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The expression of Sox17 identifies and regulates haemogenic endothelium

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Although it is well recognized that haematopoietic stem cells (HSCs) develop from a specialized population of endothelial cells known as haemogenic endothelium, the regulatory pathways that control this transition are not well defined. Here we identify Sox17 as a key regulator of haemogenic endothelial development. Analysis of *Sox17*–GFP reporter mice revealed that Sox17 is expressed in haemogenic endothelium and emerging HSCs and that it is required for HSC development. Using the mouse embryonic stem cell differentiation model, we show that Sox17 is also expressed in haemogenic endothelium generated *in vitro* and that it plays a pivotal role in the development and/or expansion of haemogenic endothelium through the Notch signalling pathway. Taken together, these findings position Sox17 as a key regulator of haemogenic endothelial and haematopoietic development.

The embryonic haematopoietic system consists of two distinct programs that differ in their potential and temporal patterns of development. Primitive haematopoiesis emerges in the yolk sac and exhibits restricted potential, generating primitive erythroblasts, macrophages, megakaryocytes but no lymphoid lineage cells or HSCs (refs 1,2). HSCs are generated during definitive haematopoiesis derived from haemogenic endothelium that is specified at different sites, the best characterized being the region comprising the developing aorta, gonads and mesonephros[3–5] (AGM). Histological analyses and imaging studies have shown that the haematopoietic cells bud from the haemogenic endothelium through a process known as the endothelial to haematopoietic transition (EHT) and subsequently form distinct clusters within the lumen of the aorta[6–8]. Numerous studies have identified key regulators of this process including HoxA3 that plays a role in the development of haemogenic endothelium and Notch1 and Runx1 that function during the EHT (refs 9–11). The SoxF family of transcription factors, Sox7, Sox17 and Sox18, have also been shown to play a role in embryonic haematopoiesis[12–14]. Sox17 is of particular interest in this context as it is expressed in the arterial vasculature[15,16], and required for the generation and maintenance of HSCs in the mouse fetal liver[17], and its expression marks progenitors that contribute to haemogenic endothelial and adult definitive haematopoiesis[18].

We previously reported that expression of Sox17 together with Flk-1 identifies a definitive haematopoietic progenitor in mouse embryonic stem cell (mESC) differentiation cultures and distinguishes it from an earlier developing Flk-1$^+$Sox17$^-$ population that represents the primitive haematopoietic program[19]. To further characterize this Sox17$^+$ population with respect to haemogenic endothelium, we used a reporter mESC line carrying a targeted *Sox17*–mCherry fusion complementary DNA (Sox17–mC; ref. 20) to track its development. When differentiated in defined culture conditions (Fig. 1a) the Sox17–mC mESC line generates a Flk-1$^+$Sox17–mC$^-$ population within 3.0 to 3.25 days (D) of culture (Fig. 1b). Although these early progenitors do not express Sox17 as they emerge, they do give rise to Sox17$^+$ cells that co-express the endothelial markers Flk-1, VE–cadherin (VEC) and CD31 following a further 24 h of culture (Supplementary Fig. S1a). Whether this Sox17$^+$ population represents the developing primitive erythroid lineage, definitive haematopoietic progenitors contaminating the D3.25 Flk-1$^+$ population[19] or emerging endothelial progenitors remains to be determined.

To examine the role of Sox17 in the generation of the definitive haematopoietic program, D3.25 embryoid bodies were dissociated and reaggregated for two days (D5.25) to induce a second population of Flk-1$^+$ cells that does express Sox17–mC. Fluorescence-activated cell sorting (FACS) analysis revealed that the D5.25 Sox17–mC$^+$ cells also expressed VEC, CD31 and AA4.1 (CD93). Most D5.25 Sox17–mC$^+$ cells did not express CD41 nor CXCR4 and none expressed Sca-1 (*Ly6a*; Fig. 1b). Real-time PCR with reverse transcription (qRT–PCR) analysis confirmed *Sox17* expression at D5.25 of differentiation (Supplementary Fig. S1b).

To determine whether the Flk-1$^+$Sox17–mC$^+$ cells exhibit haemogenic endothelial potential the F$^+$S$^+$, F$^+$S$^-$ and F$^-$S$^-$ populations were isolated from D5.25 embryoid bodies (Fig. 1c and Supplementary Fig. S1b) and plated as a monolayer with cytokines

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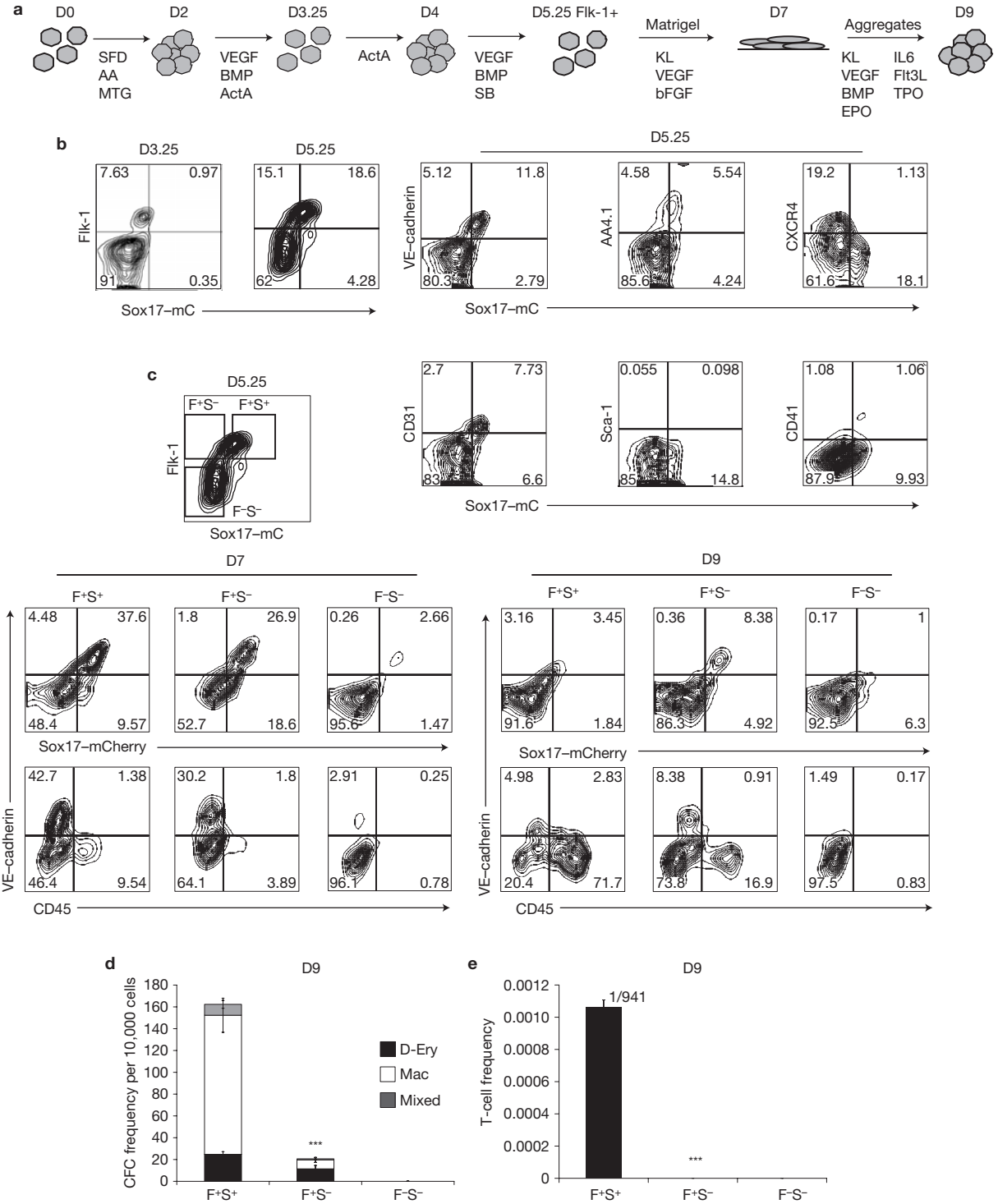


