

Supplementary Information for

Immobilization secondary to cell death of muscle precursors with a dual transcriptional signature contributes to the emu wing skeletal pattern

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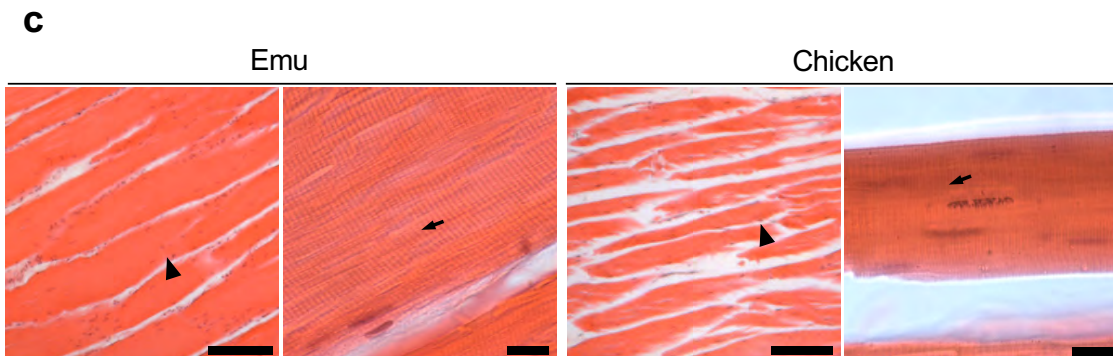
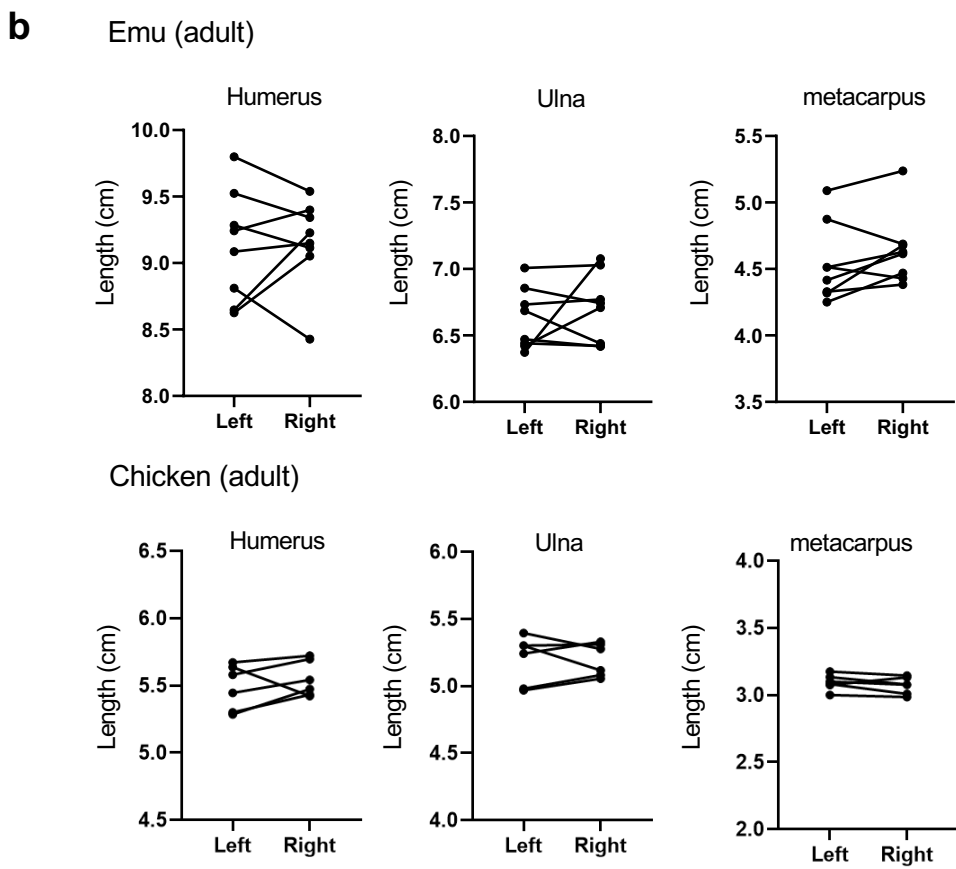
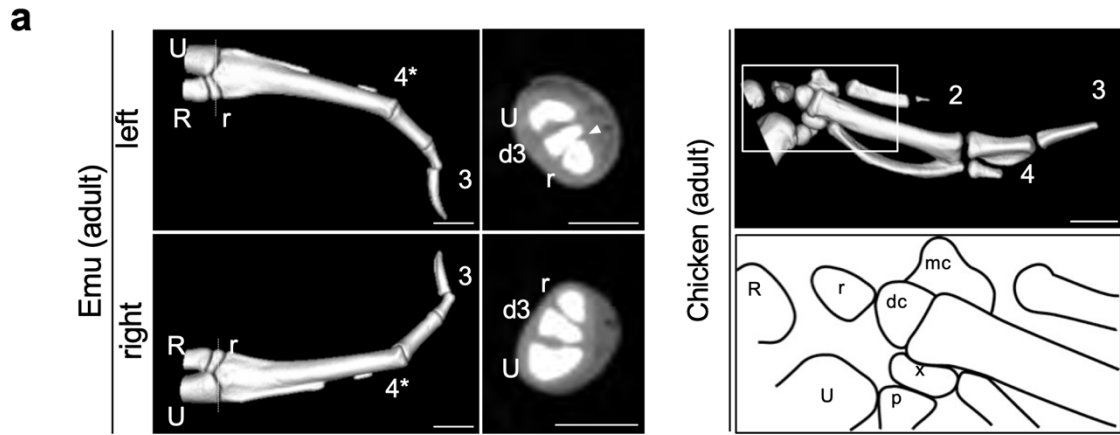
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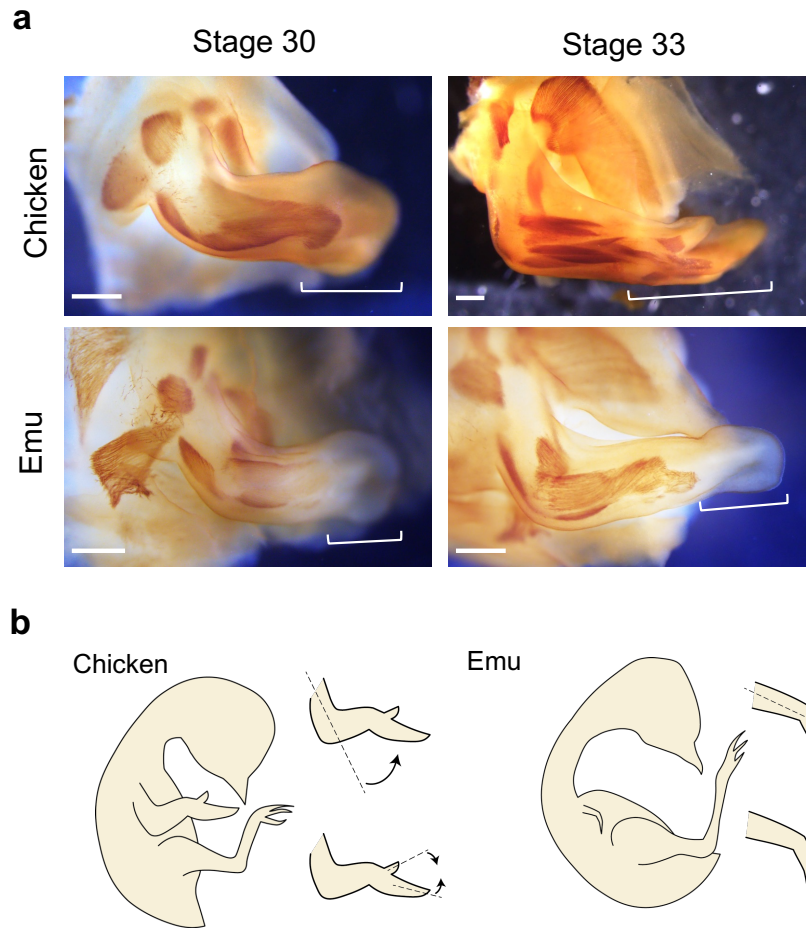
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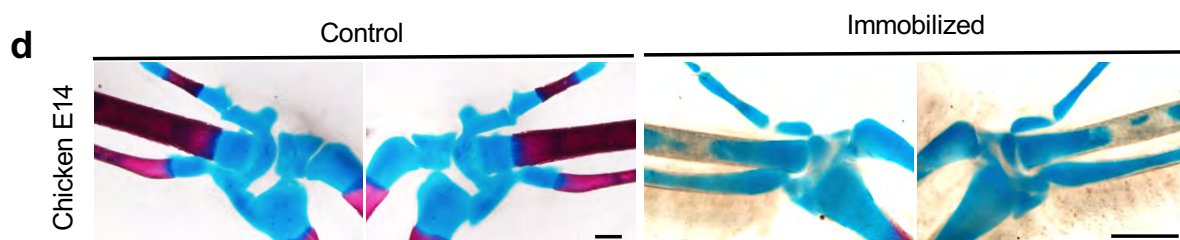
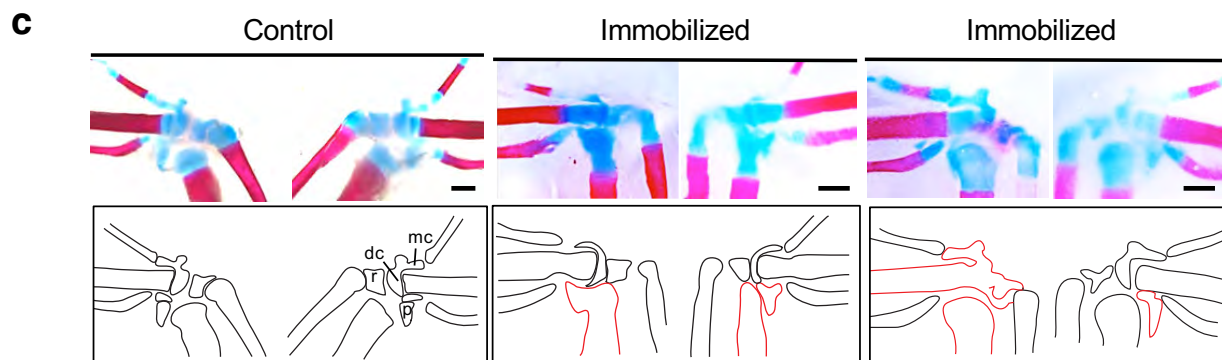
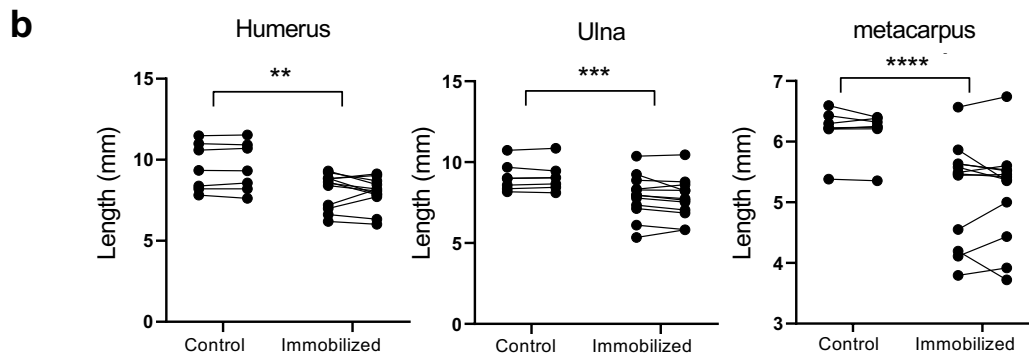
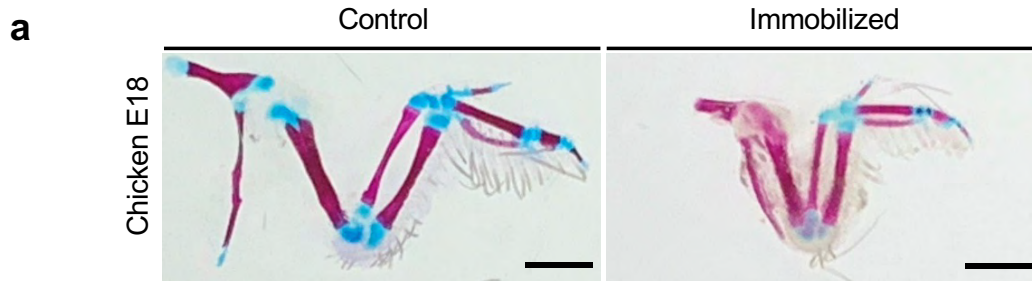
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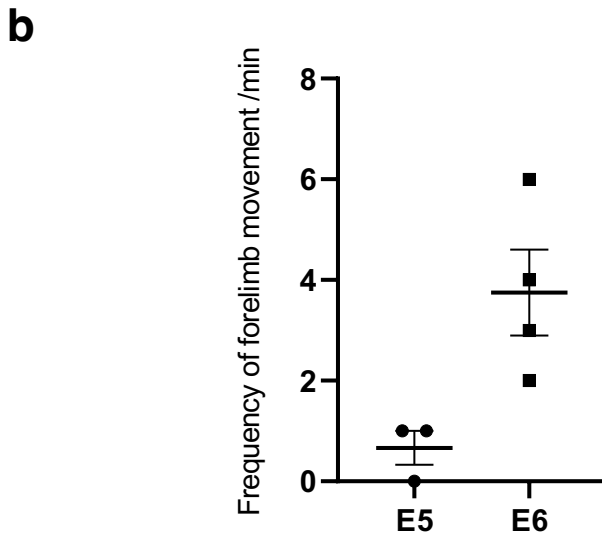
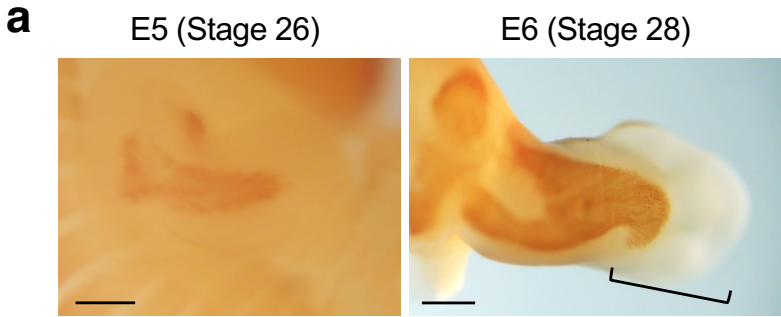
Supplementary Figure 1: Skeletal and muscular patterns of the forelimbs of emu and chicken adults. **a**, Three-dimensional rendering from CT images of the distal part of an adult emu and chicken (n=4) forelimb skeleton and transverse sections taken from the emu limb at the level of the dashed line. Arrowheads indicate the fusion of elements. 3, digits 3, 4*, rudiment of digit 4; d3, metacarpal of digit 3; r, radiale; R, Radius; U, Ulna; x, element X⁸⁴. Scale bars, 1 cm. **b**, Length of the left and right humerus, ulna and metacarpus of emu (n=8) and chicken adults (n=6). **c**, Hematoxylin and Eosin staining of muscle tissue of the wings of adult chickens (n=2) and emu (n=8). Both species displayed typical adult skeletal muscle morphology, including distinct muscle fascicles, myofibers with peripheral nuclei (arrowheads) and sarcomeric M bands (arrows). Chicken: *M. triceps brachii*. Emu: *M. flexor carpi ulnaris*. Scale bars, 100 μ m (left panels), 10 μ m (right panels).



