

Cell lineage specification and signalling pathway usage during development of the lateral plate mesoderm and forelimb mesenchyme

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Summary Statement

This study establishes a transcriptional atlas of the cell fate decisions underlying LPM specification, subdivision and development of the chicken forelimbs.

Abstract

The lateral plate mesoderm (LPM) is a transient tissue that produces a diverse range of differentiated structures, including the limbs. However, the molecular mechanisms that drive early LPM specification and development are poorly understood. In this study, we utilize single-cell transcriptomics to define the cell-fate decisions directing LPM specification, subdivision, and early initiation of the forelimb mesenchyme in chicken embryos. We establish a transcriptional atlas and global cell-cell signalling interactions in progenitor, transitional and mature cell types throughout the developing forelimb field. During LPM subdivision, somatic and splanchnic LPM fate is achieved through activation of lineage-

specific gene modules. During the earliest stages of limb initiation, we identify activation of *TWIST1* in the somatic LPM as a putative driver of limb bud EMT. Furthermore, we define a new role for BMP signalling during early limb development, revealing that it is necessary for inducing a somatic LPM fate and initiation of limb outgrowth, potentially through activation of *TBX5*. Together, these findings provide new insights into the mechanisms underlying LPM development, somatic LPM fate choice, and early initiation of the vertebrate limb.

Introduction

The lateral plate mesoderm (LPM) is a transient tissue that forms from the mesodermal germ layer during vertebrate embryogenesis. The LPM produces a remarkable diversity of cell and organ types during development, including the smooth muscle and connective tissues of the cardiovascular, respiratory and digestive system and endoskeleton of the limbs (Nishimoto and Logan, 2016; Prummel et al., 2019; Prummel et al., 2020; Selleck and Stern, 1991). LPM specification and limb morphogenesis are complex processes underpinned by coordinated signalling events and gene expression dynamics across development (Loh et al., 2016). The primitive mesoderm elongates along the anteroposterior (A-P) body axis, and undergoes mediolateral specification to form axial (notochord), paraxial (somite), intermediate and lateral plate mesoderm domains, through specific combinations of BMP, FGF and WNT signals (Loh et al., 2016; Tonegawa et al., 1997). The LPM forms as bilateral sheets, comprised of anterior (aLPM) and posterior (pLPM) domains, which are spatially defined by FGF8 and retinoic acid signalling boundaries (Cunningham et al., 2013; Zhao et al., 2009). LPM identity is further established and maintained by localized BMP4 signalling and WNT antagonism (Tonegawa and Takahashi, 1998; Tonegawa et al., 1997; Yoshino et al., 2016).

Cells of the undifferentiated LPM undergo further dorsoventral subdivision to form the somatic and splanchnic LPM layers, separated by the embryonic coelom. The somatic LPM gives rise to tissues which form the body wall, amnion, and limbs, while the splanchnic LPM forms the viscera and connective tissue lining the digestive system. Differentiation of these diverse tissues is accompanied by activation of key transcription factors during fate specification (Agarwal et al., 2003; Firulli et al., 1998; Harvey et al., 2002; Mahlapuu et al., 2001; Martin et al., 1995; Rallis et al., 2003). However, despite this understanding of LPM differentiation and tissue diversification, little is known about the mechanisms that drive

LPM subdivision and specification towards a somatic and splanchnic LPM fate (reviewed by (Prummel et al., 2020)).

Initial specification of the LPM is accompanied by activation of transcription factors *FOXF1*, *HAND1*, *OSR1* and *PRRX1* (Kuratani et al., 1994; Loh et al., 2016; Martin et al., 1995; Ormestad et al., 2004; Peterson et al., 1997). LPM subdivision is thought to occur through BMP signalling from the overlying ectoderm, through activation of somatic LPM genes (Funayama et al., 1999). In the presence of ectodermal BMPs, *PRRX1* becomes restricted to, and *IRX3* is activated in the somatic LPM (Funayama et al., 1999; Mahlapuu et al., 2001; Ocaña et al., 2012). *OSR1* becomes restricted to the intermediate mesoderm, while *HAND1* and *FOXF1* are maintained in the splanchnic LPM (Funayama et al., 1999; Mahlapuu et al., 2001). However, the mechanisms which drive somatic LPM development remain unresolved. *Prrx1*, *Irx3* and *Osr1* are dispensable for somatic LPM formation, as mouse null mutants for each of these genes are viable and do not possess aberrant LPM phenotypes (Li et al., 2014; Martin et al., 1995; Wang et al., 2005). Conversely, *Foxf1* null mutants are embryonic lethal with partial to incomplete subdivision of the LPM, misexpression of somatic LPM genes in splanchnic LPM, and gut patterning defects (Firulli et al., 1998; Mahlapuu et al., 2001). This suggests that *FOXF1* plays a role during LPM subdivision, acting to antagonize expression of somatic LPM genes which are activated by ectodermal BMP signals. However, the mechanisms responsible for establishing somatic LPM identity remain undefined and are essential to our understanding of LPM biology and early embryogenesis.

Once formed, the somatic LPM is specified into limb forming domains through retinoic acid (RA) signalling and *HOX* gene expression boundaries (Nishimoto et al., 2015; Tanaka, 2016). RA, *HOX* and β -catenin/TCF/LEF are thought to initiate limb development through activation of the T-box transcription factor *TBX5* (Agarwal et al., 2003; Logan et al., 1998; Minguillon et al., 2012; Nishimoto et al., 2015; Rallis et al., 2003). Limb outgrowth begins with *TBX5*-induced activation of *FGF10*, which together are proposed to initiate a localized epithelial to mesenchymal transition (EMT) of the somatic LPM (Gros and Tabin, 2014). *FGF10* activates *FGF8* in the overlying ectoderm, establishes a positive feedback loop, and drives proliferation of the limb bud mesenchyme and limb outgrowth (Moon and Capecchi, 2000; Nishimoto et al., 2015; Ohuchi et al., 1997). Limb patterning sees further activation of networks of transcriptional regulators and morphogens to promote outgrowth maintenance and patterning, which are well characterized (for comprehensive reviews see Tickle, 2015;

Zuniga, 2015). Yet, despite these known events, those preceding the *TBX5*-dependant limb regulatory pathway in the somatic LPM are less well understood.

In this study, we resolve the ambiguity underlying early LPM differentiation, subdivision, and limb initiation in the developing chicken forelimb field, using single-cell transcriptomics. We construct a high-resolution gene expression atlas across the developing limb field, corroborating known, and identifying previously uncharacterized tissue-specific marker genes, signalling pathways and ligand-receptor pairs active during LPM differentiation and early limb initiation. We address a long-standing gap in the literature, defining the transcriptional networks underlying subdivision and specification of the somatic and splanchnic LPM. Furthermore, we identify the sequential activation of transcription factors during limb initiation, including early activation of *TWIST1* and other genes, prior to *TBX5* and the FGF10-FGF8 feedback loop. Interrogation of *TWIST1* expression and localization in the early somatic LPM reveals a putative role during EMT of the somatic LPM. Finally, we reveal that BMP signalling is not sufficient for LPM subdivision, as previously suggested (Funayama et al., 1999), but is necessary for determining somatic LPM fate and limb initiation through activation of *TBX5* and *FGF10*. Together, these findings provide a robust overview of the developmental landscape underlying formation and specification of the LPM, representing one of the earliest events of vertebrate organogenesis.

Results

Transcriptional clustering of cell populations in the presumptive chicken forelimb field

We performed single-cell RNA sequencing of embryonic chicken forelimb fields and reconstructed the underlying cell populations. Briefly, tissues corresponding to the presumptive forelimb field, i.e. lateral to somites 20-25, were dissected from embryonic day (E) 1.5, 2.5 and 3.5 embryos, corresponding to approximate Hamburger-Hamilton stage (HH) 10, 14 and 18, respectively (Hamburger and Hamilton, 1951) (Fig. 1a). These stages cover emergence of the early LPM, subdivision and specification of the LPM into the somatic and splanchnic layers, and limb initiation and early outgrowth (Newton and Smith, 2020). Dissected tissues were dissociated to single cells, FACS-sorted to remove dead and dying cells, then processed through the 10x Chromium system. After quality filtering, a total of 15355 cells, corresponding to 5273 cells from E1.5, 6856 from E2.5 and 3226 from E3.5 embryos were recovered, with expression profiles for 16779 genes. To remove low read

count or low diversity cells, we applied an additional strict filtering threshold of 2000 UMI counts per cell, yielding a total of 3262 cells, with 1210 from E1.5, 1313 from E2.5 and 739 from E3.5 embryos. Cell transcriptomic relationships were visualized with global t-distributed Stochastic Neighbour Embedding (tSNE) dimension reduction, which showed a distinct separation of cell types according to germ layer, cell cycle phase and embryonic stage (Fig. 1b, c).

Unsupervised clustering of the chicken E1.5, E2.5 and E3.5 presumptive forelimb cell populations revealed 13 transcriptionally distinct cell clusters (c) which represented embryonic vasculature and tissues derived from the ectoderm, mesoderm, and endoderm germ layers, broadly covering known cell types within the presumptive forelimb field (Fig. 1c-d). Tissue and cell type identities were assigned to each cluster based on their differential gene expression profiles (observed as differences in log-fold change between clusters; Fig. 1e-f) and corresponding spatiotemporal expression patterns observed throughout the developing chicken embryo on the GEISHA chicken gene expression database (Bell et al., 2004; Darnell et al., 2007). Cluster-specific gene expression is shown in Fig. 1e-f and Table S1. The embryonic ectoderm comprised two clusters (c2 and c11) defined by expression of *FABP3* and *WNT6*. However, c11 showed expression of *FGF8*, revealing these cells as progenitors that contribute to formation of the Apical Ectodermal Ridge (AER). The embryonic endoderm (c7) was defined by unique expression of *GUCA2B*, *TTR*, and *SHH* (not observed in limb bud mesenchyme due to the early stages sampled). Embryonic vasculature (c8) showed unique expression of *CDH5*, and red blood cells (c13) expressed haemoglobin subunit *HBBA*.

The embryonic mesoderm was found to contribute the largest overall number of cells and was largely defined by expression of the established marker *PRRX1*. The mesoderm was composed of six clusters (c1, c3, c4, c5, c10 and c12) which represented known mesodermal tissue-types during development, separated by embryonic stage (Fig. 1b, c). The earliest identified cell type was that of the primitive mesoderm / LPM (c4), which was largely made up of E1.5 cells with expression of primitive streak and early mesodermal markers *MSGN1*, *EVX1* and *CDX4* (Alev et al., 2010). Cells of the E2.5 mesoderm largely represented more distinct tissues, including the somatic LPM (c3) – high *PRRX1* and low *TBX5*; splanchnic LPM (c5) – *COLEC10*; and paraxial mesoderm (c1) – *TCF15* (Fig. 1d, e). Finally, E3.5 cell clusters represented more differentiated tissues, such as the extraembryonic LPM / amnion (c12) – *AQP1*, and limb bud mesenchyme (c10) – high *TBX5* and *FGF10*. We also detected

two clusters (c6 and c9) which possessed ubiquitous expression of ectodermal and mesodermal markers, but also high ribosomal and mitochondrial counts. These were defined as low diversity cells which were excluded from subsequent analyses. Together, these clusters represent all of the known, major tissues types within the developing forelimb field, which for the first time, now possess detailed transcriptional profiles (Table S1).

