Supplementary Information

Initial seeding of embryonic thymus by immune-restricted lympho-myeloid progenitors

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Supplementary Figure 1 Staging of embryonic day 11 fetal thymus (Related to main Figure 1)

(**a**) Staging criteria for E11.0, 11.25, 11.5 and 11.75 embryos with the corresponding somite (S) pairs indicated. Stage criteria (in red) include morphological assessment of branchial arches (1st and 3rd row), nasal processes (2nd and 3rd row) and limb bud (4th row) development. Abbreviations: BA: branchial arches, numbered; M: Maxillary; m: mandibulary.

(**b**) Tail somite (TS) counting to stage thymus development. Somite development exhibits a faithful correlation with organ development in the embryo, however some somites especially trunk somites have already initiated their differentiation at E11, resulting in ambiguous and inconsistent somite counts. By correlating somites in the tail, which are still visible at E11, with morphology assessment of branchial arches, nasal processes and limb bud development, TS counts representing the corresponding E11 developmental stages were documented (see **Fig. 1a**). TS counting should only be undertaken in embryos after E11, when the cloaca (*) is protruding, as similar TS number may be obtained from the base of the limb bud at E10.25-10.75 when the cloaca is not yet protruding.

(**c**) At E11.5 the 3rd pouch derived primordia constituted by the thymus (light red) and parathyroid (dark red) rudiments are attached to the pharyngeal endoderm and surface ectoderm. Separation between the thymus and parathyroid primordia starts around E12.0-E12.5. Scheme adapted from[27].

(**d**) Sagittal sections were performed to reveal the fetal thymic lobes which are located in the third branchial arches (arrows) and can be visualized by cytokeratin staining (CK, green; DAPI, blue). I, II and III indicate the 1st, 2nd and 3rd branchial arches, respectively.

(**e**) At E11.5 the cytokeratin (CK, red left panel, green right panel) positive fetal thymus is not vascularized but surrounded by blood vessels as confirmed by *Vwf* eGFP (green left panel, blue right panel) and VE-Cadherin (VE-Cad, red; right panel) staining. Arrows indicate the location of the thymus rudiment.

Supplementary Figure 2 Initial seeding of the early embryonic thymus by *Rag1*-GFP expressing cells but not HSCs (Related to main Figures 1-3)

(**a**) Surrounding blood vessels (VE-Cad^{+}) are typically completely removed in dissected fetal thymus lobes (at E11.5 and E12.5) (VE-Cad, red; CK, green; DAPI, blue).

(**b**) Fetal liver cells showing overlap in staining with anti-Vwf antibody (green, left) and *Vwf* eGFP (blue, middle), as shown in the merged image (right).

(**c,d**) Representative FACS profiles (c) and summary (d) of repopulation activity of total thymocytes (fetal thymus; FT) from 4-5 pooled E11.5 embryos or total fetal liver (FL) cells from 3 pooled E12.5 embryos (CD45.2), transplanted intrafemorally into each irradiated (600Gy) $W^{41/41}$ (c-kit deficient; CD45.1) recipient. Analysis was performed 2 weeks post-transplantation, since recipients transplanted with FT (but not FL) cells at this time became severely ill and had to be terminated. Data are means (s.d.) from 5 recipient mice receiving FT and 4 recipient mice receiving FL cells.

(**e,f**) Bone marrow cells ($25x10^6$) from primary recipients (from **c,d**) were re-transplanted into lethally irradiated CD45.1 recipients (together with $2x10^5$ CD45.1 bone marrow support cells) to quantify potential stem cell activity in donor-derived (CD45.2) cells. Representative FACS profiles (**e**) and summary (**f**) of long-term FT and FL derived (CD45.2) reconstitution of blood cell lineages, 12 weeks post-transplantation, as percentage of total cells within each lineage. Dotted lines indicate the detection level of reconstitution, based on specificity of the antibodies and the number of events acquired by FACS. Mean (s.d.) data from 4 mice in each group.

(**g**) *Rag1*-GFP (green) expression in cytokeratin (CK, blue) positive thymus lobes from stained sagittal paraffin sections of TS10-15 *Rag1* GFP^+ embryos. Scale bars represent 10 μm.

(**h**) Whole mount immunofluorescence analysis of a TS14 embryo. Transverse optical sections showing localization of thymic rudiments (arrows) identified by cytokeratin (CK) staining. nt, neural tube; *, notochord. Scale bars represent 300 μm.

(i) Whole mount analysis of thymus colonization by Rag1-GFP (green) T-TIPs. Serial transverse optical sections (2.5μm) of a TS14 thymic lobe marked by cytokeratin (CK, red) staining showing GFP^+ cells both lining and inside the thymic rudiment. Scale bars represent 30 μm.

(**j,k**) Gating strategy for FACS purification of (**j**) $CD45^+Lin^-B220^-CD19^-c\text{-}Kit^+Flt3^+IL\text{-}7R\alpha^+$ E11.5 fetal liver LMPPs and (**k**) E14.5 $Lin^-CD4^-CD8^-c\text{-}Kit^+CD25^+$ DN2 and E14.5 $Lin^-CD4^-CD8^-c\text{-}Kit^-CD25^+$ DN3 thymocytes. Numbers represent the mean (from 2 biological replicates) percentage of total cells, for each gated cell population.

Supplementary Figure 3 Combined T and myeloid lineage potential in E11.5 thymus (Related to main Figures 4-5)

(**a**) Mean (s.e.m.) frequencies of FACS purified single $CD45^+Lin^-c\text{-}Kit^+CD25^-Flt3^+$ E12.5 thymic

progenitors from WT mice, revealing T cell (*n* = 48 cells) and myeloid/GM (*n* = 360 cells) lineage potential *in vitro*, from 2 and 3 independent experiments, respectively.

(**b**) Representative FACS profile of $CD4^+CD8^+$ T cells derived from single cell OP9-DL1 culture of FACS purified $CD45^+Lin^-c\text{-}Kit^+CD25^-Flt3^+$ cells from E12.5 thymus.

(**c**) Characteristic morphology of myeloid cells derived from single cell cultures of $CD45^+Lin^-c\text{-}Kit^+CD25^-Flt3^+$ E12.5 thymic progenitors. Representative cells belonging to the monocytic (top row) and granulocytic (bottom row) lineages. Scale bars: 10 μm.

(**d-f**) Three representative clones derived from single $CD45^+Lin^-c\text{-}Kit^+CD25^-Flt3^+$ E11.5 thymic progenitor cells cultured on OP9-DL1 stroma generating $CD4^+CD8^+$ T cells and myeloid cells confirmed by FACS ($CD11b^+$) and/or morphology. Asterisks indicate OP9-DL1 stromal cells. Scale bars: 20 μm.

(**g,h**) Representative clone derived from a single $CD45^+Lin^-c\text{-}Kit^+CD25^-Flt3^+Rag1\text{-}GFP^+$ cell from the peripheral blood of E11.5 embryos producing $CD4^+CD8^+$ and $Thy1.2^+CD25^+$ T cells (**g**) and myeloid (GM) cells confirmed by FACS ($CD11b^+NK1.1^-$; **g**) and morphology (**h**). Asterisk in **h**, indicate OP9-DL1 stromal cells. Scale bars: 10μm.

(**i**) Flow cytometry analysis of eYFP expression in $CD11b^+F4/80^+c\text{-}Kit^-CD25^-$ monocytes/macrophages, ETPs, DN2s and DN3s from E14.5 *Rag1*-$Cre^{tg/+}$*R26*$^{eYFP/+}$ thymuses. Data are representative of 2 independent experiments with 6 and 7 pooled embryos in each experiment.

(**j**) Flow cytometry analysis of *Rag1*-GFP expression in $CD11b^+F4/80^+c\text{-}Kit^-CD25^-$ macrophages, ETPs, DN2s and DN3s from E14.5 *Rag1*-GFP thymuses. Data are representative of 2 independent experiments with 6 and 8 pooled embryos in each experiment.

(**k,l**) Single cell immunofluorescence analysis of intracellular eYFP localization in sorted $eYFP^+$ and $eYFP^-$ $CD11b^+F4/80^+c\text{-}Kit^-CD25^-$ monocytes/macrophages (**k**) and $eYFP^+$ $CD11b^-F4/80^-c\text{-}Kit^+CD25^-$ ETPs (**l**) from E14.5 *Rag1*-$Cre^{tg/+}$*R26*$^{eYFP/+}$ thymuses. Images representative of 80, 17 and 26 single $eYFP^+$ monocytes/macrophages, $eYFP^-$ monocytes/macrophages and $eYFP^+$ ETPs analyzed, respectively. DIC, differential interference contrast.