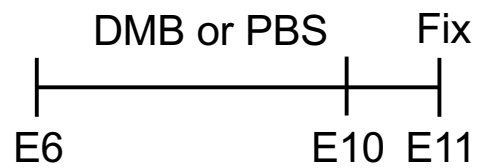
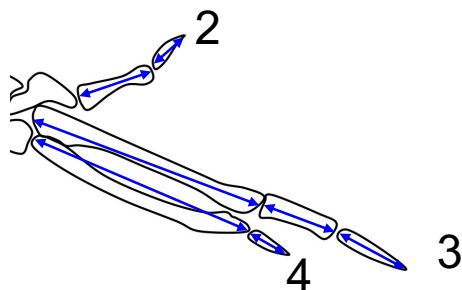
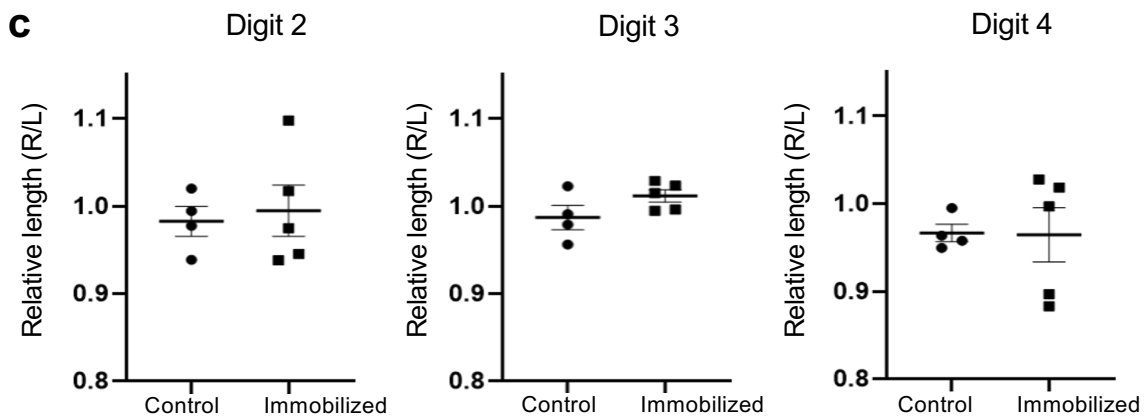
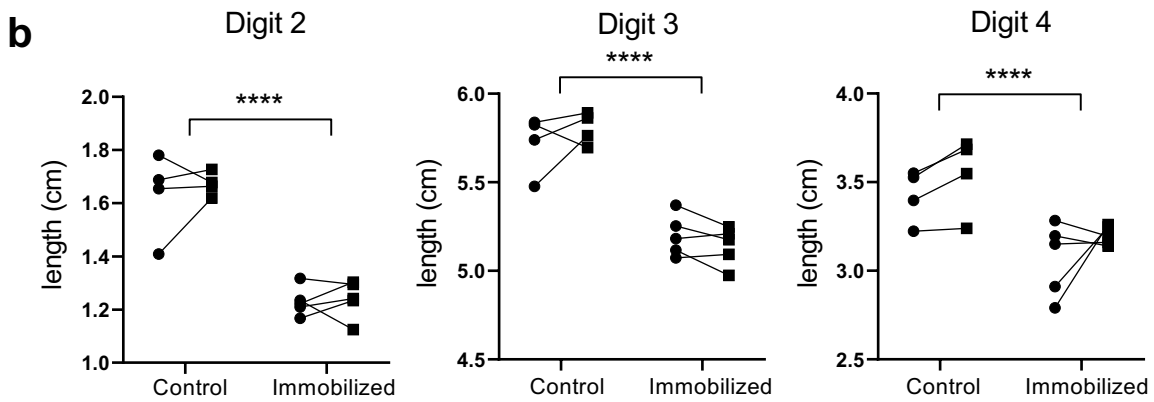
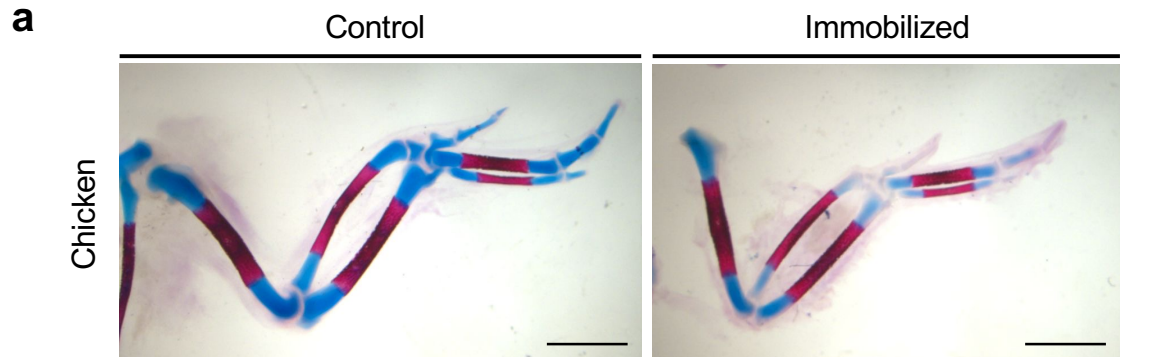
Supplementary Figure 2: Skeletal and muscular patterns of the forelimbs of emu and chicken adults. a, Immunostaining with MF20 in forelimbs of chicken and emu embryos. The formation of muscles was recognized in the autopodal regions of stage 30 (n=3) and 33 (n=4) chicken embryos, autopodial muscles were hardly detected in the forelimbs of embryos at stage 30 (n=4) and 33 (n=4). A panel of stage 30 chicken wing is flipped horizontally. Scale bars, 500 μ m. **b,** Schematic diagram of the proximal wing movement (right top) and the distal movement (right bottom) of chicken and emu embryos. See text for details.



