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Title: Inhibitory SMAD6 interferes with BMP dependent generation of muscle progenitor cells and perturbs proximodistal pattern of murine limb muscles.

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Summary (abstract)

The mechanism of pattern formation during limb muscle development remains poorly understood. The canonical view holds that naïve limb muscle progenitor cells (MPCs) invade a pre-established pattern of muscle connective tissue, thereby forming individual muscles.

Here we show that early murine embryonic limb MPCs highly accumulate pSMAD1/5/9, demonstrating active signaling of bone morphogenetic proteins (BMP) in these cells. Overexpression of inhibitory *SMAD6* in limb MPCs abrogated BMP signaling, impaired their migration and proliferation, and accelerated myogenic lineage progression. Fewer primary myofibers developed, causing an aberrant proximodistal muscle pattern. Patterning was not disturbed when *SMAD6* was overexpressed in differentiated muscle, implying that the proximodistal muscle pattern depends on BMP-mediated expansion of MPCs prior to their differentiation. We show that limb MPCs differentially express *Hox* genes, and *Hox*-expressing MPCs displayed active BMP signaling. *SMAD6* overexpression caused loss of HOXA11 in early limb MPCs. In conclusion, our data show that BMP signaling controls expansion of embryonic limb MPC as a prerequisite for establishing the proximodistal muscle pattern, a process that involves expression of *Hox* genes.

Key words

PAX3, SMAD6, BMP signaling, HOX, myogenic progenitor cell, myogenesis, muscle fiber, embryonic muscle, fetal muscle, limb muscle, patterning.

1 **Introduction**

2 As in all tetrapods, mammalian limb musculature is derived from a small number of myogenic
3 progenitor cells (MPCs) that migrate from somites into the developing limb bud, where they
4 expand in number, differentiate and form a multitude of individual muscles (Christ and Brand-
5 Saberi, 2002). Migrating limb MPCs are thought to have no positional information but rather
6 rely on signals from their new environment (Blagden and Hughes, 1999). The cues for muscle
7 patterning reside in the limb mesenchymal cells and are independent of the presence of limb
8 MPCs (Grim and Wachtler, 1991; Vallecillo-García et al., 2017). Increasing evidence suggests
9 that individual muscles are formed when MPCs invade a prepattern that is established by
10 muscle connective tissue (MCT) and controlled by a combination of transcription factors, e.g.
11 HOX (Zakany and Duboule, 2007; Swinehart et al., 2013), TBX3 (Colasanto et al., 2016), TBX4/5
12 (Hasson et al., 2010), TCF4 (Kardon et al., 2003) and OSR1 (Vallecillo-García et al., 2017).
13 However, the connective tissue of limbs without muscle does not form morphologically
14 distinguishable structures that resemble the pattern of individualized muscles. The muscle-
15 devoid space is instead filled with loosely organized mesenchyme and eventually with fat (Christ
16 et al., 1977). Initial tendon formation occurs also independently of muscle; however, the
17 tendons degenerate secondarily if they do not connect to a muscle (Huang et al., 2015).

18 Opposing the view of ‘myogenic naivety’, the expression of HOXA11 and HOXA13
19 proteins has been observed in chicken limb MPCs, suggesting that MPCs acquire positional
20 identity. Interestingly, the spatiotemporal dynamics of HOX expression in chicken MPCs are
21 influenced by cues emanating from the apical ectodermal ridge and the zone of polarizing
22 activity. In addition, ectopic application of factors such as FGFs (fibroblast growth factors) and
23 BMPs (bone morphogenetic proteins) were shown to regulate HOX expression in chicken MPCs.
24 These findings suggest that MPCs may follow similar cues during patterning as the limb
25 mesenchyme (Yamamoto et al., 1998; Hashimoto et al., 1999; Yamamoto and Kuroiwa, 2003).

26 In mice, muscle patterning starts at embryonic day (E)11.5, with successive splitting of
27 pre-muscle masses into distinct blocks. Individual muscles become distinguishable from E12.5
28 onwards, and muscle individualization is complete by E14.5 at the end of embryonic myogenesis
29 (Huang, 2017). Although non-muscle cells drive the limb muscle pattern (Kardon et al., 2002;

30 Tozer et al., 2007), MPCs first need to integrate spatiotemporal information for their
31 appropriate positioning, proliferation and differentiation. The molecular mechanisms driving
32 MPC proliferation and differentiation at the right place and time are not fully understood.

33 BMPs are involved in embryonic MPC expansion in chicken limbs (Amthor et al., 1998;
34 Wang et al., 2010). Moreover, BMP signaling displays regionalized activity within limb fetal
35 muscles at the muscle and tendon interface level, and fetal MPCs respond to BMP signaling in
36 chicken limbs (Wang et al., 2010), suggesting the appositional growth of limb muscles that is
37 maintained by direct signaling from BMP-expressing tendons. Consistent with this, BMP
38 signaling has been recently shown to promote mesoderm-derived fibroblast
39 transdifferentiation into myoblasts and their incorporation within fetal muscle fibers at the
40 muscle–tendon interface (Esteves de Lima et al., 2021). However, there is a lack of formal proof
41 for whether BMPs act directly on developing limb muscle, thereby activating a BMP-dependent
42 cell-autonomous response, at which developmental stage this interaction takes place, whether
43 it involves BMPs in physiological signaling in orthotopic positions and whether this impacts
44 muscle patterning.

45 BMPs signal on target cells via transmembrane serine/threonine kinase receptors, which
46 form a ligand-receptor complex that permits the phosphorylation of the type I receptor via the
47 constitutively active type II receptor (Nohe et al., 2002; Nohe et al., 2004). The type I receptor
48 in turn phosphorylates the BMP-responsive R-SMAD proteins 1, 5 and 9 (pSMAD1/5/9), which
49 subsequently form complexes with co-SMAD4 and translocate into the nucleus to regulate
50 transcriptional activity of target genes (Miyazawa and Miyazono, 2017). Upon BMP signaling,
51 the inhibitory SMAD6 becomes upregulated as part of a negative feedback loop. SMAD6
52 interferes with BMP signaling by blocking R-SMAD phosphorylation at the level of the receptor,
53 by antagonizing the pSMAD1/co-SMAD4 complex formation, and by increasing ubiquitin-
54 mediated proteolysis of the BMP signaling components (Goto et al., 2007; Hata et al., 1998;
55 Murakami et al., 2003).

56 We here explored the role of BMP signaling during mouse limb muscle development.
57 We employed overexpression of *SMAD6* as a mean to cell-autonomously interfere with BMP
58 signaling. We overexpressed *SMAD6* in embryonic limb MPCs and differentiated limb muscles

59 following Cre-induced recombination by crossing of *Rosa26^{LoxP-Stop-LoxP-huSMAD6-IRES-EGFP}* mice with
60 cre-driver mouse lines *Lbx1^{Cre}* and *HSA-Cre* (Miniou et al., 1999; Sieber et al., 2007; Stantzou et
61 al., 2017).

62 Results

63 BMP signaling is active in limb muscle progenitors

64 First, we identified whether limb myogenic cells respond to BMP signaling. We
65 monitored the nuclear accumulation of BMP-induced phosphorylated SMAD proteins using
66 double immunofluorescence against pSMAD1/5/9 (pSMADs) and myogenic markers in mouse
67 forelimbs at different developmental stages. In E10.5 limb buds, migrating MPCs expressed the
68 transcription factor PAX3 (**Fig. 1**), whereas the PAX7 and MYOD transcription factors were not
69 detected, thus reproducing previously published data (Lepper and Fan, 2010; Wood et al.,
70 2013). Surprisingly, all PAX3⁺ MPCs accumulated high levels of pSMADs, whereas non-myogenic
71 mesenchymal cells showed no or, if any, very weak levels of pSMADs (**Fig. 1**). One day later, at
72 E11.5, PAX3⁺ MPCs rapidly lost BMP signaling responsiveness during lineage progression. A
73 subset of emerging MYOD⁺ cells were pSMADs⁺, whereas PAX7⁺ cells were rarely pSMAD⁺ (**Fig.**
74 **1**).

75 By the end of the embryonic period, at E14.5, pSMADs were enriched at the tips of the
76 muscle fibers abutting tendons (**Fig. S1a**). Double labeling of pSMADs with either PAX7, MYOD
77 or myosin heavy chain (MHC) antibodies showed active BMP signaling in MYOD⁺ myonuclei at
78 the myotendinous junctions and notably not in tendons (**Fig. S1a**). Low levels of pSMADs were
79 also detected in rare PAX7⁺ MPCs at the muscle tips (**Fig. S1a**). By the end of the fetal period, at
80 E18.5, the pSMAD expression pattern was reversed. Indeed, the tips of fetal muscle fibers were
81 devoid of pSMADs, which had now accumulated in the nuclei of non-muscle cells at the muscle–
82 tendon interface (**Fig. S1a**). MCT cells, labeled by the marker TCF4, were rarely pSMAD⁺ (**Fig.**
83 **S1b**). Furthermore, PAX7⁺ and MYOD⁺ myogenic cells occasionally accumulated pSMADs (**Fig.**
84 **S1b**), consistent with the role of BMP signaling in postnatal satellite cells (Stantzou et al., 2017).

85

86 MPCs maintain myogenic fate following abrogation of BMP signaling

87 We abrogated BMP signaling in limb MPCs by crossing *Lbx1^{Cre}* mice (Sieber et al., 2007)
88 with *Rosa26^{LoxP-Stop-LoxP-huSMAD6-IRES-EGFP}* (*RS6*) animals (Stantzou et al., 2017). In the resulting
89 *Lbx1^{Cre};RS6* embryos, activation of the *Lbx1* promoter in migrating limb MPCs induced Cre-
90 mediated excision of the *LoxP-Stop-LoxP* cassette, leading to the expression of the inhibitory

91 human *SMAD6* (*huSMAD6*) and *EGFP*. The *Lbx1^{Cre};RS6* genotype was detected at the expected
92 frequency up to the fetal stages. However, new-born *Lbx1^{Cre};RS6* mice rarely survived, and the
93 very few that did had severe growth retardation (data not shown). We validated the activation
94 of the transgenes in *Lbx1^{Cre};RS6* embryos using *EGFP* (enhanced green fluorescent protein) as a
95 marker of successful recombination. *EGFP* fluorescence was detected in cells from the proximal
96 central field of E10.5 forelimb buds and was absent from the *RS6* controls (**Fig. S2a**). When
97 compared to whole-mount ISH against *Lbx1*, the position of the *EGFP* fluorescence
98 corresponded to that of migrating MPCs that populated the limb mesenchyme (**Fig. S2a**). In the
99 forelimb buds of E12.5 *Lbx1^{Cre};RS6* embryos, *EGFP* was present in areas corresponding to the
100 position of premuscle masses, as indicated by *Myod* mRNA expression (**Fig. S2a**).

