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Cell-autonomous Notch activity maintains the temporal specification potential of skeletal muscle stem cells

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SUMMARY

During organogenesis, a continuum of founder stem cells produces temporally distinct progeny until development is complete. Similarly, in skeletal myogenesis, phenotypically and functionally distinct myoblasts and differentiated cells are generated during development. How this occurs in muscle and other tissues in vertebrates remains largely unclear. We showed previously that committed cells are required for maintaining muscle stem cells. Here we show that active Notch signalling specifies a subpopulation of myogenic cells with high *Pax7* expression. By genetically modulating Notch activity, we demonstrate that activated Notch (NICD) blocks terminal differentiation in an Rbpj-dependent manner that is sufficient to sustain stem/progenitor cells throughout embryogenesis, despite the absence of committed progeny. Although arrested in lineage progression, NICD-expressing cells of embryonic origin progressively mature and adopt characteristics of foetal myogenic cells, including expression of the foetal myogenesis regulator *Nfix*. siRNA-mediated silencing of NICD promotes the temporally appropriate foetal myogenic fate in spite of expression of markers for multiple cell types. We uncover a differential effect of Notch, whereby high Notch activity is associated with stem/progenitor cell expansion in the mouse embryo, yet it promotes reversible cell cycle exit in the foetus and the appearance of an adult muscle stem cell state. We propose that active Notch signalling is sufficient to sustain an upstream population of muscle founder stem cells while suppressing differentiation. Significantly, Notch does not override other signals that promote temporal myogenic cell fates during ontogeny where spatiotemporal developmental cues produce distinct phenotypic classes of myoblasts.

KEY WORDS: Notch, Skeletal muscle, Spatiotemporal differentiation, Stem cells, Pax7, Myf5, Rbpj, Nfix

INTRODUCTION

How a continuum of founder stem cells generates distinct phenotypic classes of differentiating cells during organogenesis is a question of major interest in developmental biology. During *Drosophila* neuroblast divisions, the temporal expression of Pou domain and zinc finger transcription factors governs the neural identity of daughter cells, which is subject to their spatial identity (Kambadur et al., 1998; Isshiki et al., 2001; Grosskortenhaus et al., 2006). Similarly, in the vertebrate CNS, radial glial cells generate distinct neurons that are spatially specified largely via an interplay between homeodomain and bHLH transcription factors (Maurange, 2012). Although less complex in terms of cellular diversity, little is known about the generation of distinct classes of committed progeny during vertebrate skeletal muscle development.

Skeletal muscle founder stem cells in the body arise from a transient epithelial dermomyotome (DM) within somites and express the paired box/homeodomain transcription factors Pax3 and Pax7 (Ben-Yair and Kalcheim, 2005; Kassam-Duchossoy et al., 2005; Relaix et al., 2005). They undergo myogenic specification to give rise to muscle progenitor cells by sequential

expression of the bHLH myogenic regulatory factors (MRFs) *Myf5*, *Mrf4* (also known as *Myf6*) and *Myod*, and, subsequently, the differentiation gene myogenin (*Myog*) (Rudnicki et al., 1993; Kassam-Duchossoy et al., 2004; Tajbakhsh, 2009). During early embryonic stages, Pax7 marks bi-potent cells in the DM that can give rise to muscle and dermis (Ben-Yair and Kalcheim, 2005; Lepper and Fan, 2010). Dermal stem/progenitors later downregulate *Pax7* expression, in contrast to myogenic stem/progenitors, which maintain high levels of Pax7 during embryonic, foetal and postnatal myogenesis. This marker is then downregulated during myoblast differentiation (Tajbakhsh, 2009; Murphy and Kardon, 2011). Notably, embryonic [embryonic day (E) 9-14.5] and foetal (E14.5-18.5) myoblasts and differentiated cells are molecularly and phenotypically distinct (Cusella-De Angelis et al., 1994; Biressi et al., 2007a; Tajbakhsh, 2009). Embryonic myoblasts in the mouse fuse to form primary fibres from E11 and they establish the basic muscle pattern. Foetal myoblasts appear from E14.5 and subsequently form larger secondary fibres, and, unlike their embryonic counterparts, their differentiation is blocked by Tgf β and β -catenin (Cusella-De Angelis et al., 1994; Hutcheson et al., 2009). Juvenile satellite cells are then observed under the myofibre basement membrane from E16.5 and they act as reservoirs of quiescent adult muscle stem (satellite) cells from 3 weeks postnatally. Although it is thought that a continuum of stem cells that resists differentiation assures these waves of distinct myogenic populations (Gros et al., 2005; Kassam-Duchossoy et al., 2005; Relaix et al., 2005; Tajbakhsh, 2009; Murphy and Kardon, 2011) and that distinct transcriptome dynamics defines their progeny (Biressi et al., 2007b), little, if any, information is available on how these processes are regulated within muscle stem/progenitor cells. The identification of the transcription factor Nfix, which regulates

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foetal and suppresses embryonic myoblast fate, was a major step in understanding how downstream muscle progenitor cell fate decisions are mediated (Messina et al., 2010).

The Notch signalling pathway plays crucial roles in regulating the fate of stem cells in diverse tissues in metazoans (Fre et al., 2005; Androutsellis-Theotokis et al., 2006; Louvi and Artavanis-Tsakonas, 2006; Farnie and Clarke, 2007). Notch was shown to be essential for myogenic stem cell fate and differentiation throughout embryogenesis and in the adult, as both embryonic and adult muscle stem cells mutant for *Rbpj*, the downstream effector of canonical Notch signalling, differentiate precociously (Kuang et al., 2007; Schuster-Gossler et al., 2007; Vasyutina et al., 2007; Brack et al., 2008; Bjornson et al., 2012; Mourikis et al., 2012). It has been demonstrated that differentiating myogenic cells upregulate the Notch ligand delta-like 1 (*Dll1*) and stimulate Notch activity in the upstream population to assure their maintenance (Delfini et al., 2000; Hirsinger et al., 2001; Schuster-Gossler et al., 2007; Mourikis et al., 2012), in accordance with what has been shown in the nervous system (Henrique et al., 1997; Dong et al., 2012). Consistent with this notion, our previous studies have indicated that the muscle stem cell population requires the presence of differentiating cells for their maintenance, such that a lack of differentiated cells results in the loss of upstream Pax7⁺ cells in the foetus (Kassar-Duchossoy et al., 2005). Although active Notch signalling has been shown to block myogenic differentiation (Kopan et al., 1994; Shawber et al., 1996; Delfini et al., 2000; Hirsinger et al., 2001; Sun et al., 2008), a recent study in dorsal somitic muscle progenitor cells in the chick embryo reported that transient, but not sustained, activation of Notch is required for the expression of *Myod* and *Myf5* and for lineage commitment and differentiation (Rios et al., 2011). In addition, Notch signalling influences lineage diversification in the multipotent cells of the dorsal DM, where specifically in the lateral DM its activity biases progenitors toward a smooth muscle fate (Ben-Yair and Kalcheim, 2008). Therefore, although numerous studies point to Notch as a crucial regulator of myogenesis, it is not clear how this or other signalling pathways impact on the temporal release of distinct myogenic cells in vivo.

Using a combination of genetic tools and transcriptome analysis, we show that Notch is active throughout development in the muscle founder stem cell population. We demonstrate that constitutive Notch activity is sufficient to autonomously maintain and self-renew muscle stem/progenitor cells, in spite of the complete abrogation of committed and differentiated cells. Notably, we demonstrate that these replicating embryonic stem/progenitors respond to foetal environmental cues as development proceeds, and, despite the ectopic expression of markers of the adipocyte, pericyte and osteoblast lineages, they adopt a foetal muscle fate after the release of Notch activity.

MATERIALS AND METHODS

Animals

Mouse line *Tg:Pax7-nGFP* was described previously (Sambasivan et al., 2009). Reporter line *R26R^{mTomato-stop-mGFP}* (*R26R^{mT/mG}*) (Muzumdar et al., 2007) was purchased from Jackson Laboratories (stock 007576). Cre recombinase lines were described and kindly provided by the corresponding laboratories: *Myf5^{Cre}* [M. R. Capecchi (Haldar et al., 2008)]; *En1^{Cre}* [A. Joyner (Kimmel et al., 2000)]; *R26R^{stop-lacZ}* [P. Soriano (Soriano, 1999)]; *R26R^{stop-DTA}* [J. P. Martinez-Barbera (Ivanova et al., 2005)]; *R26R^{stop-NICD-nGFP}* [D. Melton; Jackson Laboratories, stock 008159 (Murtaugh et al., 2003)]; *Rbpj^{fllox/flox}* [T. Honjo (Han et al., 2002)]. Animal experiments were performed as per European Community guidelines.

Immunofluorescence and imaging

Embryos were fixed for 1 hour in 2% paraformaldehyde and 0.1% Triton X-100 and equilibrated in 15% sucrose/PBS overnight. Samples were embedded in 7% gelatin/15% sucrose, frozen in a 2-methylbutane liquid nitrogen bath and cryosectioned at 16–20 µm. Antibodies used were: GFP (chicken; Abcam 13970; 1/1000); Pax7 [rabbit (Aviva Biosystems ARP32742; 1/750) and mouse (DSHB; 1/20)]; MyoD (mouse; Dako M3512; 1/50); myogenin (DSHB F5D; 1/100); Myf5 (Santa Cruz Biotechnology sc-302; 1/1200); MyHC (rabbit; kindly provided by G. Cossu, University College London, UK; 1/750); p57 (Santa Cruz Biotechnology sc-8298; 1/200); Nfix (gift from G. Messina, University of Milan, Italy; 1/300); Mef2c (gift from S. Molinari; Cell Signaling 5030; 1/100); calcitonin receptor (AbD Serotech AHP635; 1/100); laminin (Sigma 9393; 1/1000). Confocal images were acquired using a Leica SPE confocal microscope and Leica Application Suite (LAS) or Zeiss Axioplan and Axiovision software. Images were assembled using Adobe Photoshop and Adobe InDesign.

