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► To cite this version:

Pedro Vallecillo-García, Mickael Orgeur, Glenda Comai, Sophie Poehle-Kronawitter, Cornelius Fischer, et al.. A local subset of mesenchymal cells expressing the transcription factor Osr1 orchestrates lymph node initiation. *Immunity*, 2023, 56 (6), pp.1204-1219.e8. 10.1016/j.immuni.2023.04.014 . hal-04257866

HAL Id: hal-04257866

<https://hal.science/hal-04257866v1>

Submitted on 25 Oct 2023

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“Mesenchymal Osr1 cells orchestrate lymph node initiation”

3

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25

26 **Abstract**

27 The emergence of lymph nodes (LNs) is orchestrated by lymphoid tissue organizer cells (LTos) that
28 attract and retain lymphoid tissue inducer cells (LTis) at strategic positions in the embryo. Identity and
29 mode of action applied by LTos during the first LTI attraction have remained poorly characterized.
30 Here, we show that cells expressing the gene *Osr1* are mesenchymal LTo progenitors. By dissecting
31 *Osr1*⁺ cell heterogeneity we demonstrate distinct mesenchymal LTo signatures at diverse anatomical
32 locations and reveal a common progenitor of mesenchymal LTos and LN-associated adipose tissue.
33 *Osr1* is essential for LN initiation, driving commitment of mesenchymal LTos independently of neural
34 retinoic acid, and for LN-associated lymphatic vasculature. Finally, we demonstrate that combined
35 action of CXCL13 and CCL21 is required for LN initiation. Our results substantially redefine the role and
36 identity of mesenchymal organizer cells and unify current views proposing a new model of cooperative
37 cell function in LN initiation.

38

39 **Introduction**

40 Secondary lymphoid organs (SLOs) provide infrastructure for efficient immune response and comprise
41 lymph nodes (LNs), Peyer's patches (PPs), mucosa-associated lymphoid tissue (MALT) and the spleen.
42 LNs emerge during development at strategic vascular junctions in the embryo. LN organogenesis is
43 initiated by the accumulation of so-called lymphoid tissue inducer cells (LTis) at the prospective sites
44 of LN formation as the first step in a cascade of events leading to mature LN formation. LTis belong to
45 the family of innate lymphoid cells (ILCs) (1-3), which originate from the hemogenic endothelium and
46 colonize the fetal liver (4).

47 LTis accumulate at specific sites of the embryo relying on the positional information provided by
48 lymphoid tissue organizer cells (LTos) (1, 5-7). The exact identity of LTos and their developmental origin
49 remain obscure. Of note, despite their similar functions, mesenchymal LTos in LNs and spleen have
50 different developmental origins (8-10). LNs are embedded in white adipose tissue (WAT), and
51 adipogenic progenitor cells (APCs) can be reprogrammed as mesenchymal stromal cells in LNs via LTR β
52 signaling (11). This indicates a common origin of both cell types during development.

53 Acquisition of CXCL13 expression was described as the first step in mesenchymal LTo commitment (6).
54 *Cxcl13* expression in the embryo is highly restricted to the region of LN appearance and its activation
55 has been linked to the secretion of retinoic acid (RA) by motoneurons adjacent to the LN anlage (12).
56 RA signaling is considered crucial for LN formation, as mice lacking the RA receptor-related orphan
57 receptor gamma (ROR γ t) are devoid of LNs (1, 13), and LTI differentiation in LN anlagen depends on
58 RA signaling (14).

59 Subsequently, lymphotoxin- $\alpha\beta$ (L $\alpha\beta$) expressed by LTI cells signals to LTos expressing the lymphotoxin
60 receptor beta (LT β R) (5, 6), driving LNs organogenesis. This leads to a positive feedback loop that
61 results in increased secretion of chemokines including CXCL13 and CCL21 reinforcing LTI recruitment
62 and retention, and the expression of cell adhesion molecules required for LTo differentiation (1, 5, 6).
63 The initial secretion of CXCL13 and CCL21, however, appears independent of LT β R signaling (12, 15).

64 CXCL13 is seen as a crucial factor in LN initiation, as mouse models lacking either *Cxcl13* or its receptor
65 *Cxcr5* resulted in LN initiation arrest in the majority of peripheral LNs although cervical and mesenteric
66 LNs were formed (12, 16-18). Depletion of lymphatic endothelial cells (LECs) does not impair LN
67 initiation, however LECs are important for LN formation (19). Indeed, recent studies ascribed LTo
68 functions to lymphatic and blood endothelial cells (20, 21). This suggests that, in addition to LTI
69 recruitment via the CXCL13/CXCR5 axis, additional factors emanating from LTos (mesenchymal and
70 endothelial) may be involved to achieve effective first retention of LTis in the LN anlage. Importantly,
71 LNs develop at different body locations; although similar strategies might be shared this may implicate
72 the interaction of diverse local cell types and signaling molecules. Therefore, deciphering the initial
73 steps of LN formation and assessing local tissue heterogeneities might reveal similar strategies in the
74 organogenesis of more adaptive lymphoid organs such as MALT or tertiary lymphoid organs (TLOs).

75 Here, we identify a subset of Odd-skipped related transcription factor 1-positive (*Osr1*⁺) cells in the
76 embryo as a common progenitor of mesenchymal LTos and APCs. *Osr1* is required for both,
77 mesenchymal LTo commitment and assembly of LN-associated lymphatic vasculature. Our results
78 challenge the current model of neuron-derived RA in LN initiation and redefine the initial steps of LN
79 formation, where combined chemokine-directed LTI attraction by mesenchymal and lymphatic
80 endothelial LTos results in LN initiation.

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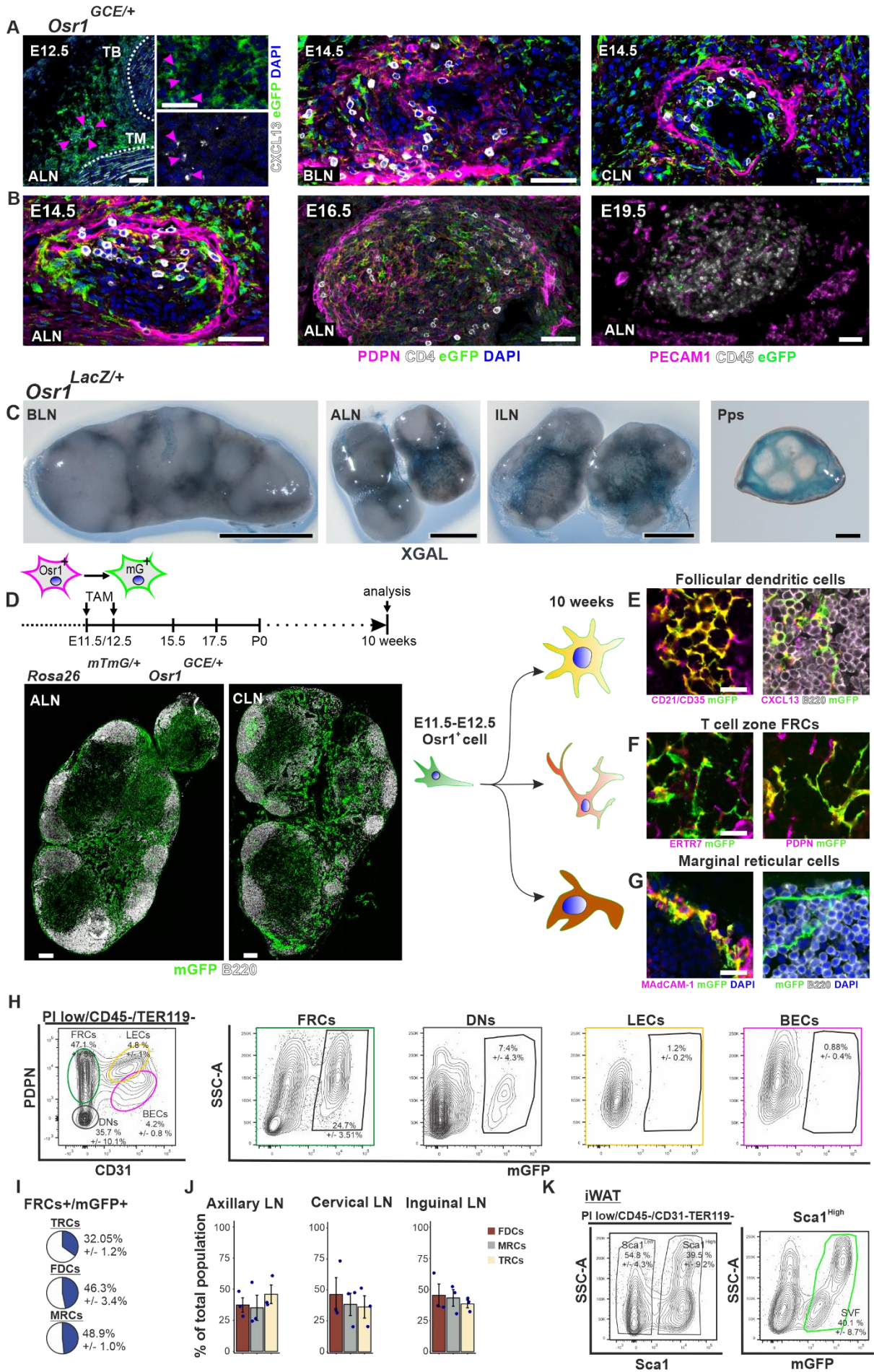
84 **Results**

85 ***Osr1* marks mesenchymal LTo progenitors and progenitors of all adult LN mesenchymal stromal** 86 **subpopulations**

87 The identity of embryonic mesenchymal precursors involved in LN formation has been hampered by
88 the lack of specific markers. We have previously shown that WAT and skeletal muscle connective tissue
89 (MCT) stromal cells share a common origin during development in a subpopulation of lateral plate
90 mesoderm-derived cells expressing the transcription factor *Osr1* (22). *Osr1* plays a key role in the
91 formation of embryonic organs such as kidney, heart, foregut and skeletal muscle (22-25), however a
92 function in LNs has not been described. *Osr1* expression, visualized via an eGFP knock-in reporter
93 (*Osr1^{GCE}*), was detected prior to LTi accumulation at stage E12.5 in the axillary area. *Osr1*⁺ cells were
94 found in the mesenchyme that surrounds the bifurcation of the lateral thoracic vein and include the
95 first CXCL13⁺ cells (Figure 1A). At E14.5, when the LN anlage organizes with the attraction of the first
96 LTi cells, *Osr1*⁺ cells localized in the cervical and axillary regions in direct contact with CD4⁺ LTi cells
97 and in association with the developing lymphatic vasculature (Figure 1A and S1A). At this stage, *Osr1*⁺
98 cells were only observed adjacent to the outer endothelial cell layer in the thymus and were absent in
99 the spleen (Figure S1B). Later, *Osr1* expression faded in the LN anlagen. At E16.5, *Osr1*⁺ cells were
100 found sparsely in close association with CD4⁺ cells and at E19.5 only few *Osr1*⁺ cells were found in the
101 LN anlage (Figure 1B). In adult peripheral and mesenteric LNs, *Osr1* expression visualized via a beta
102 galactosidase knock-in reporter (*Osr1^{LacZ}*) was restricted to the medullary region of LNs and PPs (Figure
103 1C), consistent with the *Osr1* expression found in Nr4a1⁺, Inmt⁺ and CD34⁺ stromal subpopulations
104 (26).

105 Embryonic pulse-labeling of *Osr1*⁺ cells via tamoxifen administration at E11.5 and E12.5 in
106 *Rosa26^{mTmG/+} Osr1^{GCE/+}* animals revealed that *Osr1* descendants were broadly distributed between the
107 medulla and the capsule in adult LNs (Figure 1D). *Osr1* descendants were found inside the B cell follicle
108 co-expressing CXCL13 or CD21/CD35, specific markers for follicular dendritic cells (FDCs) (Figure 1E), in
109 the cortex expressing ERTR7 or PDPN marking T cell zone fibroblastic reticular cells (TRCs) (Figure 1F),
110 and in the subcapsular sinus expressing MAdCAM-1 marking marginal reticular cells (MRCs) (Figure
111 1G). Thus, FDCs, TRCs, and MRCs can originate from a common embryonic progenitor already present
112 at stage E11.5/12.5. In addition, we found *Osr1* descendants giving rise to pericytes in close association
113 with PNA⁺ high endothelial venules (HEV), and to pericytes expressing PDGFR β associated with blood
114 endothelial cells and adjacent to LYVE1⁺ lymphatic vasculature (Figure S1C). Contrary to LNs, the
115 splenic stroma was devoid of embryonic-derived *Osr1*⁺ cells (Figure S1C). In line with the decreasing
116 expression of *Osr1* in fetal LNs, membrane GFP⁺ traced cells were rarely found in the adult LN stroma
117 after a pulse of tamoxifen at postnatal days 0 and 1 (Figure S1D) or in E18.5 axillary LN stroma after a
118 tamoxifen pulse at E15.5 (Figure S1E). Next, we measured mGFP contribution to stromal
119 subpopulations via flow cytometry analysis of peripheral LNs (PLNs) (Figure 1H). Labelling by
120 E11.5/12.5 tamoxifen pulsing, we found that approximately a quarter of the PDPN⁺ CD31⁻ FRCs were
121 *Osr1* descendants (Figure 1I). Contribution to the double negative population (PDPN⁻ CD31⁻) was low
122 and contribution to LECs (PDPN⁺ CD31⁺) and BECs (PDPN⁻ CD31⁺) was practically not detected (Figure
123 1H). Quantification of embryonic lineage contribution to TRCs, FDCs and MRCs using FACS (Figure 1I)
124 and section immunofluorescence (Figure 1J) highlighted a partial contribution to all populations. In line
125 with the partial contribution in LNs, we observed partial embryonic *Osr1*⁺ contribution to stromal
126 vascular fraction (SVF) in inguinal fat adipose tissue (Figure 1K).

127 In summary, *Osr1* marks a poorly described population of mesenchymal cells in the embryo at sites of
128 prospective LN formation and prior to mesenchymal LTo commitment or LTi attraction. Thus, *Osr1*
129 labels the first documented mesenchymal LTo progenitors, which also comprise an origin of adult
130 mesenchymal subpopulations in the LN stroma.



132 **Figure 1.** *Osr1* marks mesenchymal LTo progenitors, which are a source of adult mesenchymal stromal subpopulations. (A, B)
 133 Representative images of E12.5, E14.5, E16.5 and E19.5 *Osr1*^{GCE/+} LNs. At E12.5, the axillary region contains the first *Osr1*+
 134 cells (eGFP) expressing CXCL13 close to the lateral thoracic artery. Arrowheads point to sites of *Osr1* and CXCL13 co-expression
 135 and boxed region is shown as a magnification at the right. At the stages E14.5, E16.5 and E19.5, immunofluorescence for
 136 PDPN, CD4/CD45 and eGFP (*Osr1*) shows *Osr1* expression in cells intermingled with LTi cells and associated with the PDPN-
 137 high lymphatic vasculature. Dashed lines in (A) border teres major (TM) and triceps brachii (TB) muscles. (C) *Osr1* expression
 138 in LNs and Pps from adult *Osr1*^{LacZ} reporter animals assessed by whole-tissue X-gal staining. (D) Schematic representation of
 139 tamoxifen injection for genetic lineage tracing. Lineage contribution was assessed at 10 weeks of age. Below:
 140 immunofluorescence of 10-week-old ALN and CLN sections for mGFP (*Osr1* lineage) and B220 demarcating B cell follicle. (E-
 141 G) Representative immunofluorescence images of 10-week-old PLNs co-stained for mGFP and FDC markers CD21/CD35 and
 142 CXCL13, FRC markers ERTR7 and PDPN, and MRC marker MAdCAM-1. (H) Flow cytometry analysis from Rosa26^{mTmG/+} *Osr1*^{GCE/+}
 143 adult PLNs for *Osr1* lineage (mGFP) contribution to FRCs, double negative cells (DNs), LECs and BECs. (I) Quantification of FRCs-
 144 mGFP+ FACS isolated cells expressing the markers PDPN, MAdCAM1 and CD25/35. (J) Quantification of *Osr1* embryonic
 145 lineage contribution to adult MRCs, FDCs and TRCs quantified on sections by immunolabeling for PDPN, MAdCAM1 and
 146 CD25/35. (K) Flow cytometry analysis from Rosa26^{mTmG/+} *Osr1*^{GCE/+} adult inguinal fat pad to assess *Osr1* lineage contribution
 147 to Sca1^{high} stromal vascular fraction. Representative images have been captured from at least 3 different embryos. Values are
 148 shown as percentage of the mean and as a +/- percentage of the s.e.m. In H-K, n=3. Scale bar represents in (A) 50 μ m, (B) 1
 149 mm, (C) 200 μ m and (E-G) 10 μ m. Abbreviations: ALN, axillary LN; BLN, brachial LN; CLN, cervical LN; ILN, inguinal LN; and
 150 Pps, Peyer's patches.

151 Dissecting embryonic *Osr1*+ cell heterogeneity at the single-cell level

152 At E13.5, CXCL13 expressing LTos were only found at the location of LN formation and represented a
 153 subpopulation of mesenchymal *Osr1*+ cells in the areas of peripheral and mesenteric LN anlagen
 154 (Figure 2A and S2A). At the transcriptional level, the vast majority of *Cxcl13* expression was found in
 155 *Osr1*+ cells (Figure S2B), confirming *Osr1*+ cells as a broader population that contains mesenchymal
 156 LTos. In order to dissect the heterogeneity of the *Osr1*+ cells and to characterize early mesenchymal
 157 LTos, we performed droplet-based single-cell RNA sequencing (scRNA-seq) of E13.5 *Osr1*^{GCE/+} cells
 158 isolated by FACS (Figure 2B). To enrich for *Osr1*+ cells associated with LN primordia, E13.5 *Osr1*^{GCE/+}
 159 embryos were dissected from the axilla until the tongue including the inguinal region. Following
 160 exclusion of low-quality cells and empty droplets (Figure S2C and Methods), 6,849 E13.5 *Osr1*+ cells
 161 were further analyzed by unsupervised clustering. The 15 clusters identified were visualized by fast
 162 interpolation-based *t*-distributed stochastic neighbor embedding (FIt-SNE) on two dimensions (Figure
 163 2C). Based on the enrichment of key genes obtained after differential expression analysis (Figure S2D)
 164 and section immunofluorescence, we defined the *Osr1*+ cell subpopulations and assigned their
 165 presumptive anatomical locations (Figure 2D).