101 As the *EGFP* fluorescence was quite weak after cryosectioning, we used *Ai9* mice, a Cre
102 recombinase-dependent tandem dimer Tomato (tdTomato) reporter strain (Madisen et al.,
103 2010), to generate *Lbx1^{Cre};RS6/LoxP-Stop-LoxP-tdTomato* (*Lbx1^{Cre};RS6/Ai9*) embryos. All
104 tdTomato⁺ MPCs were also positive for *EGFP* and for *PAX3*, allowing the tracing of limb MPCs,
105 which were depleted of BMP signaling during limb mesenchyme invasion (**Fig. S2b**). We did not
106 find any tdTomato⁺/*EGFP*⁺ MPC accumulation in somites at limb level, nor aberrant migration
107 into the anterior/posterior/distal limb margins (**Fig. S2b**). At E18.5, there was strong *EGFP* and
108 tdTomato fluorescence in the limb muscles of *Lbx1^{Cre};RS6/Ai9* fetuses (**Fig. S2c**). TdTomato was
109 present in all myofibers of E18.5 forelimbs, indicating high recombination efficiency (**Fig. S2c**).
110 TdTomato expression was observed exclusively in developing muscles, indicating that MPCs
111 depleted of BMP activity differentiated exclusively into muscle cells (**Fig. S2c**).

112 As *Lbx1^{Cre}* represents a loss-of-function allele due to insertion of the *Cre* transgene into
113 the *Lbx1* exon 1 coding sequence (Sieber et al., 2007), we determined whether heterozygous
114 *Lbx1^{Cre}* mice show signs of haploinsufficiency. Myogenic marker ISH revealed similar expression
115 pattern in *Lbx1* and *Pax3* at E10.5 or *Myod* at E11.5 in *Lbx1^{Cre}* limbs compared to that in the *RS6*
116 controls (data not shown). We also did not find any significant difference in the total number of
117 myofibers, and total number and density of *PAX7*⁺ and *MYOD*⁺ cells in E18.5 *Lbx1^{Cre}* limbs
118 compared to *RS6* controls (data not shown). Furthermore, the *Lbx1^{Cre}* mice had normal viability
119 and reproduction rates. We concluded that the loss of one functional *Lbx1* allele did not cause

120 haploinsufficiency, allowing us to use *RS6* and *Lbx1^{Cre}* as controls for experiments with
121 *Lbx1^{Cre};RS6* mutants.

122 In summary, these results show that following Cre-recombination, the *huSMAD6-IRES-*
123 *EGFP* cassette was expressed exclusively in MPCs and their progeny in developing limbs of
124 *Lbx1^{Cre};RS6* mice, allowing permanent overexpression of the BMP signaling inhibitor SMAD6 in
125 cells of the myogenic lineage.

126

127 **SMAD6 overexpression abrogates BMP signaling and downregulates the marker genes of limb** 128 **muscle development**

129 We confirmed via RT-qPCR that *huSMAD6* was upregulated (3.7-fold) in the E18.5
130 forelimb muscles of *Lbx1^{Cre};RS6* fetuses compared to that of the *RS6* controls (**Fig. S2d**). Next,
131 we determined whether *huSMAD6* overexpression caused cell-autonomous abrogation of BMP
132 signaling in the myogenic lineage. Indeed, we observed the absence of pSMADs in PAX3⁺ MPCs
133 in E10.5 *Lbx1^{Cre};RS6* limb buds compared to the *RS6* controls (**Fig. 2a**). In addition, the presence
134 of pSMADs at the tips of E14.5 muscle fibers was also lost (**Fig. 2b**). Moreover, whole-mount ISH
135 revealed that *Lbx1* and *Pax3* expression was strongly reduced in E10.5 *Lbx1^{Cre};RS6* limb buds
136 compared to that in the *RS6* controls (**Fig. 2c**). Residual *Lbx1* and *Pax3* transcripts were found in
137 the proximal part of the limb buds. Similarly, *Myod* expression was strongly reduced in E11.5
138 and E12.5 limb buds from *Lbx1^{Cre};RS6* embryos compared to that from the *RS6* controls (**Fig. 2c**).
139 However, using ISH, we were unable to discriminate if the decreased gene expression was due
140 to a decrease in cell number or in the transcript number per cell.

141

142 **Abrogation of BMP signaling dampens limb MPC proliferation and distal migration**

143 We transversely cryosectioned E10.5 embryos at limb level, allowing for a proximodistal
144 sectioning plane of the developing limb bud. Double immunofluorescence for PAX3 and the
145 proliferation marker KI67 revealed a significant reduction of the entire PAX3⁺ cell population
146 and a decline in the PAX3⁺/KI67⁺ subpopulation in *Lbx1^{Cre};RS6* embryos, suggesting reduced
147 MPC proliferation after the inhibition of BMP signaling (**Fig. 3a–c**). In addition, we analyzed the
148 proximodistal distribution of the PAX3⁺ cell population in the E10.5 limb buds and found that
149 the cells were significantly reduced in the middle and distal parts of the limb bud in *Lbx1^{Cre};RS6*

150 embryos compared to that in the *RS6* controls (**Fig. 3d, e**). Together, these data suggest that the
151 lack of BMP signaling in MPCs attenuated their proliferation and distal migration.

152

153 **Abrogation of BMP signaling accelerates myogenesis progression of limb MPCs**

154 In the embryonic limb, *Pax3* controls the entry of MPCs into the myogenic program
155 (Relaix et al., 2005; Lagha et al., 2008). In E11.5 *RS6* forelimbs, we observed a transition from
156 PAX3 to PAX7 and MYOD expression. PAX7⁺ cells emerged in proximal pre-muscle masses (**Fig.**
157 **4a**). PAX3⁺ cells were located closer to the ectoderm, whereas MYOD⁺ cells were present closer
158 to the core of the limb bud (**Fig. 4b**), consistent with the myogenic lineage progression from the
159 peripheral towards central limb mesenchyme observed in developing chicken limbs (Amthor et
160 al., 1998).

161 We found a precocious loss of PAX3⁺ cells in E11.5 *Lbx1^{Cre};RS6* limbs (**Fig. 4a–c**). The loss
162 of PAX3⁺ cells was accompanied by a 68% and 46% increase in total PAX7⁺ (**Fig. 4a, d**) and MYOD⁺
163 (**Fig. 4b, e**) cell numbers, respectively. In addition, the PAX3/PAX7⁺ and PAX3/MYOD⁺ cell
164 population ratios increased by 68% and 61%, respectively, compared to that in the *RS6* controls
165 (**Fig. 4f, g**). These results suggest accelerated myogenic lineage progression in *Lbx1^{Cre};RS6* MPCs
166 due to the absence of BMP signaling, which is similar to that shown in embryonic chicken limbs
167 (Amthor et al., 1998).

168 In E12.5 *Lbx1^{Cre};RS6* limbs, the accelerated lineage progression was associated with a
169 loss in total number of MYOD⁺ cells (42%) and PAX7⁺ cells (47%) (**Fig. S3a–d**). Furthermore, we
170 detected a decline in PAX7⁺/KI67⁺ and PAX7⁺/MYOD⁺ cell populations, whereas the proportion
171 of MYOD⁺/MYOG⁺ cells increased, confirming the shift of myogenic lineage progression towards
172 differentiating myoblasts at the expense of proliferating precursors (**Fig. S3b, e–g**).

173 We did not observe a notable increase in cells expressing the apoptosis marker cleaved
174 caspase-3 in the developing pre-muscle masses (data not shown). Therefore, the failure in
175 generating limb MPCs in the absence of BMP signaling was likely caused by decreased
176 proliferation and not apoptosis.

177

178 **Abrogation of BMP signaling in MPCs disturbs *Lbx1^{Cre};RS6* limb proximodistal muscle**
179 **patterning**

180 We analyzed the consequences of decreased MPC generation on primary myofiber
181 formation after abrogation of BMP signaling by analyzing the myofiber number in transverse
182 sections at the end of the embryonic period (E14.5) of mouse forelimb development. The
183 zeugopod muscles of *Lbx1^{Cre};RS6* embryos were significantly smaller and had less than half the
184 number of primary myofibers compared to that in the *RS6* controls (**Fig. 5**). At this stage, we
185 observed defective muscle patterning in the *Lbx1^{Cre};RS6* embryos. Whereas muscle pattern was
186 normal at stylopod level, certain zeugopod muscles were either completely absent (*supinator*,
187 *extensor pollicis*, *flexor digitorum superficialis*) or fused (*extensor carpi radialis longus* and
188 *brevis*), whereas the remaining zeugopod muscles were remarkably hypoplastic (**Fig. 5a**). At the
189 autopod level, only a few remnant MHC-expressing cells were observed, while autopod muscles
190 were entirely absent (**Fig. 5a**). The anatomical changes in muscle pattern seen at the end-
191 embryonic stage (E14.5) persisted during the fetal stage (**Fig. 6**).