Global ligand-receptor signalling throughout the differentiating mesoderm

Specification of the mesoderm during embryogenesis is influenced by dynamic intrinsic and extrinsic signalling between the surrounding germ layers and developing tissues. Particularly, members of the BMP, FGF, HH and WNT signalling pathways are known to communicate between the germ layers to induce differentiation and development (Loh et al., 2016). However, the precise ligands and receptors that facilitate different aspects of mesoderm development are unclear. We therefore examined global signalling patterns and ligand-receptor crosstalk between the global cell types during specification of the forelimb field from the LPM using CellChat (Jin et al., 2021). This analysis revealed extensive signalling pathway usage between different cell types and tissues (Fig. 2a), which dynamically changed during lineage allocation of key tissue types. The E1.5 primitive MES / LPM (c4) showed the highest level of signalling pathway activity among the tissues studied, with active signalling through more than half of the predicted pathways. This included signalling through the important non-canonical WNT, FGF, HH and BMP pathways, as well as presumptive signalling through Midkine (MK) and pleiotrophin (PTN), EphrinB, Semaphorin (SEMA3,6), chemokine ligand (CXCL) and adhesion factors laminin, JAM and NECTIN.

Pathway usage was observed to significantly change during subdivision of the LPM into somatic and splanchnic LPM by E2.5, including decreases in ncWNT, FGF, CXCL and Semaphorin signalling. Splanchnic LPM (c5) formation involved HH, AGRN, FN1, CD99 and Ephrin signalling and decreased BMP signalling. In contrast, formation of the somatic LPM (c3) was characterised by decreased HH, maintained BMP, and activated collagen and NECTIN signalling. Finally, development of the limb bud mesenchyme (c10) by E3.5 was characterised by specific activation of WNT and FGF signalling, confirming known interactions, as well as strong activation of Ephrin A and ANGPTL. While these observations indicate that differentiation of multipotent LPM progenitors involves diverse signalling

crosstalk (Fig. 2a), we focused on TGF- β , WNT, BMP, FGF and HH, given their known roles influencing LPM specification, subdivision, and limb development (Loh et al., 2016).

To begin to define these complex signalling relationships, we interrogated TGF- β , WNT, BMP, FGF and HH signalling pathways active within sender and receiver cell-types, allowing construction of dynamic signalling networks (Fig. 2b). TGF- β signalling was responsible for development of the embryonic vasculature, with ligands only being received by blood vessels (c8). Early LPM development was observed to occur through BMP and HH signalling. BMPs were sent by the LPM (c4) and ectoderm, and received by the early LPM (c4) and somatic LPM / limb bud (c10), recapitulating known local and ectodermal signalling (Funayama et al., 1999; Tonegawa et al., 1997). Interestingly, the endoderm was identified as a significant sender of HH signals, being received by the early LPM, splanchnic LPM and paraxial mesoderm, affirming its known role during gut formation from splanchnic LPM (Roberts et al., 1995; Yoshino et al., 2016). During later limb initiation, the somatic LPM (c3) and limb mesenchyme (c10) were strong senders of WNT signals, received by the E3.5 limb bud mesenchyme (c10) and amnion (c12) (Takeuchi et al., 2003). The limb mesenchyme (c10) was further identified as a sender of FGF signalling, received by the AER ectoderm (c11), confirming the known role of secreted FGF10 signalling during limb initiation (Ohuchi et al., 1997). However, we did not identify the AER ectoderm (c11) as a sender of FGF signals, nor *FGF8* expression in the c11 AER cluster (Table S1), suggesting our sampling of AER was prior to establishment of the FGF10-FGF8 feedback loop.

To further define the influence of these signalling pathways, we next examined the underlying BMP, FGF, HH and WNT ligand-receptor pairs facilitating these dynamic events. This revealed disparate patterns of ligand and receptor usage amongst the different cell populations, which are detailed in Fig. 2c. During LPM subdivision, endoderm-mesoderm HH signalling occurred exclusively through *SHH* activation of *PTCH1*. Ectoderm-mesoderm BMP signalling is achieved through *BMP2* and *BMP7*, as previously described (Funayama et al., 1999), in combination with *ACVRI* and *BMPR2*, *ACVR2A* or *ACVR2B* receptors as heterodimeric complexes (Tajer et al., 2021). Interestingly, we also observed a strong source of local *BMP5* signalling from the somatic LPM, though its role in LPM development is unknown. During later limb development, there were diverse patterns of WNT ligand signalling in tissues throughout the limb field, including ectodermal *WNT3A*, 4, 6, 7A, 10A, but restricted expression of *FRZD* and *LRP6* receptors only within limb bud mesenchyme and amnion (extraembryonic LPM). Together, these data reveal that signalling throughout the

limb field is achieved through combinations of both ubiquitous and tissue-specific ligand-receptor expression patterns, which are summarized in Fig. 2d. Overall, the molecular signalling events underlying LPM development and subdivision appeared to be dependent on BMP and HH signalling, while limb development is largely driven through FGFs and WNTs. However, it is important to note that limb development is also dependent on RA signalling (Nishimoto et al., 2015; Stratford et al., 1996), though we were unable to predict these due to the complex biosynthesis pathways underlying RA signalling.

Specification and differentiation of the LPM

Next, we sought to determine the mechanisms underlying fate choice of the early LPM during differentiation, subdivision, and limb initiation. This was achieved by first sub-setting the dataset to cells of mesodermal origin. Mesodermal cells were reprocessed with UMAP dimension reduction and Leiden clustering, allocating the 6 previous mesodermal clusters (c1, c3, c4, c5, c10 and c12; Fig. 1a) into 12 new sub-clusters (mc1-12; Fig. 3a). These captured the previously determined cell types, as well additional intermediate, transitional, and terminal cell types during LPM development (Fig. 3b). Namely, these clusters labelled cells from the somitic (mc9) and intermediate mesoderm (mc10), primitive mesoderm (mc2), early (undifferentiated) LPM (mc1 and mc7), somatic (mc3) and splanchnic (mc4) LPM, visceral/gut precursors (mc11), limb bud mesenchyme (mc5), non-limb flank LPM (mc12), extra-embryonic LPM (mc8) and amnion (mc6; Fig. 3a). Diagnostic cluster gene markers and log-fold changes are listed in Table S2.

With defined cell and tissue types, differentiation pathways throughout the LPM were first explored using estimates of RNA velocity. Cell velocity estimates revealed specific, directional transcriptional trajectories between cells as they transitioned from undifferentiated E1.5 (~HH10) precursors towards their distinct tissue fates, connecting three of the four E3.5 (~HH18) cell populations (Fig. 3b, c). Interestingly, we identified a heterogeneous population of early undifferentiated LPM cells with low directional velocity compared with other neighbouring clusters, despite existing in various stages of the cell cycle (Fig. 3c). This suggests that the multipotent cells of the early LPM exist in a transiently uncommitted state, before rapidly committing towards defined cell fates by E2.5 (~HH14), likely as a response to secreted ectodermal/endodermal signals (Fig. 2d). Furthermore, the somitic and intermediate cell clusters of the paraxial mesoderm, despite forming from the primitive mesoderm, did not

show a continuum of directional RNA velocities from these precursors. As we did not intentionally sample paraxial tissues, it is unclear whether there were not enough cells isolated to represent the complete differentiation trajectory, or whether these cells possess an earlier embryonic origin to the primitive mesoderm cells captured in our data. As such, we chose to not include this lineage in subsequent analyses.

To further define the pathways of differentiation that arise throughout the LPM, we explored these through LPM lineage generation. The E1.5 primitive mesoderm (mc2) represented the earliest identified cell type so was determined as the root node of mesoderm differentiation. A principal neighbour graph was fit with monocle3 (Trapnell et al., 2014), revealing 4 major lineages (L1-4; Fig. 3d) which supported RNA velocity estimates (Fig. 3c). These lineages describe the transition from E1.5 (~HH10) primitive mesoderm cells to E3.5 (~HH18) visceral/gut precursors (mc11; L1), non-limb somatic LPM (mc6 and mc12; L2), limb bud mesenchyme (mc5; L3) and somitic/intermediate mesoderm (mc9-10; L4). Importantly, a distinct bifurcation point was observed within the early undifferentiated LPM (mc1/mc7), marking LPM subdivision and lineage specification into the somatic (mc3) and splanchnic (mc4) LPM tissue layers (Fig. 3d).

The emerging somatic and splanchnic LPM layers are accompanied by specific expression of *IRX3* and *FOXF1*, respectively (Funayama et al., 1999; Mahlapuu et al., 2001). To confirm the accuracy of this lineage bifurcation point, *IRX3* and *FOXF1* expression were examined in mesodermal cells. Indeed, cells displayed mutually exclusive expression of *IRX3* or *FOXF1* in complementary domains following the LPM bifurcation point (Fig. 3d) with *FOXF1*⁺ cells observed in the splanchnic mesoderm and viscera precursors (mc4/7/11), and *IRX3*⁺ cells in the somatopleure, flank LPM and limb bud mesenchyme (mc1/3/5) (Fig. 3d). These data confirm the accuracy of somatic and splanchnic LPM bifurcation. However, also included within this lineage trajectory was an irregular branch linking the splanchnic LPM to the extraembryonic LPM and amnion (Fig. 3d-e, dashed line), which does not accurately represent its origins *in vivo*. While we were able to resolve this branch through additional k-mean principal graphs, these each came at the expense of the somatic-splanchnic LPM bifurcation point, with neither correct topology present in a single graph without losing cluster resolution (Fig S1). Therefore, we focused solely on the somatic-splanchnic LPM branching, as confirmed by *FOXF1* and *IRX3* expression, for subsequent lineage trajectory analysis.

Gene expression dynamics underlying LPM specification and limb development

Using this defined lineage of LPM differentiation, key gene expression dynamics underlying subdivision of the somatic and splanchnic LPM and specification towards a limb or viscera fate were examined. This was achieved through calculations of pseudotime along the viscera and limb lineages (Fig. 3e) (Trapnell et al., 2014), where genes with significant expression changes along each branch were identified through Moran's I spatial autocorrelation tests (Table S3,S4). The top differentially regulated genes along a given lineage were visualized through expression heatmaps, producing gene expression modules which were repressed or activated across pseudotime. This revealed a pseudo-temporal hierarchy of dynamically expressed genes during LPM subdivision, including marker genes that were specifically activated immediately after LPM bifurcation (Fig. 3f, g; Fig S2,3). Each lineage shared co-expression of primitive mesoderm / early LPM gene modules, which were repressed prior to subdivision. Immediately following was activation of shared or lineage-specific genes and gene modules (Fig. 3f, g). During splanchnic LPM differentiation and mesodermal viscera development, 9 modules of genes were co-activated, including but not limited to, activation of transcription factors *GATA6*, *NKX2-3*, *TCF21* and *HAND1*, and repression of *PRRX1* (Fig. 3f, Fig S3). Somatic LPM differentiation featured co-activation of 6 transcriptionally distinct modules, including but not limited too, increasing *PRRX1* expression, proximal activation of *OLFML3*, *NR2F2* and the principle retinoic acid synthesis gene, *ALDH1A2* (*Raldh2*), followed by transcription factors *IRX3*, *IRX6* and *TWIST1* (Fig. 3g, Fig S3). Immediately following was activation of limb initiation gene modules, beginning with expression of *TBX5*, *TBX2* and followed by limb bud mesenchyme genes *LMX1B*, *PRRX2*, *WIF1* and *FGF10*. Interestingly, while *TWIST1* is known to be expressed in the somatic LPM and limbs (Tavares et al., 2001), its rapid activation prior to the limb initiation module indicates a previously uncharacterised role during early limb development.