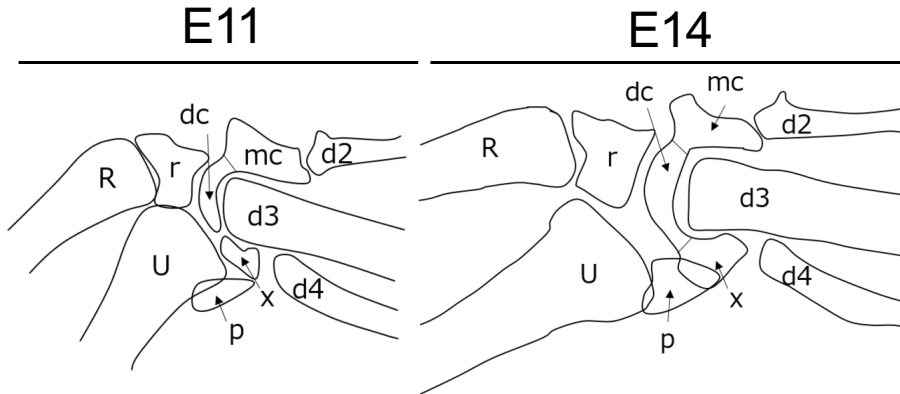
Supplementary Figure 3: Skeletal patterns of the forelimbs of immobilized chicken embryos. **a-c**, Chicken embryos were treated with PBS (n=17) or DMB (n=13) from E10 to E18. **a**, Alcian blue and Alizarin red staining of the forelimbs of chicken embryos. Scale bars, 500 μ m. **b**, Length of the left and right humerus, ulna and metacarpus of digit 3 were significantly reduced in chicken embryos immobilized between E10-18 (by 84.3%, 86.0% and 83.9% compared with controls, respectively). Welch's two-tailed t-test. **p < 0.005, ***p < 0.001, ****p < 0.0001. **c**, Wrist region of Alcian blue and Alizarin red-stained forelimbs of chicken embryos. dc, distal carpal; p, pisiform; r, radiale. Scale bars, 1 mm. **d**, Chicken embryos were treated with PBS or DMB from E6 (stage 28) to E14 (stage 39). Wrist region of Alcian blue- and Alizarin red-stained forelimbs of chicken embryos. Scale bars, 250 μ m.



Supplementary Figure 4: Formation of skeletal muscles and movement of developing forelimbs of chicken embryos. **a**, Immunostaining of MF20 in forelimb buds of E5 (stage 26, n=2) and E6 (stage 28, n=3) chicken embryos. Scale bars, 500 μ m. A bracket indicates the autopodial region. **b**, Frequency of forelimb movement per 1 minute. E5 (n=3), E6 (n=4). Mean $\pm$ SEM.