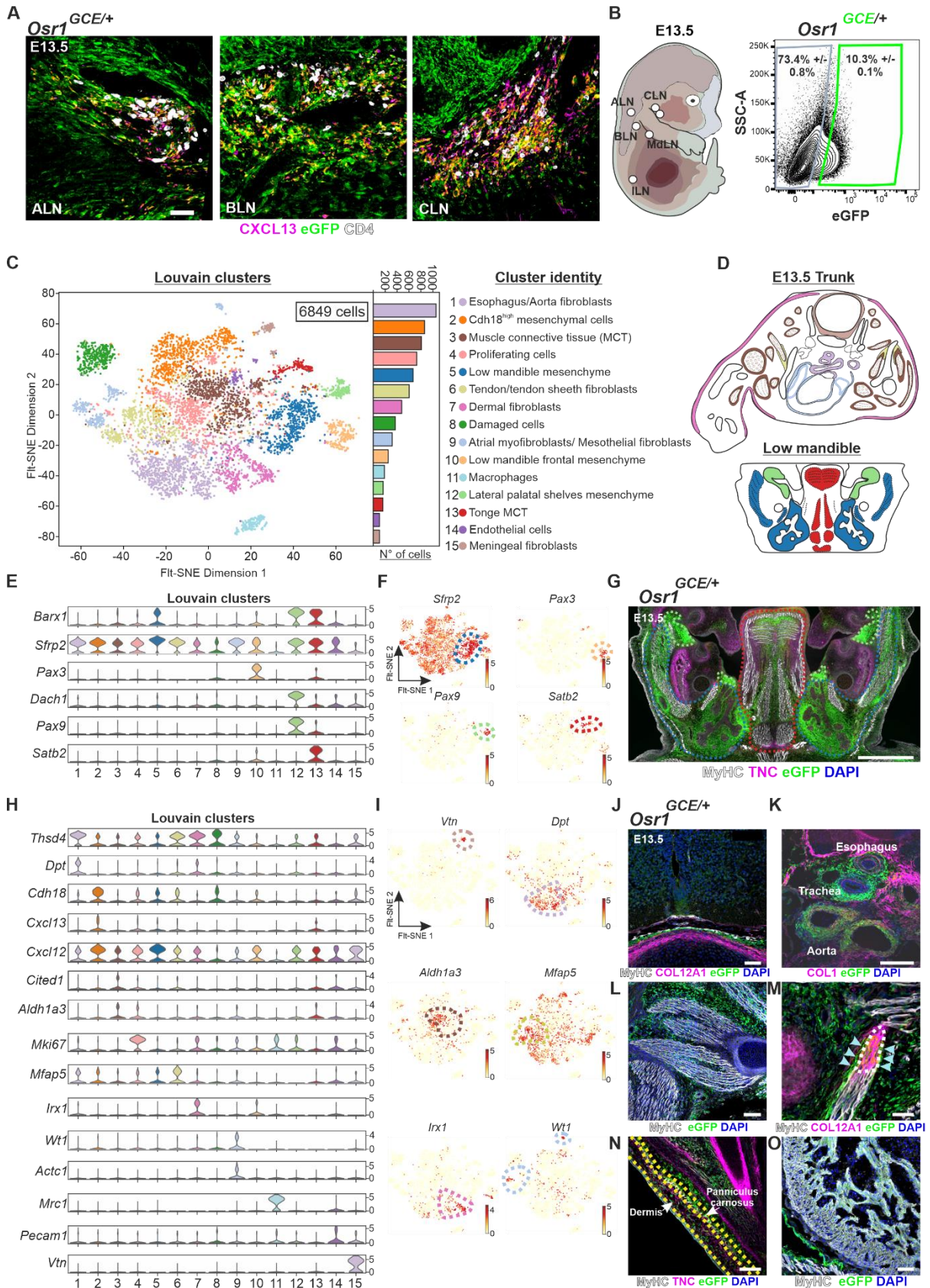
166 Given the restricted expression of the gene *Barx1* in the lower jaw and proximal limb region (27), we
 167 assigned clusters 5, 10, 12 and 13 to *Osr1*+ cells of the craniofacial region (Figure 2E and S2E).
 168 Complementary, clusters 1-4 and 6-9 contain *Osr1*+ cells anatomically located in the trunk. Cluster 5
 169 contained salivary gland mesenchymal cells and MCT of the masseter muscle enriched in *Sfrp2*
 170 expression (Figure 2E-G). Cluster 10 expressed *Pax3* suggesting mesenchymal cells of the frontal low
 171 mandible mesenchyme (28). Cluster 12 showed expression of *Pax9* and *Dach1*, which converge only at
 172 the dorsal mesenchyme of the lower jaw flanking the tongue (29), both regions rich in *Osr1*+ cells
 173 (Figure 2E-G). Cluster 13 showed expression of *Satb2*, which is expressed in the lower jaw (30),
 174 correlating with *Osr1*+ cells in the tongue MCT (Figure 2E-G).

175 Based on the expression pattern of *Osr1* (22, 31, 32), we mainly expected fibroblastic or mesenchymal
 176 cell identities. However, several clusters did not correspond to fibroblast or mesenchymal cells. Within
 177 them, cluster 14 represents a small subpopulation of *Osr1*+ cells expressing typical endothelial cell
 178 markers such as *Pecam1* (Figure 2H and S2F). Cluster 11 contains a subpopulation of *Osr1*+ cells with
 179 a macrophage signature. These cells highly express *Mrc1*, *Adgre1* or *Csf1r*, whereas genes expressed
 180 in committed LTis and LTi precursors *Cd4*, *Ilr7* and *Cxcr5* remain absent (Figure 2H and S2G). Cells from
 181 cluster 8 contain a higher proportion of mitochondrial gene expression and a reduced expression of
 182 ribosomal genes as compared to other clusters (Figure S2H). Therefore, we considered them as a

183 damaged fraction of cells generated during the process of cell isolation. By contrast, *Osr1*⁺ cells in
184 cluster 4 corresponded to proliferative cells as reflected by the higher expression of cell cycle genes
185 relative to other clusters (Figure S2H). Within the cells corresponding to mesenchymal or fibroblastic
186 cells but assigned to trunk and craniofacial regions, we found that cells in cluster 15 exclusively
187 expressed *Vtn* (Figure 2H and I) and correlated with embryonic meningeal fibroblasts (33) (Figure 2J).

188 Of the *Osr1*⁺ trunk clusters, cluster 1 contains cells with a transcriptional profile enriched in
189 extracellular matrix (ECM) markers, such as *Thsd4*, *Dpt* or *Col14a1* (Figure 2H, I, and S2I). *Osr1*⁺ cells
190 abundantly expressed COL1 in the region of the aorta and esophagus (Figure 2K). Cluster 3 likely
191 represented MCT fibroblasts of the trunk, which strongly express *Osr1* (22), given the expression of
192 *Aldh1a3* and *Cited1* (eurexpress database, <http://www.eurexpress.org>) (Figure 2H, I, L and S2J). Cluster
193 6 exclusively expressed *Tnmd* and *Tppp3* and showed an enrichment in *Mfap5* expression, suggesting
194 a tenocyte and tendon sheath fibroblast identity (34) (Figure 2H, I and S2K). *Osr1*⁺ cells were found in
195 tendons marked by collagen XII and in tendon sheaths (Figure 2M). Besides, *Osr1*⁺ cells were found
196 flanking the tendon attachment region of trunk muscles (Figure 2M). Cluster 7 was defined by exclusive
197 expression of *Irx1* and an enrichment in ECM genes, such as *Lum*, *Dcn* and *Tnc* (Figure 2H, I and S2L).
198 Embryonic skin transcriptome data (35) suggested *Osr1* and *Irx1* as specific markers for skin fibroblasts.
199 In line, we found co-expression of TNC and *Osr1* in the E13.5 dermis (Figure 2N). Cluster 9 enclosed a
200 heterogenous population of cells with a diverse transcriptional profile including *Acta1*⁺ atrial
201 myofibroblasts and *Wt1/Upk3b*⁺ epicardial/mesothelial cells (Figure 2H, I and S2M). *Osr1* was highly
202 expressed in the heart epicardial region at E13.5 (Figure 2O), and *Osr1* expression was reported in
203 atrial cardiomyocytes (36). Of note, gene enrichment analysis using available embryonic scRNA-seq
204 datasets (37) confirmed the identity for endothelial cells, macrophages and atrial myofibroblasts
205 (Figure S2N). Other cell identities were in part discordant, suggesting that our data could contribute to
206 define embryonic mesenchymal subpopulations.

207 Cluster 2 showed enriched expression of *Cdh18* and *Grm7*, which so far were not associated with
208 specific mesenchymal cell types. Of note, cluster 2 showed the highest expression of chemokines
209 *Cxcl13* and *Cxcl12* (Figure 2H and S2O). We provisionally named cluster 2 cells as *Cdh18*^{high}
210 mesenchymal cells. Due to their high chemokine production within the *Osr1*⁺ cells, we next focused
211 on this cluster possibly enriched in trunk mesenchymal LTo cells.



212

213 **Figure 2. Embryonic E13.5 *Osr1* cell heterogeneity at the single-cell level.** (A) Maximal intensity projection images of 35 μ m
 214 cross-sections of E13.5 *Osr1*^{GCE/+} peripheral LNs immunolabeled for CXCL13, CD4 and eGFP (*Osr1*). (B) FACS strategy to isolate
 215 *Osr1* cells from E13.5 *Osr1*^{GCE/+} embryos used for scRNA-seq. (C) Fit-SNE visualization of E13.5 *Osr1* cells following Louvain cell
 216 clustering. Number of cells per cluster and assigned cell identities are indicated. (D) Schematic representation of putative
 217 cluster identity and tissue distribution of *Osr1* cells of E13.5 trunk and low jaw in a cross-sectional view. (E) Violin plots of
 218 selected genes indicative of craniofacial identity depicting the distribution of gene expression within all cells per Louvain

219 cluster. (F) Fit-SNE visualization of cells expressing the indicated genes. (G) *Osr1* expression in a cross-sectional view of the
 220 low jaw. Skeletal muscles are labeled for MyHC and dense connective tissue with TNC. Putative anatomical location of
 221 craniofacial clusters is labeled by colored dashed lines. (H) Violin plots of selected genes indicative of trunk identity depicting
 222 the distribution of gene expression within all cells per Louvain cluster. (I) Fit-SNE visualization of cells expressing the indicated
 223 genes for the trunk clusters. (J-O) Representative micrographs of E13.5 *Osr1*^{GCE/+} embryos sections showing *Osr1* expression
 224 at anatomical locations corresponding to putative *Osr1*⁺ subpopulations. Skeletal muscles were labeled for MyHC and
 225 connective tissue with COL1, COL12A1 or TNC. Immunofluorescences are representative images of at least 3 different embryos.
 226 Cells in scRNA-seq analysis are derived from a single E13.5 *Osr1*^{GCE/+} embryo. In (F, I), gene expression level in cells is depicted
 227 as log-transformed normalized counts. Scale bar represents in (A) 50 μ m, (G) 600 μ m and (J-O) 100 μ m. MdLN represents
 228 mediastinal LN.

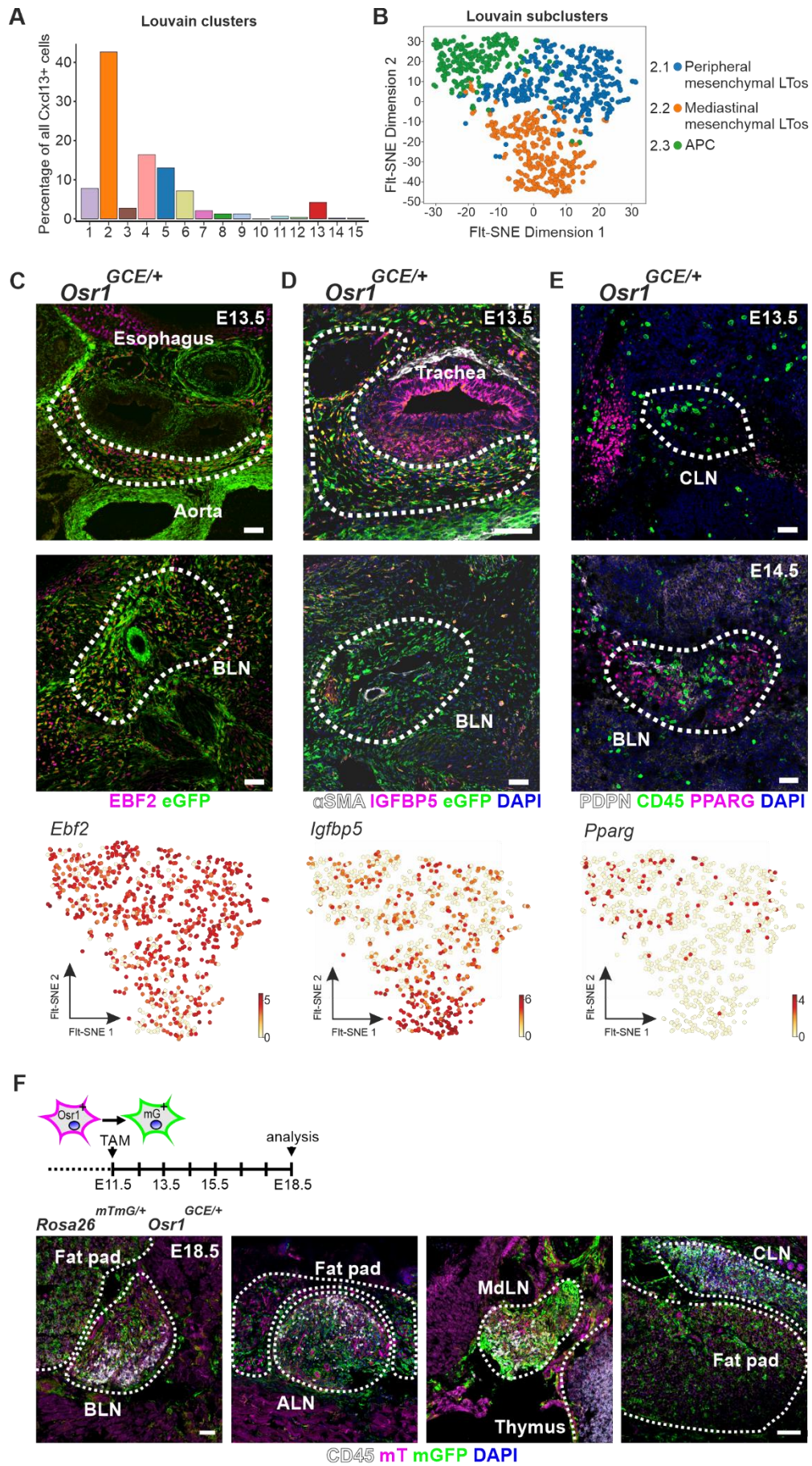
229 **A common progenitor of white adipose tissue and mesenchymal LTOs in the embryo**

230 *Cdh18*^{high} mesenchymal cells of cluster 2 were enriched for *Cxcl13*⁺ cells (Figure 3A), indicative of
 231 enriched mesenchymal LTO identity. We thus focused on this cluster and performed Louvain
 232 subclustering for *Cdh18*^{high} mesenchymal cells, revealing 3 subclusters (Figure 3B). *Cxcl13* and also
 233 *Cxcl12* expression were increased in subclusters 2.1 and 2.2 compared to the subcluster 2.3 (Figure
 234 S3A). We therefore assumed mesenchymal LTOs are mainly contained in subclusters 2.1 and 2.2. We
 235 used expression of *Ebf2* and *Igfbp5* to further distinguish these subclusters. *Ebf2* is a transcription
 236 factor found in adipogenic precursor cells (38) and is broadly expressed in *Osr1*⁺ cells (Figure S3B),
 237 however a higher expression within *Cdh18*^{high} cells was seen in subclusters 2.1 and 2.3 as compared to
 238 subcluster 2.2 (Figure 3C and S3B). EBF2 was highly abundant in *Osr1*⁺ trunk fibroblasts of the axillary
 239 region including MCT, putative mesenchymal LTOs at the location of the brachial LN and surrounding
 240 interstitial mesenchyme. *Osr1*⁺ cells with a lower expression of EBF2 were located in the interstitium
 241 between aorta and esophagus of the mediastinal cavity (Figure 3C). Conversely, *Igfbp5* expression
 242 marked subcluster 2.2 (Figure 3D and S3B). IGFBP5 expression correlated with *Osr1*⁺ cells in the
 243 mediastinal cavity but not in the axillary region (Figure 3D). We thus termed the cells from subcluster
 244 2.1 as peripheral mesenchymal LTOs and the cells from subcluster 2.2 as mediastinal mesenchymal
 245 LTOs.

246 *Osr1*⁺ cells in cluster 2.3 present an enrichment in genes involved in adipose tissue formation such as
 247 *Pparg*, *Fst*, *Sox9* and *Sox5* (39, 40) (Figures 3E, S3C). At E13.5, PPARG⁺ cells were identified in proximity
 248 to the cervical LN (Figure 3E). At E14.5, PPARG⁺ cells were found outside of the axillary and brachial
 249 LN anlagen and intermingled with CD45⁺ cells (Figure 3E and S3D). The mediastinal LN was devoid of
 250 PPARG⁺ cells (Figure S3E). *Pparg* expression was also found in subcluster 2.1 defined as peripheral
 251 mesenchymal LTOs. In line, we observed CXCL13 and PPARG double positive cells in the axillary and
 252 brachial LN anlagen (Figure S3E) indicative of a bivalent population of interstitial cells. By comparing
 253 the transcriptional signatures of *Cdh18*^{high} subclusters (Figure S3F) with the defined identities of E18.5
 254 and P3 stromal vascular fraction in the thoracic aorta-associated adipose tissue (41), we further
 255 confirmed an enriched adipocyte signature in subcluster 2.3 while subclusters 2.1 and 2.2 were
 256 enriched in a progenitor signature (Figure S3G).

257 Thus, at E13.5, *Osr1*⁺ *Cdh18*^{high} cells contain mesenchymal LTOs and APCs in the trunk. This suggests
 258 that earlier *Osr1*⁺ cells may represent bipotential progenitors giving rise to white adipocytes and
 259 mesenchymal LTOs. To assess this, we performed lineage tracing of E11.5 *Osr1*⁺ progeny until 18.5.
 260 *Osr1* descendants were found in E18.5 LN mesenchymal stroma and as adipocytes in adjacent fat pads
 261 of the axillary, brachial and cervical regions (Figure 3F). In line with the postnatal formation of visceral
 262 fat (42), we did not detect a fat pad associated with the mediastinal LN in E18.5 embryos (Figure 3F).

263 Taken together, we defined a *Cdh18*^{high} mesenchymal cell population in the E13.5 trunk containing
 264 progenitors of mesenchymal LTOs in trunk and mediastinal cavity, and also adjacent WAT in the trunk
 265 (Figure S3H). Divergent transcriptional profiles of LTOs located in mediastinal and axillary regions
 266 indicate heterogeneity of mesenchymal LTOs depending on the anatomical location.



267

268 **Figure 3.** Embryonic E13.5 *Osr1* interstitial progenitors are a source of white adipocytes and mesenchymal LTos in the trunk.269 (A) Bar plot depicting the percentage of *Cxcl13* expressing cells within all Louvain clusters. (B) Fit-SNE visualization of *Cdh18*^{high}

270 mesenchymal cells following Louvain cell subclustering with assigned cluster identities. **(C, D)** Representative micrographs of
 271 E13.5 *Osr1^{GCE/+}* mediastinal and axillary regions showing eGFP (*Osr1*), EBF2 or IGFBP5 co-expression. Below: Fit-SNE
 272 visualization of *Ebf2* and *Igfbp5* expressing cells. Mural cells are labeled in **(D)** with α SMA. **(E)** Immunofluorescence of E13.5
 273 and E14.5 *Osr1^{GCE/+}* cervical and axillary regions showing PPARG+ fat pads in association with the LN anlage immunolabeled
 274 for CD45 and PDPN. Below: Fit-SNE visualization of *Pparg* expressing cells. **(F)** Schematic representation of tamoxifen injection
 275 for genetic lineage tracing; below: images of indicated LNs and associated fat pads showing *Osr1* lineage (mGFP) contribution.
 276 CD45 cells demarcate peripheral and mediastinal LN anlage. Immunofluorescences are representative images of at least 3
 277 different embryos. In **(C-E)**, gene expression level in cells is depicted as log-transformed normalized counts and dashed lines
 278 represent regions of interest. Scale bar represents in **(C-E)** 50 μ m and in **(F)** 50 μ m (BLN) and 100 μ m (CLN).