192

193 **Normal muscle patterning following abrogation of BMP signaling in differentiated muscle**

194 We wanted to determine whether defective muscle patterning was also caused by the
195 abrogation of BMP signaling in differentiated muscle cells. We used *HSA-Cre* driver mice to
196 conditionally direct recombination in differentiated muscle cells. We first performed a time
197 course to determine the spatiotemporal occurrence of HSA-Cre-driven recombination in *HSA-*
198 *Cre;Ai9* crosses by following the onset of tdTomato expression. In E10.5 and E11.5 embryos,
199 tdTomato was found in somites but not in limb buds. TdTomato was present in developing limb
200 muscles from E12.5 onwards, which is consistent with the emergence of primary myofibers at
201 this stage (**Fig. S2e**). We then generated *HSA-Cre;RS6* mice to overexpress SMAD6 exclusively in
202 terminally differentiated muscles; a mouse model we have validated previously (Stantzou et al.,
203 2017). The forelimbs of E18.5 *HSA-Cre;RS6* fetuses developed normally, and no change was
204 detected in the muscle pattern (**Fig. 6**). This indicates that the information for the future muscle
205 pattern is already present in MPCs before their differentiation. Of note, MCT did not increase

206 at the expense of skeletal muscle, as the pattern of collagen 12 expression in the *HSA-Cre;RS6*
207 fetuses was similar to that of the controls despite the smaller muscles (**Fig. 6**).

208

209 **BMP signaling impacts *Hox* expression of myogenic cells**

210 The observed changes in the muscle pattern of *Lbx1^{Cre};RS6* mutants (**Fig. 6**) resembled
211 those previously observed in *Hoxa11/d11* double mutants (Swinehart et al., 2013), raising the
212 question of the intrinsic positional information of myogenic cells and putative regulation by
213 BMP signaling.

214 Indeed, at E10.5, PAX3⁺ MPCs, which had left the dermomyotome and migrated into the
215 limb bud, expressed HOXA11 protein. Notably, HOXA11 levels in the MPCs were higher than in
216 the surrounding limb mesenchymal cells (**Fig. 7a**). As early as one day later, at E11.5, most PAX3⁺
217 cells had lost the high HOXA11 protein levels (**Fig. 7b**). In the absence of BMP signaling in
218 *Lbx1^{Cre};RS6* embryonic limbs, the MPCs failed to accumulate high levels of HOXA11 protein (**Fig.**
219 **7c compared with 7a, S4a**).

220 To gain a global vision of *Hox* gene expression at single-cell resolution, we analyzed
221 open-access single-cell RNA sequencing (scRNAseq) datasets of early chicken and mouse whole
222 limb buds (Esteves de Lima et al., 2021; Rouco et al., 2021). Chicken and mouse limb buds have
223 comparable *Hox* patterns in limb mesenchyme and myogenic differentiation (Pownall et al.,
224 2002; Sundin et al., 1990; Yakushiji-Kaminatsui et al., 2018).

225 In chicken forelimb buds, scRNAseq showed the expression of genes of the *HOXA* and
226 *HOXD* clusters in mesenchymal cells and in muscle cluster cells (**Fig. S4b-h and S5**). As an
227 example, *HOXA11* transcripts were detected in the majority (69%) of muscle cluster cells in E4
228 limbs (E10.5 mouse stage equivalence) and more rarely (37% of cells) by E6 (E12.5 mouse stage
229 equivalence). In contrast, *HOXD13* transcripts were not detected before E6, where its expression
230 was limited to a few muscle cluster cells (**Fig. S4b**). *HOXA11* was expressed at all successive steps
231 of the myogenic process: first in PAX7⁺ and MYOD⁺ MPCs at E4 and E6, and then in MYOG⁺
232 myoblasts at E6 (**Fig. S4c-g**). There was nonetheless a drop in *HOXA11* expression during
233 myogenic lineage progression, given that at E6, 39% of PAX7⁺ muscle cluster cells co-expressed
234 *HOXA11*, while only 15% of MYOG⁺ cells co-expressed *HOXA11* (**Fig. S4h**). Interestingly, we

235 found a heterogeneous combinatorial expression of *HOXA* genes in single muscle cluster cells,
236 suggesting *HOX*-dependent positional information in chicken limb MPCs (**Fig. S5a-b**).

237 We next analyzed whether BMP response correlates with *HOXA* gene expression in
238 muscle cluster cells and found that *HOXA*⁺ cells (expressing one or several genes of the *HOXA*
239 cluster) expressed BMP downstream effectors genes *ID2* and *ID3*, but not *ID1*, in higher
240 proportion compared to *HOXA*⁻ cells (**Fig. S5c-e**). Consistently, the BMP score, which is the
241 corrected average expression of the *ID1*, *ID2* and *ID3* genes, was significantly higher in *HOXA*⁺
242 cells compared to *HOXA*⁻ cells (**Fig. S5f**).

243 scRNAseq of E12.5 mouse forelimbs showed very similar results compared to those in
244 chicken: i) Muscle cluster cells expressed genes of the *Hoxa* and *Hoxd* cluster (**Fig. S6a**); ii) a
245 subset of *Hoxa11*⁺ muscle cluster cells co-expressed *Pax3*, *Pax7*, *Myf5* and *Myod*, but only rarely
246 *Myog* (**Fig. S7**); iii) muscle cluster cells showed large heterogeneity in the expression of genes
247 of the *Hoxa* cluster (**Fig. S6b**); and iv) there was *Id1*, *Id2* and *Id3* expression in a higher proportion
248 of *Hoxa*⁺ cells than *Hoxa*⁻ cells (**Fig. S6c-e**).

249 **Discussion**

250 The current paradigm of limb muscle patterning considers limb MPCs naïve, where they develop
251 individual muscles by invading a prepattern established by MCT (Kardon et al., 2003). Our
252 results contribute to this concept by showing that BMP signaling is necessary for sufficient
253 generation of MPCs, thereby establishing the necessary cellular source for limb muscle pattern.
254 We used overexpression of *SMAD6* as an experimental tool to abrogate BMP signaling, which
255 caused precocious loss of PAX3 in MPCs and accelerated myogenic lineage progression. MPCs
256 advanced less distally, as expected, since PAX3 is a prerequisite for myogenic migration (Bober
257 et al., 1994), likely causing a mismatch between their distal progression and the local connective
258 tissue, and thus responsible for the observed defects in proximodistal muscle pattern.

259 It has been shown that impaired distal MPC migration can cause varying degrees of limb
260 muscle defects (Brohmann et al., 2000, 1; Shin et al., 2016; Vasyutina et al., 2005). A detailed
261 anatomical analysis of these mouse mutants would be required to determine whether different
262 signaling cues, e.g. SF/HGF as compared to BMPs, exert distinctive roles during muscle
263 patterning. In the absence of such comparative anatomical analysis, however, we cannot
264 exclude the possibility that migration defects, independently of the underlying molecular
265 mechanisms, result in a generic patterning defect.

266 Our data suggest that the proximodistal pattern information was already set up before
267 MPCs differentiated and before splitting of muscle masses occurred. Indeed, muscle pattern
268 remained normal when BMP signaling was directly abrogated in differentiated muscle.
269 Instructions likely occurred at the early limb bud stages, which coincided with a high BMP-
270 responsiveness of migrating PAX3⁺ MPCs. Curiously, limb MPCs, in mouse as well as in chicken,
271 expressed *Hox* genes, suggesting that MPCs acquire positional identity at early limb bud stages.
272 scRNAseq revealed: *i*) a high proportion of *Hox*-expressing MPCs in early limb buds; *ii*)
273 heterogeneity of *Hox* gene expression in the MPCs; *iii*) their sequential upregulation; and *iv*)
274 their downregulation during myogenic lineage progression. Immunohistochemistry confirmed
275 the transcriptome data: upon leaving the dermomyotome and entering limb bud mesenchyme,
276 PAX3⁺ migrating limb MPCs produced high HOXA11 protein levels, and this was dependent on
277 BMP signaling. Interestingly, the *Lbx1^{Cre};RS6* mutants resembled the muscle pattern defect

278 observed in *Hoxa11*^{-/-}/*d11*^{-/-} dKO mutants (Swinehart et al., 2013). We can only speculate about
279 the role of *Hox* genes in developing muscle, and future work, such as their conditional knockout
280 in MPCs, would be required.

281 Curiously, we show that MPCs were the only limb cells that showed robust BMP-
282 dependent pSMAD expression at early limb bud stages, implying a high dependency of MPCs on
283 BMP signaling. However, we neither explored the source of BMPs, nor which ligands of the BMP
284 family signal to limb MPCs. In previous work in chick embryos, early migrating MPCs were found
285 surrounded by BMP2/4/7-expressing cells at the limb margins, and ectopically applied BMP
286 altered the positioning of pre-muscle masses (Amthor et al., 1998). Similar expression of BMPs
287 in limb margins was also observed in mouse embryos (Michos et al., 2004). It remains to be
288 determined, whether long-range BMP signaling from limb margins could regulate MPCs.
289 Alternative sources, including expression by MPCs themselves, must be considered. Of note,
290 triple knockout of BMP2/4/7 in the apical ectodermal ridge caused polydactyly and does not
291 affect limb outgrowth, whereas overexpression of the BMP antagonist Gremlin in entire limb
292 mesenchyme prevented limb outgrowth altogether (Choi et al., 2012; Norrie et al., 2014).
293 However, muscle development has not been analysed in these mutants.

294 We would like to emphasize that our results support the MCT prepatter model. We
295 believe that mesenchymal cells that form future MCT are the source of cues, including BMPs,
296 that provide positional information to MPCs. In this scenario MCT and MPCs could be mutually
297 dependent on each other to establish the muscle pattern. Indeed, a defined MCT pattern
298 resembling a muscle pattern failed to develop in muscle-devoid limbs (Christ et al., 1977).
299 Whereas tendons initially developed autonomously in lack of muscles; they degenerated
300 secondarily (Christ et al., 1977; Schweitzer et al., 2010).

301 We here employed the overexpression of *SMAD6* as a mean to test the cell-autonomous
302 effect of abrogating BMP signaling. *SMAD6* inhibits Smad signaling by the BMP type I receptors
303 ALK-3/6 subgroup and only weakly inhibits TGF- β /activin signaling via the BMP type I receptors
304 ALK-1/2 subgroup, the latter being a preferential target of *SMAD7* (Goto et al., 2007; Miyazawa
305 and Miyazono, 2017). Since *SMAD6* is not a direct component of the BMP signaling cascade,

306 further work is required to substantiate our results, such as performing specific BMP receptor
307 knockout.