Quantitative RT-PCR (RT-qPCR)

To maximise recovery, total RNA was extracted from FACS-isolated cells directly into the cell lysis buffer (RLT) of the Qiagen RNeasy Micro Kit. The equivalent of 1.5×10^3 cells were used for transcript amplification. cDNA was prepared by random-primed reverse transcription (Super Script II, Invitrogen 18064-014) and real-time PCR was performed using SYBR Green Universal Mix (Roche 13608700) or TaqMan Universal Master Mix (ABI Prism 7700 and StepOne-Plus; Perkin-Elmer and Applied Biosystems). *Gapdh* transcript levels were used for normalisation of each target (Livak and Schmittgen, 2001). At least three biological replicates were used for each condition. For SYBR Green, custom primers were designed using Primer3Plus online software. Serial dilutions of total cDNA from E12.5 whole embryos and placentas were performed to calculate the amplification efficiency of all primers. Primer sequences are listed in supplementary material Table S1.

siRNA transfection and lentivirus transduction of primary myogenic cells

For siRNA transfections, FACS-isolated GFP⁺ cells from E18.5 control and mutant embryos were plated on Matrigel-coated 96-well dishes at 15,000 cells per well in DMEM/MCDB (1:1), 20% foetal calf serum medium without antibiotics. Cells were transfected with 10 pmol siRNAs (GFP-22 siRNA stock number 1022064; negative control siRNA stock number 1027280; Qiagen) using Lipofectamine RNAiMax reagent (Invitrogen) as per manufacturer's instructions. For longer chase periods, cells were retransfected every 2–3 days. For the production of lentiviruses, murine *Mef2c* was amplified by PCR from a plasmid kindly provided by G. Messina and cloned into the entry vector pENTR11. The expression vector was subsequently generated using the Gateway LR Clonase Kit (Life Technologies, 11791-100). For viral transduction, primary cells were incubated with lentiviruses at an MOI of 50 for 6 hours in OptiMem (Life Technologies) containing 10 µg/ml polybrene. Subsequently, medium was aspirated and cells incubated in DMEM/MCDB (1:1), 20% foetal calf serum medium.

In situ RNA hybridisation

For the detection of mRNA by in situ hybridisation (ISH) (Tajbakhsh et al., 1997), riboprobes were produced with T7 RNA polymerase from the following plasmids: for *HeyL*, IMAGE clone 40142873 was used linearised with *SacI*; for *Hes1*, a plasmid kindly provided by R. Kageyama was used as previously described (Hirata et al., 2001).

EdU administration in vivo

For proliferation experiments in vivo, 5-ethyl-2'-deoxyuridine (EdU; Invitrogen E10187) was injected twice: first at 50 µg/g body weight, then after 12 hours at 75 µg/g body weight; mice were then sacrificed 6 hours later. EdU was detected using the Click-iT EdU Cell Proliferation Assay Kit (Life Technologies C10350) as per manufacturer's instructions.

Transcriptome analysis

Expression profile data were generated using limb skeletal muscle stem/progenitor cells from transgenic *Tg:Pax7-nGFP* mice (R.S. and S.T., unpublished). The full dataset will be published elsewhere, but is available upon request. Briefly, E12.5 and E17.5 embryos were dissociated and GFP-positive stem/progenitors were isolated by FACS. Fragmented biotin-labeled cRNA samples were hybridised on GeneChip Mouse Genome 430_2 arrays (45,000 probe sets) as recommended by the manufacturer (Affymetrix). For each developmental stage, three biological replicates were hybridised. Raw data were pre-processed using the GC-Robust Multichip Analysis (GCRMA) algorithm to correct background, to adjust intensity distribution over the arrays and to convert probe intensity summarisation into a unique probe set signal. Local pooled error tests (Jain et al., 2003) were conducted in order to identify significant differences in gene expression between groups. The Benjamini-Hochberg multiple correction test (Benjamini and Hochberg, 1995) was applied to control for the number of false positives with an adjusted 5% statistical significance threshold.

Statistical analysis

For statistical comparison of Pax7-nGFP^{Hi}, Pax7-nGFP^{Mid} and Pax7-nGFP^{Lo} and quantifying cell populations, Student's *t*-test (two-tailed) was performed using PRISM software (GraphPad). A minimum of three replicates were analysed for all experiments presented.

RESULTS

A subpopulation of myogenic cells with elevated Pax7 expression is specified by active Notch signalling

To determine the specific stage of myogenic lineage progression when Notch signalling is highest, we used a transgenic mouse line that expresses nGFP under the control of the regulatory elements of *Pax7* (*Tg:Pax7-nGFP*) (Sambasivan et al., 2009) to perform a refined fractionation of myogenic cells during development (Fig. 1A; top 20% Pax7^{Hi}, intermediate 40% Pax7^{Mid}, bottom 20% Pax7^{Lo}). RT-qPCR analysis of the subfractions showed that GFP reporter expression provided a faithful readout of the lineage progression status, with the gradual loss of *Pax7* and *Myf5* and the gain of commitment and differentiation markers (*Myod*, *Myog*) in Pax7^{Lo} cells (Fig. 1B). At embryonic (E11.5) and, more prominently, at foetal (E18.5) stages, the Pax7^{Hi} population had higher Notch activity, as measured by expression of the Notch targets *HeyL*, *Hey1* and *Nrarp*, compared with the cells with lower levels of GFP (Fig. 1B). In addition, in the Pax7^{Hi} cells, higher expression of *Notch1* and *Notch3*, the ligand jagged 1 (*Jag1*), and specifically the Notch glycosyltransferase lunatic fringe (*Lfng*) was observed, whereas the *Dll1* ligand was significantly reduced (Fig. 1B). A similar pattern of expression was observed in Pax7-nGFP cells isolated from postnatal day (P) 8 and adult (post-injury) activated satellite cells (Mourikis et al., 2012). Upon analysis of purified embryonic cells overexpressing a constitutively active form of Notch1 (NICD) in vivo, a similar transcriptional response to that of the Pax7^{Hi} population was observed (supplementary material Fig. S1A). Taken together, these results indicate that irrespective of the developmental stage, the Pax7^{Hi} fraction corresponds to the stem/progenitor population and is characterised by elevated endogenous Notch activity.

Based on these observations, we hypothesised that sustained Notch activity in the DM would retain cells in a state of elevated *Pax7* expression that is compatible with muscle, but not dermal, stem/progenitor cells. To test this, we used an *En1*^{Cre} knock-in line (Kimmel et al., 2000) to target constitutive expression of NICD in cells in the central DM that produce both dermal and muscle progenitors (Fig. 1C). Immunohistological analysis of

En1^{Cre};*R26R*^{stop-NICD-nGFP} (hereafter *En1*^{Cre}-NICD) embryos showed significant expansion of the muscle founder stem cell population, manifested first by an expansion of Pax3⁺ cells in the DM, and later of Pax3⁺/Pax7⁺ cells in the myotome after epithelial-to-mesenchymal transition of the DM (Fig. 1D,E). This occurred at the expense of myogenic commitment and terminal differentiation (no *Myod* and no *Myog*, respectively; Fig. 1F; control in supplementary material Fig. S1B). Consistent with previous findings that genetic regulation of *Myf5* is distinct from that of *Myod* (Tajbakhsh et al., 1997; Tajbakhsh and Buckingham, 2000), *Myf5* continued to be co-expressed in essentially all GFP⁺/Pax7⁺ cells (Fig. 1F; designated as stem/progenitors). Interestingly, *HeyL* (upregulated ~40-fold), and not *Hes1*, was the major responding gene to NICD (supplementary material Fig. S1C).

When Notch signalling was compromised in *En1*^{Cre};*Rbpj*^{fllox/-};*R26R*^{mT/mG} embryos, *Alx4*⁺ dermal progenitors emerged at E11.5 as in wild-type embryos (supplementary material Fig. S1D), suggesting that Notch signalling is not essential for dermal lineage commitment. Accordingly, we obtained similar results using a *Myf5*^{Cre} line (Halder et al., 2008), which drives expression in some dermal progenitor cells that share a common and recent ancestry with myogenic stem/progenitors (supplementary material Fig. S1D). This observation led us to propose that Notch signalling is dispensable for the dermis/muscle cell fate choice of the founder stem cells, at least in the *En1*⁺ population found in the central portion of the DM. Instead, constitutive NICD suppressed the expression of the early dermal progenitor marker *Alx4* as well as the later marker *Dermol1* (*Twist2* – Mouse Genome Informatics) (Fig. 1G) (Scaal et al., 2001; Cinnamon et al., 2006). However, it did not impair their migration dorsally underneath the ectoderm (Fig. 1D). Therefore, elevated Notch activity in multipotent DM cells biases their fate toward skeletal myogenesis, while concomitantly blocking their differentiation.