Figure 1 Expression of Sox17 identifies the emergence of haemogenic endothelial and haematopoietic progenitors *in vitro*. (**a**) Schematic representation of the differentiation protocol for mESCs. (**b**) Left: representative experiment depicting the expression of Flk-1 and Sox17–mCherry (Sox17–mC) in day (D) 3.25 and 5.25 embryoid bodies by flow cytometry. Right: analysis of D5.25 embryoid bodies for co-expression of endothelial (CD31, AA4.1, CXCR4), haemogenic endothelial (VEC, Sca-1) and haematopoietic (CD41) markers with Sox17–mC. (**c**) D5.25 embryoid bodies were dissociated and the following fractions isolated: F^+S^+, F^+S^- and F^-S^-. The cells were cultured for 2 or 4 days as indicated in **a** and analysed for the expression of Sox17–mC, VEC and CD45. Flow cytometry plots of D7 and D9 cells showing that $CD45^+$ cells emerge only from fractions that acquired expression of Sox17–mC. (**d**) Haematopoietic progenitor potential of D5.25 sorted fractions depicted in **c** cultured for 4 days. D9 aggregates were dissociated and the cells plated in methylcellulose cultures that were scored for definitive erythroid (D-Ery), macrophage and monocyte (Mac) or granulocyte, erythrocyte, macrophage and megakaryocyte (Mixed) colonies after 8 days in culture. Bars represent standard deviation of the mean of 3 independent experiments; $^{***}P = 0.003$. (**e**) T-lymphoid potential of the D5.25 sorted fractions cultured for 4 days. D9 aggregates were dissociated and the cells plated on irradiated OP9-DL1 stromal cells in limiting dilution for 21 days. Positive wells were those that contained more than 500 cells of which more than 5% expressed the T-cell signature $CD45^+TCR\beta^+CD4^+/CD8^+$. Bars represent standard deviation of the mean of 3 independent experiments; $^{***}P = 0.0001$.

known to promote the growth of endothelial cells. Molecular analyses showed that F^+S^+ cells expressed the highest levels of *Sox17*, verifying the fidelity of the mCherry reporter (Supplementary Fig. S1b). Following two days of culture a significant proportion of the population derived from the F^+S^+ fraction downregulated expression of both VEC and Sox17–mC and upregulated the expression of CD45, suggesting that these cells initiated the EHT (Fig. 1c). The F^+S^- fraction gave rise to a population with a similar profile, including cells that co-express VEC and Sox17–mC, suggesting that the F^+S^- fraction contains progenitors of the F^+S^+ population. The F^-S^- fraction showed little capacity to generate haemogenic endothelium.

To promote haematopoietic development, the monolayers were dissociated and cultured as aggregates in haematopoietic cytokines for two days (Fig. 1a). During this time, the F^+S^+ and F^+S^--derived cells downregulated Sox17–mC expression and generated $CD45^+VEC^-$ cells. The F^-S^- fraction did not give rise to any $CD45^+$ cells, indicating that haematopoietic potential was restricted to populations that expressed or acquired expression of F^+Sox17–mC (Fig. 1c). The population derived from the F^+S^+ fraction contained the highest frequency of myeloid and multipotent progenitors and was the only one that generated T cells (Fig. 1d,e). qRT–PCR analyses revealed that cells generated from the F^+S^+ and F^+S^- fractions also expressed the highest levels of *Aml1c* (Supplementary Fig. S1c). Taken together, these findings demonstrate that Sox17 expression marks the emergence of haemogenic endothelium in mESC differentiation cultures.

To investigate the role of Sox17 in the generation of definitive haematopoiesis *in vivo*, we analysed the AGM region of embryonic day (E)11.5 $Sox17^{GFP/+}$ embryos[17] for expression of Sox17–GFP. As shown in Fig. 2a, a distinct Sox17–GFP^+ population was detected at this stage and a subset of these cells co-expressed the haemogenic endothelial markers Flk-1, VEC, CD31, CD34 and Tie2. Only a small proportion of Sox17–GFP^+ cells expressed Sca-1, CD41 and CD11b. Intriguingly, a subset of the Sox17–GFP population was $CD45^+$, suggesting that Sox17 expression marks the emerging HSCs[21]. Analyses of the yolk sac of E7.5 embryos identified a Sox17–GFP^+ population that co-expressed Flk-1 and CD31 (Supplementary Fig. S2b,c). As with the early mESC-derived population, it is unclear whether these cells represent the developing primitive erythroid lineage, the yolk sac haemogenic endothelial population recently described[22] or vascular progenitors.

