Notch signaling regulates myogenic regenerative capacity of murine and human mesoangioblasts

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Somatic stem cells hold attractive potential for the treatment of muscular dystrophies (MDs). Mesoangioblasts (MABs) constitute a myogenic subset of muscle pericytes and have been shown to efficiently regenerate dystrophic muscles in mice and dogs. In addition, HLA-matched MABs are currently being tested in a phase 1 clinical study on Duchenne MD patients (EudraCT #2011-000176-33). Many reports indicate that the Notch pathway regulates muscle regeneration and satellite cell commitment. However, little is known about Notch-mediated effects on other resident myogenic cells. To possibly potentiate MAB-driven regeneration in vivo, we asked whether Notch signaling played a pivotal role in regulating MAB myogenic capacity. Through different approaches of loss- and gain-of-function in murine and human MABs, we determined that the interplay between Delta-like ligand 1 (Dll1)-activated Notch1 and Mef2C supports MAB commitment in vitro and ameliorates engraftment and functional outcome after intra-arterial delivery in dystrophic mice. Furthermore, using a transgenic mouse model of conditional Dll1 deletion, we demonstrated that Dll1 ablation, either on the injected cells, or on the receiving muscle fibers, impairs MAB regenerative potential. Our data corroborate the perspective of advanced combinations of cell therapy and signaling tuning to enhance therapeutic efficaciousness of somatic stem cells.

Results

Dll1-Notch1 regulate in vitro differentiation of murine and human MABs. For this study, we used murine12 and human MABs,13 both isolated from somatic muscles as alkaline phosphatase+ (AP+) cells and processed as previously reported14 (Supplementary Figure 1). Consistently, with antecedent reports, AP+ murine MABs do not spontaneously undergo myogenic differentiation in vitro, unlike human AP+ cells. However, Notch-mediated effects on the regenerative potential of non-satellite resident myogenic cells are still unknown.

Mesoangioblasts (MABs) are non-satellite resident myogenic stem cells, able to circulate and regenerate dystrophic skeletal muscles.5,10 HLA-matched MABs are currently under phase 1 clinical study on Duchenne muscular dystrophy patients (EudraCT #2011-000176-33). In this view, understanding the cell-specific effects and mechanisms of myogenic cues will help improving clinical translation of MAB-based therapies in vivo. Recently, it has been shown that Notch synergizes with Pdgf-bb to convert fetal myoblasts into myogenic pericytes.11 However, knowledge about Notch-triggered effects on the regenerative potency of somatic MABs is still scant, particularly in the context of cell–cell (in vitro) and fiber–cell (in vivo) contact.

Therefore, we asked whether the Dll1-Notch1 axis regulates the myogenic potential of murine and human MABs and how to tune this pathway to ameliorate in vivo MAB-driven regeneration.

Abbreviations: Ad, adenoviral vector; AP, Alkaline phosphatase; AU, arbitrary units; cm, centimeters; CpG, cytosine guanine (linear dinucleotide); DS, human-specific DYSTROPHIN; Dll1, Delta-like ligand 1; EudraCT, European Union Drug Regulating Authorities Clinical Trials; flx, floxed, that is, comprised between two loxP sites; Gapdh, Glyceraldehyde-3-phosphate dehydrogenase; GFP, Green fluorescent protein; gsi, γ-secretase inhibitor; Hes1, Hes family bHLH transcription factor 1; HLA, human leukocyte antigen; iCreERT2, inducible Cre recombinase fused with estrogen receptor (tamoxifen-responsive); IgG, Immunoglobulin G; IP, immunoprecipitation; Jag1, Jagged 1; lucf, firefly luciferase; m, meters; MABs, mesoangioblasts; Mamf1, Mastermind-like 1; MDs, muscular dystrophies; Metf2C, Myocyte enhancer factor 2C; min, minutes; MyHC, Myosin heavy chain; NIDC, Notch1 intracellular domain; Pdgf-b, Platelet derived growth factor receptor, beta polypeptide; Pgk, Phosphoglycerate kinase; qPCR, quantitative polymerase chain reaction; Rag2, Recombination activating gene 2; RFP, Red fluorescent protein; Sgca, Sarcoglycan α; Western blot; Wnt, wingless-type MMTV integration site family; γc, Interleukin 2 receptor, gamma chain

