

Supplementary Materials for  
**TATTOO-seq delineates spatial and cell type–specific regulatory programs in  
the developing limb**

Sébastien Bastide *et al.*

Corresponding author: François Spitz, [fspitz@uchicago.edu](mailto:fspitz@uchicago.edu)

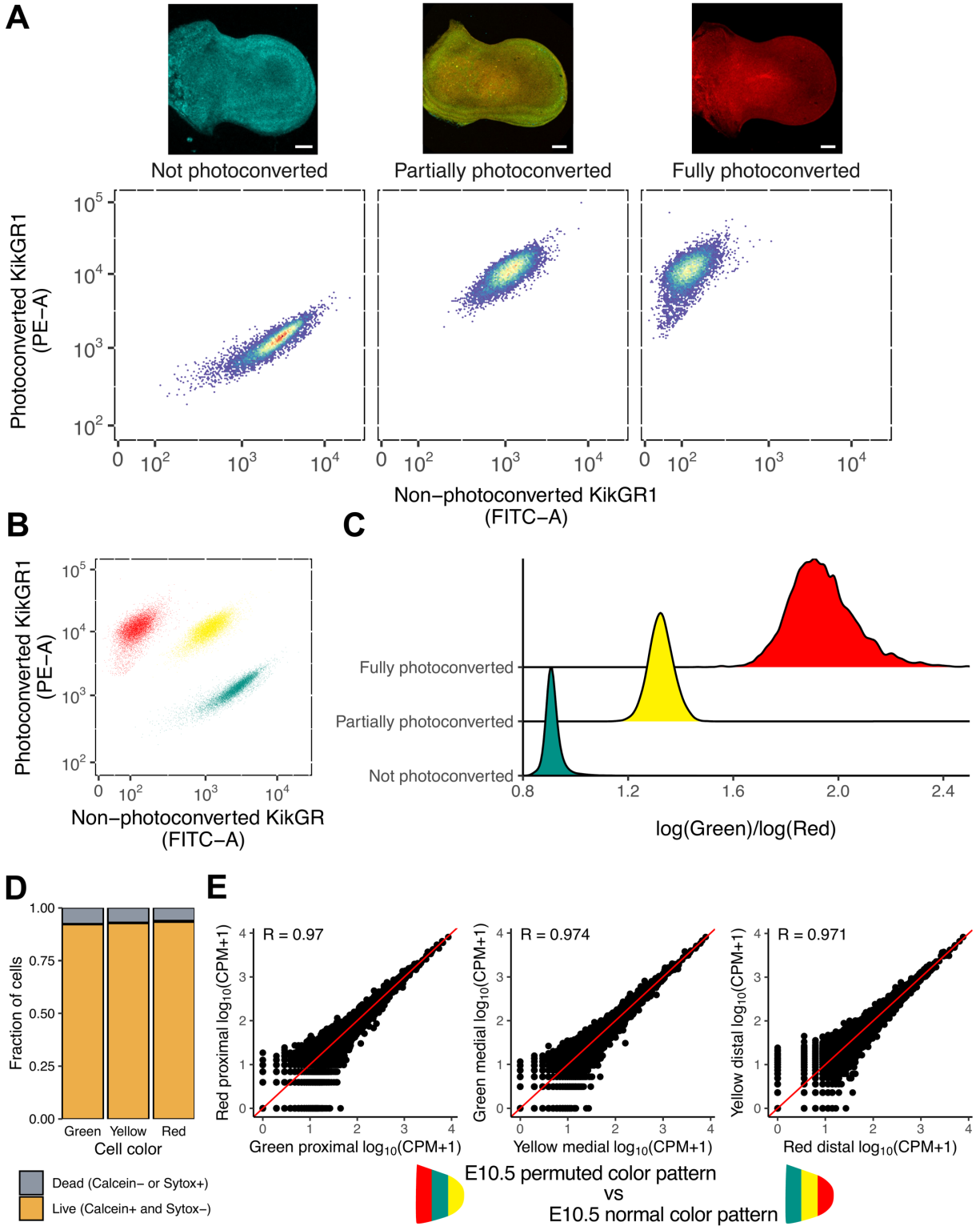
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**The PDF file includes:**

Figs. S1 to S18  
Legends for tables S1 to S5  
References

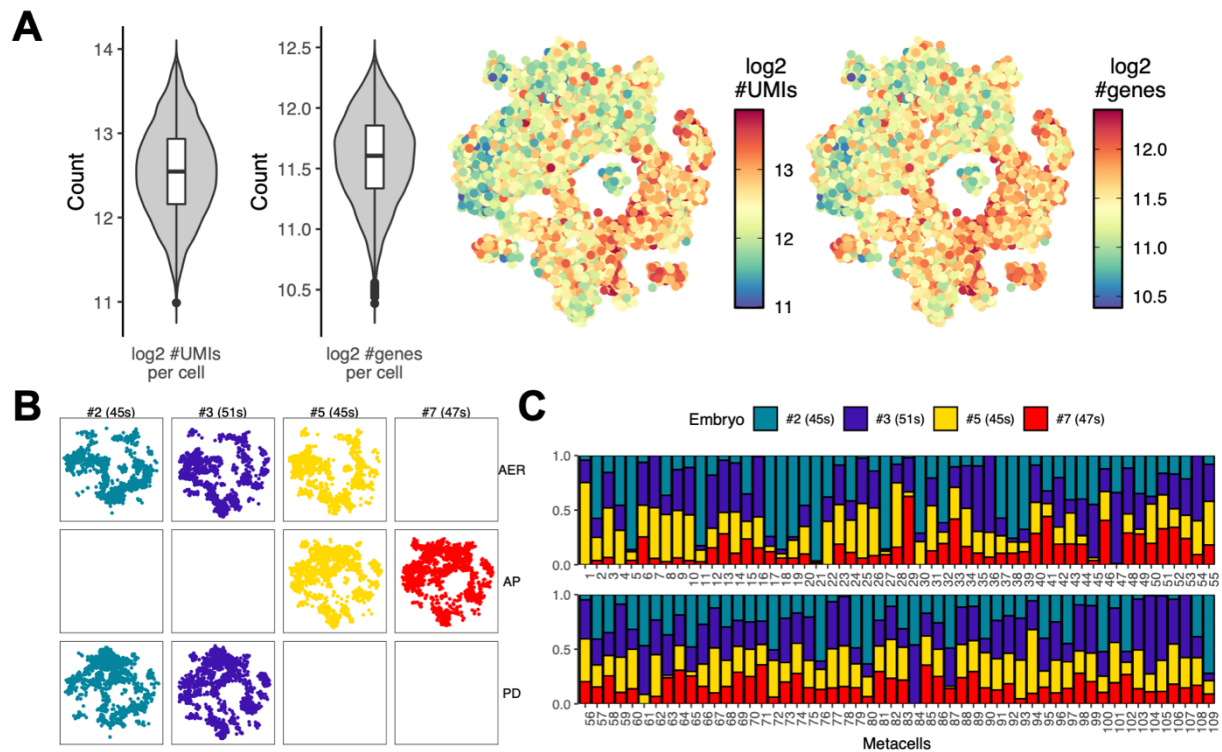
**Other Supplementary Material for this manuscript includes the following:**

Tables S1 to S5



**Fig. S1 Assessing the photoconversion efficiency and impact of the TATTOO-seq method on cell viability and transcriptome.**

