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Divergent and conserved roles of Dll1 signaling in development of craniofacial and trunk muscle

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ABSTRACT

Craniofacial and trunk skeletal muscles are evolutionarily distinct and derive from cranial and somitic mesoderm, respectively. Different regulatory hierarchies act upstream of myogenic regulatory factors in cranial and somitic mesoderm, but the same core regulatory network – MyoD, Myf5 and Mrf4 – executes the myogenic differentiation program. Notch signaling controls self-renewal of myogenic progenitors as well as satellite cell homing during formation of trunk muscle, but its role in craniofacial muscles has been little investigated. We show here that the pool of myogenic progenitor cells in craniofacial muscle of $Dll1^{LacZ/Ki}$ mutant mice is depleted in early fetal development, which is accompanied by a major deficit in muscle growth. At the expense of progenitor cells, supernumerary differentiating myoblasts appear transiently and these express MyoD. The progenitor pool in craniofacial muscle of $Dll1^{LacZ/Ki}$ mutants is largely rescued by an additional mutation of *MyoD*. We conclude from this that Notch exerts its decisive role in craniofacial myogenesis by repression of MyoD. This function is similar to the one previously observed in trunk myogenesis, and is thus conserved in cranial and trunk muscle. However, in cranial mesoderm-derived progenitors, Notch signaling is not required for Pax7 expression and impinges little on the homing of satellite cells. Thus, Dll1 functions in satellite cell homing and Pax7 expression diverge in cranial- and somite-derived muscle.

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Introduction

Approximately 60 skeletal muscle groups in the head are necessary for swallowing, eye movement and facial expression. Muscles of the head, the craniofacial muscles, are evolutionarily and developmentally distinct from trunk muscles (Noden and Francis-West, 2006; Sambasivan et al., 2011). Muscle of the trunk and limbs derive from somites that in turn are generated from the paraxial mesoderm of the trunk. Anterior paraxial mesoderm, also called cranial mesoderm, gives rise to head muscles, for instance extraocular muscles (EOM) and branchiomeric muscles (masseter and buccinator) (Grifone and Kelly, 2007; Harel et al., 2009; Nathan et al., 2008; Tzahor, 2009). Tongue and neck muscles are of mixed origin, and cells from occipital somites and cranial paraxial mesoderm contribute to their formation (Harel et al., 2009; Theis et al., 2010). In particular, the tongue muscle is in part derived from myogenic progenitor cells that delaminate from occipital somites. The delaminated cells form the hypoglossal cord, which represents a transient chain of cells that migrate to the anlage of the tongue (Hazelton, 1970; Huang et al., 1999; Noden, 1983).

The myogenic regulatory factors MyoD, Myf5 and Mrf4 cooperate to control the entry into the myogenic differentiation program, and mutants that lack the expression of all three factors fail to form muscle in the head and trunk (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993). However, different transcriptional mechanisms control expression of myogenic regulatory factors in trunk and craniofacial muscle. In particular, Pax3/7 and transcription factors of the Six family act upstream of myogenic regulatory factors during development of trunk and limb muscle (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005, 2013), whereas Pitx2 and Tbx1 take over this role in craniofacial muscle (Dong et al., 2006; Kelly et al., 2004; Kitamura et al., 1999; Sambasivan et al., 2009). In addition, cranial mesoderm does not undergo myogenesis when grafted into trunk regions, indicating that signaling pathways that control myogenic differentiation of somitic and cranial mesoderm are distinct (Mootoosamy and Dietrich, 2002). Thus, regulatory hierarchies that act upstream of the myogenic factors are different in somitic and cranial mesoderm,

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but the core transcriptional network consisting of MyoD, Myf5 and Mrf4 is used in all skeletal muscles to execute the myogenic differentiation program.

Satellite cells are the stem cells of the adult skeletal muscle and are found in muscle of the trunk and head. Satellite cells can be defined by their anatomical location between the basal lamina of the myofiber and the fiber membrane (Mauro, 1961). A Pax7-expressing progenitor population emerges in all muscle groups, and Pax7 expression marks satellite cells in the head and trunk (Gros et al., 2005; Horst et al., 2006; Kassar-Duchossoy et al., 2005; Nathan et al., 2008; Relaix et al., 2005, 2013). Like the muscle they associate with, satellite cells derive from different mesodermal lineages: the Pax3$^+$ lineage of the trunk mesoderm gives rise to satellite cells in the trunk, the Mesp1$^+$ cranial mesodermal lineage generates satellite cells in extraocular and branchiomeric muscle, and the Isl1$^+$ lineage from the anterior splanchnic lateral mesoderm generates satellite cells of branchiomeric muscles. Pax3$^+$ and Mesp1$^+$ lineages contribute to the satellite cells of tongue and neck muscle (Harel et al., 2009). Satellite cells in trunk and head muscle are also functionally different. For instance, satellite cells of jaw muscle (*musculus masseter*) display delayed differentiation and increased proliferation compared to satellite cells from leg muscle (*extensor digitorum longus*) (Ono et al., 2010). When satellite cells from extraocular muscles are transplanted into the *tibialis anterior*, they form fibers and also self-renew to generate new satellite cells. However, the new fibers produced by transplanted satellite cells no longer express markers specific to the extraocular lineage (Sambasivan et al., 2009).

The Notch signaling cascade is evolutionarily highly conserved in vertebrates and invertebrates (Artavanis-Tsakonas et al., 1999; Kimble and Simpson, 1997; Lewis, 1998). Canonical Notch signaling is activated after binding of the ligand (Dll1, Dll3, Jag1, Jag2 in mice) to the receptor (Notch 1–4 in mice), which results in receptor cleavage, release of the Notch intracellular domain (NICD) and its translocation to the nucleus. Nuclear NICD directly interacts with the transcription factor Rbpj, and the interaction is required to activate target genes like *Hey1* and *Hes1* (Jarriault et al., 1995). Ectopic activation of Notch signaling has long been known to suppress myogenic differentiation in cultured C2C12 cells, primary satellite cells, and developing chick embryos (Conboy and Rando, 2002; Delfini et al., 2000; Hirsinger et al., 2001; Kopan et al., 1994; Kuroda et al., 1999; Shawber et al., 1996). In particular, Notch signaling is known to repress *MyoD* and induce *MyoR* expression in C2C12 cells (Buas et al., 2009; Kopan et al., 1994; Kuroda et al., 1999).