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MABs\textsuperscript{9,10} (Supplementary Figure 2). We analyzed the pattern of Notch1, Dll1 and Hes1 expression and of pathway activation during spontaneous in vitro differentiation. Murine MABs showed slight upregulation of Notch1 at day 3, but progressive downregulation of Dll1 and Hes1 over time, matching Dll1 and NICD (activated receptor) protein levels (Figures 1a and b; Supplementary Figure 3a). Conversely, human MABs showed early upregulation of NOTCH1, DLL1 and HES1, paralleled by increasing protein levels of DLL1 and NiCD at day 3, followed by rapid decrease (Figures 1c and d; Supplementary Figure 3a). To investigate the role of epigenetics in the species-specific pattern of Dll1 expression, we monitored the DNA methylation propensity along the regulatory regions of the genomic Dll1/DLL1 locus. Interestingly, Dll1 regulatory regions appeared more rapidly methylated in murine than in human MABs (Figures 1e and f), being the overall methylation propensity along the murine sequences significantly higher at day 0 and 3 than in the human DLL1 locus (Supplementary Figure 3b). Thus, during spontaneous differentiation in vitro, murine MABs do not spontaneously mature into myocytes and exhibit rapid locus methylation and decreased expression of Dll1 at early stage, correlating with decreased Notch1 activation. Conversely, human MABs mature into myocytes in vitro and show

![Figure 1](image-url)
sustained Dll1 expression and Notch1 activation at early differentiation step.

We then investigated the effects of loss and gain of Notch signaling on the in vitro myogenic capacity of MABs. This was assayed in murine MABs by co-culture/fusion with C2C12 myoblasts, whereas human MABs were assayed in spontaneous differentiation toward myocytes. Specific knockdown of Notch1 and Dll1 (Supplementary Figure 4a), as well as treatment with γ-secretase inhibitor (gsi), resulted in decreased NICD levels (Supplementary Figure 4b), and in impairment of murine MAB fusion with myoblast-derived myotubes, as compared with scramble control (Figure 1g). Analogously, gsi and NOTCH1/DLL1 knockdown led to impaired maturation of human MABs into MyHC⁺ myocytes (Figure 1h). We also analyzed the involvement of other receptors and ligands, for example, Notch2 receptor and Jag1 ligand, in our in vitro system. Knockdown of Notch2/NOTCH2 or Jag1/JAG1 did not significantly alter MAB myogenic differentiation, as compared with scramble control (Supplementary Figure 5), supporting the hypothesis that the Dll1-Notch1 axis is pivotal in our experimental system. We next used a lentiviral system of doxycycline-triggered Dll1/DLL1 overexpression to evaluate the effects of gain-of-function in the early differentiation step. After application of doxycycline till day 3 of differentiation, Dll1/DLL1 overexpression dose-dependently increased in vitro myogenic maturation of both murine and human MABs (Figures 1i and j). This effect was significantly decreased in the presence of gsi (Supplementary Figure 6), thus confirming the direct involvement of Dll1-triggered Notch signaling in the early differentiation step of murine and human MABs.

As additional proof of principle, we sought to apply our dose-dependent system of transient Dll1 overexpression to a MAB model of robust myogenic differentiation. To this purpose, we used murine Sgcb-null MABs, previously reported as aberrantly and spontaneously myogenic.15,16 Transient overexpression of Dll1 dose-dependently correlated with increased Hes1 expression at day 3 (Figure 2a) and enhanced myotube formation at day 7 (Figure 2b). Thus, Dll1-Notch1 signaling during the early differentiation/commitment step sustains MAB myogenic differentiation at a later stage. We asked whether Mef2C and Maml1, reported interactors of NICD in other myogenic cell models,17,18 were involved in Dll1-dependent effects on MAB differentiation. Murine MABs showed progressive decline of gene and protein levels of Mef2C over time, whereas MEF2C was upregulated at days 3–5 in human MABs. Moreover, the levels of Maml1/MAML1 appeared stable over time in both murine and human MABs (Figures 3a and d and Supplementary Figure 7a). Notably, at day 3 of spontaneous differentiation, Mef2C and Maml1 are only partially associated to NICD and Dll1 knockout resulted in major dissociation from the residual NICD levels. Conversely, Dll1 overexpression led to complete Mef2C recruitment, whereas only after combined overexpression of Dll1 and Mef2C did Maml1 appear completely associated to the NICD complex (Figure 3e). Because Maml1 was reported as activator of Mef2C upon dissociation from NICD,17 we quantified the effects of Maml1 recruitment on Mef2C activity in our cell system at day 5 of differentiation, when NICD levels are depleted in normal conditions. Using a Mef2C-responsive luciferase system, Mef2C transcriptional activity in murine MABs was decreased after Dll1 knockdown and significantly increased after combined overexpression of Dll1 and Mef2C (Figure 3f). We obtained analogous data also from human MABs (Figure 3g and h). Thus, the Dll1-Notch1 axis appears as a major regulator of the in vitro myogenic ability of both murine and human MABs, and Dll1-triggered signaling cooperates with Mef2C and Maml1 in enhancing Mef2C activity at later stages.