279 ***Cxcl13+* cell heterogeneity defines a functional niche in the LN anlage**

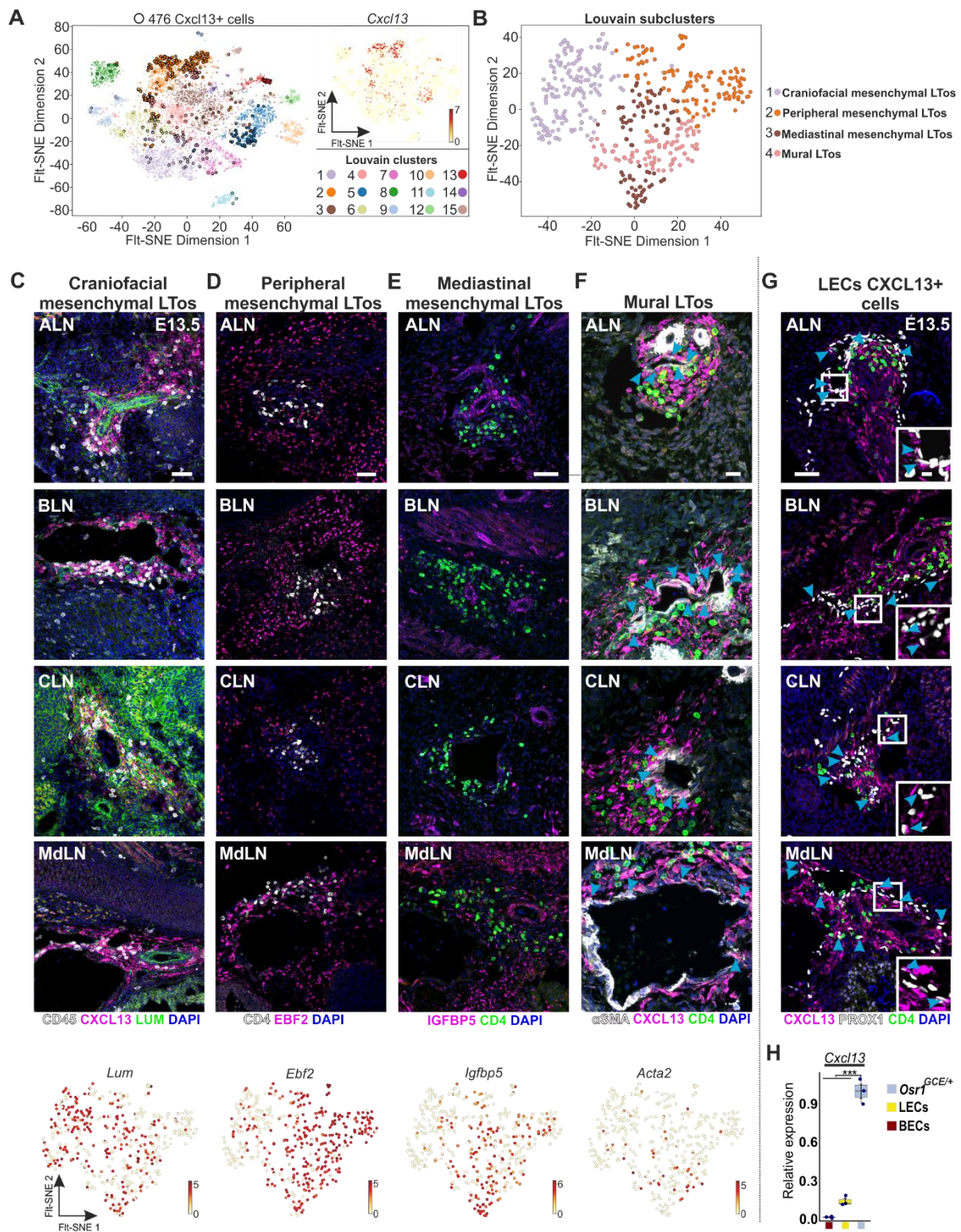
280 Additionally to cluster 2, *Cxcl13+* cells were also found in other clusters, e.g. in the craniofacial clusters,
 281 indicating a more heterogenous population of mesenchymal LTos (Figures 3A and 4A). Intriguingly,
 282 CXCL13 protein abundance itself was heterogeneous in different LN anlagen at E13.5 and E14.5 (Figure
 283 S4A). Thus, in order to resolve *Cxcl13+* cell heterogeneity, we next analyzed our scRNA-seq dataset
 284 from the perspective of all *Cxcl13* expressing cells. Cell cycle effects were regressed out to also include
 285 cells in the proliferative cluster 4. Louvain subclustering of the 476 cells with a detectable *Cxcl13*
 286 expression from all clusters (203 *Cxcl13+* cells from cluster 2 and 273 *Cxcl13+* cells from other Louvain
 287 clusters) divided them into four subclusters (Figure 4B). Based on the expression of key genes (Figure
 288 S4B, C) such as *Barx1*, subcluster 1 was assigned with a craniofacial identity representing *Cxcl13+* cells
 289 in low mandible mesenchyme (main cluster 5). *Cxcl13+* subcluster 1 cells were therefore termed
 290 craniofacial mesenchymal LTos. Craniofacial mesenchymal LTos cells presented a higher content of
 291 ECM genes such as *Lum*, *Col25a1* or *Dcn* compared to other subclusters (Figure S4B, C). In line, cervical
 292 LN anlagen showed higher abundance of LUM protein, which was not detected or low in trunk and
 293 mediastinal LNs (Figure 4C).

294 *Cxcl13+* cells in subclusters 2 and 3 shared a similar transcriptional profile to peripheral and mediastinal
 295 mesenchymal LTos (Figure S4B, C) and were identified by the expression of EBF2 and IGFBP5 (Figure
 296 4D, E). Axillary brachial and mediastinal LNs showed abundant EBF2 protein expression, which was
 297 hardly present in the cervical LN (Figure 4D and S4D). As expected, an IGFBP5 enrichment was only
 298 found in the mediastinal cavity (Figure 4E).

299 The fourth subcluster of *Cxcl13+* cells was enriched in the mural cell marker *Acta2* (43) (Figure 4B).
 300 These cells, which we termed mural LTos, also show an enriched expression of the chemokines *Ccl11*
 301 and *Ccl2* (Figure S4B, C). Immunolabeling confirmed expression of CXCL13 in ACTA2+ cells surrounding
 302 blood vessels of all LNs (Figure 4F), thus validating their mural cell identity. We note that co-expression
 303 of CXCL13 and ACTA2 was exclusively found in mural cells in LN anlagen, suggesting a specific mural
 304 cell identity in these regions.

305 Surprisingly, we also noticed expression of CXCL13 in a small proportion of LECs labelled by PROX1 or
 306 LYVE1 (Figure 4G and S4E). Similar to *Cxcl13+* mural cells, expression of CXCL13 and PROX1 in LECs was
 307 only found in lymph vessels of the LN anlage. Consistently, *Cxcl13* transcripts were mainly detected in
 308 *Osr1+* cells isolated by FACS and at lower levels in CD31+ PDPN+ LECs, but not in CD31+ PDPN- BECs
 309 (Figure 4H).

310 Altogether, subclustering of all *Osr1+/Cxcl13+* cells in combination with *in situ* immunolabeling
 311 establish that embryonic mesenchymal LTos comprise a heterogeneous population of cells exhibiting
 312 specific transcriptional profiles depending on the anatomical location. Our results are complementary
 313 to recent data that highlights mesenchymal heterogeneity in E17.5 LNs (44) and indicates that E13.5
 314 *Osr1* mesenchymal LTos represent an earlier mesenchymal population with high CXCL13 expression.
 315 Of note, CD34 is expressed only at low levels in E14.5 mesenchymal LTos (Figure S4F,G). Finally, some
 316 LECs and mural cells in the LN anlage are specified to accessory LTos expressing the chemokine CXCL13.



319 **Figure 4.** *Cxcl13* defines a functional niche in the LN anlage. (A) Fit-SNE visualizations of all *Osr1*⁺ cells highlighting the
 320 distribution of *Cxcl13*⁺ cells within Louvain clusters. Right: *Cxcl13* expression in all Louvain clusters. (B) Fit-SNE visualization of
 321 *Cxcl13*⁺ cells following Louvain subclustering with the assigned cluster identity. (C-G) Representative micrographs of E13.5
 322 *Osr1*^{GCE/+} cross-sections of axillary, brachial, cervical, and mediastinal LNs immunolabeled for LUM, EBF2, IGFBP5 or ACTA2.
 323 LTIs were labeled for CD45 or CD4, mesenchymal LTos for *CXCL13*, and LECs for PROX1. Below, Fit-SNE visualization of *Lum*,
 324 *Ebf2*, *Igfbp5* and *Acta2* in *Cxcl13*⁺ cell subclusters. In G, boxed regions are shown as higher magnification in the corner. (H)
 325 Relative expression of *Cxcl13* transcripts in FACS isolated E13.5 BECs, LECs and *Osr1*⁺ (*Osr1*^{GCE/+}) cells. Immunofluorescence
 326 images are representative of at least 3 different embryos. In (A, C-F), gene expression level in cells is depicted as log-

327 transformed normalized counts. Scale bar represents in (C-E) 50 μ m, in (F) 20 μ m and in (G) 50 μ m and 10 μ m (boxed region).
 328 In H, *** represents $p < 0.001$ and $n=3$. P values were obtained from one-way ANOVA with Dunnett's post-hoc comparison.

329

330 ***Osr1* is essential for LTi attraction to LN primordia, mesenchymal LTo commitment and LN-associated** 331 **lymphatic vasculature assembly**

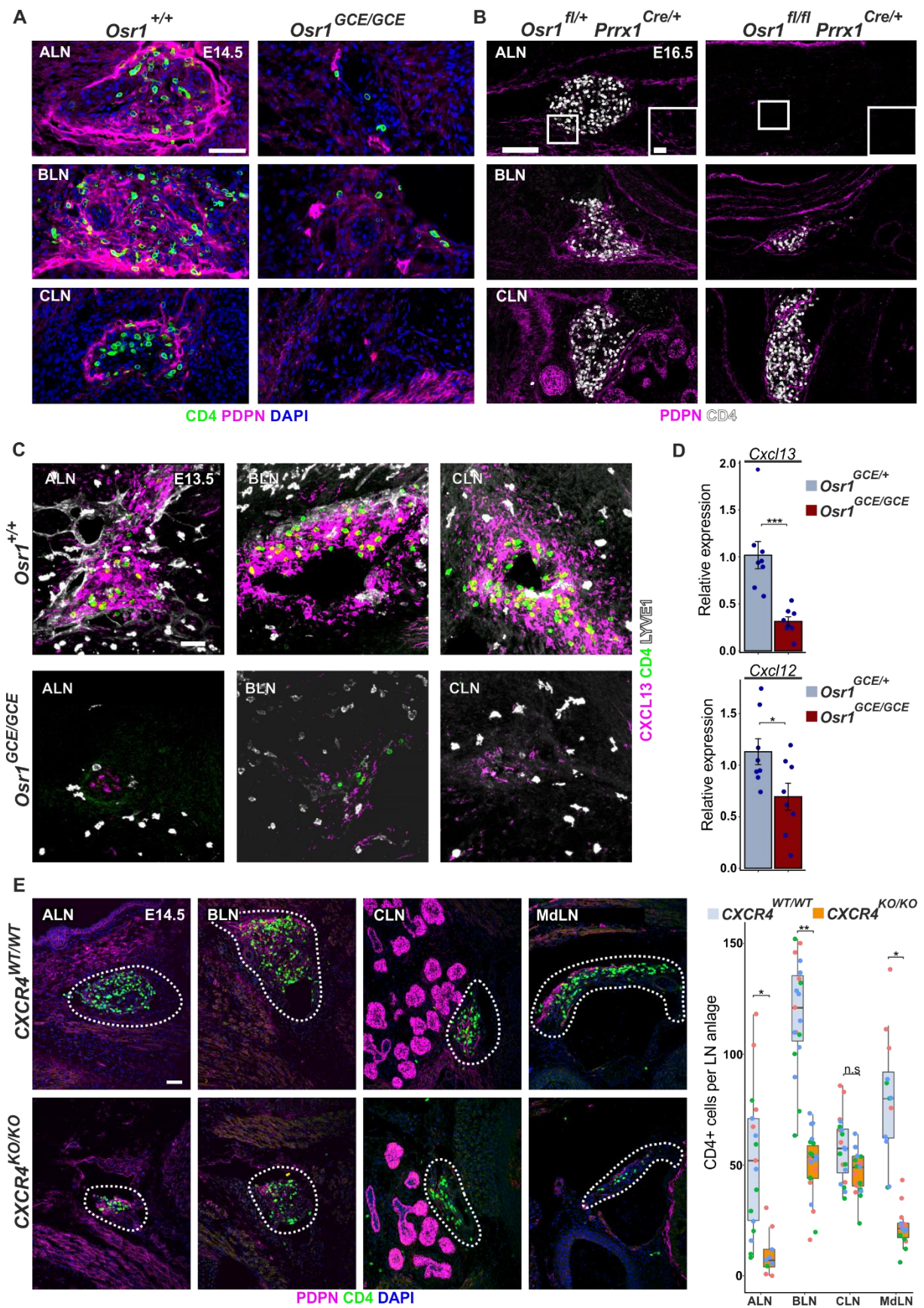
332 We then asked whether *Osr1* is necessary for LTo specification and function. Homozygous knock-out
 333 of *Osr1* (*Osr1*^{GCE/GCE}) led to an arrest in LN initiation for PLNs (cervical, axillary, brachial, inguinal,
 334 jugular) and impaired LN initiation for mesenteric LNs (mediastinal and renal), hence all LNs that can
 335 be identified at E14.5 (Figure 5A and S5A). CD4+ cells were not attracted at all or were highly reduced.
 336 Interestingly, concomitant to the defective attraction of CD4+ cells, the lymphatic vasculature network
 337 of the LN anlagen was strongly impaired, with only few PDPN-high lymphatic vessels surrounding CD4+
 338 cells in all LNs analyzed (Figure 5A and S5A). The absence of *Osr1* did not affect primary lymphoid organ
 339 development, as thymus formation was not impaired (Figure S5B), and normal spleen development
 340 has been previously reported in *Osr1*^{GCE/GCE} embryos (45). *Osr1*^{GCE/GCE} LN areas showed normal
 341 cellularity compared to controls and reduced proliferation of eGFP+ cells was observed in the cervical
 342 region, but not in other LNs (Figure S5C).

343 To bypass *Osr1*^{GCE/GCE} embryo lethality at E14/15 due to heart defects (45), we generated a conditional
 344 *Osr1* allele (*Osr1*^{fl/fl}) from the previously described *Osr1*^{LacZ/+} line (46) (Figure S5D). We used the *Prrx1*-
 345 *Cre* line mediating recombination in lateral plate mesoderm-derived mesenchymal cells (47).
 346 Recombination of *Rosa26*^{mTmG/+} or *Osr1*^{fl/fl} loci visualized by mGFP or eGFP, respectively, demonstrated
 347 *Prrx1*^{Cre/+} activity in limb, limb girdle and mediastinal region including the ALN and BLN, and low activity
 348 in the CLN region (Figure S5E, F). In line, axillary and brachial LNs were not formed or highly impaired
 349 in E16.5 *Osr1*^{fl/fl} *Prrx1*^{Cre} embryos, while cervical LNs formed normally (Figure 5B). In the axillary LN
 350 anlage, PDPN immunofluorescence demonstrated absence of associated lymphatic vasculature in
 351 E16.5 *Osr1*^{fl/fl} *Prrx1*^{Cre} embryos (Figure 5B). *Osr1*^{fl/fl} *Prrx1*^{Cre} embryos do not show edema, no embryonic
 352 or postnatal lethality was observed. This demonstrates that the defects in LTi recruitment and
 353 lymphatic vessel formation due to the loss of *Osr1* were not merely due to a developmental delay or
 354 global effects as edema appearing in *Osr1*^{GCE/GCE} embryos.

355 Disrupted early LTi recruitment pointed to an aberrant commitment of mesenchymal LTo progenitors
 356 due to the loss of *Osr1*. To assess this, we first examined CXCL13 expression in the E13.5 LN anlage.
 357 CXCL13 protein was almost absent in peripheral axillary, brachial, and cervical LNs of E13.5 *Osr1*^{GCE/GCE}
 358 embryos (Figure 5C) and reduced in renal LNs (Figure S5G). In line, *Cxcl13* expression was decreased in
 359 E13.5 *Osr1*^{GCE/GCE} cells isolated by FACS (Figure 5D). We note that residual CXCL13 signal in the
 360 *Osr1*^{GCE/GCE} LN anlage was mainly found in *Osr1*-GFP+ cells (Figure S5H), suggesting that *Cxcl13*/*Cxcl13*
 361 expression not exclusively depends on *Osr1*. In addition, *Cxcl12* expressed in all mesenchymal LTos,
 362 and *Ccl11* expressed in mural LTos, were also downregulated in E13.5 *Osr1*^{GCE/GCE} cells, while *Ccl2*
 363 expressed in mural LTos was unaltered (Figure 5D and S5I, J). Although CXCL12 and CCL11 are capable
 364 of attracting LTis *in vitro* (12), and CXCL12 has a function in B cell migration (48, 49), a specific role in
 365 LN initiation *in vivo* has not been tested yet. Therefore, we examined LN initiation in embryos deficient
 366 for the CXCL12 main receptor CXCR4 (*CXCR4*^{KO/KO}) (50). CD4+ LTi accumulation was reduced in axillary,
 367 brachial and mediastinal LNs, whereas cervical LNs appeared to form normally (Figure 5E). Contrary to
 368 *Osr1*^{GCE/GCE} embryos, we did not observe any impairment in LN lymphatic vasculature formation in
 369 E14.5 *CXCR4*^{KO/KO} embryos (Figure 5E and S5K).

370 We conclude that *Osr1*-deficient mesenchymal progenitors are arrested in their early commitment to
 371 CXCL13+ mesenchymal LTos. Furthermore, mesenchymal LTos not only rely on CXCL13 for LTi
 372 attraction, as CXCL12/CXCR4 signaling is required for proper LTi recruitment to the LN anlage. Finally,

373 lack of *Osr1* in mesenchymal cells resulted in impaired formation of lymphatic vasculature in the LN
374 anlage.