TWIST1 as a putative driver of somatic LPM specification and EMT

Limb outgrowth is first initiated by a localized EMT of the somatic LPM, proposed to be driven by *TBX5* and *FGF10* (Gros and Tabin, 2014). However, prior to *TBX5* and *FGF10* expression in the somatic LPM, we observed strong expression of *PRRX1* and activation of *TWIST1* (Fig. 3g) which are known EMT inducers during development and cancer (Fazilaty et al., 2019; Ocaña et al., 2012). We therefore further investigated *PRRX1*, *TWIST1* and *TBX5*

expression in pseudotime and by *in situ* hybridization, confirming their spatiotemporal expression profiles during limb development. *PRRX1* was expressed in the forelimb field and LPM as early as E1.5/HH10 prior to subdivision, before becoming regionalized and strongly expressed in the somatic LPM and limb bud (Fig. 4a). In comparison, *TWIST1* was specifically activated within in the somatic LPM at ~E2.0/HH12-13, immediately prior to *TBX5* which was activated in the somatic LPM by ~E2.5/HH14 (Fig. 4a).

Additional immunofluorescent labelling in HH12 chicken forelimb fields revealed strong, specific expression of TWIST1 protein in somatic LPM cells prior to limb bud EMT (Fig. 4b). At this stage, the somatic LPM existed as a pseudostratified columnar monolayer with meso-epithelial characteristics, expressing the transmembrane protein N-cadherin, but not the typical epithelial marker E-cadherin which was restricted to the ectoderm and endoderm. TWIST1 was specifically detected in cells of the somatic LPM and somite, with some TWIST1+ cells appearing to delaminate from the somatic LPM (Fig. 4b, arrows). By E2.5/HH14 the somatic LPM was no longer a distinct monolayer, observed by increased numbers of TWIST1+ cells and reduced N-cadherin which became localized to the apical edge of the somatic LPM. By ~E3.0/HH16, the early forelimb bud was distinct, populated by large numbers of TWIST1+ mesenchymal cells (Fig. 4b), which were also present in the sclerotome. We also examined whether other EMT transcription factors were co-expressed with *TWIST1* in the somatic LPM during EMT but did not detect enrichment of other EMT transcription factors in somatic LPM or limb bud mesenchyme clusters (Fig. 4c), or along the trajectory (Fig. 3c, Fig S3). As such, the early co-expression of *PRRX1* and *TWIST1* in the somatic LPM (Fig. 4a, b) suggest a putative role in somatic LPM EMT, prior to *TBX5*-induced limb initiation and FGF10 dependant outgrowth.

BMP signalling is necessary for somatic LPM fate and limb development

The BMP family of signalling factors, enriched in our receptor-ligand dataset (Fig. 2), have previously been implicated in LPM subdivision and early somatic LPM cell fate (Funayama et al., 1999). Finally, we sought to define their influence on LPM specification, subdivision, somatic LPM fate and early limb initiation through targeted BMP inhibition between the early ectoderm and developing LPM. To do this, we performed electroporation of the secreted BMP antagonist Noggin (*CAG-GFP-T2A-NOG*) into the ~HH9 forelimb field ectoderm to inhibit BMP signalling to the underlying LPM (Fig. 5a), during the window

where it is most active (Fig. 2). Control embryos electroporated with GFP (*CAG-GFP-T2A*) reproducibly developed limb buds, whereas embryos electroporated with Noggin failed to form forelimb buds (Fig. 5a), and in instances where the expression domain expanded posteriorly failed to form hindlimb buds (Fig. S4). Interestingly, inhibition of ectodermal-LPM BMP signalling was not sufficient to disrupt LPM subdivision, as previously implicated (Funayama et al., 1999). Rather, the somatic and splanchnic LPM still formed and were separated by the coelom, observed by robust *FOXF1* expression in the splanchnic LPM, albeit with a reduced limb bud (Fig. 5a, b). However, ectodermal BMP signal inhibition greatly stunted somatic LPM formation, observed by noticeable reduction of *PRRX1* expression in the limb field and somatic LPM tissue sections, compared to controls (Fig. 5b).

Inhibition of ectodermal BMP signalling via Noggin resulted in reduced outgrowth of the limb bud mesenchyme and failed formation of the developing forelimb bud (Fig. 5c). Strikingly, this was observed to occur in response to attenuated expression of *TBX5*, and failed activation of both *FGF10* in somatic LPM and *FGF8* in the AER (Fig. 5c, Fig S4b), suggesting ectodermal BMP signalling regulates the *TBX5*-*FGF10*-*FGF8* limb initiation mechanism. Conversely, we found that BMP inhibition did not influence expression of *TWIST1*, which showed similar expression and protein localization in forelimb sections compared with controls, despite reduced overall proportions of limb bud mesenchymal cells (Fig. 5a, c). Thus, *TWIST1* activation appears to be dependent on other signalling inputs which could not be determined, which may include RA signalling (Tavares et al., 2001). Together, our data confirm that ectodermal-mesodermal BMP signalling crosstalk is not required for LPM subdivision, as previously suggested (Funayama et al., 1999), but is necessary for somatic LPM specification and early limb initiation through activation of the *TBX5*, *FGF10*, *FGF8* feedback loop to regulate forelimb outgrowth.

Discussion

Single-cell transcriptomics have provided powerful methods to define cell lineage trajectories underlying multiple aspects of mesoderm and limb development (Feregrino et al., 2019; Gerber et al., 2018; Han et al., 2020; Johnson et al., 2020; Loh et al., 2016; Mahadevaiah et al., 2020; Pijuan-Sala et al., 2019; Scialdone et al., 2016). However, the initial developmental events underlying specification and diversification of the LPM were previously undefined. We add to this body of literature, revealing key mechanisms underlying specification of the

early LPM towards a limb or viscera cell fate. LPM specification originates in primitive mesodermal precursors, which follow four distinct differentiation pathways from E1.5 (~HH10) to E3.5 (~HH18) (Fig. 3). Initially, LPM progenitor cells (*FOXF1*⁺) displayed a large degree of cellular heterogeneity (Fig. 3a-d), which was accompanied by extensive signalling pathway usage (Fig. 2). This revealed that the emerging LPM forms in a transient, multipotent state, where cells are transcriptionally primed to respond to rapid changes in the extrinsic signalling environment to initiate LPM lineage specification. This was observed where the LPM heterogeneity was rapidly resolved by E2.5, with cells committing to defined differentiation lineages, including LPM subdivision, in response to dynamic changes in signalling pathway activation and repression.

Early establishment and differentiation of the LPM was predicted to occur through signalling interactions within the BMP and HH pathways, originating from the ectoderm and endoderm respectively, supporting previous observations (Funayama et al., 1999; Roberts et al., 1995; Yoshino et al., 2016). Though our analyses were unable to directly predict the transcriptional targets of these signalling interactions during LPM development, it is possible that HH is the inducing signal for early LPM specification. This hypothesis is supported where a SHH-*FOXF1*-BMP4 signalling axis drives ureter development (Bohnenpoll et al., 2017), and SHH activates BMP4 during gut development (Roberts et al., 1995). Given that we observe SHH signalling from the endoderm to PTCH receptors in the early LPM (Fig. 2) (Yoshino et al., 2016), and that *FOXF1/BMP4* are markers which specify the early LPM (Fig. 3d) (Mahlapuu et al., 2001; Tonegawa et al., 1997; Winnier et al., 1995), it is plausible that the SHH-*FOXF1*-BMP4 axis participates in initial LPM patterning and establishing its identity.

The mechanism underlying LPM subdivision have been previously suggested to occur through BMP2/7 signals derived from the ectoderm (Funayama et al., 1999). As our global signalling analysis recovered these BMP signalling events during early LPM development (Fig. 2), we investigated whether these are indeed responsible for formation of the somatic and splanchnic LPM. Targeted inhibition of ectodermal-mesodermal BMP signalling, using the secreted BMP antagonist Noggin, was not sufficient to drive LPM subdivision, observed by formation of both layers separated by the coelom (Fig. 5). This reveals that other, undefined mechanisms are responsible for LPM subdivision, though may be achieved through endodermal HH signalling through activation and maintenance of *FOXF1*. *Foxf1* null mice possess incomplete LPM subdivision, somatic-splanchnic LPM fusion and misexpression of somatic LPM genes (i.e. *Irx3*) (Firulli et al., 1998; Mahlapuu et al., 2001).

Thus, it is possible that a SHH-induced *FOXF1*-dependent mechanism initiates LPM subdivision, before ectodermal BMPs induce a somatic LPM identity on the newly formed dorsal layer.

Previously, *PRRX1* and *IRX3* were the only defining markers of the early somatic LPM (Funayama et al., 1999), yet are both dispensable for somatic LPM development (Li et al., 2014; Martin et al., 1995). Through our analyses, we have defined additional suites of genes and regulators that accompany LPM subdivision into somatic and splanchnic LPM fates (Fig. 3f-g, Fig. S2,3). LPM subdivision occurs through initial repression of a shared module of early mesoderm genes, before seeing lineage-specific activation of somatic and splanchnic gene modules (Fig. 3f, g). Of note, the first stage of LPM subdivision saw lineage-specific maintenance and activation of basic helix-loop-helix (bHLH) transcription factors *HAND1* and *TWIST1* within the splanchnic and somatic LPM lineages, respectively. *HAND1* and *TWIST1* possess known roles in later gut and limb development (Firulli et al., 1998; Krawchuk et al., 2010; Loebel et al., 2012; Wu and Howard, 2002), though our results suggest that they may play unappreciated roles during initial LPM fate specification. bHLH transcription factors form combinations of homo- and heterodimers with unique binding partners to dynamically regulate different biological processes (Fan et al., 2020). We observe expression of both ubiquitous (*HAND2*) and lineage-specific bHLH binding partners (*TCF12* and *TCF21*) (Fig. S2,S3) (Fan et al., 2020) throughout the LPM cell types. Thus lineage-specific activation of *TWIST1* and *HAND1* may mediate complementary, yet distinct roles in target gene activation during the initial stages of LPM subdivision and differentiation.

While *PRRX1* and *TWIST1* have known expression in the somatic LPM (Gitelman, 1997; Kuratani et al., 1994; Nohno et al., 1993; Tavares et al., 2001), our data reveal a potential, unreported role of these during somatic LPM development and EMT. Limb initiation begins with a localized EMT of the somatic LPM, suggested to depend on TBX5 and FGF10 (Gros and Tabin, 2014). However, *PRRX1* and *TWIST1* are known regulators of EMT in both development and cancer (Fazilaty et al., 2019; Ocaña et al., 2012; Qin et al., 2012), suggesting they may possess an uncharacterized role during early EMT or initial induction of the forelimb mesenchyme. As noted above, *TWIST1* elicits different biological functions and activity thresholds through dimerization with other transcription factors (Fan et al., 2020; Krawchuk et al., 2010; Loebel et al., 2014), which notably includes *PRRX1* in neural crest cells (Fan et al., 2021). Indeed, we identify robust, tissue-specific co-expression of *TWIST1* (and *PRRX1*, not shown) in the meso-epithelial somatic LPM monolayer, before and after

EMT and proliferation of the limb bud mesenchyme (Fig. 4). This putative, cooperative role is supported where exogenous *prx1a* drives aberrant EMT and migration of the LPM in zebrafish, which can be rescued by co-knockdown of *twist1b* (Ocaña et al., 2012). Furthermore, *Twist1* null mouse mutants possess severely atrophied forelimb buds, as a potential consequence of reduced TWIST1-induced EMT of LPM precursors (Chen and Behringer, 1995). Together, these data implicate *PRRX1* and *TWIST1* as early mediators of somatic LPM specification and EMT, though this requires further validation *in vivo*. Of note, it is unclear whether *PRRX1* and/or *TWIST1* may cooperate with *TBX5* to facilitate EMT and induction of limb bud outgrowth.