Maintenance of early myogenic stem/progenitors throughout development by constitutive Notch activity

Given that constitutive allelic expression of NICD maintains the upstream myogenic stem/progenitor population cell-autonomously, we used NICD as a tool to interrogate the distinct spatiotemporal behaviours of these cells and their relationship with other myogenic subpopulations. Since *Myf5* is the earliest MRF to be expressed in somitic and cranial mesoderm, we crossed constitutively expressing *Myf5*^{Cre} mice (Halder et al., 2008) to trigger the expression of NICD in these muscle progenitors. *Myf5*^{Cre/+};*R26R*^{stop-NICD-nGFP/+} embryos (hereafter *Myf5*^{Cre}-NICD) were highly oedemic (Fig. 2A; starting at E16.5), lacked skeletal muscles and died perinatally. Analysis of head and trunk musculature in these embryos at E18.5 showed a total lack of skeletal muscles, and, instead, a dramatic expansion of Pax7⁺/*Myf5*⁺/*Myog*⁻ muscle cells that we refer to as stem/progenitors. Interestingly, the patterning of these cells followed that of the presumed muscle masses in both head and trunk regions (Fig. 2B). This demonstrates that, in contrast to Wnt and Shh signalling, which inhibit myogenesis in the head but promote myogenesis in the trunk (Tzahor et al., 2003), there is no such disparity in the effect of Notch signalling on cranial and somitic myogenic cells. Furthermore, it shows that expression of NICD is sufficient to maintain their viability and self-renewal capacity in the absence of downstream committed progeny.

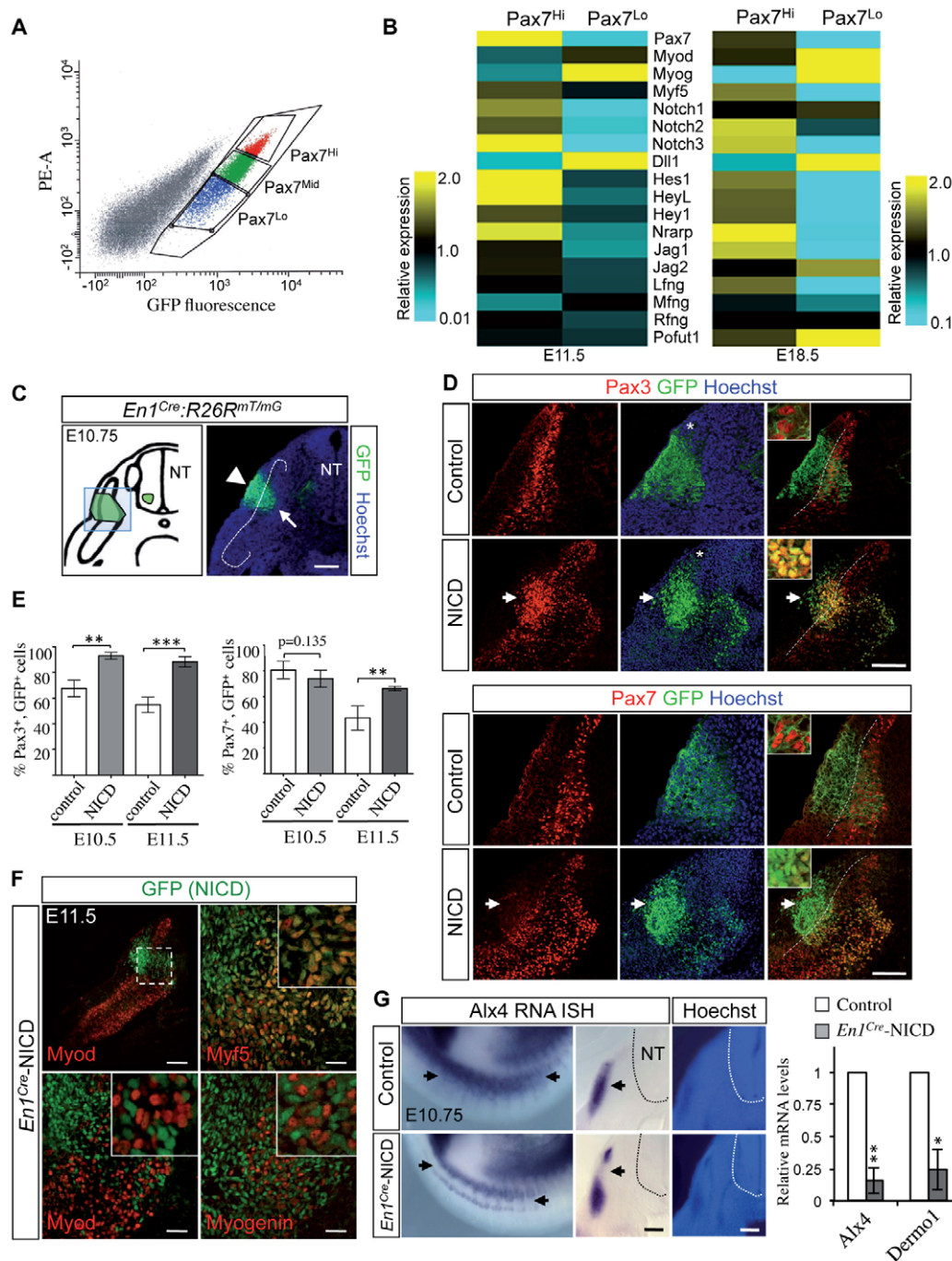


Fig. 1. High Notch activity defines an upstream, undifferentiated myogenic population with elevated *Pax7* expression. (A) Fractionation by FACS of GFP⁺ cells from E11.5 *Tg:Pax7-nGFP* mouse embryos into Pax7^{Hi} (20% of population), Pax7^{Mid} (40%) and Pax7^{Lo} (20%). Intensity of the GFP signal reflects activity of the *Pax7* promoter. (B) Expression of muscle and Notch signalling factors in the Pax7^{Hi} and Pax7^{Lo} fractions measured by RT-qPCR. Data are presented as a heatmap, relative to expression in Pax7^{Mid} cells (2^{-ΔΔCt}). All genes listed were differentially expressed between Pax7^{Hi} and Pax7^{Lo} samples with statistical significance (*n*=3 embryos/genotype). (C) Distribution of *En1*^{Cre} targeted cells in an interlimb transverse section of an E10.75 *En1*^{Cre}:R26R^{mT/mG} embryo. Marked GFP⁺ cells are detected in both dermal (arrowhead) and muscle (arrow) lineages. Dotted line delineates dermomyotome (DM) from myotome. The boxed region in the diagram indicates the anatomical location. (D) Anti-Pax3 and anti-Pax7 immunostaining on serial sections of *En1*^{Cre}-NICD (nuclear GFP) and control (*En1*^{Cre}:R26R^{mT/mG}; membrane GFP) embryos. NICD overexpression results in expansion of the Pax3 but not the Pax7 expression domain (see arrows and insets). Asterisks indicate GFP⁺ cells in the dermal compartment. (E) Quantification of the effect of NICD overexpression on Pax3 and Pax7 at E10.5 and E11.5. Values are presented as percentage of Pax3 or Pax7 co-expression with GFP over total number of GFP⁺ cells. (F) Interlimb sections of E11.5 *En1*^{Cre}-NICD embryos stained with antibodies against Myf5, Myod and Myog. The boxed region in the upper left panel designates the anatomical location shown in the other panels. (G) (Left) Whole-mount RNA in situ hybridisation (ISH) for *Alx4* on E10.75 embryos. The *En1* targeting domain is indicated by arrows. (Right) RT-qPCR for the dermal markers *Alx4* and *Dermo1*, using FACS-isolated cells from *En1*^{Cre}-NICD and control (*En1*^{Cre}:R26R^{mT/mG}) E10.75 embryos. Error bars indicate s.e.m.; *n*=3 embryos/genotype. **P*<0.05, ***P*<0.01, ****P*<0.001. NT, neural tube; PE-A, phycoerythrin. Scale bars: 100 μm in C and in F upper left; 80 μm in D; 40 μm in remainder of F; 30 μm in D insets; 15 μm in F insets.

