
Figures and figure supplements

Hypertrophic chondrocytes serve as a reservoir for marrow-associated skeletal stem and progenitor cells, osteoblasts, and adipocytes during skeletal development

Jason T Long *et al*

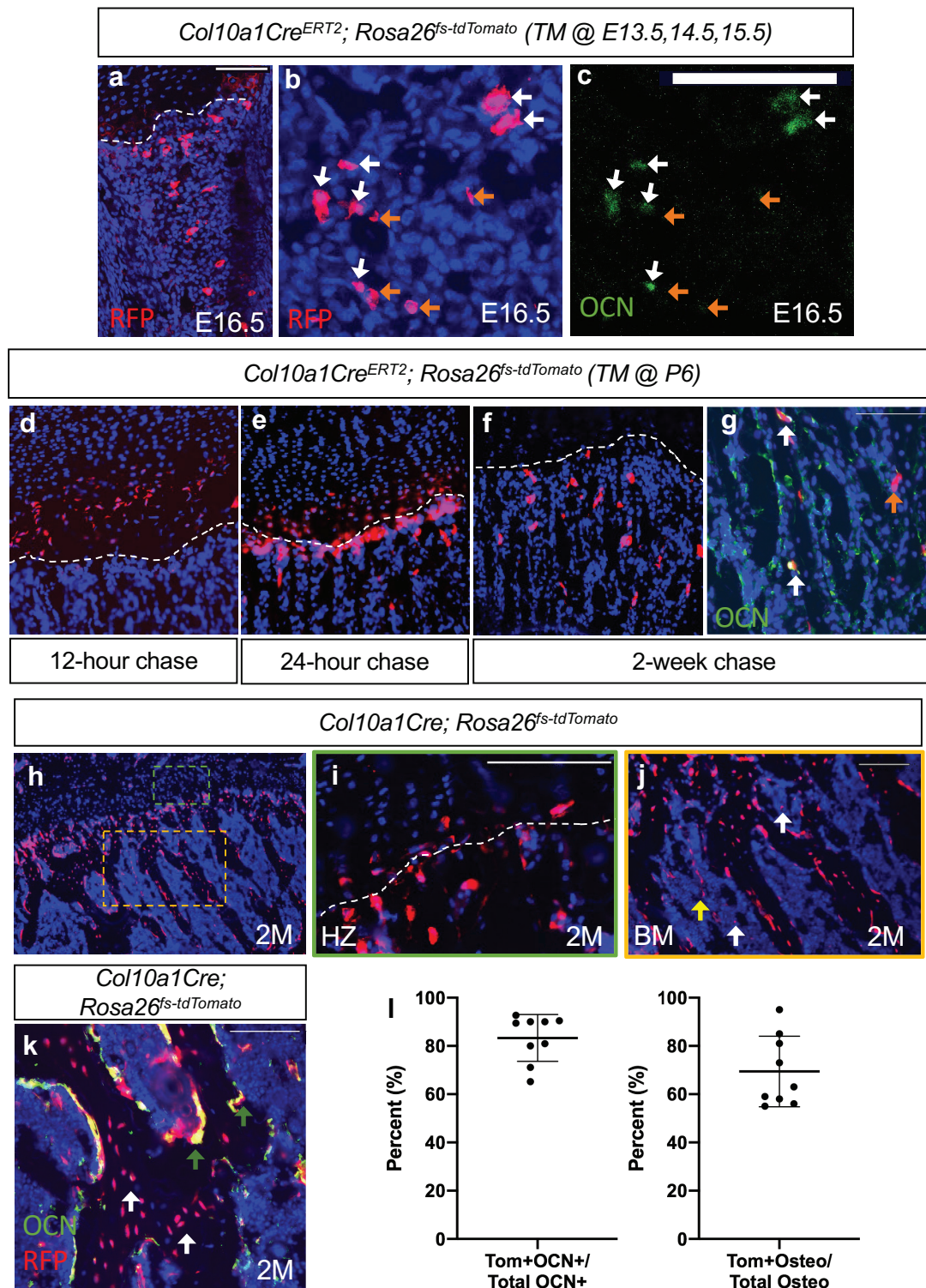


Figure 1. Hypertrophic chondrocytes are the primary source of osteoblasts/osteocytes in trabecular bone. Tibia sections of *Col10a1Cre^{ERT2}; Rosa26^{fs-tdTomato}* mice injected with tamoxifen at E13.5, 14.5, and 15.5 and subsequently sectioned at E16.5 and stained for (a) DAPI/RFP. (b–c) Higher magnification with OCN⁺/tdTOMATO⁺ descendants marked with white arrows and tdTOMATO⁺ descendants not associated with bone (OCN⁻) are indicated by orange arrows. Tibia sections of *Col10a1Cre^{ERT2}; Rosa26^{fs-tdTomato}* mice injected with tamoxifen at P6 and subsequently sectioned at (d) 12 hour chase, Figure 1 continued on next page

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(e) 24 hr chase, and (f) 2 week chase. (g) OCN immunostaining of *Col10a1CreERT2; Rosa26^{fs-tdTomato}* bone sections following 2 week chase of TM. OCN⁺/tdTOMATO⁺ descendants marked with white arrows. tdTOMATO⁺ descendants not associated with bone (OCN⁻) are indicated by orange arrows. (h) Section of 2 M old *Col10a1Cre; Rosa26^{fs-tdTomato}* tibia with higher magnifications of (i) hypertrophic zone (green box) and (j) bone marrow cavity (orange box). Non-bone lining TOMATO⁺ descendants marked with white arrows and potential vessel associated tdTOMATO⁺ descendants indicated by yellow arrow. (k) OCN and RFP immunostaining of 2 M *Col10a1Cre; Rosa26^{fs-tdTomato}* tibia. OCN⁺/tdTOMATO⁺ osteoblasts represented by green arrows and tdTOMATO⁺ osteocytes with white arrows with quantifications shown in (l – **Figure 1—source data 1**). Scale bars = 100 μ m. N = 3 slides from three biologic replicates, SD \pm 9.7% for OCN stain, SD \pm 14.8% for osteocytes. Dotted line demarks the chondro-osseous junction.

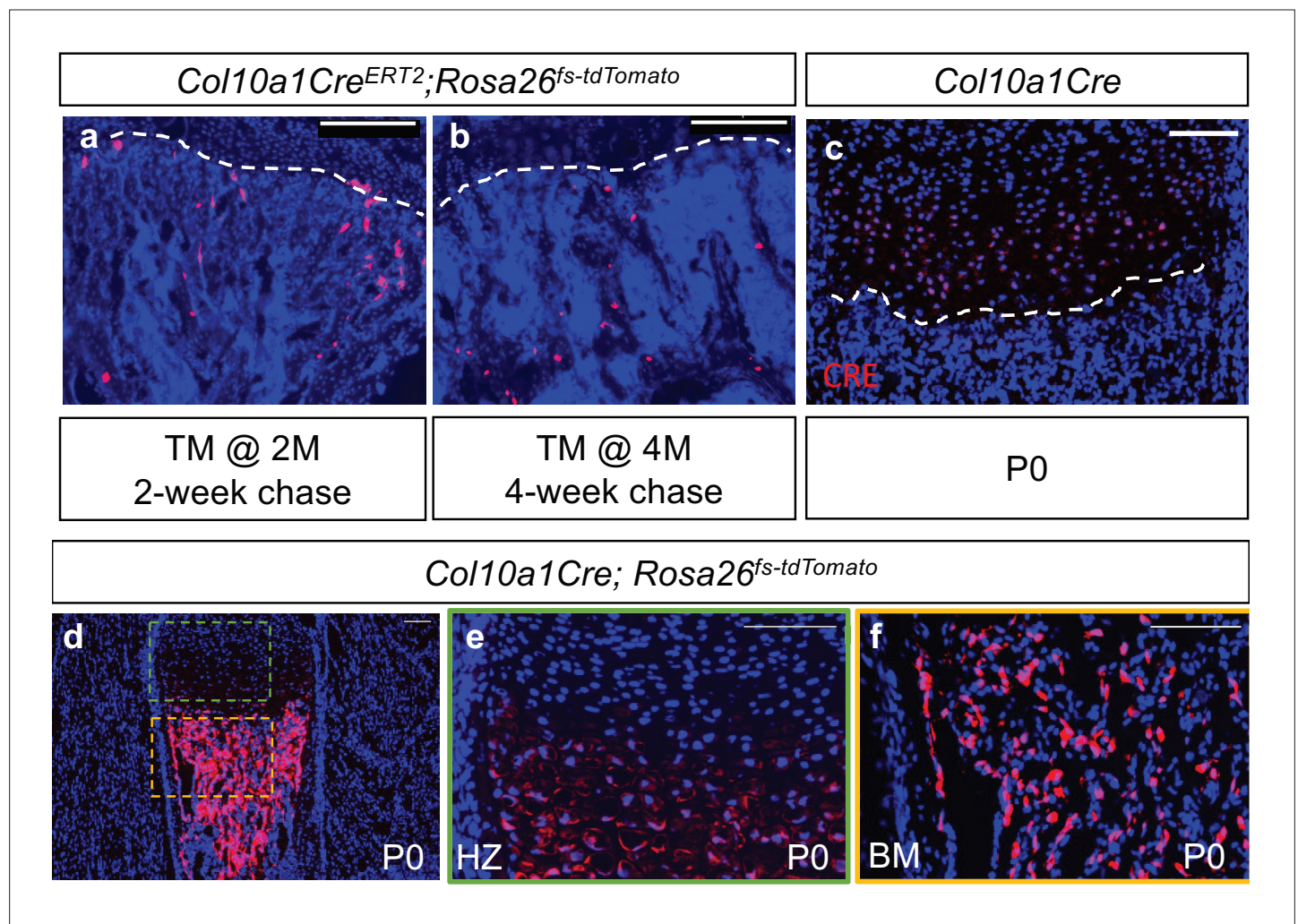


Figure 1—figure supplement 1. Contribution of hypertrophic chondrocytes to the bone marrow are observed into skeletal maturity and CRE expression is restricted to hypertrophic chondrocytes in *Col10a1Cre* mice. (a) Tibia sections of *Col10a1Cre^{ERT2}; Rosa26^{fs-tdTomato}* mice injected with tamoxifen at 2 M of age and subsequently sectioned at 2-week chase. (b) Tibia sections of *Col10a1Cre^{ERT2}; Rosa26^{fs-tdTomato}* mice injected at 4 months of age and subsequently sectioned at 1-month chase. (c) CRE immunostaining of P0 bone sections from *Col10a1Cre* mouse line. (d) Section of P0 *Col10a1Cre; Rosa26^{fs-tdTomato}* mouse tibia with higher magnifications regions of (e) hypertrophic zone (green box) and (f) bone marrow cavity (orange box). Chondro-osseous junction represented by white dotted line. Scale bar = 100 μm.

