Supplementary Note 1

We first sought to apply Monocle 3 to a single major cell type, cluster 25, whose 26,559 cells we annotate as limb bud mesenchyme on the basis of *Hoxd13, Fgf10* and *Lmx1b* expression (**Supplementary Table 4**). Visualizing the trajectory of cells of this cluster illustrates the dramatic expansion of limb mesenchymal cells over developmental time, with the main outgrowth between E10.5 and E12.5 (**Extended Data Fig. 7a**). Gene expression is highly dynamic during this expansion, with the levels of 4,763 protein-coding genes changing (FDR of 1%; **Supplementary Table 7**). The early stages of limb mesenchyme development are characterized by expression of some expected genes such as *Tbx15*[1], and *Gpc3*[2] and the later stages by *Msx1*[3], *Epha4*[4] and *Dach1*[5] (**Extended Data Fig. 7b**), but the vast majority of dynamically expressed genes are novel. Transcription factors significantly upregulated during limb mesenchyme development included those with roles in chondrocyte differentiation (*e.g. Sox9*[6] and *Yap1*[7]), muscle differentiation (*e.g. Tead4*[8]), and wound healing and limb regeneration (*e.g. Smarcd1*[9]) (**Extended Data Fig. 7c**).

Interestingly, forelimb and hindlimb cells were not obviously separated by unsupervised clustering (**Extended Data Fig. 7d**) or trajectory analysis (**Extended Data Fig. 7e**), but could be distinguished by the mutually exclusive expression of *Tbx5* in forelimb (2,085 cells, 7.9% of all limb mesenchyme cells) and *Pitx1* in hindlimb (1,885 cells, 7.1% of all limb mesenchyme cells) with only 22 cells expressing both markers (0.08% of all limb mesenchyme cells vs. ~0.6% expected if they were independent; **Extended Data Fig. 7f**)[10]. 285 genes were differentially expressed between cells assigned to the forelimb and hindlimb in this way (**Extended Data Fig. 7g**, **Supplementary Table 8**). Known marker genes such as *Tbx4* and the genes of the Hoxc cluster (*Hoxc4-10*)[11] were upregulated in hindlimb cells as expected, but we also identified genes not previously shown to be differentially expressed. For example, we observed *Epha3* and *Hs3st3b1* to be 5-fold enriched in forelimb, and *Pcdh17* and *Igf1* to be 3-fold enriched in hindlimb.

Although developmental time is a major axis of variation in the limb mesenchyme trajectory (**Extended Data Fig. 7a**), there is clearly additional structure. At least some of this appears to correspond to the two main spatial axes of limb development: the proximal-distal axis (the primary direction of outgrowth) and the anterior-posterior axis (corresponding to the five digits)[10]. With Monocle 3, we applied Moran's I test[12] to detect genes exhibiting autocorrelation across the limb mesenchyme trajectory (*i.e.* genes expressed in similar regions of the principal graph). We found, for example, that cells expressing *Sox6* and *Sox9* (proximal markers)[13,14], *Hoxd13* and *Tfap2b* (distal markers)[15], *Pax9* and *Alx4* (anterior markers), and *Shh* and *Hand2* (posterior markers), were differentially distributed across the trajectory (**Extended Data Fig. 7h**, **Extended Data Fig. 7i**). Whole-mount *in situ* hybridization of *Hoxd13* (a known distal marker) and *Cpa2* (a novel marker whose distribution in the Monocle 3 trajectory was similar to that of known distal markers), confirmed that both genes are expressed in the distal limb mesenchyme between E10.5 and E13.5 (**Extended Data Fig. 7j-l**). Altogether, we identified 1,783 genes exhibiting variable expression across the limb mesenchymal trajectory (FDR of 1%; Moran's I > 0.01). These genes clustered into eight patterns of expression, several of which matched the distributions of known markers for the proximal-distal and anterior-posterior axes (**Extended Data Fig. 7m**, **Supplementary Table**

9). These analyses illustrate how this single cell atlas of mouse organogenesis can be used to characterize the spatiotemporal dynamics of gene expression in specific systems.

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