(**m,n**) Representative FACS profiles and mean (s.d.) frequency (**m**) of CD45.2 reconstitution of thymocytes in CD45.1 lethally irradiated recipient mice, 3 weeks post-transplantation with a mixture of *Rag1*-$Cre^{Tg/+}$ and *R26*-stop-$eYFP^{FL/FL}$ (1:1) CD45.2 bone marrow cells. (**n**) Frequency of $eYFP^+$ cells within $CD11b^+F4/80^+CD4^-CD8^-c\text{-}Kit^-CD25^-$ macrophages. Data are means (s.d.) of 4 recipient mice. Values in (**m**) are mean frequencies of total thymocytes (left plot) and of total CD45.2 thymocytes

(right plot). Values in (**n**) are mean frequencies of total CD45.2 thymus cells (left plot) and of total CD45.2 macrophages (right plot).

Supplementary Figure 4 Lineage potentials in E11.5 thymus (Related to main Figures 4 and 5)

(**a**) Morphology of representative myeloid cells from cultures of E11.5 embryonic thymus. White arrows indicate monocytic cells, and black arrows granulocytic cells. Asterisk indicates OP9/OP9-DL1 stromal cells. Scale bars: 10 μm.

(**b**) Representative acetylcholinesterase stained CFU-Mk colonies from E11.5 fetal liver. Scale bars represent 100 μm.

(**c**) Mean (s.d.) number of DAF^+ erythroid colonies generated from E11.5 unfractionated fetal liver cells cultured in semi-solid methylcellulose (*n* = 4; 50,000 cells/biological replicate).

(**d,e**) Representative flow cytometry analyses of T cell (**d**) and B cell (**e**) producing cultures of individual thymic lobes from *Rag1*-GFP E11.5 embryos.

(**f**) Gating strategy for FACS sorting of $Lin^-CD45^{lo}VE\text{-}Cad^+c\text{-}Kit^+$ AGM stem/progenitor cells (HSPC). Numbers represent mean frequencies of total cells, from 3 litters analysed in 2 independent experiments.

(**g**) Representative FACS profiles of pooled E12.5 thymuses. Virtually all *Rag1*-GFP^+ cells express CD45 and progenitor markers c-Kit and Flt3 but are negative for lineage markers and CD25. The number in the first plot reflects the percentage of total cells, whereas subsequent plots show frequencies relative to total GFP^+ cells. All frequencies are means of 3 litters (6-7 embryos pooled in each litter) from 2 independent experiments.

Supplementary Figure 5 Global gene expression analysis of T-IPs/ETPs, LMPPs and HSCs at different stages of development (Related to main Figure 7)

Hierarchical clustering by genes (**a**) or by cell populations (**b**) indicating the relationship between the different cell populations analyzed, according to their global gene expression profile. In **b**, hierarchical clustering was performed using the multi-scale bootstrap resampling method. Red values denote Approximately Unbiased (AU) *p*-values (%), and green values denote Bootstrap Probability (BP) *p*-values (%).

Supplementary Figure 6 Embryonic *Ccr*, *Pir* and Notch related gene expression in T-IPs, ETPs and candidate thymus seeding progenitors (Related to main Figure 7 and 8)

(a**)** Representative flow cytometry analysis of the expression of CCR6, 7 and 9 in E11.5 T-IPs. Numbers represent mean percentages of data from 3 experiments, with 7-9 pooled embryos per experiment. Grey, isotype control; Red, specific anti-CCR antibody.

(**b**) Heatmap for co-expression (ΔCt values, relative to *Hprt*) of the highest expressed chemokine receptor genes in single E11.5 $CD45^{+}Lin^{-}c\text{-}Kit^{+}CD25^{-}Flt3^{+}$ T-IPs (*n* = 85 cells, from 2 biological replicates (each a pool of 5 and 9 embryos). Red indicates high, white intermediate and blue low expression levels. Grey indicates below detection level. Three cells were excluded due to absence of *Flt3* amplification.

(**c**) Expression of *Pir* genes in E11.5 T-IPs (*n* = 6), as compared to E11.5 AGM HSPCs (*n* = 3) and E11.5 FL LMPPs (*n* = 3), as well as intra-thymic E12.5 (*n* = 3), neonatal (NN; 1 week; *n* = 3) and Adult (8 weeks; *n* = 3) ETPs. Expression is presented as mean (s.d.) RPKM. Significant differences between different populations are marked with asterisks (*p<0.05, **p<0.01, ***p<0.001); 0, below detection level. n represents biological replicates.

(**d**) Mean (s.d.) percentage of single E11.5 $CD45^{+}Lin^{-}c\text{-}Kit^{+}CD25^{-}Flt3^{+}$ T-IPs co-expressing *Pir*, GM and lymphoid affiliated genes while not expressing Mk or E genes. Only cells that amplified *Flt3*, *c-Kit* and *Hprt* (corresponding to 97% of total cells analyzed) were included. (*n*=85 cells, from 2 biological replicates, each using a pool of 5-9 embryos).

(**e**) Representative flow cytometry analysis PIRA/B expression in E11.5 fetal liver $CD45^{+}Lin^{-}B220^{-}CD19^{-}c\text{-}Kit^{+}Flt3^{+}IL\text{-}7R\alpha^{+}$ LMPPs (*n*=2), E11.5 $CD45^{+}Lin^{-}c\text{-}Kit^{+}CD25^{-}Flt3^{+}$*Rag1*-$GFP^{+}$ cells in circulation (*n*=3), $CD45^{+}Lin^{-}c\text{-}Kit^{+}CD25^{-}Flt3^{+}$*Rag1*-$GFP^{+}$ T-IPs (*n*=3) and E12.5 $CD45^{+}Lin^{-}c\text{-}Kit^{+}CD25^{-}Flt3^{+}$*Rag1*-$GFP^{+}$ ETPs (*n*=3). Numbers in plots indicate average percentages of all biological replicate (*n* = 2-3), each replicate from a pool of 5-9 embryos.

(**f**) Myeloid and/or T cell generation from single $CD45^{+}Lin^{-}c\text{-}Kit^{+}CD25^{-}Flt3^{+}$ PIRA/B^{+} or PIRA/B^{-} cells isolated from circulation of E11.5 embryos and cultured on OP9-DL1 (*n*=254 cells). Mean (s.d.) frequencies from 3 independent experiments are shown.

Supplementary Figure 7 Thymus rudiment colonization by T-IPs in E11.5 *Rbpj* deficient embryos (Related to main Figure 8)

(**a,b**) Whole mount imaging of a *Rbpj*$^{Fl/Fl}$ *Vav*-$Cre^{+/+}$ *Rag1*-$GFP^{Tg/+}$ TS11 embryo. (**a**) Transverse

optical section showing localization of thymic rudiments (arrows) identified by cytokeratin (CK, red). nt, neural tube; *, notochord. Scale bars represent 300 µm. (**b**) Three-dimensional image of the left and right thymic lobes showing the thymus rudiment surrounded by *Rag1*-GFP+ (green) T-IPs. Note that the thymus rudiment is connected to the third pharyngeal pouch on one side and to the external ectoderm on the other side. Scale bars represent 50 µm.

(**c-e**) Using the "surfaces" function of Imaris software the thymus rudiment was 3D-reconstructed to allow the quantification of the distances to Rag1-GFP^{+} cells. Image segmentation (**d, e**) was made based on cytokeratin fluorescence intensity (**c**) and morphology (see also Supplementary Fig. 1). Scale bars represent 50 µm.