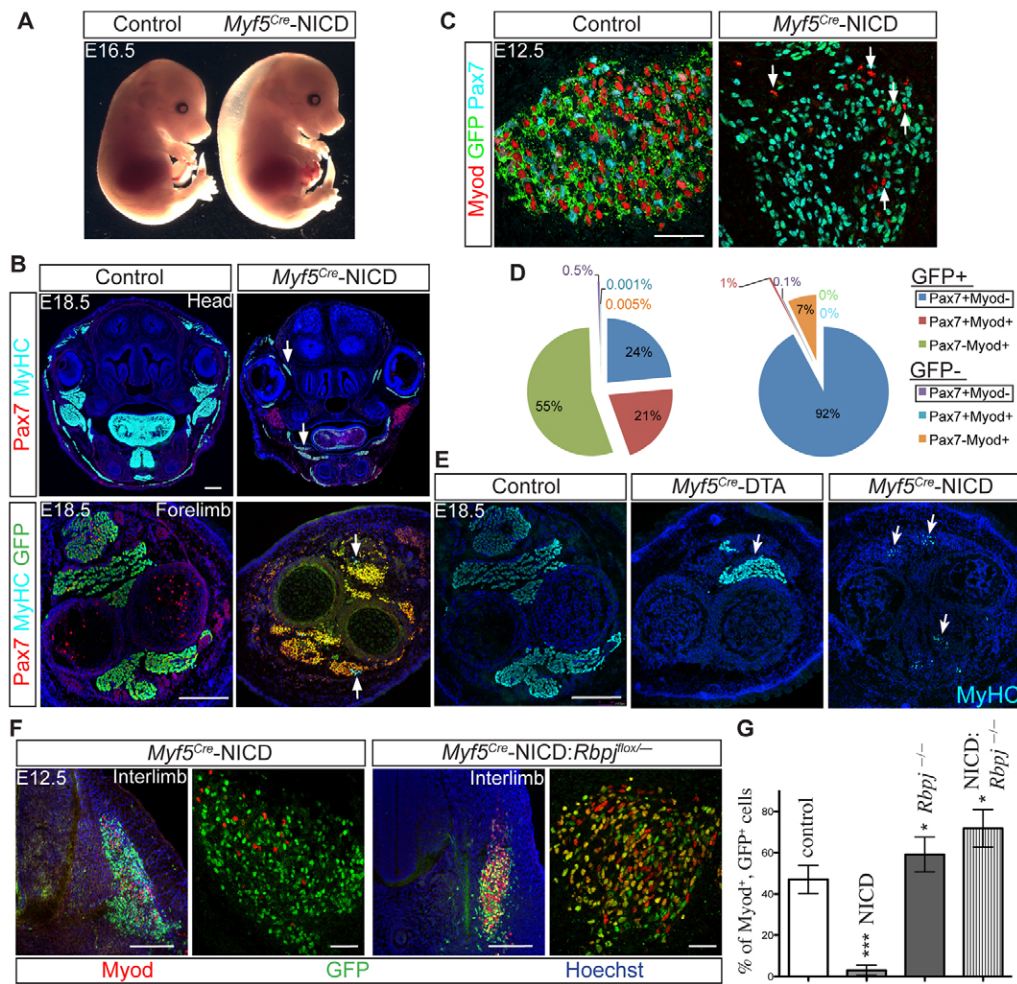


Fig. 2. Activated Notch cell-autonomously sustains a stem/progenitor state during development in head and trunk via *Rbpj*. (A) E16.5 *Myf5*^{Cre}-NICD (*Myf5*^{Cre/+}; *R26R*^{stop}-NICD-nGFP/+) mouse embryo with severe oedema, as compared with control. (B) (Top) Head coronal sections of E18.5 control (*Myf5*^{Cre/+}; *R26R*^{stop}-mT-mG/+) and *Myf5*^{Cre}-NICD embryos. Residual myosin heavy chain (MyHC) staining in *Myf5*^{Cre}-NICD samples is due to non-targeted (GFP⁻) muscle cells (arrows). (Bottom) Forelimb transverse sections showing that essentially all NICD⁺ cells (GFP⁺) are Pax7⁺. Arrows indicate rare MyHC⁺/GFP⁻ myofibres. (C) Immunostaining of transverse forelimb sections of E12.5 control and *Myf5*^{Cre}-NICD embryos. Residual Myod⁺ cells are indicated (arrows). (D) Quantification of marker expression in cells from transverse forelimb sections of E12.5 control and *Myf5*^{Cre}-NICD embryos. NICD-targeted GFP⁺ cells showed substantial expansion of the Pax7⁺/Myod⁻ population (blue: 24% in control, 92% in NICD, $P=0.0015$), whereas the non-targeted (GFP⁻) Pax7⁺ population was not altered significantly (Pax7⁺/Myod⁻, $P=0.0572$ or GFP⁻/Pax7⁺/Myod⁺, $P=0.4226$) and only a modest increase in the proportion of the Myod⁺ population was observed (GFP⁻/Pax7⁺/Myod⁺, $P=0.0048$). $n=3$ embryos/genotype. (E) Partial rescue of muscle mass by *Myf5*^{Cre}-independent cells in forelimbs of E18.5 *Myf5*^{Cre}:*R26R*^{stop}-DTA embryos, as shown by the presence of MyHC-marked myofibres (middle panel, arrow). Weak rescue was detected in embryos expressing NICD (right panel, arrows). (F) NICD-mediated inhibition of differentiation is reversed in the absence of *Rbpj* in *Myf5*^{Cre}:*R26R*^{stop}-NICD-nGFP;*Rbpj*^{flax/-} embryos, as shown by the presence of cells that are double positive for NICD (nGFP) and Myod (right panels). See also supplementary material Fig. S2A. (G) Quantification of Myod⁺/GFP⁺ cells in control (*Myf5*^{Cre/+}; *Rbpj*^{flax/+}; *R26R*^{stop}-mT-mG/+), NICD (*Myf5*^{Cre/+}; *R26R*^{stop}-NICD-nGFP/+), *Rbpj*^{flax/-} (*Myf5*^{Cre/+}; *Rbpj*^{flax/-}; *R26R*^{stop}-mT-mG/+) and NICD;*Rbpj*^{flax/-} (*Myf5*^{Cre/+}; *R26R*^{stop}-NICD-nGFP/+ ; *Rbpj*^{flax/-}). Values are presented as percentage of Myod⁺/GFP⁺ cells over total number of GFP⁺ cells. Error bars indicate s.e.m.; $n=3$ embryos/genotype. * $P<0.05$, *** $P<0.001$. Scale bars: 50 μ m in C,F; 250 μ m in E and in B forelimb; 500 μ m in B head.

The complete lack of musculature in *Myf5*^{Cre}-NICD embryos was unexpected given two previous reports showing the presence of a *Myf5*-independent Myod⁺ subpopulation that rescues myogenesis after diphtheria toxin ablation (DTA) of *Myf5*⁺ cells (Gensch et al., 2008; Haldar et al., 2008). Quantification of forelimb musculature of *Myf5*^{Cre}-NICD embryos at E12.5 showed an initial modest increase in non-targeted (GFP⁻) myoblasts that were Pax7⁺/Myod⁺ to $7\pm0.7\%$ (essentially undetectable in control), whereas $92\pm0.3\%$ of the GFP⁺ population was Pax7⁺/Myod⁻ (Fig. 2C,D; $24\pm2.6\%$ in control). Further analysis revealed that the small fraction of GFP⁻/Myod⁺ cells was neither sustained nor

expanded at later stages of development, but instead differentiated to form rudimentary myofibres (Fig. 2B, bottom right, arrows). That the Myod⁺ subpopulation did not rescue myogenesis in this model was evident in a comparison of *Myf5*^{Cre/+}; *R26R*^{stop}-mT-mG/+ (GFP marker control), *Myf5*^{Cre/+}; *R26R*^{stop}-DTA/+ (cell ablation and Myod rescue) and *Myf5*^{Cre/+}; *R26R*^{stop}-NICD-nGFP/+ (skeletal muscle agenesis) foetuses. In the DTA ablation model, a partial rescue of muscle differentiation was observed in the body, whereas in the constitutive NICD model no significant rescue of skeletal myogenesis was detected by the *Myf5*-independent population (Fig. 2E). Taken together, these results demonstrate that