In normal muscle development, a resident pool of myogenic progenitor cells is established in the fetal muscle, and this pool is constantly replenished and maintained into adulthood. When Notch signaling is ablated in mice, the progenitor pool is lost due to uncontrolled myogenic differentiation. In particular, the pool of myogenic progenitor cells begins to decline early in fetal development, which is accompanied by a transient boost of MyoD$^+$ myoblasts. The loss of progenitors results in formation of tiny trunk muscle (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). Dominant-negative Maml (DnMaml) downregulates Notch signaling by forming an inactive Rbpj complex, and expression of *DnMaml* impairs fetal and adult muscle growth (Bröhl et al., 2012; Lin et al., 2013). Furthermore, in the adult mutation of *Rbpj* interferes with the maintenance of quiescent satellite cells (Bjornson et al., 2012; Mourikis et al., 2012b). We recently showed that in trunk muscle, premature differentiation and progenitor depletion caused by mutation of *Dll1*, *Rbpj* or by *DnMaml* expression are rescued by an ablation of *MyoD*. Thus, during fetal myogenesis a major role of Notch is to repress MyoD expression and/or function, thereby ensuring that the progenitor pool is appropriately maintained. Such double mutant mice can be used as a tool to assess roles of Notch in late myogenesis. Interestingly, rescued myogenic progenitors in the trunk are unable to assume a satellite cell position in MyoD-rescued *Dll1*, *Rbpj* or *DnMaml* mutant mice. In addition, Pax7 expression is impaired in *Dll1;MyoD*$^{-/-}$ and *Rbpj;MyoD*$^{-/-}$ mutants but remains unaffected in *DnMaml;MyoD*$^{-/-}$ animals. Expression of *DnMaml* attenuates Notch signals, whereas mutation of *Dll1* and *Rbpj* strongly reduces or eliminates it. This indicates that Pax7 expression and satellite cell homing require different thresholds of Notch signals (Bröhl et al., 2012). Together, these data show that Notch signals do suppress myogenic differentiation, control the colonization of the stem cell niche and Pax7 expression in satellite cells derived from somites.

Cranial and somitic mesodermal cells enter myogenesis and initiate expression of myogenic regulatory factors by distinct mechanisms. The role of Notch has been extensively analyzed in trunk muscle development in mice, and is known to suppress myogenic differentiation in craniofacial muscle (Mourikis et al., 2012a; Schuster-Gossler et al., 2007; Vasyutina et al., 2007). Here we use mouse genetics to show that in *Dll1* mutant mice, craniofacial myogenesis is severely perturbed. In normal development of craniofacial muscle, a Pax7$^+$ progenitor pool is established and maintained. In head muscle of *Dll1* mutants, the Pax7$^+$ myogenic progenitor pool is depleted in early fetal development which is accompanied by a transient appearance of supernumerary MyoD$^+$ myoblasts. This indicates that progenitor cells differentiate in an uncontrolled manner. The loss of the progenitor pool leads to major deficits in muscle growth in late fetal development. The additional mutation of *MyoD* largely rescues cranial myogenesis and the depletion of Pax7$^+$ progenitors. Emerging satellite cells in cranial mesoderm-derived muscles of such *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ double mutants express Pax7 and colonize the stem cell niche. Thus, the Notch-dependent suppression of differentiation via MyoD regulation is conserved in development of head and trunk skeletal muscles. However, in cranial mesoderm-derived progenitors, Dll1 signaling is neither required for satellite cell homing nor for Pax7 expression.

Material and methods

Mouse strains

The *MyoD*$^{-/-}$, *Dll1*^{LacZ}, *Dll1*^{Ki}, *Met*$^{-/-}$, *Rosa26*^{LacZ}, *Lbx1*^{Cre} and *Mesp1*^{Cre} strains have been described previously (Bladt et al., 1995; Hrabe de Angelis et al., 1997; Rudnicki et al., 1992; Saga et al., 1999; Schuster-Gossler et al., 2007; Sieber et al., 2007; Soriano, 1999). As controls, we used either heterozygous *Dll1*$^{Ki/+}$, *MyoD*$^{+/-}$ or double heterozygous *Dll1*$^{Ki/+}$;*MyoD*$^{+/-}$ littermates. For lineage tracing experiments *Lbx1*^{Cre};*Rosa26*^{LacZ} or *Mesp1*^{Cre};*Rosa26*^{LacZ} mice on a heterozygous or homozygous *Met* mutant background were used.

Immunohistochemistry, FACS and in situ hybridization

Immunohistology was performed on 12 μm cryosections of tissues fixed in Zamboni's fixative for 2 h. To assess muscle size, 3–6 consecutive sections of at least 3 animals/genotype/age were examined. For quantification of MyoD$^+$ cells, at least 6 consecutive sections of at least 3 animals/genotype/age were examined. For counting of MyoD$^+$ cells, the desmin-positive area of a particular muscle group was outlined, cells in this area were counted, and the numbers of cells/area were determined using ImageJ. Variance was assessed by determining the standard error of the mean (SEM) by using the two sample *t*-test. The following antibodies were used: mouse anti-skeletal fast myosin and rabbit anti-laminin (Sigma-Aldrich, St. Louis, USA),

goat anti-desmin and rabbit anti-MyoD (Santa Cruz Biotechnology, Santa Cruz, USA), rabbit anti-β-galactosidase (MP Biomedicals, Santa Ana, USA) guinea-pig anti-Pax7 (Bröhl et al., 2012) as well as secondary antibodies conjugated with Cy2, Cy3, Cy5 (Dianova, Hamburg, Germany) or with Alexa Fluor 488 and 555 (Invitrogen, Karlsruhe, Germany). DAPI (4′,6-diamidino-2-phenylindole) (Sigma-Aldrich) was used as a nuclear counterstain. Satellite cells were isolated from P21 wild type mice by FACS as described (Bröhl et al., 2012).

In situ hybridization was performed essentially as described (Brohmann et al., 2000). Tissue was either embedded into OCT compound (Sakura Finetek, Torrance, USA) and cryosectioned, or the embryos were fixed with 4% paraformaldehyde over night for whole-mount *in situ* hybridization (Wilkinson, 1992). At least 3 animals/genotype/age were examined. Fragments amplified from cDNA were used to generate DIG-labeled riboprobes.

qRT-PCR

Total RNA was isolated from FACS-isolated satellite cells in three independent experiments. cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) and PCR was performed using Absolute qPCR SYBR Green Mix (Thermo Scientific, Waltham, USA). PCR primers:

Notch1 (fw caagaggcttgagatgctcc, rev aaggattggagtcctggcat),
Notch3 (fw actgcagtgctggcgtctcttcaa, rev catcccagccgcattcctcagtgtt),
Rbpj (fw ctcagcaagcggataaaggtca, rev gatgtaaaatgctccccactgttg),
Hey1 (fw gccgacgagaccgaatcaataaca, rev tcccgaaaccccaaactccgatag),
Dll1 (fw gatacacacagcaaacgtgacacc, rev ttccatcttacacctcagtcgcta),
Hes1 (fw cagacattctggaaatgactgtgaa, rev cgcggtatttccccaacac),
Pax7 (fw agcaatggcctgtctcctc, rev acgtgggcaagctgtctcctg),
β-actin (fw gtccacacccgccaccagttc, rev ggcctcgtcacccacatag).

qRT-PCR was quantified by duplicate analysis of samples from three animals. Relative transcript levels were normalized to transcript levels in satellite cells of the leg. Variance was assessed by determining the standard error of the mean (SEM) by using the two sample *t*-test.