308 In conclusion, our data suggest that BMP signaling controls embryonic limb MPCs to
309 maintain PAX3-expressing precursor status and to acquire positional identity by expressing HOX
310 code, thereby coordinating MPC migration and proliferation, which establishes the muscle
311 pattern prior to the splitting of pre-muscle masses into individual muscles.

312 **Materials and methods**

313 **Mouse lines used for embryo generation**

314 We conducted all animal experiments according to national and European legislation as well as
315 institutional guidelines for the care and use of laboratory animals as approved by the French
316 government.

317 The following mouse lines have been previously described: *Lbx1^{Cre}* mice (Sieber et al.,
318 2007), *HSA-Cre* transgenic mice (Miniou et al., 1999), *Rosa26^{LoxP-Stop-LoxP-huSMAD6-IRES-EGFP}* mice (i.e.
319 *RS6*) (Stantzou et al., 2017), and *Ai9* mice, which contain an insertion in the *Rosa26* locus of a
320 strong and ubiquitous CAG promoter, followed by a floxed-Stop cassette-controlled *tdTomato*
321 (Madisen et al., 2010).

322 *Lbx1^{Cre}*, *HSA-Cre* and *RS6* mice were interbred to obtain *Lbx1^{Cre};RS6* and *HSA-Cre;RS6*
323 embryos. *Lbx1^{Cre}* and *Ai9* mice were interbred to obtain heterozygous *Lbx1^{Cre};Ai9* mice, which
324 were crossed with *RS6* mice to obtain *Lbx1^{Cre};RS6/Ai9* embryos.

325 Genomic DNA isolated from ear clippings postnatally or from either yolk sacs or parts of
326 the non-limb tissues prenatally were genotyped. The PCR primers are described in **Table S1**.

327 **Embryonic and fetal forelimb collection and processing**

328 Embryos and fetuses were collected in ice-cold phosphate-buffered saline (PBS) at different
329 stages (plug date: E0.5). Embryos used for whole-mount ISH experiments were fixed overnight
330 in 4% paraformaldehyde (PFA) at 4°C, washed twice in PBS-T (0.1% Tween-20, P9416, Sigma)
331 and dehydrated using a methanol series of 50% methanol (15 minutes × 2) and 100% methanol
332 (15 minutes), after which they could be stored at -20°C for whole-mount ISH.

333 For immunostaining, forelimbs from E10.5 and E11.5 embryos were dissected as pairs
334 connected with the rostral (thoracic) body segment to preserve the structure of the forelimbs
335 and forelimb-level somites. At the later stages, the forelimbs were individually dissected. The
336 tissues were fixed at 4°C in either 1% PFA for 1 hour (E10.5–E12.5) or in 4% PFA for 2 hours
337 (E14.5 and E18.5), washed thrice for 10 minutes and then dehydrated overnight at 4°C in either
338 15% sucrose (E10.5–E12.5) or 30% sucrose (E14.5 and E18.5). The forelimbs were embedded in
339 Optimum Cutting Temperature compound (Qpath) in disposable plastic moulds (Dutscher),
340 frozen in liquid nitrogen and stored at -80°C for sectioning.

341 **RNA isolation and RT-qPCR**

342 Total RNA from frozen E18.5 forelimb muscle tissue was extracted using TRIzol (Life
343 Technologies Ambion) in combination with an RNeasy Mini kit (Qiagen). Traces of DNA in the
344 RNA extract were removed with an RNase-Free DNase Set (Qiagen). The isolated RNA was
345 quantified using a NanoVue Plus GE HealthCare spectrophotometer (Dutscher). Next,
346 complementary DNA (cDNA) was synthesized using reverse transcriptase (SuperScript™ III First-
347 Strand Synthesis SuperMix kit, Invitrogen). RT-qPCR was performed according to the SYBR Green
348 protocol (Bio-Rad) in triplicate on a CFX96 Touch Real-Time detection system (Bio-Rad) using
349 iTaq Universal SYBR Green Supermix (Bio-Rad) and primers for *huSMAD6* and the housekeeping
350 gene *Gapdh* as described previously (Stantzou et al., 2017).

351 **Whole-mount ISH**

352 Whole-mount ISH with digoxigenin-labeled probes was used for visualizing the expression of
353 *Lbx1*, *Pax3* or *Myod*. ISH was performed as previously described (Murgai et al., 2018) (Tajbakhsh
354 et al., 1997).

355 **Immunofluorescence staining**

356 Serial sections of frozen forelimbs on SuperFrost Plus adhesion slides (Thermo Fisher Scientific)
357 were obtained at 10-µm thickness using a cryostat at -24°C (Leica CM3050S). E10.5 and E11.5
358 forelimbs were longitudinally sectioned, which allowed 2D visualization of forelimb sections in
359 the proximodistal and dorsoventral axes. E12.5, E14.5 and E18.5 forelimbs were sectioned in
360 the transverse plane (except stated otherwise), which allowed 2D visualization of forelimb
361 sections in the dorsoventral and anteroposterior axes. The forelimb sections on the slides were
362 directly used for immunofluorescence staining experiments or were stored at -80°C for future
363 use.

364 Immunofluorescence staining was performed using the following protocols: *i*) 5-minute
365 rehydration of slides in PBS; *ii*) permeabilization with 0.1% (E10.5–E12.5) or 0.5% (E14.5 and
366 E18.5) Triton X-100 (Sigma-Aldrich) (in the case of nuclear protein staining, e.g. PAX3, PAX7,
367 MYOD, Myogenin (MYOG), pSMAD1/5/9, HOXA11, KI67) or with methanol at -20°C (for non-
368 nuclear proteins, e.g. MHC, laminin alpha-2, DsRed, collagen 12); *iii*) three 5-minute washes in
369 PBS; *iv*) antigen retrieval by 20-minute immersion of the slides in boiled 10 mM citric acid

370 solution kept at 60°C in a water bath; the slides were cooled at room temperature in the citric
371 acid solution, and three PBS washes were performed (only for E14.5 and E18.5 nuclear staining);
372 v) up to 1.5-hour blocking with 10% normal goat serum (Abcam); vi) overnight incubation with
373 primary antibodies (dilutions prepared in blocking solution, **Table S2**) at 4°C; vii) three 5-minute
374 washes in PBS; viii) up to 1.5-hour incubation with secondary antibodies (dilutions prepared in
375 blocking solution, **Table S2**); ix) three 5-minute washes in PBS; x) 10-minute incubation with
376 DAPI for nuclear staining (dilution 1:5000); xi) 5-minute washing in PBS; xii) coverslip mounting
377 with Fluoromount-G (Southern Biotech). **Tables S2 and S3** detail the primary and secondary
378 antibodies used in this study.

379 **Imaging**

380 Embryos were dissected and whole limbs in the unfixed state were immediately imaged for
381 native EGFP and tdTomato fluorescence using a stereomicroscope (SteREO Lumar.V12, Zeiss).
382 Native EGFP and tdTomato fluorescence was subsequently imaged on fresh unfixed
383 cryosections, and fluorescence immunohistochemistry was captured under 20×, 40× or 63×
384 objective using a fluorescence microscope (Zeiss Axio Imager) with an Orkan camera
385 (Hamamatsu). Images were acquired with AxioVision software. Mosaic images of
386 immunostained whole limbs were obtained after stitching together multiple individual images
387 captured with a 20× objective of all the different regions in the whole limb for all fluorescence
388 channels of interest. Masson's trichrome staining images were acquired using a digital slide
389 scanner (Leica) and analyzed with ImageScope software. Images were exported and saved as
390 TIFF files for further analyzes or for illustration in the figures.

391 **Morphometric studies**

392 The captured fluorescent images were analyzed by applying morphometric studies using ImageJ
393 (Schneider et al., 2012).

394 The populations of cell and nuclear markers (PAX3, MYOD, PAX7, MYOG, KI67, HOXA11,
395 DAPI) were quantified on immunostained cryosections as detailed above by superimposing
396 fluorescence channels to visualize signal colocalization.

397 Proximodistal migration of PAX3-expressing MPCs in E10.5 forelimbs was quantified by
398 dividing the forelimb into 10 equally sized proximodistal zones and counting the PAX3⁺ cells in
399 each zone.

400 Myofibers on the transverse sections of E14.5 forelimb zeugopods were quantified
401 following co-immunostaining against laminin alpha-2 and MHC. Total muscle CSA was
402 determined as the sum of the CSA of all individual zeugopod muscles.

403 Morphometric studies at E10.5, E11.5 and E12.5 were conducted on three consecutive
404 sections of each forelimb, and $n = 5$ forelimbs were analyzed for each genotype except if stated
405 otherwise. Morphometric studies at E14.5 were conducted on one transverse section through
406 the proximal region of forelimb zeugopods, assuring measurements at the maximal size of the
407 zeugopod muscle.

408 **scRNAseq analysis of whole limb cells**

409 The scRNAseq protocol for E12.5 mouse whole limb cells is described by Rouco *et al.* (Rouco *et*
410 *al.*, 2021); that for chicken whole limb cells is described by Esteves de Lima *et al.* (Esteves de
411 Lima *et al.*, 2021). Briefly, scRNAseq datasets were generated from whole forelimbs from two
412 different E4 embryos and three different E6 embryos using a 10X Chromium Chip (10X
413 Genomics) followed by sequencing with a High Output Flow Cell using an Illumina Nextseq 500
414 and by sequence analysis with Cell Ranger Single Cell Software Suite 3.0.2 (10X Genomics). Only
415 mononucleated muscle cells are included in the datasets, as plurinucleated myotubes are
416 excluded by the single-cell isolation protocol. Downstream clustering analysis of scRNAseq data
417 was performed using the Seurat package (v3.0) (Stuart *et al.*, 2019) under R (v3.6.1) (Macosko
418 *et al.*, 2015; R: The R Project for Statistical Computing). We then extracted the clusters identified
419 as muscle clusters by the differential expression of the classical myogenic markers (*PAX7*,
420 *MYOD*, *MYOG*) and performed the remaining analysis on these muscle clusters only. Gene
421 expression was defined by 'gene log-normalized count > 0'. The scRNAseq datasets were
422 analyzed using Seurat tools: FeaturePlot and Violin plots. Custom feature plots highlighting gene
423 co-expression were generated using the R package ggplot2 v3.3.3 (Wickham, 2016). Population
424 intersection plots were generated with the R package UpSetR v1.4.0 (Conway *et al.*, 2017).