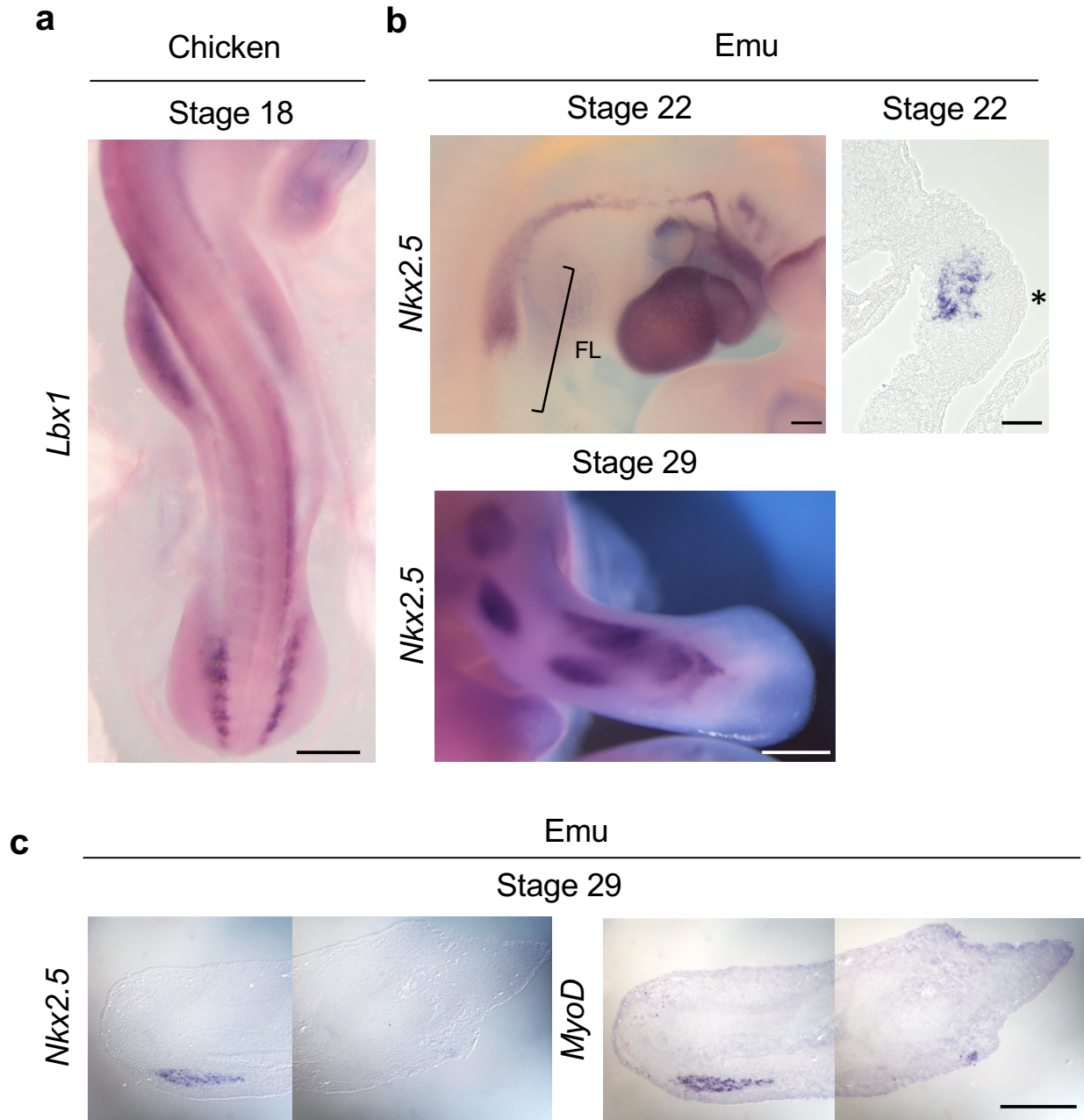


Supplementary Figure 5: Skeletal patterns of the forelimbs of immobilized chicken embryos. **a-b**, Chicken embryos were treated with PBS (n=4) or DMB (n=5) from E6 to E10. **a**, Alcian blue and Alizarin red staining of the forelimbs of chicken embryos. Scale bars, 2 mm. **b**, Total length of left and right skeletal elements of digits (metacarpus and phalanges) of immobilized chicken embryos was significantly reduced compared to that in controls (by 74.2%, 89.7% and 89.9%, respectively) for all digits. Mean $\pm$ SEM. Welch's two-tailed t-test. **p < 0.01, ***p < 0.001. **c**, Ratio of left to right total bone length (coefficient of variation: 1.39%, 7.08%, 1.17%, 4.66%, 1.37%, and 5.93% for control digit 2, immobilized digit 2, control digit 3, immobilized digit 3, control digit 4, and immobilized digit 4, respectively). Mean $\pm$ SEM.

a**b**

	left	right
1	p + U	d4 + x ; p + U
2	-	dc + d3
3	p + U	-
4	-	-
5	-	dc + mc ; U + r
6	dc + mc	dc + mc ; U + r

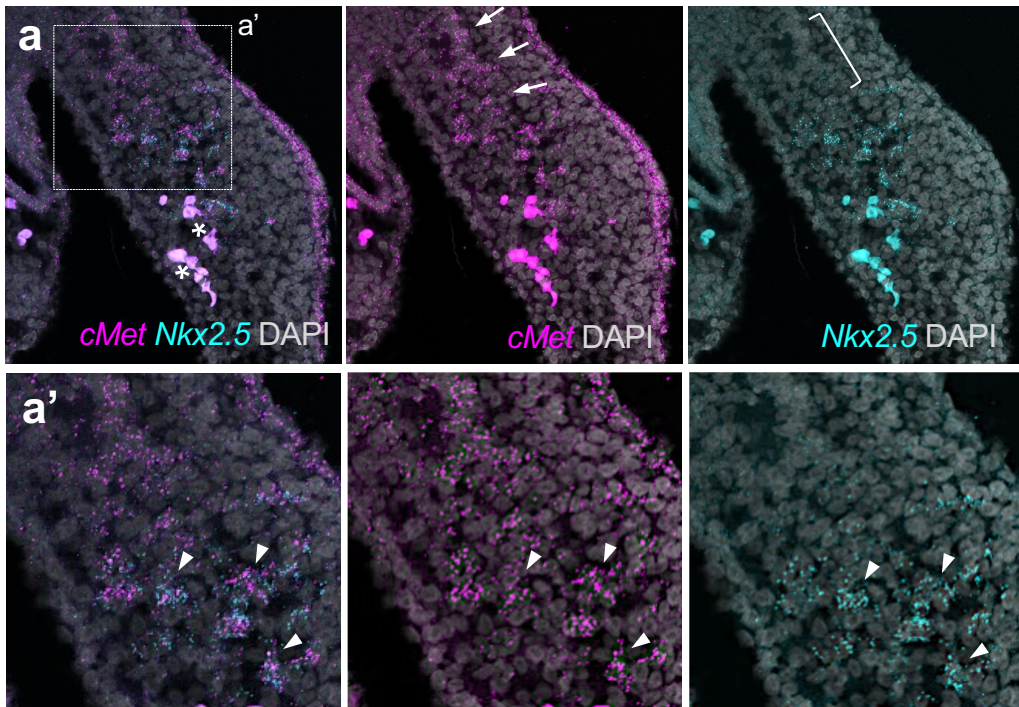
Supplementary Figure 6: Fusion of skeletal elements caused by immobilization in chicken embryos. **a**, Schematic diagram of the skeletal pattern at the wrist region of E11 and E14 chicken embryos. dc, distal carpal; d2-4, metacarpal of digit 2-4; mc, metacarpal; p, pisiform; r, radiale; R, radius; U, ulna; x, element X⁸⁴. **b**, Fused elements observed in chicken embryos immobilized from E6 to E14 (Fig. 2b, c).



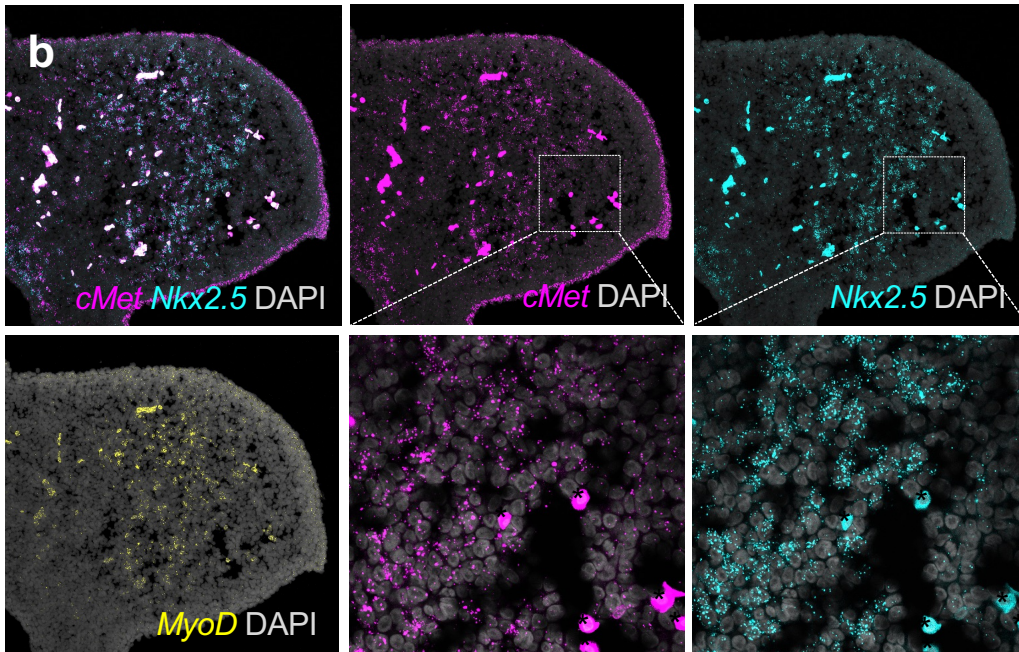
Supplementary Figure 7: *Nkx2.5* expression in emu wings. **a**, *Lbx1* expression in stage 18 chicken embryos (n=2). **b**, *Nkx2.5* expression in emu forelimb buds (stage 22 (n=2); stage 29 (n=2)). *Nkx2.5* was expressed in a group of cells in the forelimb buds, as observed in cross-sections (stage 22 (n=3)). Asterisk, apical ectodermal ridge. **c**, In situ hybridization of adjacent sections revealed that *Nkx2.5* expression overlapped with *MyoD* expression, a muscle cell marker (n = 1). Scale bars, 500 μ m in (a), (b, stage 29), (c); 50 μ m in (b, stage 22).