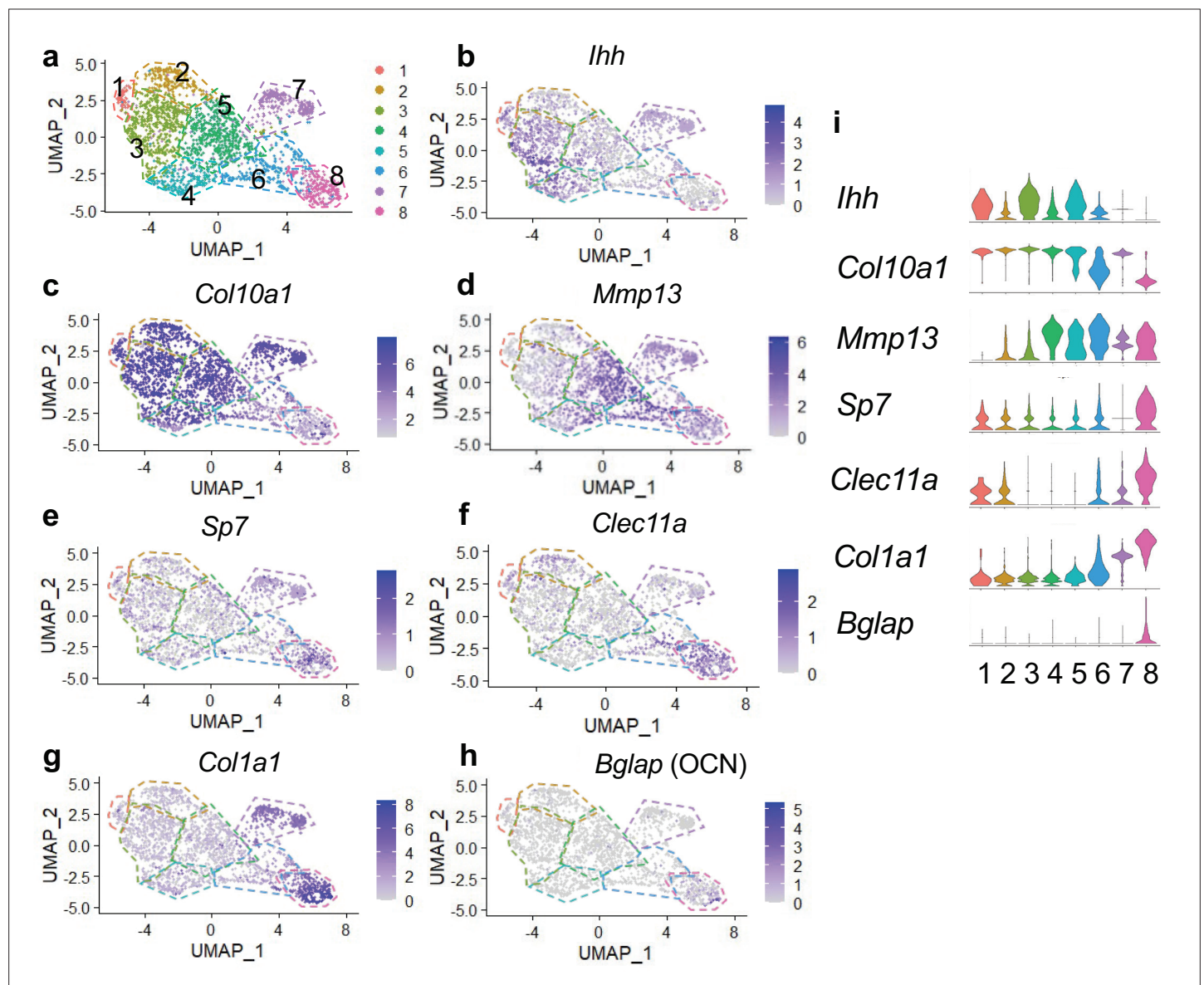


Figure 2. Single cell transcriptomics at E16.5 captures hypertrophic chondrocytes and osteoblasts. (a) UMAP shown in two-dimensional space produced using Seurat 4 package of R from single-cell RNA-sequencing of cleaned skeletal rudiments from *Col10Cre; Rosa26^{fs-tdTomato}* mice at E16.5. (b–d) Feature plots of hypertrophic chondrocyte associated genes identified in clusters 1–7. (e–h) Feature plots of osteoprogenitor/osteoblast specific genes identified in clusters 7–8. (i) Violin plot representing the relative level of chondrocyte and osteoblast associated gene expression (b–h).

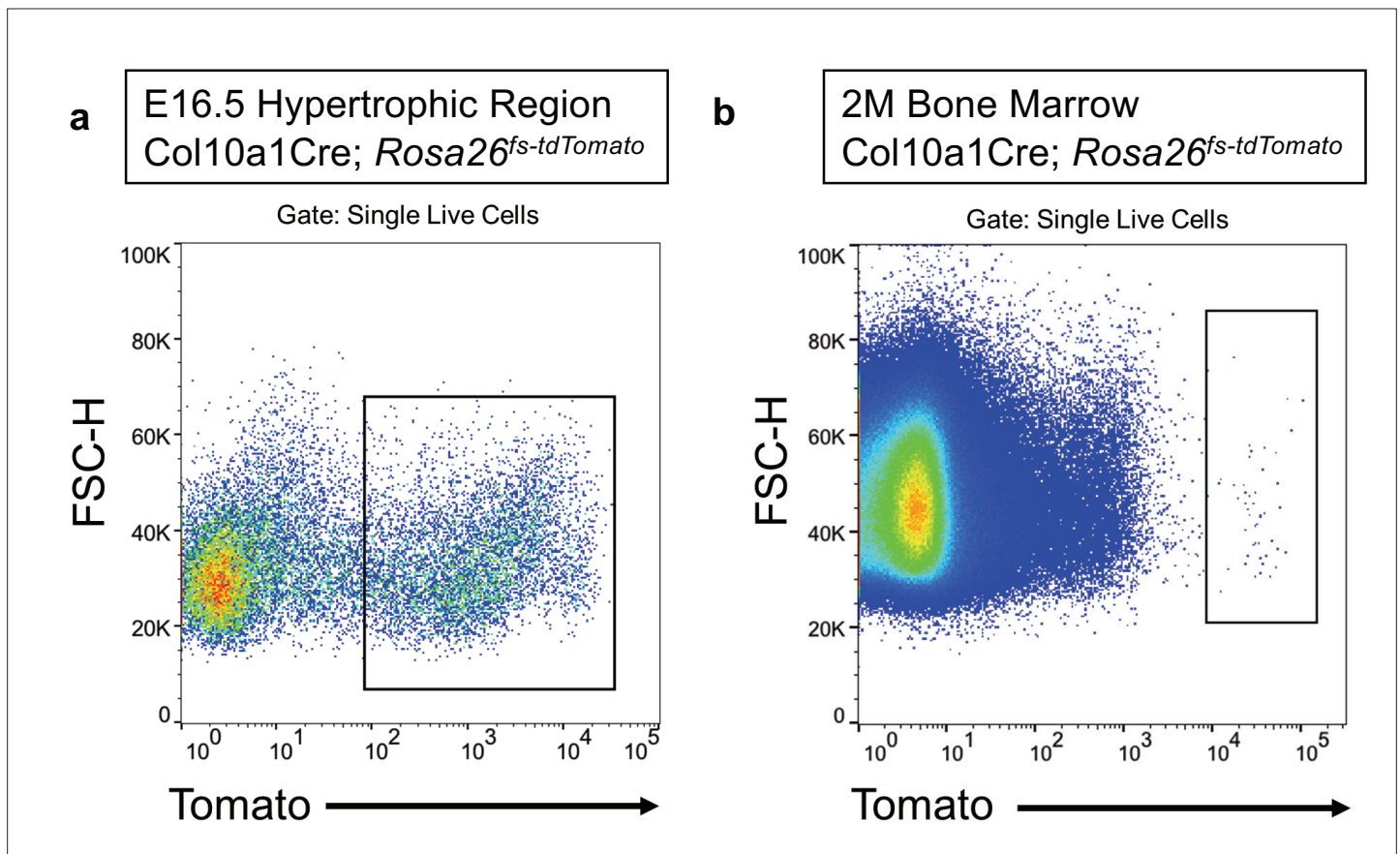


Figure 2—figure supplement 1. FACS plots for isolation of tdTOMATO⁺ cells at E16.5 and 2 M for scRNA-sequencing. (a) E16.5 FACS plot identified tdTOMATO⁺ expressing cells isolated by total bone digestion and utilized for 10 X Genomics scRNA-sequencing represented in **Figure 2**. (b) 2 M FACS plot identified tdTOMATO⁺ expressing cells isolated by bone marrow flush and digestion and utilized for 10 X Genomics scRNA-sequencing represented in **Figure 3**.