The unexpected finding of this study was the severe inhibitory effect of BMP antagonism on early somatic LPM fate and limb specification and outgrowth. Inhibition of ectodermal BMP signalling, through ectopic expression of Noggin, both reduced commitment and proportions of somatic LPM cells through inhibition of *PRRX1*, and severe atrophy of limb bud outgrowth through attenuation of *TBX5*, *FGF10* and *FGF8* (Fig. 5b). Activation of *TBX5* in the somatic LPM has been previously suggested to depend on RA, with cooperative activation through *HOX* genes and β -CAT/TCF/LEF through the canonical WNT signalling pathway (Minguillon et al., 2012; Nishimoto et al., 2015). However, the WNT ligand for this activation has not been reported, and our data shows no evidence of active WNT signalling in the early LPM / somatic LPM (Fig. 2). Supporting this, BMP-induced differentiation of iPSCs into LPM-like cells, defined by induction of *TBX5*, occurs in the absence of both WNT and RA (Loh et al., 2016). Instead, our data reveals a novel role for ectodermal BMP signalling in limb initiation through somatic LPM fate determination and *TBX5* activation. Addition of BMP2 beads to chick hindlimb buds can induce ectopic *TBX5* (Rodriguez-Esteban et al., 1999), supporting our finding that BMP induces activation of *TBX5* in the forelimb. BMP antagonism is known to affect limb outgrowth by reducing AER formation via attenuated activation of *FGF8* and *MSX2* in the ectoderm (Capdevila and Johnson, 1998; Pizette and Niswander, 1999; Pizette et al., 2001). However, our data reveal that this is likely an indirect effect of attenuated *TBX5* activation and reduced maintenance of the FGF10-FGF8 feedback loop. Together, these observations establish a new model of early forelimb development, where ectodermal BMPs regulate somatic LPM formation and limb initiation (Fig. 6) within the predefined HOX and RA-specified forelimb forming domain.

A limitation of this study was our inability to predict the precise function of *TWIST1*, nor the role of RA signalling during LPM specification and limb outgrowth (Cunningham et al., 2013; Gibert et al., 2006; Nishimoto et al., 2015; Zhao et al., 2009) due to its complex biosynthesis pathway. However, our somatic LPM lineage analysis revealed activation of the RA synthesis gene *ALDH1A2* and RA-responsive transcription factor *NR2F2* in the first module of somatic LPM differentiation, immediately followed by activation of *TWIST1* (Fig. 3, S3). As *TWIST1* was not responsive to BMP signalling (Fig. 5c), it is possible that *TWIST1* is activated through a RA mechanism. In support of this, *TWIST1* is induced in limb buds by ectopic RA soaked beads (Tavares et al., 2001). While additional data is required to determine the role of *TWIST1* during somatic LPM differentiation and early limb initiation, these data suggest that somatic LPM fate is specified through the combined action of BMP and RA signalling (Fig. 6).

This study fills a long-standing gap in the literature, establishing the first transcriptional atlas of multipotent LPM progenitors and their differentiation through transitional and maturing cell types in the embryonic chicken forelimb field. We have defined the global signalling pathways and gene expression dynamics during early LPM development and shed light on the early cell-fate decisions preceding initiation of the vertebrate forelimb. Furthermore, though we were unable to identify the signal that initiates LPM subdivision, we defined a necessary role of ectodermal BMP signals in somatic LPM identity and limb bud formation. While we have begun to characterize the gene regulatory networks underlying LPM specification, prior to limb outgrowth (Fig 6.), more work is needed to define the role of key genes and signalling pathways during early specification of the LPM. Nevertheless, in this study we present the detailed overview of full cellular and developmental events from LPM specification to forelimb initiation, provide exciting new avenues to scrutinize the molecular events underlying fate specification, development and evolution of the vertebrate limbs.

Methods

Egg incubation, tissue collection, single cell sampling, sequencing

Chicken eggs were collected at embryonic day (e) 1.5 (stage 10), E2.5 (HH14) or E3.5 (HH18). Embryos were dissected away from extra-embryonic membranes, rinsed in ice-cold DPBS then the LPM dissected. LPM tissues were digested with 0.05% Trypsin / EDTA and incubated at 37°C for 15 minutes, with mechanical dissociation every 5 minutes until no

clumps were visible. Enzymatic activity was stopped with addition of 10% FCS. The dissociated cells were spun at 400g for 5 minutes, then resuspended in 1x EDTA / Propidium Iodide in DMEM (Gibco). Cells were filtered through a 70um Flowmi Cell Strainer (Scienceware), and viable cells were isolated through flow cytometry (Flowcore, Monash University). Samples were submitted to Micromon Genomics (Monash University) for analysis using the 10X Genomics Chromium Single Cell 3' V3.1 chemistry, as per the manufacturer's instructions. Samples were subjected to 10 cycles of PCR for cDNA amplification and 16 cycles for library amplification. The prepared libraries were sequencing using on the MGITech MGISEQ2000RS platform with MGIEase V3 chemistry, on two lanes with 100bp paired end reads.

Bioinformatic analysis

Pre-processing

Reads were aligned to the chicken GRCg6a reference using CellRanger (v4.0.0, using option: `--force-cells 15000`). Due to the number of reads observed just downstream of annotated genes, the gene annotation (from Ensembl release 100, gene biotypes: protein coding, lincRNA and antisense) was edited to include 1000bp downstream each gene. Single cell analysis was performed in R using packages `scrn` (Lun et al., 2016), `scater` (McCarthy et al., 2017) for QC, `monocle3` (Trapnell et al., 2014) for trajectory analysis, and `iSEE` for interactive viewing (Lun et al., 2018). Gene names were used for analysis and, where they mapped to multiple Ensembl ids, the Ensembl ID with the highest number of counts was kept. Cells with low total UMI counts (<2000) were excluded. Cell cycle was annotated with `cyclone` in the `scrn` package (Lun et al., 2016) using the mouse reference from (Scialdone et al., 2015) mapped to its one-to-one chicken orthologs.

The top 1000 genes with the highest biological variance were identified with `modelGeneVar` function of `scrn` (Lun et al., 2016), blocked on the sequencing sample, and excluded mitochondrial genes or genes on the Z or W chromosomes to minimise sex effects. PCA was calculated on these, and the first 15 PCs used to generate a global chicken tSNE layout. Clusters were defined with the `walktrap` method, on a SNN graph (k=10) (Lun et al., 2016), and cluster identities were determined from gene logFC changes and spatial expression

profiles in the Gallus Expression *In Situ* Hybridization Analysis (GEISHA) database (Bell et al., 2004; Darnell et al., 2007).

Global signalling pathway usage and ligand-receptor crosstalk

Global signalling patterns throughout the chicken forelimb field were examined using the R package CellChat (Jin et al., 2021), where signalling communication networks were constructed based on 1:1 gene orthology with a curated *Homo sapiens* database and default parameters. Visualizations of pathway and ligand receptor signalling were generated with CellChat and edited with Adobe Illustrator.

Mesoderm analysis and lineage reconstruction

Mesodermal cell clusters were subset from the full tSNE for additional, focused analyses. Briefly, mesodermal clusters were subset to a new object, PCA and UMAP dimension reduction was recalculated using the previously determined highly variable genes and corrected for cell cycle effects. Next, the object was imported into Monocle3 (Cao et al., 2019; Trapnell et al., 2014) for clustering (k=4). Cluster labels were confirmed by identifying differentially expressed marker genes through regression analysis implemented in *monocle_fit_models* function, producing distinct 12 sub-clusters covering all known cell types within the developing limb field mesoderm.

Estimations of RNA velocity were calculated where reads were aligned to the reference a second time with STAR solo (v2.7.5) to identify proportions of spliced and unspliced transcripts (Dobin et al., 2013). Velocity analysis with performed with *velocyto.R* (La Manno et al., 2018), and directional transcriptional velocities between cells were visualized. Lineage trajectories throughout the mesoderm were additionally constructed in Monocle3 using reverse graph embedding (k=4, minimum branch length = 15, *rann.k* = 50), which produced 4 major lineages that originated in E1.5 cells and terminated in E3.5 cells. Lineage bifurcation points were corroborated using known LPM marker gene expression through the *plot_cells* function, then pseudotime was calculated by selecting the origin of the lineages using the *order_cells* function. To identify genes that dynamically changed in expression across

pseudotime, key lineages throughout the mesoderm were subset using the *chose_graph_segments* function and graph tests were run to identify lineage-specific, differentially regulated genes and filtered based on Moran's I statistic and q value. Modules of genes that significantly changed across pseudotime were visualized by hierarchical clustering through the R package ComplexHeatmap. Gene expression in individual cells across pseudotime were further visualized using the *plot_gene_in_pseudotime* function in Monocle3.

Functional experimentation

Gene expression analysis by in situ hybridization and immunofluorescence

Whole mount *in situ* hybridization for spatial mRNA expression was carried out as described previously (Smith et al., 2016) with minor modifications. Briefly, whole HH8-HH22 chicken embryos were fixed overnight in 4% paraformaldehyde, dehydrated in methanol, and rehydrated in PBTX (PBS + 0.1% Triton X-1000). Tissues were permeabilized in 10µg/mL proteinase K for up to 1 hour, depending upon stage, then re-fixed in glutaraldehyde/ 4% PFA. Tissues underwent pre-hybridization (50% formamide, 5x SSC, 0.1% Triton X-100, 0.5% CHAPS, 5mM EDTA, 50mg/mL Heparin, 1mg/mL yeast RNA, 2% blocking powder) overnight at 65°C. Riboprobe templates were provided as gifts, generated from public sources, or designed and synthesised in house. Primer sequences and/or source are listed in Table S5. Where applicable, templates were amplified from limb and whole embryo cDNA using gene specific primers. Fragments were resolved by 1% agarose electrophoresis, excised, and purified using a Nucleospin PCR clean-up kit and subcloned into p-GEM T-easy (Promega). Antisense RNA probes were synthesized using T3, T7 or SP6 RNA polymerases and the DIG-labelling kit (Roche, #11277073910) as per the manufacturer's instructions. Precipitated probes were added to pre-hybridized tissues (approx. 5mL/ tube) and hybridization was carried out overnight at 65°C. Tissues were then subjected to stringency washes, blocked in BSA, then treated overnight with anti-DIG antibody conjugated with alkaline phosphatase. Tissues were exposed to BCIP/NBT colour reaction at room temperature for up to 3 hours (340mg/mL NBT and 175 mg/mL BCIP in NTMT (100mM NaCl, 100mM Tris-HCl, pH9.5, 50mM MgCl₂, 0.1% Tween-20).

Chicken embryos were fixed in 4% PFA/PBS for 15 minutes at room temperature then cryo-protected in 30% sucrose. Embryos were snap frozen in OCT and 10mm frozen sections were cut. For immunofluorescent detection of TWIST1, antigen retrieval was performed by the Monash Histology platform. Briefly, slides were heated at 60 degrees for 30 minutes followed by retrieval in citrate solution (pH 6). All other sections were left in PBS. For co-detection of TWIST1 and other markers (Table S5) in the LPM, antibody incubations were performed on successive tissue sections. Sections were blocked and permeabilised in 1% Triton X-100, 2% BSA/PBS for 1-2hr at room temperature, then incubated with primary antibody (1:100) in 0.5% Triton X-100, 1% BSA/PBS incubation overnight at 4°C. Secondaries (1:1000) were added the following day and left at room temperature for one hour. Slides were washed once with DAPI diluted in PBS, followed by two washes in PBS.