Emu

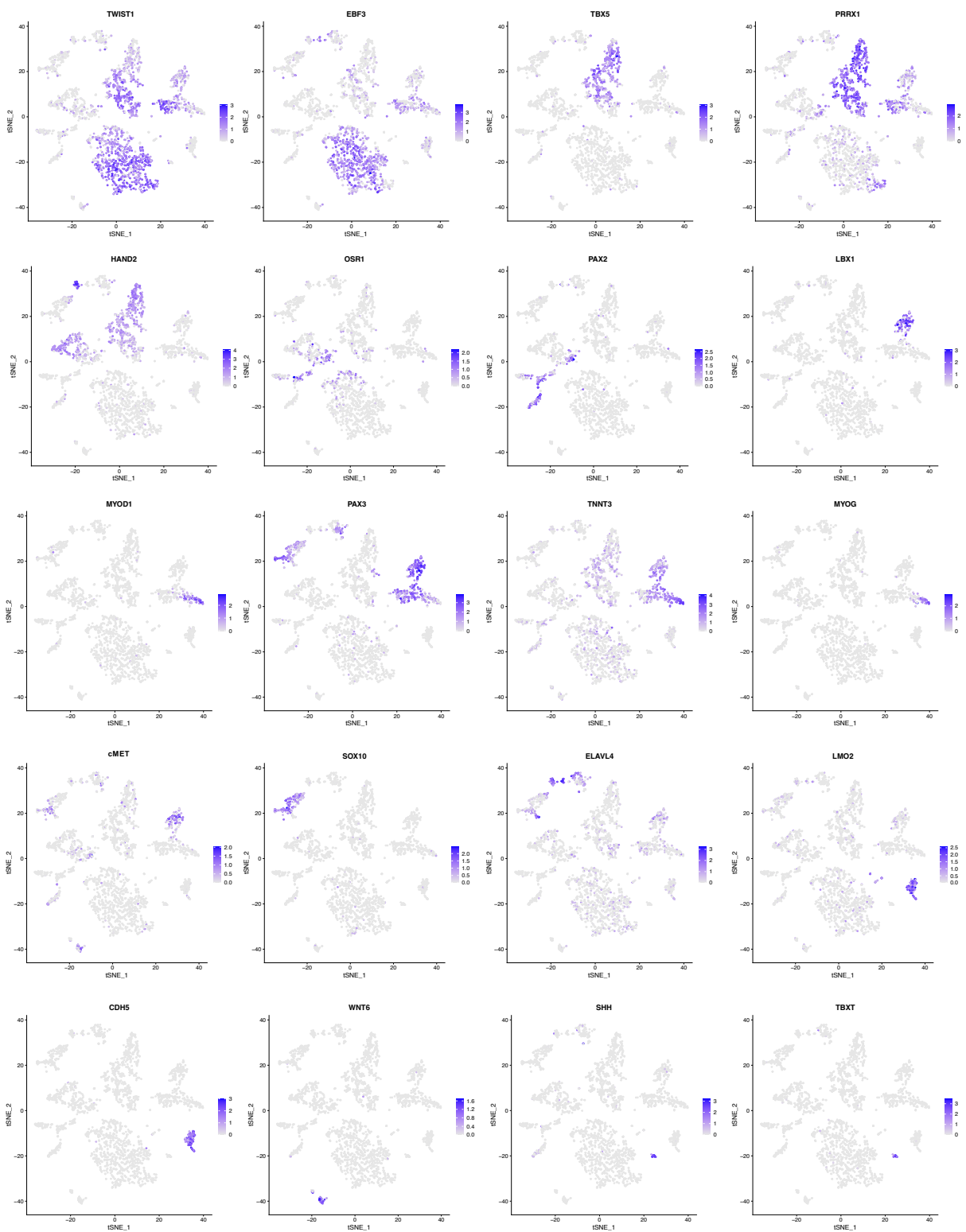
Stage 19



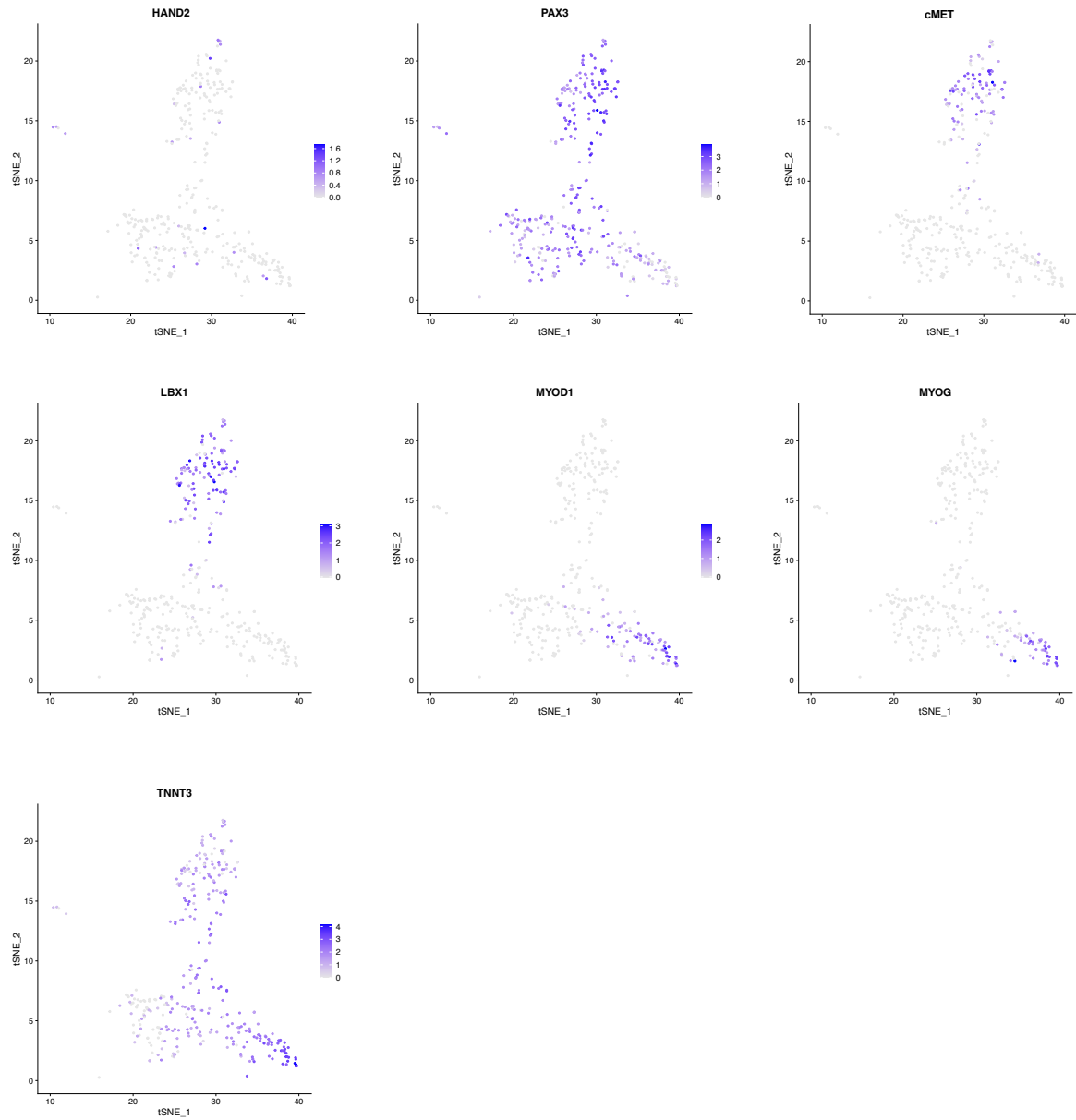
Stage 25



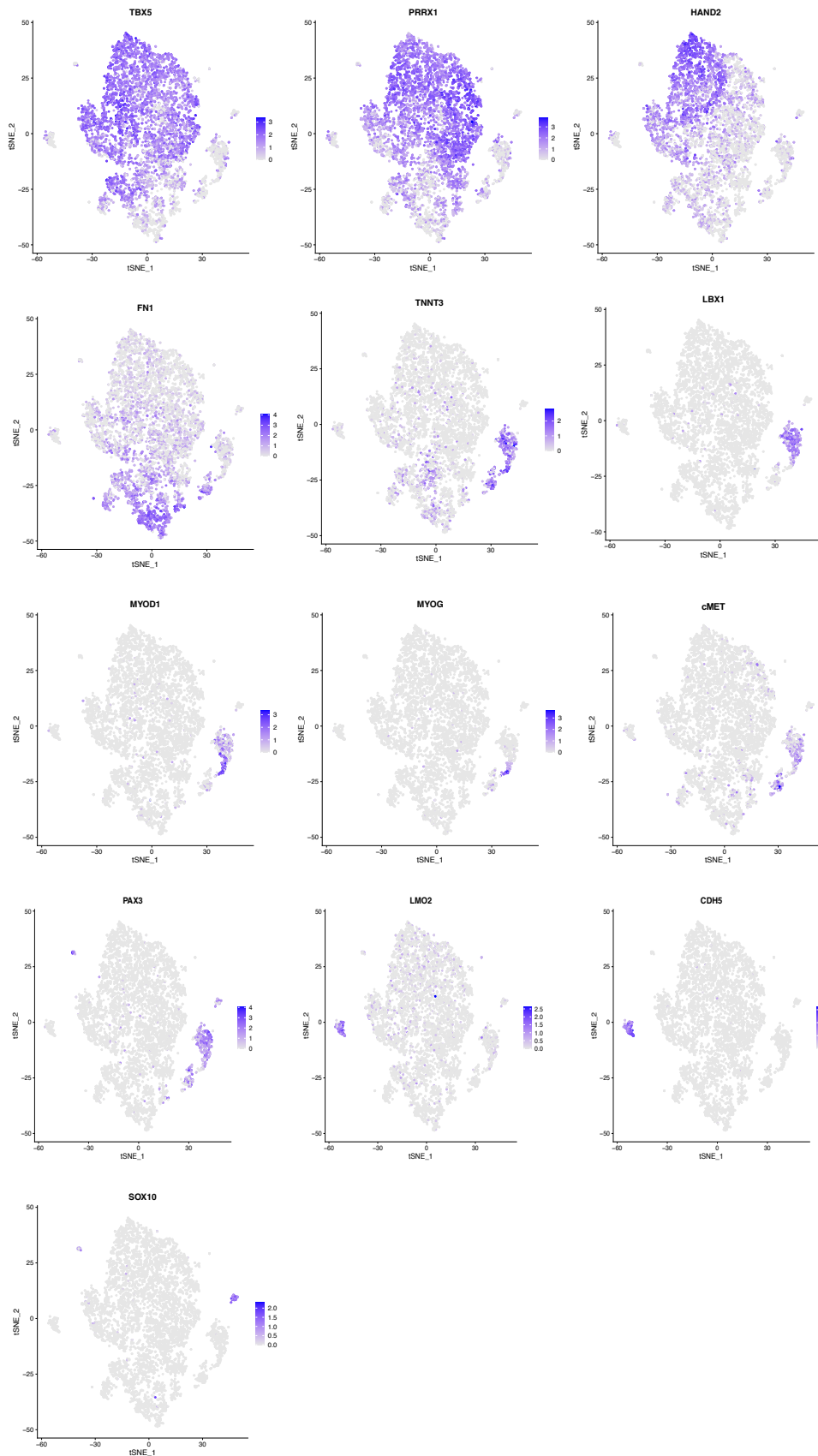
Supplementary Figure 8: Distribution of transcripts of *cMet*, *Nkx2.5* and *MyoD* in emu forelimb buds. **a, a'**, Distribution of *cMet* and *Nkx2.5* transcripts in developing emu forelimb buds at stage 19 (n=4). Arrows indicate the *cMet* transcripts in muscle precursors derived from the dermomyotome. A square bracket indicates the *Nkx2.5*-negative region. Note that *cMet* transcripts co-localized with *Nkx2.5* transcripts (arrowheads). Asterisks indicate blood cells. **b**, Distribution of *Nkx2.5*, *cMet* and *MyoD* in the stage 25 forelimb buds shown in Fig. 2d.