**Primbing murine and human MABs for enhanced in vivo regeneration.** Subsequently, we sought to determine whether tuning the signaling without genomic integrations could ameliorate the in vivo regenerative potential of murine and human MABs. We used an adenoviral-based, non-integrative system of temporary overexpression of Dll1 or Mef2C (Ad-Dll1 and Ad-Mef2C, respectively, Supplementary Figure 4c). This allowed us to substitute for the endogenous NICD expression after Dll1 or Mef2C transduction, and thus to interact with endogenous Notch signaling. Accordingly, analyses of NICD levels and displacement of Notch1 from the NICD complex were performed in both murine and human MABs (Figures 3a and d and Supplementary Figure 7a). As expected, the Dll1-Notch1 axis appeared stable over time in both murine and human MABs, whereas only after combined overexpression of Dll1 and Mef2C did Maml1 appear completely associated to the NICD complex (Figure 3e). Because Maml1 was reported as activator of Mef2C upon dissociation from NICD,17 we quantified the effects of Maml1 recruitment on Mef2C activity in our cell system at day 5 of differentiation, when NICD levels are depleted in normal conditions. Using a Mef2C-responsive luciferase system, Mef2C transcriptional activity in murine MABs was decreased after Dll1 knockdown and significantly increased after combined overexpression of Dll1 and Mef2C (Figure 3f). We obtained analogous data also from human MABs (Figure 3g and h). Thus, the Dll1-Notch1 axis appears as a major regulator of the in vitro myogenic ability of both murine and human MABs, and Dll1-triggered signaling cooperates with Mef2C and Maml1 in enhancing Mef2C activity at later stages.
Figures 7b and c), priming the cells in vitro prior to bilateral intra-arterial injection into dystrophic mice. After injection into Sarcoglycana(Sgca)-null mice, combined priming of green fluorescent protein (GFP)+ murine MABs with Ad-Dll1 and Ad-Mef2C resulted in enhanced levels of engraftment into GFP+ chimeric fibers and restoration of Sgca, as shown by immunostaining, western blotting (WB) and quantitative polymerase chain reaction (qPCR) at 4 weeks (Figures 4a and b and Supplementary Figure 8) and 8 weeks post injection (data not shown). Accordingly, fibrotic scars appeared also reduced in treated mice (Supplementary Figure 9a). We then assessed the functional relevance of histological and molecular data by gait analysis and treadmill assay. In both tests, combined priming of injected MABs resulted in enhanced levels of engraftment into MEF2C+ muscle fibers (Figures 7b and c), priming the cells in vitro prior to bilateral intra-arterial injection into dystrophic mice. After injection into Sarcoglycana(Sgca)-null mice, combined priming of green fluorescent protein (GFP)+ murine MABs with Ad-Dll1 and Ad-Mef2C resulted in enhanced levels of engraftment into GFP+ chimeric fibers and restoration of Sgca, as shown by immunostaining, western blotting (WB) and quantitative polymerase chain reaction (qPCR) at 4 weeks (Figures 4a and b and Supplementary Figure 8) and 8 weeks post injection (data not shown). Accordingly, fibrotic scars appeared also reduced in treated mice (Supplementary Figure 9a). We then assessed the functional relevance of histological and molecular data by gait analysis and treadmill assay. In both tests, combined priming of injected MABs significantly ameliorated the functional outcome, both at 4 and 8 weeks post injection, as assayed by gait analysis and treadmill test (Figure 5e and Supplementary Figure 13c). To examine the requirement of Dll1 presentation by the homing cell, we isolated and characterized flx MABs from post-natal muscles of transgenic mice, as previously described.14 We then tested their myogenic potential in vitro by fusion with C2C12-derived myotubes, and in vivo by intra-arterial delivery into Sgca-null mice (Figure 5a). After tamoxifen-driven Dll1 knockout, fix GFP+ MABs showed significant impairment in the contribution to GFP+ myotubes in vitro (Figure 5b) and to Sgca+/GFP+ fibers in vivo (Figures 5c and d and Supplementary Figures 12a and b), as compared with vehicle-treated cells. Interestingly, Dll1 knockout in homing fix MABs resulted also in significant reduction of the functional outcome at 4 and 8 weeks post injection, as assayed by gait analysis and treadmill test (Figure 5e and Supplementary Figure 13c). To validate our transgenic model, we also assayed fix satellite cells, which, as expected, showed reduced levels of Pax7 and increased spontaneous differentiation after tamoxifen addition (Supplementary Figures 14a and b). Subsequently, we asked whether Dll1 presentation by the host fiber is also