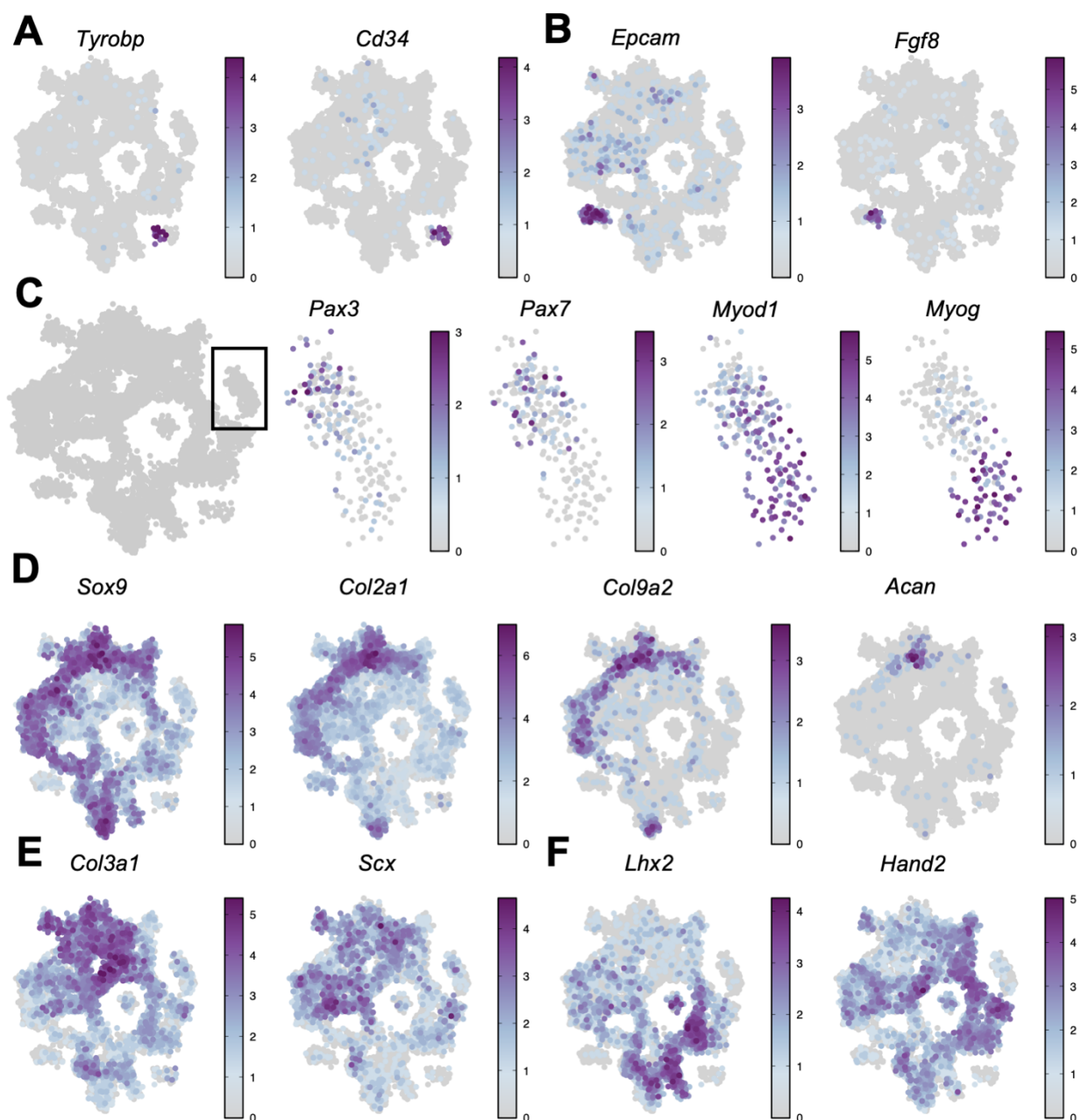
**(A)** Homogeneous distribution of green and red fluorescence in non-photoconverted, partially photoconverted and fully photoconverted whole limbs show the efficiency of photoconversion along the dorsal-ventral axis of the limb bud. Pictures of the corresponding limbs are shown above the flow cytometry plots. Scale bars = 100  $\mu\text{m}$ . **(B)** Overlay of the three samples depicted in **(A)** show no overlap between the three colors. **(C)** Ridge plot of the ratio of the log-green and red fluorescence. **(D)** Fraction of live and dead cells (using Calcein and Sytox) assessed by flow cytometry for each color. The fraction of dead cells is independent from the color. **(E)** Comparison of aggregated gene expression for different degrees of photoconversion on the same spatial compartment in E10.5 wild-type mouse limbs processed with TATTOO-seq.



**Fig. S2 Quality check for the TATTOO-seq data.**

(A) Violin and 2D projection showing the distribution of the number of genes and number of UMIs detected per cell. The number of detected genes and UMIs is slightly higher in distal cells which are more actively proliferating. Median UMI count per cell = 5980, median number of detected genes = 3115. (B) 2D projection split by sample (columns) and photoconversion pattern (rows). (C) Bar chart showing the contribution of each embryo to each metacell.

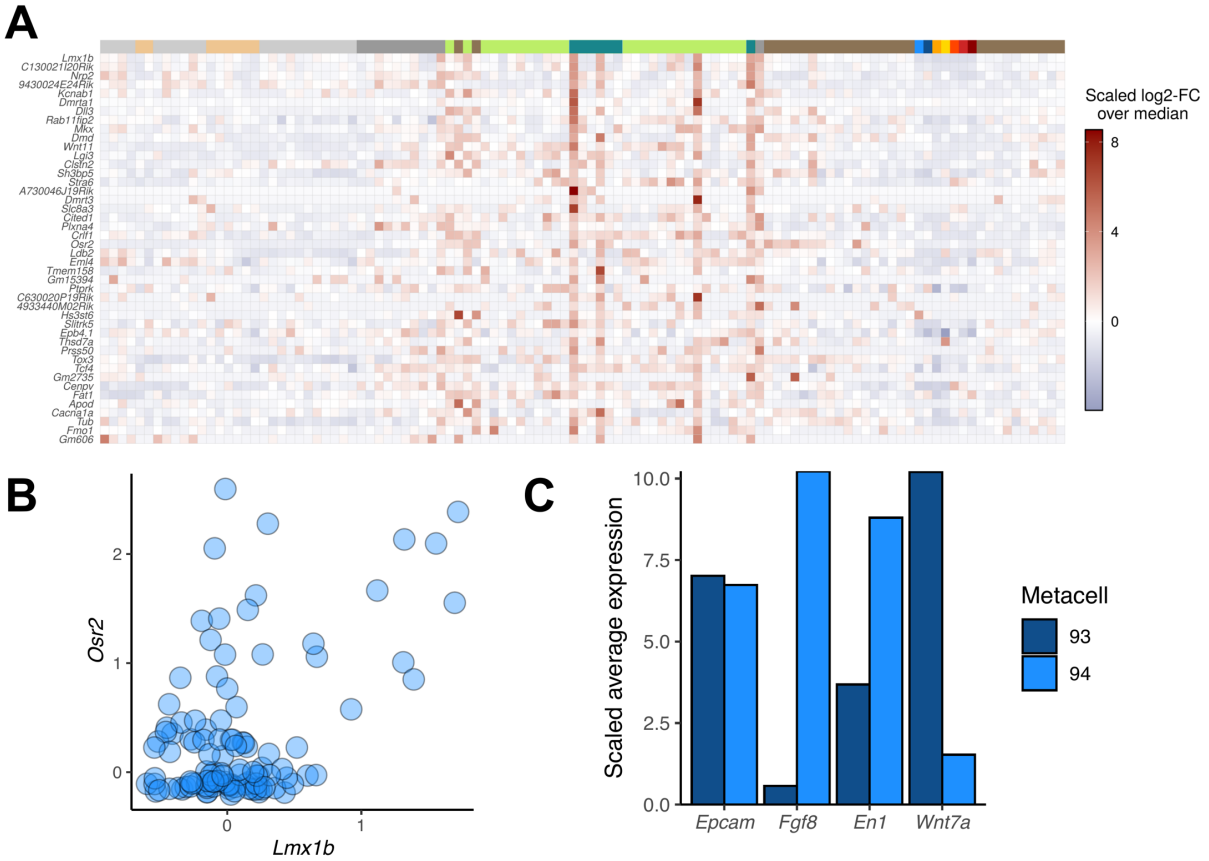




**Fig. S3 Expression of cell type specific marker genes.**

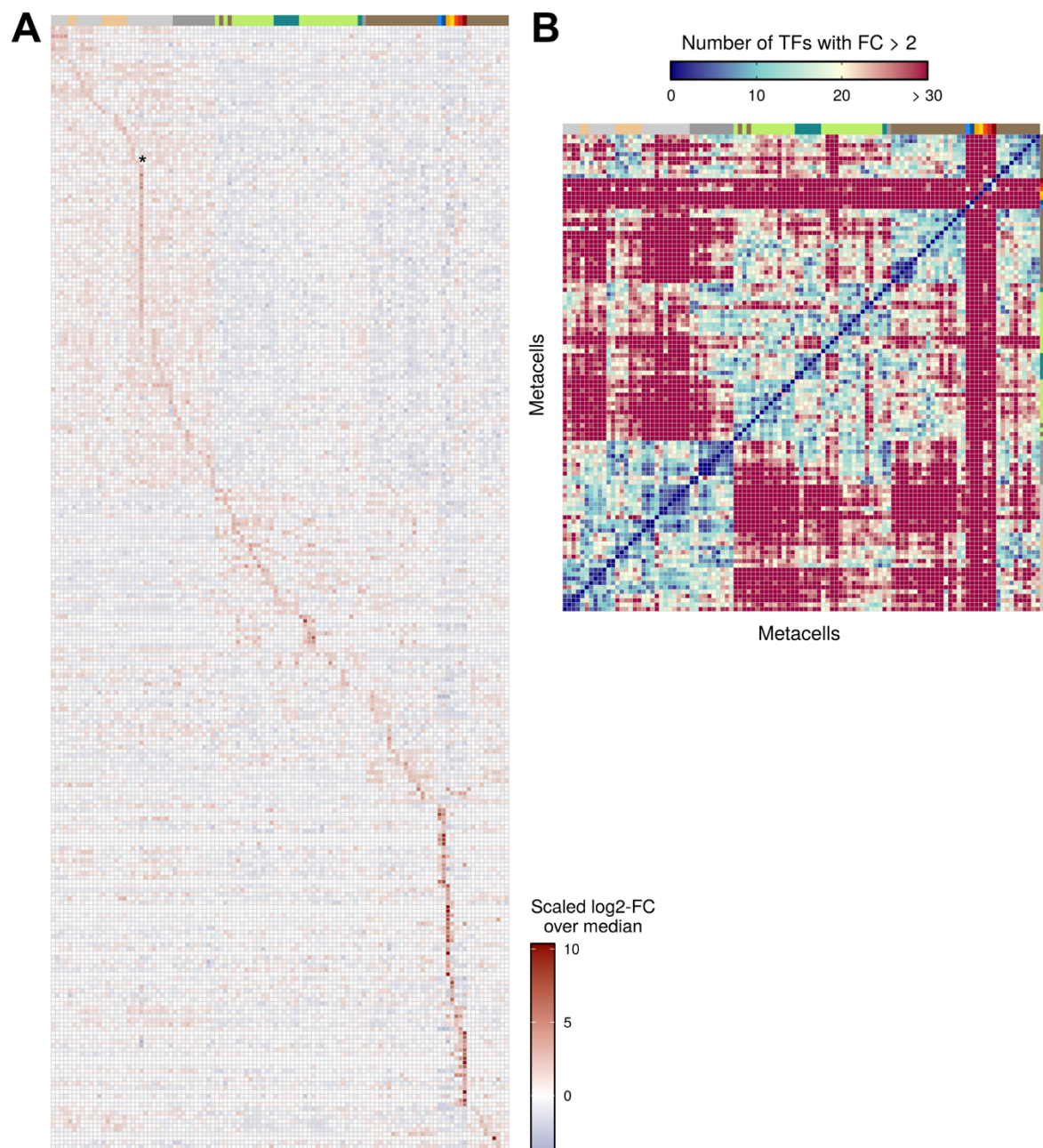
2D projection of the metacell graph. Cell color represents  $\log_{10}$  depth-normalized counts for marker genes of (A) immune and endothelial cells, (B) epithelial cells (*Epcam*<sup>+</sup>) are divided into two metacells: the *Fgf8*-expressing AER and the *Fgf8*-negative dorsal and ventral ectoderm, (C) three myogenic progenitor cell states corresponding to different stages of differentiation are detected, from *Pax3*-expressing progenitors migrating from the somites into the limb bud, to *Myod1*-expressing early differentiating myoblasts and *Myog*-expressing differentiated myocytes,

