

*Inhibitory SMAD6 interferes with BMP-dependent generation of muscle progenitor cells and perturbs proximodistal pattern of murine limb muscles*

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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 premuscle 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;

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

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

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

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

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

## Results

### BMP signaling is active in limb muscle progenitors

First, we identified whether limb myogenic cells respond to BMP signaling. We monitored the nuclear accumulation of BMP-induced phosphorylated SMAD proteins using double immunofluorescence against pSMAD1/5/9 (pSMADs) and myogenic markers in mouse forelimbs at different developmental stages. In E10.5 limb buds, migrating MPCs expressed the transcription factor PAX3 (**Fig. 1**), whereas the PAX7 and MYOD transcription factors were not detected, thus reproducing previously published data (Lepper and Fan, 2010; Wood et al., 2013). Surprisingly, all PAX3<sup>+</sup> MPCs accumulated high levels of pSMADs, whereas non-myogenic mesenchymal cells showed no or, if any, very weak levels of pSMADs (**Fig. 1**). One day later, at E11.5, PAX3<sup>+</sup> MPCs rapidly lost BMP signaling responsiveness during lineage progression. Emerging MYOD<sup>+</sup> cells showed pSMADs in varying levels, some were negative and others showed a continuum from faintly to strongly positive. PAX7<sup>+</sup> cells, however, were rarely pSMAD<sup>+</sup> (**Fig. 1**). Of note, pSMAD<sup>+</sup> non-myogenic cells were also found in the progress zone of E11.5 limb buds (**Fig. 1**).

By the end of the embryonic period, at E14.5, pSMADs were enriched at the tips of the muscle fibers abutting tendons (**Fig. S1A**). Double labeling of pSMADs with either PAX7, MYOD or myosin heavy chain (MHC) antibodies showed active BMP signaling in MYOD<sup>+</sup> myonuclei at the myotendinous junctions and notably not in tendons (**Fig. S1A**). Low levels of pSMADs were also detected in rare PAX7<sup>+</sup> MPCs at the muscle tips (**Fig. S1A**). This confirms, in mouse, the presence of BMP-responsive myonuclei and MPCs at the tips of primary myofibers facing tendons, reminiscent to previous work in chick (Esteves de Lima et al., 2021).

By the end of the fetal period, at E18.5, the pSMAD expression pattern was reversed. Indeed, the tips of fetal muscle fibers were devoid of pSMADs, which had now accumulated in the nuclei of non-muscle cells at the muscle–tendon interface (**Fig. S1A**). MCT cells, labeled by the marker TCF4, were rarely pSMAD<sup>+</sup> (**Fig. S1B**). Furthermore, PAX7<sup>+</sup> and MYOD<sup>+</sup> myogenic cells occasionally accumulated pSMADs (**Fig. S1B**), consistent with the role of BMP signaling in postnatal satellite cells (Stantzou et al., 2017).



## MPCs maintain myogenic fate following abrogation of BMP signaling

We abrogated BMP signaling in limb MPCs by crossing *Lbx1<sup>Cre</sup>* mice (Sieber et al., 2007) with *Rosa26<sup>LoxP-Stop-LoxP-huSMAD6-IRES-EGFP</sup>* (*RS6*) animals (Stantzou et al., 2017). In the resulting *Lbx1<sup>Cre</sup>;RS6* embryos, activation of the *Lbx1* promoter in migrating limb MPCs induced Cre-mediated excision of the *LoxP-Stop-LoxP* cassette, leading to the expression of the inhibitory human *SMAD6* (*huSMAD6*) and *EGFP*. The *Lbx1<sup>Cre</sup>;RS6* genotype was detected at the expected frequency up to the fetal stages. However, new-born *Lbx1<sup>Cre</sup>;RS6* mice rarely survived, and the very few that did had severe growth retardation (data not shown). We validated the activation of the transgenes in *Lbx1<sup>Cre</sup>;RS6* embryos using EGFP (enhanced green fluorescent protein) as a marker of successful recombination. EGFP fluorescence was detected in cells from the proximal central field of E10.5 forelimb buds and was absent from the *RS6* controls (**Fig. 2A**). When compared to whole-mount ISH against *Lbx1*, the position of the EGFP fluorescence corresponded to that of migrating MPCs that populated the limb mesenchyme (**Fig. 2A**). In the forelimb buds of E12.5 *Lbx1<sup>Cre</sup>;RS6* embryos, EGFP was present in areas corresponding to the position of premuscle masses, as indicated by *Myod* mRNA expression (**Fig. 2A**).