(**f,g**) Serial transverse optical sections (2.5 µm) of the thymic rudiment region from a *Rbpj*$^{Fl/Fl}$ *Vav*-Cre$^{+/+}$ *Rag*1-GFP$^{Tg/+}$ (**c,** *Vav*-Cre$^{+/+}$) and a *Rbpj*$^{Fl/Fl}$ *Vav*-Cre$^{Tg/+}$ *Rag1*-GFP$^{Tg/+}$ (**d,** *Vav*-Cre$^{Tg/+}$) embryo. Images are representative of 8 and 6 thymic lobes from 4 Vav-Cre$^{+/+}$ and 3 Vav-Cre$^{Tg/+}$ embryos, respectively. Scale bars represent 50 µm. (c-g) TPP, thymus-parathyroid primordium; PE, pharyngeal endoderm; SE, surface ectoderm

Supplementary Figure 8 E11.5 *Rbpj*-deficient T-IPs show unaffected expression of Notch related and lineage affiliated genes (Related to main Figure 8)

(**a-d**) Single cell gene expression analysis of E11.5 CD45^{+}Lin^{-}c-Kit^{+}CD25^{-}Flt3^{+} T-IPs from *Rbpj*$^{Fl/Fl}$ *Vav*-Cre$^{Tg/+}$ *Rag1*-GFP$^{Tg/+}$ (*n*=46 cells from 6 embryos) and *Rbpj*$^{Fl/Fl}$ *Vav*-Cre$^{+/+}$ *Rag1*-GFP$^{Tg/+}$ (*n*=36 cells from 8 embryos) littermate controls from 2 different litters. Only cells that amplified *Flt3*, *c-Kit* and *Hprt* (corresponding to 98% of total cells analyzed) were included. (**a**) *Rbpj* (**b**) Notch receptors and Notch target genes (**c**) frequency of single cells co-expressing GM and early lymphoid affiliated genes. (**d**) T cell related genes. (**b-d**) For additional gene expression analysis of *Vav*-Cre^{+} cells only cells in which *Rbpj* had been deleted were included. For panels (a-d) numbers indicate the frequencies of positive cells. For panels b-d no differences between Cre^{-} and Cre^{+} cells were statistically significant ($p<0.05$).

(**e,f**) Impact of *Rbpj*-deficiency on thymocyte progenitor development during early stages of embryonic development. Representative flow cytometry analysis of thymus rudiments from E13.5 (**e**) and E14.5 (**f**) *Rbpj*$^{Fl/Fl}$*Vav*-Cre$^{Tg/+}$*Rag1*-GFP$^{Tg/+}$ (*Vav*-Cre$^{Tg/+}$; *n*=3 and n=4, respectively) and *Rbpj*$^{Fl/Fl}$*Vav*-Cre$^{+/+}$*Rag1*-GFP$^{Tg/+}$ (*Vav*-Cre$^{+/+}$; *n*=7 and n=11, respectively) littermate control embryos. Numbers represent frequencies of total CD45^{+} cells.

(**g**) *In vitro* T cell differentiation of E11.5 *Rbpj*$^{Fl/Fl}$*Vav*-Cre$^{Tg/+}$ (*Vav*-Cre$^{Tg/+}$) and *Rbpj*$^{Fl/Fl}$*Vav*-Cre$^{+/+}$*Rag1*-GFP$^{Tg/+}$ (*Vav*-Cre$^{+/+}$) T-IPs on OP9-DL1 stroma. Data are representative of 4 *Vav*-Cre$^{+/+}$ and 6 *Vav*-Cre$^{Tg/+}$ embryos analysed following 14 days of culture. Numbers represent frequencies of total CD45^{+} cells.

SUPPLEMENTARY TABLES

Supplementary Table 7 Antibodies used for immunofluorescence staining

Antigen	Clone	Host	Species reactivity	Supplier	Application
Primary Antibodies					
Cytokeratin	Polyclonal	Rabbit	Mouse	Dako	*Paraffin section* *Whole mount*
GFP	Polyclonal	Chicken	Jelly fish	Abcam	Paraffin sections
GFP	Polyclonal	Rabbit	Jelly fish	Life technologies	Whole mount
Vwf	Polyclonal	Rabbit	Mouse	Millipore	Paraffin sections
VE-Cadherin	Polyclonal	Goat	Mouse	R&D	Paraffin sections
F4/80	CI:A3-1	Rat	Mouse	Abcam	Paraffin sections
Secondary Antibodies					
IgG Cy3	-	Donkey	Goat	Jackson Immu. Laboratories	Paraffin sections
IgG DyLight 488	-	Donkey	Chicken	Jackson Immu. Laboratories	Paraffin sections
IgG DyLight 549	-	Donkey	Rat	Jackson Immu. Laboratories	Paraffin sections
IgG DyLight 649	-	Donkey	Chicken	Jackson Immu. Laboratories	Paraffin sections
IgG AlexaFluor 488	-	Donkey	Rabbit	Invitrogen	Paraffin sections
IgG AlexaFluor 633	-	Goat	Rabbit	Invitrogen	Paraffin sections
IgG AlexaFluor 488	-	Goat	Chicken	Life technologies	Whole mount
IgG AlexaFluor 555	-	Goat	Rabbit	Life technologies	Whole mount

Supplementary Table 8 Antibodies and viability stains used for FACS

Antibody	Clone	Supplier	Application
7-aminoactinomycin D (7-AAD)	-	Sigma	Viability dye
B220 PE-Cy5	RA3-6B2	BioLegend	Lineage cocktail for BM LSKFLT3 staining and AGM HSPC staining
B220 APC	RA3-6B2	BD Biosciences	Lineage cocktail for NN and adult ETP staining
B220 PE-Cy7	RA3-6B2	BioLegend	Lineage cocktail for T-IP staining in fetal thymus, LMPP staining in fetal liver
CCR9 APC	eBioCW-1.2	E-bioscience	T-IP staining in fetal thymus
CCR6 PE	29-2L17	BioLegend	T-IP staining in fetal thymus
CCR7 PE	RB12	BD Biosciences	T-IP staining in fetal thymus
CCR9 PECy7	CW-1.2	BioLegend	T-IP staining in fetal thymus
CD11c APC	N418	E-bioscience	Lineage cocktail for NN and adult ETP staining
CD16/32 purified	2.4G2	Hybridoma	Fc-block
CD150 APC	TC15-12F12.2	BioLegend	Platelet analysis
CD19 APC	1D3	BD Biosciences	Lineage cocktail for NN and adult ETP, and BM LSKFLT3 staining
CD19 Pacific blue	1D3	E-bioscience	In vitro B cell readout
CD19 PE	1D3	BD Biosciences	In vitro B cell readout
CD19 PE-Cy5	1D3	E-bioscience	Lineage cocktail for BM LSKFLT3 staining and AGM HSPC staining
CD19 PE-Cy7	1D3	E-bioscience	Lineage cocktail for T-IP staining in fetal thymus; LMPP staining in fetal liver
CD25 PE	3C7	BD Biosciences	In vitro T cell readout
CD25 PerCP-Cy5.5	PC61	BD Biosciences	T-IP/ETP and DN staining
CD3e APC	145-2C11	E-bioscience	Lineage cocktail for NN and adult ETP, BM LSKFLT3 staining and fetal liver LMPP staining
CD31 PE-Cy7	390	E-bioscience	AGM HSPC staining
CD4 AlexaFluor700	RM4-5	E-bioscience	NN and adult ETP staining
CD4 APC-eFluor780	RM4-5	E-bioscience	In vitro T cell readout and peripheral blood analysis
CD4 PE-Cy7	RM4-5	BD Biosciences	Lineage cocktail for T-IP staining in fetal thymus
CD4 PE-Cy5	RM4-5	BD Biosciences	Lineage cocktail for BM LSKFLT3 staining
CD41 PE	MWReg30	E-bioscience	Platelet analysis
CD44 APC	IM7	BioLegend	In vitro T cell readout
CD45 AlexaFluor700	30-F11	E-bioscience	ETP staining in E12.5 thymus, LMPP staining in fetal liver
CD45 PE	30-F11	E-bioscience	AGM HSPC staining
CD45 Pacific Orange	30-F11	Invitrogen	In vitro hematopoietic readout
CD45.1 biotin	A20	BD Biosciences	Peripheral blood reconstitution analysis
CD45.1 PE	A20	BD Biosciences	Peripheral blood reconstitution analysis
CD45.2 AF700	104	BioLegend	Peripheral blood reconstitution analysis
CD45.2 FITC	104	BD Biosciences	Peripheral blood reconstitution analysis
CD45.2 PerCP-Cy5.5	104	BD Biosciences	Peripheral blood reconstitution analysis
CD5 PE-Cy5	53-7.3	BioLegend	Lineage cocktail for BM LSKFLT3 staining and AGM HSPC staining
CD8α APC-eFluor780	53-6.7	E-bioscience	In vitro readout T cell readout and peripheral blood analysis
CD8α PE-Cy7	53-6.7	E-bioscience	NN and adult ETP staining, and lineage cocktail for T-IP staining in fetal thymus
CD8α PE-Cy5	53-6.7	BD Biosciences	Lineage cocktail for BM LSKFLT3 staining and AGM HSPC staining
c-kit APC-eFluor780	2B8	E-bioscience	T-IP/ETP, LSKFLT3, and AGM HSPC stainings, LMPP staining in fetal liver