**Analysis of Dll1 requirement using transgenic MABs and muscles.** To further examine the directionality of the ligand presentation at the interface between the homing cell and the host fiber, we examined MABs and muscle fibers of Dll1lox/lox, Rosa26::iCreERT2+/− (fix) transgenic mice, allowing conditional tamoxifen-driven Dll1 knockout (Supplementary Figures 12a and c). To examine the requirement of Dll1 presentation by the homing cell, we isolated and characterized flx MABs (Supplementary Figures 12d and e) from post-natal muscles of transgenic mice, as previously described.14 We then tested their myogenic potential in vitro by fusion with C2C12-derived myotubes, and in vivo by intra-arterial delivery into Sgca-null mice (Figure 5a). After tamoxifen-driven Dll1 knockout, fix GFP+ MABs showed significant impairment in the contribution to GFP+ myotubes in vitro (Figure 5b) and to Sgca+/GFP+ fibers in vivo (Figures 5c and d and Supplementary Figures 13a and b), as compared with vehicle-treated cells. Interestingly, Dll1 knockout in homing fix MABs resulted also in significant reduction of the functional outcome at 4 and 8 weeks post injection, as assayed by gait analysis and treadmill test (Figure 5e and Supplementary Figure 13c). To validate our transgenic model, we also assayed fix satellite cells, which, as expected, showed reduced levels of Pax7 and increased spontaneous differentiation after tamoxifen addition (Supplementary Figures 14a and b). Subsequently, we asked whether Dll1 presentation by the host fiber is also
required. We therefore tested the engraftment levels of wild-type GFP+ MABs after intra-arterial delivery into Dll1-knockout adult flx muscles (Figure 5f). In the absence of acute damage, tamoxifen-treated mice did not present significant alterations or lesions in skeletal muscles, as compared with vehicle-treated controls (Supplementary Figure 14c). In Dll1-knockout muscles, the rate of MAB engraftment was significantly reduced as compared with vehicle-treated controls, as shown by immunostaining, qPCR and WB (Figures 5g and i). Thus, Dll1 presentation by both the homing cell and the host fiber appears a positive regulator of MAB commitment and engraftment.

Figure 4  Combined priming with Dll1/DLL1 and Mef2C/MEF2C enhances the in vivo regenerative capacity of both murine and human MABs. (a, b) Immunofluorescence staining on gastrocnemius muscle slides of Sgca-null mice at 4 weeks after bilateral femoral artery injection of adenoviral-primed GFP+ murine MABs. Laminin/GFP staining (a) has been used to evaluate engraftment and Sgca/GFP staining (b) for regeneration capacity. (c, d) Functional assessment of the adenoviral-based priming on the murine MAB-driven muscle regeneration of dystrophic muscles at 4 weeks post injection. The gait analysis (c) has been performed on the stride length of mice spontaneously walking along a 1 m path. The treadmill assay (d) has been performed on mice running on a 10°-uphill oriented treadmill belt with 1 m/min² acceleration on 10 m/min starting speed. Data points depict the value of each assayed mouse, bars indicate the average values; *P<0.05 versus sham; §P<0.05 versus Ad-Dll1 and versus Ad-Mef2C (n = 6 mice/group). (e, f) Immunofluorescence staining on gastrocnemius muscle slides of Rag2-null/γc-null mice at 4 weeks after bilateral femoral artery injection of adenoviral-primed GFP+ human MABs. Laminin/GFP staining (e) has been used to evaluate engraftment and hDystrophin (hDYS)/GFP staining (f) for regeneration capacity. Sham, vehicle-injected control mice; +Ad-mock, MABs primed with Ad-LacZ; adenoviral-treated control cells with unperturbed Notch signaling. Scale bars indicate 100 μm.
Figure 5  Analysis of Dll1 requirement for MAB engraftment/regenerative capacity using Dll1^{flx/flx};Rosa26::iCreERT2^{+/−} (flx) transgenic MABs and muscles. (a) Representative scheme of the experiment assessing tamoxifen-driven Dll1 knockout in the homing flx MABs, using the vehicle-treated cells as control. (b) Immunofluorescence staining on co-cultures of C2C12 myoblasts with GFP^{+} flx MABs after Dll1 knockout. White arrows indicate chimeric MyHC^{+} myotubes and MAB myogenic differentiation. (c) Immunofluorescence staining on gastrocnemius muscle slides to evaluate the regenerative capacity of Dll1-knockout GFP^{+} flx MABs at 8 weeks after intra-arterial injection into Sgca-null mice. (d) qPCR-based quantification of engraftment (GFP) and regeneration (Sgca) in the tibialis anterior muscles at 8 weeks post injection; *P<0.05 versus vehicle-treated flx MABs (n=5 mice/group). (e) Functional assessment of conditional Dll1 knockout on MAB-driven regeneration of dystrophic muscles at 8 weeks after bilateral injection, by means of gait analysis (stride length) and treadmill assay (running time, distance). Data points depict the value of each assayed mouse, bars indicate the average values; *P<0.05 versus vehicle-treated flx MABs (n=5 mice/group). (f) Representative scheme of the experiment assessing tamoxifen-driven Dll1 depletion in the flx muscles, using the vehicle-treated mice as control. (g) Immunofluorescence staining on gastrocnemius muscle slides to evaluate the engraftment ability of homing GFP^{+} MABs into Dll1-knockout muscles at 4 weeks after intra-arterial injection. (h, i) Expression and protein level analyses of GFP to quantify reduced homing ability of MABs into tibialis anterior fibers after conditional Dll1 knockout at 4 weeks post injection; *P>0.05 (n=3 mice/group). Expression and protein levels in (d, h, i) are reported in arbitrary units (AU) as fold change versus vehicle-treated mice. Scale bars indicate 100 μm.
Discussion