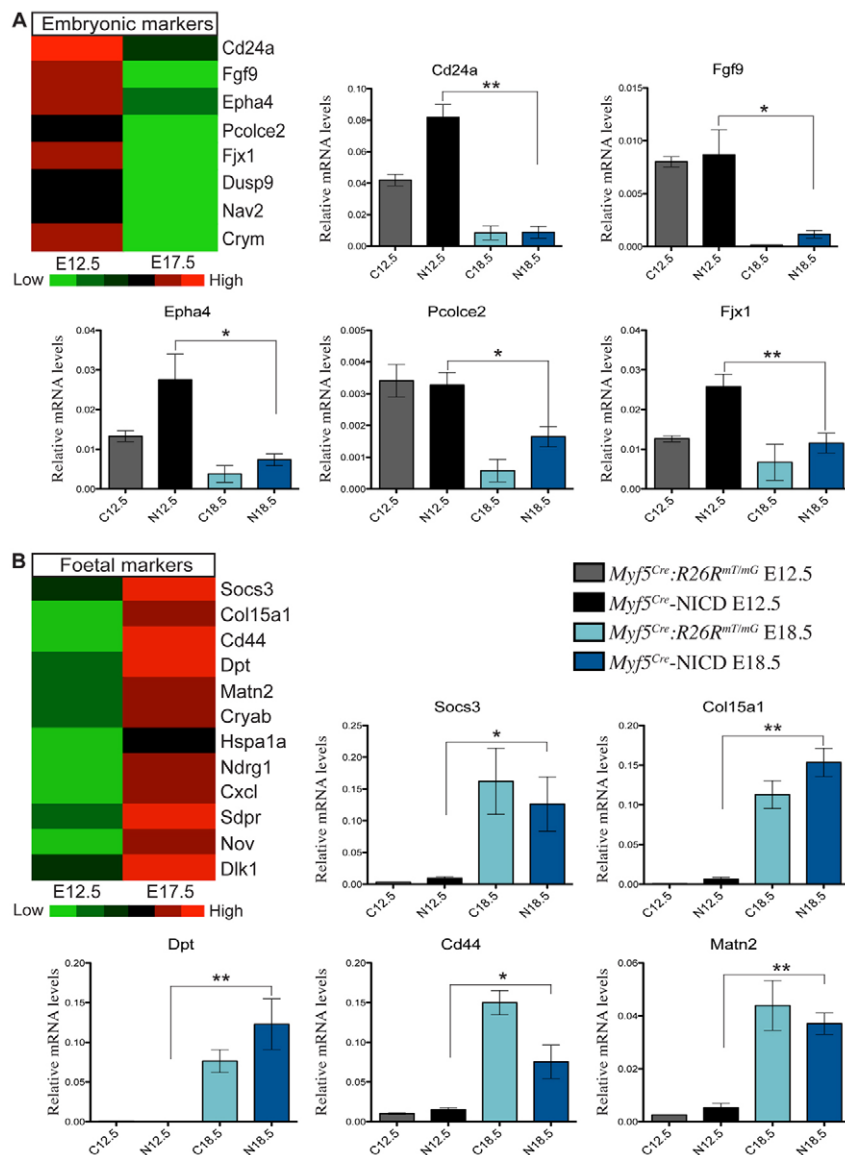


Fig. 3. Differentiation-arrested stem/progenitor cells in the embryo respond to developmental cues and acquire foetal characteristics in the foetus. (A,B) Microarray expression data from embryonic (E12.5) and foetal (E17.5) *Tg:Pax7-nGFP* mouse limbs. Differential expression of selected embryonic (A) and foetal (B) markers is shown in the heatmaps (green is low expression, red is high). Bar charts indicate relative transcript levels of specific embryonic (A) and foetal (B) markers in E12.5 and E18.5 control (Myf5^{Cre/+};R26R^{mT-mG/+}) and Myf5^{Cre}-NICD FACS-isolated cells assessed by RT-qPCR. Data are normalised to *Gapdh* (2^{-ΔCt}). Error bars indicate s.e.m.; n=3 embryos/genotype. *P<0.05, **P<0.01.

constitutive Notch is sufficient to maintain a self-renewing population of muscle stem/progenitors while inhibiting differentiation throughout embryogenesis. Although undifferentiated, these Myf5-NICD⁺ cells inhibited the expansion of the presumed Myf5-independent Myod⁺ population, indicating that negative cross-regulation between these subpopulations can occur primarily at the stem/progenitor cell level, thereby resulting in failure of *Myf5* myogenic rescue in this scenario.

The Myf5^{Cre}-NICD model also permitted us to address a longstanding question in the field of Notch signalling and myogenesis (Shawber et al., 1996; Nofziger et al., 1999): is the block of differentiation by Notch accomplished via canonical signalling or independently of Rbpj? To resolve this issue, we generated compound mice that express NICD in an *Rbpj* null background (Myf5^{Cre/+};R26R^{stop-NICD-nGFP/+};Rbpj^{fllox/-}) and scored for the expression of *Myod* and *Myog* in the myotome. In the absence of Rbpj, constitutively activated Notch1 did not inhibit cell differentiation in the trunk or head muscles. Instead, cells differentiated more rapidly than controls, reproducing the phenotype that we observed in *Rbpj* null mice (Fig. 2F,G; supplementary material Fig. S2A-C). Therefore, an Rbpj-

independent pathway does not appear to be manifested for muscle progenitor cell differentiation in this scenario.

Self-renewing embryonic myogenic stem/progenitor cells arrested in lineage progression adopt foetal phenotypes in the foetus

Given that Notch signalling blocked differentiation and sustained the muscle stem/progenitor population from embryo to foetus, we examined whether this population would be responsive to the foetal environment and adopt foetal cell fate characteristics. The shift from embryonic to foetal development is a critical step during skeletal myogenesis, in which molecularly and phenotypically distinct foetal myoblasts generate secondary myofibres (Biressi et al., 2007b). We showed previously, using a differential transcriptome comparison between embryonic and foetal Myf5^{GFP-P/+} myogenic cells from the limbs, that different classes of genes distinguish these two myoblast populations (Biressi et al., 2007b). This led to the identification of the transcription factor Nfix as a crucial determinant of the foetal myoblast fate (Messina et al., 2010). To complement this study and identify novel markers of the ancestral Pax7⁺ muscle stem/progenitor population, we performed

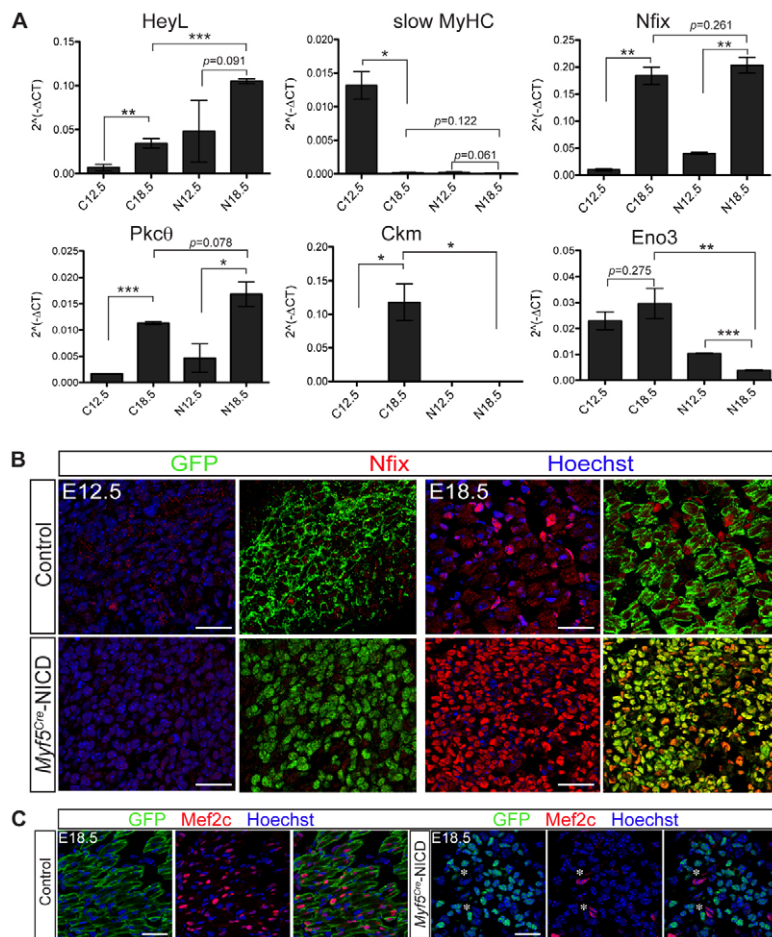


Fig. 4. Nfix fails to induce its downstream targets in the presence of NICD. (A) Relative transcript levels in E12.5 and E18.5 purified cells from control and *Myf5^{Cre}*-NICD samples for *Nfix*, its interacting partners [*Pkc theta*, *Mef2a* (see also supplementary material Fig. S4)] and downstream targets (*Ckm*, *Eno3*). *HeyL* is a reporter of Notch activity and slow MyHC (*Myh7*) is an embryonic marker. Error bars indicate s.e.m.; *n*=3 embryos/genotype. **P*<0.05, ***P*<0.01, ****P*<0.001. (B) Control and *Myf5^{Cre}*-NICD E12.5 and E18.5 mouse forelimbs stained for *Nfix*. (C) Control and *Myf5^{Cre}*-NICD E18.5 forelimbs stained for *Mef2c*. Scale bars: 40 μm in B; 25 μm in C.

a differential transcriptome analysis between FACS-isolated embryonic (E12.5) and foetal (E17.5) Pax7-nGFP cells. We identified a number of differentially expressed genes that were not observed previously (Fig. 1A,B). RT-qPCR analysis of control cells isolated by FACS from E12.5 and E18.5 *Myf5^{Cre/+};R26R^{mT-mG/+}* samples confirmed the temporal specificity of these transcripts in embryonic and foetal progenitors (Fig. 3A,B; supplementary material Fig. S3A,B).

To assess the phenotype of *Myf5^{Cre}*-NICD cells that persist in the foetus, we used lineage priming (Enver and Greaves, 1998) as an assay to determine their temporal state and commitment status. Strikingly, and in accordance with the control, *Myf5^{Cre}*-NICD cells isolated from the foetus (E18.5) showed an upregulation of all foetal markers, but not embryonic markers (Fig. 3B; supplementary material Fig. S3A,B). Notably, the embryonic (E12.5) *Myf5^{Cre}*-NICD cells expressed the embryonic markers at levels comparable to the control and did not express any of the foetal markers at this stage (Fig. 3A). These results suggest that the upstream embryonic population of Pax7⁺/Myf5⁺ cells maintained by constitutive allelic expression of NICD was receptive to the changing environmental signals of the foetus, and consequently adopted a foetal phenotype while concomitantly losing embryonic molecular markers.

Embryonic to foetal myogenic specification is regulated through Nfix-mediated transcriptional regulation. Analysis of *Nfix* as well as members of its transcriptional core regulatory group showed that *Nfix* and *Pkcθ* (*Prkcg* – Mouse Genome Informatics) transcripts were upregulated both in control and *Myf5^{Cre}*-NICD cells at E18.5 compared with E12.5 (Fig. 4A). Importantly, embryonic slow

myosin heavy chain, a marker specific for embryonic but not foetal myoblasts, was not expressed in *Myf5^{Cre}*-NICD cells at E18.5 (Fig. 4A). Interestingly, however, the downstream targets of Nfix, such as muscle creatine kinase (*Ckm*) and β-enolase (*Eno3*), were not significantly induced in the *Myf5^{Cre}*-NICD cells at E18.5 in spite of the presence of robust levels of Nfix protein (Fig. 4B), its co-factor Pkcθ and myocyte-specific enhancer factor 2a (*Mef2a*) (supplementary material Fig. S4A). We also found that another Mef paralogue, *Mef2c*, which promotes myogenic differentiation, was strongly repressed in the *Myf5^{Cre}*-NICD cells (Fig. 4C; supplementary material Fig. S4A). Although it normally does not bind to Nfix (Messina et al., 2010), we tested whether restoring *Mef2c* expression in the presence of NICD would rescue myogenesis and permit the induction of the differentiation marker *Ckm*. Exogenous overexpression of *Mef2c* did not promote the expression of *Ckm*, although some upregulation of *Eno3* was observed (supplementary material Fig. S4B,C), suggesting that activated Notch interferes with the transcriptional activity of Nfix at different levels. These findings indicate that *Myf5^{Cre}*-NICD cells arising in the embryo undergo normal ontogeny and adopt the correct temporal fate. However, their lineage progression to the myoblast and differentiation stages is abrogated by the constitutive expression of NICD.