Supplementary Figure 9: Expression heatmap of marker genes visualized on tSNE plot from stage 20/21 emu trunk cells at the forelimb level. The expression heatmap of marker genes of each cell cluster is visualized on a tSNE plot (see Fig. 3a, b). Note that several genes known to be expressed in limb mesenchyme, such as *Prrx1*, *Hand2*, and *Tbx5* showed loosely restricted expression, suggesting a largely undifferentiated state for the corresponding cell clusters. We also identified cell populations enriched for markers of mesenchyme (*Twist*, *Ebf3*), intermediate mesoderm (*Osr1*, *Pax2*), muscle progenitors (*Lbx1*, *MyoD1*, *Pax3*, *Tnnt3*, *Myog*, *Met*), neural crests (*Sox10*), neuron (*Elav4*), notochord (*Shh*, *TbxT*), and ectoderm (*Wnt6*).



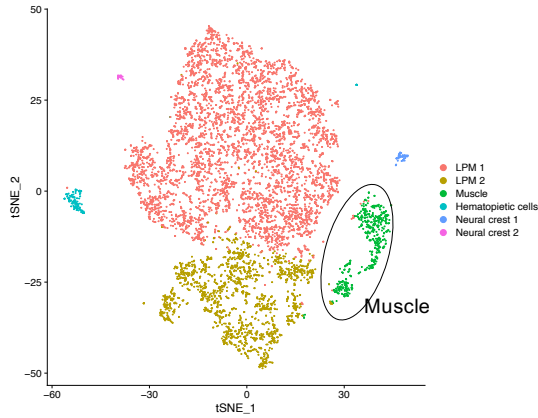
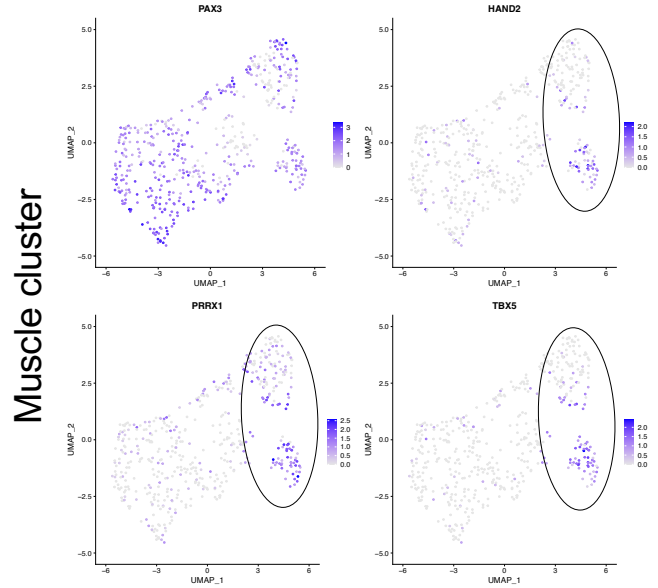
Supplementary Figure 10: Expression heatmap of marker genes on a re-clustering tSNE plot of muscle cluster from stage 20/21 emu trunk tissue at the forelimb level. The expression heatmap of marker genes is visualized on a re-clustering tSNE plot of muscle cluster (see Fig. 3a, b).



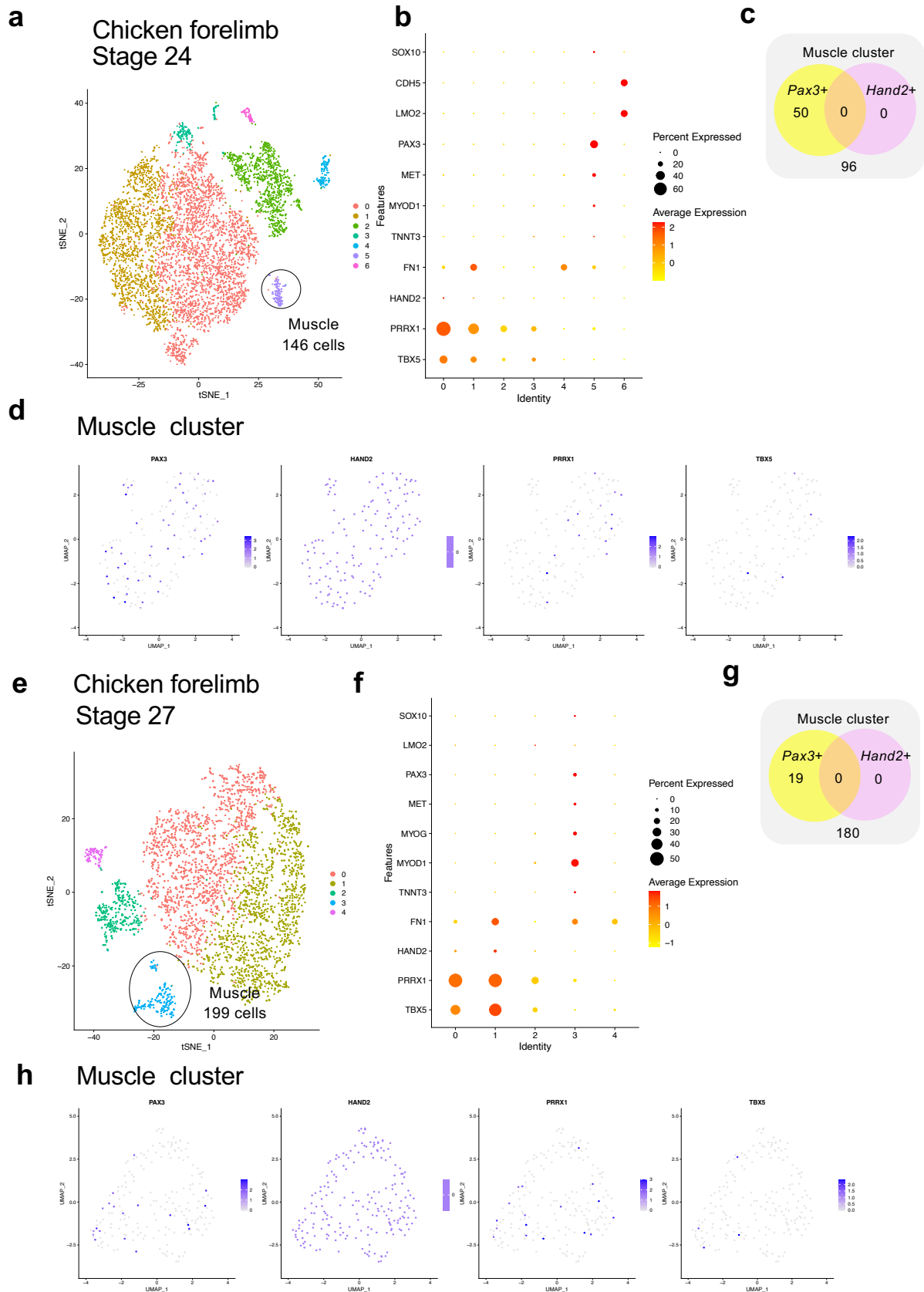
Supplementary Figure 11: Expression heatmap of marker genes visualized on tSNE plot from stage 25 emu forelimb bud cells. The expression heatmap of marker genes of each cell cluster is visualized on a tSNE plot (see Fig. 3d, e). Cell populations enriched for markers of the lateral plate mesoderm (*Tbx5*, *Prrx1*, *Hand2*, *Fn1*), muscle progenitors (*Lbx1*, *MyoD1*, *Pax3*, *Tnnt3*, *Myog*, *Met*), hematopoietic cells (*Lmo2*, *Chn5*) and neural crests (*Sox10*) were identified.