Targeted electroporation

Electroporation of chicken ectoderm was performed using custom parameters. Briefly, eggs were incubated for ~36 hours until stage HH8-HH10. Here, a solution containing TOL2 Transposase and *Tol2-CAG-GFP-T2A* or *Tol2-CAG-GFP-T2A-NOG* plasmids (available on request) at final concentrations of 1µg/µl were mixed with 0.1% fast green and injected between the vitelline membrane and embryo. Electroporation was performed by placing the negative electrode above the presumptive forelimb field on the right side of the embryo, and positive electrode under the embryo above the yolk, and delivered through 3x 10V, 60ms width, 50ms space pulses (Intracel TSS20 Ovodyne Electroporator). Eggs were sealed with tape and the embryos were incubated for a further 48-72h. Embryos were then harvested and GFP fluorescence were captured on a Fluorescence dissecting microscope. GFP positive embryos were then fixed in 4% PFA overnight at 4°C. Electroporation's were performed across multiple independent experiments to ensure consistent results.

In situ hybridization was performed on GFP positive embryos, as per above. For co-detection of *in situ* gene expression and GFP immunofluorescence, positive embryos were cryosectioned at 10µm sections and subject to antigen retrieval. Then, sections were processed for immunofluorescence, as above, using an anti-GFP (Table S5) antibody. Tissue sections were imaged on a brightfield / fluorescent microscope.

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Author contributions

A.H.N and C.A.S conceptualized and designed the study. A.H.N performed the experiments and wrote the manuscript. A.T.M assisted with flow cytometry. S.M.W performed bioinformatic pre-processing. A.H.N and S.M.W performed computational analysis. All authors assisted with interpretation of the data. All authors contributed to editing of the final manuscript.

Competing interests

The authors declare no competing interests.

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Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files. Raw count matrices have been uploaded to GEO under the identifier - provided upon acceptance.

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Figures

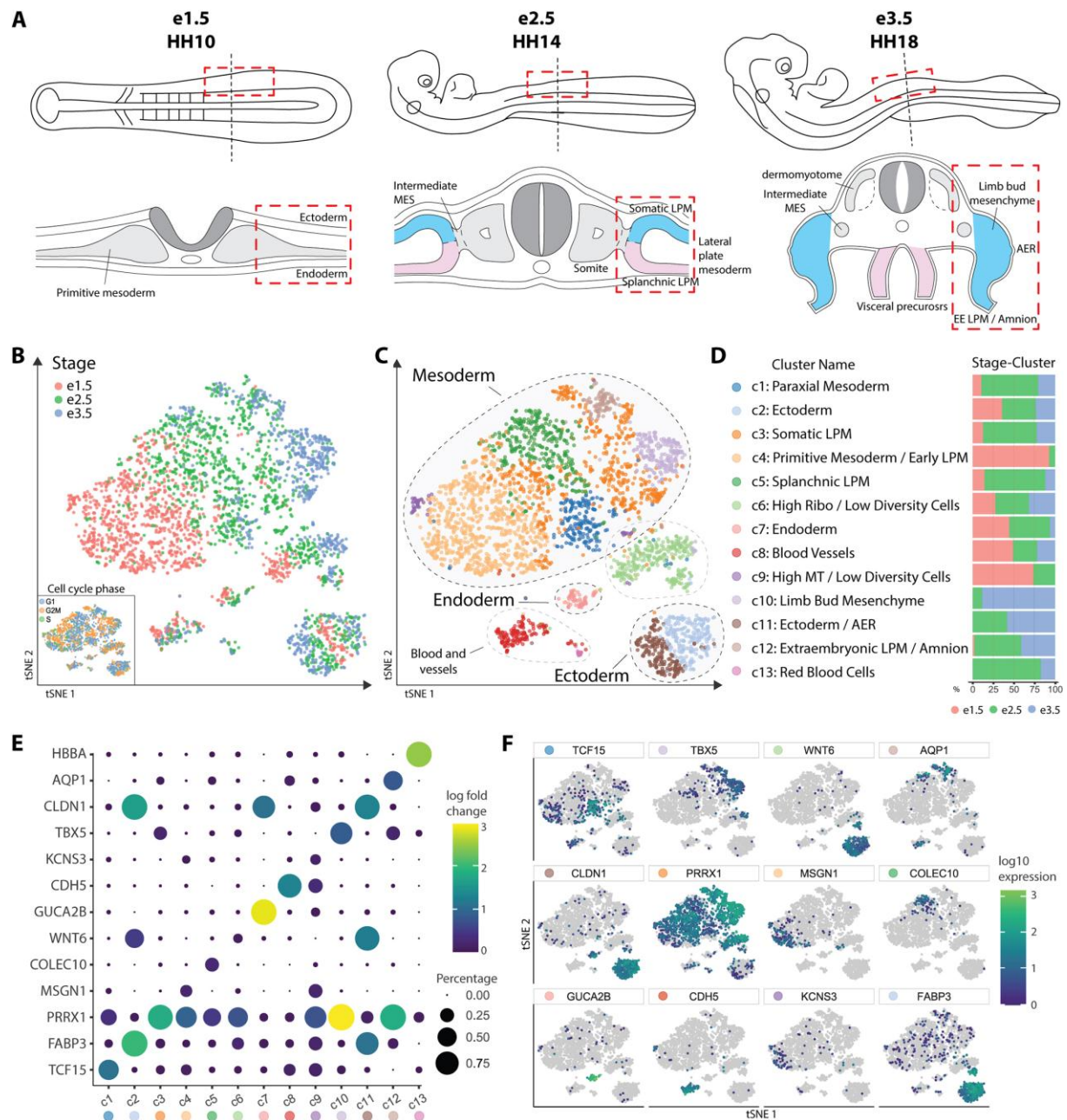


Fig. 1. Identification of cell types in the avian forelimb field

(A) Cells were isolated from chicken embryonic day (e) 1.5, e2.5 and e3.5 to sample all major tissues in the developing forelimb field. (B) tSNE visualisation separated cells based on stage, and (C) germ layer origin. (D) Unsupervised clustering revealed 13 distinct clusters covering all major cell types in the developing forelimb field. (E) Unique gene expression profiles were detected for each major cluster, (F) and were largely specific to each cell population.

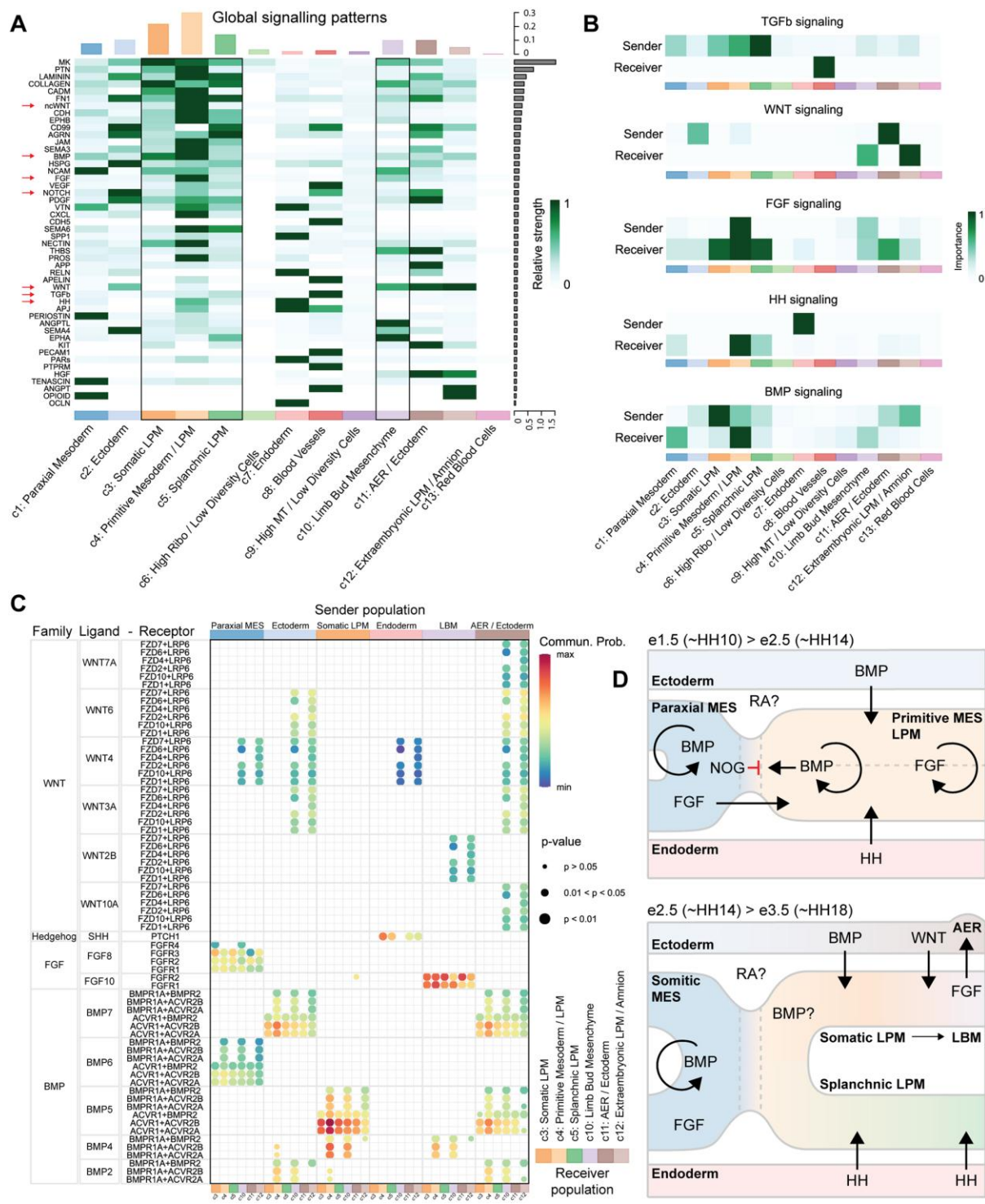


Fig. 2. Cellular signalling and ligand-receptor crosstalk in the forelimb field

(A) Predictions of active signalling pathways utilized by cell type clusters revealed diverse pathway usage and enriched signalling in the primitive mesoderm, LPM, ectoderm and limb bud. Major signalling pathways are highlighted by red arrows, emphasizing tissue-specific differences in pathway usage. (B) Identification of sender and receiver cell types utilizing major signalling pathways. TGF β signalling was enriched between the splanchnic LPM and vasculature, WNT signalling between the limb and AER ectoderm (c11), FGF in the primitive mesoderm, HH in the endoderm and BMPs in the LPM and dorsal ectoderm (c2). (C) Identification of key ligand-receptor pairs facilitating tissue-specific signalling from major signalling pathways. This revealed broad, tissue-specific patterns of ligand and receptor heterodimer usage between cell types. For example, BMP7 was identified as the main ligand facilitating ectoderm-LPM signalling, while several WNTs were expressed between the ectoderm and limb bud. FGF10 was confirmed to signal between the limb and ectoderm. (D) Diagrammatic summary of signalling pathways active between tissues in the developing limb field.