DAPI (4',6-diamidino-2-phenylindole, dihydrochloride)	-	Invitrogen	Viability dye
F4/80 PE-Cy7	BM8	BioLegend	In vitro myeloid readout
F4/80 APC	BM8	Invitrogen BioLegend	In vitro myeloid readout, Lineage cocktail for fetal liver LMPP staining. Fetal thymus macrophage staining
Flt3 biotin	A2F10	E-Bioscience	T-IP/ETP staining, LMPP staining in fetal liver
Flt3 PE	A2F10	BD Biosciences, BioLegend	T-IP/ETP staining, LSKFLT3 staining in BM
Gr-1 APC	RB6-8C5	E-bioscience	Lineage cocktail for NN and adult ETP, BM LSKFLT3 staining and fetal liver LMPP
Gr-1 Pacific Orange	RB6-8C5	Invitrogen	In vitro myeloid readout and peripheral blood analysis
Gr-1 PE	RB6-8C5	E-bioscience	In vitro myeloid readout
Gr-1 PE-Cy5	RB6-8C5	BioLegend	Lineage cocktail for BM LSKFLT3 staining and AGM HSPC staining
IL-7R biotin	A7R34	E-bioscience	LMPP staining in BM, TI-P staining in fetal thymus rudiment
IL-7R PE	A7R34	E-bioscience	LMPP staining in fetal liver
Mac-1 AlexaFluor700	M1/70	E-bioscience	In vitro myeloid readout
Mac-1 APC	M1/70	BioLegend	Peripheral blood reconstitution analysis
Mac-1 PE-Cy5	M1/70	BioLegend	Lineage cocktail for BM LSKFLT3 staining
Mac-1 PE	M1/70	E-bioscience	Fetal thymus macrophage staining
NK1.1 APC	PK136	E-bioscience	Lineage cocktail for NB ETP and LMPP in fetal liver
NK1.1 FITC	PK136	E-bioscience	In vitro NK cell readout
NK1.1 Pacific blue	PK136	BioLegend	In vitro NK cell readout and peripheral blood analysis
NK1.1 BV605	PK136	BioLegend	In vitro T cell readout
PirA/B APC	10-1-PIR	E-bioscience	LMPP staining in fetal liver, TI/ETP-P staining in fetal thymus rudiment and fetal blood staining
Sca-1 FITC	E13-161.7	BD Biosciences	LSKFLT3 staining in BM, LMPP staining in fetal liver
Sca-1 Pacific Blue	E13-161.7	BioLegend	LSKFLT3 staining in BM
Streptavidin PE-TXR	-	BD Biosciences	NN and adult ETP staining
Streptavidin QD655	-	Invitrogen	LMPP staining in fetal liver
Sytox Blue	-	Invitrogen	Viability dye
Tcrβ APC	H57-597	E-bioscience	Lineage cocktail for NN and adult ETP staining
Tcrγδ APC	eBioGL3	E-bioscience	Lineage cocktail for NN and adult ETP staining
Ter119 PE-Cy5	TER119	BioLegend	Lineage cocktail for BM LSKFLT3 staining and AGM HSPC staining
Ter119 PE-Cy5.5	TER119	E-bioscience	Platelet analysis (negative marker)
Ter119 APC	TER119	E-bioscience	Lineage cocktail for fetal liver LMPP staining
Thy1.2 APC	53-2.1	E-bioscience	In vitro T cell readout
Thy1.2 PE-Cy5	30-H12	BioLegend	In vitro T cell readout
VE-Cad (CD144) APC	BV13	E-bioscience	AGM HSPC staining

Supplementary Table 9 Cytokines used for *in vitro* assays

Growth factor	Supplier	Assay	Final conc. (ng/ml)
Murine stem cell factor mSCF	*R&D Systems*	*Mk liquid culture* *GM liquid culture* *B cell OP9 co-culture* *T cell OP9-DL1 co-culture* *Myeloid OP9 co-culture* *T cell/myeloid combined OP9-DL1 co-culture*	50 ng/ml 25 ng/ml 25 ng/ml 25 ng/ml 10 ng/ml 10ng/ml
Murine interleukin 3 mIL-3	*R&D Systems*	*Mk liquid culture* *GM liquid culture* *Myeloid OP9 co-culture* *Megacult culture* *T cell/myeloid combined OP9-DL1 co-culture*	20 ng/ml 10 ng/ml 10 ng/ml 10 ng/ml 10 ng/ml
Murine granulocyte/macrophage colony stimulating factor mGM-CSF	*Immunex*	*GM liquid culture* *Myeloid OP9 co-culture* *T cell/myeloid combined OP9-DL1 co-culture*	25 ng/ml 10 ng/ml 10 ng/ml
Human granulocyte colony stimulating factor hG-CSF	*Amgen*	*T cell/myeloid combined OP9-DL1 co-culture* *GM liquid culture* *Myeloid OP9 co-culture*	10 ng/ml 25 ng/ml 10 ng/ml
Human colony stimulating factor 1 hCSF-1	*Peprotech*	*GM liquid culture* *Myeloid OP9 co-culture* *T cell/myeloid combined OP9-DL1 co-culture*	25 ng/ml 10 ng/ml 10 ng/ml
Human erythropoietin hEPO	*Janssen-Cilag*	*Mk liquid culture*	5 U/ml
Human FLT3 ligand hFLT3L	*Immunex*	*Mk liquid culture* *GM liquid culture* *B cell OP9 co-culture* *T cell OP9-DL1 co-culture* *Myeloid OP9 co-culture* *T cell/myeloid combined OP9-DL1 co-culture*	50 ng/ml 25 ng/ml 25 ng/ml 25 ng/ml 5 ng/ml 5 ng/ml
Human interleukin 6 hIL-6	*Genetics Institute Inc*	*Myeloid OP9 co-culture* *Megacult culture* *T cell/myeloid combined OP9-DL1 co-culture*	10 ng/ml 20 ng/ml 10 ng/ml
Human interleukin 7 hIL-7	*R&D Systems*	*B cell OP9 co-culture* *Myeloid OP9 co-culture* *T cell/myeloid combined OP9-DL1 co-culture*	20 ng/ml 1 ng/ml 1 ng/ml
Human interleukin 11 hIL-11	*Genetics Institute Inc*	*Megacult culture*	50 ng/ml
Human thrombopoietin hTHPO	*Peprotech*	*Mk liquid culture* *GM liquid culture* *Megacult culture*	50 ng/ml 25 ng/ml 50 ng/ml

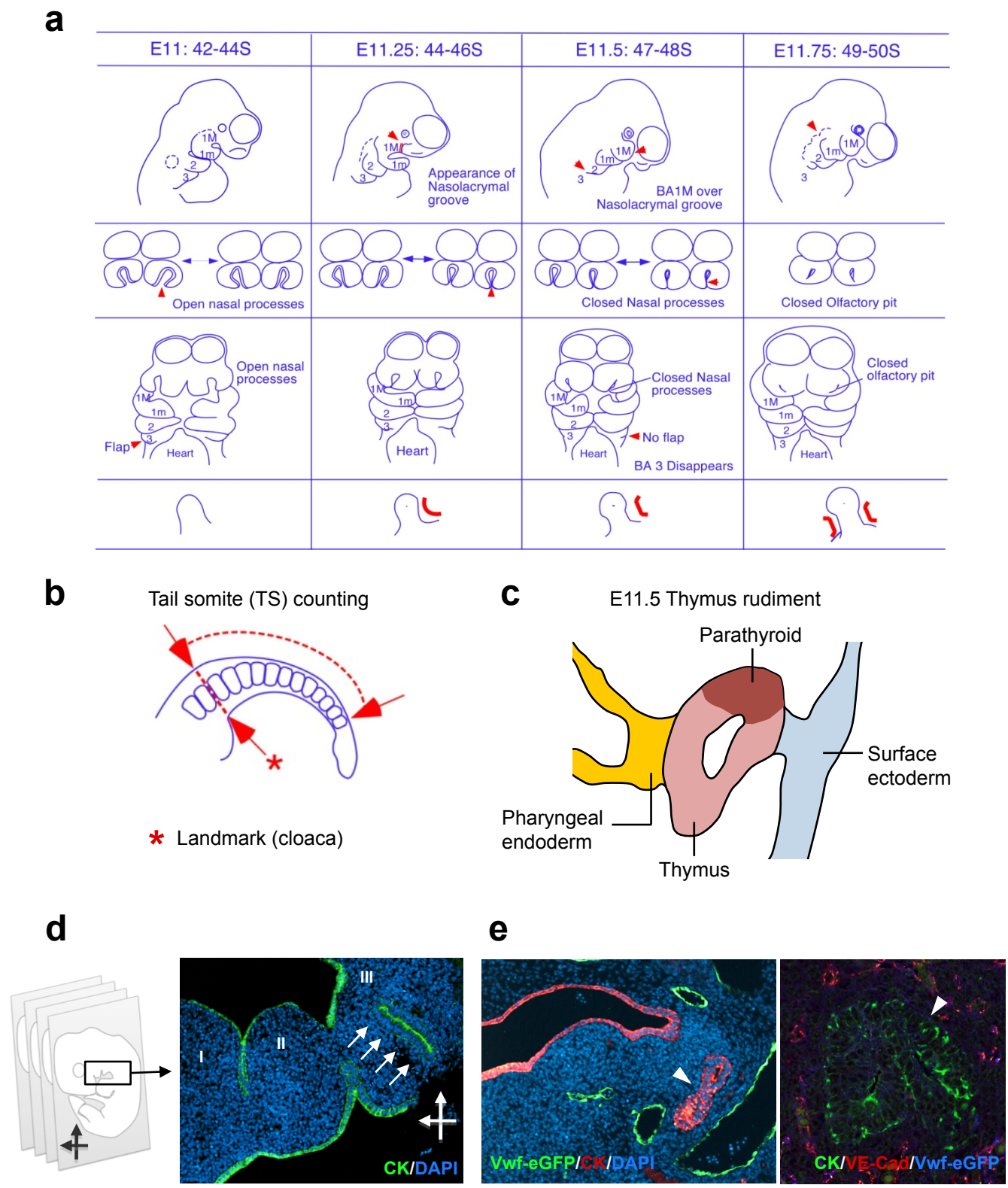
Supplementary Table 10 Taqman assays used for multiplex quantitative single cell PCR