As the EGFP fluorescence was quite weak after cryosectioning, we used *Ai9* mice, a Cre recombinase-dependent tandem dimer Tomato (tdTomato) reporter strain (Madisen et al., 2010), to generate *Lbx1<sup>Cre</sup>;RS6/LoxP-Stop-LoxP-tdTomato* (*Lbx1<sup>Cre</sup>;RS6/Ai9*) embryos. All tdTomato<sup>+</sup> MPCs were also positive for EGFP and for PAX3, allowing the tracing of limb MPCs, which were depleted of BMP signaling during limb mesenchyme invasion (**Fig. 2B**). We did not find any tdTomato<sup>+</sup>/EGFP<sup>+</sup> MPC accumulation in somites at limb level, nor aberrant migration into the anterior/posterior/distal limb margins (**Fig. 2B**). At E18.5, there was strong EGFP and tdTomato fluorescence in the limb muscles of *Lbx1<sup>Cre</sup>;RS6/Ai9* fetuses (**Fig. 2C**). TdTomato was present in all myofibers of E18.5 forelimbs, indicating high recombination efficiency (**Fig. 2C**). TdTomato expression was observed exclusively in developing muscles, indicating that MPCs depleted of BMP activity differentiated exclusively into muscle cells (**Fig. 2C**).

As *Lbx1<sup>Cre</sup>* represents a loss-of-function allele due to insertion of the *Cre* transgene into the *Lbx1* exon 1 coding sequence (Sieber et al., 2007), we determined whether heterozygous *Lbx1<sup>Cre</sup>* mice show signs of haploinsufficiency. Myogenic marker ISH revealed similar expression

pattern in *Lbx1* and *Pax3* at E10.5 or *Myod* at E11.5 in *Lbx1<sup>Cre</sup>* limbs compared to that in the *RS6* controls (data not shown). We also did not find any significant difference in the total number of myofibers, and total number and density of PAX7<sup>+</sup> and MYOD<sup>+</sup> cells in E18.5 *Lbx1<sup>Cre</sup>* limbs compared to *RS6* controls (data not shown). Furthermore, the *Lbx1<sup>Cre</sup>* mice had normal viability and reproduction rates. We concluded that the loss of one functional *Lbx1* allele did not cause haploinsufficiency, allowing us to use *RS6* and *Lbx1<sup>Cre</sup>* as controls for experiments with *Lbx1<sup>Cre</sup>;RS6* mutants.

In summary, these results show that following Cre-recombination, the *huSMAD6-IRES-EGFP* cassette was expressed exclusively in MPCs and their progeny in developing limbs of *Lbx1<sup>Cre</sup>;RS6* mice, allowing permanent overexpression of the BMP signaling inhibitor SMAD6 in cells of the myogenic lineage.

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

We confirmed via RT-qPCR that *huSMAD6* was upregulated (3.7-fold) in the E18.5 forelimb muscles of *Lbx1<sup>Cre</sup>;RS6* fetuses compared to that of the *RS6* controls (**Fig. 2D**). Next, we determined whether *huSMAD6* overexpression caused cell-autonomous abrogation of BMP signaling in the myogenic lineage. Indeed, we observed the absence of pSMADs in PAX3<sup>+</sup> MPCs in E10.5 *Lbx1<sup>Cre</sup>;RS6* limb buds compared to the *RS6* controls (**Fig. 3A**). In addition, the presence of pSMADs at the tips of E14.5 muscle fibers was also lost (**Fig. 3B**). Moreover, whole-mount ISH revealed that *Lbx1* and *Pax3* expression was strongly reduced in E10.5 *Lbx1<sup>Cre</sup>;RS6* limb buds compared to that in the *RS6* controls (**Fig. 3C**). Residual *Lbx1* and *Pax3* transcripts were found in the proximal part of the limb buds. Similarly, *Myod* expression was strongly reduced in E11.5 and E12.5 limb buds from *Lbx1<sup>Cre</sup>;RS6* embryos compared to that from the *RS6* controls (**Fig. 3C**). However, using ISH, we were unable to discriminate if the decreased gene expression was due to a decrease in cell number or in the transcript number per cell.