381 *LTis were labeled for CD4 and LECs for LYVE1. (D) RT-qPCR for Cxcl13 and Cxcl12 in FACS isolated Osr1-GFP+ cells from E13.5*
 382 *Osr1^{GCE/+} and Osr1^{GCE/GCE} embryos (n=8). (E) Representative images of peripheral and mediastinal LNs of E14.5 CXCR4^{WT/WT} and*
 383 *CXCR4^{KO/KO} embryos labeled for CD4 and PDPN. Dashed lines demarcate LN anlagen. At the right, quantification of CD4+ cells*
 384 *in LN anlagen per section is shown (n=3). Dot color represents measurements from different embryos. Immunofluorescence*
 385 *images have been captured from at least 3 independent experiments. Scale bar in (A) 50 μm, in (B) 100 μm and in (C, E) 50*
 386 *μm. (D, E) Error bar represents s.e.m. and * p<0.05, ** p<0.01, *** p<0.001 or n.s not significant. P values were obtained from*
 387 *student's t-test.*

388 **Mesenchymal retinoic acid signaling is required for LTo commitment**

389 In the current view of LN initiation, Cxcl13 expression is triggered by RA secretion from neurons at the
 390 site of the LN primordium (12). However, transcriptome analysis of Osr1+ cells (22) showed robust
 391 expression of *Aldh1a2* encoding the main RA producing enzyme during development, which was
 392 decreased in *Osr1^{GCE/GCE}* cells. Conversely, expression of *Cyp26b1* encoding the enzyme cytochrome
 393 P450 26B1 catabolizing all-trans RA was increased in *Osr1^{GCE/GCE}* cells. This suggests that Osr1+ cells
 394 may themselves be a source and modulator of RA signaling. We first analyzed ALDH1A2 expression
 395 from E12.5 until E19.5 on tissue sections. High expression of ALDH1A2 was detected in meningeal and
 396 epicardial fibroblasts (Figure S6A), in line with previous reports (33, 51, 52), validating antibody fidelity.
 397 ALDH1A2 expression was observed at E12.5 in the axillary LN region in venous-associated
 398 mesenchymal LToS (Figure 6A and S6B). From this stage until E15.5, ALDH1A2 expression was restricted
 399 to mesenchymal LToS intermingling with CD45+ cells in peripheral and mesenteric LN anlagen (Figure
 400 6A and S6C). ALDH1A2 expression decreased at late fetal stages and at E19.5 in the axillary LN only few
 401 cells expressed ALDH1A2 (Figure 6A). Comparing *Aldh1a2* expression between Osr1+ cells, BECs, LECs
 402 and CD45+ cells separated by FACS suggested that Osr1+ cells are the major source of RA production
 403 in the LN anlage (Figure 6B). Expression of ALDH1A2 in mesenchymal cells at E13.5 was corroborated
 404 by PDGFRα staining (Figure S6D). Next, we confirmed *Aldh1a2* downregulation and *Cyp26b1*
 405 upregulation in E13.5 *Osr1^{GCE/GCE}* cells by RT-qPCR (Figure 6B). Despite the mild reduction of *Aldh1a2*
 406 mRNA expression, ALDH1A2 protein was severely reduced in LN anlagen of E13.5 *Osr1^{GCE/GCE}* embryos,
 407 and almost absent in ALN and BLN (Figure 6C). Importantly, motoneurons in the vicinity of most PLNs
 408 and mesenteric LNs did not express high levels of ALDH1A2 (Figure 6C). Expression of ALDH1A2 in
 409 TUJ1+ motoneurons was in fact only observed close to the BLN (Figure 6C and S6E). These
 410 motoneurons however did not show reduced ALDH1A2 in E13.5 *Osr1^{GCE/GCE}* embryos (Figure 6C and
 411 S6E). These results suggest that in LN anlagen RA is mainly produced by mesenchymal LToS and that
 412 mesenchymal RA signaling may be severely impaired in *Osr1^{GCE/GCE}* embryos.

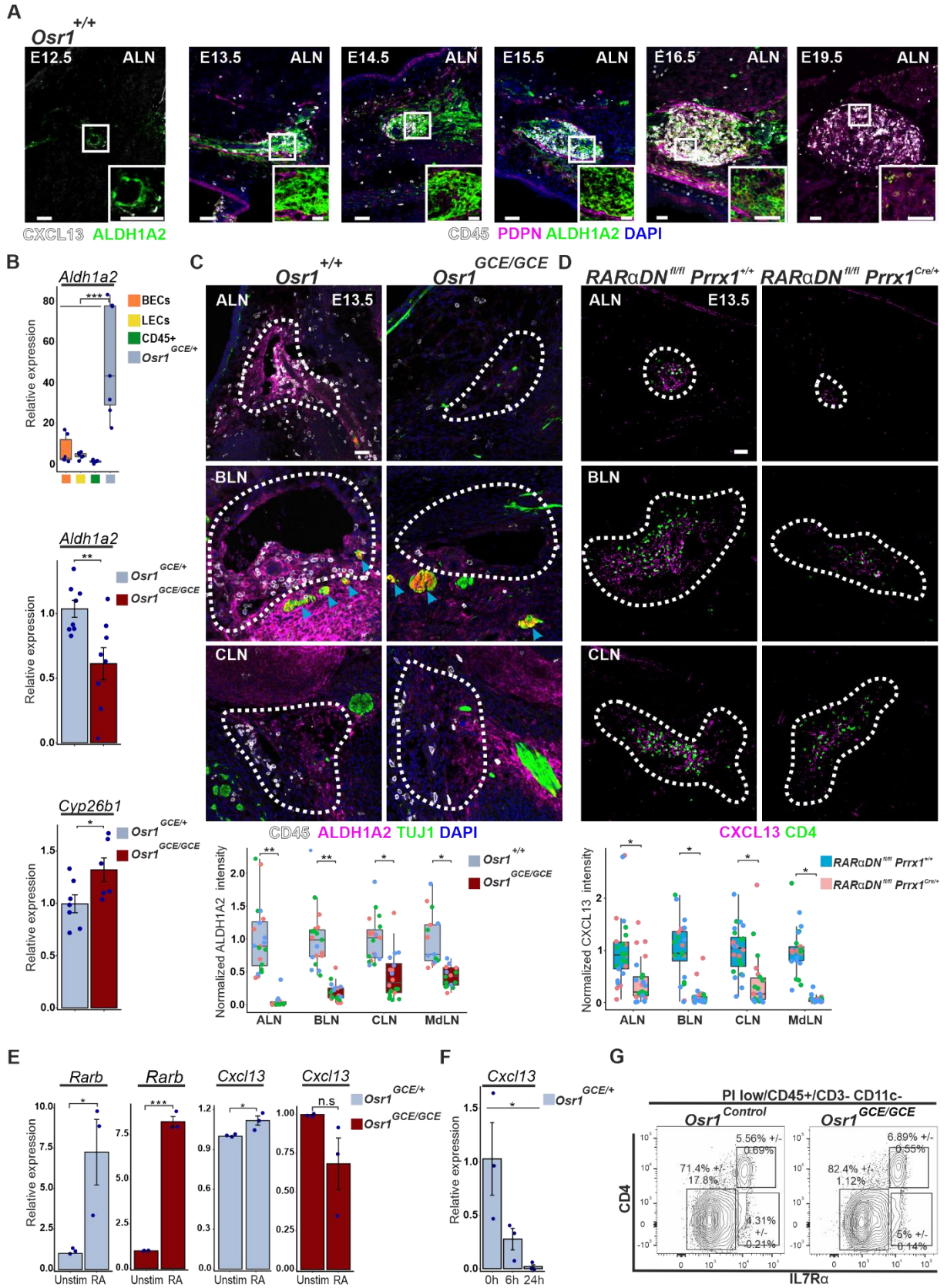
413 RA activates *Cxcl13* expression *in vitro* (12), suggesting that lack of RA signaling in *Osr1^{GCE/GCE}* embryos
 414 causes failed mesenchymal LTo commitment. However, a link between RA receptor (RAR) inactivation
 415 and CXCL13 expression *in vivo* during LN initiation has not been established. We first treated pregnant
 416 mice with the RAR inhibitor BMS493. At E13.5, expression of RA target *Rarb* was decreased after
 417 BMS493 treatment, but not that of other RAR genes (Figure S6F). A reduction specifically of *Cxcl13*, but
 418 not other chemokine genes, was confirmed in E13.5 embryos treated with BMS493 (Figure S6F).
 419 CXCL13 protein abundance and CD4+ cell accumulation were severely decreased in axillary, brachial
 420 and mediastinal LNs of E13.5 BMS493 treated embryos (Figure S6G). To specifically inactivate RA
 421 signaling in mesenchymal cells, we analyzed *RARαDN^{f/f} Prrx1^{Cre/+}* embryos (53). At E13.5, these
 422 embryos show a reduced expression of CXCL13 and a concomitant reduced CD4+ LTI accumulation in
 423 LN anlagen (Figure 6D). This establishes that RA signaling in mesenchymal LToS is required for initial
 424 *Cxcl13* expression and thus LTo commitment and LN initiation.

425 As both, absence of *Osr1* or reduction of RA signaling in LToS disrupts *Cxcl13* expression, we analyzed
 426 whether *Cxcl13* activation by RA (12) *in vitro* depends on *Osr1*. Although robust upregulation of *Rarb*
 427 confirmed efficacy of RA treatment, RA stimulation triggered only a very mild upregulation of *Cxcl13*
 428 in *Osr1^{GCE/+}* cells, which was, however, abrogated in *Osr1^{GCE/GCE}* cells (Figure 6E), suggesting that *Osr1* is

429 required for RA-mediated *Cxcl13* activation. Interestingly, we observed a fast downregulation of *Cxcl13*
430 expression *in vitro* (Figure 6F). We hypothesized that downregulation of *Cxcl13* expression might be
431 caused by a lack of 3-dimensional cell environment, and cultivated *Osr1^{GCE/+}* cells in embryoid bodies.
432 This, however, also led to rapid downregulation of *Cxcl13* (Figure S6H). In 24h embryoid bodies,
433 lentiviral infection using an *Osr1*-FLAG construct did not stimulate *Aldh1a2* expression. In addition, RA
434 stimulation and/or *Osr1*-FLAG lentiviral infection, neither separately or in conjunction, could sustain
435 *Cxcl13* expression (Figure S6H), despite robust *Rarb/Osr1* upregulation (Figure S6H).

436 We next tested whether loss of *Osr1*, in addition to LTi attraction, also affected LTi differentiation. Flow
437 cytometric analysis showed that CD45+ cell abundance in the liver of E13.5 *Osr1^{GCE/GCE}* was comparable
438 to control littermates (Figure S6I). However, we measured a reduction of CD45+ cells in tissue
439 comprising PLNs in E13.5 *Osr1^{GCE/GCE}* embryos (Figure S6I). To investigate if CD45+ cell reduction in PLNs
440 was accounted exclusively by a LTi reduction, we performed flow cytometric analysis of the
441 CD45+/CD3- CD11b-cells containing LTi precursors and mature LTis in PLN enriched tissue. We
442 detected no alteration in IL7R α + CD4+ LTis or IL7R α + CD4- LTi precursors in E14.5 *Osr1^{GCE/GCE}* mice
443 compared to controls (Figure 6G). In addition, *Rorc* and *Cd4* transcript levels were unchanged in both
444 CD45+ isolated cells from E13.5 *Osr1^{GCE/GCE}* peripheral tissues and whole E13.5 tissues from BMS493-
445 treated embryos (Figure S6J). These results suggest that lack of mesenchymal ALDH1A2 in *Osr1^{GCE/GCE}*
446 embryos did not affect LTi precursor differentiation into CD4+ LTi cells.

447 We conclude that mesenchymal LTOs are the main producers of RA in most LN anlagen, and that RA
448 production is affected by loss of *Osr1*. Mesenchymal RA signaling controls in part CXCL13 expression
449 dependent on *Osr1*, but not the expression of other chemokines involved in LN initiation. Finally, our
450 results in *Osr1^{GCE/GCE}* and BMS493-treated embryos suggest that mesenchymal RA is only involved in
451 mesenchymal LTO commitment and not in LTi differentiation.



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Figure 6. *Osr1*⁺ LTis are a source of RA, and RA signaling is required for mesenchymal LTO commitment. **(A)** Representative immunofluorescence images of the axillary region at indicated stages showing ALDH1A2 expression by mesenchymal LTos. At E12.5 in the axillary region first CXCL13⁺ cells are found in the proximity of the thoracic artery co-expressing ALDH1A2. LTis are labeled for CD45 and LECs with PDPN. Boxed regions are shown as higher magnifications. **(B)** RT-qPCR of *Aldh1a2* expression in FACS isolated E13.5 *Osr1*⁺ cells, BECs, LECs or CD45⁺ cells. Below: *Osr1* E13.5 *Osr1*^{GCE/GCE} FACS isolated cells present a reduction in *Aldh1a2* and an increase in *Cyp26b1* transcripts assessed by RT-qPCR (n=6-8). **(C)** Representative micrographs of ALN, BLN, CLN and mediastinal LNs of *Osr1*^{+/+} and *Osr1*^{GCE/GCE} embryos labelled for ALDH1A2, CD45 for immune

460 cells and TUJ1 for motoneurons. Motoneurons only show ALDH1A2 expression at the BLN, which was not decreased in
 461 *Osr1^{GCE/GCE}* embryos (blue arrowheads). Below, normalized ALDH1A2 intensity in the LN anlage quantified on sections (**D**)
 462 CXCL13 expression and CD4+ LTI accumulation is reduced in LNs of E13.5 *RARαDN^{fl/fl} Prrx1^{Cre/+}* embryos as compared to
 463 controls. Below, normalized CXCL13 intensity in the LN anlage quantified on sections (n=3). (**E**) Stimulation of E13.5 *Osr1^{GCE/+}*
 464 and *Osr1^{GCE/GCE}* cells isolated by FACS with all-trans retinoic acid (100 nM). A very mild upregulation of *Cxcl13* transcripts is
 465 seen after 6h of retinoic acid activation only in E13.5 *Osr1^{GCE/+}* cells, whereas a robust *Rarb* upregulation is seen in *Osr1^{GCE/+}*
 466 and *Osr1^{GCE/GCE}* cells (n=3). (**F**) Analysis of *Cxcl13* expression measured by RTqPCR in E13.5 *Osr1+* isolated by FACS from LNs
 467 enriched regions (n=3). (**G**) Flow cytometry to discriminate LTI precursors (CD4-) and mature LTis (CD4+) from E14.5 *Osr1*
 468 controls and *Osr1^{GCE/GCE}* peripheral tissue. CD45+/CD3- CD11c-/Il7Rα+ CD4+ cells were not reduced in *Osr1^{GCE/GCE}* embryos
 469 (n=3-4). Immunofluorescence images were captured from at least 3 independent experiments. Dot colors in graphs of (**C**, **D**)
 470 represent measurements from different embryos. Scale bar in (**A**) 50 μm and in (**C**, **D**) 50 μm. In (**B**, **C**, **D**, **E** and **F**) error bar
 471 represents s.e.m. and * p<0.05, ** p<0.01, *** p<0.001 or n.s nor significant. In (**B** and **F**), P values were obtained from one-
 472 way ANOVA with Dunnett's post-hoc comparison, or by using a student's t-test (*Osr1^{GCE}* vs *Osr1^{GCE/GCE}*). In (**C**, **D** and **E**), P values
 473 were obtained from student's t-test. In (**G**) data are shown as a percentage of the mean and as a +/- percentage of the s.e.m.

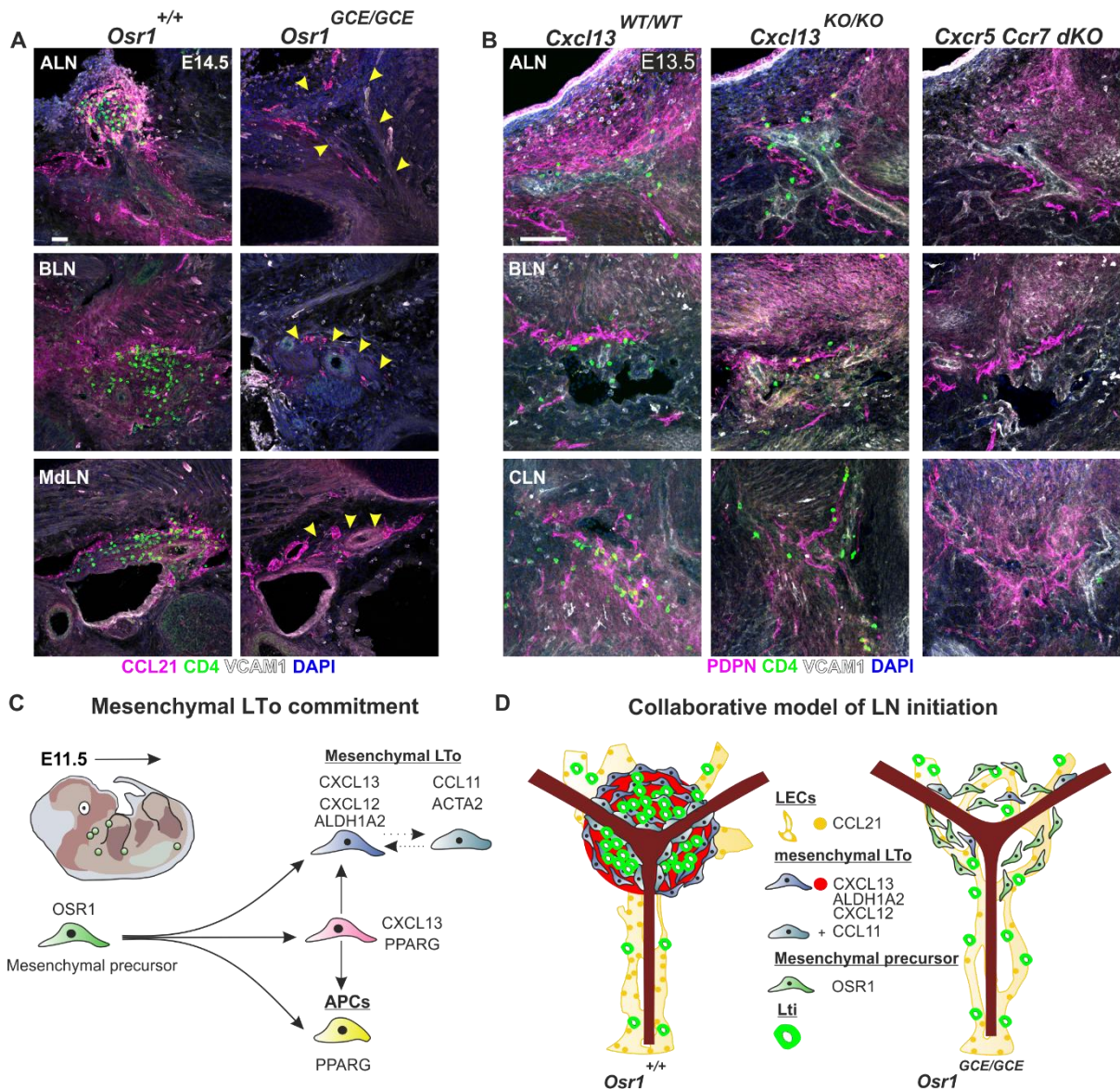
474 LN initiation requires the combined action of mesenchymal LTos and lymphatic vasculature

475 Expression of CXCL13 and its receptor CXCR5 are essential for the formation of most peripheral LNs,
 476 however cervical and mesenteric LNs still develop in single KO mutant embryos (17, 54). In *Osr1^{GCE/GCE}*
 477 deficient embryos, initiation is arrested in all PLNs and mesenteric LNs are strongly impaired, thus
 478 additional factors must contribute to LN initiation. CCL21, which is expressed in lymphatic endothelial
 479 cells, attracts LTis via its receptor CCR7 (12). Intriguingly, *Osr1* knockout embryos present defects in LN
 480 lymphatic vasculature.