Results

Genetic analysis in mice show that myogenesis in the trunk is repressed by Notch signaling through repression of MyoD (Bröhl et al., 2012). To analyze the role of Notch signaling during head myogenesis, we examined components of the Notch signaling cascade in craniofacial muscles. *Dll1* was strongly expressed in developing muscle of the tongue, the cheek, and in extraocular muscle at E11.5 (Fig. 1A and B). *Dll1* was also present in E18 and P21 head muscle, as determined by qRT-PCR (Fig. 1C). We isolated satellite cells from craniofacial muscles at P21 by FACS to further define expression of Notch signaling components (Fig. 1D). Cytospin analysis indicated that 85–90% of the isolated cells were myogenic progenitor cells as defined by the expression of Pax7. A small subpopulation co-expressed Pax7 and MyoD (shown for cells from the masseter in Fig. 1E and F). Comparison of satellite cells isolated from limbs, masseter, tongue and extraocular muscle indicated that *Notch1/3*, *Rbpj* and *Hey1* were expressed at comparable levels in satellite cells independent of their origin. *Dll1* and *Hes1* were expressed at higher levels in satellite cells from masseter and tongue than in satellite cells from leg and extraocular muscle (Fig. 1G).

We analyzed craniofacial muscle mass in $Dll1^{LacZ/Ki}$ mice at E12 and E13. We observed little difference in the size and shape of emerging craniofacial muscle groups at E12, but at E13 their overall size was clearly reduced (Fig. 2A and B and data not shown). The size of the masseter was strongly affected, whereas in comparison tongue and extraocular muscles appeared little changed at this stage (Fig. 2A and B). At E18, all muscle groups were severely reduced in size in $Dll1^{LacZ/Ki}$ compared to control mice (Fig. 2C and D). In particular, masseter, buccinator and extraocular muscles were tiny in $Dll1^{LacZ/Ki}$ mutant animals (Fig. 2D). The tongue muscle was considerably smaller but appeared less strongly affected than other craniofacial muscle groups (Fig. 2D).

We noted that $Pax7^+$ progenitor cells were detectable in craniofacial muscles of control mice at E13 and E18, but they were absent in $Dll1^{LacZ/Ki}$ mutants (Fig. 2A–D and E–H for higher magnifications). In addition, the number of differentiating myoblasts defined by expression of MyoD was transiently increased in craniofacial muscle, but this became apparent at distinct stages in different muscle groups. In particular, in branchiomeric muscle $MyoD^+$ myoblasts were observable in control mice at E10.5, and they were increased in number in $Dll1^{LacZ/Ki}$ mutants (Fig. 3A and B; see C for a quantification). At E11.5, similar numbers of $MyoD^+$ cells were present in branchiomeric muscle of control and mutant mice (Fig. 3D and E; see F for a quantification). At E15, $MyoD^+$ cells were absent in branchiomeric muscles of $Dll1^{LacZ/Ki}$ mutants but not in control mice (Fig. 3G and H; see I for a quantification). In addition, the mutant branchiomeric muscle was small (Fig. 3G and H). In the anlage of the tongue, $MyoD^+$ cells were absent at E10.5 in control and $Dll1^{LacZ/Ki}$ mutant mice. At E11.5, $MyoD^+$ cells were observable in control mice, and their number was increased in $Dll1^{LacZ/Ki}$ mutant tongues. At E15, $MyoD^+$ cell numbers were significantly reduced in the tongue of mutant compared to control mice, and the size of the muscle was reduced (Fig. 3D–I). Thus, supernumerary myoblasts appeared transiently at the expense of progenitor cells in craniofacial muscle of $Dll1^{LacZ/Ki}$ mice. This is very similar to the phenotype observed previously in the developing trunk muscle (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). The transcription factor MyoR participates in the control of craniofacial myogenesis, and its expression is controlled by Notch in C2C12 cells (Buas et al., 2009; Lu et al., 2002). We examined *MyoR* expression in $Dll1^{LacZ/Ki}$ mutant animals by *in situ* hybridization. *MyoR* was expressed in somites and branchial arches of control animals and was strongly downregulated in $Dll1^{LacZ/Ki}$ mutants at E11 and E11.5 (Fig. 3J–M). *MyoR* expression in the limbs and *Tbx1*, *Myf5* and *Pitx2* expression in the head were unchanged (Fig. 3J–M, Fig. S1). We conclude that mutation of *Dll1* results in an early differentiation of craniofacial progenitor cells, *i.e.* a transient appearance of supernumerary $MyoD^+$ myoblasts at the expense of $Pax7^+$ progenitor cells. In addition, *MyoR* expression was downregulated.

We noted that the tongue muscle was least affected by mutation of *Dll1*. Tongue muscle derives from cranial as well as somitic mesoderm, and we aimed to define the relative contribution of the two lineages to assess whether the *Dll1* mutation might interfere with only one lineage. To analyze this, we used lineage tracing, *i.e.* an indicator strain ($Rosa26^{LacZ}$) that expresses β-galactosidase after Cre-meditated recombination, as well as $Lbx1^{Cre}$ and $Mesp1^{Cre}$ expressed in muscle progenitors derived from somitic and cranial mesoderm, respectively. This analysis showed that progenitor cells from somites and cranial mesoderm contribute to extrinsic and intrinsic tongue muscle (Fig. 4A and B). The tyrosine kinase receptor Met is required for delamination of muscle progenitor cells from somites (Bladt et al., 1995; Dietrich et al., 1999). $Lbx1^{Cre}$-dependent lineage tracing on a $Met^{-/-}$ mutant background demonstrated a complete absence of $\beta\text{-gal}^+$ cells in the tongue (Fig. 4C), verifying that $Lbx1^{Cre}$ marks the somitic lineage that depends on Met for migration. In addition, the