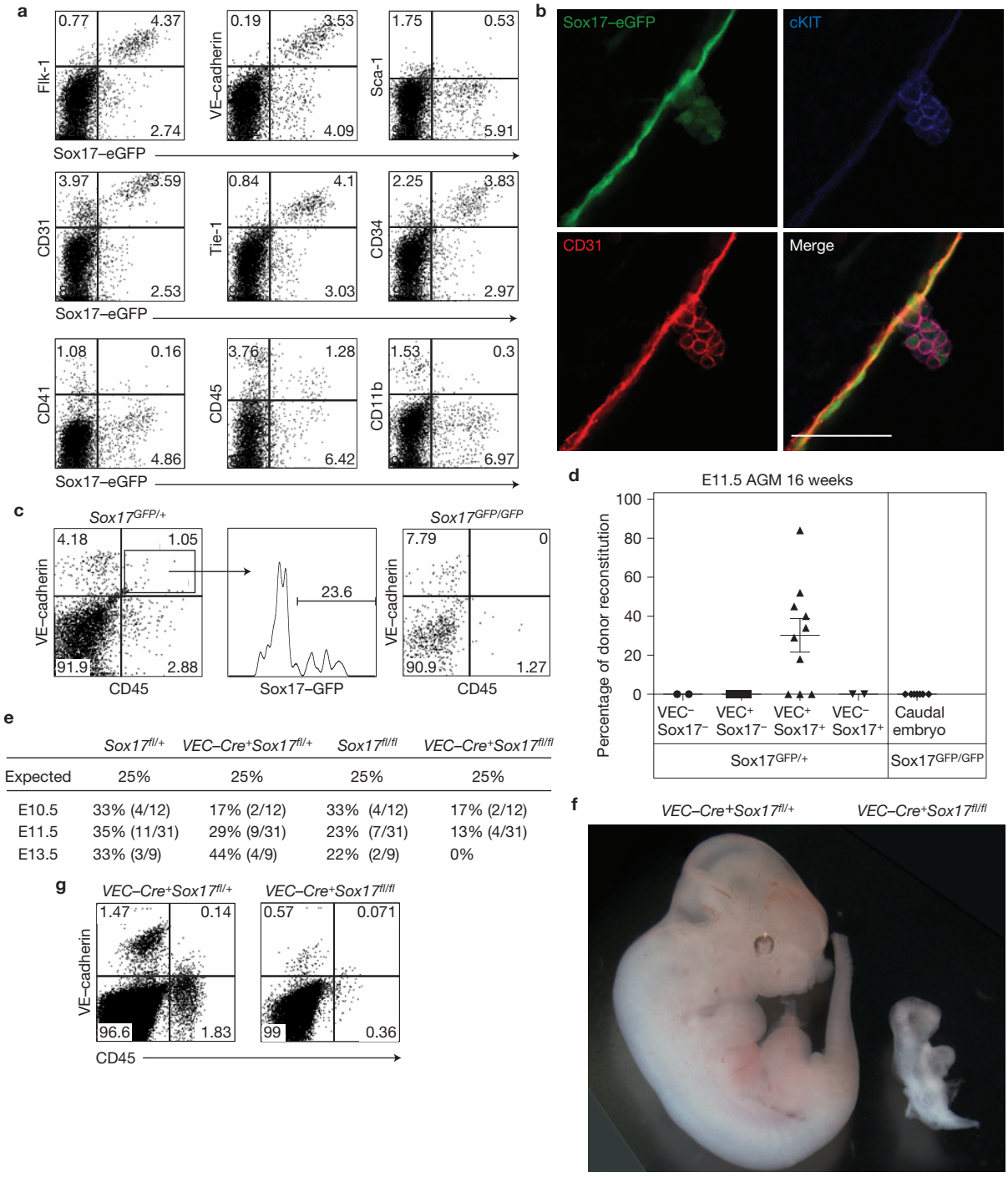
Analyses of E10.5 embryos by whole-mount immunostaining revealed that Sox17–GFP was present in the endothelial cells lining the arteries but not the veins (Supplementary Fig. S2a) as well as in all cells of the emerging haematopoietic clusters found in the dorsal aorta (Fig. 2b). These observations clearly show that Sox17 is expressed in the newly forming haematopoietic cells and the haemogenic endothelium from which they differentiate. To formally demonstrate that Sox17–GFP marks emerging HSCs the VEC^+Sox17–GFP^+, VEC^+Sox17–GFP^-, VEC^-Sox17–GFP^+ and VEC^-Sox17–GFP^- fractions were isolated by FACS from E11.5 $Sox17^{GFP/+}$ embryos ($CD45.2^+$) and the cells were transplanted into $CD45.1^+$ recipients (Fig. 2d). Seven out of ten recipients transplanted with VEC^+GFP^+ cells showed significant levels of donor-derived multilineage haematopoietic reconstitution (Supplementary Fig. S2d). In contrast, no donor-derived haematopoietic cells were detected in the recipient mice transplanted with VEC^+GFP^-, VEC^-GFP^+ or VEC^-GFP^- cells, indicating that all HSCs at this stage are Sox17–GFP^+.

Flow cytometric analyses of the AGM region of E11.5 $Sox17^{GFP/+}$ embryos revealed that 23% of the VEC^+CD45^+ population, previously shown to contain all HSC activity[21], expressed Sox17–GFP. These observations are consistent with the above transplantation studies and suggest that expression of Sox17 marks the HSCs within the VEC^+CD45^+ population (Fig. 2c). In contrast to the heterozygous embryos, the AGM region of $Sox17^{GFP/GFP}$ (null) embryos had no detectable VEC^+CD45^+ cells, suggesting that they are deficient in HSCs. Transplantation studies confirmed the deficiency in HSC development as none of the eight recipient mice transplanted with $Sox17^{GFP/GFP}$ cells showed donor cell reconstitution (Fig. 2d). These findings suggest that *Sox17* is required for the generation of HSCs. Alternatively, the lack of HSCs may be a secondary effect of the posterior patterning defects resulting from the deletion of Sox17 in all lineages.