**(D)** chondrogenic progenitors, **(E)** dense regular connective tissue progenitors and **(F)** different populations of undifferentiated mesenchyme.



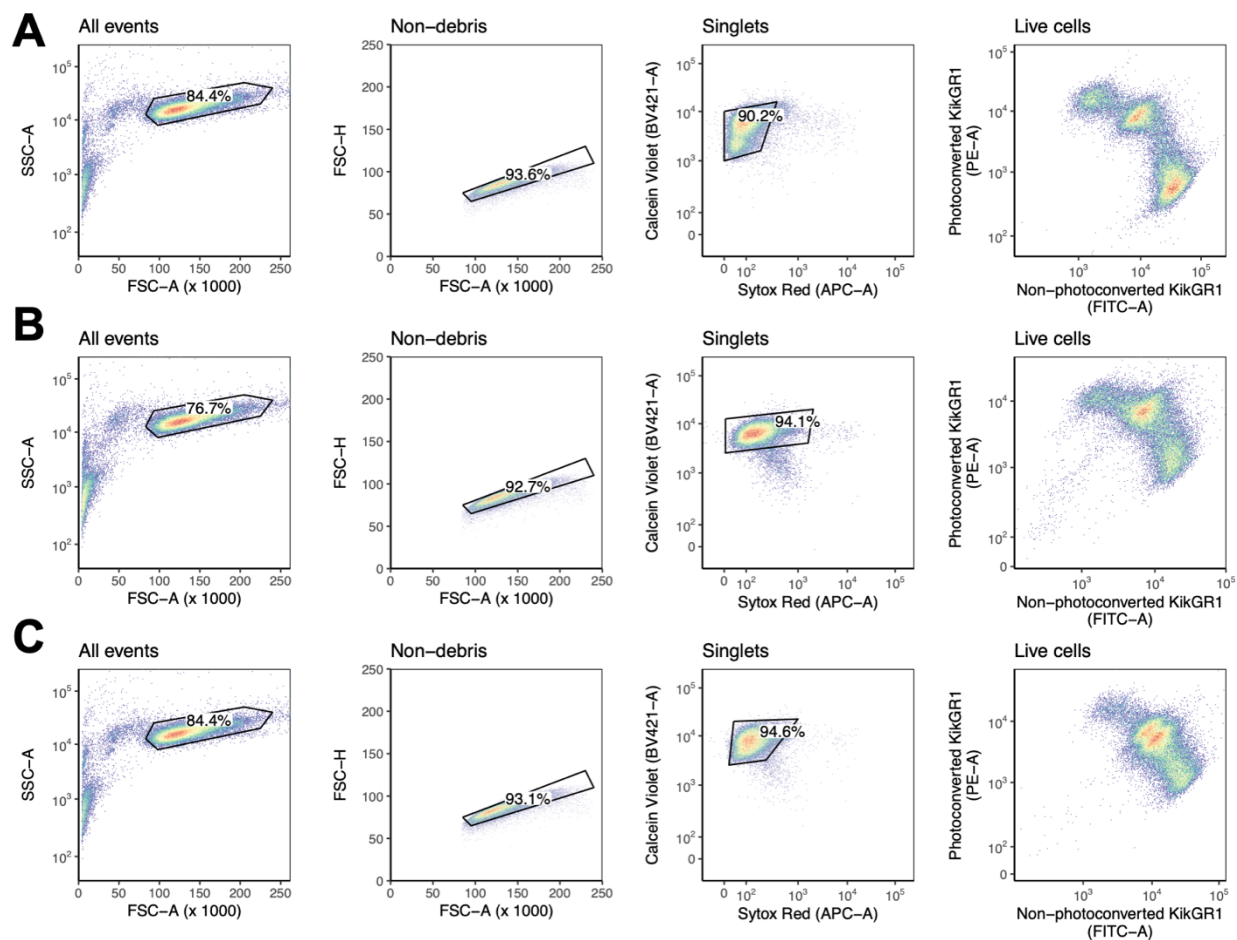
**Fig. S4 DV organization of the limb cell states.**

(A) *Lmx1b* was used as a marker of the dorsal mesenchyme (81). Heatmap showing the normalized expression of genes whose expression is loosely correlated or anti-correlated to that of *Lmx1b* ( $|R| > 0.4$ ). (B) Scatter plot showing the expression of *Lmx1b* and *Osr2* for all metacells. Metacells exhibiting high levels of *Lmx1b* showed high levels of *Osr2*, a key regulator in connective tissue differentiation, suggesting that connective-tissue differentiation at this stage is preferentially occurring dorsally, as suggested by *Osr2 in situ* experiments (82). (C) Barplot of the expression of the AER marker *Fgf8*, the ventral marker *En1* and the dorsal marker *Wnt7a* in the *Epcam*<sup>+</sup> ectodermal metacells.



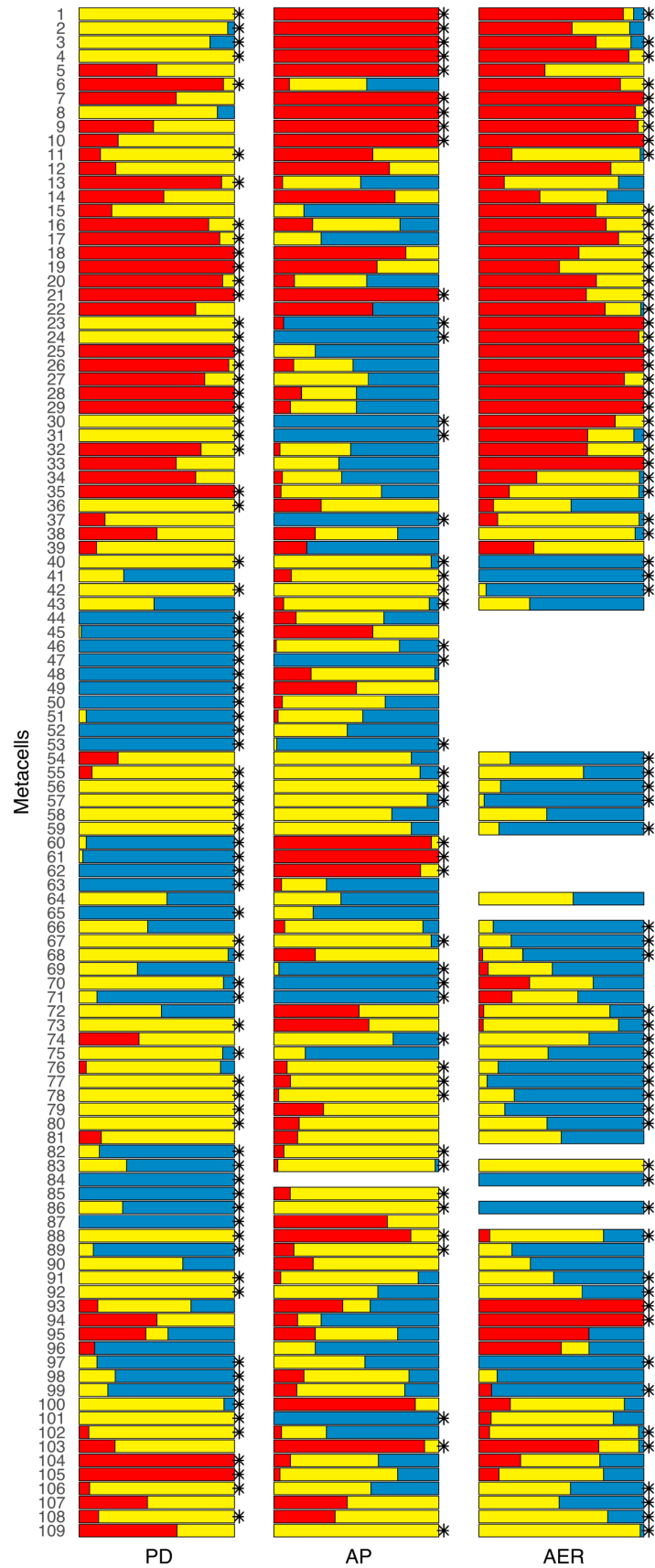
**Fig. S5 Metacells exhibit a hierarchical and specific combinatorial code of TFs.**

**(A)** Heatmap showing the normalized expression of genes with loosely specific gene expression ( $AUC > 0.5$ ) across all metacells. \* marks metacell 22 which groups cells of overall lower quality. **(B)** Heatmap showing the pairwise number of TFs that show different expression levels ( $FC > 2$ ). Cell types are annotated using the same color code as in **Fig. 1**.