481 CCL21 is produced exclusively by lymphatic vasculature independent of their position in the embryo
 482 (Figure S7A). At E14.5, lymphatic vasculature impairments also resulted in an interrupted production
 483 of the chemokine CCL21 in E14.5 *Osr1^{GCE/GCE}* peripheral and mesenteric LN anlagen (Figure 7A). To
 484 validate a synergistic function of LECs expressing CCL21 and mesenchymal LTos expressing CXCL13, we
 485 analyzed early LN initiation in E13.5 *Cxcl13^{KO/KO}* compared to *Cxcr5/Ccr7* double KO mutants.
 486 Surprisingly, *Cxcl13* KO embryos showed an initial normal accumulation of LTis in peripheral LNs at
 487 E13.5 (Figure 7B). At E14.5 axillary and brachial LNs in *Cxcl13^{KO/KO}* did not contain LTis (Figure S7B)
 488 suggesting a failure in LTI retention. This phenotype is maintained during development and *CXCL13^{KO/KO}*
 489 embryos at E18.5 do not develop axillary and brachial LNs and show strongly reduced cervical LNs
 490 (Figure S7C). In contrast, the combined lack of *Cxcr5* and *Ccr7*, thus disrupting CXCL13 and CCL21
 491 signaling simultaneously, led to complete absence of LTI recruitment to all LNs at E13.5 (Figure 7B and
 492 S7D) thus phenocopying lack of *Osr1*.

493 Collectively, our data indicate that the initial recruitment of LTis is not solely dependent on CXCL13,
 494 but needs the coordinated action of lymphatic vasculature and mesenchymal LTos as a general
 495 mechanism in the formation of PLNs and mesenteric LNs.

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498 **Figure 7. Mesenchymal cells and LECs cooperate in LN initiation.** (A) Maximal intensity projection of 35- μ m sections showing
 499 defective lymphatic vasculature in E14.5 *Osr1*^{GCE/GCE} ALN, BLN and mediastinal LN with an interrupted CCL21 expression
 500 (arrowheads). CD4 and VCAM1 label LTis and blood vasculature. (B) Immunofluorescence of 35 μ m sections of ALN, BLN and
 501 CLN in E13.5 *CXCL13*^{WT/WT}, *CXCL13*^{KO/KO} and *CXCR5/CCR7* double KO mice. Note normal CD4⁺ cell accumulation in E13.5
 502 *CXCL13*^{KO/KO} but no CD4⁺ cell accumulation in *CXCR5/CCR7* double KO. PDPN and VCAM1 label lymphatic and blood
 503 vasculature. (C) Schematic representation of mesenchymal LTo commitment and (D) *Osr1* function in LN initiation.
 504 Immunofluorescence images were captured from at least 3 independent experiments. Scale bar represents in (A) 50 μ m and
 505 in (B) 100 μ m.

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513 **Discussion**

514 In mice, LN initiation starts at E12.5 (2, 5), however visualization and analysis of LN anlagen earlier than
515 E14.5 in the literature are rare, leaving cell types involved in the first attraction of LTIs poorly
516 characterized. We show that *Osr1*⁺ mesenchymal cells represent the first defined precursor of
517 mesenchymal LTos and are an early origin for the three known types of adult mesenchymal LN cells:
518 FDCs, TRCs, and MRCs. Quantitative analysis showed only a partial contribution of *Osr1* progenitors to
519 these cell pools, however our analysis might underappreciate the contribution of *Osr1* cells as (i) *Osr1*⁺
520 cells were tamoxifen-labelled only in a very short time frame thus probably not encompassing all LTo
521 progenitors during the early phase of LN initiation; and (ii) possible low recombination efficacy of the
522 *Osr1*-CreERT2 allele (compare *Osr1*-eGFP expression at E14.5/16.5 and lineage mGFP labeling at E15.5
523 in Figures 1A and S1E). Recently characterized subpopulations of mesenchymal stromal cells (26) are
524 probably also derived at least in part from *Osr1*⁺ embryonic cells, as embryonic *Osr1*⁺ traced cells are
525 broadly distributed in the LNs, however this remains to be tested. Interestingly, *Osr1* expression
526 remains active in the medullary region of adult LNs and PPs, indicating a yet undefined function in
527 adult LNs.

528 Embryonic and adult APCs have been proposed as a source of LN stromal cells (11). However, a
529 common embryonic progenitor for both tissue types has not been addressed yet. By disentangling
530 E13.5 *Osr1*⁺ cell heterogeneity via genetic lineage tracing and scRNA-seq, we defined a new
531 *Osr1*⁺/*Cdh18*^{high} mesenchymal subpopulation in the trunk with enriched expression of *Cxcl12* and
532 *Cxcl13*. Further bioinformatic and *in vivo* expression analysis subdivided these cells into adipogenic
533 progenitors and mesenchymal LTos. Genetic lineage tracing of E11.5 *Osr1*⁺ cells confirmed their
534 bifurcated potential thus strongly suggesting a common cellular origin for mesenchymal LN stromal
535 cells and adipogenic progenitors (Figure 7C). Of note, some cells in axillary and brachial LN anlagen
536 displayed a bivalent signature co-expressing CXCL13 and PPARG at E14.5. This suggests that, at least in
537 these LNs, progenitors of LN-associated WAT and mesenchymal LTos are committed during the same
538 developmental time frame including a phase of cellular plasticity towards either lineage (Figure 7C).
539 Activation of the *Ltβr* signaling pathway in mesenchymal LTos is crucial for their late differentiation
540 and inhibits adipocyte differentiation of isolated APCs (11). However, early mesenchymal LTo
541 commitment occurs independently of this signaling cascade (12, 15). This indicates that commitment
542 of APCs at E13.5 might also occur independently of *Ltβr* signaling and that *Ltβr* signaling may rather be
543 necessary to maintain mesenchymal LTo identity at later stages by continuous suppression of
544 adipogenic fate.

545 Formation of LNs at diverse locations of the body requires commitment of mesenchymal precursors to
546 LTos in different tissue environments. In line, the formation of ectopic lymphoid tissue under
547 fingolimod treatment in mice highlights LN emergence as an adaptive process (20). Thus, cellular
548 adaptation in different cell types or even the reprogramming of differentiated cells under certain
549 conditions might be necessary. We show that *Osr1*⁺/*CXCL13*⁺ mesenchymal LTos in the cervical,
550 peripheral and mediastinal region presented distinct transcriptional signatures, **which resemble**
551 **transcriptional profiles of mesenchymal cells in designated anatomical locations.** They also expressed
552 a localized ECM, which might contribute to LN diversity and local adaptation. Complementary to recent
553 data (44) and *Cxcl13*-EYFP lineage contribution (20), *Osr1*⁺/*CXCL13*⁺ mesenchymal LTos represent an
554 early precursor population with low expression of CD34 suggesting that E17.5 populations of
555 mesenchymal precursors have a common origin in *CXCL13*⁺ cells. In line with transcriptional
556 heterogeneity found in *Osr1*/*CXCL13*⁺ mesenchymal LTos, E17.5 mesenchymal subpopulations are
557 heterogeneously distributed within inguinal, brachial and mesenteric LNs (44).

558 In the current view of LN initiation, localized production of RA by motoneurons adjacent to the LN
559 anlage was proposed to activate *Cxcl13* expression in mesenchymal LTos (12). However, motoneurons

560 are broadly distributed along the embryo and RA production by neurons does not follow a pattern
561 similar to LN distribution in the body (55-57). We show that (i) at several LN anlagen motoneurons do
562 not express ALDH1A2, while (ii) in all LN anlagen mesenchymal LTos express high levels of ALDH1A2,
563 suggesting LTos themselves are the predominant producers of RA in the area of LN formation.
564 Furthermore, in *Osr1^{GCE/GCE}* embryos, motoneurons in the vicinity of brachial LNs show normal
565 ALDH1A2 expression, nevertheless CXCL13 expression and brachial LN formation are severely
566 impaired, altogether arguing against a significant role of neural-derived RA and pointing to
567 mesenchymal cells as a main RA source.

568 *Osr1*-deficient LTos show reduced expression of *Aldh1a2*/ALDH1A2 and increased expression of
569 *Cyp26b1*, which very likely limits local mesenchyme-derived RA availability impinging on CXCL13
570 expression. In line, inhibition of RA signaling either globally (BMS493 treatment) or specifically in
571 mesenchymal cells (*RARαDN^{fl/fl} Prrx1^{Cre/+}*) led to both reduction of *Cxcl13* expression and LTi attraction,
572 demonstrating the crucial role of RA signaling in mesenchymal LTo commitment and LN initiation.
573 Neither BMS493 treatment or lack of RA signaling nor lack of *Osr1* completely abrogated CXCL13
574 production by mesenchymal LTos, suggesting additional players in the control of *Cxcl13* expression. In
575 line with this, we surprisingly found a dynamic downregulation of *Cxcl13* after embryonic cell isolation
576 and *in vitro* culture, which could not be recovered by lentiviral *Osr1*-FLAG overexpression and/or RA
577 signaling activation. Of note, lack of local mesenchymal RA production in *Osr1^{GCE/GCE}* embryos did not
578 affect LTi differentiation, in line with a dependence of the last differentiation step from LTi precursors
579 to mature LTis on maternal RA (14).

580 In summary, our data suggest that motoneuron-derived RA is not involved in LN initiation but
581 demonstrate a crucial role for mesenchymal RA signaling in LN initiation, challenging the current model
582 of LN initiation.

583 The transcription factor *Osr1* has been involved in the formation of several organs, cell-autonomously
584 controlling mesenchymal cell differentiation, directly regulating expression of ECM genes or
585 transcription factors such as *Sox9* or *Tcf7l2* and the chemokine *Cxcl12* (22, 25, 58-60) or acting in a non-
586 cell autonomous manner influencing cell behavior (22). Despite being required for *Aldh1a2* and *Cxcl13*
587 expression *in vivo*, we could not establish a direct regulation of both genes by *Osr1*.

588 The critical function of *Osr1* in mesenteric and peripheral LN initiation in the embryo cannot be only
589 explained by reduced CXCL13 expression observed in *Osr1^{GCE/GCE}* embryos. In the absence of CXCL13 or
590 its receptor CXCR5, cervical LNs and mesenteric LNs still develop (26, 52). Furthermore, our analysis of
591 *Cxcl13^{KO/KO}* embryos at E13.5 revealed normal initial LTi attraction to all LN anlagen. Mesenchymal LTos
592 showed high expression of *Cxcl12* and *Ccl11*, encoding two chemokines attracting LTis *in vitro* (12).
593 Indeed, we showed here that the CXCL12 receptor CXCR4 is required for proper attraction of LTis
594 during LN initiation in peripheral and mesenteric LNs. However, cervical LNs in *CXCR4^{KO/KO}* embryos
595 develop normally. These results point to a differential requirement for chemokine factors in the
596 initiation of cervical, mesenteric, and peripheral LNs. We show that in the LN anlage an additional
597 subpopulation of *Osr1+*/CXCL13+ mural cells acquired mesenchymal LTo functions. *Osr1+*/CXCL13+
598 mural cells remain attached to the vascular vessels and express α SMA, and also showed expression of
599 *Ccl11* and *Ccl2*. Whether these chemokines contribute to, and are required for, LN initiation at specific
600 locations remains to be tested. In addition, a small proportion of lymphatic endothelial cells at the LN
601 anlage acquired CXCL13 expression *in situ*. Thus, the production of CXCL13 in different cell types
602 together with the assistance of other chemokines might create a multifactorial microenvironment
603 necessary for LTi recruitment during development.

604 Mesenchymal cells are an unappreciated actor in lymphatic vasculature formation during development
605 and in adult tissues. We reveal a critical function for *Osr1+* mesenchymal cells in lymphatic vasculature

606 assembly in all LNs. Lymphatic vessels play an essential role collecting CD4+ cells from peripheral
607 tissues and conducting them to the LN anlage (21). In addition, lymphatic vasculature produces a
608 potent LTi-attracting chemokine, CCL21 (12). *Cxcr5/Ccr7dKO* embryos recapitulate the global defects
609 seen in *Osr1* mutants demonstrating a requirement for combined CXCL13 and CCL21 signaling. This
610 suggests that the combined action of lymphatic vasculature and mesenchymal LTos via CXCL13 and
611 CCL21 is a general mechanism in LTi recruitment necessary for LN initiation across divergent
612 anatomical locations. In *Osr1^{GCE/GCE}* embryos, both, disrupted lymphatic vessel assembly and
613 subsequent impaired CCL21 expression in the prospective LN region might contribute to defects in all
614 LNs we observed, thus exacerbating the defects seen in *Cxcl13^{KO/KO}* embryos. This places the
615 mesenchymal transcription factor *Osr1* upstream of two processes crucial for LN initiation,
616 orchestrating both mesenchymal LTo commitment and formation of LN-associated lymphatic
617 vasculature. Of note, the initial expression of CXCL13 by mesenchymal LTos and CCL21 expression by
618 lymphatic vasculature are independent of LTβR signaling (12, 15).

619 Current views of LN initiation are based on the isolated function of mesenchymal or endothelial
620 organizer cells. Together, our data substantially revise the identity and function of mesenchymal cells
621 involved in LN initiation. They unify the divergent actual views of LN initiation adapting the prevailing
622 paradigm of LN initiation to a new model that incorporates the pivotal role of mesenchymal cells in
623 lymphatic vessel formation to achieve LTi attraction at the LN anlage.

624

625 **Acknowledgements**

626 This work was funded by the Deutsche Forschungsgemeinschaft (DFG; grant VA 1272/1-1) and the
627 Freie Universität Berlin. We are gratefully to Andrew P. McMahon (Keck School of Medicine of USC,
628 USA) and Andreas Kispert (Hannover Medical School, Germany) for providing *Osr1^{GCE}* and *R26^{mTmG}*
629 mouse lines. We thank Carmen Birchmeier and Ines Lahmann (Max Delbrück Center for Molecular
630 Medicine, Berlin, Germany) for providing *Cxcr4^{+/-}* mice and technical support. We gratefully thank
631 Caroline Bräuning (Max Delbrück Center for Molecular Medicine, Berlin, Germany) for assisting scRNA-
632 seq experiments. We thank Uta Marchfelder and Erwin Weiß (Max Planck Institute for Molecular
633 Genetics, Germany) for FACS sorting support. We thank Stefan Mundlos, Nobert Brieske and Thorsten
634 Mielke for their support (Max Planck Institute for Molecular Genetics, Germany). We also thank Petra
635 Knaus (Freie Universität, Berlin) for the support with lentiviral infection.

636 **Author contributions:**

637 Conceptualization P.V.G and S.S. P.V.G performed the majority of experiments and data collection.
638 G.C, M.G and S.P-K performed data collection. C.G-T supervised FACS experiments. C.F and S.Sa.
639 supervised the scRNA-seq procedure. M.O performed scRNA-seq data analysis. G.C, S.T and U.H
640 provided reagents and mouse embryos. G.C performed BMS493 treatment. Formal analysis and
641 interpretation were performed by P.V.G, M.O and S.S. P.V.G, M.O, U.H and S.S wrote the manuscript.

642 All authors critically reviewed and approved the final version of the manuscript.

643 **Methods**

644 **Animals**

645 Mice were maintained in an enclosed, pathogen-free facilities, and experiments were performed in
 646 accordance with European Union regulations and under permission from the Landesamt für
 647 Gesundheit und Soziales (LaGeSo) Berlin, Germany (Permission numbers ZH120, G0346/13, G0240/11,
 648 G0268-16) and the ethics committee of the Institute Pasteur (CETEA) (licence 2015-008). Mouse lines
 649 were described previously; *Osr1^{GCE}* (23), *Prrx1^{Cre}* (47), *R26R^{mTmG}* (61), *Osr1^{LacZ}* (46), *Cxcr4^{+/-}* (50), CXCR5
 650 (18), CXCL13 (breeding pairs derived from Jackson Laboratories, Bar Harbor, ME, USA), CCR7 (62), and
 651 CXCR5/CCR7 DKO (63).

652 To transform the *Osr1^{LacZ/+}* locus in a *Osr1^{fl/fl}* locus, we bred *Osr1^{LacZ/+}* animals with animals expressing
 653 ubiquitous flippase recombinase (64). Then, we bred *Osr1^{fl/fl}* animals with *Prrx1^{Cre}* animals (47) to
 654 achieve constitutive excision of *Osr1* exon2 in mesenchymal cells of most peripheral LNs, but not
 655 affecting *Osr1* function in heart development (45, 65) (a schematic representation of the *Osr1* locus is
 656 depicted in figure S5C).

657 **Tamoxifen and Progesterone administration for *Osr1*⁺ cell lineage tracing**

658 As we described previously (22), tamoxifen (Sigma Aldrich) was dissolved in a 1:10 ethanol/sunflower
 659 oil mixture. For embryonic lineage tracing, we bred *R26R^{mTmG/mTmG}* females to *Osr1^{GCE/+}* males. Pregnant
 660 females were injected with 150 µl of a 20 mg mL⁻¹ tamoxifen stock. Tissues were collected at E18.5.

661 For lineage tracing experiments inducing at embryonic stages and analysing adult SLNs, we bred
 662 *Osr1^{GCE/+}* females to *R26R^{mTmG/mTmG}* males. To ameliorate negative effects of tamoxifen during delivery
 663 a mixture of 60 µl was injected intraperitoneally to the pregnant female containing 20 mg mL⁻¹
 664 tamoxifen and 16,6 mg mL⁻¹ Progesterone (Sigma Aldrich). Adult tissues were collected at 8-12 weeks
 665 of age.

666 For perinatal lineage tracing, new born *Osr1^{GCE/+}; R26R^{LacZ/+}* pups were injected subcutaneously into the
 667 neck fold with 25 µl of a 3 mg mL⁻¹ tamoxifen stock. Adult tissue was collected at 12 weeks of age. For
 668 successive injections, only 1 injection was performed in 24h.

669 **BMS493 treatment**

670 To inhibit retinoic acid signalling *in vivo*, we used the pan-RAR inverse agonist BMS493 (Tocris, 3509).
 671 As described in (66), pregnant females were injected intraperitoneally with 10 mg kg⁻¹ of BMS493.
 672 BMS493 was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich) at a concentration of 5 mg mL⁻¹ and
 673 stored at -20 °C in tight cap tubes. Immediately prior injection, 200 µl sterile phosphate buffer (PBS)
 674 was added per 50 µl BMS493 aliquot. BMS493 and DMSO injections were performed every 12h as
 675 described in schematic representation.

676 **Retinoic acid treatment**

677 To activate retinoic acid signalling *in vitro*, cells were treated with all-trans retinoic at a concentration
 678 of 100 nM (Sigma Aldrich) added to cell culture medium.

679 **Tissue preparation**

680 Embryonic tissues were fixed in 4% PFA for 2h on ice or overnight at 4°C in case eGFP has to be detected
 681 after antigen retrieval. Tissues were dehydrated in two steps using 15% and 30% (w/v) sucrose (Roth)
 682 solutions before O.C.T. (Sakura) cryo-embedding in a chilled ethanol bath. Embryonic tissue was

683 sectioned at 12 or 35 μm thickness. Adult LNs were collected, freed from remaining fatty tissue and
684 fixed in 4% PFA solution at 4°C overnight. Adult tissue was sectioned at 12 μm thickness.