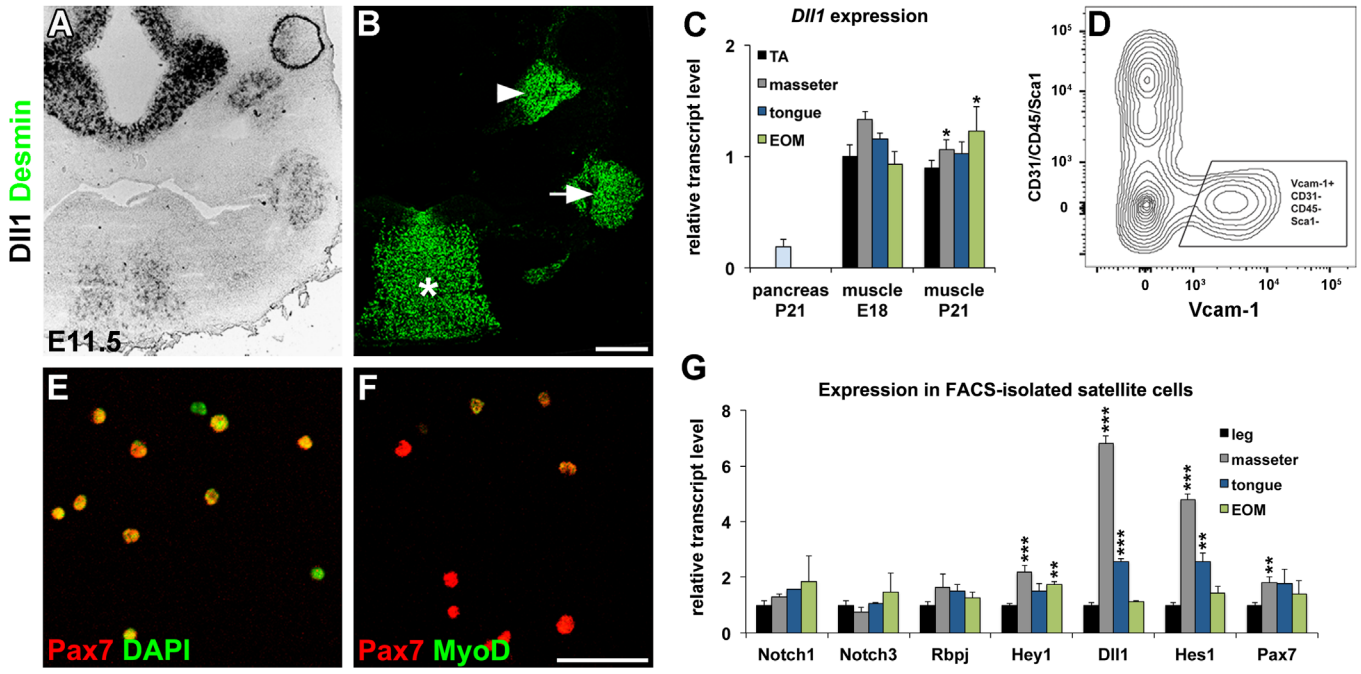

Fig. 1. Notch signaling in craniofacial muscle satellite cells. (A) *In situ* hybridization using a *Dll1* probe demonstrates the expression of *Dll1* in the developing craniofacial muscle at E11.5. (B) Anti-desmin immunohistology was used to define the exact location of craniofacial muscle groups. Branchiomeric muscle (arrow), extraocular muscle (arrow head), and tongue muscle (asterisk) are indicated. (C) Quantification of *Dll1* mRNA isolated from *tibialis anterior* (black), masseter (grey), tongue (blue) and extraocular muscle (green) at E18 and P21 by qRT-PCR. mRNA isolated from P21 pancreas was used as a negative control. (D) FACS analysis of muscle cells using antibodies directed against Vcam-1, CD31, CD45, and Sca1. The gate used to isolate cells is indicated. (E,F) Immunohistological analysis of sorted satellite cells that were cytospun after isolation and stained using anti-Pax7 and anti-MyoD antibodies as indicated. Nuclei were counterstained with DAPI. (G) qRT-PCR analysis of various components of the Notch signaling pathway in FACS-isolated satellite cells from different muscle groups. Relative gene expression levels in satellite cells from leg (black), masseter (grey), tongue (blue) and extraocular muscle (green) are shown. Error bars, SEM. Statistical significance is indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Scale bars: 300 μm in (B), 50 μm in (F).

extrinsic tongue muscle was present and was little changed in size, indicating that somite-derived cells contribute a minor proportion of cells to this muscle. *Mesp1*^{Cre}-dependent lineage tracing on the *Met* mutant background demonstrated that residual muscle and myogenic progenitors in the tongue of *Met* mutants indeed derive from cranial mesoderm (Fig. 4D). In summary, cranial- and somite-derived mesoderm contributes to both, intrinsic and extrinsic tongue muscle. However, the majority of the extrinsic tongue muscle derives from cranial mesoderm, whereas the hypoglossal cord contributes most precursors to the intrinsic tongue muscle. The *Dll1* mutation affects both, ex- and intrinsic tongue muscle groups, and impinges thus on development of progenitor cells that derive from somitic and craniofacial mesoderm (cf. Fig. 2C and D).

We next tested whether craniofacial muscle development in *Dll1*$^{LacZ/Ki}$ mice is rescued by *MyoD* ablation. Compared to *Dll1*$^{LacZ/Ki}$ mice (Fig. 5A), the size of craniofacial muscle groups was markedly increased in *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ mutants (Fig. 5B). Overall, craniofacial muscle size was indistinguishable in *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ and *MyoD*$^{-/-}$ mutants (Fig. 5C). Thus, the absence of *MyoD* rescued the deficits in growth of craniofacial muscle caused by mutation of *Dll1*. This indicates that a major role of Notch during fetal myogenesis is to repress MyoD expression and/or function, thereby ensuring that the progenitor pool is appropriately maintained. However, *MyoR* expression was not restored (Fig. S2).

We also defined the number of Pax7$^+$ progenitor cells associated with cranial mesoderm-derived muscles in *Dll1*$^{LacZ/Ki}$ and MyoD-rescued *Dll1*$^{LacZ/Ki}$ mice (Fig. 5D–H). Pax7$^+$ cells were detectable in all cranial mesoderm-derived muscles groups of *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ but not *Dll1*$^{LacZ/Ki}$ mutants (Fig. 5E–E″, F–F″). It should be noted that craniofacial muscles of *MyoD*$^{-/-}$ mutants contain supernumerary Pax7$^+$ progenitor cells compared to control mice, and we therefore compared in subsequent experiments *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ *and MyoD*$^{-/-}$ mutants (Fig. 5G–G″). In the masseter, numbers of Pax7$^+$ progenitor cells in the *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ and *MyoD*$^{-/-}$ mutants were similar (Fig. 5H). The overall size of intrinsic and extrinsic tongue muscle was rescued in *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ mice. However, we detected only few Pax7$^+$ cells in the intrinsic tongue muscle, *i.e.* in the part of the muscle derived from the somite. Pax7$^+$ cells were however abundant in the extrinsic tongue muscle, *i.e.* the part derived from cranial mesoderm (Fig. 5A–C; see 5H′ for a quantification of Pax7$^+$ cells in the extrinsic tongue muscle). The rescue of the progenitor pool in extraocular muscles was extensive but not complete (Fig. 5H″). In conclusion, the loss of the muscle size and the progenitor pool caused by mutation of *Dll1* in cranial mesoderm-derived muscles is substantially rescued by *MyoD* ablation.