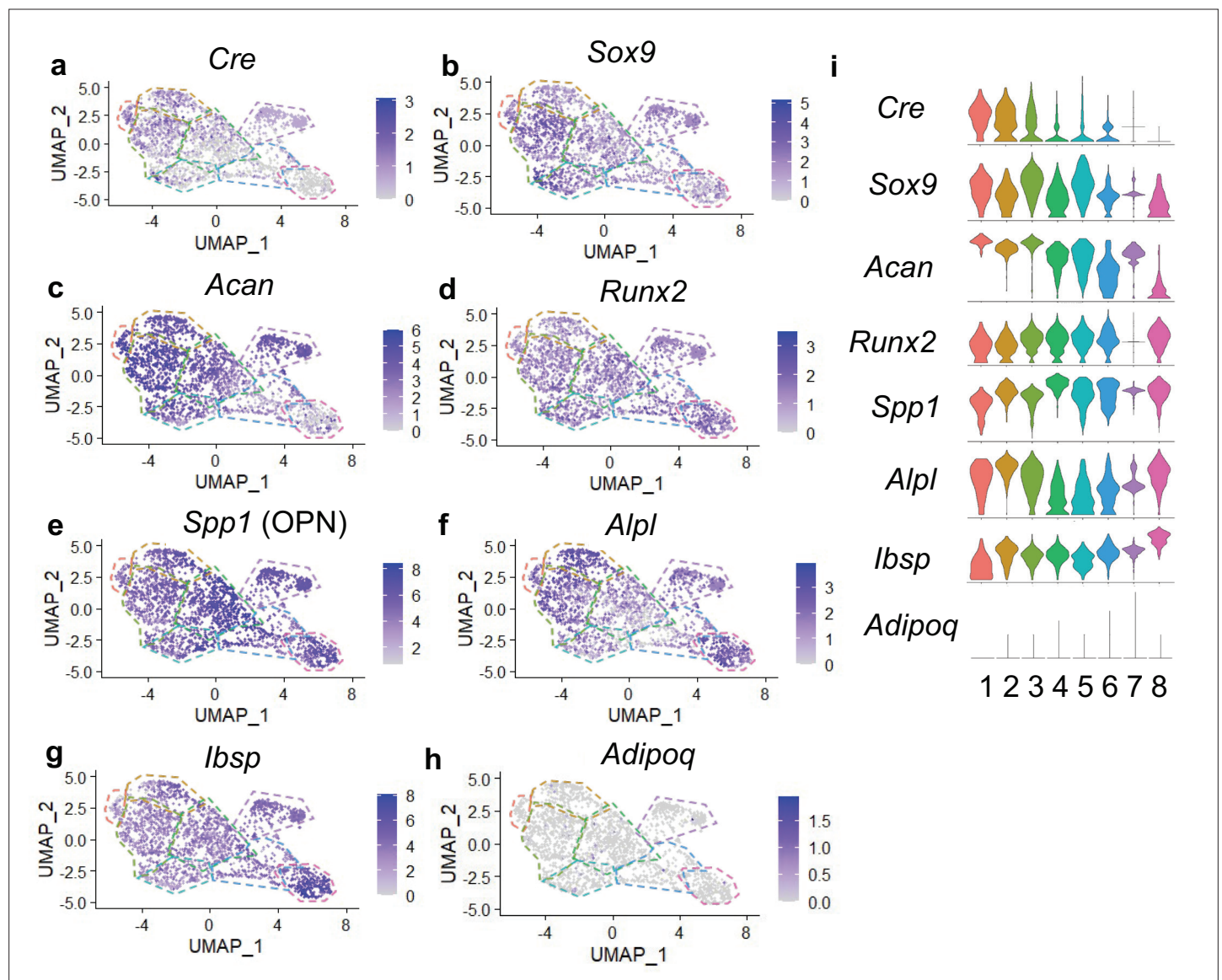


Figure 2—figure supplement 2. Additional genes of interest expressed in hypertrophic chondrocytes and descendants at E16.5. (a) Feature plot of *Cre* expression was enriched in clusters 1–2 at E16.5. (b–c) Feature plot of genes associated with chondrocytes were observed enriched in clusters 1–7. (d–g) Feature plot of genes associated with hypertrophic and osteoblast differentiation were observed throughout all clusters. (h) Feature plot of *Adipoq* had very limited expression in any cluster. (i) Violin plot of genes of interest (a–h).

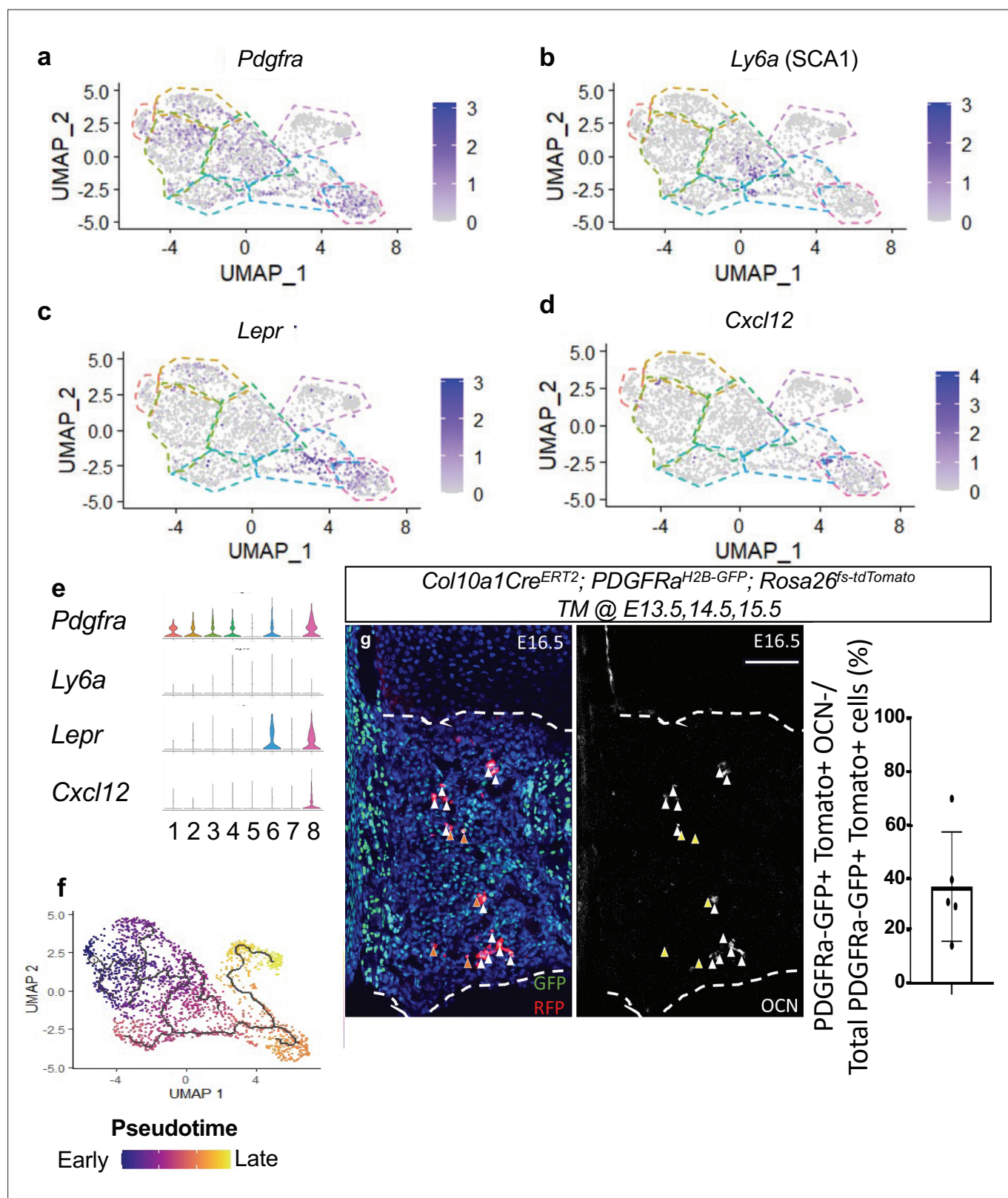


Figure 3. Single-cell transcriptomics of E16.5 hypertrophic chondrocytes and descendants reveals an intermediate SSPC upstream of osteoblasts. (a–d) Feature plot of SSPC-associated genes identified between hypertrophic chondrocyte and osteoblast clusters (e) Violin plot representing the relative level of SSPC associated gene expression (a–d). (f) Monocle three trajectory analysis throughout pseudotime. (g) E16.5 tibia sections of *Col10a1Cre^{ERT2}; Rosa26^{fs-tdTomato}; PDGFRα^{H2B-GFP}* mice injected with tamoxifen at E13.5, 14.5, and 15.5 and quantification (Figure 3—source data 1).

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Orange arrows represent tdTOMATO⁺/PDGFRa^{H2B-GFP+} cells that are OCN⁻. White arrows represent tdTOMATO⁺/PDGFRa^{H2B-GFP+} cells that co-express OCN. Scale bar = 100 μ m. N = 1/2 slides for three biological replicates, Average = 36.7%, SD \pm 20.7%. Dotted line demarks the chondro-osseous junction.

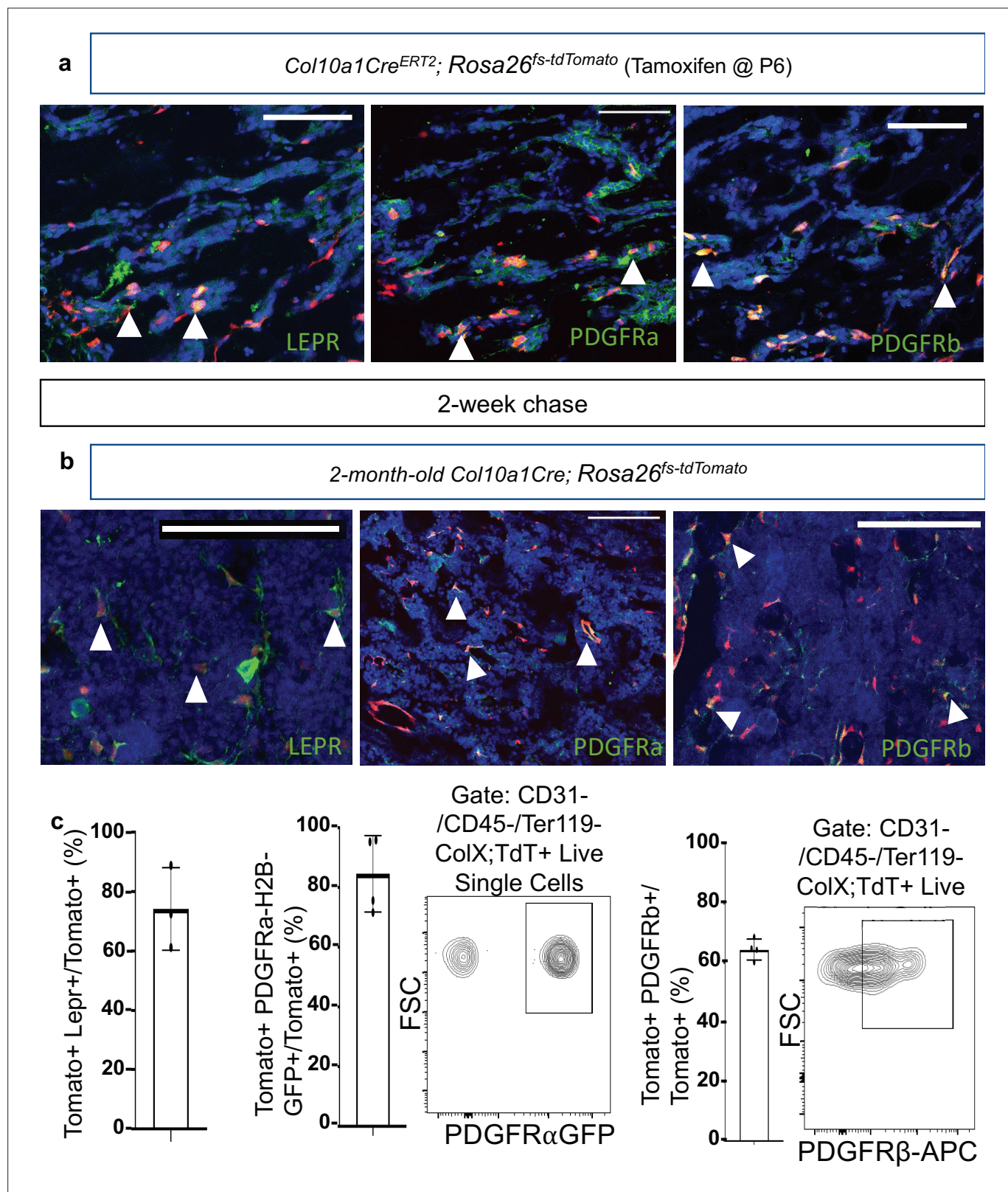


Figure 3—figure supplement 1. SSPC associated proteins are detected in hypertrophic chondrocyte-derived marrow-associated cells.