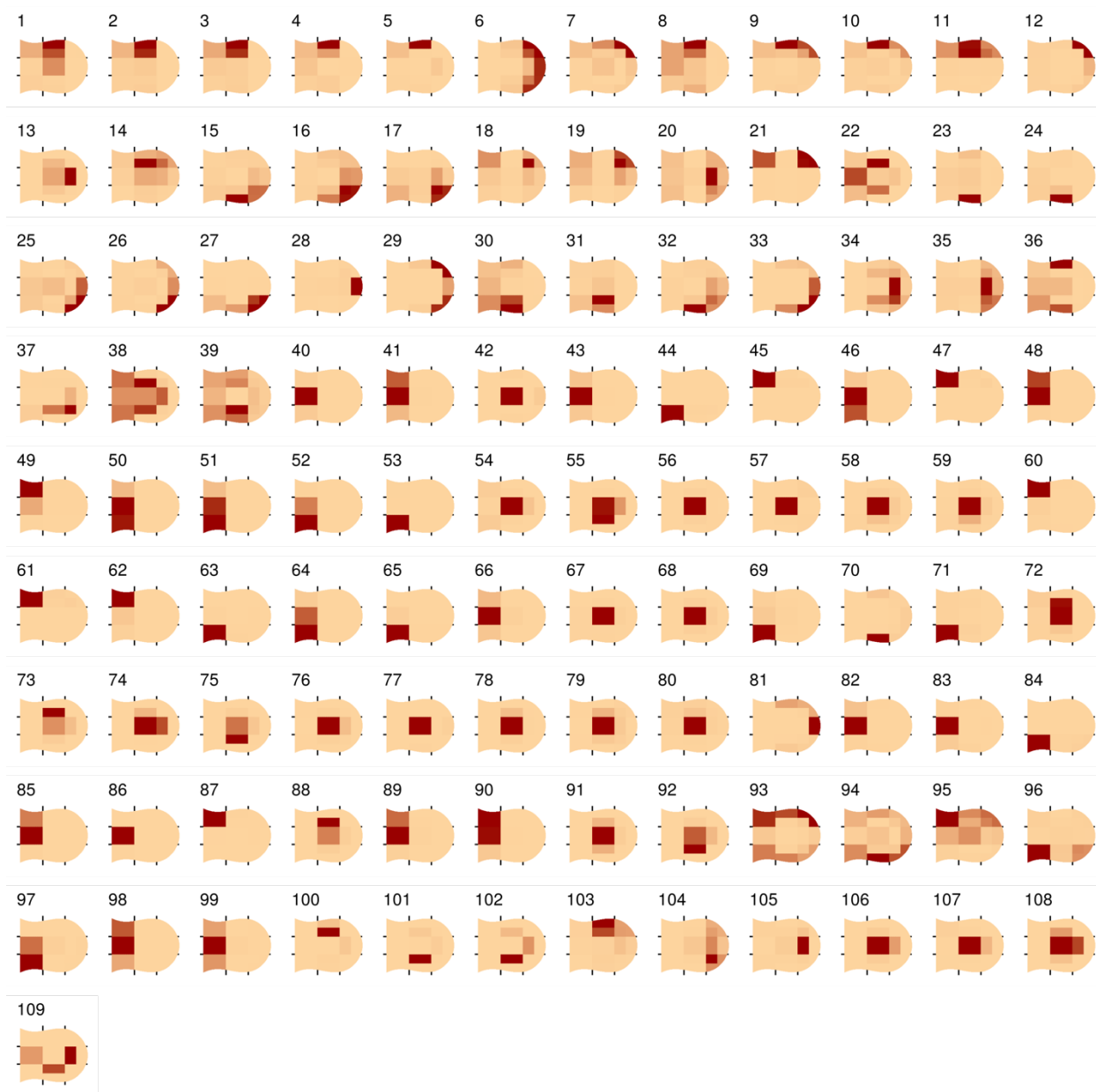
**Fig. S6 Gating strategy for TATTOO-seq.**

The gating strategy used for the PD (A), AP (B) and AER (C) photoconversion patterns. Debris are first filtered out using SSC-A vs FSC-A, then singlets are recovered by looking at FSC-H vs FSC-A. Finally, live cells are recovered by inspecting Calcein Violet vs Sytox Red. Non-green and non-red cells are then excluded. No further gating on Red vs Green is performed.



**Fig. S7 Metacells are spatially homogeneous.**

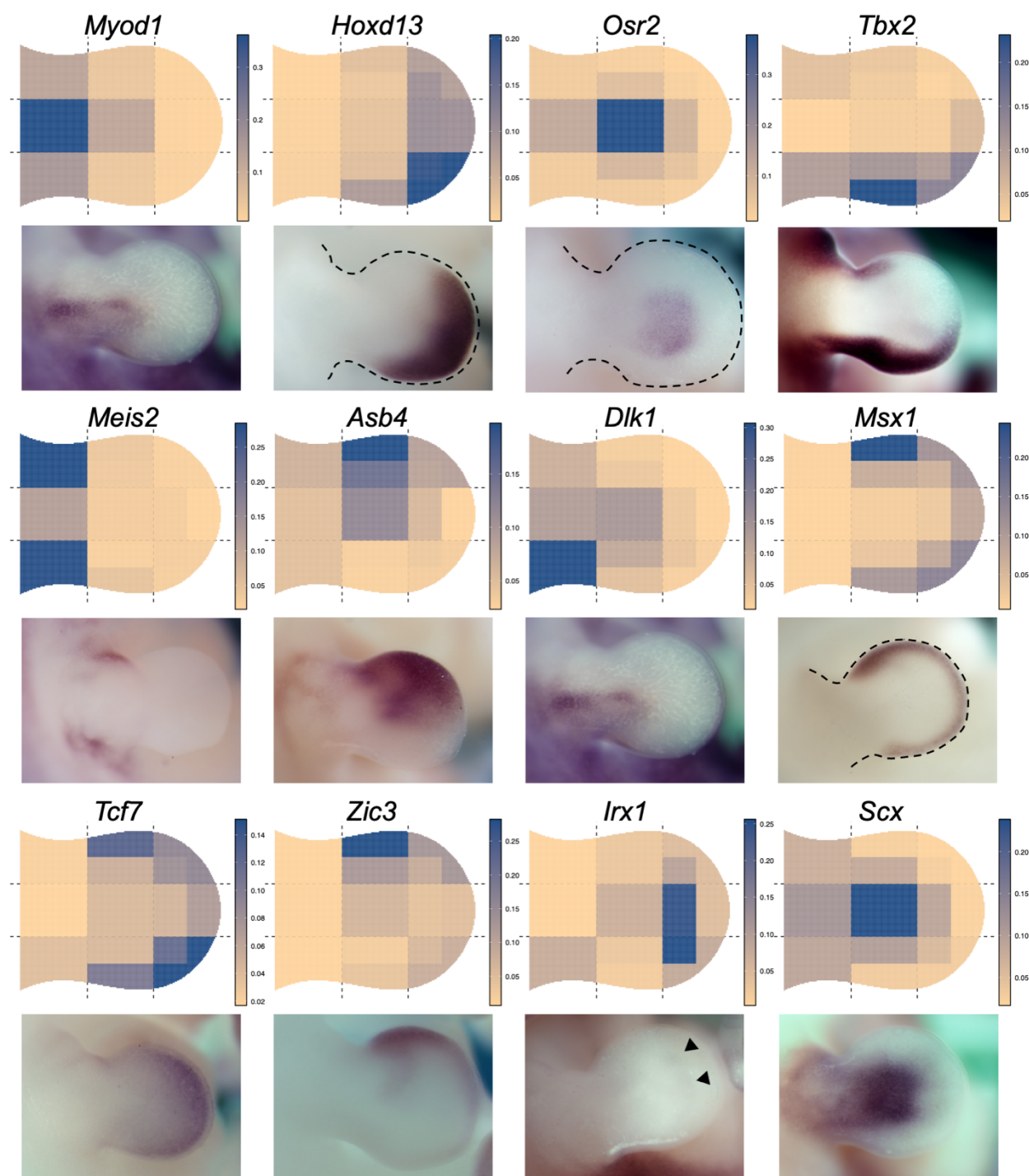
We employed Shannon entropy as a metric for measuring color heterogeneity. Monte Carlo simulations ( $n = 100000$ ) were used to test for positional homogeneity. Stacked bar plots showing the distribution of color for each metacell in all three photoconversion patterns (PD, AP and AER). Clusters labeled with a star (\*) display significantly lower Shannon entropy than expected by chance (Bonferroni corrected p-value  $\leq 0.01$ ).