Satellite cells are defined by their anatomical position, *i.e.* they locate between the basal lamina and the sarcolemma of muscle fibers (Mauro, 1961). We quantified the proportion of Pax7$^+$ progenitor cells below the basal lamina and in the interstitial space (Fig. 6). In control animals at E18, the majority of muscle progenitor cells in cranial mesoderm-derived muscles are located below the basal lamina (87%, 85%, 80% in the masseter, extrinsic tongue muscle and EOM, respectively), and the remainder resides in the interstitial space (Fig. 6A–A″). In *Dll1*$^{LacZ/Ki}$ mutants, Pax7$^+$ cells were very rare (Fig. 6B–B″). In *MyoD*$^{-/-}$ mutants, the overall numbers of Pax7$^+$ cells were increased, and many of the supernumerous cells located to the interstitial space (Fig. 6C–C″). In particular, the proportion of cells below the lamina decreased (63%, 65% and 69% in the masseter, extrinsic tongue muscle and EOM, respectively; Fig. 6F). This indicates that interstitial myogenic progenitors are stabilized by the mutation of *MyoD* (cf. Bröhl et al., 2012). In rescued *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ mice, very moderate changes in the proportion of cells located below the lamina were observed (63%, 51% and 45% in the masseter, extrinsic tongue muscle and EOM, respectively; Fig. 6F). This contrasts the severe

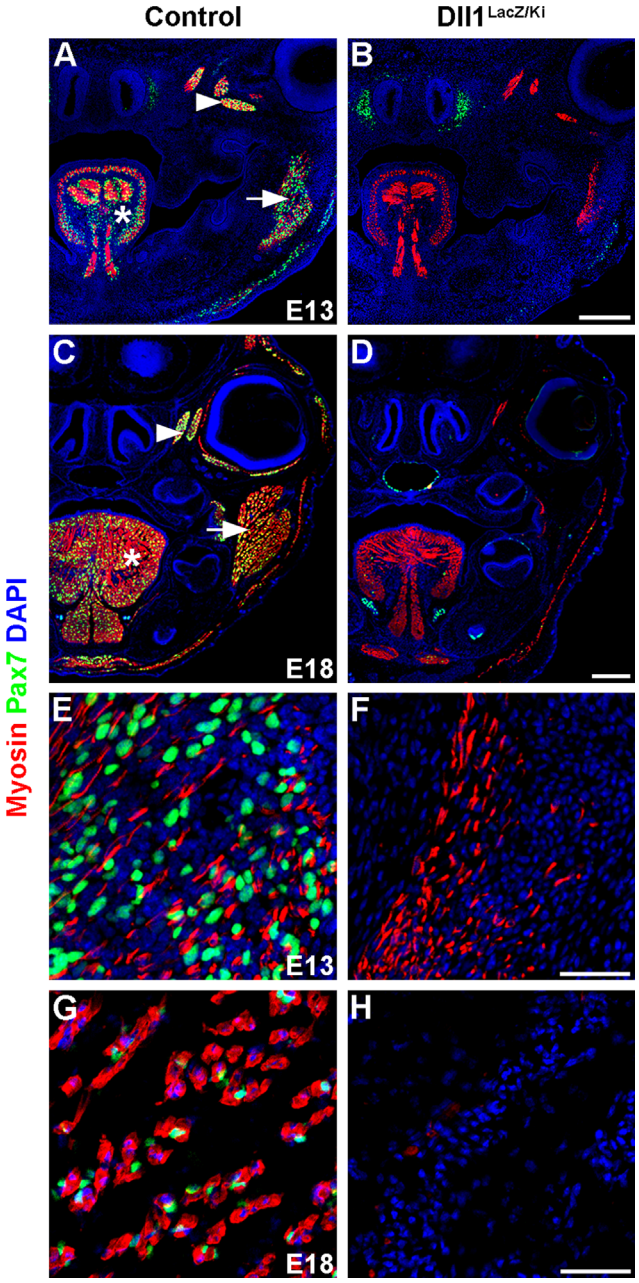

Fig. 2. Disrupted muscle growth and loss of Pax7^{+} progenitors in craniofacial muscle of *Dll1* mutants. (A–D) Immunohistological analysis of control and *Dll1*$^{LacZ/Ki}$ mutant mice at E13 (A,B) and E18 (C,D) revealed a decrease in muscle size and a loss of Pax7^{+} progenitors; muscle and myogenic progenitor cells in the head are visualized by anti-myosin (red) and anti-Pax7 (green) antibodies. DAPI (blue) was used as a counterstain. (E-H) Higher magnification of the masseter at E13 (E,F) and E18 (G,H). Arrow, arrowhead and asterisk indicate masseter, extraocular and tongue muscle. Scale bars: 300 μm in (B), 500 μm in (D), 50 μm in (F,H).

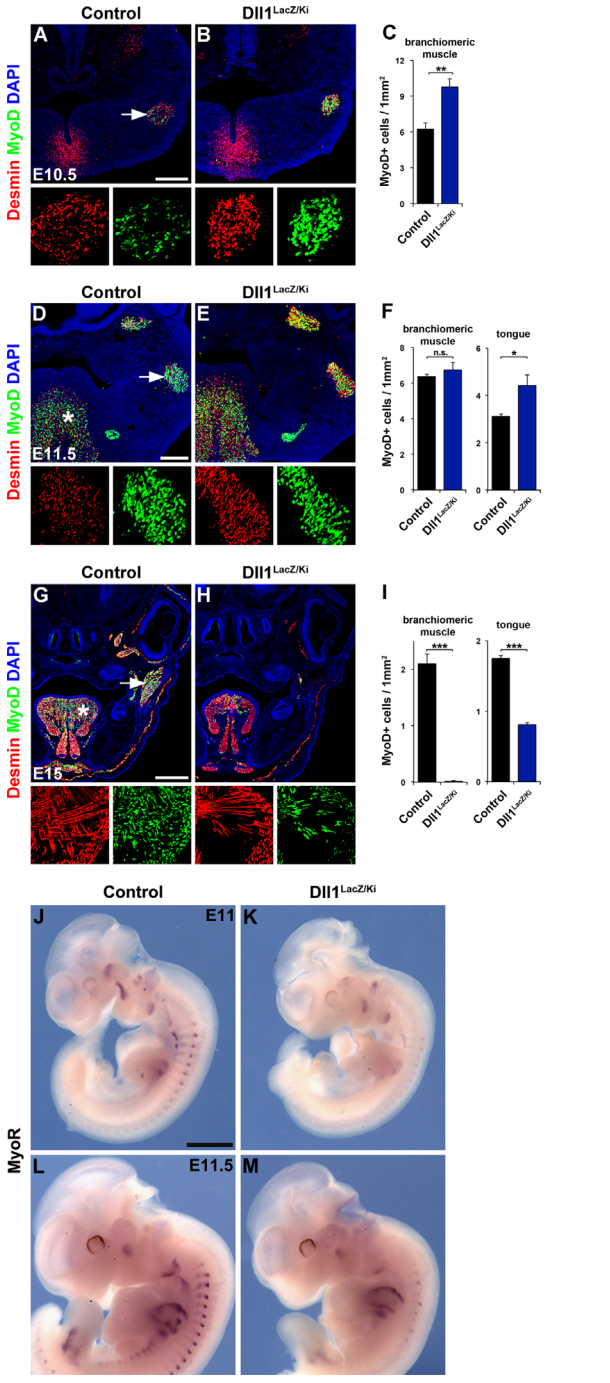

Fig. 3. Transient appearance of supernumerary MyoD^{+} cells in craniofacial muscle of *Dll1*$^{LacZ/Ki}$ mice. (A,B; D,E; G,H) Immunohistological analysis of craniofacial muscles in control and *Dll1*$^{LacZ/Ki}$ mice at E10.5 (A,B), E11.5 (D,E) and E15 (G,H) using antibodies against desmin (red), MyoD (green); DAPI (blue) was used as a counterstain. (C,F,I) Quantification of MyoD^{+} cells/1 mm^2 cells in branchiomeric (C,F,I) and ;tongue muscle (F,I). Branchiomeric muscle was small at E15 and MyoD^{+} cells were absent. (J-M) Whole-mount *in situ* hybridization using a *MyoR*-specific probe on control and *Dll1*$^{LacZ/Ki}$ mice at E11 (J,K) and E11.5 (L,M). Error bars, SEM. Statistical significance is indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., not significant). Arrow and asterisk indicate branchiomeric muscle and tongue muscle. Scale bars: 200 μm in (A,D), 500 μm in (G), 1 mm in (J).