Bcl11b	B-cell leukemia/lymphoma 11B	Mm00480516_m1
Ccr2	chemokine (C-C motif) receptor 2	Mm04207877_m1
Ccr3	chemokine (C-C motif) receptor 3	Mm00515543_s1
Ccr5	chemokine (C-C motif) receptor 5	Mm01963251_s1
Ccr7	chemokine (C-C motif) receptor 7	Mm01301785_m1
Ccr9	chemokine (C-C motif) receptor 9	Mm02620030_s1
Cd3d	CD3 antigen, delta polypeptide	Mm00442746_m1
Cd3e	CD3 antigen, epsilon polypeptide	Mm00599683_m1
Cd3g	CD3 antigen, gamma polypeptide	Mm00438095_m1
CD79a	CD79A antigen (immunoglobulin-associated alpha)	Mm00432423_m1
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	Mm00514283_s1
Csf1r	colony stimulating factor 1 receptor	Mm01266652_m1
Csf2ra	colony stimulating factor 2 receptor, alpha	Mm00438331_g1
Csf3r	colony stimulating factor 3 receptor (granulocyte)	Mm00432735_m1
Cxcr4	chemokine (C-X-C motif) receptor 4	Mm01996749_s1
Dtx1	deltex 1 homolog (Drosophila)	Mm00492297_m1
Ebf1	early B cell factor 1	Mm00432948_m1
Epor	erythropoietin receptor	Mm00438760_m1
Esam	endothelial cell-specific adhesion molecule	Mm00518378_m1
Evi1	MDS1 and EVI1 complex locus	Mm00491303_m1
Fcgr3	Fc receptor, IgG, low affinity III	Mm00438882_m1
Flt3	FMS-like tyrosine kinase 3	Mm00439016_m1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1
Gata1	GATA binding protein 1	Mm01352636_m1
Gata2	GATA binding protein 2	Mm00492301_m1
Gata3	GATA binding protein 3	Mm00484683_m1
Hes1	hairy and enhancer of split 1 (Drosophila)	Mm01342805_m1
Hes5	hairy and enhancer of split 5 (Drosophila)	Mm00439311_g1
Hey1	hairy/enhancer-of-split related with YRPW motif 1	Mm00468865_m1
Hey2	hairy/enhancer-of-split related with YRPW motif 2	Mm00469280_m1
Hoxb4	homeobox B4	Mm00657964_m1
Hprt1	hypoxanthine guanine phosphoribosyl transferase 1	Mm01545399_m1
Igmh	immunoglobulin heavy constant mu	Mm01718955_g1
Ikzf1	IKAROS family zinc finger 1	Mm00456421_m1
Il2ra	interleukin 2 receptor, alpha chain	Mm00434261_m1
Il7r	interleukin 7 receptor	Mm00434295_m1
Kit	kit oncogene	Mm00445212_m1
Klf1	Kruppel-like factor 1 (erythroid)	Mm04208330_g1
Lck	lymphocyte protein tyrosine kinase	Mm00802897_m1
Lilrb3	leukocyte immunoglobulin-like receptor, subfamily B (Pirb)	Mm01700366_m1
Mpl	myeloproliferative leukemia virus oncogene	Mm00440310_m1
Mpo	Myeloperoxidase	Mm01298424_m1
Notch1	Notch gene homolog 1 (Drosophila)	Mm00435249_m1
Nrarp	Notch-regulated ankyrin repeat protein	Mm00482529_s1
Pax5	paired box gene 5	Mm00435501_m1
Pf4	platelet factor 4	Mm00451315_g1
Pira2	paired-Ig-like receptor A2	Mm02768273_g1
Pira6	paired-Ig-like receptor A2	Mm01705006_sH
Procr	protein C receptor, endothelial	Mm00440992_m1
Ptcra	pre T-cell antigen receptor alpha	Mm00478363_m1
Rag1	recombination activating gene 1	Mm01270936_m1
Rag2	recombination activating gene 2	Mm00501300_m1

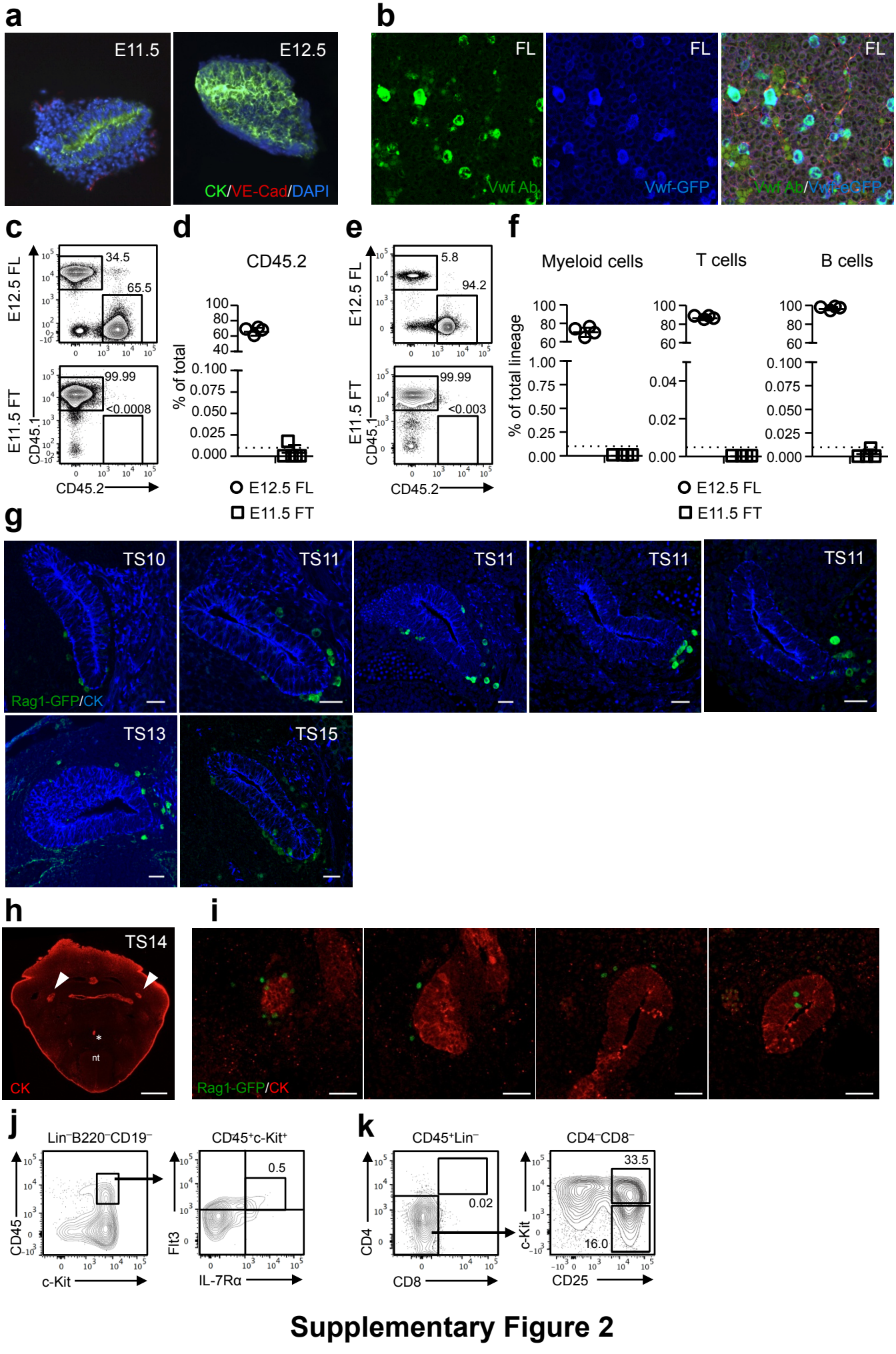
Rbpj	recombination signal binding protein for immunoglobulin kappa J region	Mm01217627_g1
Slgh	Sterile IgH Forward primer: GGACTTTGGGATGGGTTTGGTT Reverse primer: CCCTGGTCCTAGACATCAGAGTAAT Reporter sequence: CCCAGATGAAGGGCTAC	Custom assay
Tcf7	transcription factor 7, T-cell specific	Mm00493445_m1
Themis	thymocyte selection associated	Mm00724485_m1
Vpreb1	pre-B lymphocyte gene 1	Mm00785614_sH
Vwf	Von Willebrand factor homolog	Mm00550376_m1