a

Emu forelimb
Stage 25

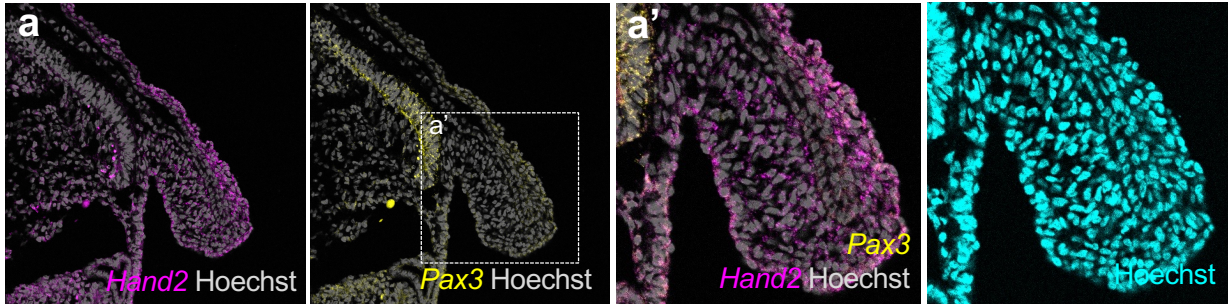
**b**

Supplementary Figure 12: Expression heatmap of marker genes visualized on the UMAP plot of the extracted muscle cluster from stage 25 emu forelimb bud cells. **a**, tSNE plots of stage 25 emu forelimb buds shown in Figure 3d. **b**, The expression heatmap of marker genes is visualized on the UMAP plot of the extracted muscle cluster from stage 25 emu forelimb buds using multidimensional reduction. Note that cell clusters enriched with the LPM markers, such as *Hand2*, *Prrx1* and *Tbx5* (circled), were identified.

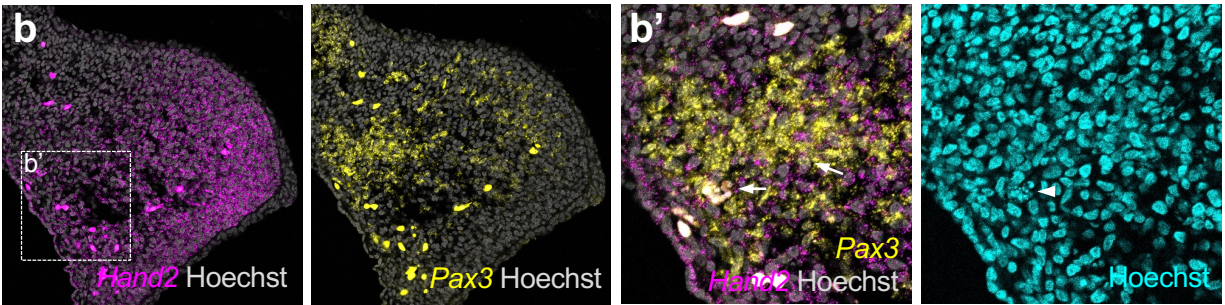


Supplementary Figure 13: Expression heatmap of marker genes visualized on the UMAP plot of the extracted muscle clusters from stages 24 and 27 chicken forelimb bud cells. **a, d,** UMAP plots of a publicly available single-cell RNA-seq dataset obtained from stage 24 (a) and 27 (e) chicken forelimb buds⁵⁴, respectively. **b, f,** Dot plots of subcluster marker gene expression of stage 24 data (b) and stage 27 data (f), respectively. Dot color represents the average expression level, and dot size represents the percentage of cells expressing marker genes. **c, g,** Venn diagram showing number of cells expressing *Pax3* and/or *Hand2* in the muscle cluster of stage 24 data (c) and stage 27 data (g), respectively. **d, h,** The expression heatmap of marker genes is visualized on the UMAP plot of the extracted muscle cluster from stages 24 (d) and 27 (h) chicken forelimb bud cells using multidimensional reduction. Note that *Hand2* expression was not observed, and the expression of other LPM markers were not enriched.