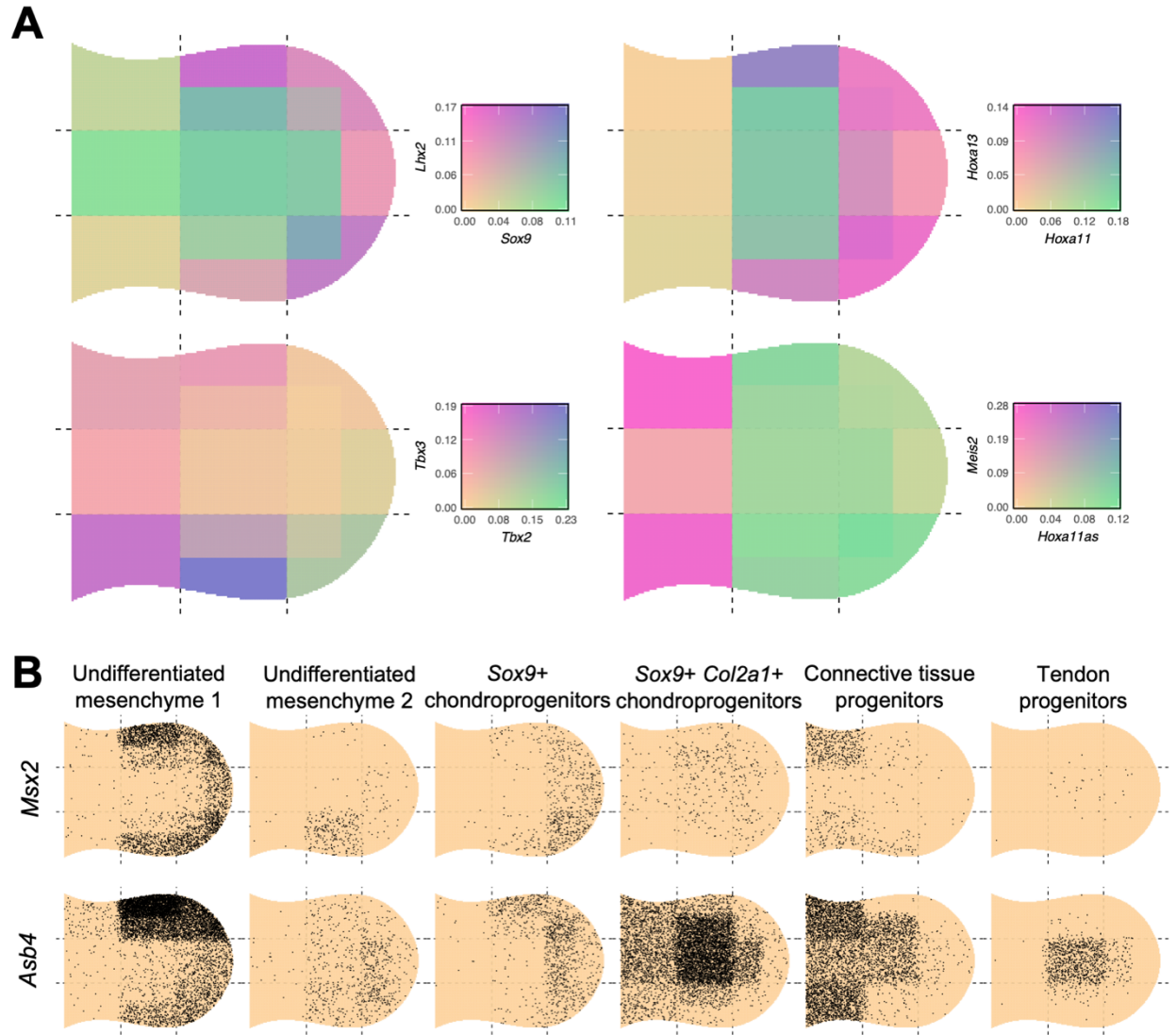
**Fig. S8 Mouse limb bud metacell positions.**

Spatial distribution for all mouse limb bud metacells.





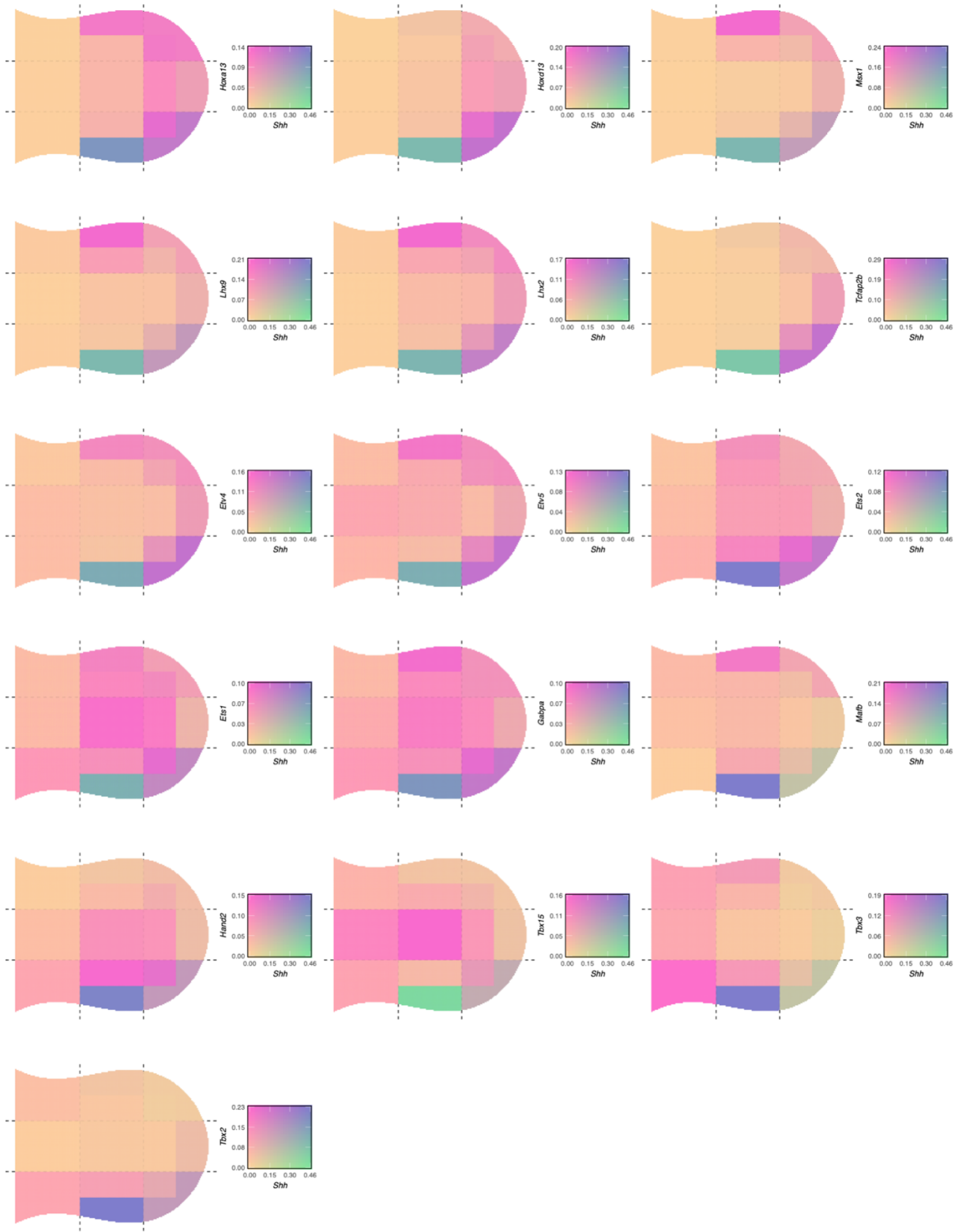
**Fig. S9 vISH match the gene expression patterns obtained by classical *in situ* hybridization.** vISH and the corresponding classical ISH patterns for twelve TFs involved in limb development. ISH images were obtained from the EMBRYO database (79).