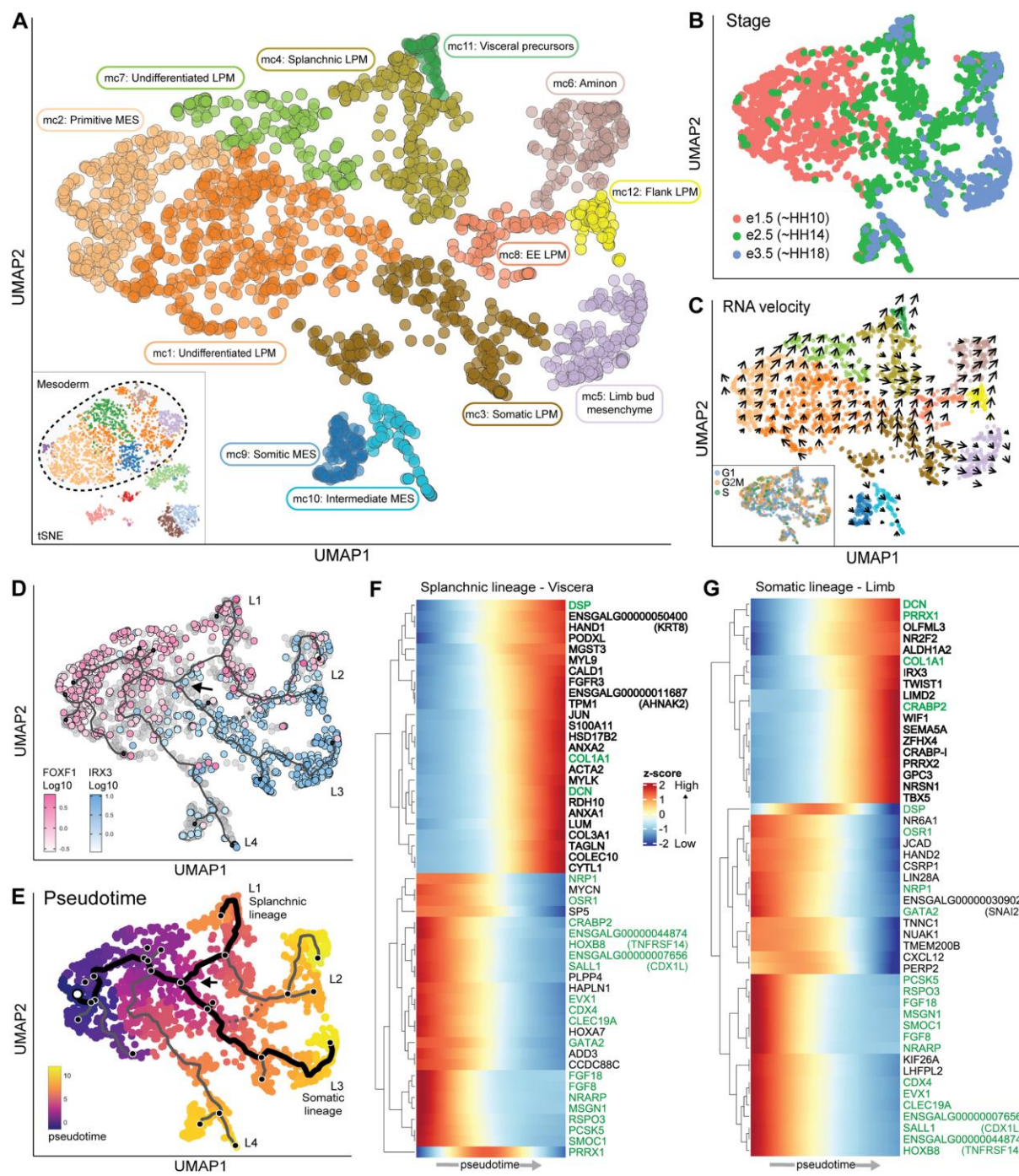


Fig. 3. Lineage reconstruction and gene expression dynamics underlying LPM differentiation. (A) Subsampling, UMAP projection and re-clustering of mesodermal cell types revealed 12 distinct sub-clusters (mc1-12) with greater resolution of lineage choices and transitional cell types in the forelimb mesoderm, which clearly separated by stage (B). (C) Transcriptional trajectories were identified through estimates of RNA velocity, revealing distinct lineages of differentiation across different stages of the cell cycle (insert). (D) Trajectory inference was computed with Monocle3 further describing 4 lineages (L1-L4) of differentiation. The somatic-splanchnic LPM bifurcation (black arrow) was calibrated using expression of known markers *IRX3* and *FOXF1*, respectively. (E) The root node was set in the primitive MES (white circle), and pseudotime calculated to identify gene expression dynamics along the splanchnic and somatic LPM lineages (bold lines). Gene expression dynamics were calculated along the splanchnic (F) and somatic (G) LPM lineages, revealing activation and repression of distinct gene modules accompanying their differentiation pathways. Unique lineage-specific genes in shown in black while shared genes are in green.

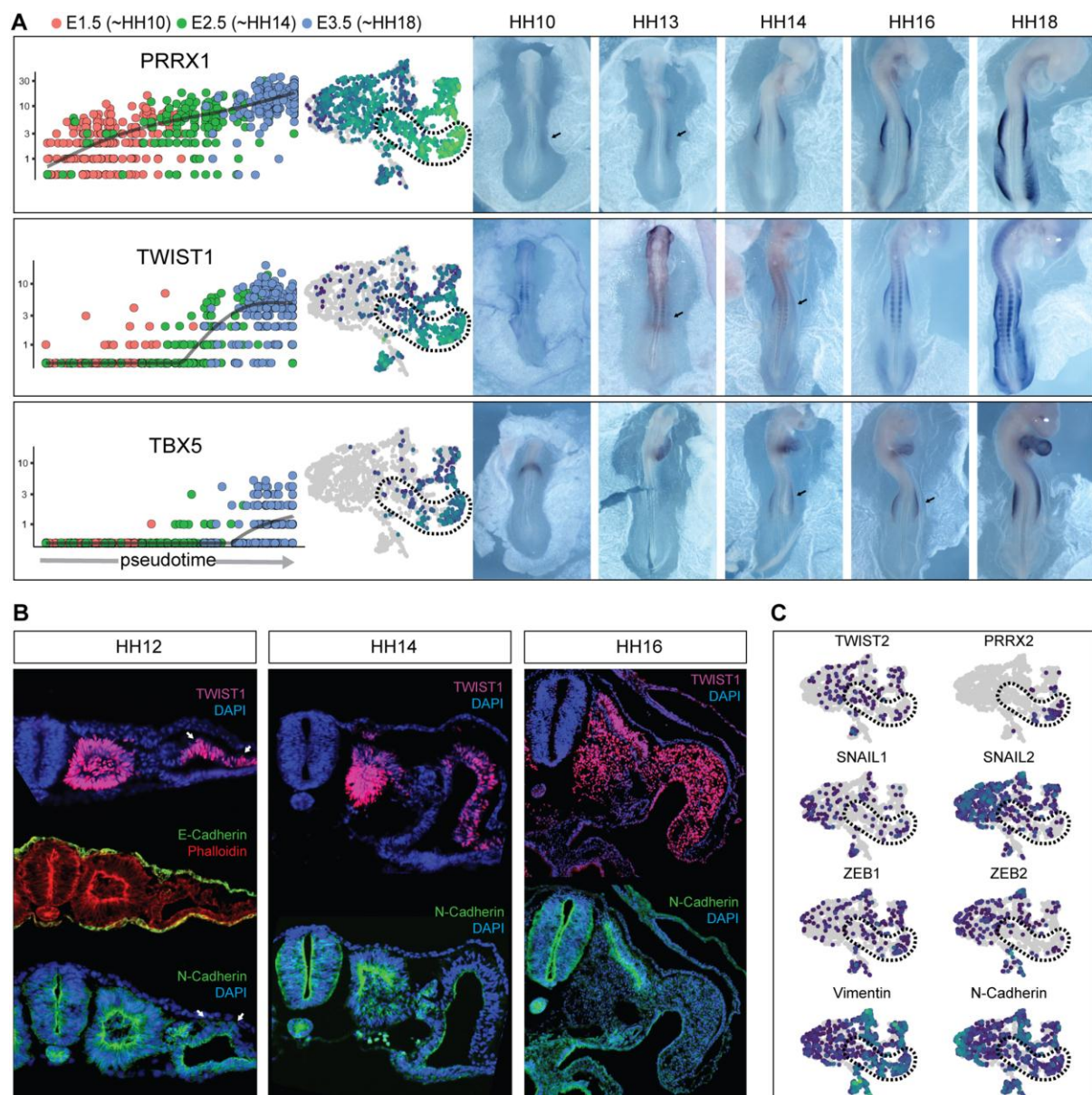


Fig. 4. TWIST1 is a putative regulator of somatic LPM and limb bud EMT

(A) Expression dynamics of *PRRX1*, *TWIST1* and *TBX5* during somatic LPM lineage specification during pseudotime, and their *in situ* spatiotemporal gene expression profiles during early chicken development. *PRRX1* demarks formation and development of the LPM, while *TWIST1* is activated in the somatic LPM immediately prior to the onset of *TBX5*. Black arrows denote the limb field and onset of gene expression. (B) Immunofluorescent labelling in the developing forelimb field revealed the LPM possesses meso-epithelial characteristics, shown by absence of E-Cadherin and presence of N-Cadherin. *TWIST1* is observed in the stage (HH) 12 somatic LPM after subdivision, but prior to EMT and proliferation of the limb bud mesenchyme by stage (HH) 16. White arrows show *TWIST1*⁺ cells migrating out of the somatic LPM cell layer. (C) Single-cell expression of EMT markers. *TWIST1* (and *PRRX1*) appears as the major candidates underlying somatic LPM EMT, due to lack of enrichment of other EMT transcription factors in the somatic LPM.

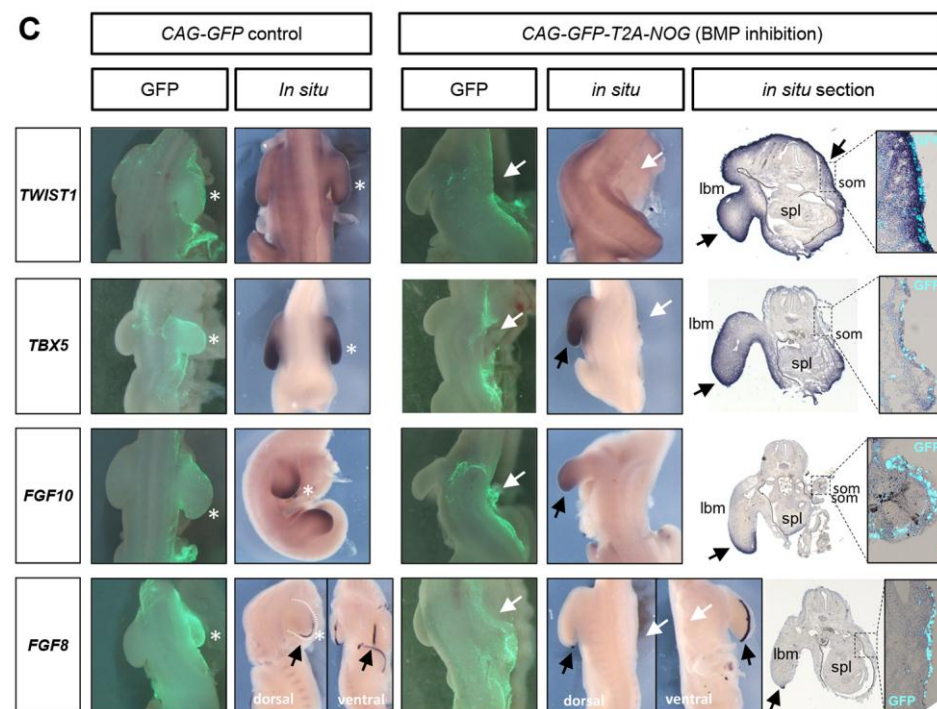
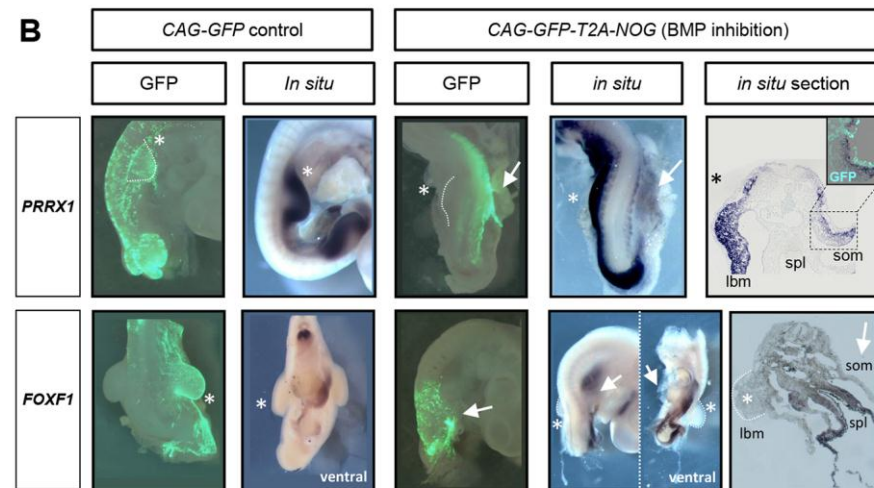
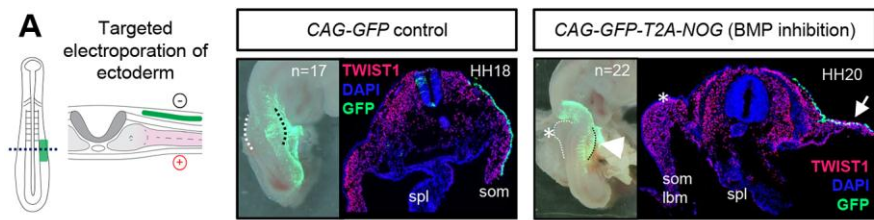