Supplementary Table 11 Primers and UPL probes used for RT-qPCR

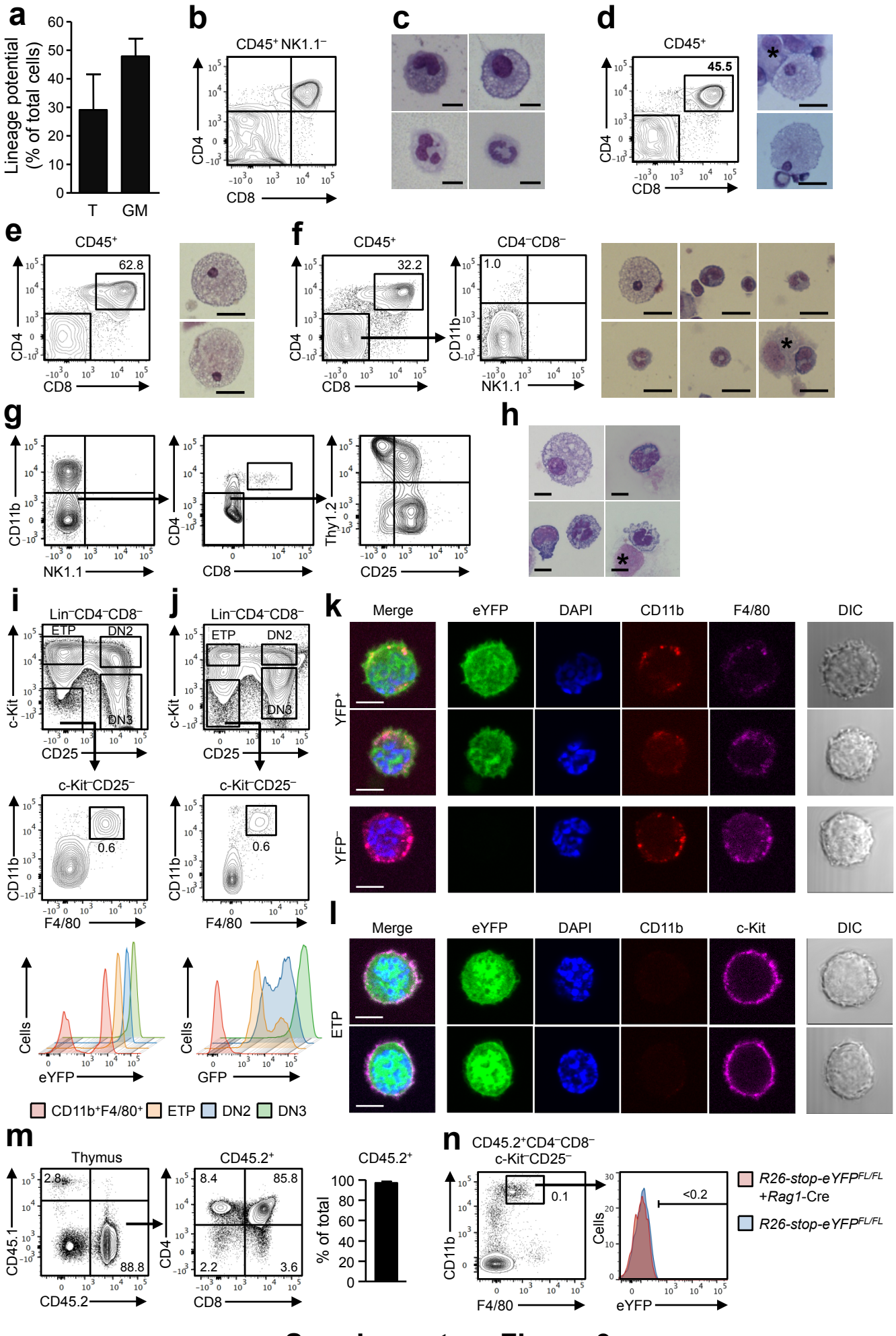
Dll4	Fwd: AGGTGCCACTTCGGTTACAC Rev: GGGAGAGCAAATGGCTGATA	UPL Probe # 106
Actb	Fwd: AAGGCCAACCGTGAAAAGAT Rev: GTGGTACGACCAGAGGCATAC	UPL probe # 56
Hmbs	Fwd: TCCCTGAAGGATGTGCCTAC Rev: AAGGGTTTTCCCGTTTGC	UPL probe # 79
Tbp	Fwd: GGGGAGCTGTGATGTGAAGT Rev: CCAGGAAATAATTCTGGCTCA	UPL probe # 97