To determine whether endothelial expression of Sox17 is required for establishment of definitive haematopoiesis, we next conditionally deleted *Sox17* in VEC^+ cells by crossing $Sox17^{fl/fl}$ and *VEC–Cre* mice[11,17]. *VEC–*Cre^+ $Sox17^{fl/fl}$ embryos were observed at expected numbers at E11.5. However, by E13.5 no live *Sox17*-deleted embryos were detected (Fig. 2e and Supplementary Fig. S2e). As observed in embryos in which *Sox17* was deleted in $Tie2^+$ endothelial cells[17], the E11.5 *VEC–*Cre^+ $Sox17^{fl/fl}$ embryos showed pronounced growth retardation when compared with their heterozygous littermates (Fig. 2f). FACS analysis revealed that the AGM region of the *VEC–*Cre^+ $Sox17^{fl/fl}$ embryos has reduced VEC^+CD45^-, VEC^+CD45^+, VEC^-CD45^+ and Sca-1^+CD45^+ populations, indicating a defect in haemogenic endothelial and HSC development (Fig. 2g and Supplementary Fig. S2f). Collectively, the findings from these *in vivo* studies demonstrate that expression of Sox17–GFP marks the developing haemogenic endothelium and HSCs in the AGM and that it is required for HSC development at this site.

To further investigate the role of Sox17 in the establishment of haematopoiesis, we analysed the effects of deleting its expression *in vitro* using a $Sox17^{-/-}$ mESC line (Supplementary Fig. S3a). Although cell proliferation seemed to be lower in the $Sox17^{-/-}$ cultures when compared with the Sox17–mC controls (Supplementary Fig. S3b), $Sox17^{-/-}$Flk-1^+ populations were detected at D3.25 and D5.25 (Fig. 3a). The $Sox17^{-/-}$ D3.25 Flk-1^+ population exhibited normal primitive erythroid and myeloid potential, indicating that Sox17 is not required for establishment of the primitive haematopoietic program (Fig. 3b). Analyses of the $Sox17^{-/-}$ D5.25 Flk-1^+ cells showed that they retained the capacity to generate adhesive populations that had haemogenic endothelial cell surface marker profiles similar to those derived from the Sox17–mC control cells (Fig. 3c, left). Both wild-type and Sox17-null D5.25 Flk-1^+ cells also gave rise to $CD31^+CXCR4^-$ and $CD31^+CXCR4^+$ populations thought to represent venous and arterial endothelium, respectively[23]. qRT–PCR analysis showed that the D7 $Sox17^{-/-}$ cells expressed higher levels of *Sox7* than the controls, suggesting that its upregulation may compensate for the loss of Sox17. Expression levels of *Sox18*, as well as of genes associated with arterial (*EphrinB2*), venous (*COUP-TFII*) and haemogenic endothelial (*HoxA3, Aml1c*) development, and notch signalling (*Notch1, Jagged1*) were unaffected by the loss of *Sox17* (Fig. 3d, left).

We next sorted the D7 VEC^+ fraction and cultured the cells as a monolayer for 48 h to determine whether the $Sox17^{-/-}$ cells could undergo the EHT. Both the $Sox17^{-/-}$ and Sox17–mC control



	$Sox17^{fl/+}$	$VEC\text{–}Cre^{+}Sox17^{fl/+}$	$Sox17^{fl/fl}$	$VEC\text{–}Cre^{+}Sox17^{fl/fl}$
Expected	25%	25%	25%	25%
E10.5	33% (4/12)	17% (2/12)	33% (4/12)	17% (2/12)
E11.5	35% (11/31)	29% (9/31)	23% (7/31)	13% (4/31)
E13.5	33% (3/9)	44% (4/9)	22% (2/9)	0%

Figure 2 Sox17 expression marks haemogenic endothelium *in vivo* and is required for the generation of long-term repopulating HSCs. (**a**) Representative flow cytometric profiles of AGM regions isolated from E11.5 $Sox17^{GFP/+}$ embryos stained with endothelial (Flk-1, CD31, Tie2), haemogenic endothelial (VEC, CD34, Sca-1) and haematopoietic (CD41, CD45, CD11b) markers. (**b**) Whole-mount immunofluorescence micrographs of an intra-arterial haematopoietic cluster from the dorsal aorta of a 36–39 somite pair $Sox17^{GFP/+}$ embryo (×20) following staining with antibodies against GFP (Sox17), cKIT and CD31. Scale bar, 50 μm. (**c**) Representative flow cytometric profiles of AGM regions isolated from E11.5 $Sox17^{GFP/+}$ heterozygote and $Sox17^{GFP/GFP}$-null embryos demonstrating the absence of the $VEC^{+}CD45^{+}$ population that contains HSCs. (**d**) Proportion of donor-derived haematopoietic cells detected in the peripheral blood of recipients 4 months following transplantation of the indicated populations. Three embryo equivalents were used for transplantation. Each dot represents an individual transplant recipient. Error bars represent s.e.m. (**e**) Progeny derived from mating $VEC\text{–}Cre^{+}\,Sox17^{fl/+}$ males with $Sox17^{fl/fl}$ females: conditional deletion of Sox17 using VEC–Cre was lethal by E13.5. (**f**) Compared with control embryos, the $VEC\text{–}Cre^{+}\,Sox17^{fl/fl}$ embryos were growth retarded, pale and lacked visible haematopoiesis. (**g**) Representative flow cytometric profiles of AGM regions isolated from $VEC\text{–}Cre^{+}\,Sox17^{fl/+}$ and $VEC\text{–}Cre^{+}\,Sox17^{fl/fl}$ embryos depicting the total decrease in VEC and CD45 staining and the absence of the $VEC^{+}CD45^{+}$ population that contains HSCs.