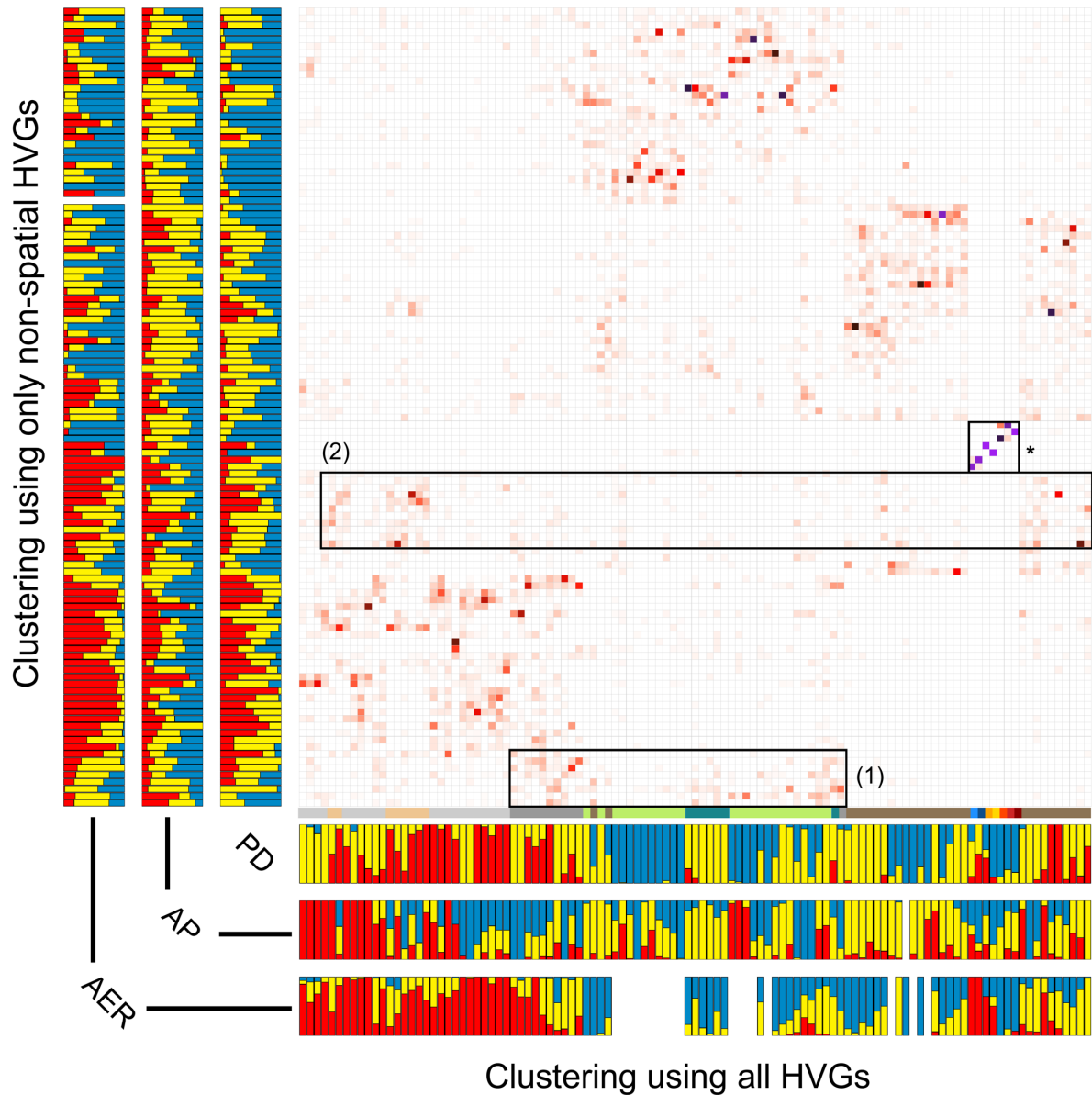
**Fig. S10 Double virtual *in situ* hybridization (vISH) and cell type-specific vISH.**

**(A)** Double vISH uses a bivariate color scale to represent the spatial overlap of expression patterns.

**(B)** vISH can be deconvolved into cell type-specific vISH. To take into account the different dynamic ranges across cell types, up to 10,000 UMIs are sampled according to the expected number of counts for each spatial bin.



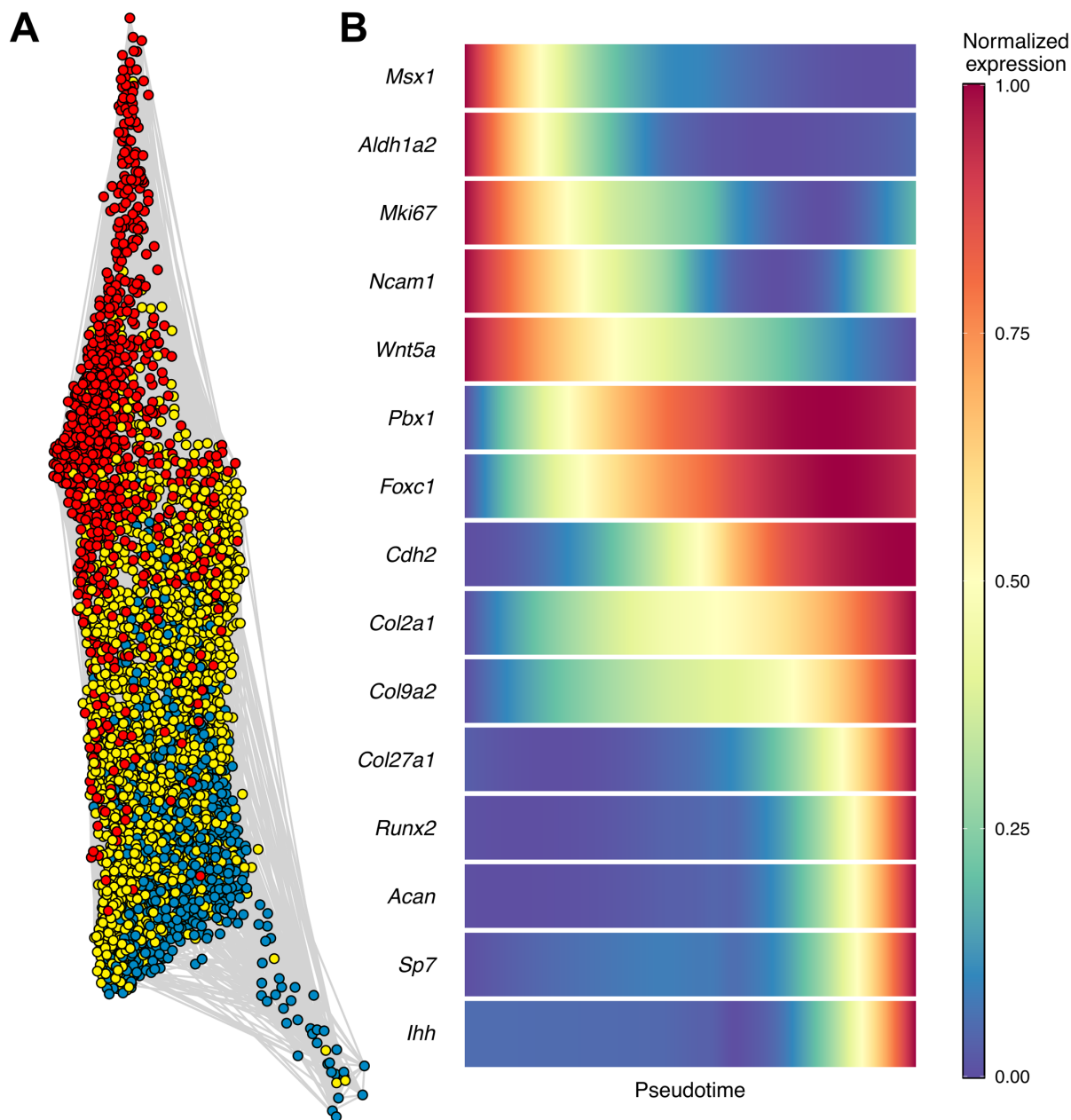
**Fig. S11 Spatial domains of expression of potential regulators of *Shh* expression in the ZPA. Double vISH for *Shh* and transcription factors shown in Fig. 2.**



**Fig. S12 Confusion matrix between the two dataset clusterings.**

Confusion matrix representing the redistribution of cells between the clustering using all the highly variable genes and the clustering using only non-spatial highly variable genes. Darker values (black, purple) indicate high correspondence between metacells of the two clusterings. The color distribution is indicated on the side of the heatmap. Non-mesenchymal clusters are unaffected (\*) while some mesenchymal clusters are split and merged. Some no-HVGs metacells clusters receive contribution from type 2 undifferentiated mesenchyme and dense regular connective tissue progenitors metacells (1), and from distal chondrogenic progenitors and proximal *Sox9<sup>+</sup> Col2a1<sup>+</sup>*