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

We transversely cryosectioned E10.5 embryos at limb level, allowing for a proximodistal sectioning plane of the developing limb bud. Double immunofluorescence for PAX3 and the

proliferation marker KI67 revealed a ~ 40% reduction of the entire PAX3<sup>+</sup> cell population and a decline in the PAX3<sup>+</sup>/KI67<sup>+</sup> subpopulation in *Lbx1<sup>Cre</sup>;RS6* embryos, suggesting reduced MPC proliferation after the inhibition of BMP signaling (**Fig. 4A–C**). The cell death marker cleaved Caspase-3 was absent in E10.5 limb mesenchyme in both genotypes, whereas it was present at trunk level and, as expected, at interdigital positions of E12.5 autopods (**Fig. S2A**). In addition, we analyzed the proximodistal distribution of the PAX3<sup>+</sup> cell population in the E10.5 limb buds and found that total cell numbers were significantly reduced in the middle and distal parts of the limb bud in *Lbx1<sup>Cre</sup>;RS6* embryos compared to that in the *RS6* controls (**Fig. 4D, E**). As total PAX3<sup>+</sup> cell number in *Lbx1<sup>Cre</sup>;RS6* limbs was lower than in *RS6* limbs, we also analyzed the normalized distribution of PAX3<sup>+</sup> cells along the proximo-distal axis. Such analysis revealed a decreased presence of normalized PAX3<sup>+</sup> cell numbers in the distal parts of the limb, whereas there was a tendency towards increased cell numbers in the proximal parts (**Fig. S2B**). Next we determined the distribution of PAX3<sup>+</sup> cell in dorsal and ventral pre-muscle masses. We found a ~ 40% reduction in cell numbers within the dorsal and ventral pre-muscle masses when comparing *Lbx1<sup>Cre</sup>;RS6* limbs with *RS6* limbs (**Fig. S2C**), which accords with the loss in total PAX3<sup>+</sup> cell number in *Lbx1<sup>Cre</sup>;RS6* limbs (**compare with Fig. 4B**). Cell numbers, however, were similar when comparing dorsal and ventral muscle masses of the same genotype (**Fig. S2C**). Together, these data suggest that the lack of BMP signaling in MPCs attenuated their proliferation and distal migration, and data argue against a loss of MPCs by apoptosis or a rerouting of migration.

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

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

We found a precocious conversion of PAX3<sup>+</sup> cells towards PAX7<sup>+</sup> and MYOD<sup>+</sup> cells in E11.5 *Lbx1<sup>Cre</sup>;RS6* limbs: the total number of PAX3<sup>+</sup> cells decreased by 85%, whereas the total number of PAX7<sup>+</sup> cells increased by 64% and the MYOD<sup>+</sup> cells by 46% (**Fig. 5A-E**), thus the total PAX3/PAX7/MYOD population remained stable. In addition, the PAX3<sup>-</sup>/PAX7<sup>+</sup> and PAX3<sup>-</sup>/MYOD<sup>+</sup> cell population ratios increased by 68% and 61%, respectively, compared to that in the *RS6* controls (**Fig. 5F, G**). These results suggest accelerated myogenic lineage progression in *Lbx1<sup>Cre</sup>;RS6* MPCs due to the absence of BMP signaling, which is similar to that shown in embryonic chicken limbs (Amthor et al., 1998).

In E12.5 *Lbx1<sup>Cre</sup>;RS6* limbs, the accelerated lineage progression was associated with a loss in total number of MYOD<sup>+</sup> cells (42%) and PAX7<sup>+</sup> cells (47%) (**Fig. S3A-D**). Furthermore, we detected a decline in PAX7<sup>+</sup>/KI67<sup>+</sup> and PAX7<sup>+</sup>/MYOD<sup>+</sup> cell populations, whereas the proportion of MYOD<sup>+</sup>/MYOG<sup>+</sup> cells increased, confirming the shift of myogenic lineage progression towards differentiating myoblasts at the expense of proliferating precursors (**Fig. S3B, E-G**).