(a) Immunostaining of SSPC associated proteins in both bone sections from *Col10a1Cre^{ERT2}; Rosa26^{fs-tdTomato}* mice injected with tamoxifen at P6 and chased for 2 weeks and (b) 2 M old *Col10a1Cre; Rosa26^{fs-tdTomato}* bone sections. Representative co-labeled (LEPR, PDGFRα, or PDGFRβ and tdTOMATO) cells are identified by white arrows. (c) Quantification of contribution to SSPC-like cells by hypertrophic chondrocyte descendants by

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immunofluorescence of 2-month-old *Col10a1Cre; Rosa26^{ls-tdTomato}* stained with LEPR antibody (left – Average = 74.21%, **Figure 3—figure supplement 1—source data 1**), flow cytometric analysis of bone marrow cells from 1-month-old *Col10a1Cre; Rosa26^{ls-tdTomato}; PDGFRa^{H2B-GFP}* (middle – Average = 84.6%, **Figure 3—figure supplement 1—source data 2**), and 2-month-old *Col10a1Cre; Rosa26^{ls-tdTomato}* stained with PDGFRb antibody (right – Average = 64.33%, **Figure 3—figure supplement 1—source data 3**) N = 3 biologic replicates (LEPR), 4 biologic replicates (PDGFRa), and four biologic replicates (PDGFRb). SD LEPR = $\pm 13.92\%$, PDGFRa = $\pm 12.81\%$, and PDGFRb = $\pm 3.48\%$. Scale bars = 100 μm .

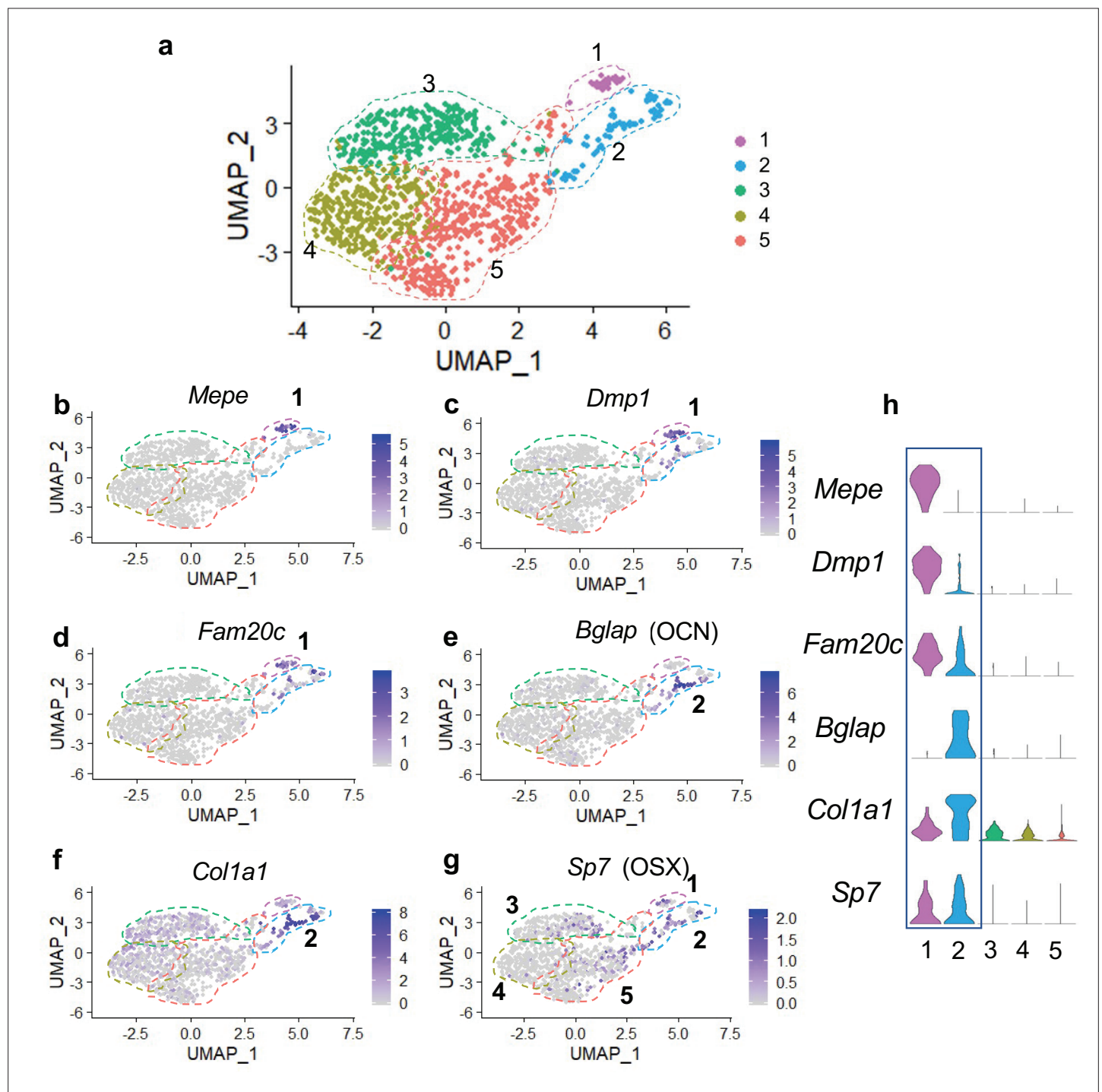


Figure 4. Single cell transcriptomics of hypertrophic chondrocyte descendants following FACS and 10 X Genomics sequencing at 2 M of age. (a) UMAP shown in two-dimensional space produced using Seurat 3 package of R from single-cell RNA-sequencing of bone marrow digest from *Col10Cre*; *Rosa26^{ls-tdTomato}* mice at 2 M. (b–f) Feature plots of osteoblast specific genes identified in cluster 1 (b–d) and cluster 2 (e–f). (g) Feature plot of the osteoprogenitor associated gene, *Sp7*. (h) Violin plot representing the relative level of osteoblast-associated gene expression (b–g).

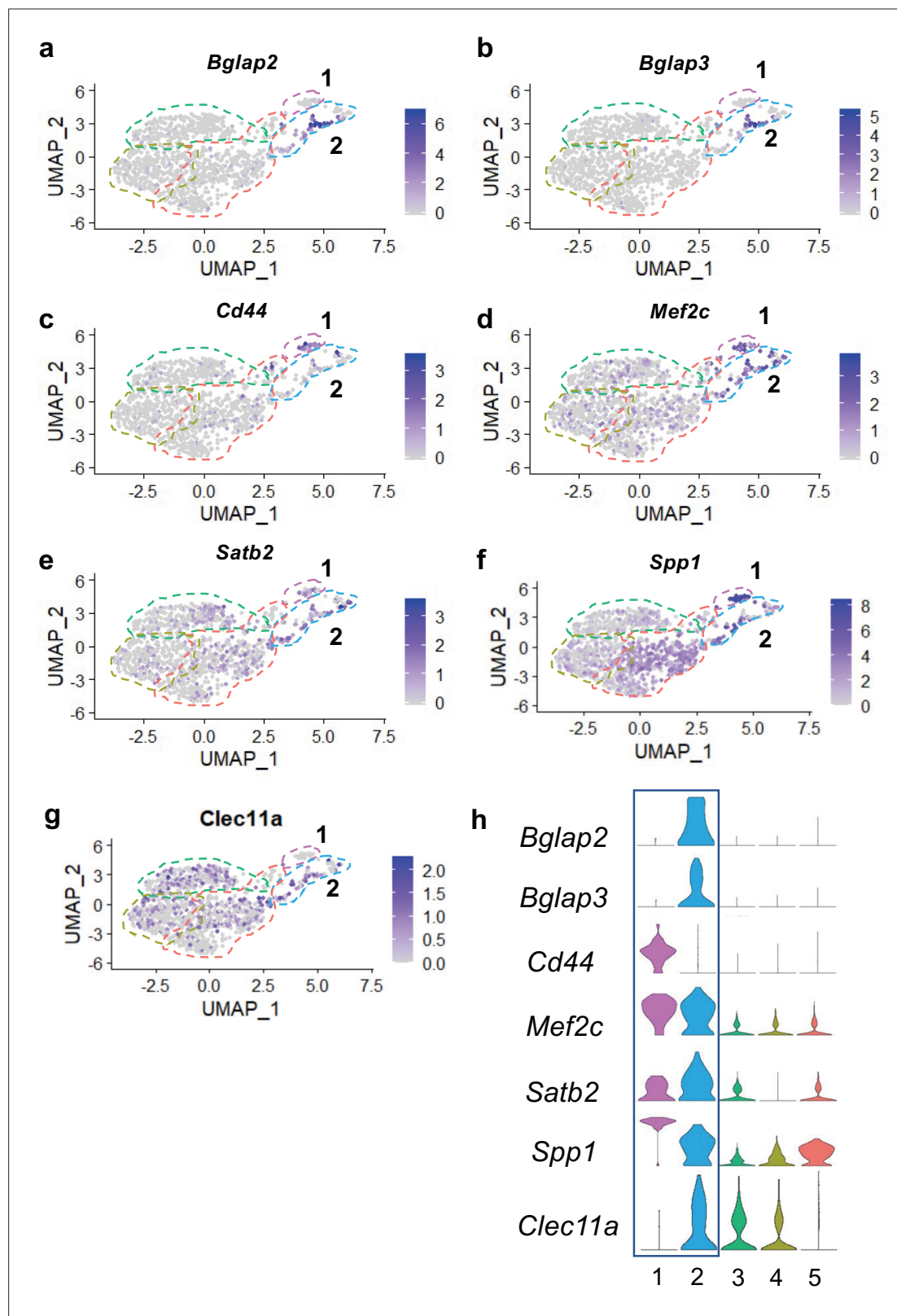


Figure 4—figure supplement 1. Additional osteoblast associated genes observed in clusters 1 and 2 at 2 months of age. (a–g) Feature plots of genes associated with osteoblasts were observed in clusters 1 and 2 with minimal expression in clusters 3–5. (h) Violin plots representing relative gene expression levels in (a–g) among the five identified clusters.