Foetal myoblasts arrested during lineage progression retain robust myogenic potential

We showed above that myoblasts expressing activated Notch remain undifferentiated and respond effectively to ontologically

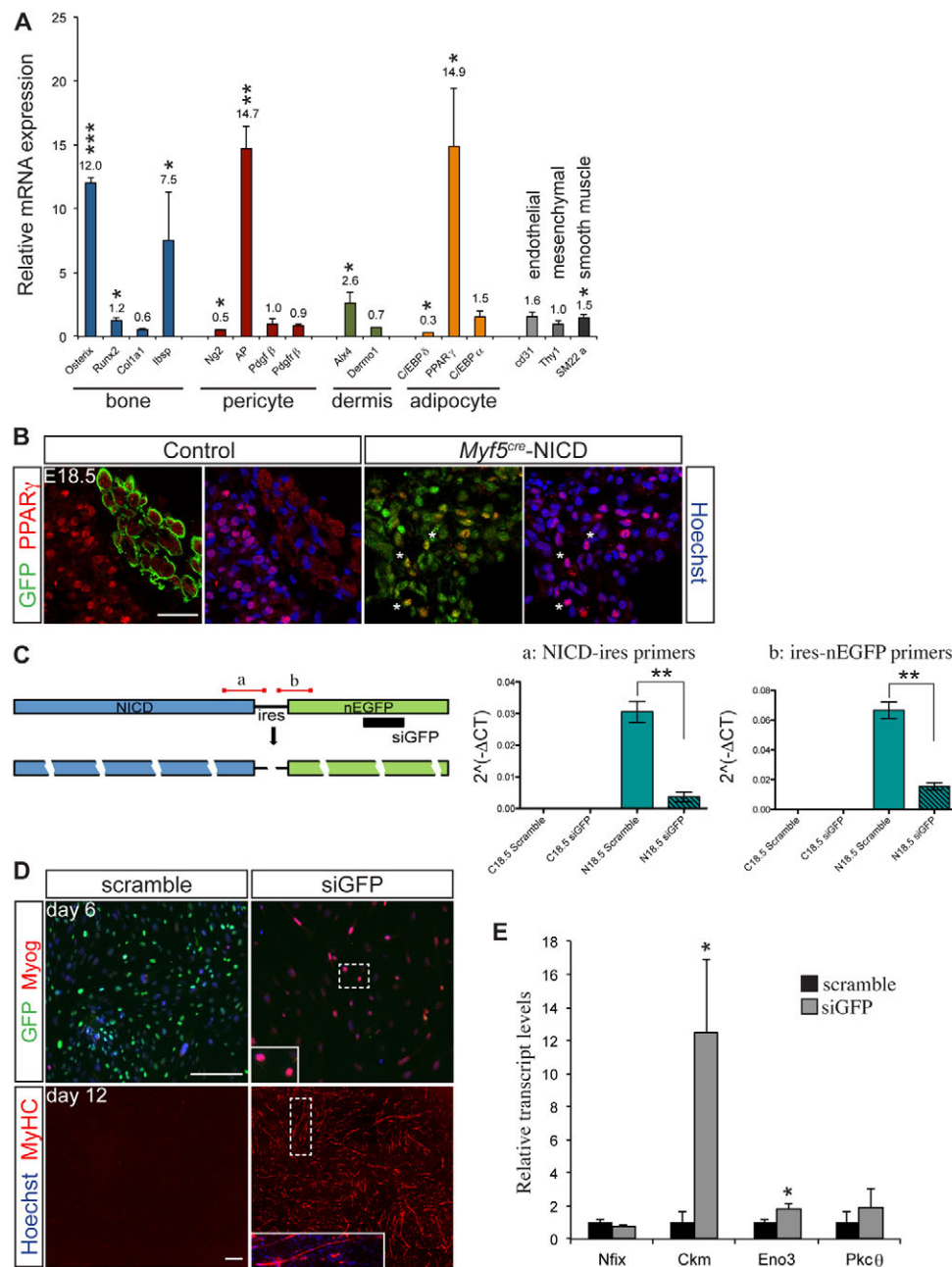


Fig. 5. Foetal NICD-expressing cells retain their myogenic potential despite the expression of markers of diverse lineages. (A) Relative expression of osteoblast (blue), pericyte (dark red; AP, alkaline phosphatase), dermoblast (green) and adipocyte (orange) markers and also of markers of endothelial cells (*Cd31*; also known as *Pecam1*), mesenchymal cells (*Thy1*) and smooth muscle cells (*Sm22a*; also known as *Tagln*). Transcript levels in *Myf5^{Cre}-NICD* are presented relative to control ($2^{-\Delta\Delta Ct}$). Error bars indicate s.d.; $n=3$ embryos/genotype. (B) Anti-Ppary nuclear staining of E18.5 control (*Myf5^{Cre};R26R^{mT-mG/+}*) and *Myf5^{Cre}-NICD* at the forelimb level. Note the presence of NICD-expressing cells co-expressing Ppary (asterisk). (C) (Left) Silencing the *NICD-ires-nEGFP* transgene by siRNA. The siGFP targets the EGFP sequence of the bicistronic transcript, leading to the degradation of the entire mRNA. (Right) The levels of *NICD-ires-nEGFP* transcripts as determined using primers targeting the NICD-ires (a) or the ires-nEGFP (b) junction sequences. Scrambled siRNA was used as transfection controls. Samples were collected after two 48-hour pulses of siRNA transfections. Error bars indicate s.e.m.; $n=3$ embryos/genotype. (D) siGFP transfection of FACS-isolated cells from *Myf5^{Cre}-NICD* E18.5 foetuses. Scrambled siRNA was used as negative control. Efficient EGFP knockdown specifically in siGFP-treated samples resulted in the appearance of *Myog*⁺ cells by day 6 and the formation of multinucleated fibres by day 12. (E) Induction of the *Nfix* targets, but not *Nfix*, after silencing of the *NICD* transgene. Values of scrambled control are represented as 1. Data are presented as ratios of siGFP-treated over scrambled siRNA-treated samples ($2^{-\Delta\Delta Ct}$). Error bars indicate s.d.; $n=3$ embryos/genotype. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Scale bars: 25 μ m in B; 100 μ m in D, top; 300 μ m in D, bottom.

changing environmental signals for their temporal specification. Given that these cells arose in the somitic DM and that they were initially multipotent for several lineages, we wondered whether this

property was retained after their exit from the DM under continuous NICD expression. We isolated *Myf5^{Cre}-NICD* cells at E18.5 and examined the expression of specific markers for multiple

lineages by RT-qPCR or immunofluorescence. Notably, pericyte, adipocyte and osteoblast markers genes were detected in the *Myf5^{Cre}*-NICD cells (Fig. 5A). Interestingly, although we observed the expression of more upstream lineage markers for these cell types, this was not accompanied by induction of the downstream differentiation cascade. For example, peroxisome proliferator-activated receptor gamma (*Pparγ*), which marks differentiating adipocytes, was induced robustly in the *Myf5^{Cre}*-NICD cells. However, the preadipocyte factor *C/EBP-δ* (*Cebpδ*) was slightly reduced, whereas no induction was seen for the *Pparγ* target *C/EBP-α* (*Cebpα*), indicating that further differentiation was blocked. Strikingly, *Pparγ* protein was also observed in *Myf5^{Cre}*-NICD cells in vivo, but these cells were located only on the periphery of the myogenic zone, sometimes adjacent to adipogenic regions (Fig. 5B; data not shown). This suggests that other local signals promote further transdifferentiation and cell commitment of this pre-myogenic population.

To assess the myogenic differentiation potential of these cells, NICD expression from the *R26^{NICD-nGFP}* locus was suppressed by targeting the NICD-ires-nGFP bi-cistronic transcript with siGFP. This approach allowed the knockdown of the transgene expressing NICD (Fig. 5C) while leaving endogenous Notch signalling intact. After 12 days in culture, Myog⁺ cells and multinucleated myotubes were observed in the siGFP-treated cells, but not in the cells with control/scrambled siRNA (Fig. 5D). Notably, many of these myotubes were larger than embryonic myotubes, which typically have only a few differentiated nuclei. Furthermore, these siGFP-treated cells expressed genes specific for foetal myogenesis (Fig. 5E; *Ckm* and *Eno3*). Taken together, these results indicate that constitutive NICD expression does not compromise the ability of myogenic stem/progenitor cells to adopt foetal myoblast and differentiated phenotypes, in spite of their initial expression of genes of multiple cell lineages.