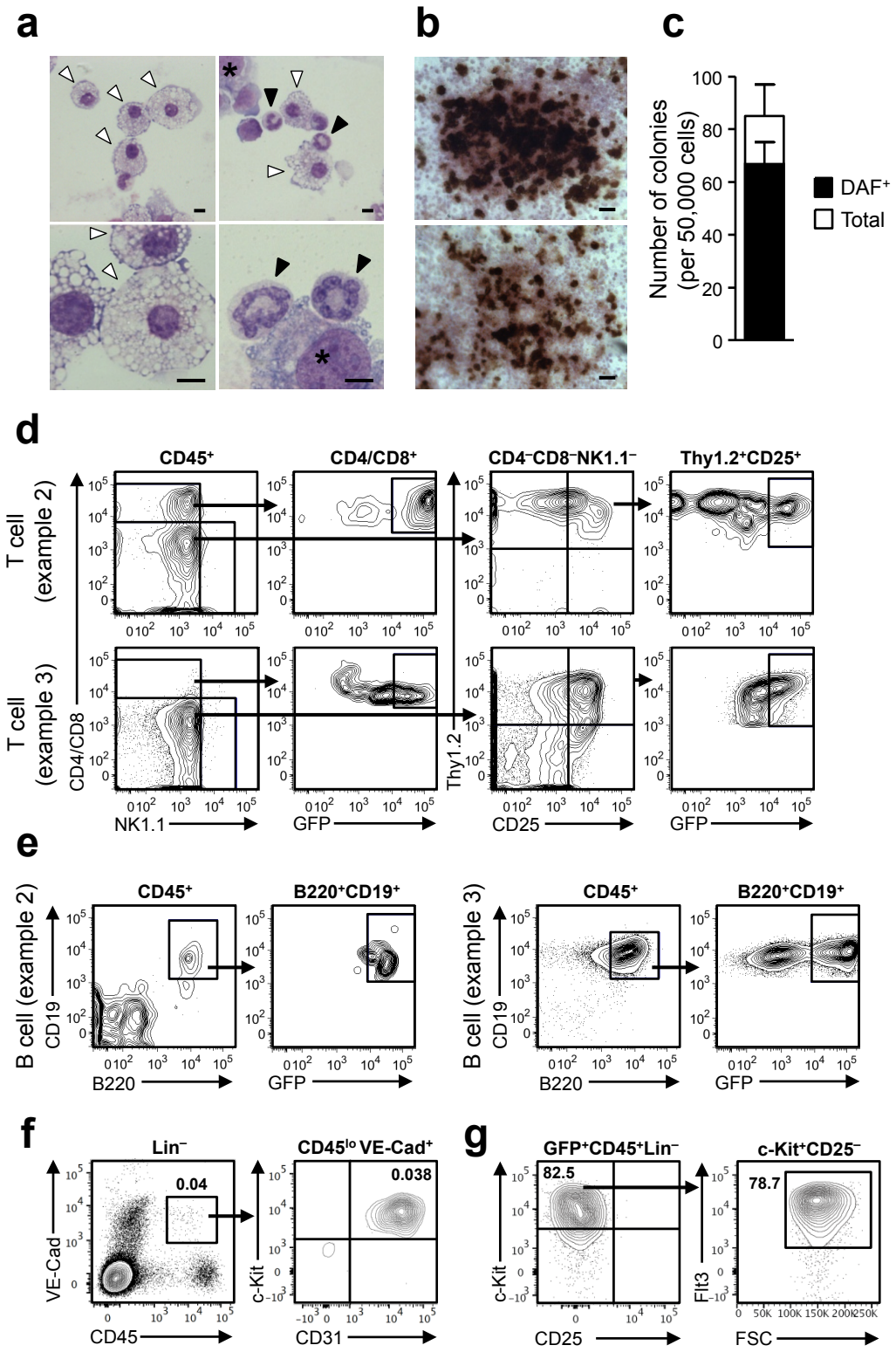
Supplementary Figure 1



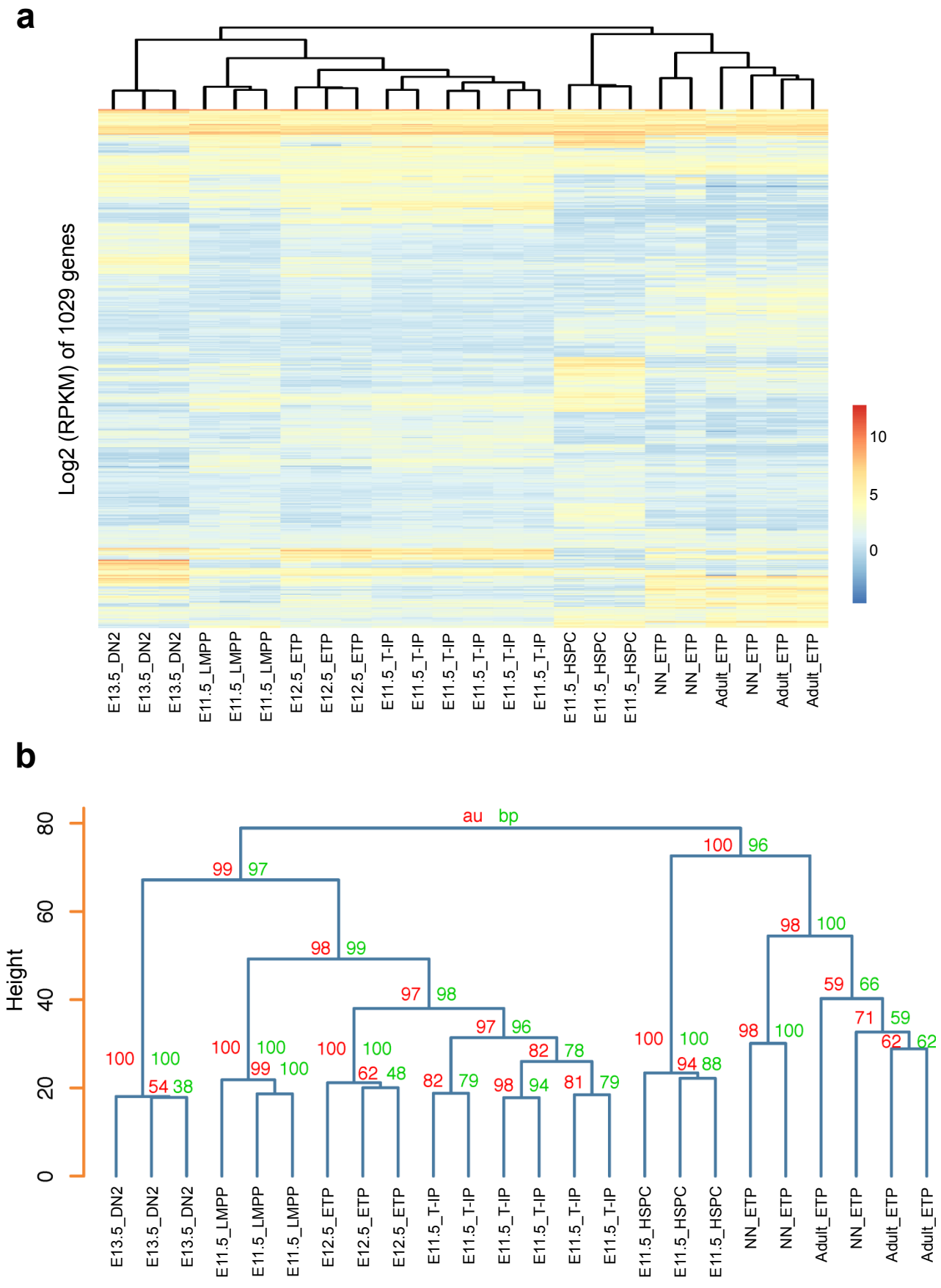
Supplementary Figure 2



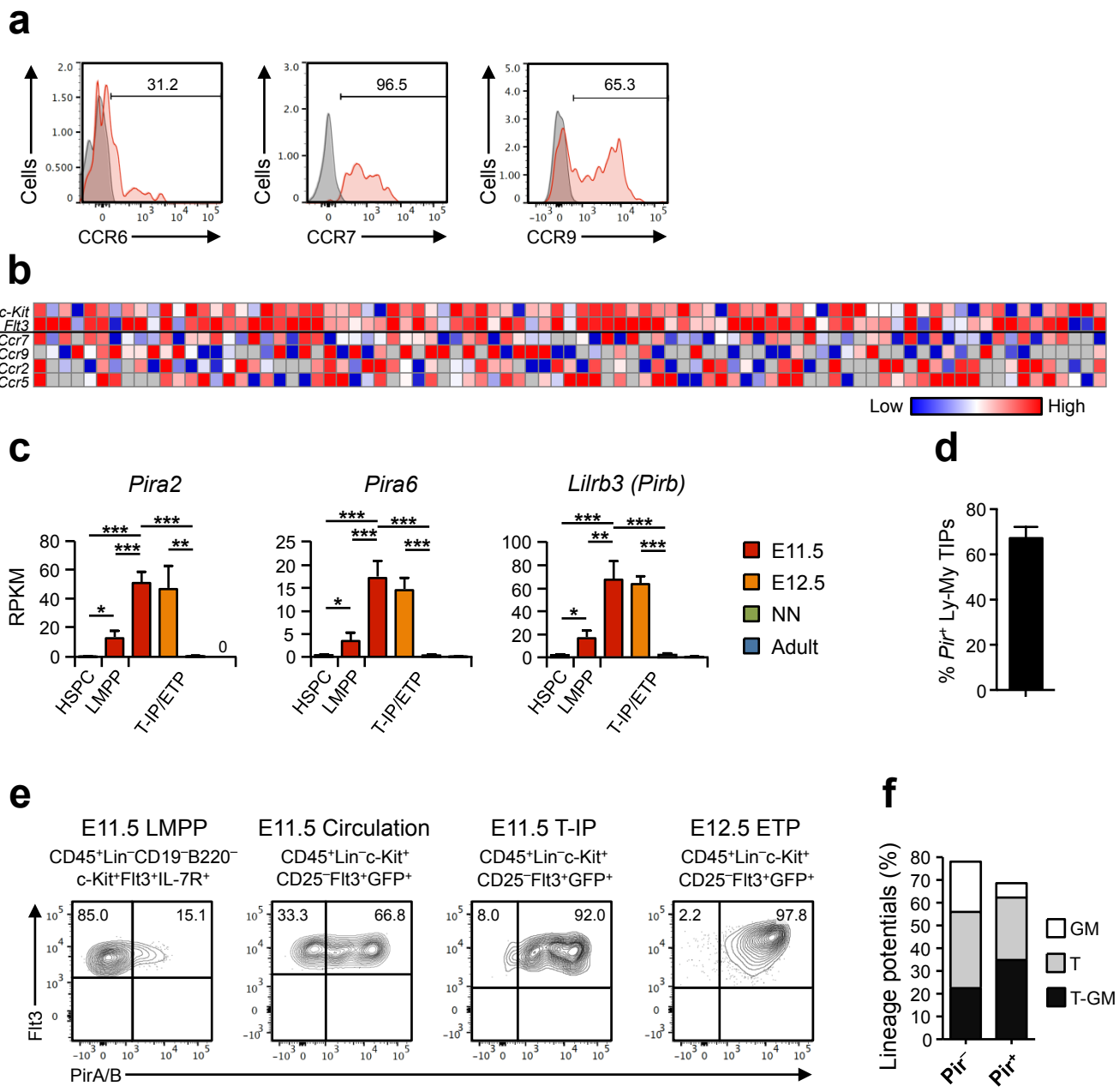