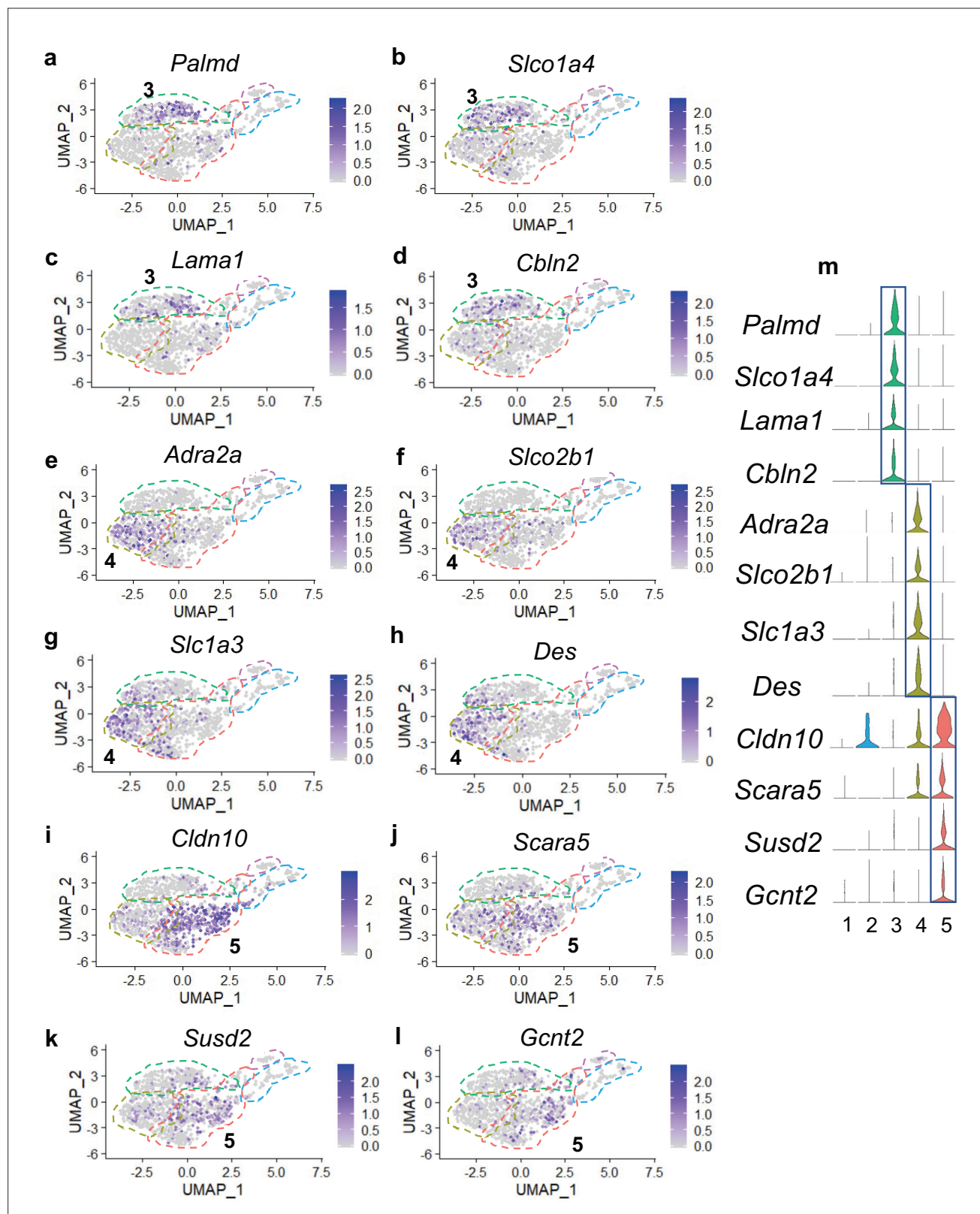


Figure 4—figure supplement 2. Differentially expressed genes mostly unique to clusters 3, 4, and 5. (a–j) Feature plots of genes identified by differential gene expression in cluster 3 (a–d), cluster 4 (e–h), and cluster 5 (i–l). (m) Violin plots of differentially expressed genes represented in (a–l).

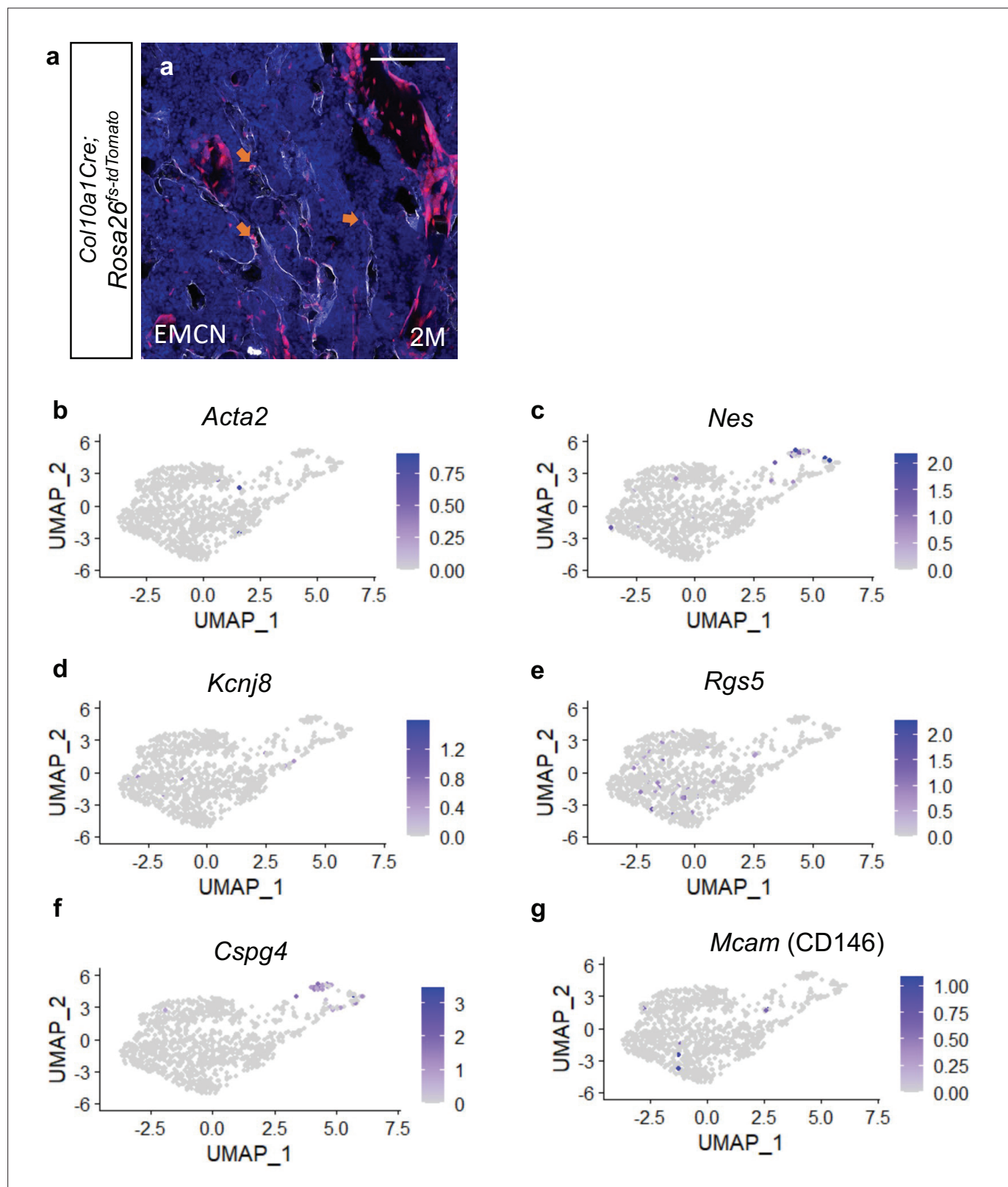


Figure 4—figure supplement 3. Hypertrophic chondrocyte-derived cells can associate with blood vessels; however, do not express genes associated with pericytes or vascular smooth muscle cells. **(a)** Immunostaining for endothelial-cell-associated protein, ENDOMUCIN, on 2-month-old *Col10a1Cre; Rosa26^{fs-tdTomato}* bone sections show adjacent tdTOMATO⁺ cells indicated by orange arrows. Feature plots for **(b–c)** vascular smooth muscle cell associated genes and **(d–g)** pericyte-associated genes indicate that these genes are rarely expressed in any cell cluster. Scale bar = 100 μm.

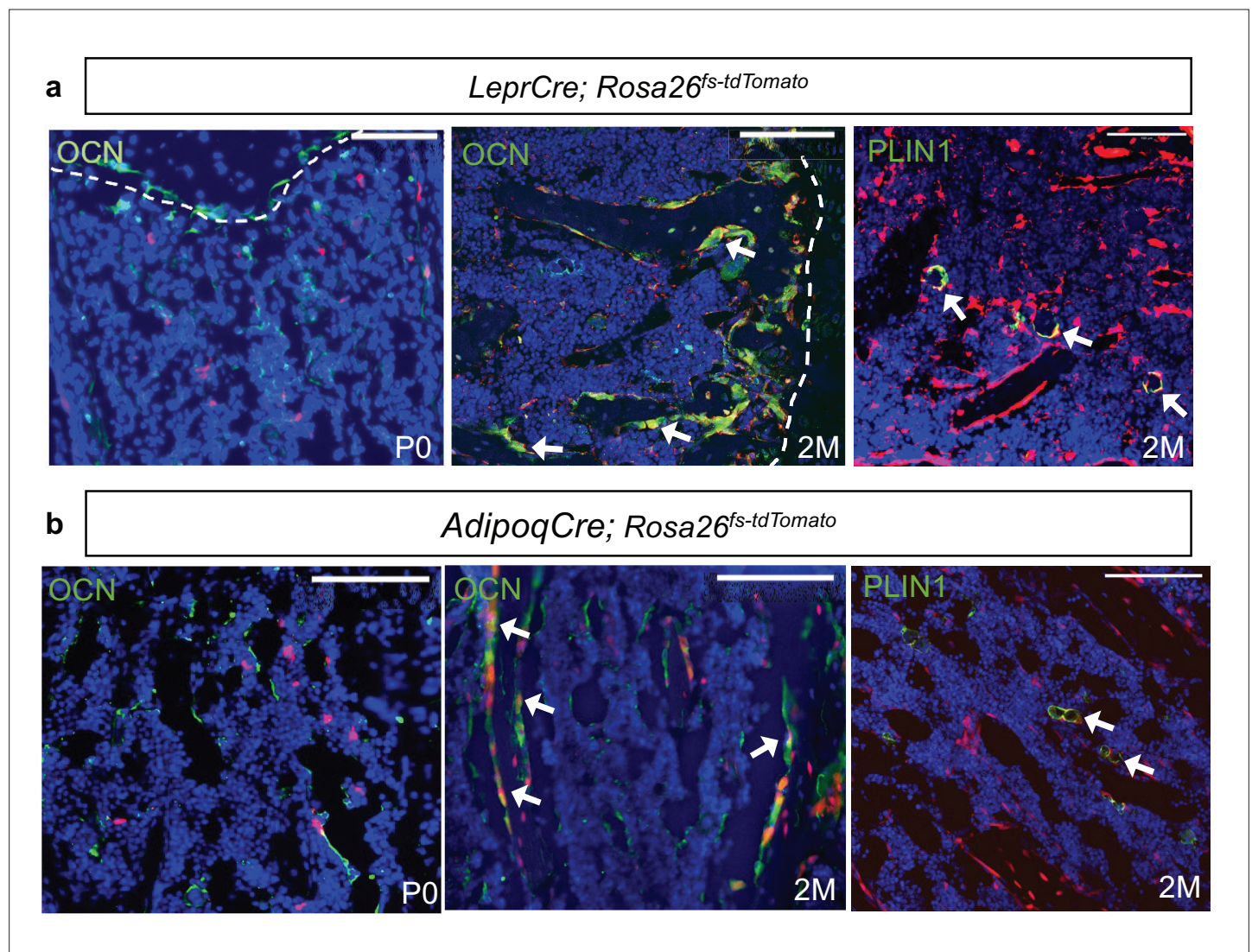


Figure 4—figure supplement 4. *LeprCre; Rosa26^{fs-tdTomato}* and *AdipoqCre; Rosa26^{fs-tdTomato}* mice exhibit reporter expression in marrow associated cells, osteoblasts, and adipocytes with age. **(a)** Immunostaining of bone sections from *LeprCre; Rosa26^{fs-tdTomato}* mice exhibit tdTOMATO⁺ marrow-associated cells; however, do not display tdTOMATO⁺, OCN⁺ osteoblasts at P0. Immunostaining at 2 months of age reveals both tdTOMATO⁺, OCN⁺ osteoblasts and tdTOMATO⁺, PERILIPIN⁺ adipocytes. **(b)** Immunostaining of bone sections from *AdipoqCre; Rosa26^{fs-tdTomato}* mice also exhibit tdTOMATO⁺ marrow-associated cells; however, do not display tdTOMATO⁺, OCN⁺ osteoblasts at P0. Immunostaining at 2 M of age reveals both tdTOMATO⁺, OCN⁺ osteoblasts and tdTOMATO⁺, PERILIPIN⁺ adipocytes.