homing deficit of emerging satellite cells that we observed in trunk muscle (Fig. 6G,H; cf. Bröhl et al., 2012). We conclude that Dll1 signaling impinges little on satellite cell homing in cranial mesoderm-derived muscles.

Discussion

Cranial and trunk muscles are evolutionarily distinct and for instance they vary in the expression of skeletal muscle specific protein isoforms. This might account for the fact that myopathies can differentially affect muscle groups in the head and trunk. Furthermore, the regulatory network that governs expression of myogenic regulatory factors differs in trunk and craniofacial muscle (Dong et al., 2006; Kelly et al., 2004; Kitamura et al., 1999; Rudnicki et al., 1993; Sambasivan et al., 2009; Tajbakhsh

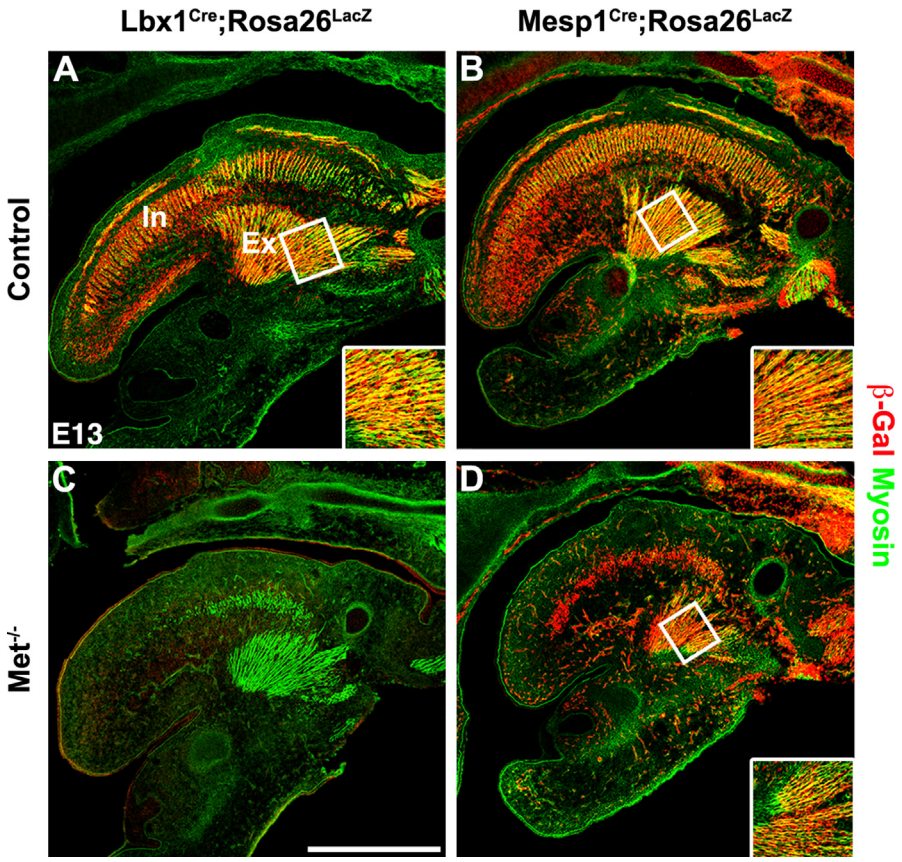

Fig. 4. Mixed origin of tongue muscle progenitor cells. Lineage tracing using *Lbx1*^{Cre};*Rosa26*^{LacZ} and *Mesp1*^{Cre};*Rosa26*^{LacZ} in a control (A,B) and *Met*$^{-/-}$ mutant background (C,D). Cells in the tongue that derive from the hypoglossal cord express β-galactosidase in (A,C) and are shown in red. Cells that derive from cranial mesoderm express β-galactosidase in (B,D) and are shown in red. Analysis was performed on E13 embryos, and anti-myosin antibodies were used to stain differentiated muscle (green). In—intrinsic tongue muscle, Ex—extrinsic tongue muscle. Scale bar: 500 μm.

et al., 1997). Notch signaling suppresses the entry into the myogenic program in trunk muscle, but the function of Notch in craniofacial myogenesis has been little investigated. We show here that the progenitor pool in craniofacial muscle of *Dll1*$^{LacZ/Ki}$ mutant mice is depleted early, which is accompanied by a transient appearance of supernumerary differentiating cells. The depletion of the pool is largely rescued by the additional mutation of *MyoD*. We conclude from this that Notch exerts a decisive role in craniofacial myogenesis by suppressing progenitor cell differentiation via regulation of MyoD. This function is similar to the one observed previously in trunk myogenesis, and is thus conserved in head and trunk muscle development. However, other Notch functions differ. In particular, homing of emerging satellite cells and Pax7 expression in progenitor cells do not depend on Dll1 signaling in cranial mesoderm-derived muscle.

Notch signaling and MyoD

In *Dll1*$^{LacZ/Ki}$ mice, masseter and extraocular muscles were very small and lacked Pax7^{+} progenitor cells at E13 or E18. The tongue muscle, which originates from both, occipital somites and head mesenchyme, lacked Pax7^{+} cells and was reduced in size but was less strongly affected than other craniofacial muscles. We conclude that due to the lack of myogenic progenitor cells, fetal muscle growth is severely impaired when *Dll1* is mutated.

Notch signaling suppresses myogenic differentiation both *in vivo* and *in vitro* (Bjornson et al., 2012; Conboy and Rando, 2002; Kopan et al., 1994; Kuroda et al., 1999; Mourikis et al., 2012b; Schuster-Gossler et al., 2007; Shawber et al., 1996; Vasyutina et al., 2007). Several molecular mechanisms by which Notch mediates this were discussed (Buas and Kadesch, 2010). We found that ablation of *MyoD* in *Dll1*$^{LacZ/Ki}$ mutants rescued the deficits in growth of all craniofacial muscles, indicating that the major function of Notch in craniofacial myogenesis is to repress MyoD. This mechanism is thus conserved in head and trunk muscle (this work and (Bröhl et al., 2012).

