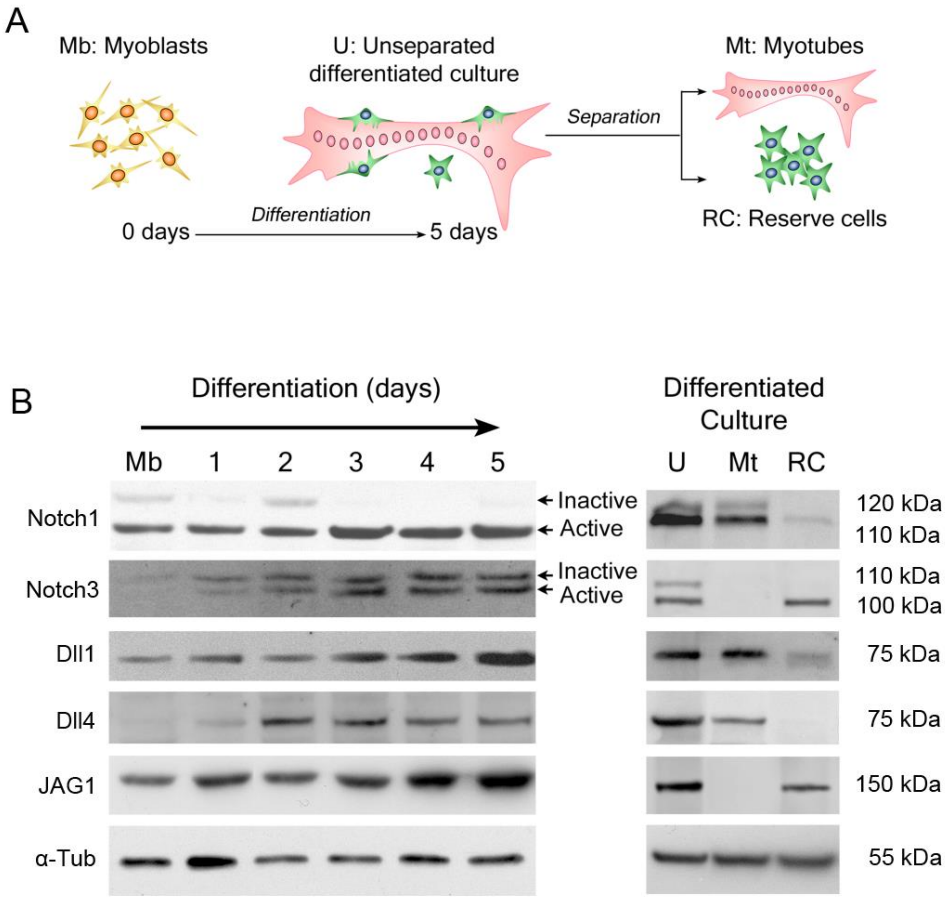

FIGURE 2 Low et al.