685 **Immunolabelling**

686 Cryo-sections were warmed up for at least 30 minutes at room temperature (RT). Antigen retrieval was
687 performed for the antibodies ALDH1A2, LUM and PPARG. In this case, sections were shortly washed
688 with PBS and subsequently immerse in 1x Target Retrieval Solution (Dako) pH 6. Heat-induced epitope
689 retrieval was achieved using a steamer (IHCWorld) for 30 min. Retrieval solution was thoroughly rinsed
690 out with PBS. Sections were blocked with 5% (v/v) Horse Serum (Vector Laboratories) in 0.1% (v/v)
691 Triton X-100 (Sigma Aldrich) PBS for 1h at RT. Primary antibodies dissolved in blocking solution were
692 incubated at 4 °C overnight, followed by secondary antibody staining of 1h at RT. Antibodies used for
693 these experiments are listed in supplementary tables 1 and 2. Specimens were counterstained with
694 5 μg μL^{-1} 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and mounted with FluoromountG
695 (SouthernBiotech).

696 **Cell isolation and flow cytometry**

697 For cell isolation of embryonic tissues, E13.5 *Osr1*^{GCE/+} or *Osr1*^{GCE/GCE} tissues were enriched in LNs
698 dissecting the embryos from the axilla until the tongue and including the inguinal region. Embryonic
699 tissue was minced using a small scissor in 1 ml high-glucose Dulbecco's modified eagle medium
700 (DMEM, Pan Biotech) containing 10% foetal bovine serum (FBS, Pan Biotech) and 1%
701 penicillin/streptomycin (P/S) solution. Further enzymatic digestion of the tissue was performed using
702 0,7 mg ml⁻¹ of Collagenase (Collagenase A, Roche) in DMEM medium at 37 °C for 45 min. Antibody
703 labelling (antibodies see supplementary table 3) was performed for 20 min on ice. For the isolation
704 and FACS analysis of LTis and their precursors, LNs enriched tissues were dissected from E14.5
705 *Osr1*^{controls} and *Osr1*^{GCE/GCE} embryos and treated as above described for E13.5 embryos. Antibody
706 labelling (antibodies see supplementary table 3) was performed for 15 min at RT.

707 For stromal cell isolation of adult LNs, PLNs and mesenteric LNs from *Rosa26*^{mTmG/+} *Osr1*^{GCE/+} and
708 *Rosa26*^{mTmG/+} *Osr1*^{+/+} animals were dissected and shortly minced with a small scissor in 5 ml DMEM
709 medium containing 10% FBS and 1% P/S. LNs were further enzymatic digested adding 0,8 mg ml⁻¹
710 collagenase and 0,2 mg ml⁻¹ Dispase II (Roche) for 45 min at 37 °C. Cell suspensions were collected by
711 centrifugation at 400g for 8 min and washed with a solution containing PBS and 2% FBS. Cell suspension
712 were enriched in stromal cells by depleting CD45+ hematopoietic cells using MACS microbeads
713 (Miltenyi). For that, hematopoietic cells were bound to CD45+-microbeads (10 μl /10⁷ cells) and
714 depleted using LS columns (Miltenyi). Unbound cell suspension was collected and used for further
715 antibody labelling performed for 20 min on ice.

716 Before flow cytometry, cell suspensions were washed using a solution containing PBS, 0,4% FBS and 2
717 mM EDTA, collected by centrifugation at 300 g for 5 min and passed through a 35- μm cell strainer filter
718 (BD Biosciences). To assess viability, cells were stained with propidium iodide (2 μg ml⁻¹, eBioscience)
719 immediately before sorting or analysis.

720 Sorts and analyses were performed on a FACS Aria II and FACS Aria fusion (BD Biosciences). Data were
721 collected using FACSDIVA software. Further analyses were performed using FlowJo 10 (FlowJo LLC)
722 software. Sorting gates were defined based on unstained and fluorescence negative controls. Cells
723 were collected into 400 μl high-glucose DMEM.

724 Lentiviral infection

725 Lentiviral particles were purchased from AMsBio (Abingdon, UK). For embryoid bodies infections,
726 E13.5 C57Bl6 LNs enriched tissues were isolated as described above (Cell isolation and flow cytometry).
727 4×10^4 E13.5 cells were resuspended in pre-warmed growth medium (high glucose DMEM
728 supplemented with 10% FBS) to a concentration of 1000 cells per μl . Osr1-FLAG virus particles were
729 added to the cells in a multiplicity of infection of 1 and RNA was isolated 24 h after. Mouse embryonic
730 fibroblast (MEFs) infections of E13.5 C57Bl6 cells were conducted as previously described (22) and
731 achieved by spinoculation. Virus particles and cells were centrifuge for 60 min at 300 g before seeding.

732 Chromatin immunoprecipitation

733 Chromatin immunoprecipitation (ChIP) using E13.5 MEFs infected with Osr1-FLAG was performed as
734 previously described (22). Briefly, cells were fixed with 1% formaldehyde (Sigma Aldrich) for 10 min.
735 Chromatin cross-linking was quenched by adding 550 μl of 2.5 M Glycine. Next, cells were washed
736 twice with DPBS and collected with a scratcher. After cell lysis, nuclei were collected in 900 μl
737 sonication buffer and sonicated for 20 cycles in a Bioruptor (Biogenade). For ChIP, 30 μg sonicated
738 chromatin was incubated with an anti-FLAG antibody under rotation at 4°C overnight. Protein G beads
739 (Invitrogen) was added to the chromatin-antibody mixture and incubated under rotation at 4°C
740 overnight. Dynabeads-antibody complex was isolated from the chromatin using vigorous shaking at
741 65°C for 30 min and further de-crosslinked overnight at 65°C. Proteins bound to the chromatin were
742 removed with a treatment of 60 min at 55°C using Poteinase K (500mg/ml) and subsequently a
743 treatment of 30 min at 37°C with RNase A (Sigma Aldrich). Genomic DNA used as an input was obtained
744 using de-crosslinked chromatin without antibody pulldown. Primer sequences are provided in
745 supplementary table.

746

747 Imaging

748 X-Gal staining of whole LNs were documented with a Zeiss SteREO Discovery V12 stereomicroscope.
749 Confocal images of immunolabelled sections were taken using the confocal laser scanning microscope
750 systems LSM710, LSM810 (Zeiss) or Leica DMI8 microscopes. Images were captured using Zen 2010
751 (Zeiss) and LAS Life System (Leica). For the quantifications shown in figures 5E, S5C, S5H, 6C, D and
752 S6G, consecutive images of the LN region were selected leaving 12 μm (1 section) between quantified
753 images. ALDH1A2 and CXCL13 protein intensity on sections were quantified by the ImageJ algorithm
754 “integrated density” as sum of pixels values in the LN anlage. In Figure S5C and H, percentage of
755 Ki67/eGFP and eGFP/CXCL13 overlapping signals were obtained using the ImageJ tool image
756 calculator-subtract. The percentage of overlapping signals were quantified by the ImageJ algorithm
757 “integrated density”.

758 Quantitative real-time PCR

759 Total RNA extraction from FACS isolated cells was performed using Direct-zol™ RNA MicroPrep and
760 MiniPrep kits (Zymo Research) following manufacturer’s protocol. Reverse transcription was
761 conducted using the M MuLV Reverse Transcriptase Kit (Biozym). Relative gene expression analyses
762 were performed using GoTaq® qPCR kit (Promega) or Blue S’Green qPCR kit (Byozim) on a 7900HT Real
763 Time PCR system or QuantStudio 7 Flex Real-Time-PCR-System (Applied Biosystems). Primer sequence
764 information is provided in supplementary table 4. Data were acquired and analysed using SDS 2.0 and
765 QuantStudio™ Real-Time PCR softwares (Applied Biosystems).

766 Droplet-based single-cell RNA-seq analysis

767 E13.5 *Osr1*^{GCE/+} cells from LNs enriched tissues were isolated via FACS and 10,000 collected cells were
768 run using the 10x Chromium (10x Genomics) system according to the manufacturer's
769 recommendations (Chromium Single Cell 3' chemistry V3 Kit). After generation of Gel Beads-in-
770 Emulsion (GEM), cDNA was amplified in a thermal cycler using 10 cycles (98°C for 3 minutes, 98°C for 15
771 seconds, 63°C for 20 seconds and 72°C for 1 minute). The libraries were constructed performing a
772 sample index PCR of 11 cycles (98°C for 45 seconds, 98°C for 20 seconds, 54°C for 30 seconds and 72°C
773 for 20 seconds). Final libraries had a concentration of 17.1 ng/μl and 504 bp on average. Libraries were
774 sequenced on an Illumina HiSeq 4000 device using the following read specifications: 8-bp index, 28-bp
775 read 1 (16-bp barcode and 12-bp UMI), and 98-bp strand-specific read 2.

776 The ~58 M sequencing reads generated were mapped against the primary assembly of the mouse
777 genome mm10, for which we included an additional synthetic sequence encompassing the
778 eGFP-CreERT2 and PGK-Neo cassettes corresponding to the *Osr1*^{GCE} allele, using the STARsolo algorithm
779 from STAR v2.7.5c (67) (parameters: --solotype CB_UMI_Simple; --sjdbOverhang 97; --soloCBstart 1; -
780 --soloCBlen 16; --soloUMIstart 17; --soloUMIlen 12; --soloStrand Forward; --outSAMtype BAM
781 SortedByCoordinate; --outMultimapperOrder Random; --outSAMattributes NH HI NM MD AS nM CR
782 CB CY UR UB UY GX GN). The primary comprehensive gene model from the GENCODE vM25, excluding
783 pseudogenes and small non-coding RNA genes but including the additional *Osr1*^{GCE} allele, was used as
784 reference annotation (--sjdbGTFfile) and the 10X Chromium barcode whitelist v3 (3M February 2018)
785 was provided for error correction and demultiplexing of cell barcodes (--soloCBwhitelist), while
786 allowing multiple matches in whitelist with no more than one mismatched base (--soloCBmatchWLtype
787 1MM_multi). Following mapping, multi-mapped reads were extracted from the alignment map
788 according to the NH tag using SAMtools v1.10 (68) and intersected against the gene annotation model
789 using BEDtools bamtobed (parameters: -tag HI; -split) and intersect (parameters: -s; -wa; -wb; -loj)
790 v2.29.2 (69). Multi-mapped reads that were found to overlap with a single gene were retrieved and
791 merged with the uniquely-mapped reads, resulting in a total of ~48M aligned reads (83.1% of initial
792 sequencing reads).

793 Next, reads mapped on genes were counted using featureCounts v2.0.1 (70) (parameters: -M; -s 1)
794 according to first exon features (-t exon) and then transcript features (-t transcript) to include reads
795 that originated from introns (pre-mRNAs) as well. Gene counting at the UMI level per cell was
796 performed using STARsolo error-corrected barcodes and UMI-tools count v1.0.0 (71), while using the
797 directional method to group reads per UMI with a maximum hamming distance of 1 (parameters: --
798 extract-umi-method=tag; --umi-tag=UR; --cell-tag=CB; --method=directional; --per-gene; --per-cell).
799 The resulting count matrix was then parsed to exclude low-quality cells (< 600 counts, < 500 genes and
800 ≥ 10% mitochondrial counts) and sparse genes (expressed in < 0.05% cells, i.e. < 4 cells). The 6849 cells
801 that remained after filtering, with a median number of counts per cell of 3473 and covering 21,412
802 genes for a median number of genes per cell of 1844 (Figure S2C), were further analyzed using Pegasus
803 v1.0.0 (72). Gene counts were first normalized to 10⁵ to obtain an identical sum per cell and scaled
804 using a logarithmic transformation after adding a pseudocount of 1. The top 2000 genes with the
805 highest variance were then selected for downstream analysis. Principal component analysis (PCA) was
806 used to reduce the dimensionality of the data by computing 50 principal components (PCs). A *k*-nearest
807 neighbors (kNN) graph was constructed from the 50 PCs by fixing the local neighborhood number at
808 100. Clustering was performed using the Louvain algorithm with a resolution set at 1.3 and the fast
809 interpolation-based *t*-distributed stochastic neighbor embedding (Fit-SNE) on two dimensions was
810 chosen for visualization with a perplexity of 30 and an early exaggeration of 12. Differential expression

811 analysis between Louvain clusters was performed using a Mann-Whitney *U* test and a q-value
812 threshold (alpha) of 0.05 for significance.

813 To identify putative cell-type-specific gene markers, differentially expressed genes detected as being
814 upregulated in each cluster were filtered out according to a percentage fold change of at least 2
815 between the percentage of expressing cells in a given cluster relative to all other clusters, resulting in
816 a list of 7730 upregulated genes across all Louvain clusters. Hierarchical clustering of upregulated
817 genes was performed by averaging their expression level across all cells for each cluster and by using
818 the clustermap function from the Python package seaborn v0.11.0 (73) with the average linkage
819 method and the correlation distance metric. Cell type enrichment analysis of Louvain clusters was
820 assessed by comparing the upregulated genes detected for each cluster against the 2861 gene markers
821 identified for the 38 cell types defined from E9.5-E13.5 mouse embryos (37). Statistical significance of
822 marker gene overlaps was computed using a hypergeometric test followed by a false discovery rate
823 (FDR) correction with the Benjamini-Hochberg method.

824 To further investigate the cell population corresponding to the Louvain cluster 2, the 860 cells
825 contained exclusively within this cluster were analyzed using Pegasus v1.0.0 (72) by adjusting the
826 following parameters: PCA was computed on the top 400 genes with the highest variance and 5 PCs;
827 the number of nearest neighbors was fixed at 80 to construct the kNN graph; and the resolution was
828 set at 0.5 for Louvain subclustering.

829 To explore the heterogeneity within the *Cxcl13*+ cell population, the 476 cells with at least 1 UMI count
830 for *Cxcl13* were extracted. 203 (42.6%) cells were included in the Louvain cluster 2, while the 273
831 (57.4%) remaining cells were spread among the 14 other Louvain clusters except for the Louvain cluster
832 10. Analysis of these 476 *Cxcl13*+ cells was performed using Pegasus v1.3.0 (72). The 400 most highly
833 variable genes were selected and PCA was computed on 5 PCs. To include the 78 (16.4%) *Cxcl13*+ cells
834 contained within the Louvain cluster 4 of proliferative cells, cell-cycle scores were calculated based on
835 the cell cycle genes defined in (74). Cell cycle effects were then regressed out on the PCs according to
836 the predicted G1/S and G2/M phases. kNN graph was constructed from the regressed PCs by fixing the
837 local neighborhood at 50 and a resolution of 0.7 was used for Louvain subclustering.

838 **Statistical analysis**

839 Student's t-test and one-way ANOVA with Dunnett's post-hoc comparison were performed using Prism
840 8 (GraphPad) software. Error bars in all figures, including supplementary information, represent the
841 mean \pm standard error of the mean (s.e.m.). In figures 5E, 6C, 6D and S6G, student's t-test were
842 performed using calculated averages from single measurements obtained from the same embryo.

843 **Data availability**

844 Raw fastq and count data from scRNA-seq sequencing experiments have been deposited in the Gene
845 Expression Omnibus (GEO) database under the accession number GSE199851.

846

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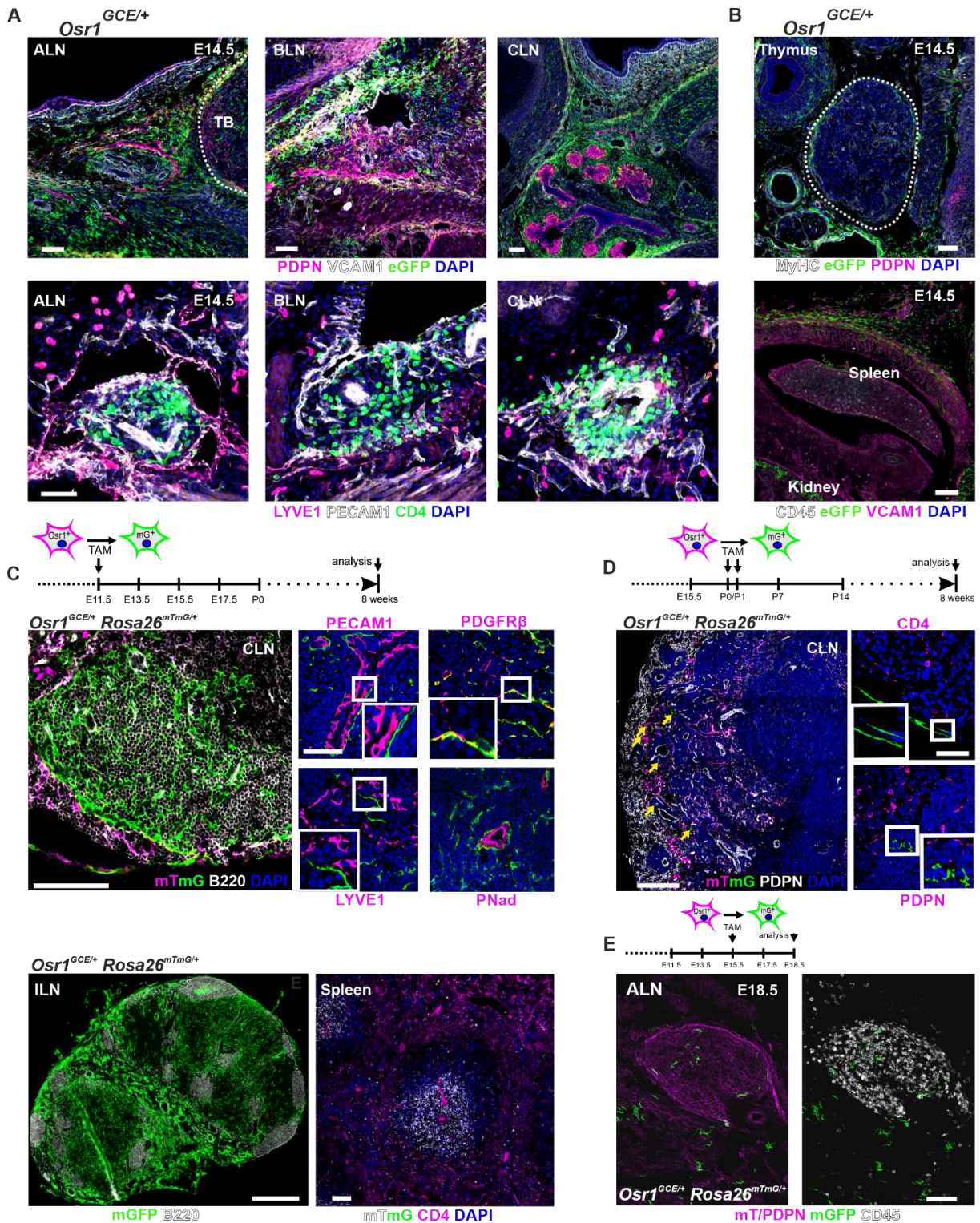
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“Mesenchymal Osr1 cells orchestrate lymph node initiation”