Notch signals, Pax7 expression and homing of emerging satellite cells

In trunk muscle derived from the somites, myogenic progenitor cells were rescued in *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ mice, but these progenitors no longer expressed Pax7 and their identification relied thus on the use of other additional markers like Pax3 (Bröhl et al., 2012). Similarly, we observed only rare Pax7^{+} cells in the rescued intrinsic tongue muscle, which is largely generated from somites. In contrast, in other cranial muscle groups Pax7^{+} cells were abundant in *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ mice, indicating that Pax7 expression is Notch-independent in cranial mesoderm-derived progenitor cells. We conclude that Pax7 expression is differentially controlled by Notch in progenitor cells that derive from cranial and trunk mesoderm.

Satellite cells locate below the basal lamina of muscle fibers. The lamina starts to appear around E15.5, and emerging satellite cells are first detectable at this stage (Bröhl et al., 2012). We show here that Dll1 signals impinge little on homing of satellite cells in head muscle. In trunk muscle of *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ mutants,

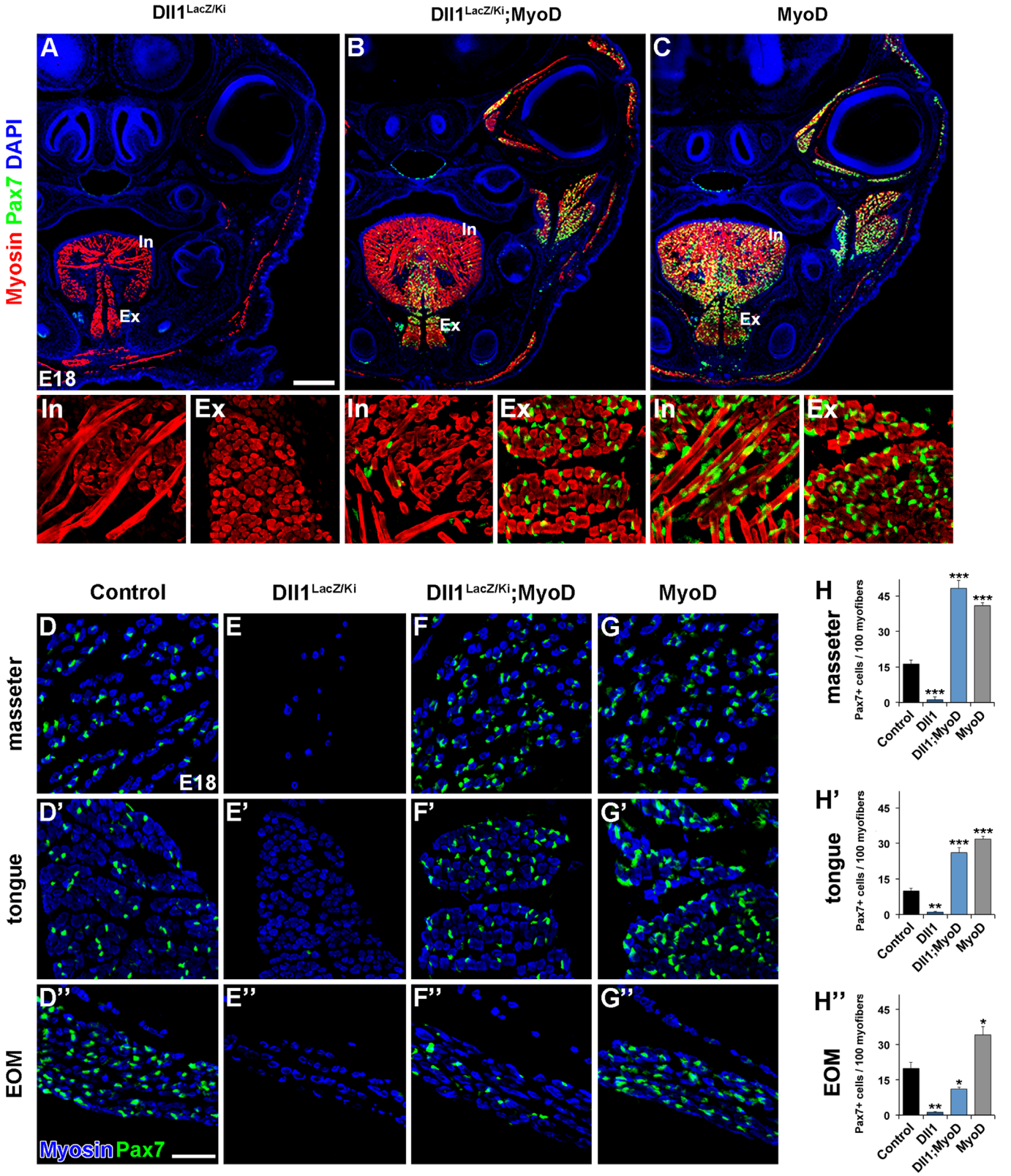

Fig. 5. Rescue of muscle progenitors in *Dll1* mutant mice by additional mutation of *MyoD*. (A-C) Immunohistological analysis of craniofacial muscles in $Dll1^{LacZ/Ki}$ (A), $Dll1^{LacZ/Ki}$;$MyoD^{-/-}$ (B) and $MyoD^{-/-}$ (C) mice at E18 using DAPI (blue) and antibodies against myosin (red) and Pax7 (green). Note the marked increase in the size of the muscle in $Dll1^{LacZ/Ki}$;$MyoD^{-/-}$ compared to $Dll1^{LacZ/Ki}$ mice. In—intrinsic part of the tongue, Ex—extrinsic part of the tongue. (D-G) Analysis of $Pax7^{+}$ cells (green) in masseter (D-G), extrinsic tongue (D'-G') and extraocular (D''-G'') muscle at E18 of control, $Dll1^{LacZ/Ki}$, $Dll1^{LacZ/Ki}$;$MyoD^{-/-}$ and $MyoD^{-/-}$ mice. (H-H'') Quantification of $Pax7^{+}$ cells/100 myofibers in masseter (H), extrinsic tongue (H′) and extraocular (H″) muscle. Error bars, SEM. Statistical significance is indicated ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Scale bars: 500 μm in (A), 50 μm in (D).