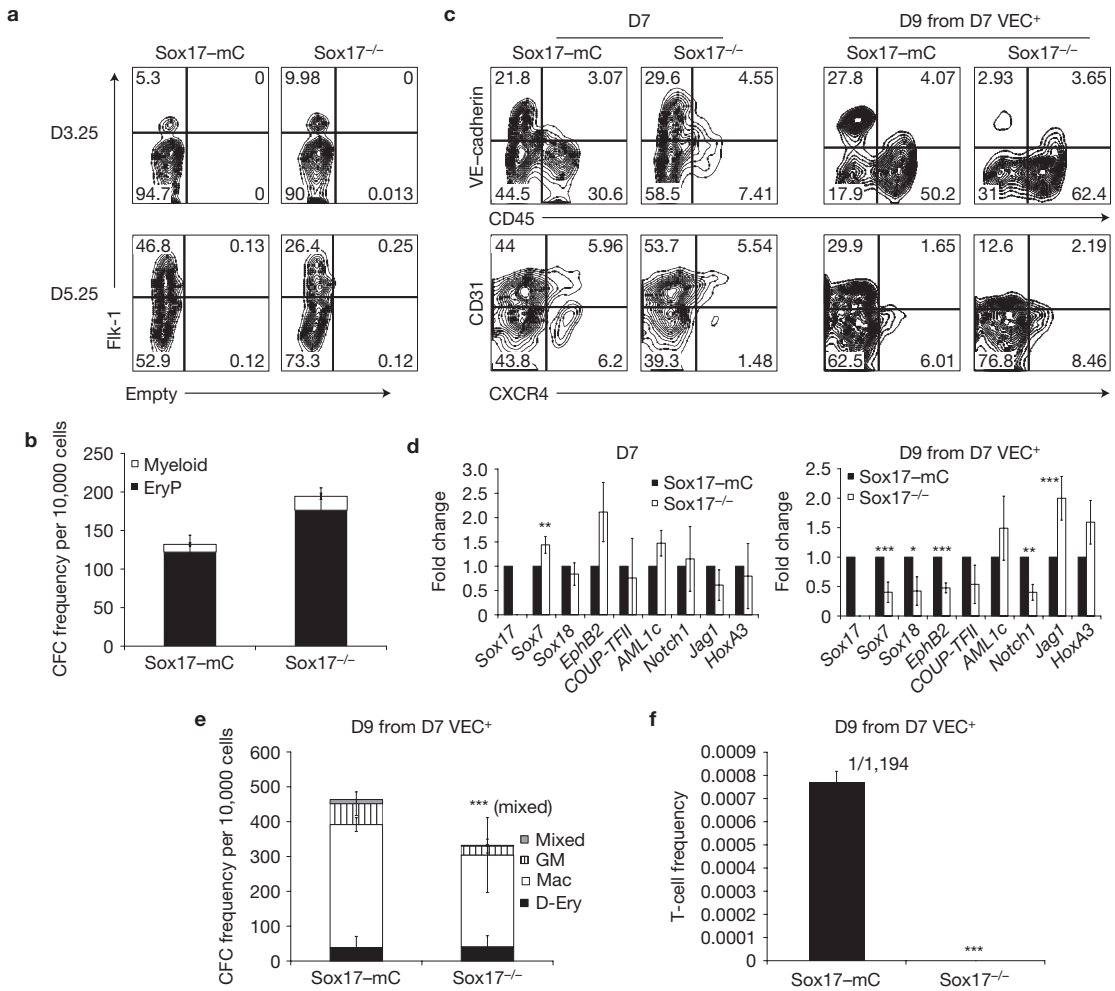


Figure 3 Expression of Sox17 is required for the EHT and definitive haematopoiesis in mESC differentiation cultures. (**a**) Flow cytometric analyses showing the proportion of Flk-1$^+$ cells in D3.23 and D5.25 embryoid bodies generated from Sox17–mC and Sox17$^{-/-}$ ESCs. (**b**) Primitive erythroid (EryP) and myeloid (Myeloid) progenitor potential of D3.25 Flk-1$^+$ cells that were aggregated for 24 h. There is no significant change in primitive haematopoietic output with the loss of Sox17 expression. Bars represent standard deviation of the mean of 3 independent experiments. (**c**) Flow cytometric analyses showing the proportion of VEC$^+$, CD45$^+$, CD31$^+$ and CXCR4$^+$ cells in D7 from Sox17–mC and Sox17$^{-/-}$ embryoid bodies. To generate D9 cultures the D7 VEC$^+$CD45$^-$ population was isolated by FACS and reaggregated as depicted in Fig. 1a. (**d**) qRT–PCR-based analyses showing expression of the indicated genes in D7 monolayers and D9 aggregates generated from SOX17–mC and Sox17$^{-/-}$ embryoid bodies. Values shown are relative to *Act*β. For comparison of the 2 populations, the values for the expression levels in the Sox17–mC cells are set to 1. Bars represent standard deviation of the mean of 3 independent experiments. D7: *Sox7* $^{**}P = 0.01$. D9: *Sox7* $^{***}P = 0.003$, *Sox18* $^{*}P = 0.014$, *EphrinB2* $^{***}P = 0.0004$, *Notch1* $^{**}P = 0.01$, *Jagged1* $^{***}P = 0.003$. (**e**) Haematopoietic progenitor potential of the Sox17–mC- and Sox17$^{-/-}$-derived D9 aggregates. D9 cells were dissociated and plated in methylcellulose cultures, and scored for definitive erythroid (D-Ery), macrophage and monocyte (Mac), granulocyte and macrophage (GM) or granulocyte, erythrocyte, macrophage and megekaryocyte (Mixed) colonies after 8 days in culture. Bars represent standard deviation of the mean of 3 independent experiments. For mixed colonies $^{***}P = 0.001$. (**f**) T-lymphoid progenitors measured on OP9-DL1 stromal cells. Bars represent standard deviation of the mean of 3 independent experiments. $^{***}P = 0.0001$.

VEC$^+$ cells generated large haematopoietic CD45$^+$ populations, demonstrating their capacity to transition to a haematopoietic fate (Fig. 3c, right). With this transition, the size of the VEC$^+$ and CD31$^+$ populations in the Sox17$^{-/-}$ cultures decreased markedly when compared with controls, suggesting that Sox17 is required for the maintenance of the haemogenic vasculature *in vitro*. qRT–PCR analyses supported this interpretation in demonstrating that expression of genes associated with endothelial development, including *Sox7, Sox18, EphrinB2* and *Notch1* was significantly downregulated in the Sox17$^{-/-}$ population when compared with controls (Fig. 3d, right). No differences were detected in the expression of *Runx1*, *HoxA3* or *COUP-TFII*. Colony-forming cell (CFC) assays revealed that the Sox17$^{-/-}$ VEC cells were able to generate myeloid and erythroid progenitors at numbers comparable to those from the control population. In contrast, the Sox17$^{-/-}$ cells generated fewer multipotential progenitors than the control cells (Fig. 3e) and showed a complete lack of T-lymphoid potential (Fig. 3f). Collectively, these findings indicate that Sox17 is dispensable for erythroid and myeloid development from the D7 VEC$^+$ haemogenic endothelium but is required for the generation of multipotential and T-lymphoid progenitors from this population.