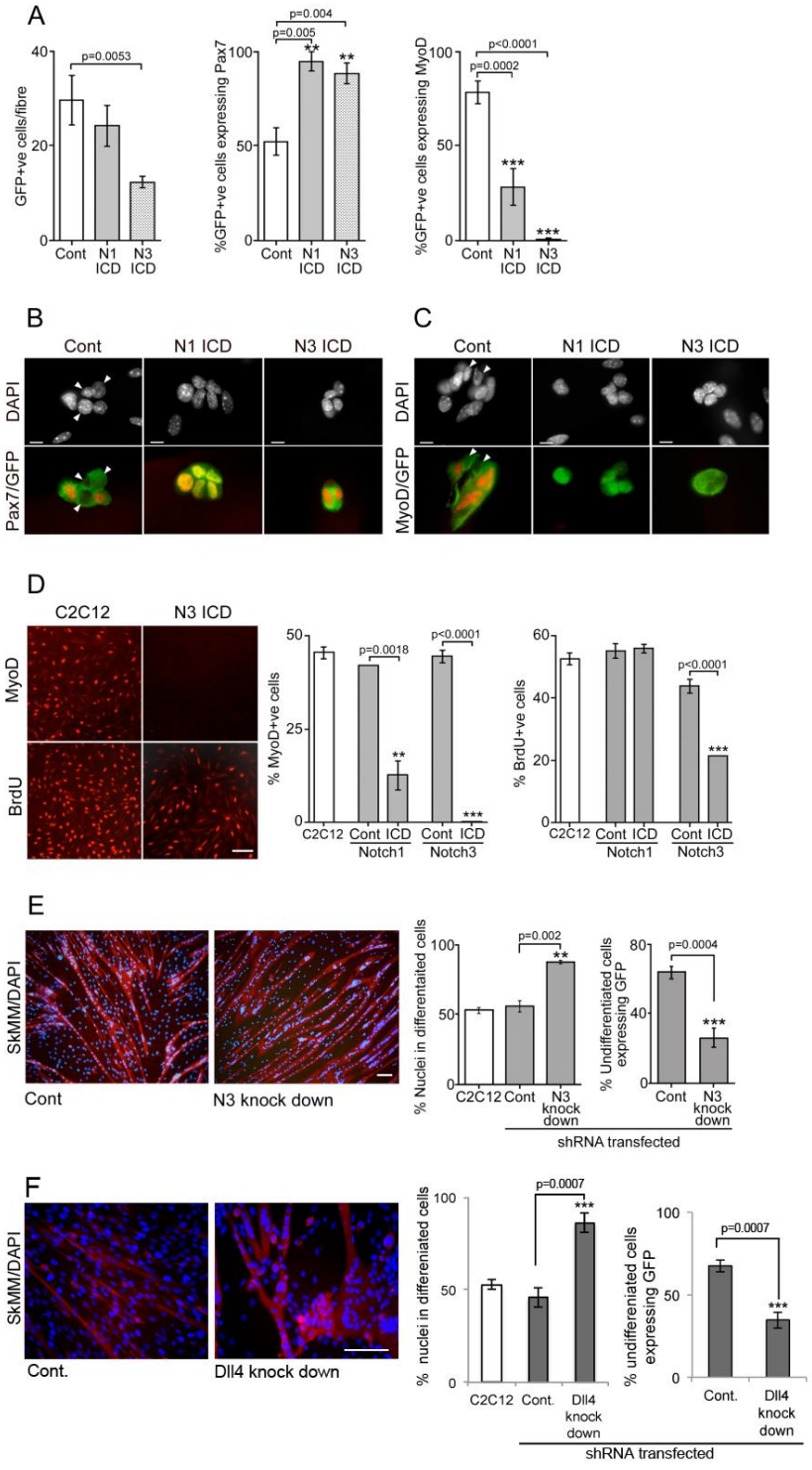

FIGURE 3 Low et al.

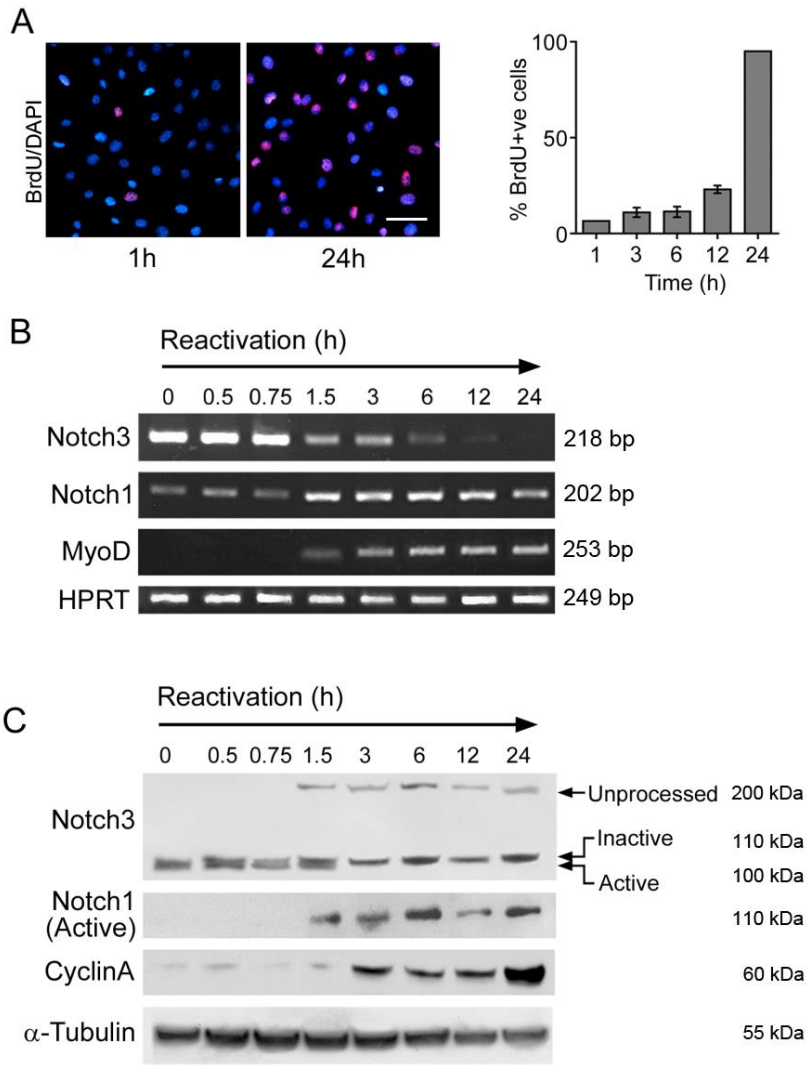

FIGURE 4 Low et al.