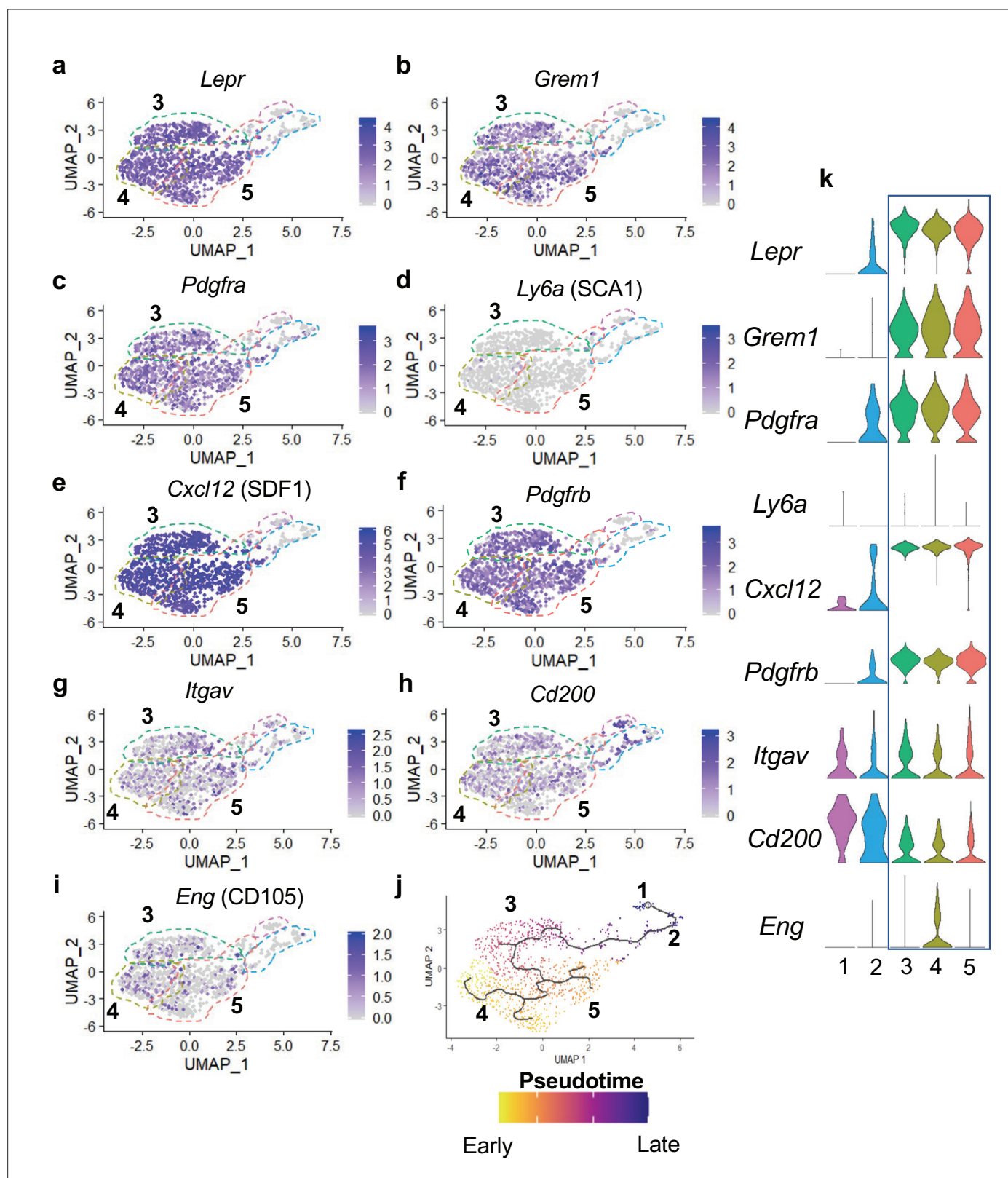


Figure 5. Many hypertrophic chondrocyte derived cells express genes associated with SSPCs. (a–f) Feature plots of genes previously identified as SSPC markers in genetic mouse models. (g–i) Feature plots of genes previously identified as SSPC markers for use in flow cytometry and FACS. (j) Monocle three trajectory analysis with clusters noted throughout pseudotime. (k) Violin plots of SSPC-associated genes in (a–i).

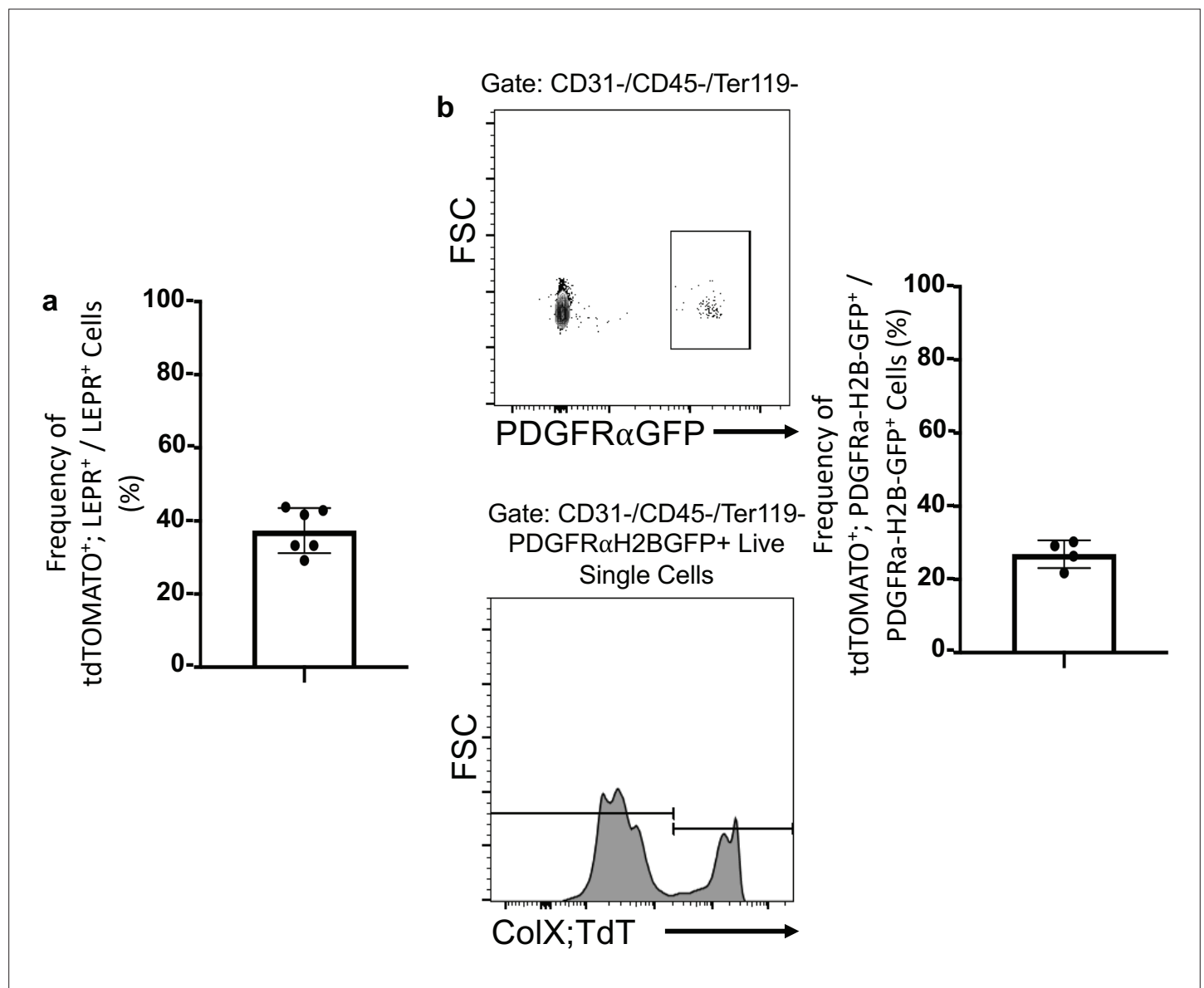


Figure 5—figure supplement 1. Contribution of hypertrophic chondrocyte descendants to total SSPC populations. (a) Quantitation of immunostaining for LEPR on bone sections from 2 M old *Col10a1Cre; Rosa26^{fs-tdTomato}* mice exhibit 37.4% LEPR⁺; tdTOMATO⁺ as compared to total LEPR⁺ marrow-associated cells (**Figure 5—figure supplement 1—source data 1**). N = 2 technical replicates of three biologic replicates, SD ±6.2%. (b) Flow cytometric analysis of 1-month-old *Col10a1Cre; Rosa26^{fs-tdTomato}; PDGFRα^{H2B-GFP}* mice exhibit 26.93% PDGFRα⁺; tdTOMATO⁺ as compared to total PDGFRα⁺ marrow-associated cells (**Figure 5—figure supplement 1—source data 2**). N = 4 biologic replicates, SD ±3.79%.