To gain further insight into the mechanism by which Sox17 is regulating definitive haematopoiesis we investigated the consequences of enforced expression, using a mESC line containing a doxycycline-inducible *Sox17* cDNA (ref. 24). For these studies, Sox17 was induced

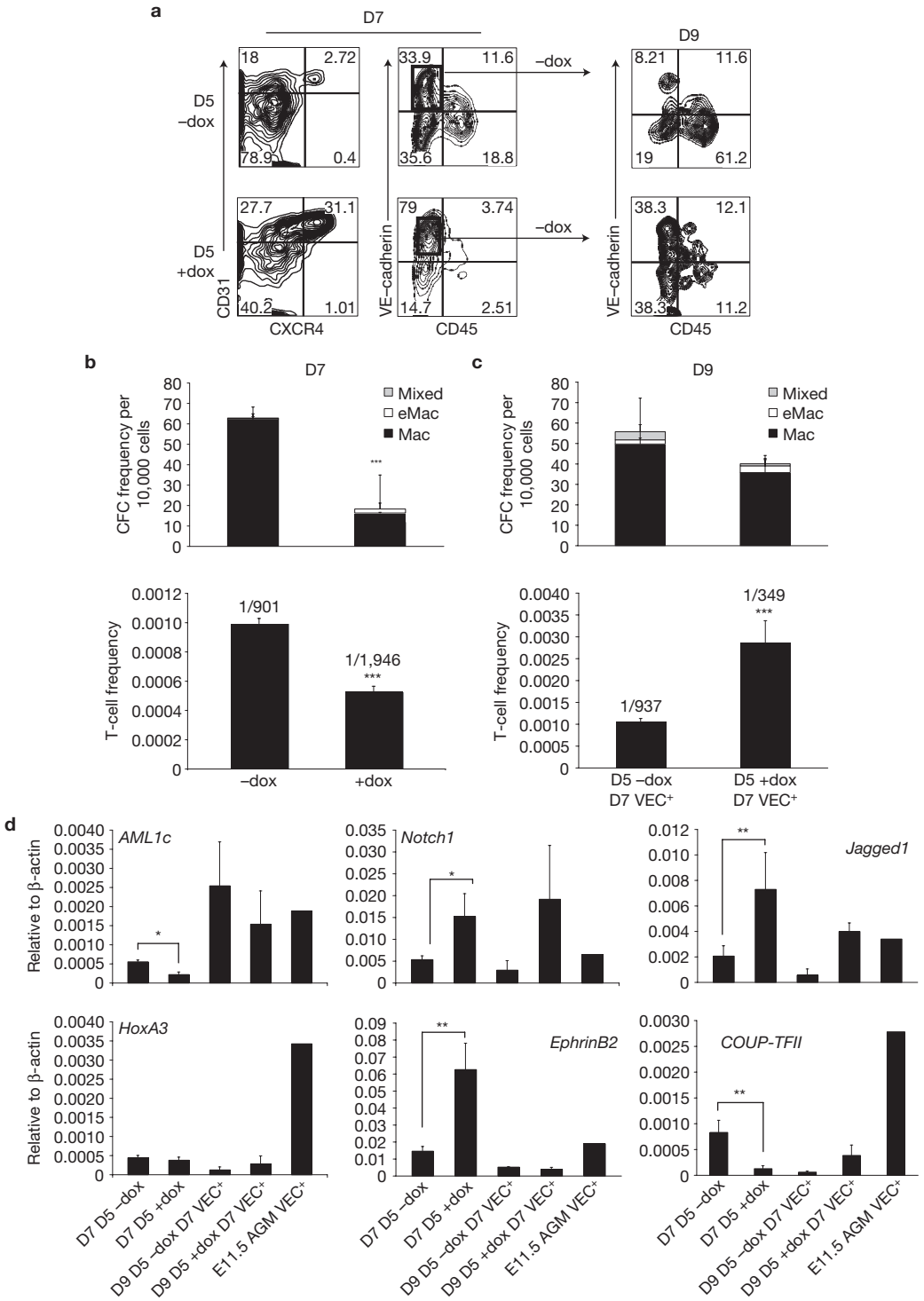


Figure 4 Effects of enforced Sox17 expression on haemogenic endothelial and haematopoietic development. (**a**) Left and middle, flow cytometric analyses showing the proportion of $CD31^+CXCR4^+$ and VEC^+CD45^+ cells in D5.25 Flk-1^+-derived monolayers cultured for 2 days in the presence (+dox) or absence (−dox) of 1 μg ml^{-1} doxycycline. Right: flow cytometric analysis showing the proportion of VEC^+ and $CD45^+$ cells in aggregates generated from the D7 induced and non-induced VEC^+CD45^- population cultured for 2 days without doxycycline in the presence of haematopoietic cytokines. (**b**) Myeloid and erythroid (top) and T-lymphoid (bottom) progenitor potential of the D7 induced and non-induced monolayers. Bars represent standard deviation of the mean of 3 independent experiments; for myeloid and erythroid $^{***}P = 0.003$, and for T lymphoid $^{***}P = 0.0006$. (**c**) Myeloid and erythroid (top) and T-lymphoid (bottom) progenitor potential of aggregates generated from VEC^+CD45^- cells isolated by FACS from the D7 induced and non-induced monolayer populations. Cells were cultures as aggregates for 2 days (D9) in the absence of doxycycline before analyses. Bars represent standard deviation of the mean of 3 independent experiments. For T-lymphoid progenitors $^{***}P = 0.0004$. (**d**) qRT–PCR analyses of the indicated genes in D7 induced and non-induced monolayer and in D9 aggregates generated from them; VEC^+ cells isolated from the AGM of an E11.5 wild-type embryo are shown as a control. Values shown are relative to *Act*β. Bars represent standard deviation of the mean of 3 independent experiments; *Aml1c* $^{*}P = 0.012$, *Notch1* $^{*}P = 0.03$, *Jagged1* $^{**}P = 0.038$, *EphrinB2* $^{**}P = 0.006$, *COUP-TFII* $^{**}P = 0.006$.

in the D5.25 Flk-1$^+$ population during the 2 days of monolayer culture that promotes the expansion of haemogenic endothelium. During this culture period, untreated Flk-1$^+$ cells gave rise to an adherent haemogenic endothelial population that initiated the EHT, as demonstrated by the emergence of round haematopoietic cells (Supplementary Fig. S4a). In contrast, the haemogenic endothelial population generated from the doxycycline-induced Flk-1$^+$ contained no budding haematopoietic cells. These differences were not due to an increase in cell death as total cell numbers were equal in both groups (Supplementary Fig. S4b). The morphological changes induced by overexpression of Sox17 were mirrored by a pronounced increase in the size of the VEC$^+$ population, a significant decrease in the size of the CD45$^+$VEC$^-$ population and a reduction in the frequency of myeloid and T-cell progenitors at D7 (Fig. 4a,b). Interestingly, expression of Sox17 also led to an increase in the size of the arterial-like CD31$^+$CXCR4$^+$ population. These observations suggest that enforced expression of *Sox17* results in an increase in the size of the haemogenic endothelial population and an impairment in its ability to undergo the EHT.