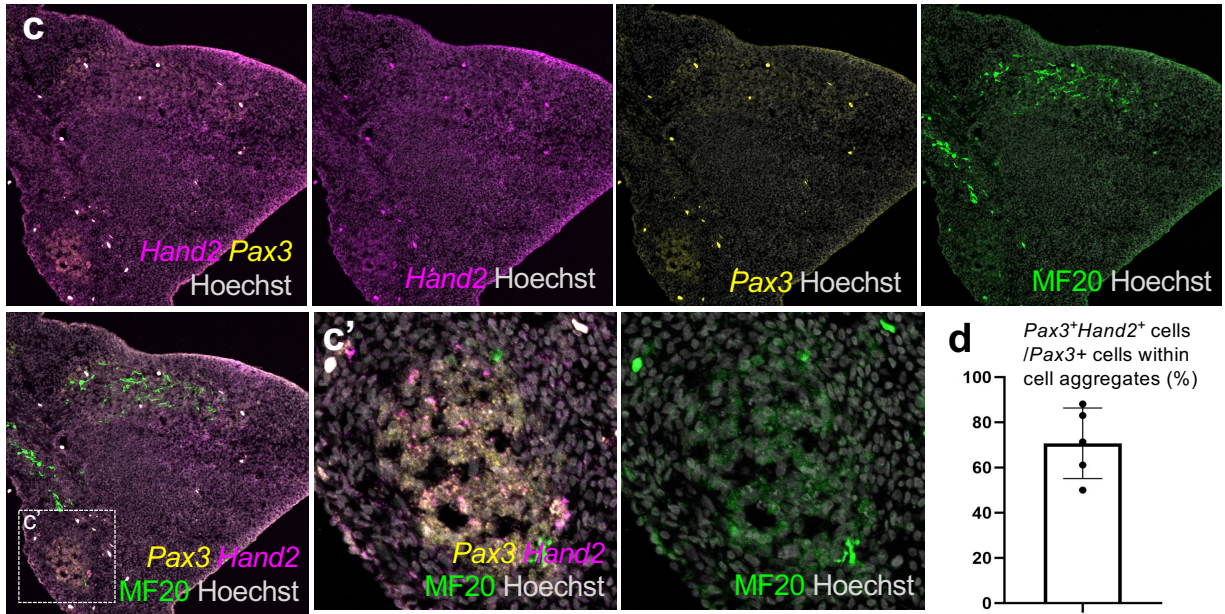
Stage 19



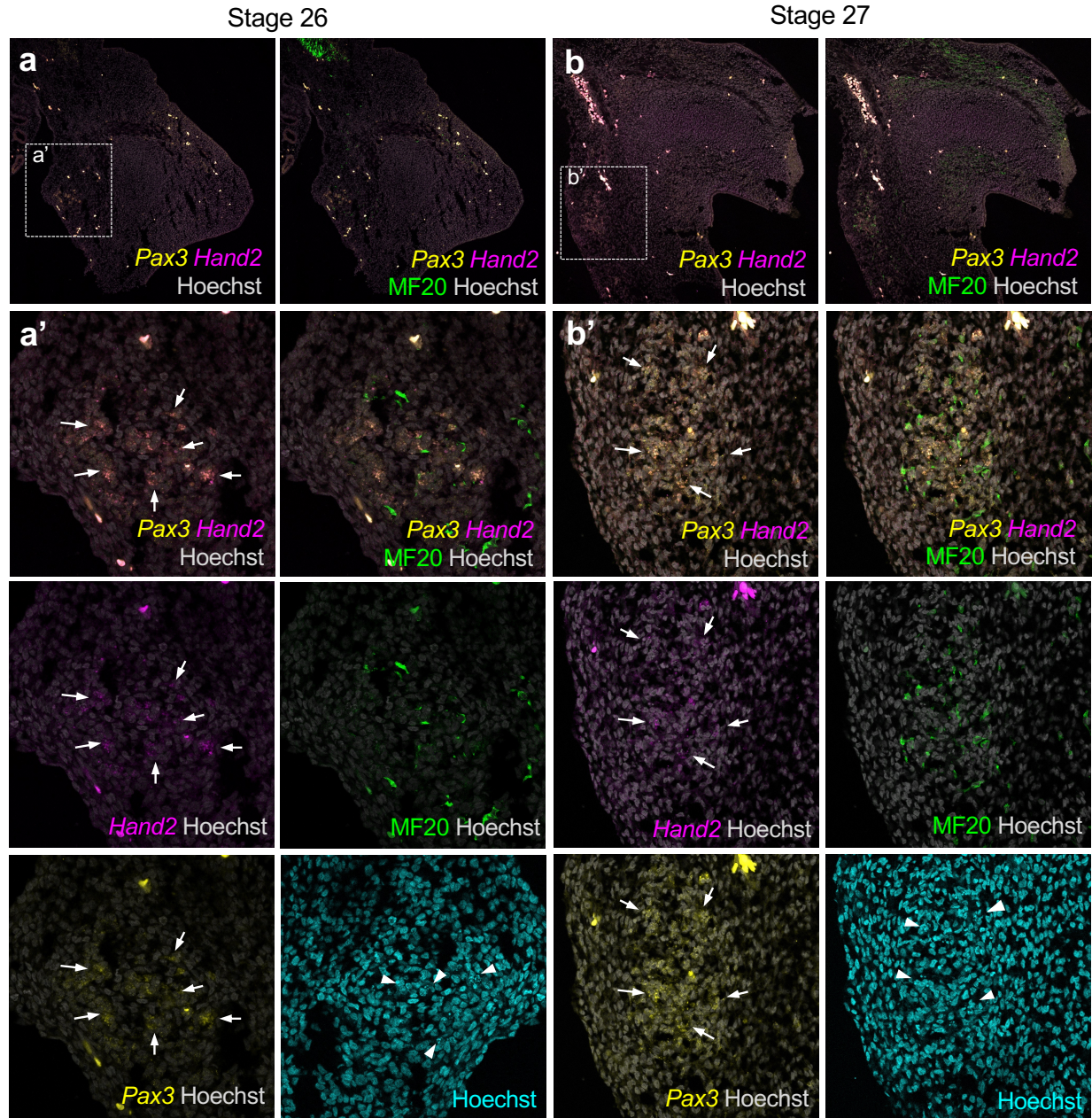
Stage 23



Stage 25



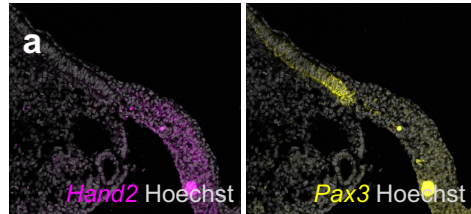
Supplementary Figure 14. HCR for *Pax3* and *Hand2* and immunostaining for myosin heavy chain in emu forelimb buds. a-c, HCR for *Pax3* and *Hand2* and immunostaining for myosin heavy chain (MF20) in emu forelimb buds at stages 19, 23 and 25 shown in Fig. 4a-c. **a', b', c'**, Enlarged images indicated in (a), (b), and (c). Note that co-localization of *Pax3* and *Hand2* transcripts and fragmented nuclei were detected in a few cells at the proximal region by stage 23. **d**, Percentage of *Pax3*+/*Hand2*+ cells in *Pax3*+ cells among aggregated myogenic cells at the proximal region at stage 25. Panels of (a) and (c) are flipped horizontally.



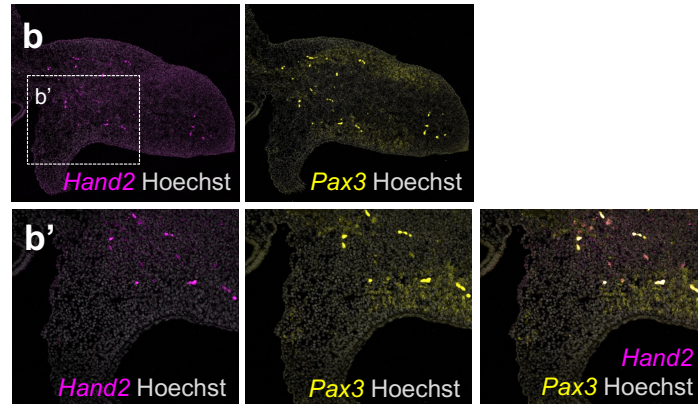
Supplementary Figure 15. HCR for *Pax3* and *Hand2* and immunostaining for myosin heavy chain in emu forelimb buds. a-b, HCR for *Pax3* and *Hand2* and immunostaining for myosin heavy chain (MF20) in emu forelimb buds at stages 26 and 27. **a', b'**, Enlarged images indicated in (a) and (b). Note that a small number of *Pax3* and *Hand2* transcripts and fragmented nuclei were detected in cells at the proximal region at stage 26 and 27. Panels of (a) are flipped horizontally.

Chicken

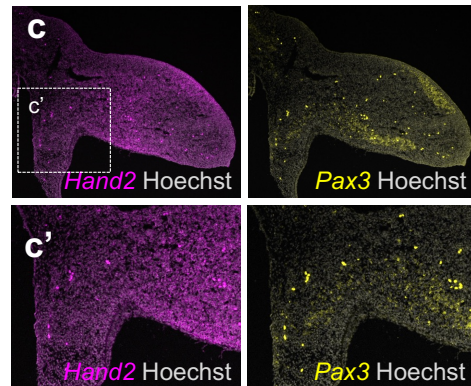
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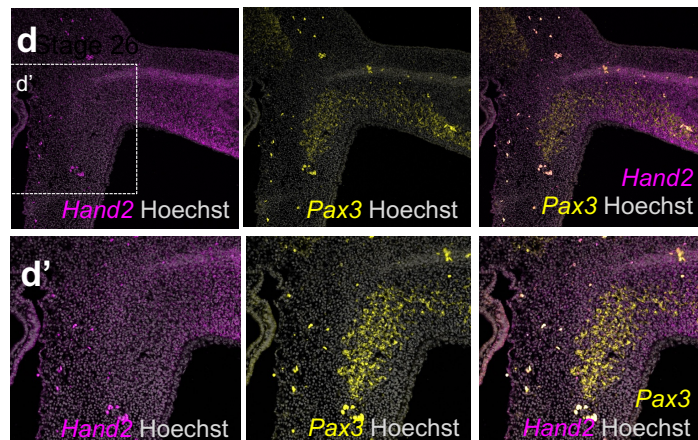
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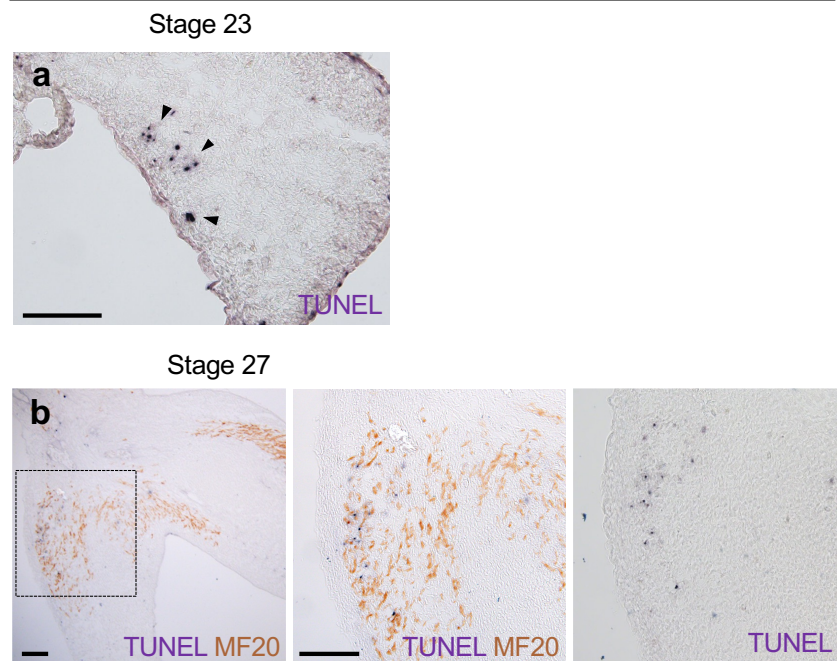
Stage 25



Stage 26



Supplementary Figure 16. HCR for *Pax3* and *Hand2* in chicken forelimb buds. a-d, HCR for *Pax3* and *Hand2* in chicken forelimb buds at stages 19 (a, n=3), 23 (b, n=4), 25 (c, n=2) and 26 (d, n=3). **b', c', d'**, Enlarged images indicated in (b), (c) and (d). Unlike emu forelimb buds, no aggregated muscle progenitors are observed at the proximal part of chicken forelimb buds at stages 23, 25 or 26. Panels of (b) and (d) are flipped horizontally.



Supplementary Figure 17. A subpopulation of muscle progenitors aggregated at the proximal region of emu forelimb buds undergoes cell death at least from stage 23, and were surrounded by MF20-positive cells at least by stage 27. a, TUNEL staining in the aggregated cell population at the proximal region of stage 23 (n=2) emu forelimb buds. **b,** TUNEL staining, and immunostaining for MF20 in the cell population at the proximal region of stage 27 emu forelimb buds (n=6). Note that TUNEL-positive cells were surrounded by MF20-positive cells. These are serial sections of the forelimb buds used for Figure S15b. All panels are flipped horizontally. Scale bars, 100 μ m.

Reference

- 84 James, F., Pourtless, J. & Swain, L. Cladistics and the origin of birds: A review and two new analyses. *Ornithol Monogr* **66**, 1-78, (2009).