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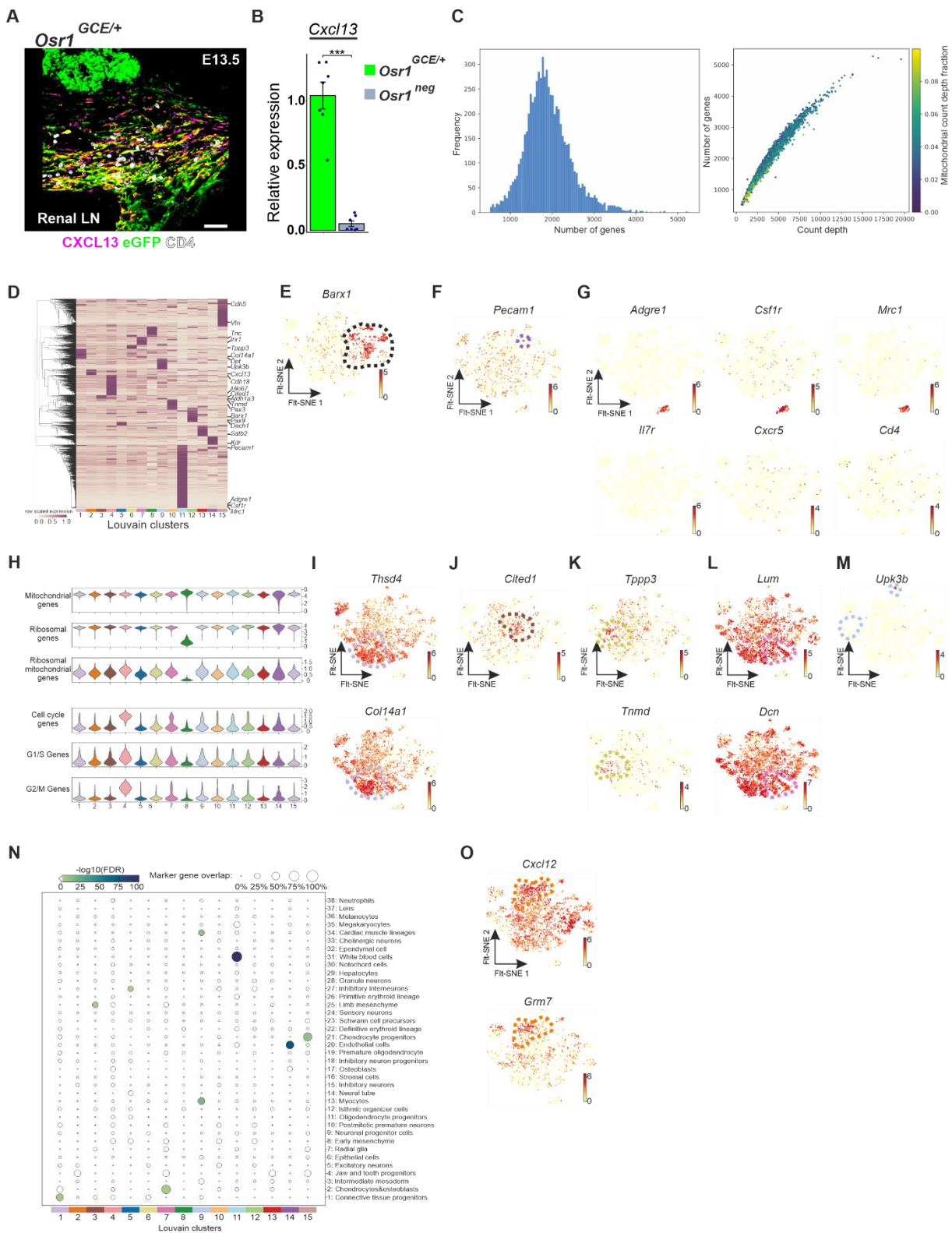
Supplementary figure 1



S1 related to figure 1. *Osr1* embryonic expression and lineage contribution to primary and secondary lymphoid organs. **(A)** Immunofluorescence of E14.5 *Osr1*^{GCE/+} peripheral LN regions identifying LN anlagen. Antibodies against PDPN, LYVE1, PECAM1, VCAM1, CD4 and eGFP (*Osr1*) were used to label lymphatic vasculature, blood vasculature, mesenchymal LTos and LTis. **(B)** *Osr1* is not expressed in E14.5 *Osr1*^{GCE/+} thymus and spleen; co-staining for MyHC, *Osr1*, PDPN in thymus and CD45, *Osr1* and VCAM1 in the spleen. Dashed line demarcates the thymus. **(C)** Schematic representation of Cre recombinase induction at the stage E11.5. Lineage contribution was assessed at 8 weeks of age. Immunofluorescence of CLN sections for membrane GFP and B220 demarcates B cell follicle. Embryonic E11.5 *Osr1* cells contribute to vasculature associated fibroblasts, in the proximity of blood vessels (PECAM1), lymphatic vessels (LYVE1), high endothelial venules (PNad) and mural cells (PDGFRβ). Higher magnification of the boxed regions is shown at the bottom corner. Below, E11.5/E12.5 *Osr1* descendants contribute to inguinal LN but are not found in the splenic stroma. **(D)** Contribution of *Osr1* cells to adult

mesenchymal stromal cells decreases at late stages of development. Schematic representation of perinatal (P0 and P1) tamoxifen induction. mGFP cells are sparsely found in the CLN. Higher magnification of the boxed regions is shown at the bottom. (E) Low E15.5 Osr1 contribution is shown in the axillary LN of E18.5 embryos. Sparse mGFP+ cells are found in the LN anlage and surrounding tissues. Representative immunofluorescence images were captured from at least 3 independent experiments. Scale bar represents in (A) 200 μm , (B) 50 μm (thymus) and 100 μm (spleen), (C, D) 100 μm and (E) 200 μm . CLN represents cervical LN.

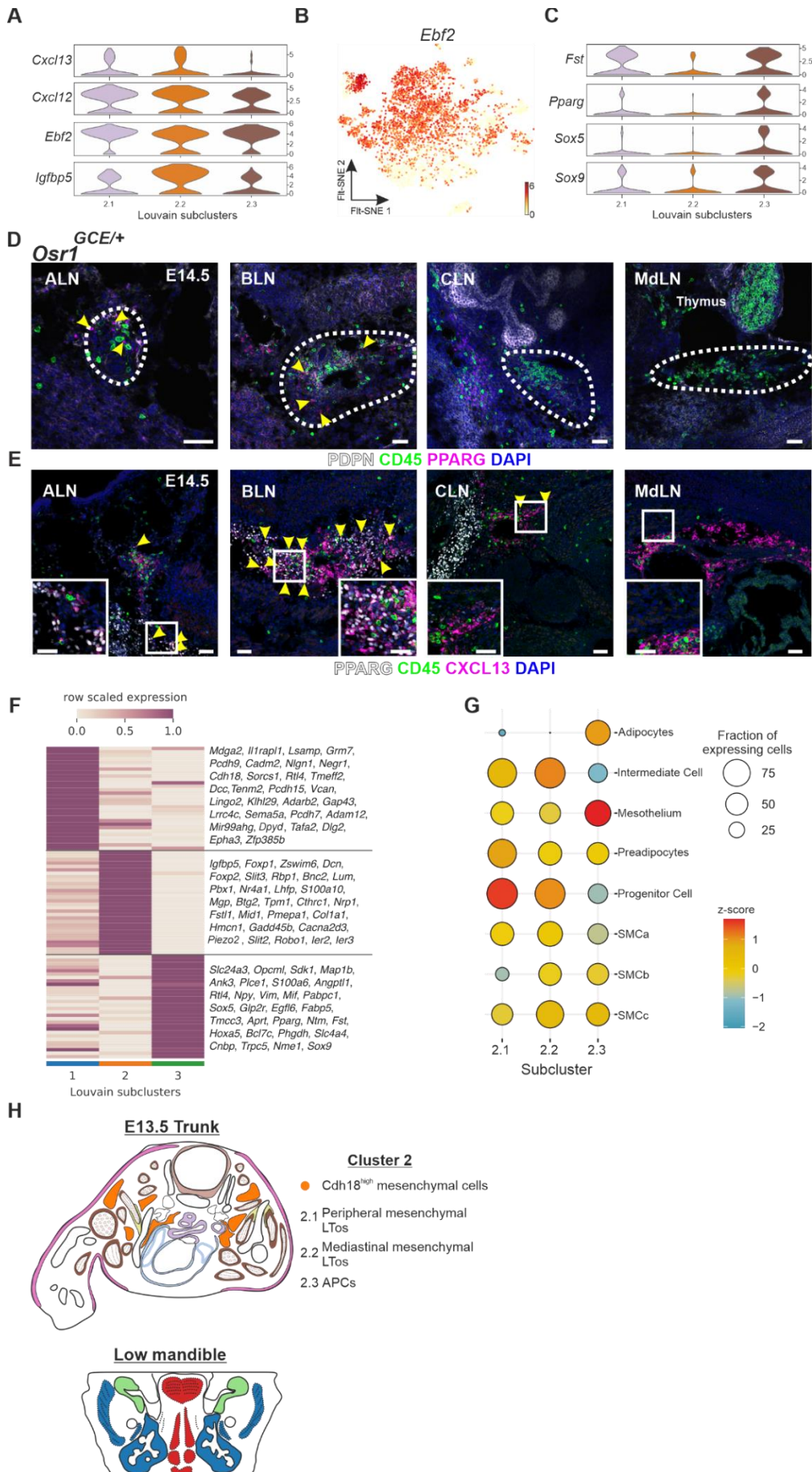
Supplementary figure 2



S2 related to figure 2. E13.5 *Osr1* scRNA-seq unravels *Osr1*⁺ cell heterogeneity. (A) 3D reconstruction of E13.5 *Osr1*^{GCE} renal LN. Immunofluorescence of 35 μ m cross-sections shows CXCL13 and *Osr1* co-expression in close association with CD4 LTI cells. Immunofluorescence is representative image of at least 3 independent experiments. Scale bar represents 50 μ m. **(B)** *Cxcl13* expression is restricted to *Osr1*^{GCE/+} cells. Relative *Cxcl13* expression from FACS sorted E13.5 *Osr1* positive (*Osr1*^{GCE/+}) and *Osr1* negative cells. **(C)** Quality plots showing the number of genes (left) and counts (right) detected per cell in single cell sequencing of *Osr1*⁺ E13.5 cells. **(D)** Hierarchical clustering of 7730 upregulated genes detected across all Louvain clusters with a percentage fold change of at least 2 between the percentage of expressing cells in a given cluster relative to all other clusters. Gene expression level was averaged across all cells for each cluster. Key genes used to define the 15 clusters are indicated on

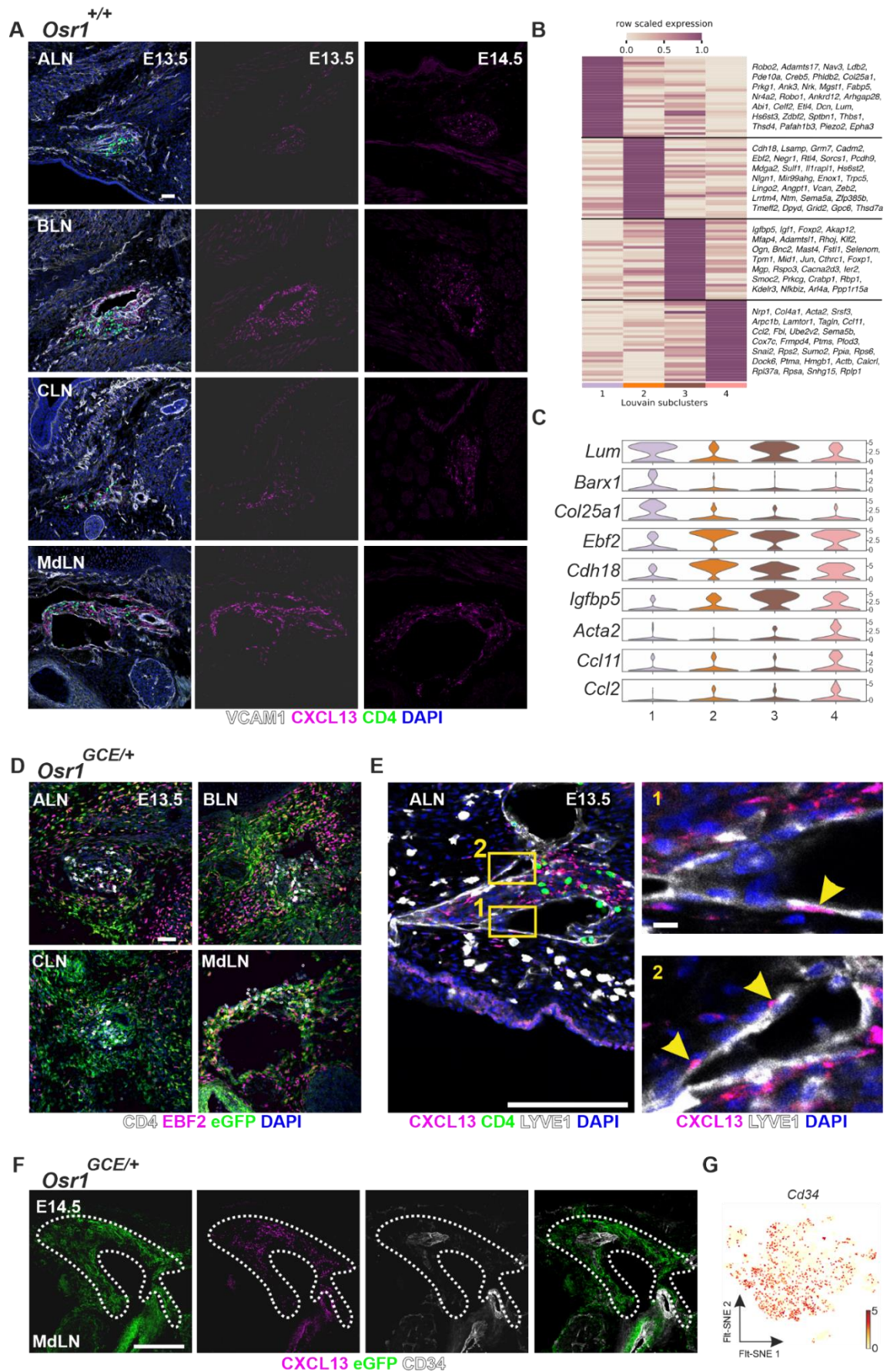
the right. **(E-G)** Fit-SNE visualization of cells expressing the indicated genes. **(H)** Violin plots depicting the distribution of averaged gene expression in all cells per Louvain cluster for mitochondrial, ribosomal and cell cycle genes. **(I-M)** Fit-SNE visualization of cells expressing the indicated genes. **(N)** Cell type enrichment analysis of Louvain clusters with embryonic cell populations defined from E9.5-E13.5 mouse embryos based on marker gene overlap. Overlaps with a false discovery rate (FDR) below 0.01 were considered as significant. **(O)** Fit-SNE visualization of cells expressing the indicated genes. In **(E-G, I-M, O)**, gene expression level in cells is depicted as log-transformed normalized counts. Cells for the scRNA-seq analysis were derived from a single E13.5 *Osr1*^{GCE/+} embryo.

Supplementary figure 3



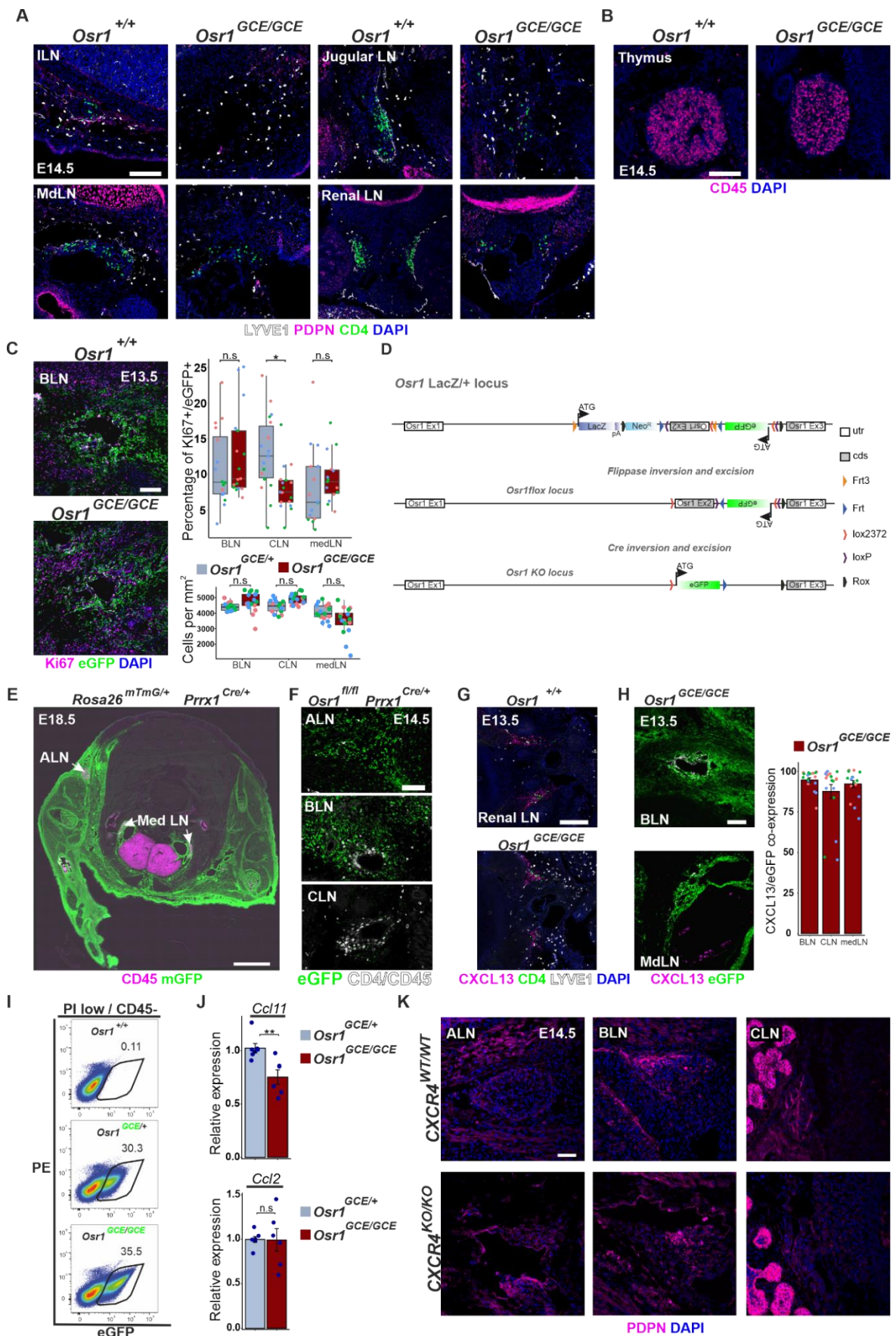
S3 related to figure 3. *APCs and mesenchymal LTos are derived from a common Osr1 embryonic progenitor in the trunk. (A)* Violin plots of selected genes depicting the distribution of gene expression within all cells per Louvain subcluster of cluster 2. **(B)** *Flt-SNE visualization of cells expressing Ebf2 in all Osr1 cells. Color bars represent the gene expression level in cells as log-transformed normalized counts. (C)* Violin plots of selected genes depicting the distribution of gene expression within all cells per Louvain subcluster of cluster 2. **(D)** *PPARG positive cells are found in close association to LNs. Representative micrographs of E14.5 Osr1^{GCE/+} peripheral and mediastinal LN anlage. CD45 are confined to the LN anlage (dashed line) in close proximity to PPARG positive APCs (E)* Immunofluorescence of E14.5 LNs for CXCL13 and PPARG shows partial co-expression in ALN and BLN. Yellow arrows are pointing to PPARG nuclei surrounded by CXCL13 expression in BLN, ALN and CLN. Higher magnification of the boxed region is shown as inserts. **(F)** *Clustering of 30 most upregulated genes in each Louvain subcluster. Genes used to define the 3 subclusters are indicated on the right. (G)* Dot plot depiction of cell type enrichment analysis for Louvain subclusters 2.1, 2.2 and 2.3. Enrichment analysis was performed using transcriptional signatures of E18.5/P3 aortic stromal vascular fraction and the 35 most upregulated genes in each Louvain subcluster. Expression levels of all upregulated genes were averaged across all cells for each cell subpopulation. **(H)** *Schematic representation of putative cluster identity including cluster 2 subpopulations in the trunk and mediastinal cavity. Immunofluorescence are representative images of at least 3 independent experiments. Scale bar represents in (D, E) 50 μm.*

Supplementary figure 4



S4 related to figure 4. A heterogenous cell population contributes to LT_i attraction in the LN anlage (A) Heterogenous CXCL13 expression on E13.5 and E14.5 ALN, BLN, CLN and mediastinal LNs. CD4 (green) labels LT_is, CXCL13 labels mesenchymal LTos and VCAM1 is highly expressed in blood vessels. **(B)** Clustering of 30 most upregulated genes in each Louvain subcluster. Genes used to define the 4 subclusters are indicated on the right. **(C)** Violin plots of selected genes depicting the distribution of gene expression within all Cxcl13+ cells per subcluster. **(D)** Immunofluorescence of E13.5 ALN, BLN, CLN and mediastinal LN showing EBF2 and Osr1 co-expression in ALN, BLN and mediastinal LN. In the CLN, mesenchymal LTos express low amount of EBF2. **(E)** In the axillary LN anlage of E13.5 embryos, CXCL13 is found in some LYVE1 positive LECs. LT_is are labeled in green for CD4. Higher magnification of the boxed regions is shown on the right. Arrows are pointing to cells co-expressing CXCL13 and LYVE1. **(F)** CD34 is expressed at low level by E14.5 mesenchymal LTos in the mediastinal LN anlage. Low CD34 expression is found in few Osr1+ cells in the CXCL13+ area (dashed lines). **(G)** Fit-SNE visualization of cells expressing Cd34 in all Osr1 cells. Color bars represent the gene expression level in cells as log-transformed normalized counts. Immunofluorescence are representative images of at least 3 independent experiments. Scale bar represents in **(A, C)** 50 μ m and in **(D)** 200 μ m and 20 μ m (magnification).