425 Within the muscle clusters, cells were grouped according to two identities, i.e. whether
426 or not they expressed *HOXA* (*HOXA*⁺ and *HOXA*⁻, respectively). The *HOXA*⁺ identity was defined
427 by the expression (i.e. gene log-normalized count > 0) in a cell of at least one of the seven *HOXA*
428 genes found in the muscle clusters (*HOXA4*, *HOXA5*, *HOXA6*, *HOXA7*, *HOXA9*, *HOXA10*, *HOXA11*).
429 *HOXA*⁻ identity was conferred to cells that expressed none of the seven *HOXA* genes. A BMP
430 score was calculated using the AddModuleScore function for the well-characterized BMP
431 transcriptional read-out genes *ID1*, *ID2* and *ID3*. Response to BMP signaling was then compared
432 between these two identities using the Seurat tool Violin plots and the ggplot2 tool boxplots.

433 **Data availability**

434 Both chicken and mouse scRNAseq datasets have been deposited in the National Center for
435 Biotechnology Information Gene Expression Omnibus database
436 (<https://www.ncbi.nlm.nih.gov/geo/>) respectively under accession numbers GSE166981 and
437 GSE168633.

438 **Statistical analyses**

439 Numerical data are presented as the mean ± standard deviation (SD). The probability for
440 statistical differences between experimental and control groups was determined by calculating
441 the exact *p*-value using the non-parametric two-tailed Mann-Whitney *U* test. GraphPad Prism
442 Software version 7.00 for Windows (La Jolla, CA, USA, www.graphpad.com) was used for all
443 statistical analyses and graphs.

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448

449 **Declaration of interests**

450 The authors do not have any financial or non-financial competing interests.

451

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461

462 **Author Contribution**

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Figures and legends

Figure 1. BMP signaling activity in developing limbs. Longitudinal sections of E10.5 and E11.5 forelimbs depict immunofluorescence signal of pSMAD1/5/9 (red) and PAX3, PAX7 or MYOD (green) following co-immunohistochemistry. Nuclei are stained with DAPI (blue). Left images show entire forelimbs, which are outlined by white dotted lines (pro: proximal; dis: distal; dor: dorsal; ven: ventral). Insets (white boxes) are shown at higher magnification on the right side of the respective entire forelimb and depict individual and merged fluorescence channels. White arrowheads show the few PAX7⁺ cells positive for pSMAD1/5/9. *n* = 5 biological replicates for each immunostaining and embryonic stage. Scale bars = 200 μ m.

Figure 1

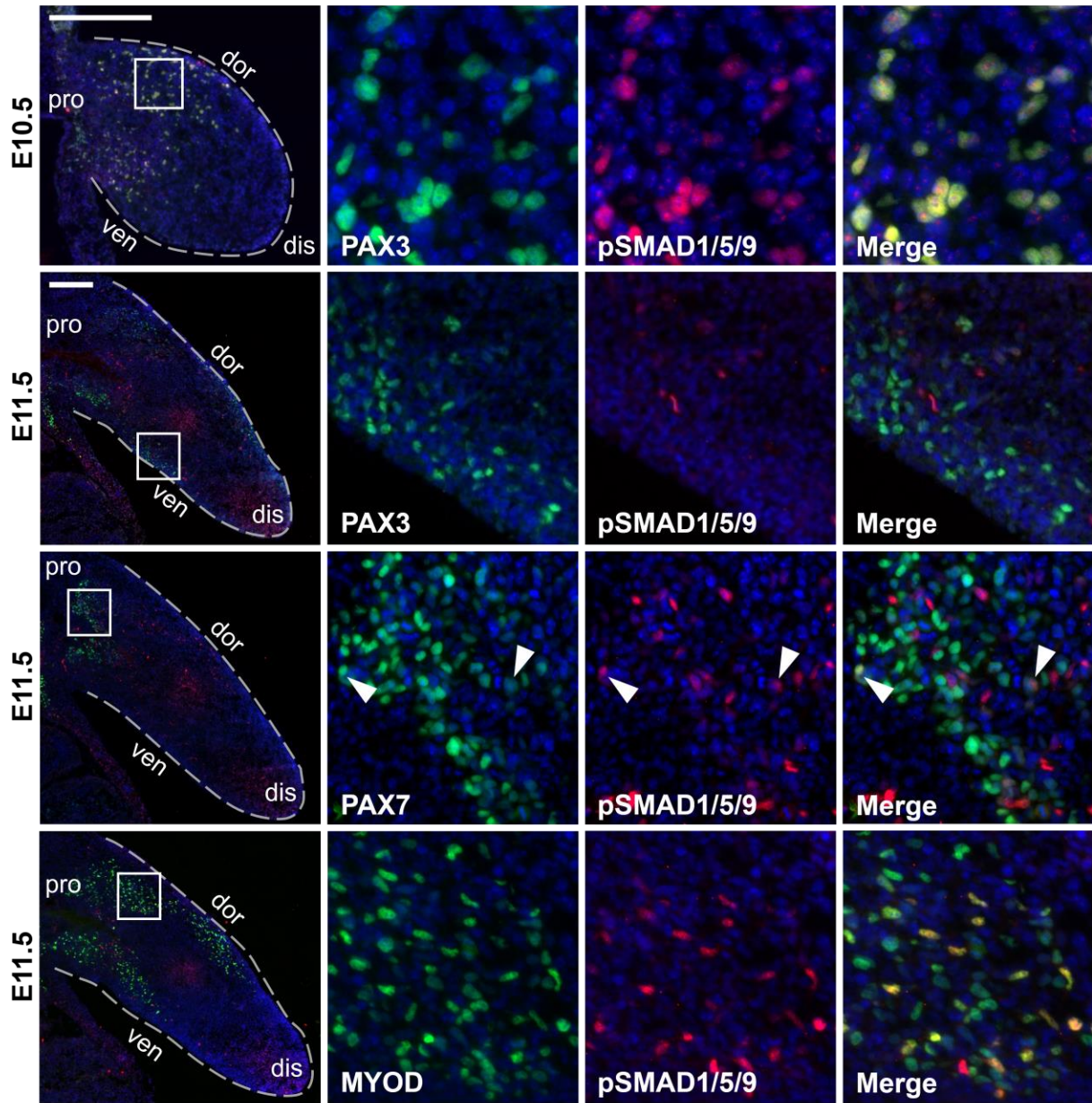


Figure 2. Effect of SMAD6 overexpression in the developing limb muscles. (a-b) Effect of SMAD6 overexpression on BMP signaling. **(a)** Left images depict the immunofluorescence signals of PAX3 (green) and pSMAD1/5/9 (red) following co-immunohistochemistry on longitudinal sections of E10.5 entire forelimbs of *RS6* and *Lbx1^{Cre};RS6* embryos. Nuclei are stained with DAPI (blue). Forelimbs are outlined by white dotted lines. Insets (white boxes) are shown at higher magnification on the right side of the respective entire forelimb and depict individual and merged fluorescence channels. **(b)** Left images depict immunofluorescence signals of MHC (green) and pSMAD1/5/9 (red) following co-immunohistochemistry on transverse sections at mid-zeugopod level of E14.5 forelimbs of *RS6* and *Lbx1^{Cre};RS6* embryos. Nuclei are stained with DAPI (blue). Insets (white solid lines) are shown at higher magnification on the right side of the respective cross-sectioned forelimb and depict the *pronator teres* muscle in individual and merged fluorescence channels. *n* = 5 biological replicates for all stages and immunostaining (pro: proximal; dis: distal; dor: dorsal; ven: ventral; ant: anterior; pos: posterior). Scale bar = 200 μm. **(c)** Effect of SMAD6 overexpression on the transcription of early markers of limb muscle development. The images show the expression patterns of *Lbx1*, *Pax3* and *Myod* transcripts (purple) following whole-mount ISH of E10.5, E11.5 and E12.5 *Lbx1^{Cre};RS6* embryos compared to *RS6* controls. Images show dorsal view of the forelimbs (outlined by grey dotted line) (pro: proximal; dis: distal).