### **Abrogation of BMP signaling in MPCs disturbs *Lbx1<sup>Cre</sup>;RS6* limb proximodistal muscle patterning**

We analyzed the consequences of decreased MPC generation on primary myofiber formation after abrogation of BMP signaling by visualizing myofibers on transverse sections at the end of the embryonic period (E14.5) of mouse forelimb development. The zeugopod muscles of *Lbx1<sup>Cre</sup>;RS6* embryos were significantly smaller and contained about half the number of primary myofibers compared to that in the *RS6* controls (**Fig. 6**). At this stage, we observed defective muscle patterning in the *Lbx1<sup>Cre</sup>;RS6* embryos. Whereas muscle pattern was normal at stylopod level, certain zeugopod muscles were either completely absent (*supinator*, *extensor pollicis*, *flexor digitorum superficialis*) or fused (*extensor carpi radialis longus* and *brevis*), whereas the remaining zeugopod muscles were remarkably hypoplastic (**Fig. 6**). At the autopod level, only a few remnant MHC-expressing cells were observed, while autopod muscles were entirely absent (**Fig. 6**). The anatomical changes in muscle pattern seen at the end-embryonic stage (E14.5) persisted during the fetal stage (**Fig. 7**).

## Normal muscle patterning following abrogation of BMP signaling in differentiated muscle

We wanted to determine whether defective muscle patterning was also caused by the abrogation of BMP signaling in differentiated muscle cells. We used *HSA-Cre* driver mice to conditionally direct recombination in differentiated muscle cells. We first performed a time course to determine the spatiotemporal occurrence of HSA-Cre-driven recombination in *HSA-Cre;Ai9* crosses by following the onset of tdTomato expression. In E10.5 and E11.5 embryos, tdTomato was found in somites but not in limb buds. TdTomato was present in developing limb muscles from E12.5 onwards, which is consistent with the emergence of primary myofibers at this stage (**Fig. S4**). We then generated *HSA-Cre;RS6* mice to overexpress SMAD6 exclusively in terminally differentiated muscles; a mouse model we have validated previously (Stantzou et al., 2017). The forelimbs of E18.5 *HSA-Cre;RS6* fetuses developed normally, and no change was detected in the muscle pattern (**Fig. 7**). These results may indicate that the information for the future muscle pattern is already present in MPCs before their differentiation. An alternative explanation may be that sufficient MPCs reached their destination (as migration and/or proliferation were not affected), allowing them to be exposed to patterning cues. Of note, MCT did not increase at the expense of skeletal muscle, as the pattern of collagen 12 expression in the *HSA-Cre;RS6* fetuses was similar to that of the controls despite the smaller muscles (**Fig. 7**).

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

The observed changes in the muscle pattern of *Lbx1<sup>Cre</sup>;RS6* mutants (**Fig. 7**) resembled those previously observed in *Hoxa11/d11* double mutants (Swinehart et al., 2013), raising the question of the intrinsic positional information of myogenic cells and putative regulation by BMP signaling.

Indeed, at E10.5, PAX3<sup>+</sup> MPCs, which had left the dermomyotome and migrated into the limb bud, expressed HOXA11 protein. Notably, HOXA11 levels in the MPCs were higher than in the surrounding limb mesenchymal cells (**Fig. 8A**). As early as one day later, at E11.5, most PAX3<sup>+</sup> cells had lost the high HOXA11 protein levels (**Fig. 8B**). In the absence of BMP signaling in

*Lbx1<sup>Cre</sup>;RS6* embryonic limbs, the MPCs failed to accumulate high levels of HOXA11 protein (**Fig. 8C compared with 8A, S5A**).

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

In chicken forelimb buds, scRNAseq showed the expression of genes of the *HOXA* and *HOXD* clusters in mesenchymal cells and in muscle cluster cells (**Fig. S5B-H and S6**). As an example, *HOXA11* transcripts were detected in the majority (69%) of muscle cluster cells in E4 limbs (E10.5 mouse stage equivalence) and more rarely (37% of cells) by E6 (E12.5 mouse stage equivalence). In contrast, *HOXD13* transcripts were not detected before E6, where its expression was limited to a few muscle cluster cells (**Fig. S5B**). *HOXA11* was expressed at all successive steps of the myogenic process: first in *PAX7<sup>+</sup>* and *MYOD<sup>+</sup>* MPCs at E4 and E6, and then in *MYOG<sup>+</sup>* myoblasts at E6 (**Fig. S5C-G**). There was nonetheless a drop in *HOXA11* expression during myogenic lineage progression, given that at E6, 39% of *PAX7<sup>+</sup>* muscle cluster cells co-expressed *HOXA11*, while only 15% of *MYOG<sup>+</sup>* cells co-expressed *HOXA11* (**Fig. S5H**). Interestingly, we found a heterogeneous combinatorial expression of *HOXA* genes in single muscle cluster cells, suggesting *HOX*-dependent positional information in chicken limb MPCs (**Fig. S6A-B**).