Taken altogether, our data suggest that Dll1-triggered Notch1 activation plays a pivotal role in the myogenic commitment of post-natal murine and human MABs. The difference in Dll1 epigenetic/transcriptional regulation and in Notch1 activation during the early in vitro differentiation step could account, at least partially, for the intrinsic difference in spontaneous in vitro myogenic capacity between murine and human MABs. Moreover, Dll1-triggered positive influence on MAB differentiation seems confirmed in the proof of principle of Dll1 overexpression with the aberrantly myogenic Sgcb-null MABs. The aforementioned observations, in combination with the IP and luciferase analyses at days 3 and 5 of differentiation, suggest a time-dependent threshold effect of Dll1-activated NICD interplay with Mef2C and Maml1 (Figure 6a). According to this hypothetical model, in the absence of Dll1-presenting myoblasts, murine MABs exhibit low levels of Dll1 and Mef2C at early differentiation stage and, therefore, Mef2C activity at later stages is likely insufficient to corroborate further progression. Differently, human MABs present sufficient levels of DLL1 and MEF2C to trigger MEF2C activity and myocyte maturation at later stage. Furthermore, in both murine and human MABs, transient overexpression of Dll1/DLL1 and Mef2C/MEF2C during the early stage supposedly saturates complex formation and correlates with enhanced myogenic capacity.

This in vitro model is reinforced by our adenoviral-based system of MAB priming prior to injection. Adenoviruses do not integrate in the genome of the engrafting MABs, still promoting the overexpression pulse of the transgenes and enhancing the commitment. Once transduced cells are engrafted, the vector is likely diluted in the fibers, discontinuing the signaling and ameliorating the regeneration outcome of MABs in targeted muscles.

The directionality of Dll1 requirement for MAB homing and differentiation in vivo constitutes an interesting question, considering that Notch signals have been directly linked to the niche engraftment by satellite cells. We addressed this point by means of a transgenic system of conditional Dll1 deletion. Together with the data previously discussed, the results obtained from flx MABs and flx muscles suggest a potential, yet incomplete, model of the basal role of Notch on MAB engraftment and differentiation in vivo (Figure 6b). According to this model, Notch1 activation in the homing MABs by Dll1-exposing cells or fibers may result in NICD-mediated recruitment of Mef2C and Maml1. Once engrafted in the fiber, potentially also through Dll1-mediated interactions, NICD levels would decrease in the absence of receptor stimulation and Mef2C would then exert its transcriptional activity, promoting the expression of mid-late myogenic factors. Noteworthy, this model might also point to Notch-based recruitment of MABs by Dll1-exposing activated satellite cells in the context of muscle injury.

Our study cannot exclude the contribution of other ligands or receptors of the Notch family, probably accounting for the remaining engraftment of Dll1-knockout MABs or in Dll1-depleted muscles. However, other interactions appeared less significant than the Dll1-Notch1 axis in our experimental setup, considering that knockdown of Notch2/NOTCH2 or Jag1/JAG1 did not impair the myogenic ability of murine and human MABs

Figure 6 Hypothetical models of Notch-based tuning of MAB myogenic differentiation. (a) The intrinsic difference in the levels of Dll1-triggered Notch1 activation and of Mef2C could explain, at least partially, the difference in spontaneous myogenic capability between murine and human MABs in our experimental system. According to this hypothesis, murine MABs present low levels of Dll1-triggered signaling and of Mef2C during the commitment stage. At a later step of differentiation, once NICD is removed from the complex, Mef2C activity levels are therefore minimal. Human MABs present intrinsically higher levels of DLL1 and MEF2C in vitro. As a consequence, murine MABs fail to spontaneously produce mature myocytes in vitro, whereas human MABs differentiate, although with low efficiency, spontaneously. Transient overexpression of Dll1/DLL1 and Mef2C/MEF2C during the commitment stage induces complete recruitment of Mef2C/MEF2C and Maml1/MAML1 by NICD, thereby reinforcing Mef2C activity and myogenic capability of both murine and human MABs, as observed in vivo. (b) Representative scheme of the hypothetical model regarding Dll1 involvement during the commitment-differentiation transition of MABs in vivo, according to our experimental setup with transgenic MABs and muscles. Dll1-Notch1 interactions between the dystrophic fiber and the homing MABs trigger their commitment and engraftment, possibly through Maml1/Mef2C interplay. After engraftment, the signaling is discontinued, NICD removed and the engrafted MABs activate the myogenic program, likely through sustained levels of Mef2C activity in vitro. Furthermore, our in vivo data indicate that ligand-based tuning of the Notch pathway and its interactors may constitute a feasible integration-free strategy to potentiate the outcome of intra-arterial MAB therapy, possibly in combination with approaches to ameliorate microcirculation in dystrophic muscles. To potentially accelerate clinical translation of such approaches, it will be fundamental to MAB-specifically assess how the Notch pathway is involved in the interaction with other resident cell types and how it intertwines with other cascades, such as Wnt and Bone morphogenetic protein pathways.
Materials and Methods