Figure 2

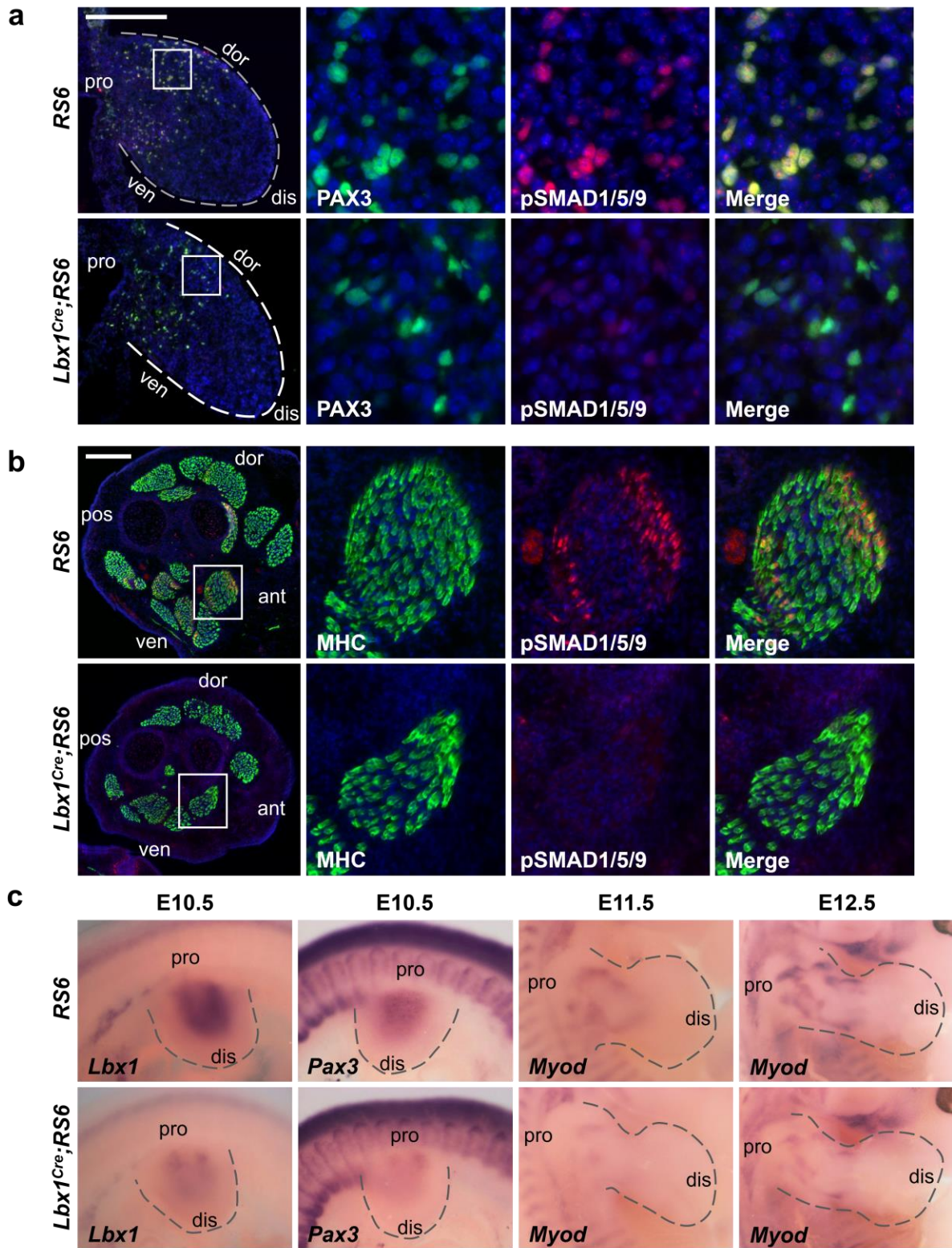


Figure 3. Effect of *SMAD6* overexpression on limb MPC proliferation and migration. (a) Left images depict immunofluorescence staining of PAX3 (green) and KI67 (red) following co-immunohistochemistry on longitudinal sections of E10.5 entire forelimbs of *RS6* control and *Lbx1^{Cre};RS6* embryos. Nuclei are stained with DAPI (blue). Insets (white solid lines) are shown at higher magnification on the right side of the respective entire forelimb and depict individual and merged fluorescence channels (pro: proximal; dis: distal; dor: dorsal; ven: ventral). Scale bar = 200 μ m. **(b)** Dot-plotted bar graph shows the number of PAX3⁺ cells in the forelimbs of both genotypes. **(c)** Stacked bar graph depicts the percentages of PAX3⁺/KI67⁺ (orange) ($p = 0.0079$) and PAX3⁺/KI67⁻ (green) ($p = 0.0079$) MPCs in the forelimbs of both genotypes. **(d)** Immunofluorescence staining of PAX3 (green) and DAPI (blue) on longitudinal sections of E10.5 forelimbs of *RS6* control and *Lbx1^{Cre};RS6* embryos. The limb was divided into 10 equal zones along the proximodistal axis. Scale bar = 200 μ m. **(e)** Histogram depicts the number of PAX3⁺ MPCs based on their position along the proximodistal limb axis as depicted in (d). $n = 5$ biological replicates for each genotype. Each replicate represents the mean of three consecutive serial sections. Data are the mean \pm SD.

Figure 3

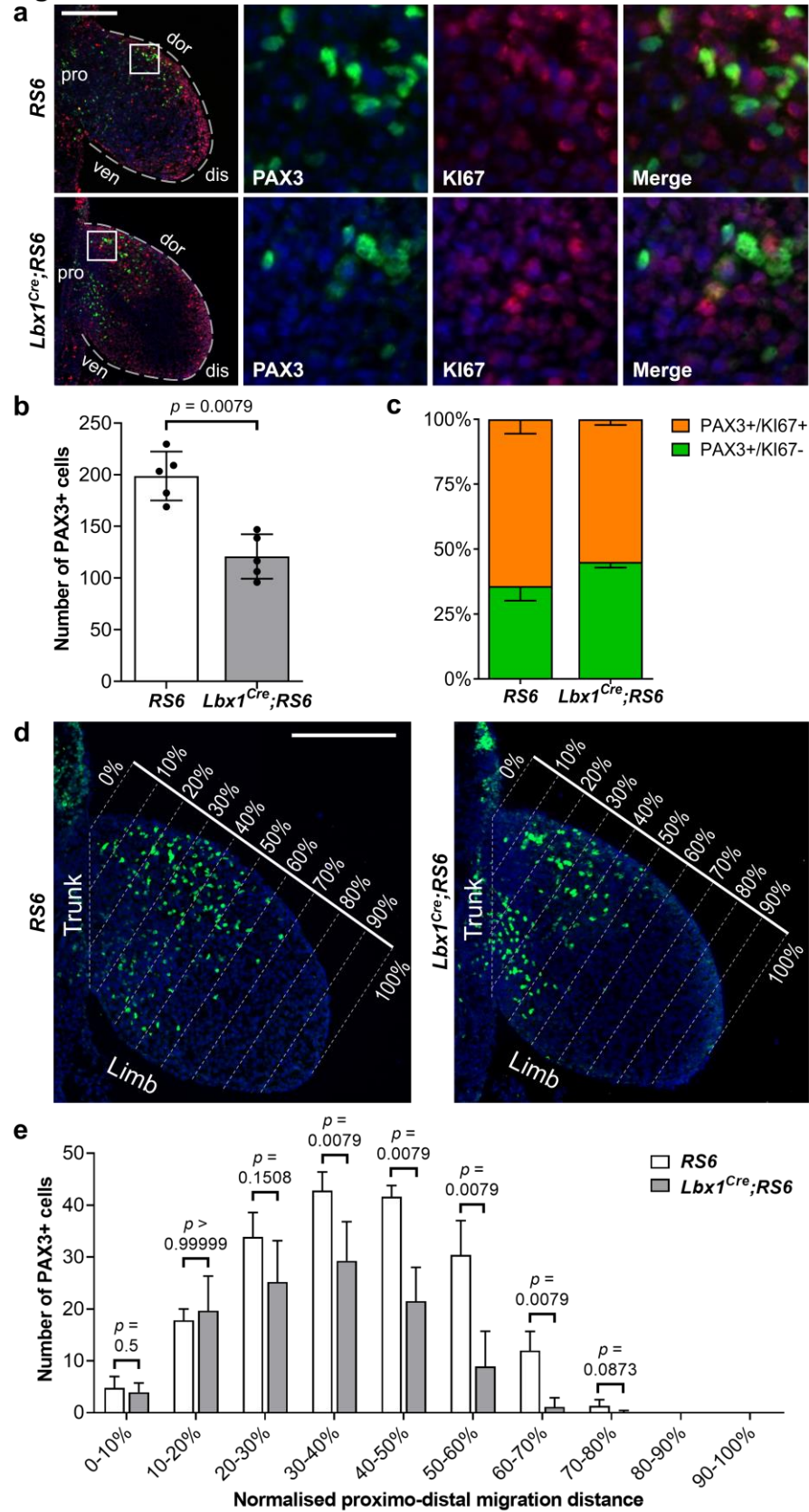


Figure 4. Effect of SMAD6 overexpression on myogenic lineage progression. (a and b) Left images depict immunofluorescence staining of PAX3 (green) and either PAX7 or MYOD (red) after co-immunohistochemistry on longitudinal sections of E11.5 entire forelimbs of *RS6* control and *Lbx1^{Cre};RS6* embryos. Nuclei are stained with DAPI (blue). Limbs are outlined by white dotted lines (pro: proximal; dis: distal; dor: dorsal; ven: ventral). Scale bar = 200 μ m. Insets (white boxes) are shown at higher magnification on the right side of the respective entire forelimb and depict individual and merged fluorescence channels. **(c–e)** Dot-plotted bar graphs show the total number of PAX3⁺, PAX7⁺ and MYOD⁺ cells. **(f)** Stacked bar graph depicts the percentages of PAX3⁺/PAX7⁻ cells (green) ($p = 0.0159$), PAX3⁺/PAX7⁺ cells (orange) ($p = 0.1905$) and PAX3⁻/PAX7⁺ cells (red) ($p = 0.0159$). **(g)** Stacked bar graph depicts the percentages of PAX3⁺/MYOD⁻ cells (green) ($p = 0.0159$), PAX3⁺/MYOD⁺ cells (orange) ($p = 0.0159$) and PAX3⁻/MYOD⁺ cells (red) ($p = 0.0159$) cells. $n = 4$ biological replicates for *RS6* and $n = 5$ for *Lbx1^{Cre};RS6*. Each replicate represents the mean of three consecutive serial sections. Data are the mean \pm SD.