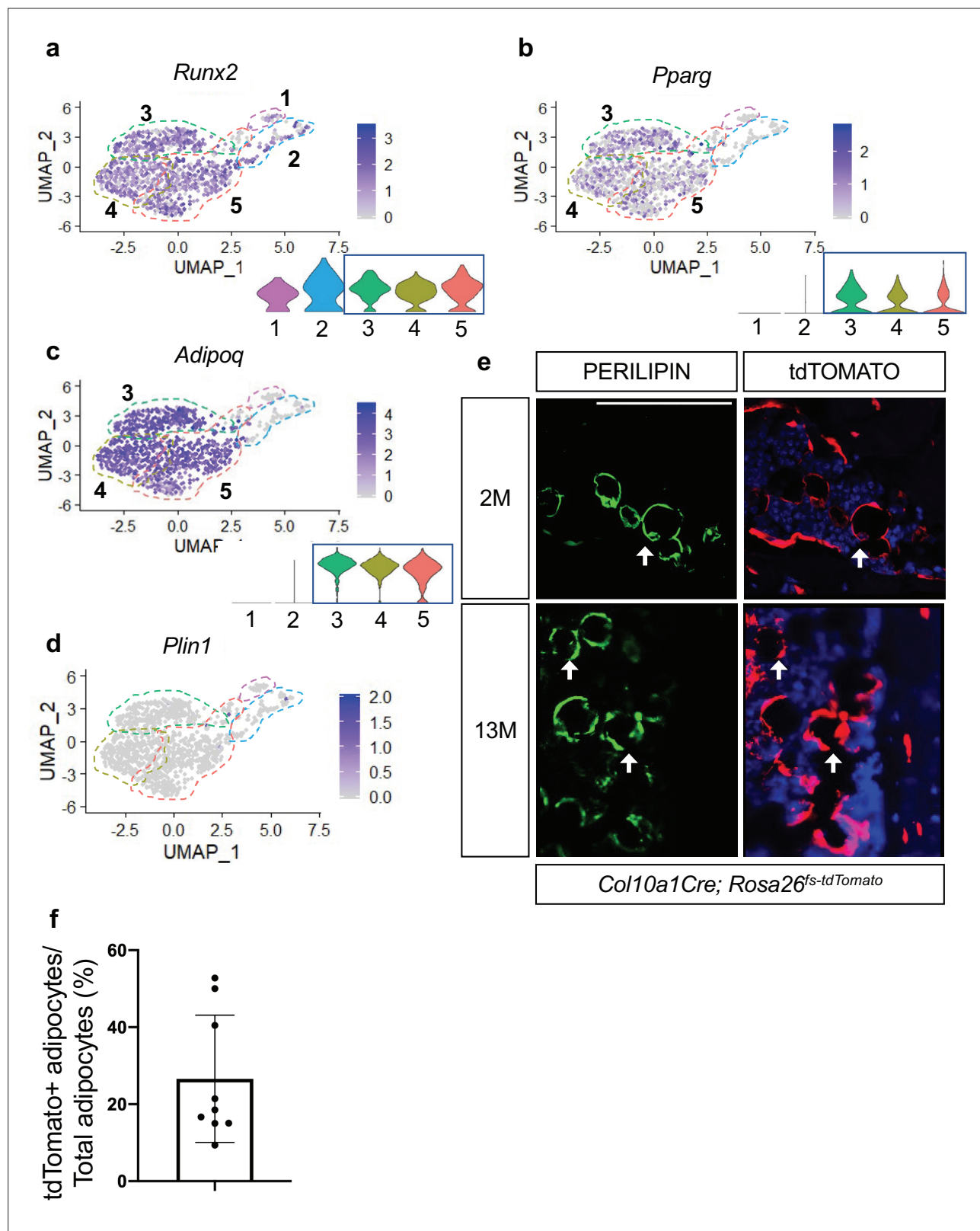


Figure 6. Hypertrophic chondrocyte derived SSPCs exhibit osteogenic and adipogenic differentiation capacities. (a) Feature plot and violin plot of the osteoblast specification gene, *Runx2*. (b–c) Feature plots and violin plots of the adipogenic specification gene, *Pparg*, and adipogenic associated gene, *Adipoq*. (d) Feature plot indicating a lack of expression of the mature lipid laden adipocyte gene, *Perilipin*. (e) Immunostaining for PERILIPIN in *Col10a1Cre;R26-tdTomato* mice at 2 months and 13 months of age. PERILIPIN⁺, tdTOMATO⁺ adipocytes noted with white arrows. Scale bar = 100 μ m

Figure 6 continued

(f) Quantification of PERILIPIN⁺, tdTOMATO⁺ adipocytes at 13 months of age in (e) Average = 27.59% (**Figure 6—source data 1**). N = 3 slides from three biologic replicates, SD \pm 16.2%.

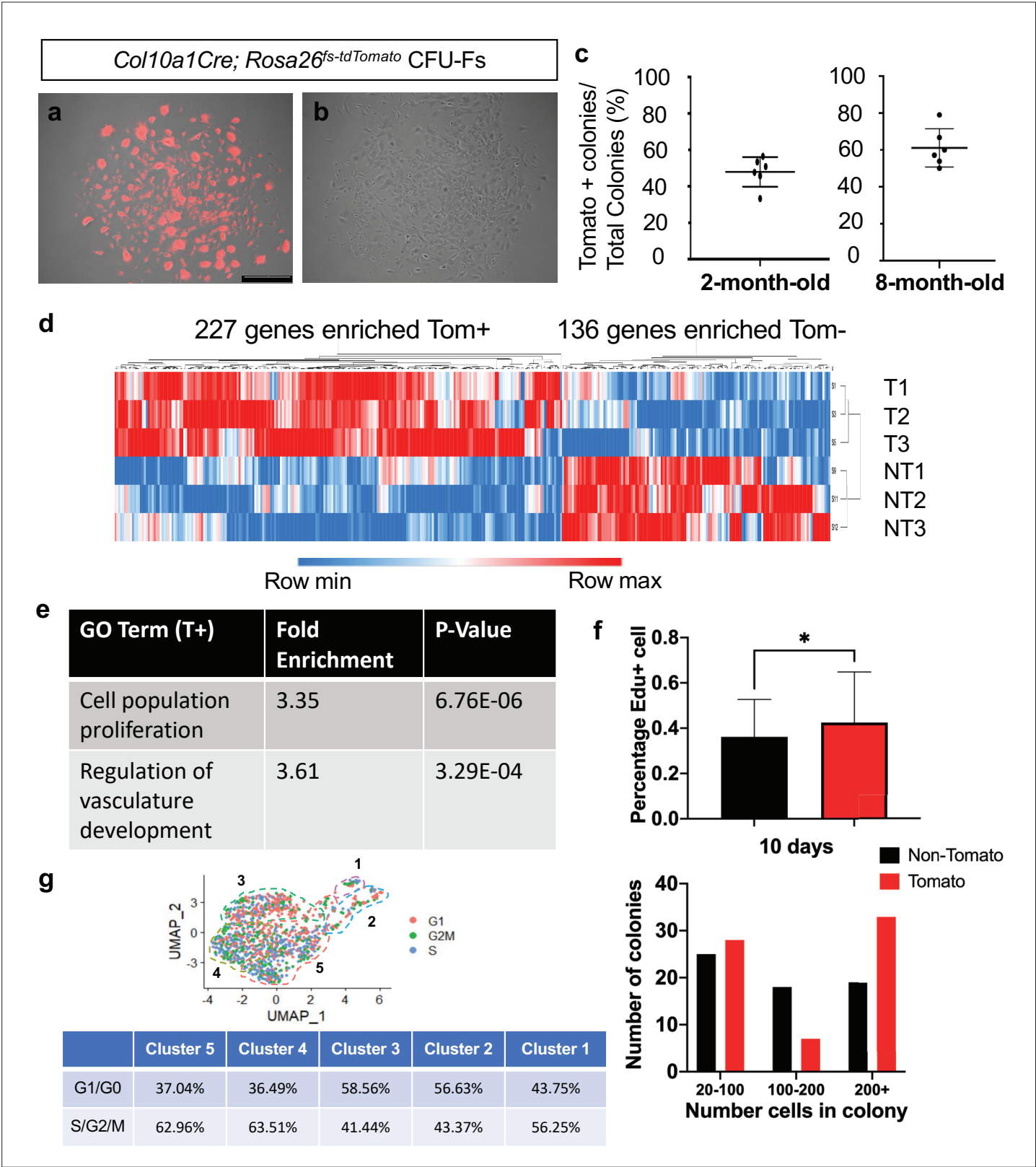


Figure 7. CFU-Fs derived from hypertrophic chondrocytes are similar to CFU-Fs derived from other cell sources, but contain SSPCs with enhanced proliferative capacities. (a–c) CFU-Fs derived from hypertrophic chondrocytes (tdTOMATO⁺) (a) and those derived from other cell sources (tdTOMATO⁻) (b) are established and develop at similar frequencies at 2- and 8 months of age (c – **Figure 7—source data 1**). Scale bar = 500 μ m (d) Heat-map from bulk RNA-seq data from three tdTOMATO⁺ CFU-Fs and 3 TOMATO⁻ CFU-Fs (**Figure 7—source data 2**). (e) Gene ontology terms associated with

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tdTOMATO⁺ CFU-Fs (**Figure 7—source data 3** and **Figure 7—source data 4**). (f) EdU incorporation within tdTOMATO⁺ and tdTOMATO⁻ CFU-Fs (top - **Figure 7—source data 5**) and differences in cell numbers between tdTOMATO⁺ and tdTOMATO⁻ CFU-Fs (bottom - **Figure 7—source data 6**). N = 2 technical replicates of three biologic replicates, SD tdTOMATO⁻ ± 16.5%, SD tdTOMATO⁺ ± 22.3%, p-value = 0.044. (g) Cell cycle analysis of the SSPCs associated with clusters 1–5 using Seurat 3.