Supplementary Figure 3



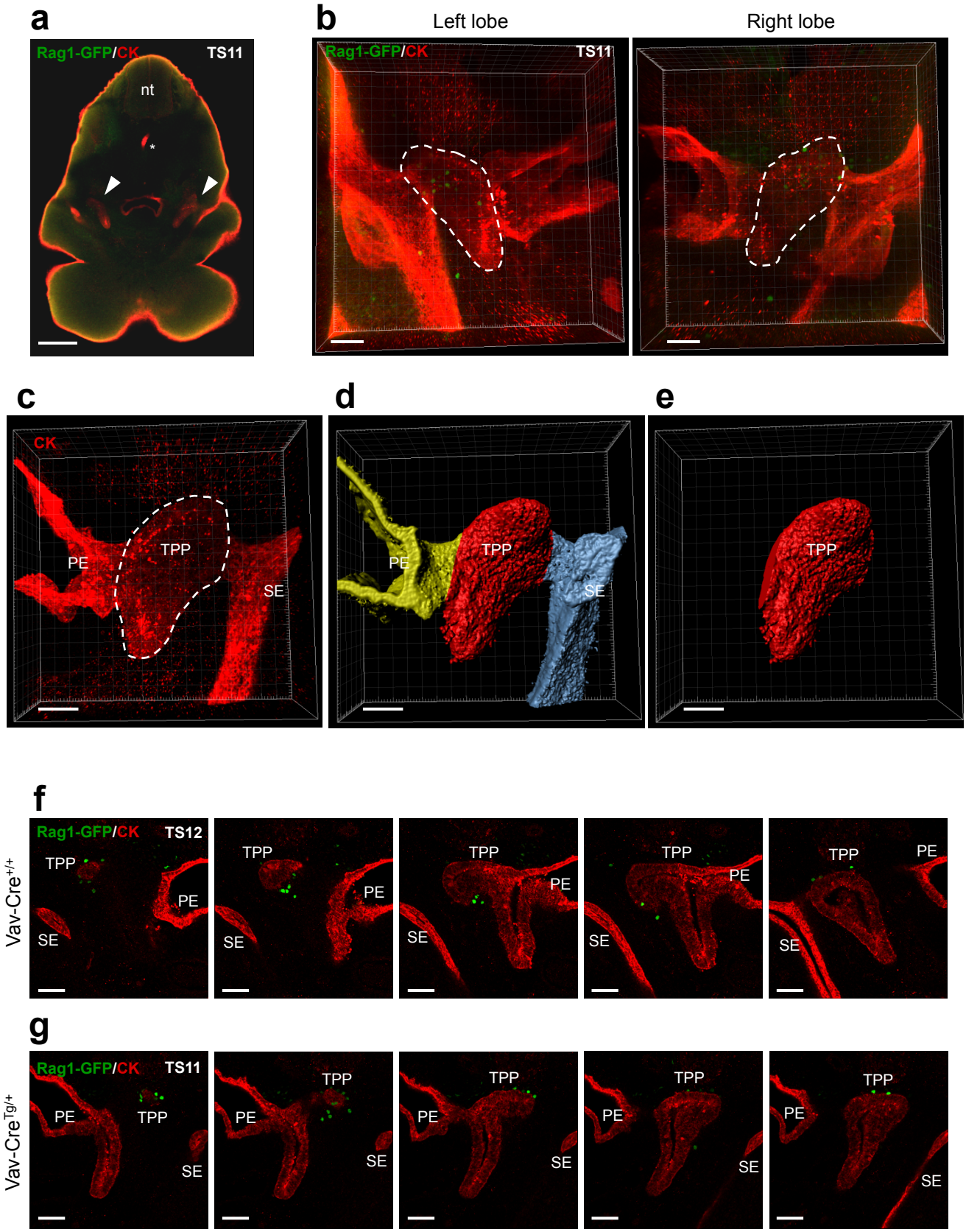
Supplementary Figure 4



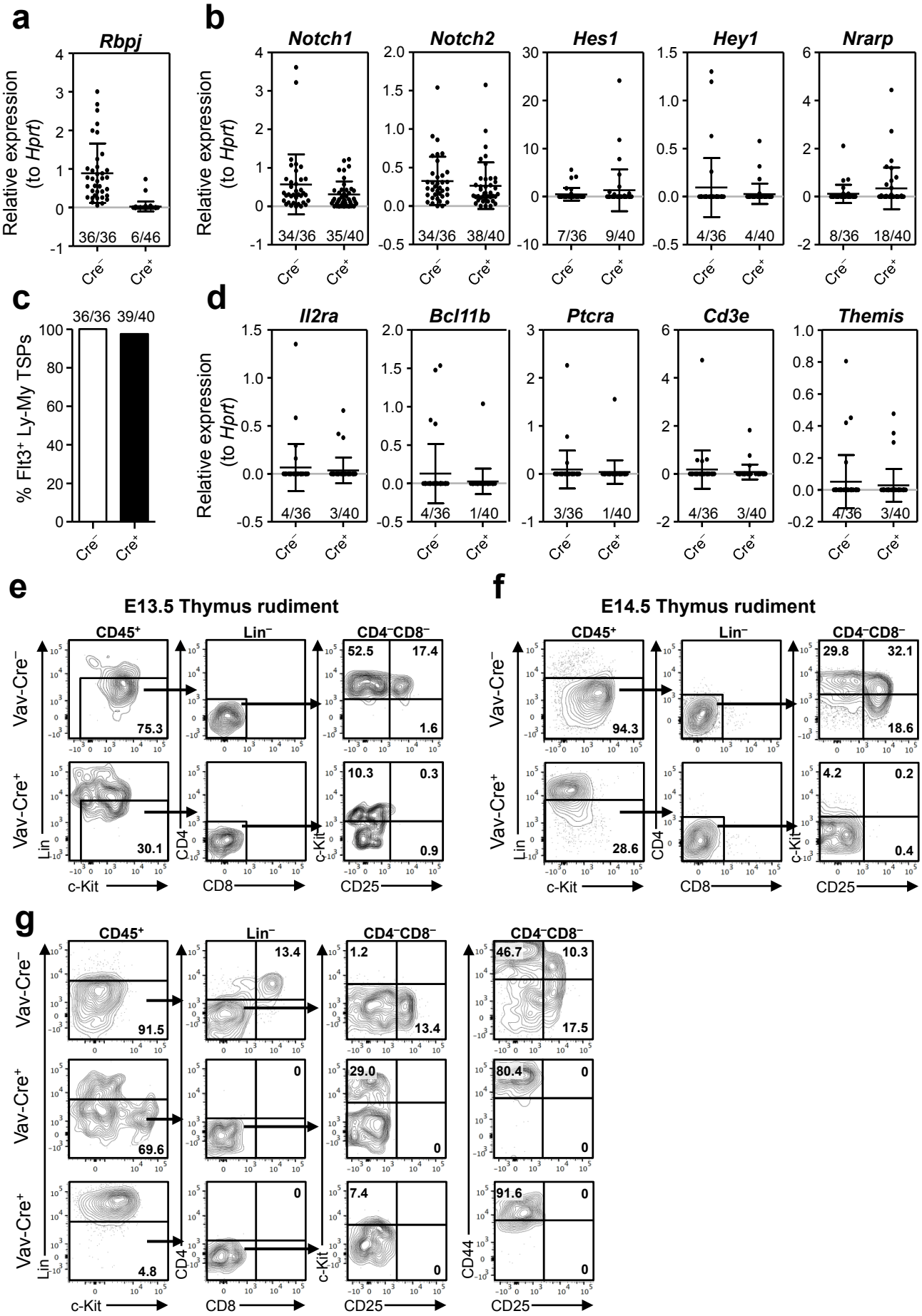
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure 8