Figure 4

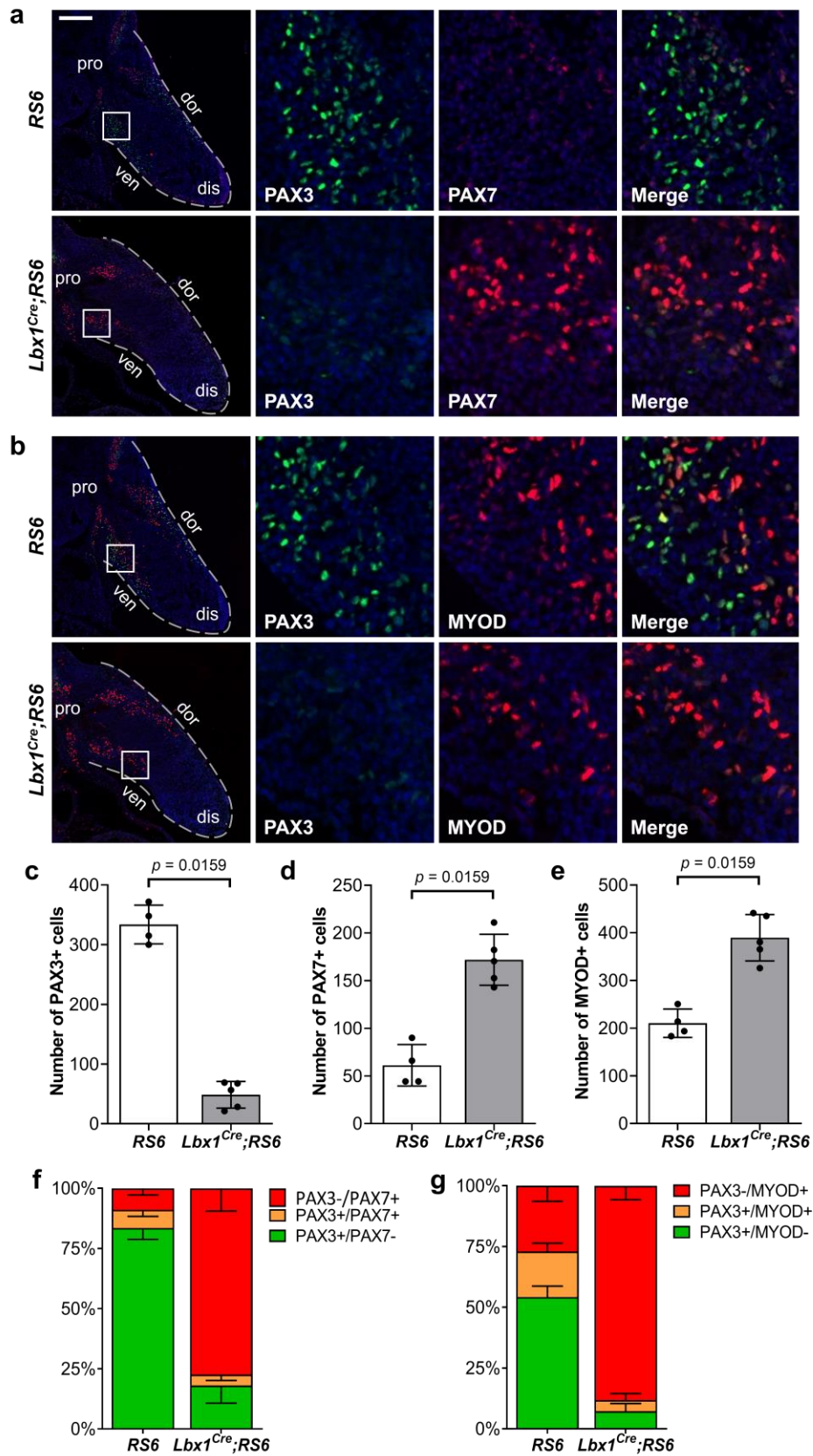


Figure 5. Effect of SMAD6 overexpression on embryonic muscle pattern. (a) Immunostaining for MHC (red) on transverse sections at the zeugopod (upper images) and autopod (lower images) level of E14.5 forelimbs from *RS6* and *Lbx1^{Cre};RS6* embryos. Nuclei are stained with DAPI (blue). Inset (yellow dotted lines) is shown at higher magnification to depict remnants of MHC-expressing cells in the *Lbx1^{Cre};RS6* forelimb autopod. Muscles, which are numbered in yellow in *RS6* embryos, are absent from the *Lbx1^{Cre};RS6* embryos (yellow asterisks). Letters indicate the bones: r: *radius*; u: *ulna*; m: *metacarpals*. Numbers indicate the muscles: 9: *extensor carpi radialis longus*; 10: *extensor carpi radialis brevis*; 11: *extensor digitorum communis*; 12: *extensor digitorum lateralis*; 13: *extensor carpi ulnaris*; 14: *supinator*; 15: *extensor pollicis*; 16: *extensor indicis proprius*; 17: *pronator teres*; 18: *flexor carpi radialis*; 19: *palmaris longus*; 20: *flexor carpi ulnaris*; 21: *flexor digitorum superficialis*; 22/23/24/25: *flexor digitorum profundus* (superficial 's', humeral 'h', ulnar 'u' and radial 'r' heads); 26: *pronator quadratus*; 27: *thenars*; 28: *hypothenars*; 29: *lumbricals*; 30: *interossei*. Scale bar = 200 μ m. **(b and c)** Dot-plotted bar graphs show CSA **(b)** and total myofiber number **(c)** of zeugopod muscles of both genotypes at E14.5. The muscle CSA measurement and myofiber count were conducted on transverse sections at the proximal zeugopod level. *n* = 5 biological replicates. Data are the mean \pm SD.

Figure 5

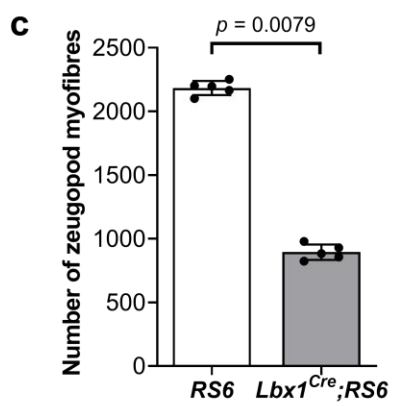
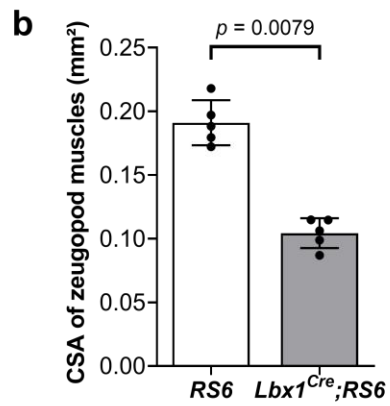
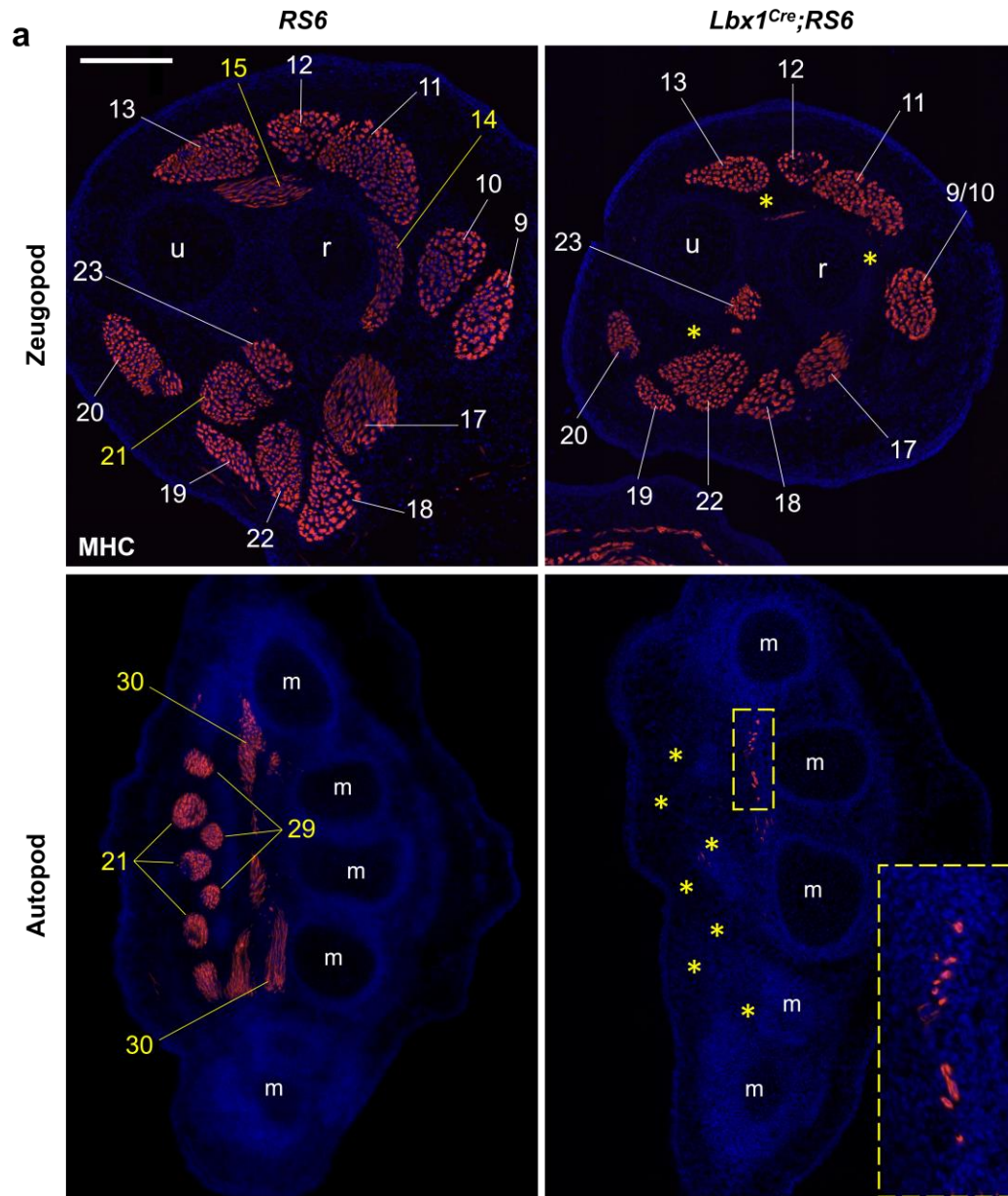


Figure 6. Effect of *SMAD6* overexpression on fetal muscle pattern. Immunostaining for MHC (red) and collagen 12 (green) on transverse sections at the zeugopod (upper images) and autopod (lower images) level of E18.5 forelimbs from *RS6*, *Lbx1^{Cre};RS6* and *HSA-Cre;RS6* embryos. Row 1 and 3 show merged images; row 2 and 4 show collagen 12. Muscles, which are numbered in yellow in *RS6* embryos, are absent from the *Lbx1^{Cre};RS6* embryos (yellow asterisks). Letters indicate the bones: r: *radius*; u: *ulna*; m: *metacarpals*. Numbers indicate muscles as well as the corresponding MCT compartments and tendons: 9: *extensor carpi radialis longus*; 10: *extensor carpi radialis brevis*; 11: *extensor digitorum communis*; 12: *extensor digitorum lateralis*; 13: *extensor carpi ulnaris*; 14: *supinator*; 15: *extensor pollicis*; 16: *extensor indicis proprius*; 17: *pronator teres*; 18: *flexor carpi radialis*; 19: *palmaris longus*; 20: *flexor carpi ulnaris*; 21: *flexor digitorum superficialis*; 22/23/24/25: *flexor digitorum profundus* (superficial 's', humeral 'h', ulnar 'u' and radial 'r' heads); 26: *pronator quadratus*; 27: *thenars*; 28: *hypothenars*; 29: *lumbricals*; 30: *interossei*. Scale bar = 500 μ m. *n* = 5 biological replicates.