Cell culture, isolation and differentiation. Murine AP2+ MABs (n=3 clones from 3 individuals; p10-20) were cultured on collagen (Sigma-Aldrich, St. Louis, MO, USA)-coated plastic vessels (Nunc, Penfilnd, NY, USA) in DMEM20% medium (DMEM 4.5 g/l glucose, supplemented with 20% heat-inactivated fetal bovine serum, 1% Pen-Strp, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential aminoacids, 0.2% 2-mercaptoethanol; all reagents by Life Technologies, Carlsbad, CA, USA) at 37°C/5% CO2. Human AP2+ MABs (n=3 clones from 3 individuals; p8-15) were isolated by fluorescence-activated cell sorting (FACS) from human MAB populations by AP-PE antibody (R&D Systems, Minneapolis, MN, USA; 2 μl/10^6 cells) at FACS Aria III (BD, Franklin Lakes, NJ, USA). Human AP2+ MABs were cultured on collagen (Sigma-Aldrich)-coated plastic vessels (Nunc) in IMDM10% medium (IMDM medium, supplemented with 10% heat-inactivated fetal bovine serum, 1% Pen-Strp, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential aminoacids, 0.2% 2-mercaptoethanol, 5 ng/ml human FGF2 (Repeotech, Rocky Hill, NJ, USA) at 37°C/5%CO2. Antigen profiling was performed with APC-conjugated antibodies (eBioscience, San Diego, CA, USA), following antibody specifications and appropriate isotypes, at FACS Canto (BD), then data were charted by FlowJo software (Tree Star, Ashland, OR, USA). AP enzymatic staining was performed incubating for 30 min at 37°C 2% PFA-fixed cells with SigmaFast BCIP-NBT (Sigma-Aldrich) tablets dissolved in distilled water. Spontaneous differentiation was performed on collagen-coated vessels in DMEM2% medium (DMEM 4.5 g/l glucose, supplemented with 20% heat-inactivated horse serum, 1% Pen-Strp, 1% L-glutamine, 1% sodium pyruvate) at 37°C/5%CO2. C2C12 myoblasts (p5-12) were cultured in DMEM10% medium (DMEM 4.5 g/l glucose, supplemented with 10% heat-inactivated fetal bovine serum, 1% Pen-Strp, 1% L-glutamine, 1% sodium pyruvate) on plastic vessels (Corning, Corning, NY, USA) at 37°C/5%CO2. Differentiation in co-culture was performed seeding C2C12 myoblasts with MABs at 1:1 ratio, then applying DMEM2% medium after 24 h. GFP+ MABs were obtained by integration of a GFP-bearing transposon, as previously described.12

WB, IP, qPCR and immunofluorescence/histological staining analyses. WB analyses were performed on 50 μg starting protein extracts, previously obtained homogenizing cells or tissues in RIPA buffer supplemented with 1% PMSF 1 mM, 1% sodium fluoride 10 mM, 1% protease inhibitor cocktail, 1% sodium orthovanadate 0.5 mM (all reagents by Sigma-Aldrich). Gels were polymerized with 30% acrylamide (Sigma-Aldrich) according to assayed protein size (8% for MyHC; 10% for DI1, NIDC, Mamt1; 12% for Gapdh, Me2C, GFP) and electrophoresis/transfer/detection was performed on gels in parallel when assaying the same samples for multiple proteins. Incubation with primary antibody was performed in 5% skim milk TBS-T overnight at 4°C, whereas HRP-conjugated secondary antibodies (1:5000, Santa Cruz, Dallas, TX, USA) were applied for 1 h at room temperature. Bands were revealed after 15 min incubation with SuperSignal Dura Chemiluminescence substrate (Thermo Scientific, Waltham, MA, USA) and acquired at GelDoc setup (Bio-Rad, Hercules, CA, USA). Quantitation was performed on gels loaded and blotted in parallel. Densitometric values of protein bands were quantitated through QuantityOne software (Bio-Rad), then normalized versus background noise and averaged values of the related Gaph/GAPD internal control bands. Primary antibodies, dilutions; mouse anti-MyHC (Developmental Studies Hybridoma Bank, Iowa, IA, USA; clone MF20), 1:10; rabbit anti-Dll1 (Cell Signaling, Beverly, MA, USA), 1:50; rabbit anti-Notch1 (Abcam, Cambridge, UK), 1:500; rabbit anti-NIDC (Abcam), 1:500; rabbit anti-Mamt1 (Millipore, Billenica, MA, USA), 1:500; rabbit anti-Gapdh (Sigma-Aldrich), 1:1000; rabbit anti-Me2C (LifeSpan Biosciences, Providence, RI, USA), 1:500; rabbit anti-GFP (Life Technologies), 1:300.