We next analyzed whether BMP response correlates with *HOXA* gene expression in muscle cluster cells and found that *HOXA<sup>+</sup>* cells (expressing one or several genes of the *HOXA* cluster) expressed BMP downstream effectors genes *ID2* and *ID3*, but not *ID1*, in higher proportion compared to *HOXA<sup>-</sup>* cells (**Fig. S6C-E**). Consistently, the BMP score, which is the corrected average expression of the *ID1*, *ID2* and *ID3* genes, was significantly higher in *HOXA<sup>+</sup>* cells compared to *HOXA<sup>-</sup>* cells (**Fig. S6F**).

scRNAseq of E12.5 mouse forelimbs showed very similar results compared to those in chicken: *i*) Muscle cluster cells expressed genes of the *Hoxa* and *Hoxd* cluster (**Fig. S7A**); *ii*) a subset of *Hoxa11<sup>+</sup>* muscle cluster cells co-expressed *Pax3*, *Pax7*, *Myf5* and *Myod*, but only rarely *Myog* (**Fig. S8**); *iii*) muscle cluster cells showed large heterogeneity in the expression of genes

263 of the *Hoxa* cluster (**Fig. S7B**); and iv) there was *Id1*, *Id2* and *Id3* expression in a higher proportion  
264 of *Hoxa*<sup>+</sup> cells than *Hoxa*<sup>-</sup> cells (**Fig. S7C-E**).

## Discussion

The current paradigm of limb muscle patterning considers limb MPCs as naïve, where they develop individual muscles by invading a prepattern established by MCT (Kardon et al., 2003). Our results contribute to this concept by showing that BMP signaling (produced by limb connective tissue cells surrounding developing muscles [Amthor et al., 1998; Esteves de Lima et al., 2021]) is necessary for the generation of MPCs responsive to BMP, thereby establishing the necessary cellular source for limb muscle pattern. We used overexpression of *SMAD6* as an experimental tool to abrogate BMP signaling, which caused precocious loss of PAX3 in MPCs and accelerated myogenic lineage progression. MPCs advanced less distally, as expected, since PAX3 is a prerequisite for myogenic migration (Bober et al., 1994), likely causing a mismatch between their distal progression and the local connective tissue, and thus responsible for the observed defects in proximodistal muscle pattern.

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

We demonstrated that limb MPCs expressed *Hox* genes in mouse as well as in chicken embryos. scRNAseq revealed: *i*) a high proportion of *Hox*-expressing MPCs in early limb buds; *ii*) heterogeneity of *Hox* gene expression in the MPCs; *iii*) their sequential upregulation; and *iv*) their downregulation during myogenic lineage progression. Immunohistochemistry confirmed the transcriptome data: upon leaving the dermomyotome and entering limb bud mesenchyme, PAX3<sup>+</sup> migrating limb MPCs produced high HOXA11 protein levels, and this was dependent on BMP signaling. Interestingly, the *Lbx1<sup>Cre</sup>;RS6* mutants resembled the muscle pattern defect observed in *Hoxa11<sup>-/-</sup>/d11<sup>-/-</sup>* dKO mutants (Swinehart et al., 2013). Swinehart et al. showed that *Hoxa11* was not expressed by differentiated muscle cells at E14.5, but in cells surrounding primary muscle fibers, such as TCF4<sup>+</sup> connective tissue cells. Whether HOXA11 colocalizes with