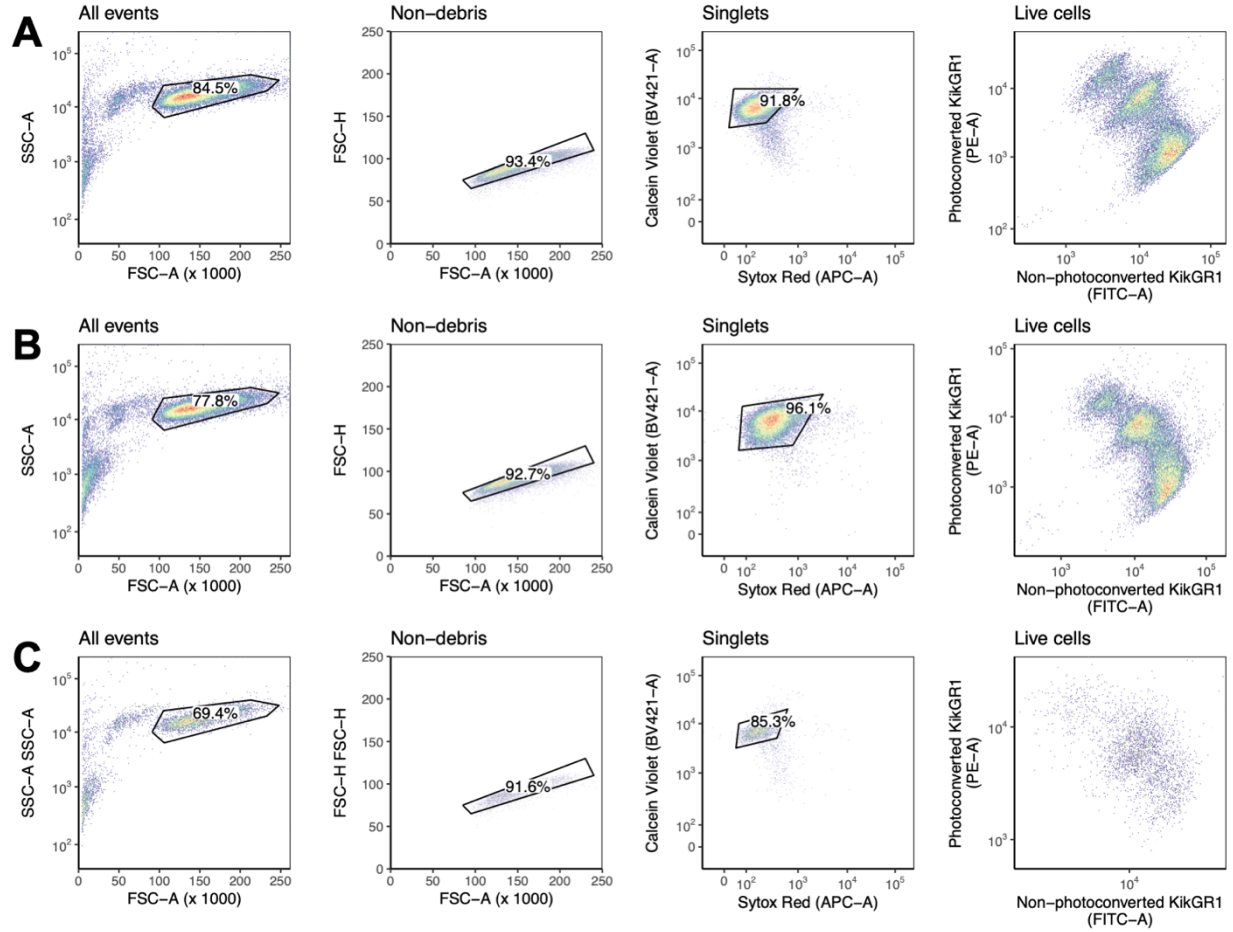
chondroprogenitors (2). Blacklisting of spatial HVGs additionally results in extensive cell type internal reorganization. Abrogating spatial information potentially leads to artefactual clustering if cell types are homogeneous.



**Fig. S13 Eliminating confounder effects of cell position reveals overlapping chondrogenic transcriptomic states.**

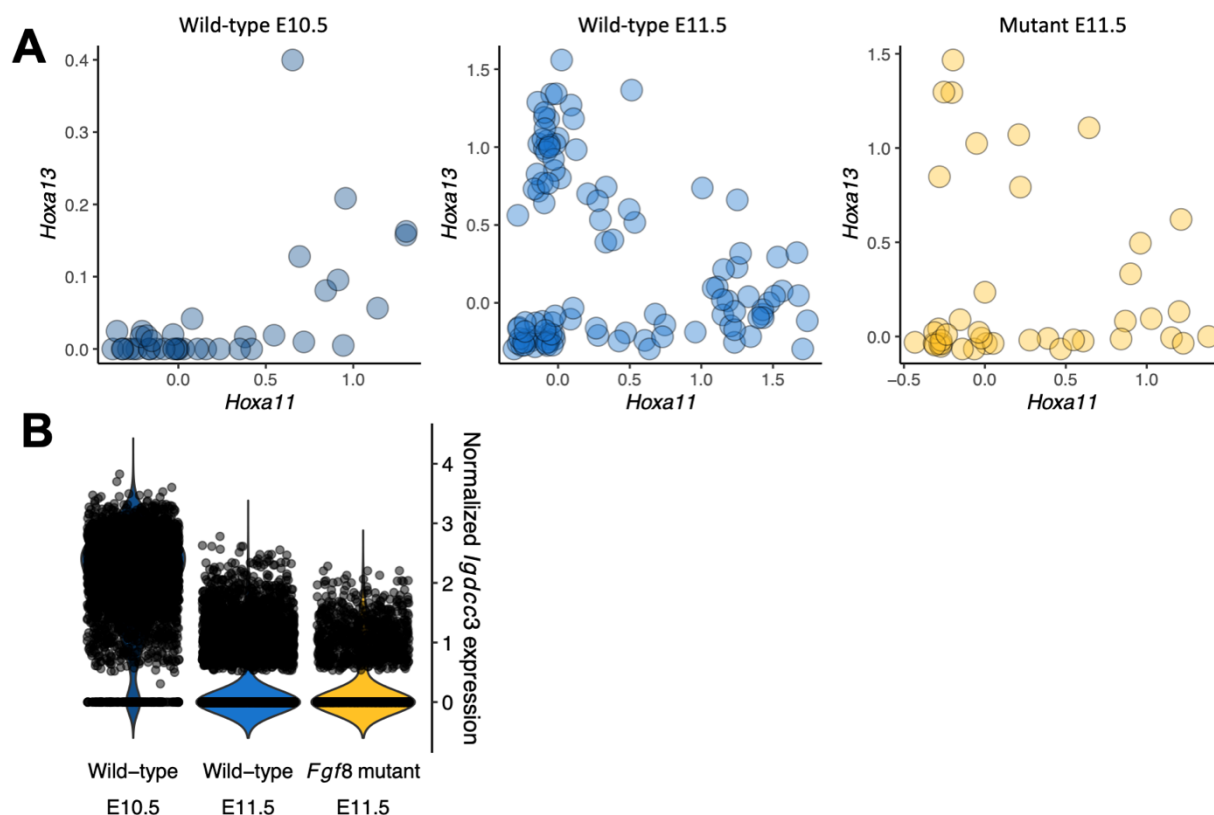
**(A)** 2D projection of the k-NN ( $k = 10$ ) graph of chondrocytes using the force-directed Distributed Recursive (Graph) Layout algorithm. The procedure used to generate the graph is described in the **methods** section. **(B)** Pseudotime was assessed using ELPiGraph (77) and gene expression as a function of pseudotime was estimated and smoothed using a loess regression for various stage-specific chondrogenesis markers.





**Fig. S14 Gating strategy for TATTOO-seq on mutant embryos.**

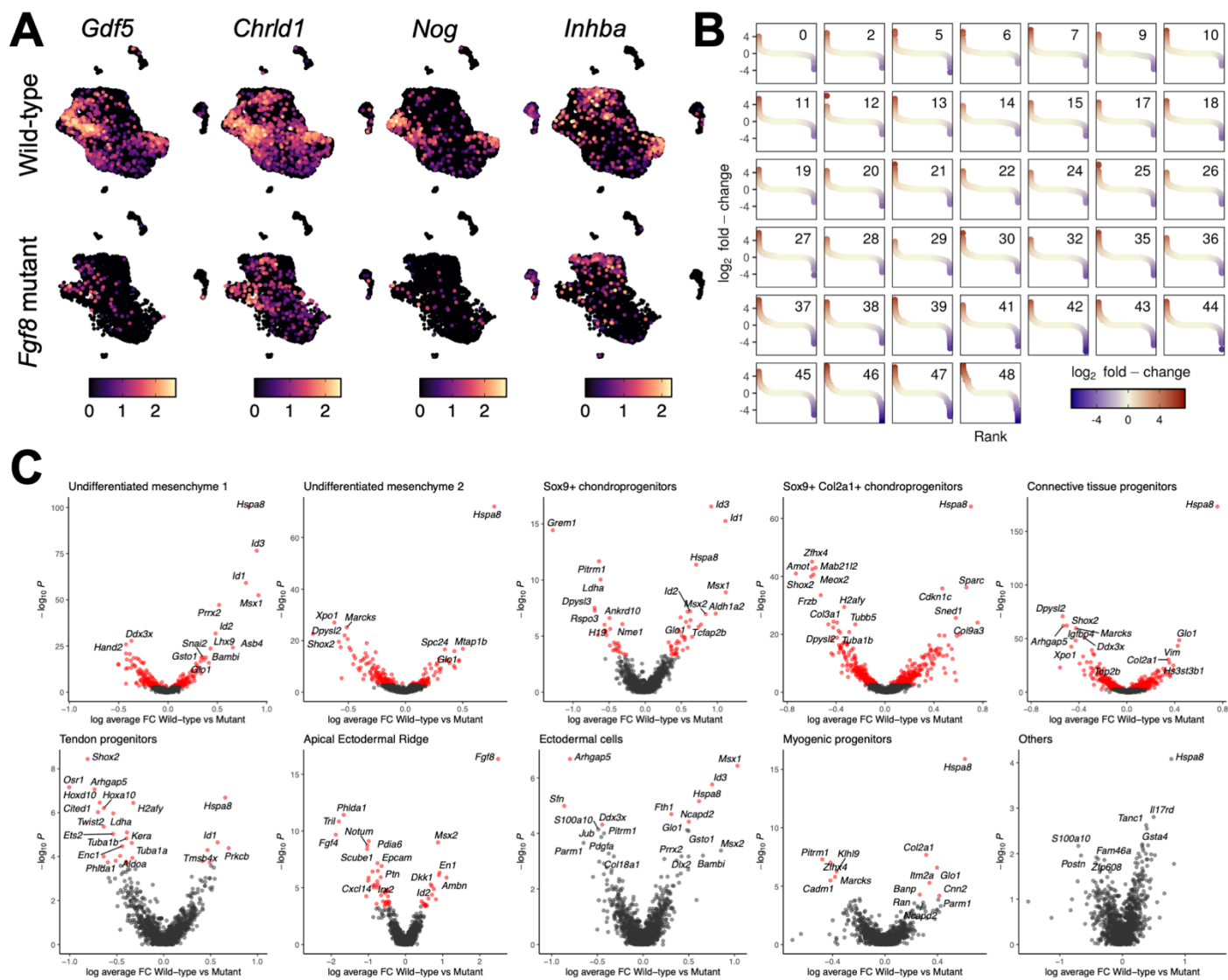
The gating strategy used for the PD (A), AP (B) and AER (C) photoconversion patterns. Debris are first filtered out using SSC-A vs FSC-A, then singlets are recovered by looking at FSC-H vs FSC-A. Finally, live cells are recovered by inspecting Calcein Violet vs Sytox Red. Non-green and non-red cells are then excluded. No further gating on Red vs Green is performed.