Figure 6

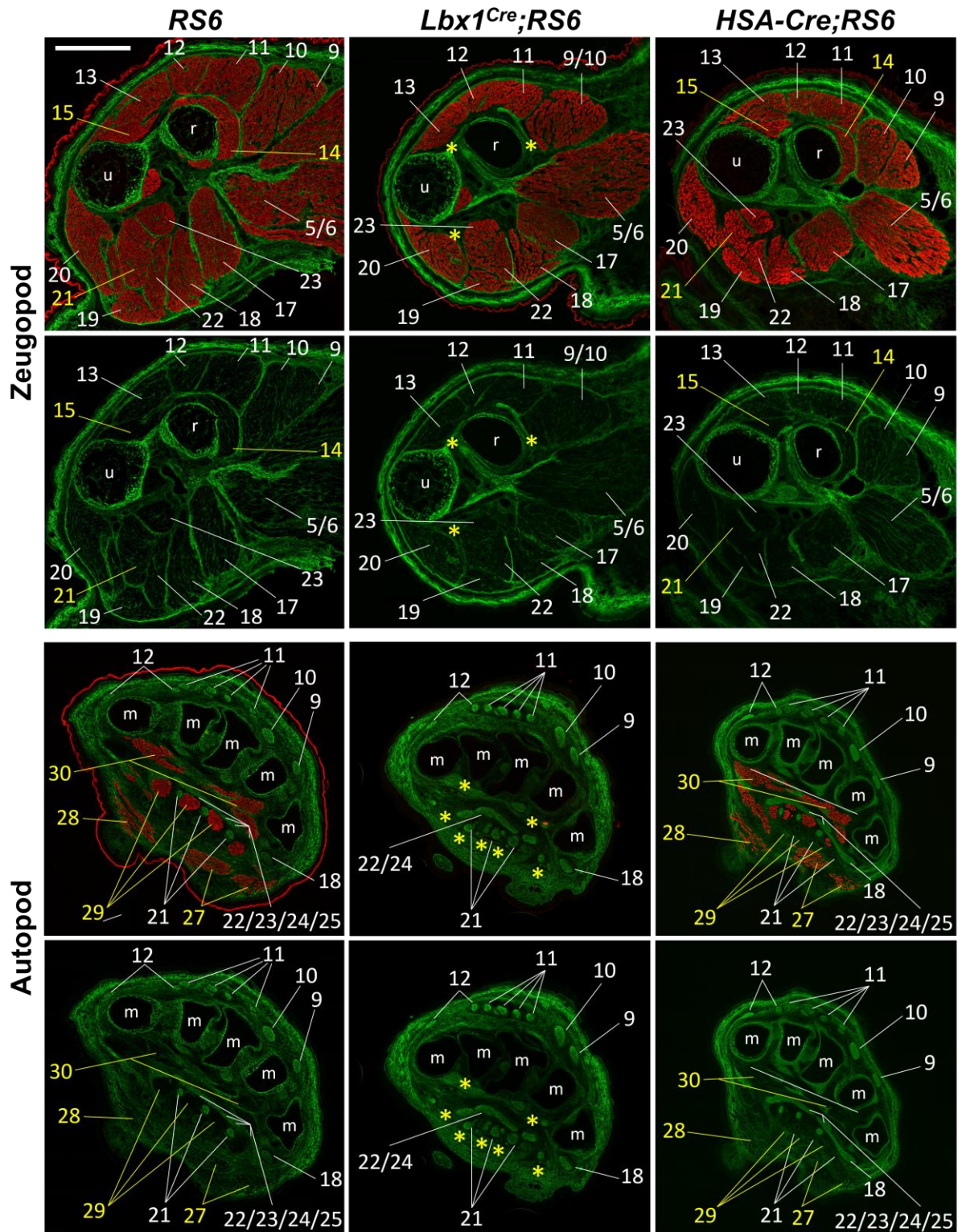
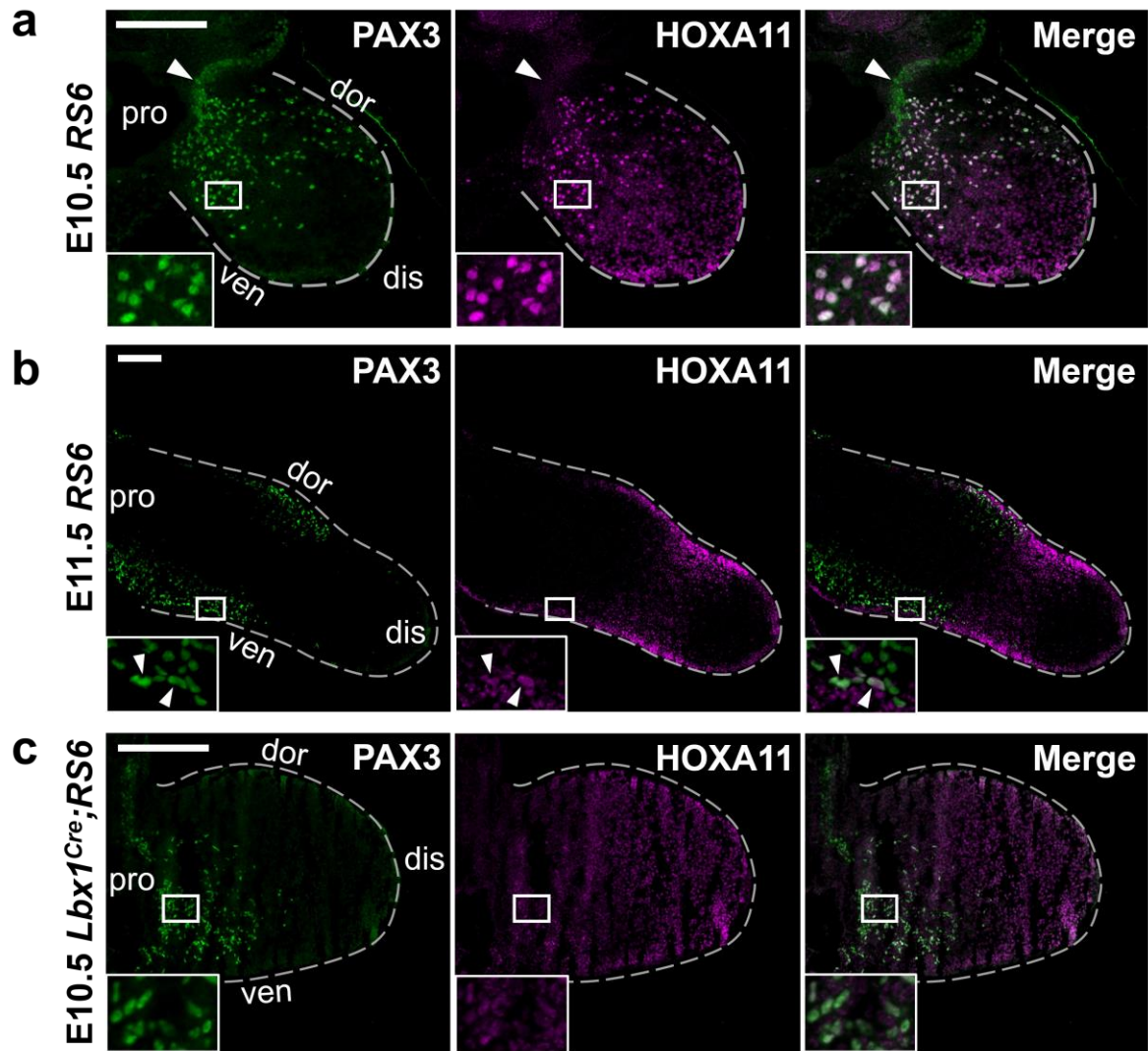


Figure 7. HOX proteins in MPCs relies on BMP signaling. Co-immunohistochemistry of longitudinal sections of embryonic limbs. Forelimbs are outlined by white dotted lines. Insets (white boxes) are shown at higher magnification on the right side of the respective entire forelimb and depict individual and merged fluorescence channels. (pro: proximal; dis: distal; dor: dorsal; ven: ventral). **(a)** PAX3 (green) and HOXA11 (magenta) of control *RS6* forelimbs at E10.5. Insets depict representative *RS6* forelimb MPCs highly positive for HOXA11. White arrowhead indicates ventral lip of the dermomyotome. **(b)** PAX3 (green) and HOXA11 (magenta) of *RS6* forelimbs at E11.5. Insets depict a portion of the mid-ventral pre-muscle mass. White arrowheads indicate the few PAX3⁺ cell remaining positive for HOXA11. **(c)** PAX3 (green) and HOXA11 (magenta) of *Lbx1Cre;RS6* forelimbs at E10.5. Insets depict representative *Lbx1Cre;RS6* forelimb MPCs weakly positive for HOXA11. Scale bars = 200 μ m. *n* = 4-5 biological replicates for *RS6* limbs and *n* = 5 for *Lbx1^{Cre};RS6* limbs.

Figure 7



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