MPCs (which also surround primary muscle fibers), however, was not investigated (Swinehart et al., 2013). It has also been shown that *Hoxa13*<sup>-/-</sup> KO and *Hoxa13*<sup>-/-</sup>/*d13*<sup>-/-</sup> dKO disturb autopod development (Fromental-Ramain et al., 1996). We here found both, *Hoxa13* and *Hoxd13*, being expressed by MPCs. However, here we examined scRNAseq data sets from whole limb buds, which did not allow us to specify which MPC subpopulation (e.g. autopod MPCs) expressed which HOX code. Thus, it remains to be determined whether *Hox* gene expression in MPC follows the collinearity in the developing limb. We can therefore only speculate about the exact role of *Hox* genes in developing muscle, and how their expression relates to BMP signaling. Previous work on chick limb MPCs showed that *Hoxa11* and *Hoxd13* blocked expression of *MyoD*, and that *Hox* gain-of-function experiments resulted in distorted limb muscle patterning (Yamamoto and Kuroiwa, 2003). Many questions, however, remain unresolved: does the HOX code control MPC proliferation, myogenic lineage progression and muscle splitting? Do MPCs, through HOX code, acquire positional identity and establish a muscle pre-pattern? Alternatively, herein observed loss in specific muscles may simply result from a tissue default that is caused by insufficiently generated precursors.

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

The majority of MPCs is derived from migratory and *Pax-3* dependent MPCs of somite origin. However, recent work demonstrated a dual origin of MPCs in the developing limb: a small population of MCT cells are integrated into myotubes at muscle tips close to tendons in chicken and mouse muscles (Esteves de Lima et al., 2021, Yaseen et al., 2021), a process being promoted by BMP signaling (Esteves de Lima et al., 2021). BMP gain- and loss-of-function experiments in chicken embryos demonstrated that BMP signaling balances the fibroblast-myoblast conversion and consequently the muscle pattern (Esteves de Lima et al., 2021). We show here, that BMP signaling also regulates the somite-derived *Pax-3* dependent MPC lineage in mouse limbs. Cell-autonomous inhibition of BMP signaling in somite-derived MPCs caused absence of entire muscles. Therefore, MCT depends on the presence of somite-derived MPCs and are lost secondarily when muscle fails to develop. Further, the generation of the somite-independent muscle lineage depends on the presence of somite-derived muscle.

We found that the patterning defect in *Lbx1<sup>Cre</sup>;RS6* limbs persisted from embryonic to fetal stages, showing i) that secondary myogenesis cannot compensate for embryonic muscle defects, and ii) remaining muscles continue to grow despite persistent inhibition of BMP signaling.

We would like to emphasize that our results support the MCT prepatterning model (see model in **Fig. S9**). We believe that mesenchymal cells that form future MCT are the source of cues, including BMPs, that inform MPCs of where to migrate and proliferate. MCT and MPCs could be mutually dependent on each other to establish the muscle pattern. Indeed, a defined MCT pattern resembling a muscle pattern failed to develop in muscle-devoid limbs (Christ et al., 1977). Whereas tendons initially developed autonomously in lack of muscles; they degenerated secondarily (Christ et al., 1977; Schweitzer et al., 2010).

We here employed the overexpression of *SMAD6* as a mean to test the cell-autonomous effect of abrogating BMP signaling. *SMAD6* inhibits Smad signaling by the BMP type I receptors ALK-3/6 subgroup and only weakly inhibits TGF- $\beta$ /activin signaling via the BMP type I receptors ALK-1/2 subgroup, the latter being a preferential target of *SMAD7* (Goto et al., 2007; Miyazawa and Miyazono, 2017). Since *SMAD6* is not a direct component of the BMP signaling cascade, further work is required to substantiate our results, such as performing specific BMP receptor

351 knockout. In previous work, we showed that satellite cell-specific overexpression of *SMAD6* or  
352 knockout of *Alk3*, or overexpression of the BMP antagonist *Nog* in postnatal mice decreased  
353 proliferation of satellite cells, diminished their accretion during myofiber growth and retarded  
354 muscle growth, whereas overexpression of *SMAD6* exclusively in terminally differentiated  
355 myofibers did not affect satellite cell dependent muscle growth (Stantzou et al., 2017). Together  
356 with herein presented results, this confirms that BMP signaling acts in a similar cell-autonomous  
357 manner in MPCs during prenatal and postnatal development.

358         In conclusion, our data suggest that BMP signaling controls embryonic limb MPCs to  
359 maintain PAX3-expressing precursor status, coordinates MPC migration, proliferation and  
360 myogenic lineage progression, thereby providing the cellular source that is required for building  
361 the correct muscle pattern. The expression of the HOX code in MPCs may indicate that  
362 positional identity is established prior to the splitting of premuscle masses into individual  
363 muscles. Future loss- and gain-of-function experiments are required to directly test the function  
364 of *Hox* gene expression in MPCs.