Distinct roles for Notch signalling during maturation of muscle stem cells

We showed above that constitutive expression of NICD maintains muscle stem/progenitors at the expense of differentiation. These cells continued to proliferate and prefigured muscle mass formation. Given that these cells lose embryonic characteristics and adopt those found in the foetus, we asked whether they adopted later properties that characterise the emerging satellite cell population. A prominent feature of satellite cells is their quiescent state. We examined the cell cycle status of control and *Myf5^{Cre}*-NICD cells (*Pax7⁺/GFP⁺*) by exposing E12.5 and E18.5 embryos to the nucleotide analogue EdU (see Materials and methods) and measured cells that were double positive for Pax7 and EdU. In accordance with the phenotypes described above during early myogenesis, activated Notch sustained stem/progenitor cell proliferation (Fig. 6A) at the expense of differentiation (Fig. 1C; Fig. 2B-D), with 71.7±3.8% and 83.9±2.9% of *Pax7⁺/EdU⁺* cells in *Myf5^{Cre}*-NICD and control embryos, respectively (Fig. 6A,B). Strikingly, at E18.5, activated Notch continued to block cell differentiation, yet unexpectedly it had a strong negative impact on proliferation such that the majority of *Pax7⁺/GFP⁺* cells did not take up EdU (Fig. 6A,B; 18.9±1.0% and 75.5±2.1% of *Pax7⁺/EdU⁺* cells in *Myf5^{Cre}*-NICD and control, respectively). Consistent with the proliferation profile, examination of the cell cycle inhibitor p57 (*Cdkn1c* – Mouse Genome Informatics) showed that, whereas at E12.5 the majority of the *Myf5^{Cre}*-NICD⁺ cells were p57[−] and actively proliferating (EdU⁺), by E18.5 most of the cells expressed robust levels of p57 and were EdU[−] (Fig. 6C-E). Therefore,

sustained expression of activated Notch is compatible with foetal progenitors eventually adopting a *Pax7⁺/Myod[−]/EdU[−]* state, which is characteristic of satellite cells.

In addition to their quiescence, satellite cells are characterised by the expression of specific markers, including calcitonin receptor (CTR; *Calcr*), which is highly expressed in postnatal quiescent satellite cells but not in myogenic cells in proliferation (Fukada et al., 2007). This marker showed high levels of expression in most of the *Myf5^{Cre}*-NICD cells at E18.5, but not during embryonic stages (Fig. 7A; data not shown). Furthermore, a defined feature of the muscle stem cell niche is the expression of laminin in the basement membrane surrounding muscle fibres, and emerging satellite cells are ensheathed by laminin staining. Remarkably, in the total absence of myofibres, laminin staining was observed surrounding these *Myf5^{Cre}*-NICD cells at E18.5, but not at E12.5 (Fig. 7B), suggesting that these NICD-expressing cells were capable of constructing a component of the adult stem cell niche. Taken together, our data suggest that, at E18.5, *Myf5^{Cre}*-NICD cells have both foetal and satellite cell characteristics.

DISCUSSION

The capacity of stem cells to generate distinct classes of committed progeny spatiotemporally, although well investigated in the nervous system, remains largely undefined for most tissues and organs. Key to understanding this process is interpreting the relative roles of powerful signalling pathways that impinge on these events. Using different combinations of genetically modified mice, we identify Notch signalling as necessary and sufficient for maintaining muscle stem cells and allowing them to be receptive to specification and differentiation cues throughout development. Remarkably, cell-autonomous expression of the intracellular fragment of Notch in muscle stem/progenitors was sufficient to allow their maintenance, temporal specification and recapitulation of ontology in the absence of committed progeny and differentiation.

We demonstrate that myoblasts with high Notch activity fail to terminally differentiate, both in the embryonic and foetal trunk and head. This property of canonical Notch signalling is unique, as other crucial signalling molecules have been shown to impact differently on the myogenic programme, either temporally (embryo/foetus) (Cusella-De Angelis et al., 1994; Hutcheson et al., 2009) or spatially (trunk/head) (Tzahor et al., 2003). We also show for the first time for skeletal muscle in vivo that Notch signalling occurs via an Rbpj-dependent pathway, and that an Rbpj-independent pathway is either not significant or lacking in the somite. Whether this is also the case for muscle stem/progenitor cells in other contexts requires further investigation.

Interestingly, Notch activity is sufficient to autonomously maintain embryonic *Pax7⁺/Myog[−]* stem/progenitor cells throughout development, even in the absence of differentiating cells, which we showed previously to be required for their survival (Kassar-Duchossoy et al., 2005). Therefore, this reinforces the notion that the differentiating cells might signal to and sustain the upstream population by direct cell-cell interactions. We propose that Notch signalling fulfils the requirements as the principal pathway, and we show experimentally that it is sufficient. This notion is supported by the transcriptional signature of upstream (*Pax7^{Hi}*) and differentiating (*Pax7^{Lo}*) myogenic cells, which shows high Notch activity in the former, and elevated Dll1 ligand expression in the latter. Hence, in the experimental situation in which the stem/progenitor cells have sustained NICD expression, the downstream population becomes dispensable for their survival.

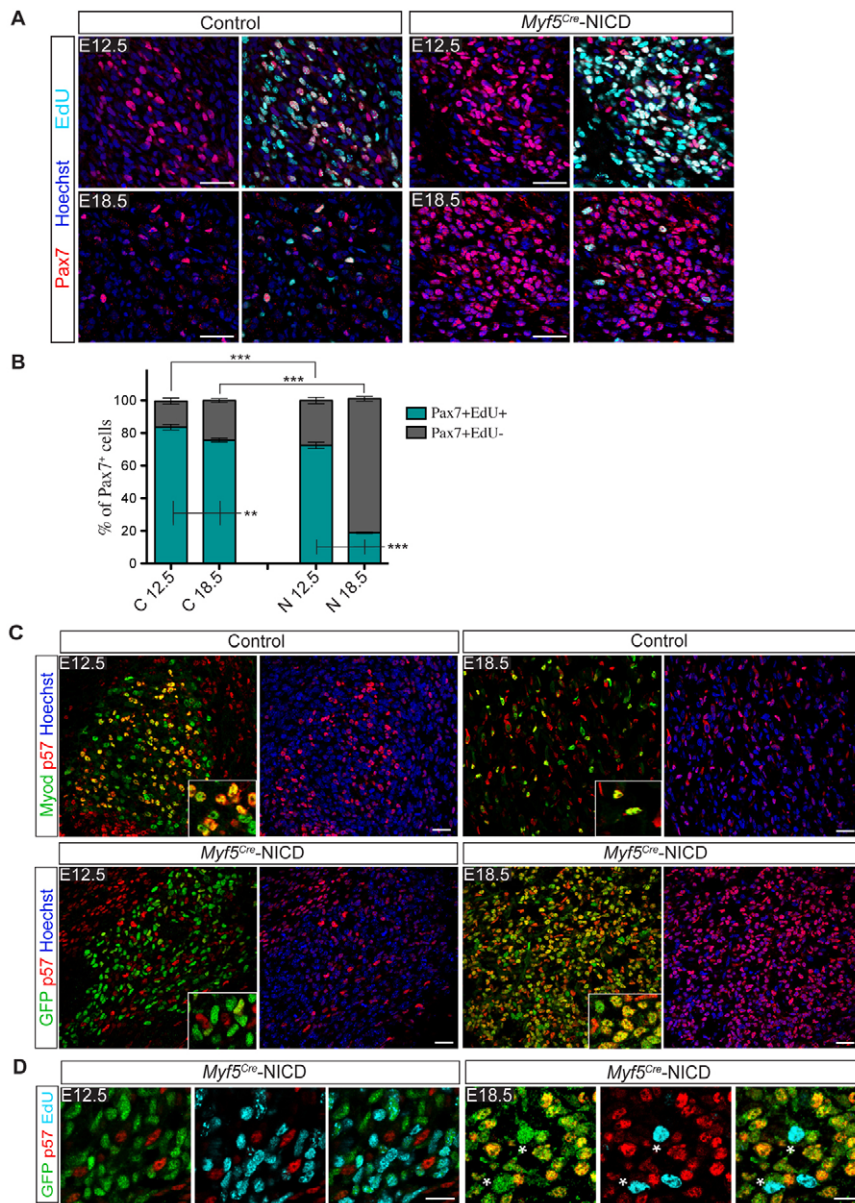


Fig. 6. Differential effect of NICD on proliferation of myogenic cells. (A) Cell division kinetics of control (*Myf5^{Cre/+}:R26^{mT-mG/+}*) and *Myf5^{Cre}-NICD*-expressing cells in E12.5 and E18.5 mouse forelimbs. Cell cycling of the Pax7⁺ cells was measured by incorporation of EdU. (B) The percentage of total Pax7⁺/EdU⁺ cells at E12.5 and E18.5 in control and *Myf5^{Cre}-NICD* embryos. Error bars indicate s.e.m.; *n*=3 embryos/stage. ***P*<0.01, ****P*<0.001. (C) Immunostaining of control (top) or *Myf5^{Cre}-NICD* (bottom) E12.5 and E18.5 embryo forelimbs for Myod and p57 (top) or GFP and p57 (bottom). (D) Immunostaining of *Myf5^{Cre}-NICD* cells at E12.5 and E18.5 for p57 and EdU. Non-cycling, EdU⁻ cells in *Myf5^{Cre}-NICD* are p57⁺. Scale bars: 50 μ m in A,C; 15 μ m in C inset; 20 μ m in D.