Fig. 5. Inhibition of ectodermal BMP signalling severely impacts limb bud development

(A) Targeted electroporation of the e1.5 (~HH9-10) limb field ectoderm. Electroporation of GFP (n=17) produced green fluorescence in the limb field ectoderm, overlaying the somatic LPM / forelimb buds observed by TWIST1 protein localization. Electroporation of GFP-T2A-NOG into the ectoderm (n=22) reproducibly inhibited somatic LPM formation and limb bud outgrowth (Fig. S4), confirmed by reduced proportions of TWIST1+ cells. White arrows show reduced somatic LPM thickness and limb outgrowth while asterisks show normal limb buds. (B) BMP antagonism by Noggin was not sufficient to disrupt LPM subdivision. *PRRX1* expression and somatic LPM commitment was greatly reduced in GFP-T2A-NOG electroporated embryos, though the LPM still underwent subdivision observed by restricted expression of *FOXF1* in the splanchnic LPM and the presence of the coelom. (C) BMP antagonism inhibits limb formation and outgrowth. After 72h, stage (HH) 22 embryos electroporated with GFP-T2A-NOG failed to form forelimbs (white arrows) compared to GFP controls (asterisks). Inhibited limb outgrowth was accompanied by reduced activation of *TBX5*, and absent *FGF10* and *FGF8* expression in whole mount embryos. This was further visualized in forelimb field tissue sections, where reduced gene expression was observed in somatic LPM cells underlying Noggin-GFP positive ectoderm. *TWIST1* was not affected by BMP inhibition, showing no obvious reductions in mRNA expression (C) or protein localization (A) compared to GFP controls. som = somatic LPM; spl = splanchnic LPM; lbm = limb bud mesenchyme.

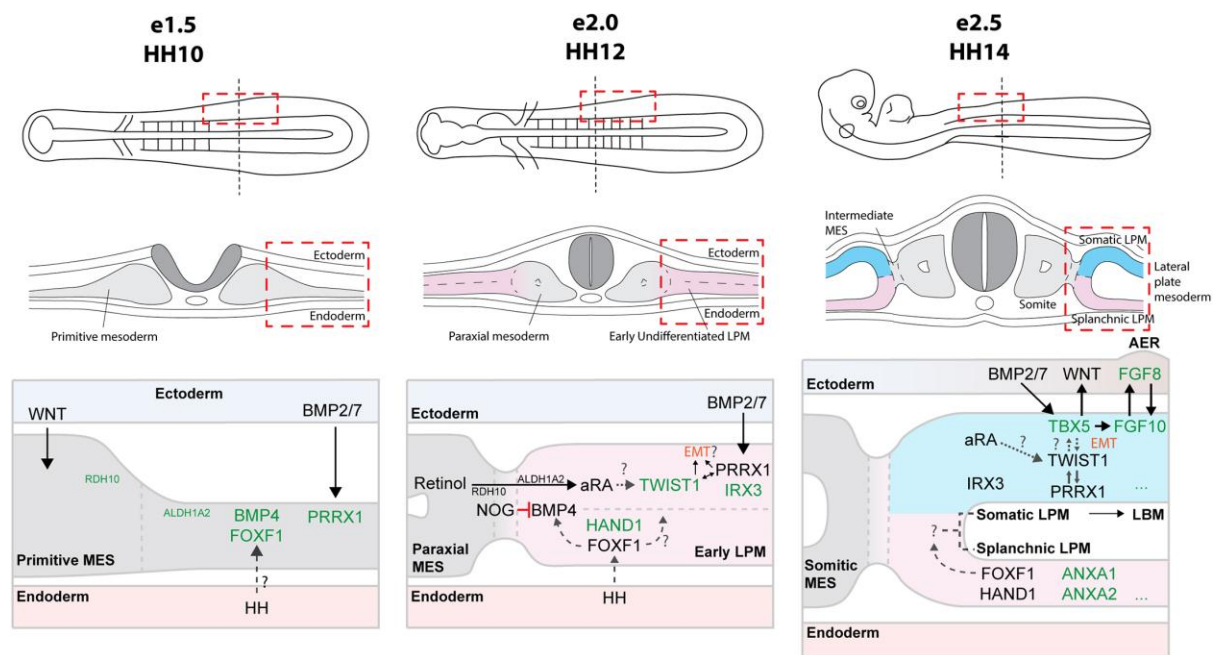


Fig 6. Overview of the signalling inputs and GRNs driving LPM specification and limb initiation

Mechanisms underlying specification of the LPM towards a somatic and splanchnic cell fate at stages (HH) 10, 12 and 14. Initially, ectodermal BMP2/7 signals, and putative HH signals from the endoderm, define somatic and splanchnic cell fates, respectively, through activation lineage-specific gene modules. *TWIST1* is rapidly activated in the somatic LPM, which may cooperatively influence EMT of the early meso-epithelial somatic LPM monolayer. After the somatic LPM is established, ectodermal BMPs activate *TBX5*, establishing the FGF10-FGF8 feedback loop to drive limb bud outgrowth.

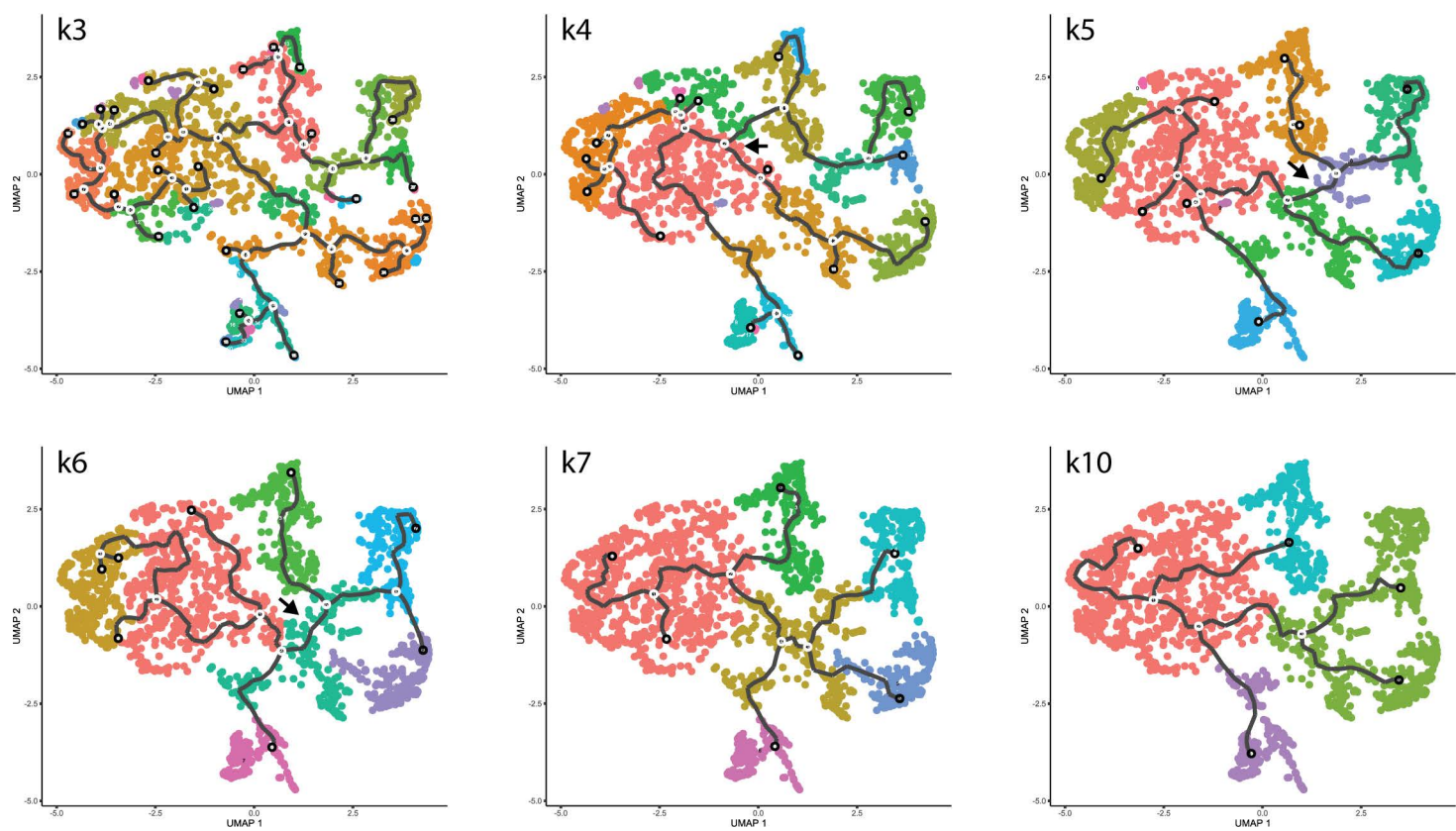


Fig. S1. Calibration of alternate LPM lineage topologies

Influence of k-mean values on lineage topologies throughout the LPM. Various K-mean values were sequentially applied until lineages recapitulated known IRX3/FOXF1 marker gene expression patterns in Figure 3. k4 produced a biologically relevant branch between somatic and splanchnic cell clusters (arrow), whereas k5 produced a relevant branch between the limb and non-limb somatic LPM (arrow). Neither branch structure was represented within a single graph.