To determine whether the Sox17-induced haemogenic endothelial population can generate haematopoietic cells, VEC$^+$CD45$^-$ cells were isolated from the induced and non-induced D7 populations and cultured in the absence of doxycycline (Fig. 4a, right panel). As expected, the non-induced VEC$^+$ cells generated a substantial VEC$^-$CD45$^+$ population by D9. The VEC$^+$ cells from the D7 induced monolayer also produced CD45$^+$ haematopoietic cells, although the size of the population was considerably smaller than that generated by the non-induced cells. The frequency of myeloid and multipotent progenitors in the non-induced and induced D9 populations was comparable, consistent with the observation that both contained CD45$^+$ cells (Fig. 4b). In contrast, the induced population showed a threefold higher frequency of T-lymphoid progenitors (Fig. 4c), indicating that it exhibits enhanced definitive haematopoietic potential. Molecular analyses revealed that expression levels of *EphrinB2*, *Notch1* and *Jagged1* were significantly upregulated whereas that of *COUP-TFII* was reduced in the induced population at D7 of culture (Fig. 4d). Expression of *Aml1c* was lower in the induced compared to non-induced population at D7. By D9, the levels had increased in both populations. Enforced expression of Sox17 had no effect on the expression of *HoxA3*. Collectively, these findings demonstrate that enforced expression of Sox17 leads to an increase in the size of the haemogenic endothelial population able to generate T lymphocytes and suggest that this effect may be mediated through the Notch pathway.

To investigate the role of Notch signalling in the Sox17-induced expansion of haemogenic endothelium, induced and non-induced D5 Flk-1$^+$ cells were cultured (48 h) in the presence and absence of the pathway inhibitor γ-secretase inhibitor (γSI). The addition of γSI reduced total cell numbers as well as the size of the CD31$^+$CXCR4$^+$ arterial-like endothelial population generated from both the Sox17-induced and non-induced populations (Fig. 5a and Supplementary Fig. S5a). Manipulation of the Notch pathway did not, however, impact the size of the induced VEC$^+$ population. The addition of γSI did prevent the Sox17-induced changes in *EphrinB2* and *COUP-TFII* expression (Fig. 5b). D7 induced and non-induced VEC$^+$CD45$^-$ cells from γSI-treated or control cultures were next isolated, cultured as aggregates for 2 days and the resulting populations assayed for the presence of CD45$^+$ cells, for *Aml1c* expression and for myeloid/erythroid and T-cell potential. The addition of γSI to the non-induced progenitors had no effect on the CD45 profile, the levels of *Aml1c* expression or the T-cell potential of the D9 population (Fig. 5c,d). In contrast, Notch inhibition in Sox17-induced cultures markedly reduced the size of the CD45$^+$ population, the expression levels of *Aml1c* and the frequency of T-cell progenitors (Fig. 5c,d and Supplementary Fig. S5b). The addition of γSI did not, however impact the generation of myeloid and erythroid progenitors (Supplementary Fig. S5c).

To determine whether Sox17 can directly activate Notch1, we analysed the *Notch1* promoter region for motifs that can be recognized by Sox17. The JASPER database identified four Sox17-binding sites in the mouse *Notch1* promoter, two (BS1 and BS2) of which are evolutionarily conserved (Fig. 5e and Supplementary Fig. S5d)[25]. To determine whether Sox17 can activate *Notch1,* U2OS cells were transfected with a Sox17 expression plasmid together with a reporter construct containing a 660-base-pair (bp) region of the *Notch1* promoter including the BS1 and BS2 sites upstream of luciferase. Sox17 induced luciferase activity in a dose-dependent fashion, demonstrating that Sox17 can activate the *Notch1* promoter (Fig. 5f). Mutations of the Sox-binding motif of BS1 and BS2 reduced luciferase activity, demonstrating that these sites are necessary for the Sox17-mediated activation of the *Notch1* promoter.

To determine whether the loss of *Sox17* also affected the levels of *Notch1* expression *in vivo*, the AGM regions from E11.5 *VEC–Cre*$^+$*Sox17*$^{fl/+}$ and *VEC–Cre*$^+$*Sox17*$^{fl/fl}$ embryos were isolated and analysed for gene expression by qRT–PCR (Supplementary Fig. S5e). The expression of *Notch1* but not other members of the Notch family (*Notch4, Jagged1)* was significantly decreased in the *VEC–Cre*$^+$*Sox17*$^{fl/fl}$ cells, suggesting that *Notch1* is also a target of Sox17 during embryogenesis.

Deciphering the regulatory pathways that control the establishment of definitive haematopoiesis is essential for modelling this program in ESC differentiation cultures and ultimately to generate HSCs *in vitro*. Our findings have provided new insights into this process and position Sox17 as a key regulator of haemogenic endothelium. Specifically, our mESC data show that Sox17 is required for the development and/or maintenance of haemogenic endothelium able to generate T cells. Interestingly, the Sox17$^{-/-}$VEC$^+$ haemogenic endothelium did retain myeloid and erythroid potential, indicating that the specification of a subset of these progenitors is Sox17 independent. These observations suggest that Sox17 specifically regulates the subpopulation of haemogenic endothelium that gives rise to the T-lymphoid lineage, a measure of the definitive haematopoiesis, and that dependence on Sox17 distinguishes it from the haemogenic endothelium that generates myeloid and erythroid progenitors. The existence of distinct definitive progenitor populations in the mESC model is consistent with *in vivo* studies that showed that the formation of erythroid–myeloid progenitors and HSCs can be uncoupled on the basis of the temporal and spatial expression of CBFβ in *Ly6a*- expressing cells[26].