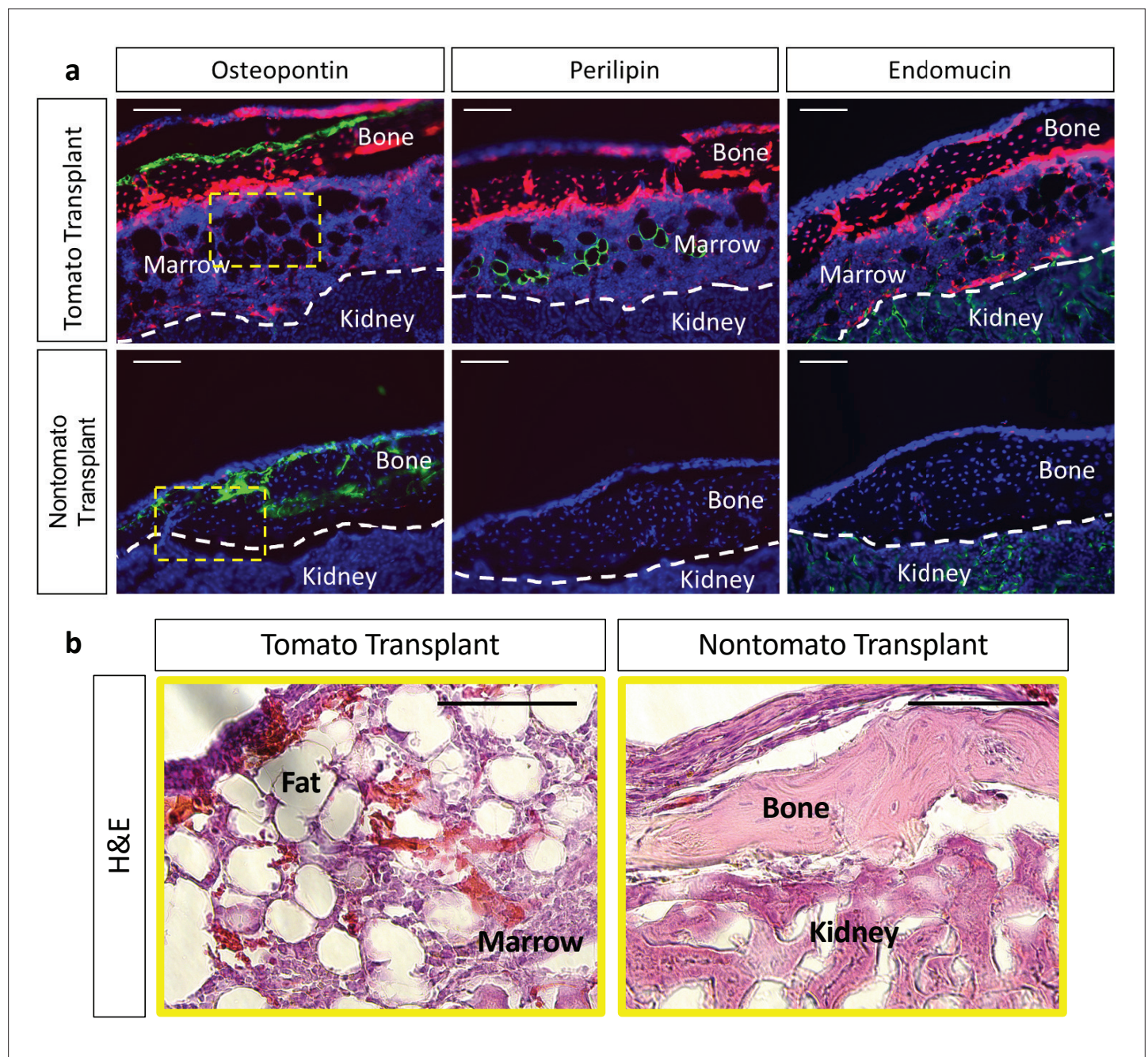


Figure 8. Kidney capsule transplantations of tdTOMATO⁺ cells exhibit complete ossicle formation with bone, adipocytes, and bone marrow compared to tdTOMATO⁻ resulting in only bone formation. **(a)** Immunofluorescent stain for bone (osteopontin), adipocytes (perilipin), and vessels (endomucin) for tomato⁺ transplant (top) and tomato⁻ transplant (bottom). **(b)** Hematoxylin and Eosin stain for marrow establishment in tdTomato⁺ transplant and tdTOMATO⁻ transplant.

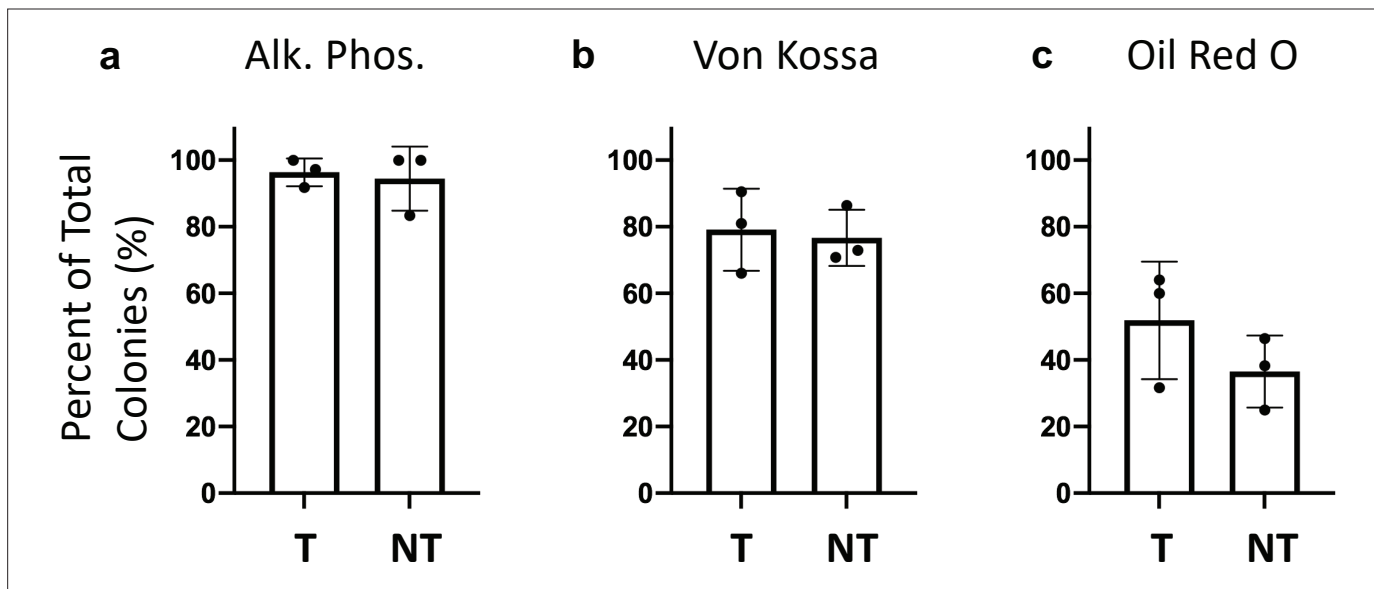


Figure 8—figure supplement 1. In vitro differentiation of colonies reveals relatively similar osteogenic and adipogenic differentiation capacities. (a–b) CFUs from *Col10a1Cre; Rosa26^{fs-tdTomato}* bone marrow differentiate into osteoblasts stained with alkaline phosphatase (a) and von kossa (b) (CFU-OB). alkaline phosphatase: SD tdTOMATO⁺ (T) \pm 4.2%, SD tdTOMATO⁻ (NT) \pm 9.6%, p-value = 0.63. von kossa: SD tdTOMATO⁺ \pm 12.3%, SD tdTOMATO⁻ \pm 8.4%, p-value = 0.67. (c) CFUs from *Col10a1Cre; Rosa26^{fs-tdTomato}* marrow differentiate into adipocytes stained with oil red o (CFU-AD). SD tdTOMATO⁺ \pm 17.6%, SD tdTOMATO⁻ \pm 10.8%, p-value = 0.11. **Figure 8—figure supplement 1—source data 1.**

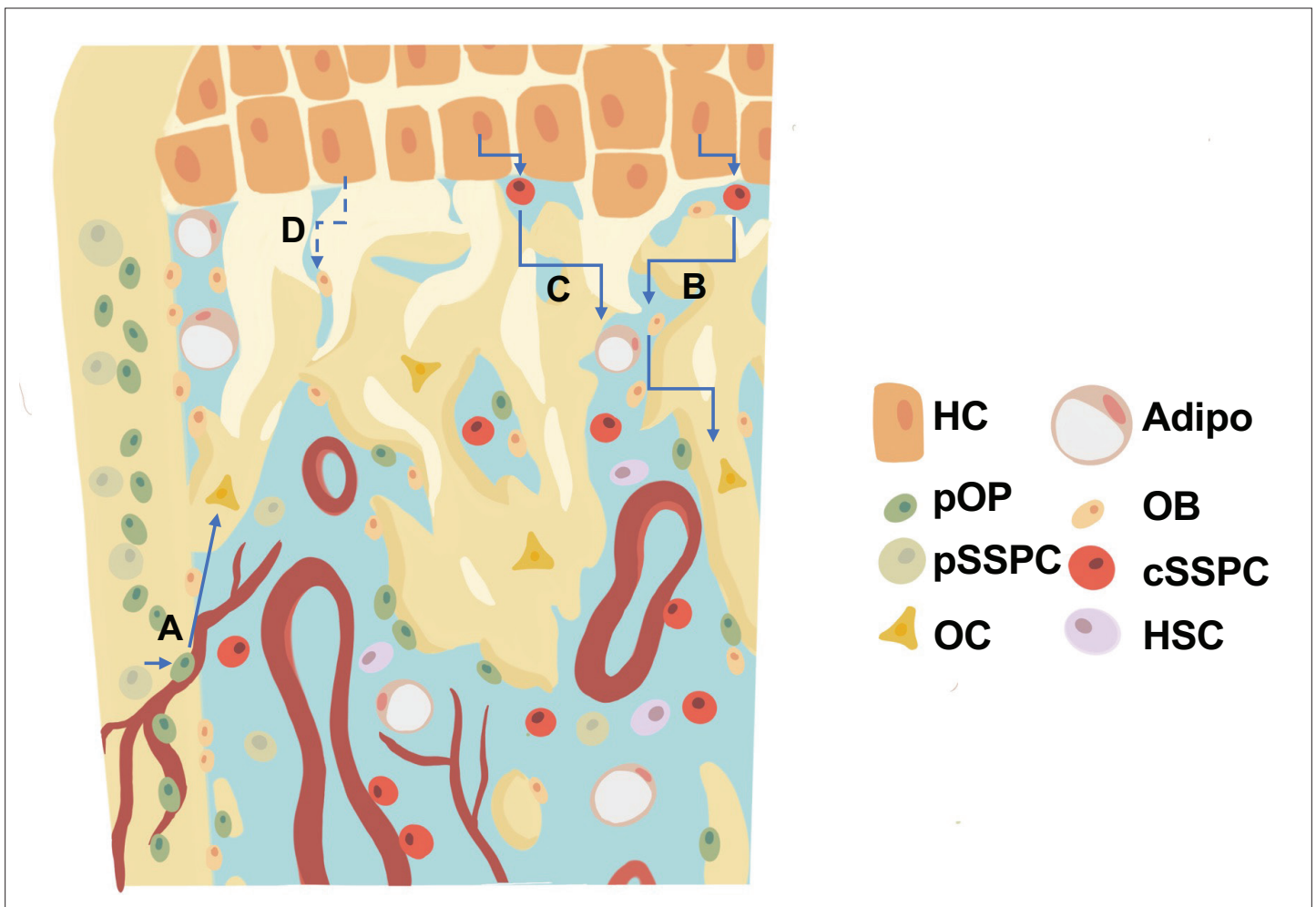


Figure 9. Revised model for the generation of trabecular/endocortical bone associated osteoblasts/osteocytes from multiple sources. **(a)** Perichondrial or periosteal SSPCs/osteoprogenitors (pSSPC/pOP) migrate into the marrow utilizing blood vessels and possess the ability to further differentiate into osteoblasts/osteocytes (OB/OC). **(b–c)** Hypertrophic chondrocytes (HC) dedifferentiate into chondrocyte derived SSPCs (cSSPC) with the capacity to generate osteoblasts/osteocytes **(b)** and adipocytes (Adipo) **(c)**. **(d)** Data presented does not rule out the potential for the transdifferentiation of hypertrophic chondrocytes directly into osteoblasts/osteocytes. HSC = hematopoietic stem cells.