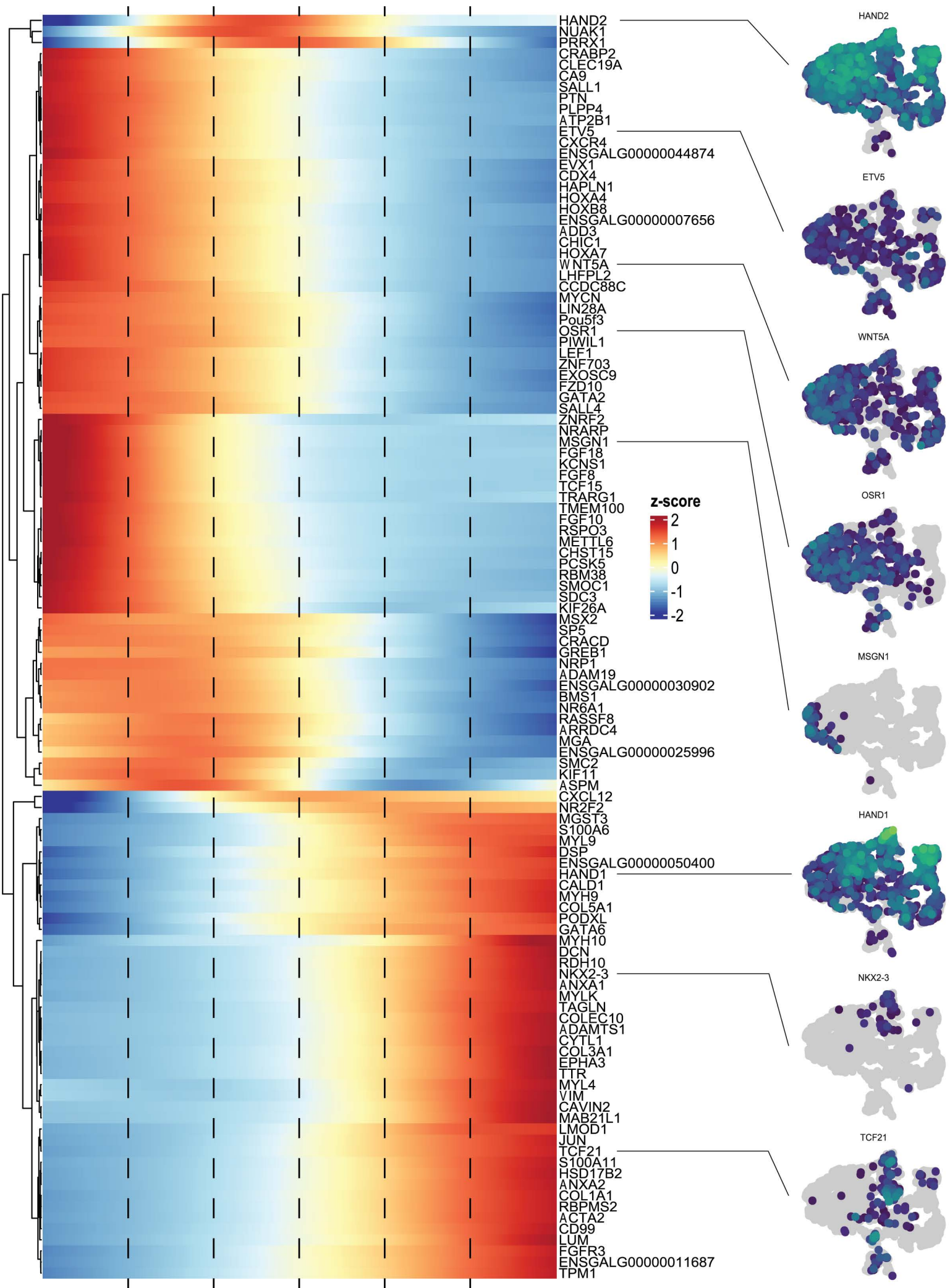


Fig. S2. Splanchnic LPM expression dynamics.

Extended pseudotime heatmap of significant differentially regulated genes along the splanchnic LPM lineage, with select expression plots. Bottom half of the heatmap revealed modules of genes that are specifically activated during splanchnic LPM specification and subsequent differentiation. The full list of significant genes are found in table S3.

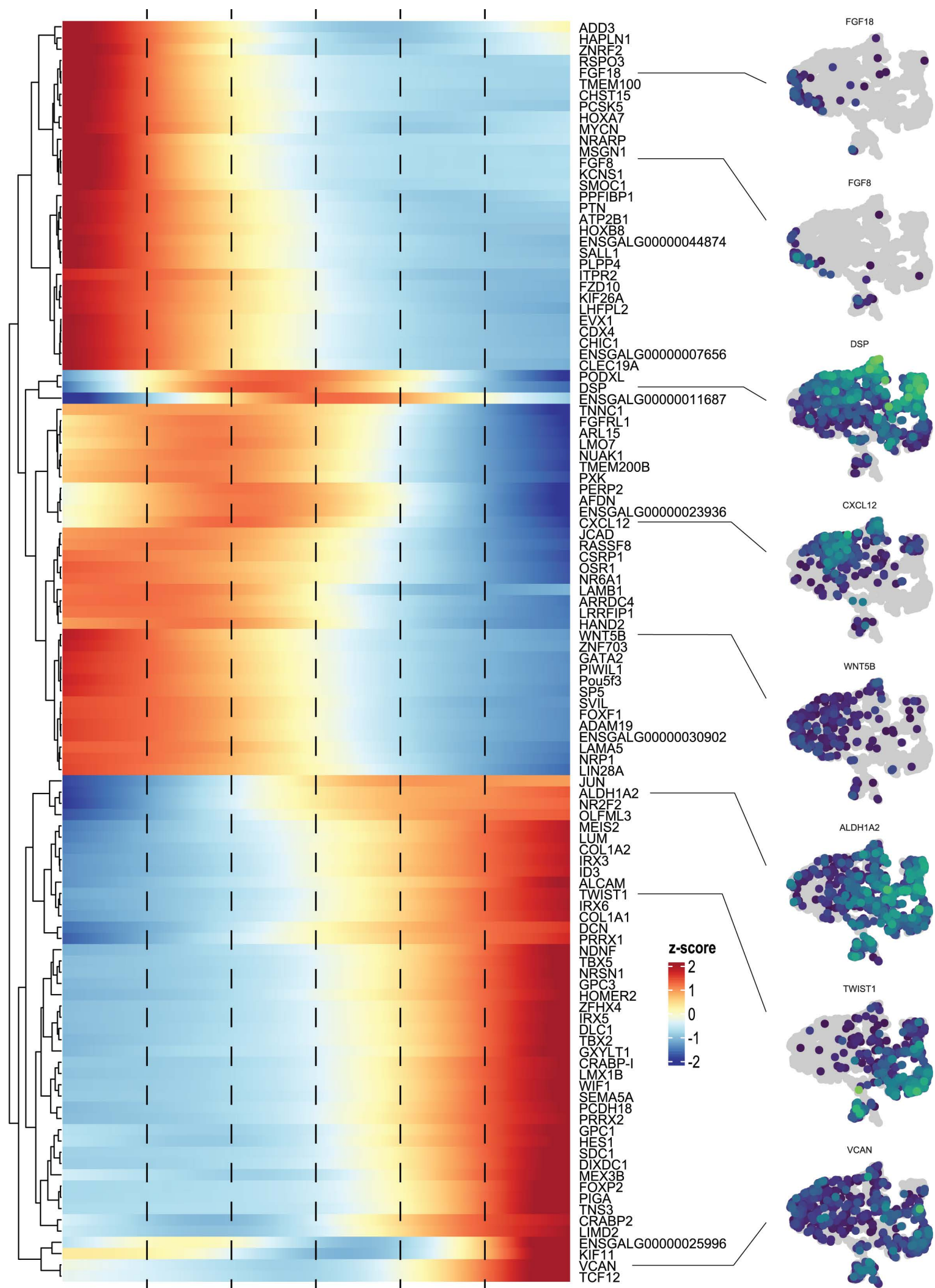


Fig. S3. Somatic LPM expression dynamics.

Extended pseudotime heatmap of significant differentially regulated genes along the somatic LPM lineage, with select expression plots. Bottom half of the heatmap revealed modules of genes that are specifically activated during somatic LPM specification and subsequent limb bud differentiation. The full list of significant genes are found in table S4.

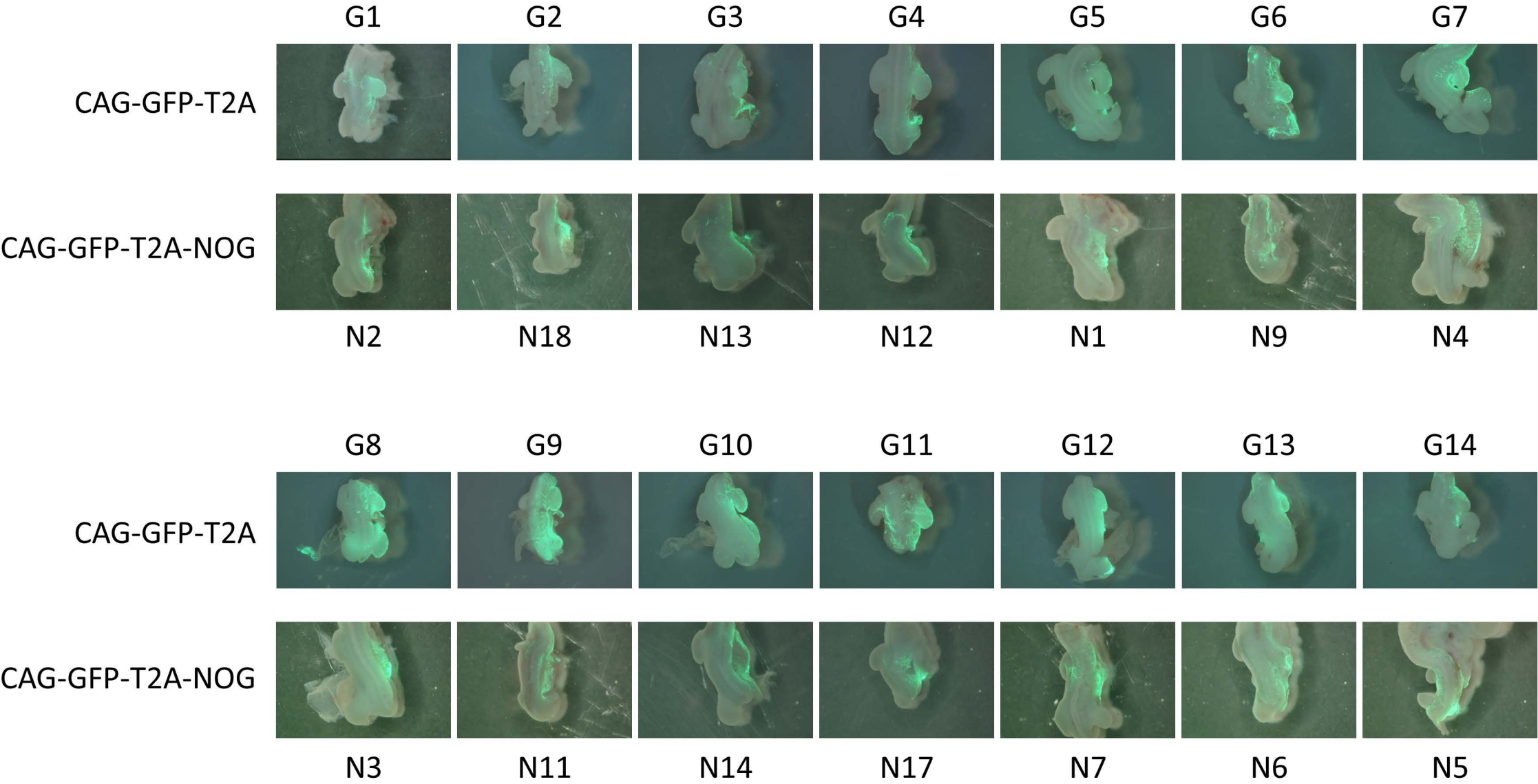


Fig. S4. BMP antagonism inhibits forelimb outgrowth.

Extended images from Figure 5, showing the effect of Noggin on limb development compared with GFP controls. Note, in instances where GFP-T2A-NOG field is extended posteriorly, hindlimbs additionally fail to develop.

Table S1. Global limb field cluster differentially expressed genes.

[Click here to download Table S1](#)

Table S2. LPM-specific cluster differentially expressed genes.

[Click here to download Table S2](#)

Table S3. Splanchnic LPM lineage significant spatially correlated genes.

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Table S4. Somatic LPM lineage significant spatially correlated genes.

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Table S5. Antibodies and plasmids used in the study.

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