IP was performed on 100 μg protein extracts, previously obtained homogenizing cells in IP buffer (Tris-HCl pH 8.5 50 mM, NaCl 150 mM, 1% NP40, 0.5% sodium deoxycolate, 1% PMSF 1 mM, 1% sodium fluoride 10 mM, 1% protease inhibitor cocktail, 1% sodium orthovanadate 0.5 mM (all reagents by Sigma-Aldrich)). Immunoprecipitation was performed by overnight incubation with 0.5 μg/reaction mouse anti-NIDC (Abcam) in a total volume of 500 μl IP buffer, while rotating at 4°C. Bound fraction was precipitated by proteinG-sepharose beads (GE Healthcare, Billerica, MA, USA) and separated from the unbound. Query proteins were then assayed on parallel membranes by rabbit primary antibodies.

For qPCR analyses, RNA was isolated through RNA mini kit, removing gDNA traces and plate reader by Life Technologies). PGK-PGK was used as internal normalizer. The list of primers can be found in the Supplemental Materials and Methods.

Immunofluorescence staining was performed on PFA-fixed cells or cross-sectional 10 μm-thick muscle slices, obtained by cryoembedding muscles in Tissue-Tek OCT (Sakura, Alphen aan den Rijn, The Netherlands) and cutting at Cryostar NX70 (Thermo Scientific) cryostat (−16 °C sample, −22 °C blade). Incubation with primary antibodies was performed in PBS supplemented with 5% BSA overnight at 4°C, whereas Alexa Fluor-conjugated secondary antibodies (1:500, Life Technologies) were applied for 1 h at room temperature. Nuclei were counterstained with 10 μg/ml Hoechst (Sigma-Aldrich). Images were acquired at Eclipse Ti (Nikon, Tokyo, Japan) fluorescence inverted microscope, using NIS Elements AR 4.1.0.11 (Nikon) software with background ROI calibration optimized on isotype controls. Primary antibodies, dilutions; goat anti-GFP (Abcam), 1:500; rabbit anti-RFP (Bio-Connect, Huisven, The Netherlands), 1:200; mouse anti-Myc (DSHB, clone MF20), 1:3; rabbit anti-laminin (Sigma-Aldrich), 1:300; mouse anti-Sqa (Leica Biosystems, Nussloch, Germany), 1:100; mouse anti-human DYSTROPHIN (Leica Biosystems, clone Dys3), 1:100; rabbit anti-human sarcomeric ACTININ (Abcam), 1:250. Masson’s trichromic staining was conducted on 5 μm-thick cross-sectional paraffin-embedded muscle slices by means of staining kit (Sigma-Aldrich) according to manufacturer’s instructions.

DNA methylation assay. CpG islands were defined by submitting the 10 kb gDNA sequence upstream of the transcription start to CpG island searcher (http://cpgislands.usc.edu). lower limits, CpG% = 55. O2csGpcIExpGp = 0.65, length = 300, distance = 100; CpG island in murine Dll1 locus, −4257 to −3246 bp from transcription start. Dll1 islands in human DLL1 locus, −5908 to −3112 bp and −503 to −0 bp from transcription start. gDNA was isolated from 106 cells in proliferative conditions by means of genomic DNA Mini Kit (Life Technologies) and 1 μg gDNA was randomly fragmented into fragments of 200-300 bp in 50 μl TE buffer in Bioruptor sonication bath (Diagenode, Liège, Belgium) at 4°C for 15 cycles (30 μsec sonication/30’ rest) at high intensity. Sheared gDNA (200 ng) was then enriched for fragments containing ≥ 5 me-CpGs using MethyCollector Ultra kit (Active Motif, Carlsbad, CA, USA; low salt conditions) and purified via MinElute Reaction Cleanup Up kit (Qiagen, Venlo, The Netherlands). Purified DNA was then assayed for specific amplification of sequential fragments of regulatory regions through Sybr green-based qPCR, using APC promoter (negative control, unmethylated) and NBR2 promoter (positive control, methylated) as standards for relative quantification of the methylation propensity of the single fragments. The list of primers can be found in the Supplemental Materials and Methods.