Supplementary figure 5

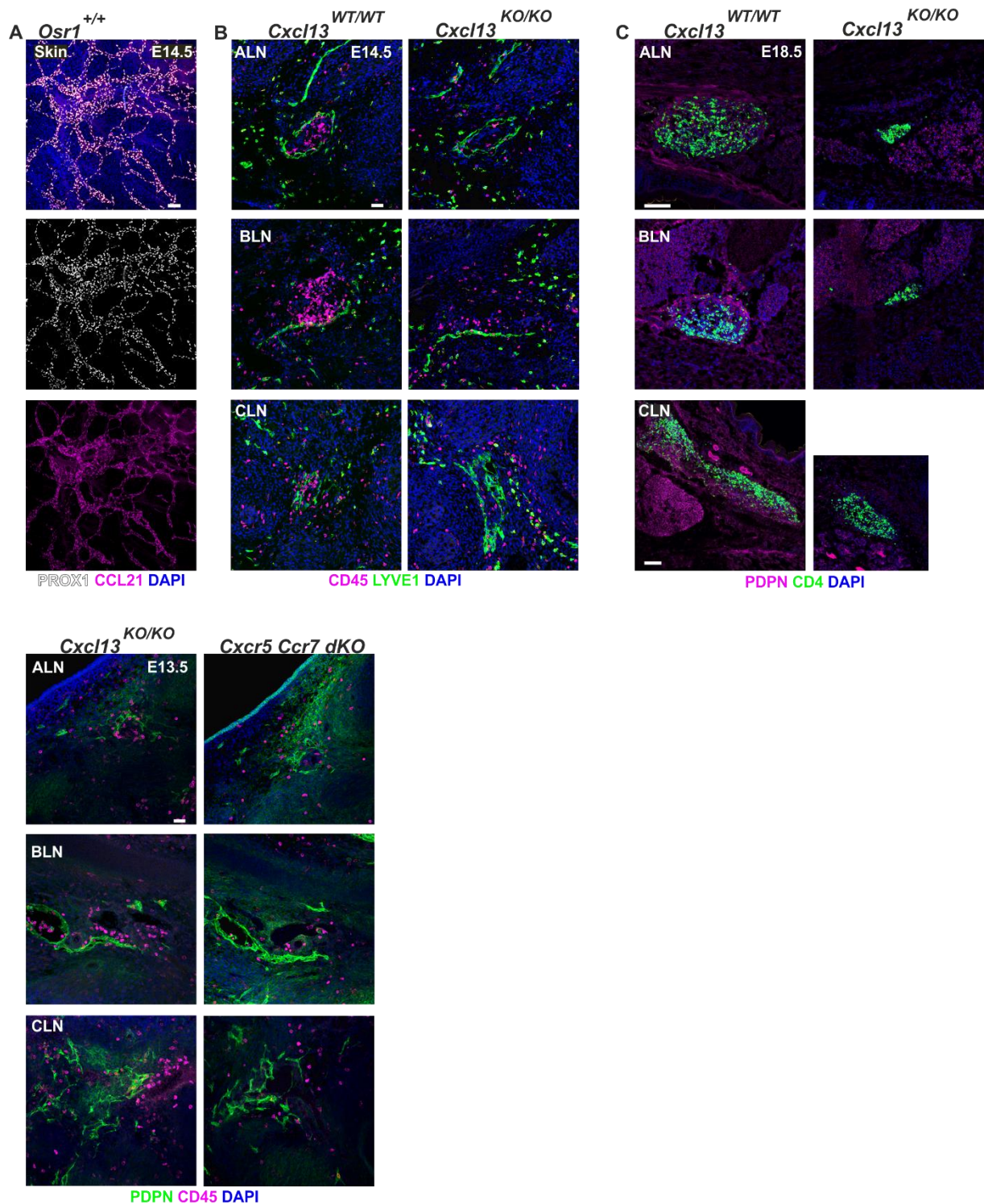


S5 related to figure 5. *Osr1* controls mesenchymal LTo commitment and formation of accessory lymphatic vasculature. (A) Representative immunofluorescence of inguinal, mediastinal, jugular and renal LN at E14.5 in *Osr1*^{+/+} and *Osr1*^{GCE/GCE}

embryos using anti CD4 and PDPN antibodies showing impaired LN initiation. **(B)** Thymus morphology assessed by CD45 immunofluorescence is normal in E14.5 $Osr1^{GCE/GCE}$ embryos as compared to $Osr1^{+/+}$ littermates. **(C)** Cell proliferation and cellularity were assessed in E13.5 $Osr1^{GCE/+}$ and $Osr1^{GCE/GCE}$ embryos. Left, representative images of immunofluorescences using anti Ki67 and anti GFP antibodies. Right, quantification of Ki67+ eGFP+ cells at the designated LN anlage and nuclei per area in E13.5 $Osr1^{GCE/+}$ and $Osr1^{GCE/GCE}$ sections (n=3). **(D)** Schematic representation of $Osr1^{LacZ}$ locus transformation into an $Osr1^{fl}$ locus showing flippase and Cre recombinase events and reorganization of the eGFP cassette. **(E, F)** Recombination of the $Osr1^{fl}$ locus via $Prrx1$ -Cre driven expression assessed via immunofluorescence in E14.5 ALN, BLN and CLN of $Osr1^{fl/fl} Prrx1^{Cre}$ embryos. An anti GFP antibody was used to label recombined $Osr1^{+}$ cells and CD45/CD4 for LTis. **(G)** CXCL13 reduction in E13.5 $Osr1^{GCE/GCE}$ embryos at the renal LN anlage. CD4 labels LTis and LYVE1 LECs. **(H)** Representative images of E13.5 $Osr1^{GCE/GCE}$ LN anlage labeled for CXCL13 and $Osr1$. In E13.5 $Osr1^{GCE/GCE}$ embryos, residual CXCL13 expression is restricted to mesenchymal $Osr1$ -eGFP+ cells. Right, quantification of CXCL13 signal expressed by $Osr1^{+}$ cells (n=3). **(I)** FACS strategy for mesenchymal $Osr1^{+}$ cell isolation from E13.5 $Osr1^{GCE/+}$ and E13.5 $Osr1^{GCE/GCE}$ embryos (n=5). **(J)** $Ccl11$ expression was reduced and $Ccl2$ remained unchanged in E13.5 $Osr1^{GCE/GCE}$ homozygous FACS sorted cells when compared to $Osr1^{GCE/+}$ heterozygous cells, n=6. **(K)** Lymphatic vasculature labeled with PDPN is not significantly impaired in LN anlage of E14.5 $CXCR4^{KO/KO}$ embryos. Immunofluorescence are representative images of at least 3 independent experiments. Scale bar represents in **(A, B, C, F, G, H, K)** 100 μ m and in **(E)** 1 mm. Error bar represents SEM. P-values were obtained from student's t-test, ** $p < 0.01$ or n.s not significant.

ALDH1A2 reduction in mesenchymal LTos of E13.5 *Osr1^{GCE/GCE}* embryos. Motoneurons stained with TUJ1 do not present reduced ALDH1A2 expression. CD45 was used to label LTis. **(F)** RA inverse agonist BMS493 treatment only affects *Rarβ* expression. Relative measurement of gene expression of RAR genes in E13.5 control (DMSO) and BMS493 treated embryos (n=3-4). Below, only the expression of *Cxcl13* is compromised in BMS493 treated embryos. Relative expression assessed by RT-qPCR from whole embryonic tissue (n=3-4). Schematic representation of BMS493 administration is shown on the right. **(G)** Representative immunofluorescence of E13.5 BLN treated with the RA inverse agonist BMS493 or DMSO. CXCL13 expression is reduced in ALN, BLN and mediastinal LN as quantified below. Normalized CXCL13 intensity in the LN anlage quantified on sections (n=3). **(H)** *Osr1*-FLAG lentiviral infection did not activate *Aldh1a2* expression. All-trans retinoic acid (100 nM) activation alone or in conjunction with *Osr1*-FLAG lentiviral infection did not rescue *Cxcl13* downregulation in E13.5 embryonic bodies, whereas *Osr1/Rarβ* are robustly induced (n=2-4). **(I)** FACS analysis of CD45+ cells from liver and peripheral tissues of E13.5 *Osr1^{Control}* (+/+ and GCE/+) and E13.5 *Osr1^{GCE/GCE}* embryos. A reduction of CD45+ cells was only observed in peripheral tissues (n=6-7). **(J)** Relative expression of *Rorc* and *Cd4* transcripts measured via RT-qPCR in CD45+ cells from E13.5 *Osr1^{GCE/+}* or *Osr1^{GCE/GCE}* embryos and E13.5 tissues of control (DMSO) and BMS493 treated embryos (n=3-4). *Rorc* and *Cd4* expression remains unchanged in all conditions. Immunofluorescence images are representative images of at least 3 independent experiments. Scale bar represents in **(A, D, G)** 200 μm and **(A)** 50 μm (below), **(B)** 50 μm, **(C)** 10 μm and 25 μm (below) and **(E)** 20 μm. In **(F, G, H, I and J)**, error bar represents SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or n.s not significant. In **(H)**, P-values were obtained from one-way ANOVA with Dunnett's post-hoc comparison and in **(F, G, I and J)**, P-values were obtained from student's t-test.

Supplementary figure 7



S7 related to figure 7. Combined action of mesenchymal LTos and LECs is required for LTi attraction in LNs. **(A)** Skin whole-mount immunofluorescence depicting lymphatic vasculature labeled by PROX1 and CCL21. **(B)** At the stage E14.5, ALN and BLN anlagen show dissolved LTi-accumulation in *CXCL13*^{KO/KO} embryos. CD45 labels immune cells and LYVE1 LECs. **(C)** In E18.5 *CXCL13*^{KO/KO} embryos, LN impairments are maintained during development. ALN and BLN are not formed and CLN presents a reduced size. Antibodies against CD4 and PDPN were used to label LTis and LECs. **(D)** Combined *CXCR5* and *CCR7* deficiency leads to an arrest in LTi accumulation in all PLNs at the stage E13.5. Antibodies against PDPN and CD45 were used to stain lymphatic vasculature and immune cells. Immunofluorescence are representative images of at least 3 independent experiments. Scale bar represents in **(A, C)** 100 μ m and **(B, D)** 50 μ m.

Supplementary table 1

Primary antibodies:

Antibody	Clone	Conjugate	Concentration/ Dilution	Source
Mouse anti-MyHC	Monoclonal	Unconjugated	1:500	Chemicon
Chicken anti-GFP	Polyclonal	Unconjugated	1:1000	Aves
Rabbit anti- α -SMA	Polyclonal	Unconjugated	2 $\mu\text{g ml}^{-1}$	Abcam
Hamster anti-Pecam1	Polyclonal	Unconjugated	1:250	DSHB (2H8)
Rabbit anti-Col12a1	Polyclonal	Unconjugated	1:500	M. Koch
Goat anti-collagen I	Polyclonal	Unconjugated	1:400	Novus Biologicals
Hamster anti PDPN	Polyclonal	Unconjugated	1:100	DSHB (8.1.1)
Rat anti CD4	GK 1.5	Unconjugated	1:100	Biologend
Rat anti B220	RA3-6B2	Alexa Fluor $\text{\textcircled{R}}$ 594	1:100	Biologend
Rat anti CD21/35	7E9	Alexa Fluor $\text{\textcircled{R}}$ 594	1:100	Biologend
Rat anti MadCAM1	MECA-367	Unconjugated	1:100	Biologend
Rat anti PNad	MECA-79	Unconjugated	1:100	Biologend
Rat anti CD45	30-F11	Unconjugated	1:100	Biologend
Rat anti ERTR7	ER-TR7	Unconjugated	1:100	BioRad (MCA2402)
Rabbit anti TNC	Polyclonal	Unconjugated	1:200	Chemicon
Rabbit anti LUM	Monoclonal	Unconjugated	1:50	CUSABIO
Goat anti IGFBP5	Polyclonal	Unconjugated	1:200	R&D System (AF578)
Sheep anti EBF2	Polyclonal	Unconjugated	1:20	R&D System (AF7006)
Rabbit anti PPARG	81B8	Unconjugated	1:150	Cell Signaling (81B8)
Goat anti CXCL13	Polyclonal	Unconjugated	10 $\mu\text{g ml}^{-1}$	R&D System (AF470)
Rabbit anti PROX1	Polyclonal	Unconjugated	1:200	ReliaTech
Rabbit anti LYVE1	Polyclonal	Unconjugated	1:200	Abcam (33682)
Mouse anti VCAM1	Polyclonal	Unconjugated	1:100	DSHB (P3C4)
Goat anti CCL21	Polyclonal	Unconjugated	10 $\mu\text{g ml}^{-1}$	R&D System (AF457)
Rabbit ALDH1A2	Polyclonal	Unconjugated	1:100	Abcam (75674)
Rat anti PDGFR β	APB5	PE	1:100	Biologend
Mouse anti TUJ1	TUJ1	Unconjugated	1:200	Biologend
Goat anti PDGFR α	Polyclonal	Unconjugated	1:100	Cell Signaling
Mouse anti CD34	RAM34	Unconjugated	1:100	BD PharMingen
Rabbit anti-Ki67	Polyclonal	Unconjugated	1:100	Abcam

Supplementary table 2

FACS antibodies

Antibody	Clone	Conjugate	Concentration/ Dilution	Source
Rat anti-T119	TER-119	APC	1:100	eBioscience
Rat anti CD31	390	APC	1:100	eBioscience
Rat anti IL-7R	A7R34	Alexa Fluor 488	1:100	Biolegend
Rat anti CD4	GK 1.5	APC-Cy7	1:100	Biolegend
Rat anti CD3	17A2	PE	1:100	Biolegend
Hamster anti CD11c	N418	PE	1:100	Biolegend
Rat anti PDPN		Alexa Fluor 594	1:100	Biolegend
Rat anti-CD45	30-F11	APC	1:100	eBioscience
Anti-Sca1	D7	APC-Cy7	1:100	eBioscience
Anti-PDPN	8.1.1	APC-Cy7	1:100	Biolegend

Supplementary table 3

Secondary antibodies:

Antibody	Conjugate(s)	Source
Donkey anti-mouse	Alexa Fluor 488, 568 and 680	Molecular Probes
Donkey anti-rabbit	Alexa Fluor 488, 568 and 647	Molecular Probes
Donkey anti-goat	Alexa Fluor 488, 568 and 680	Molecular Probes
Goat anti-hamster	Alexa Fluor 488, 568	Molecular Probes
Donkey anti-rat	Alexa Fluor 488, 568, 647	Molecular Probes
Donkey anti-chicken	Alexa Fluor 488	Molecular Probes
Donkey anti-sheep	Alexa Fluor 488, 568	Molecular Probes

Supplementary table 4

Primer sequences (RT-qPCR):

Gene	Forward	Reverse
<i>Gadph</i>	CTGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTCT
<i>Cxcl13</i>	CAACGCTGCTTCTCCTCT	CAGGGGGCGTAACTTGAAT
<i>Cxcl12</i>	GCTCCACCCACAAGGTTAAG	CTGGCAGAAGGCCTGAATA
<i>Cxcl10</i>	CATCCTGCTGGGTCTGAGTG	TGATCTCAACACGTGGGCAG
<i>Ccl2</i>	AGGTCCTGTCTATGCTTCTG	GCTGCTGGTGATCCTCTTGT
<i>Ccl11</i>	TCCATCCCAACTTCTGCTG	TGGGGTCAGCACAGATCTCT
<i>Osr1</i>	GCACACTGATGAGCGACCT	TGTAGCGTCTTGTGGACAGC
<i>Aldh1a2</i>	GGATGCGTCTGAAAGAGGAC	CCCAGCCTGCATAATACCTC
<i>Cyp26b1</i>	GCTCATCGGAGAGACTGGTC	CCAGTAGGATCTTGCGCACA
<i>Rarb</i>	CTTCAAAGCAGGAATGCACA	GGCCAGCTCACTGAATTTGT
<i>Rara</i>	AAATCATCCGGCTACCACT	TCTGGATGCTTCGTCGGAA
<i>Rxra</i>	TCAGTACTGCCGCTACCAGA	GTCTCAGTCTTGGGCTCGAC
<i>Rxrb</i>	CTGCAAGGGTTTCTTCAAGC	ATCTCCATCCCCGTCTTTGT
<i>Rorc</i>	CAGAATGTGCAGGGCCTACA	GAAAAACACAGGGCGCTGAG
<i>Cd4</i>	TTCACCTGGAAGTTCTCTGACC	AACGATCCTTTCTCCCATGC
<i>Il7ra</i>	GGACGATCACTCCTTCTGGT	TTGCAGCTTGTTAAGAGTTAGGC
<i>Osr1</i>	GCACACTGATGAGCGACCT	TGTAGCGTCTTGTGGACAGC
<i>Cl-Cxcl13</i>	TTCAAGTCTTTGCCGAAGGT	TGAGACTCTTTGTTGCTGTTGG
<i>P-Cxcl13</i>	GATGGTTGTGTGTATGACAAGGA	GAGGGTGTGTCTCCATTGCT
<i>Cr-Cxcl13</i>	GTGCAGGCAGGTGTAGAGC	GAATAGGTGTGGCCTTGCTG
<i>Cl-Aldh1a2</i>	CATGGGGATAGAGGCAGAAA	AGAATCTCCGGTTGAGCTT
<i>P-Aldh1a2</i>	GAGGAAGGCAGATGGGAAAT	CTTGGAAGGTAAGGGAGTGTG