## Materials and methods

### Mouse lines used for embryo generation

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

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

*Lbx1<sup>Cre</sup>*, *HSA-Cre* and *RS6* mice were interbred to obtain *Lbx1<sup>Cre</sup>;RS6* and *HSA-Cre;RS6* embryos. *Lbx1<sup>Cre</sup>* and *Ai9* mice were interbred to obtain heterozygous *Lbx1<sup>Cre</sup>;Ai9* mice, which were crossed with *RS6* mice to obtain *Lbx1<sup>Cre</sup>;RS6/Ai9* embryos.

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

### Embryonic and fetal forelimb collection and processing

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

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

### RNA isolation and RT-qPCR

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

### Whole-mount ISH

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

### Immunofluorescence staining

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

Immunofluorescence staining was performed using the following protocols: *i*) 5-minute rehydration of slides in PBS; *ii*) permeabilization with 0.1% (E10.5–E12.5) or 0.5% (E14.5 and E18.5) Triton X-100 (Sigma-Aldrich) (in the case of nuclear protein staining, e.g. PAX3, PAX7, MYOD, Myogenin (MYOG), pSMAD1/5/9, HOXA11, KI67, Caspase-3) or with methanol at -20°C (for non-nuclear proteins, e.g. MHC, laminin alpha-2, DsRed, collagen 12); *iii*) three 5-minute washes in PBS; *iv*) antigen retrieval by 20-minute immersion of the slides in boiled 10 mM citric

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

### **Imaging**

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

### **Morphometric studies**

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

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

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

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

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

#### **scRNAseq analysis of whole limb cells**

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

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

#### **Data availability**

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

#### **Statistical analyses**

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



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## Declaration of interests

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

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## Author Contribution

Conceptualization: H. Amthor, A.S., F.R., K.P., S. Stricker, D.D., G.A.; Methodology: H. Amthor, T.B., H. Asfour, A.S., S.H., F.Z., S. Swist, D.D., E.H., R.R., G.A.; Validation: H. Amthor, A.S., H. Asfour, D.D., E.H., R.R., G.A.; Formal analysis: H. Amthor, H. Asfour, A.S., D.D., E.H., R.R., G.A.; Investigations: H. Amthor, H. Asfour, A.S., F.Z., S.H., D.D., E.H., R.R., G.A.; Resources: H. Amthor, F.R., S. Swist, T.B., A.S., H. Asfour, D.D., E.H., R.R., G.A.; Writing-original draft preparation: H. Amthor, A.S., H. Asfour; Writing-review and editing: H. Amthor, A.S., H. Asfour, F.Z., S. Stricker, S. Swist, S.H., T.B., K.P., F.R., D.D., E.H., R.R., G.A.; Visualisation: H. Amthor, A.S., H. Asfour, R.R.; Supervision: H. Amthor, A.S., F.R., D.D., G.A.; Project administration: H. Amthor, A.S., H. Asfour; Funding acquisition: H. Amthor, H. Asfour, F.R., G.A..

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## Figure 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<sup>+</sup> cells positive for pSMAD1/5/9. MyoD<sup>+</sup> cells negative for pSMAD1/5/9 are depicted with white arrows, faintly positive ones with orange arrows, strongly positive ones with light blue arrows. Yellow arrowheads depicts pSMAD1/5/9 expression in the progress zone of E11.5 limb buds. *n* = 5 biological replicates for each immunostaining and embryonic stage. Scale bars = 200  $\mu$ m.