**Fig. S15 Differences between wild-type and *Fgf8* mutant cells do not reflect a global developmental delay in mutant limbs.**

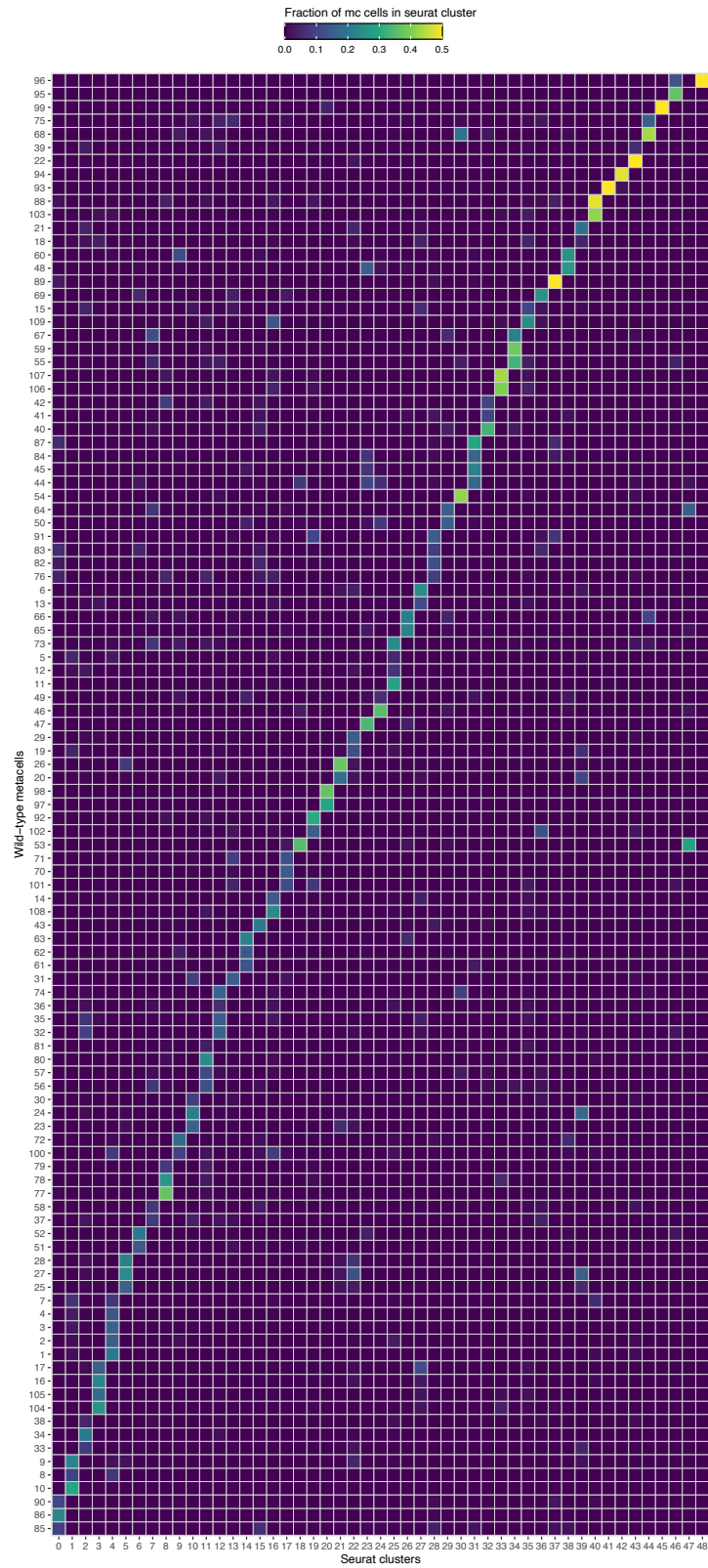
**(A)** Scatter plot showing the normalized expression of *Hoxa13* and *Hoxa11* in E10.5 and E11.5 wild-type metacells and E11.5 mutant metacells. In the mutant limbs, *Hoxa13* and *Hoxa11* were expressed in distinct cell-states, corresponding to the largely mutually exclusive territories observed in wild-type E11.5 limbs, and contrasting with the nested expression found earlier at E10.5 (78, 83). **(B)** Violin plot showing the log<sub>10</sub> depth-normalized expression of *Igdcc3* in E10.5 and E11.5 wild-type cells and E11.5 mutant cells. *Igdcc3* has been reported to be a sensitive temporal marker of limb development, showing strong expression at E10.5 but minimal expression at E11.5 (84). We found its expression greatly reduced in both wild-type and mutant E11.5 scRNA-seq data, while it is robustly detected in E10.5 samples.





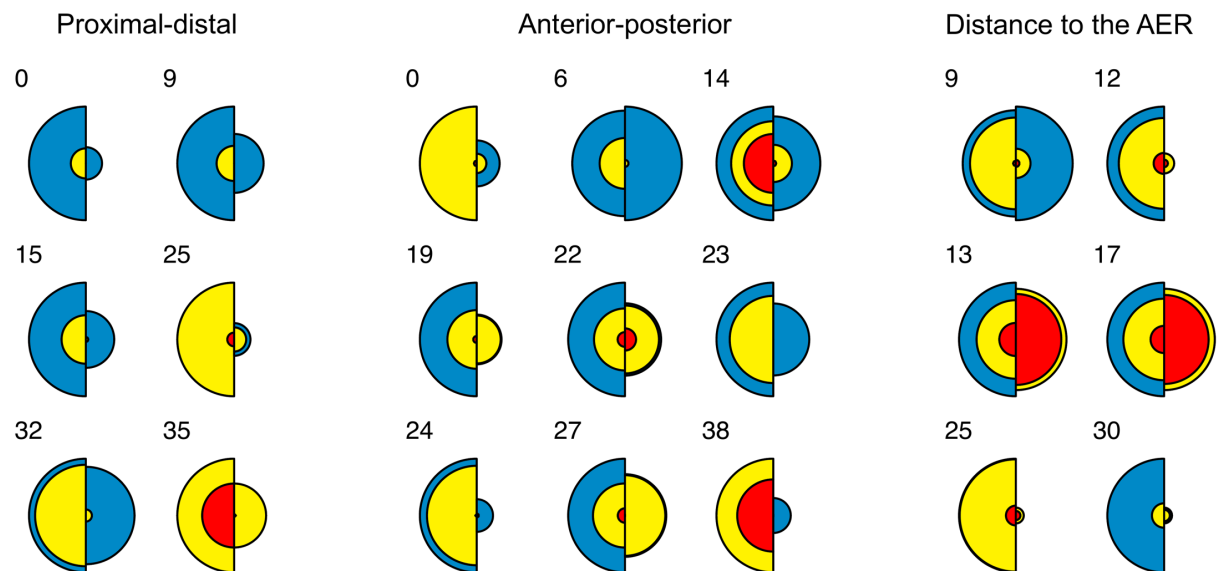
**Fig. S16 Transcriptional differences between wild-type and mutant limb cells.**

(A) UMAP showing  $\log_{10}$  depth-normalized counts for some genes associated with the TGF $\beta$  superfamily signaling showing a reduction of expression in mutant cells. The data is split by genotype: wild-type (top row) and *Fgf8* mutant (bottom row). (B) Plot showing the distribution of  $\log_2$  fold-changes between wild-type and mutant cells within each Seurat cluster. (C) Volcano plot showing the p-values of statistical tests for differential expression as a function of  $\log_2$  fold-change between wild-type and mutant cells in each cell type. Genes for which BH-corrected p-value < 0.01 are represented in red and genes names are indicated for the top 20 most significant genes.



**Figure S17 Correspondence between wild-type metacells and Seurat clusters**

Contingency table describing the relationship between wild-type metacells and Seurat clusters.  
Each cell shows the fraction of cell from a given metacell in each Seurat cluster.



**Fig. S18 Spatial distribution changes between wild-type and mutant limb cells.**

Circular histograms representing the distribution of colors for wild-type and mutant cells in Seurat clusters that show altered spatial distributions.

**Other Supplementary Materials for this manuscript include the following:**

Table S1. Gene expression in the metacells of the wild-type forelimb bud

Table S2. “Spatially regulated” genes in the forelimb bud

Table S3. Differentially accessible peaks in the *Sox9* regulatory domain

Table S4. Gene expression in the Seurat clusters comprising wild-type and DEL(PoIL-SHFM) forelimb cells

Table S5. Cell type-specific differential expression in DEL(PoIL-SHFM) mutant forelimbs

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