Knockdown, overexpression and luciferase reporter vectors. For knockdown of Dll1/DLL1 and Notch1/NOTCH1, specific shRNA-mimicking oligonucleotides were PCR-amplified and cloned at the 3′ of the fluorescent tracer into psi-CHECK2 reporter vector (efficient in murine MABs; reporter, RFP) backbones (Open Biosystems, Huntsville, AL, USA). Vectors carrying the scramble shRNA control were purchased. Knockdown vectors were then used to produce lentiviral particles in 293T cells, using 2nd generation packaging plasmids. At 24 h after application of the viral supernatant, transduced MABs were sorted for the tracer and then plated for knockdown efficiency check and for differentiation. MABs pre-treated for 48 h with medium supplemented with 1:500 γ-secretase inhibitor X (Millipore) were also then incorporated in the differentiation experiment as control of chemical inhibition of the signaling. The list of oligonucleotides can be found in the Supplemental Materials and Methods.

For doxycycline-driven overexpression of Dll1/DLL1 and Mel2C2/MEF2C, full-length cDNAs were purchased as in between recombining sequences compatible for Gateway system (Genecoopoeia, Rockville, MD, USA) or cloned into pENTR11 shuttle vector (Life Technologies) from non-compatible plasmids (Open Biosystems). cDNA sequences were then recombined into pLOVE lentiviral backbone using LR Clonase II kit (Life Technologies), according to manufacturer’s instructions. Lentiviral particles were produced in 293T cells, using 3rd generation packaging plasmids (5 ml viral suspension/75 cm² 293T cells). Two milliliters of viral suspension were used to transduce 5 × 105 cells. Twenty-four hours after transduction, doxycycline was added for 72 h according to the different concentrations, and then removed during the second part of differentiation. In case of gsi-treated control, gsi was added in combination with the doxycycline. Doxycycline-driven overexpression of Dll1/DLL1 and Mel2C2/MEF2C has been used for all in vitro experiments reported in Figures 1.2-3 and related Supplementary Figures. Luciferase activity was monitored by transfecting transduced cells with RSRF-luc-2W (Addgene, Cambridge, MA, USA) and renilla luciferase plasmids by means of
Effectene (Qiagen). Luciferase activity was quantified at day 5 of in vitro spontaneous differentiation, normalizing firefly luciferase activity to renilla luciferase read per sample (Dual Reporter Luciferase assay kit by Promega, Madison, WI, USA) at EG&G microplate luminometer LB96V (Berthold, Wildbad, Germany).

For the adenoviral-based strategy, CDNAs in PENTR-11 shuffle vector were recombined, as mentioned before, into pAd-CMV-VS-DEST, whereas pAd-CMV-VS-GW-lacZ was used as Ad-mock control (both vectors by Life Technologies). Adenoviral particles were produced by transfecting PacI-linearized pAd vectors into 25 cm² 293A cells (final volume = 2 ml). Upon 100% mortality, I supernatant was used to transduce 150 cm² 293A cells (final volume = 10 ml). Upon 100% mortality, II supernatant was frozen in 2 ml aliquots and stored at -80 °C. One vial was used to transduce 5 × 10⁸ proliferating MABs and, after 48 h, cells were carefully washed, collected and resuspended in the appropriate number/volume conditions for injection.

In vivo experiments and evaluation of the functional outcome.

All animal protocols were conducted in compliance with Ethical Committee Guidelines of KU Leuven (project 095/2012) and Belgian legislation. Sgca-null (C57/B6 background) dystrophic mice (6-months old) were generated by the group of K.P. Campbell (University of Iowa, IA, USA) by PCR on 1 ng gDNA 5 days after last injection (primers, Fw: 5′-agagctcgtgagccgctgctc-3′, Rev: 5′-agtgactgtggagcccgtc-3′). Conditional Dll1 (5.1 cm²) mice were isolated from the skeletal muscle following the culture conditions.

Fix mice derivation, flx cell isolation and conditional Dll1 knockout. Dll1flx/+ mice were rederived from frozen 2-cell stage embryos, obtained from Prof. Julian Lewis (Cancer Research UK) with Flx SCs were isolated from proliferating MABs and, after 48 h, cells were carefully washed, collected and resuspended in the appropriate number/volume conditions for injection.

Kruskal–Wallis and Mann–Whitney U test were used. Significance was accepted when P < 0.05 was scored in both tests. All statistical tests were performed by means of Prism software (GraphPad, La Jolla, CA, USA).

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Author contributions

MQ designed and performed experiments, analyzed data, drafted the manuscript; DC, GG and JC performed experiments; MS designed experiments, provided funding, drafted the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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