Although not formally proven, a similar mechanism appears to operate for the maintenance of quiescent satellite cells. Satellite cells have high Notch activity that is required for their maintenance (Bjornson et al., 2012; Mourikis et al., 2012) and are in direct contact with the basement membrane and the myofibre. The myofibre is the most likely source of ligand, which, like the Notch receptor, is a transmembrane molecule. One unexpected finding from our work is the observation that allelic expression of Notch was compatible with Myf5 protein expression, as shown also in the chick (Delfini et al., 2000; Hirsinger et al., 2001). A recent study in the chick reported that transient Notch activation was necessary to prime muscle stem cells in the dorsal DM to enter the myogenic lineage (positive for Myf5 and Myod), but sustained NICD overexpression results in the eventual downregulation of Myf5 and Myod and a failure to enter the myotome region (Rios et al., 2011). By contrast, our present study shows that muscle stem/progenitors with sustained NICD continuously express Myf5 and actively transit into the myotomal region. The differences in these results could be

related to the levels of NICD expressed or to the specific location of the stem/progenitors in the somites that were examined.

Independent studies have shown that the early embryonic Pax7⁺ cells give rise to foetal and then adult muscle stem cells (Gros et al., 2005; Kassam-Duchossoy et al., 2005; Relaix et al., 2006; Lepper and Fan, 2010). Although these studies have established the embryonic origins of foetal and adult muscle stem cells, it remains unclear if there is a single self-renewing population in the embryo that is sustained to adulthood, or whether intermediate, stage-specific populations are produced that can give rise to the populations of the corresponding developmental stages. The data presented in this study support the former scenario because embryonic myogenic cells with sustained NICD expression are arrested during lineage progression, yet they acquire a foetal signature and eventually the characteristics of satellite cells. Likewise, during development, a Notch-high cell population escapes differentiation, while adapting to the growing organism. To fulfil

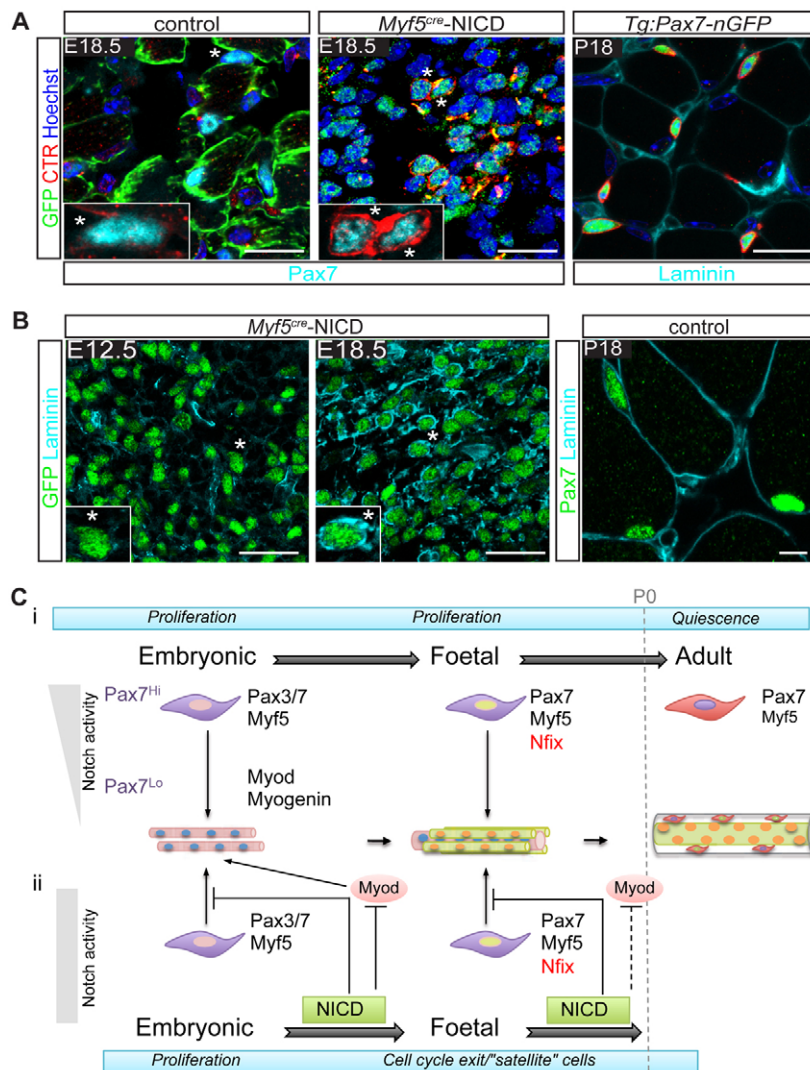


Fig. 7. Foetal NICD-expressing cells acquire satellite cell characteristics. (A) Immunostaining of E18.5 *Myf5^{Cre/+};R26^{RmT-mG/+}*, *Myf5^{Cre}-NICD* mouse forelimb and P18 *Tg:Pax7-nGFP* tibialis anterior muscle sections for calcitonin receptor (CTR). (B) Immunostaining of sections from E12.5 and E18.5 *Myf5^{Cre}-NICD* forelimb for laminin and GFP. Control P18 tibialis anterior muscle stained for laminin and Pax7. Asterisks indicate cells in the inset. Scale bars: 20 μm. (C) Cell-autonomous activated Notch (NICD) maintains the temporal specification potential of skeletal muscle stem/progenitor cells. (i) During skeletal myogenesis, the upstream myogenic population (*Pax7^{Hi}*) is maintained by high Notch activity. Differentiation is induced by muscle regulatory factors such as Myod, resulting in downregulation of Notch and Pax7 (*Pax7^{Lo}*) to form myofibres of the corresponding developmental stage. The transcriptional regulator Nfix is necessary for the acquisition of foetal myogenic identity. In the adult, muscle stem (satellite) cells are quiescent and are maintained by active Notch signalling. (ii) Sustained Notch activity cell-autonomously maintains the stem/progenitor population and inhibits differentiation. Notably, it does not interfere with the developmental specification of myogenic cells during ontogeny; embryonic markers are downregulated and foetal markers are upregulated in a temporal fashion, and differentiation continues to be inhibited. Notably, the majority of the myogenic population remains *Myod⁻*; cells exit the cell cycle to yield a quiescent satellite-like cell state in the absence of committed progeny. Activated Notch sustains proliferation of myogenic cells in the embryo and promotes reversible cell cycle exit in the foetus.

a continuous requirement for Notch, this pathway should not be dominant over other developmental cues, but at the same time it must exert specificity for the cells to assume their appropriate temporal fates. Indeed, we find that foetal myoblasts with sustained activated Notch retain robust myogenic potential in spite of expressing determinant markers of other lineages. Similarly, the dermomyotomal NICD-expressing cells downregulate *Pax3* and *HeyL* when they migrate towards the dermis, whereas they maintain *Pax3* and *HeyL* and also express *Pax7* and *Myf5* when they enter the myotome (Fig. 1D,E; supplementary material Fig. S1C). Therefore, activated Notch, in combination with tissue-specific factors, specifies and maintains muscle stem cells throughout development.

The outcome of Notch activation is notoriously unpredictable and depends on the spatiotemporal context in which it is acting, i.e. it is influenced by cross-talk with other signalling pathways and/or the cellular state (Hurlbut et al., 2007; Schwanbeck et al., 2011). In the present study, we observe actively dividing NICD-expressing stem/progenitor cells during embryonic stages and cell cycle-arrested cells during late foetal myogenesis, although differentiation is blocked at both stages. Strikingly, late foetal myogenic cells enter cellular quiescence (*Pax7⁺/Myog⁻/EdU⁻*), indicating that the entire ontological development of muscle stem cells from embryo to reversible cell cycle exit can be recapitulated

by constitutive expression of Notch. We and others reported recently the unexpected decrease in Notch activity as soon as satellite cells exit quiescence and enter the cell cycle, and the continued low activity in proliferating myoblasts until they return to quiescence (Bjornson et al., 2012; Mourikis et al., 2012). A recent study confirmed this notion by showing that NICD overexpression in freshly isolated satellite cells decreases their proliferation capacity (Wen et al., 2012). These studies combined point to the importance of Notch signalling in quiescent satellite cells and that downregulation of this pathway is necessary for transit amplification of the satellite cell population after cell cycle re-entry.

Our observation of foetal and satellite cell characteristics in *Myf5^{Cre}-NICD* fetuses at the single-cell level warrants further investigation. We predict that these cells represent a transition state. Intriguingly, the *NICD⁺/EdU⁻* cells at E18.5 are positive for the cell cycle inhibitor p57, consistent with their non-cycling nature, even though satellite cells do not express p57 (data not shown). This observation supports the notion that these cells are in a transition phase and we speculate that p57 is acting as a brake to the proliferating myoblasts to drive them into a terminal (differentiated) or reversible (quiescent) arrested state.

In summary, we show here that Notch signalling regulates muscle stem cell self-renewal as well as their temporal

specification. Although Notch activity declines progressively as these myogenic cells differentiate, muscle stem cells can be maintained cell-autonomously with allelic expression of Notch, independently of committed cells. Remarkably, this constitutive expression of NICD does not override environmental signals, thereby allowing the muscle stem cells to integrate cues from the changing environment to progress ontologically.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at

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