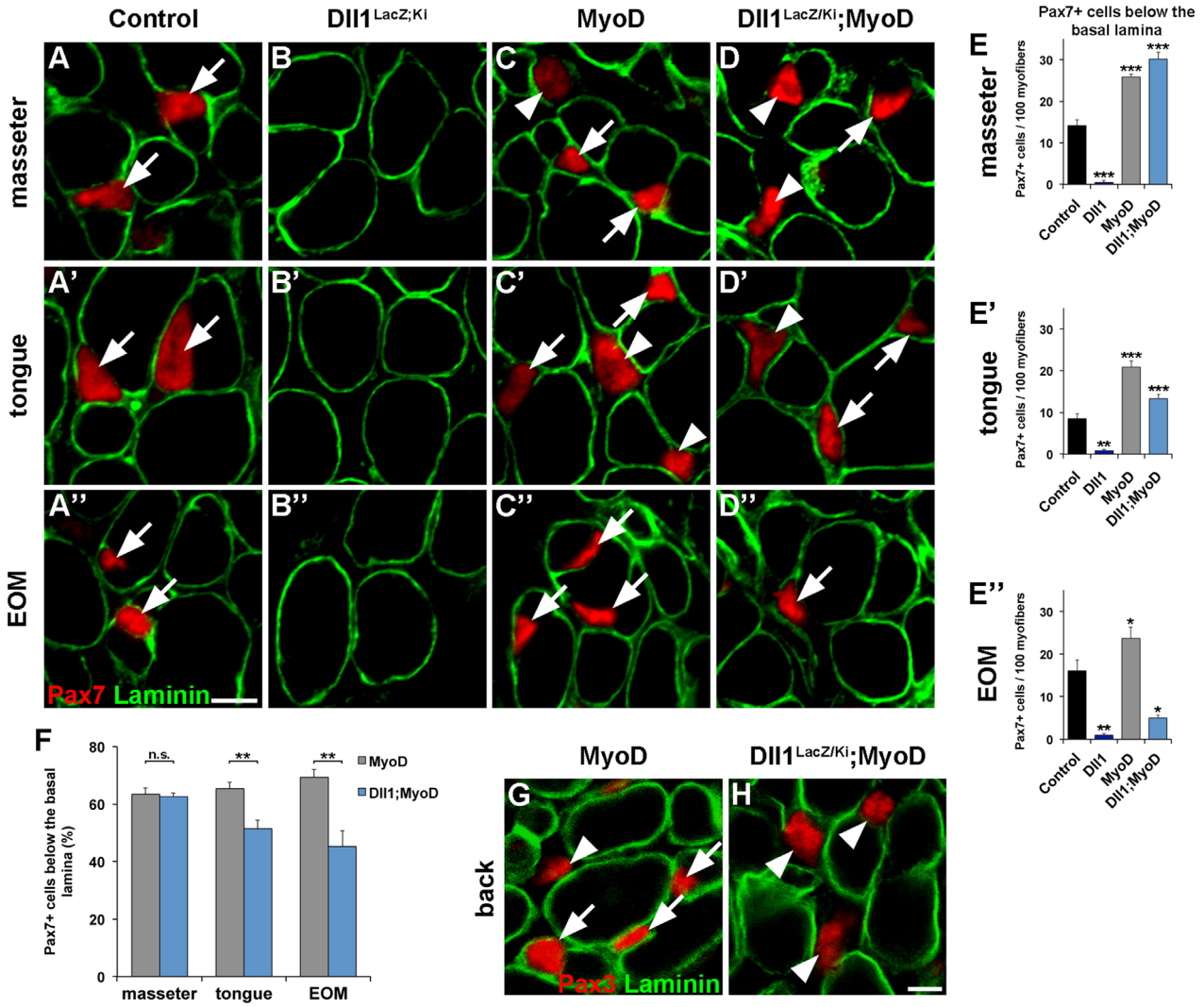

Fig. 6. Homing of emerging satellite cells in cranial mesoderm-derived muscles is independent of Dll1. (A-D) Location of emerging satellite cells in their niche was analyzed in different cranial mesoderm-derived muscle groups of control (A-A''), *Dll1*$^{LacZ/Ki}$ (B-B''), *MyoD*$^{-/-}$ (C-C'') and *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$(D-D'') mice at E18. The analysis was performed by immunohistology using anti-laminin (green) and anti-Pax7 (red) antibodies. Pax7^{+} cells located below the basal lamina (arrows) and in the interstitial space (arrowheads) are indicated. (E-E'') The number of Pax7^{+} progenitor cells located below the basal lamina was quantified in the different cranial mesoderm-derived muscle groups as number of cells/100 myofibers. (F) Quantification of the proportion of Pax7^{+} cells that locate below the basal lamina in cranial mesoderm-derived muscles. (G,H) Location of emerging Pax3^{+} satellite cells in back muscle of *MyoD*$^{-/-}$ (G) and *Dll1*$^{LacZ/Ki}$;*MyoD*$^{-/-}$ (H) mutant mice at E17.5. Arrows and arrowheads point toward emerging satellite cells located below the basal lamina and in the interstitial space, respectively. Error bars, SEM. Statistical significance is indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s., not significant). Scale bars: 5 μm.

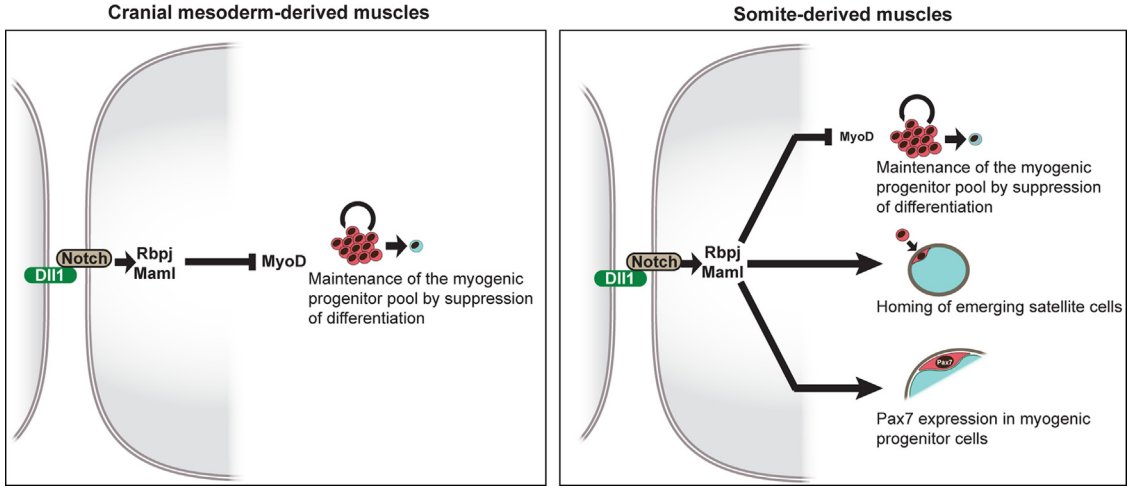

Fig. 7. Notch functions in craniofacial and trunk myogenesis. Distinct functions of Dll1 signals in cranial mesoderm-derived (left) and somite- derived (right) muscle. Dll1-dependent suppression of differentiation via MyoD regulation is conserved in all skeletal muscle groups. However, in cranial mesoderm-derived progenitors, Dll1-signaling is neither required for satellite cell homing nor for Pax7 expression.

progenitor cells fail to assume a satellite cell position (Bröhl et al., 2012). We conclude that Dll1 signaling differentially affects colonization of the satellite cell niche in head and trunk muscle (see Fig. 7 for a summary of Notch functions in craniofacial and trunk myogenesis).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.09.005.

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