**Figure 2. Fate mapping of limb MPCs. (A)** Images depict the dorsal view of forelimbs (outlined by dotted lines), where there is native EGFP fluorescence (green) in recombined cells from E10.5 and E12.5 forelimbs from *Lbx1<sup>Cre</sup>;RS6* mice as compared to the position of pre-muscle masses as revealed by *Lbx1* and *Myod* transcripts (purple) following whole-mount ISH. *n* = 5 biological replicates for each condition. Scale bar = 500  $\mu$ m. **(B)** Dorsal view of an E10.5 limb bud of *Lbx1<sup>Cre</sup>;RS6/Ai9* embryos depicts native fluorescence of EGFP (green) and tdTomato (red) at low magnification (left column) and at higher magnification (middle column). The right column depicts co-immunostaining for PAX3 (green) and DsRed (red) on cryosections of E10.5 *Lbx1<sup>Cre</sup>;RS6/Ai9* forelimbs. Nuclei are stained with DAPI (blue). *n* = 5 biological replicates for each condition and genotype. Scale bar = 500  $\mu$ m. **(C)** Left images depict dorsal view of an E18.5 forelimb of an *Lbx1<sup>Cre</sup>;RS6/Ai9* fetus showing native fluorescence of EGFP (green) and tdTomato (red) in the limb muscles. The right images depict co-immunostaining for MHC (magenta), collagen type 12 (white) and tdTomato (red) in transverse sections of forelimbs at zeugopod level. *n* = 5 biological replicates for each condition/immunostaining. Scale bars = 500  $\mu$ m. **(D)** Dot-plotted bar graph shows the relative quantified mRNA expression of *huSMAD6* per 1 million

*Gapdh* mRNA in E18.5 forelimb muscles in *RS6* and *Lbx1<sup>Cre</sup>;RS6* fetuses using RT-qPCR. *n* = 5 biological replicates. Data are the mean  $\pm$  SD.

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**Figure 3. 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<sup>Cre</sup>;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<sup>Cre</sup>;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  $\mu$ m. **(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<sup>Cre</sup>;RS6* embryos compared to *RS6* controls. Images show dorsal view of the forelimbs (outlined by grey dotted line) (pro: proximal; dis: distal).

**Figure 4. 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<sup>Cre</sup>;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  $\mu$ m. **(B)** Dot-plotted bar graph shows the number of PAX3<sup>+</sup> cells in the forelimbs of both



genotypes. The number of cells was determined as average from three consecutive longitudinal sections. **(C)** Stacked bar graph depicts the percentages of PAX3<sup>+</sup>/KI67<sup>+</sup> (orange) ( $p = 0.0079$ ) and PAX3<sup>+</sup>/KI67<sup>-</sup> (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<sup>Cre</sup>;RS6* embryos. The limb was divided into 10 equal zones along the proximodistal axis. Scale bar = 200  $\mu$ m. **(E)** Histogram depicts the number of PAX3<sup>+</sup> 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 5. 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<sup>Cre</sup>;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  $\mu$ 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<sup>+</sup>, PAX7<sup>+</sup> and MYOD<sup>+</sup> cells. **(F)** Stacked bar graph depicts the percentages of PAX3<sup>+</sup>/PAX7<sup>-</sup> cells (green) ( $p = 0.0159$ ), PAX3<sup>+</sup>/PAX7<sup>+</sup> cells (orange) ( $p = 0.1905$ ) and PAX3<sup>-</sup>/PAX7<sup>+</sup> cells (red) ( $p = 0.0159$ ). **(G)** Stacked bar graph depicts the percentages of PAX3<sup>+</sup>/MYOD<sup>-</sup> cells (green) ( $p = 0.0159$ ), PAX3<sup>+</sup>/MYOD<sup>+</sup> cells (orange) ( $p = 0.0159$ ) and PAX3<sup>-</sup>/MYOD<sup>+</sup> cells (red) ( $p = 0.0159$ ) cells.  $n = 4$  biological replicates for *RS6* and  $n = 5$  for *Lbx1<sup>Cre</sup>;RS6*. Each replicate represents the mean of three consecutive serial sections. Data are the mean  $\pm$  SD.

**Figure 6. Effect of *SMAD6* overexpression on embryonic muscle pattern.** 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<sup>Cre</sup>;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<sup>Cre</sup>;RS6* forelimb autopod. Muscles, which are numbered in yellow in *RS6* embryos, are absent from the *Lbx1<sup>Cre</sup>;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: *hypothennars*; 29: *lumbricals*; 30: *interossei*. Scale bar = 200  $\mu$ m. *n* = 5 biological replicates.

**Figure 7. 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<sup>Cre</sup>;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<sup>Cre</sup>;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: *hypothennars*; 29: *lumbricals*; 30: *interossei*. Scale bar = 500  $\mu$ m. *n* = 5 biological replicates.

**Figure 8. *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<sup>+</sup> 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  $\mu$ m. *n* = 4-5 biological replicates for *RS6* limbs and *n* = 5 for *Lbx1<sup>Cre</sup>;RS6* limbs.