Our enforced expression studies indicate that sustained levels of Sox17 expression lead to an increase in the size of the haemogenic endothelial population able to generate T lymphocytes. A recent study using human ESCs has also provided evidence that enforced expression of Sox17 results in expansion of a population with properties of haemogenic endothelium (ref. 27). However, as only

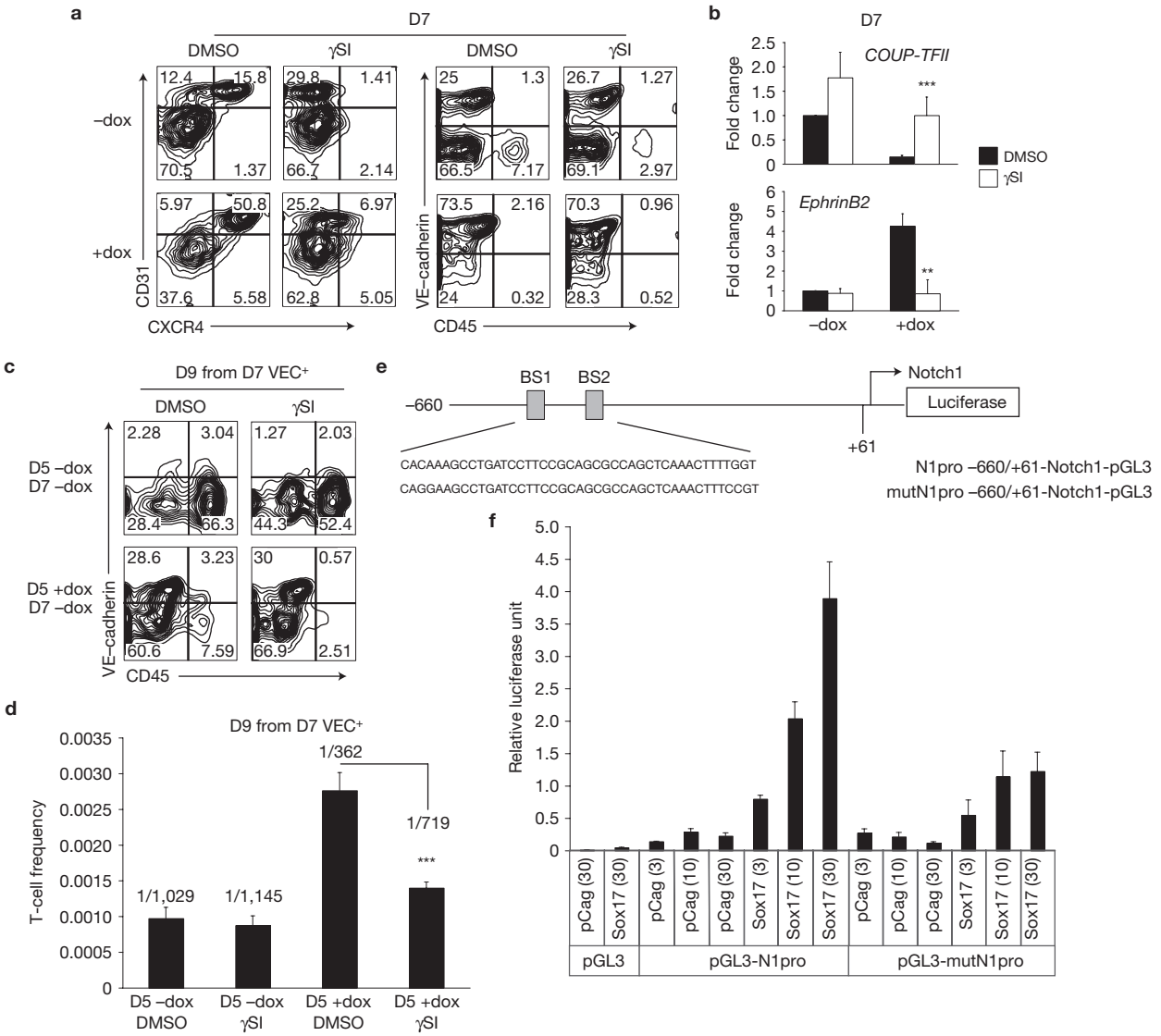


Figure 5 The effects of enforced Sox17 expression are mediated through Notch signalling. (**a**) Flow cytometric analyses of D5.25 Flk-1$^+$-derived monolayer cells cultured for 2 days (D7) with (+dox) or without (−dox) 1 μg ml^{-1} doxycycline in the presence or absence of γ-secretase inhibitor (γSI, L-685458; 10 μM, Tocris). Cells cultured in the vehicle dimethylsulphoxide (DMSO) represent the control. (**b**) qRT–PCR-based analysis of *EphrinB2* and *COUP-TFII* expression in the different D7 populations described in **a**. Values shown are relative to *Actβ*. Bars represent standard deviation of the mean of 3 independent experiments; *COUP-TFII* $^{***}P = 0.002$, *EphrinB2* $^{**}P = 0.003$. (**c**) Flow cytometric analyses showing the proportion of VEC$^+$ and CD45$^+$ in aggregates generated from VEC$^+$CD45$^-$ cells isolated by FACS from the different D7 monolayer populations indicated in **a**. The sorted cells were cultured for 2 days as aggregates in the absence of doxycycline and γ-secretase inhibitor. (**d**) T-lymphoid progenitor potential in the D9 aggregates generated from the D7 monolayer populations grown under the indicated conditions. Bars represent standard deviation of the mean of 3 independent experiments, $^{***}P = 0.0008$. (**e**) Schematic diagram depicting the wild-type and mutated version of the BS1 and BS2 Sox17-binding sites in the *Notch1* promoter. The numbers indicate the position relative to the transcriptional start site. (**f**) Luciferase assays using U2OS cells co-transfected with pGL3 control vector or the indicated Notch1 promoter constructs and increasing concentrations of the pCAG expression plasmids. Bars represent mean luciferase intensity relative to pGL3-empty ±s.d., $n = 3$, $P = 0.001$.

erythroid and myeloid potential was analysed, it is unclear whether the observed effect was on haemogenic endothelium with definitive haematopoietic potential. The demonstration that regulation of haemogenic endothelium by Sox17 may be mediated by Notch signalling is consistent with the known role of this pathway in definitive haematopoietic development[10] and positions the requirement for Notch signalling at the specification stage of haemogenic endothelium. Previous studies have identified *HoxA3* as a transcriptional regulator of haemogenic endothelium and provided evidence that it functions upstream of Sox17 (ref. 9). The observation that *HoxA3* expression was not impacted following overexpression or deletion of Sox17 in our mESC-derived populations is consistent with this interpretation.

The findings in our study support the emerging body of work demonstrating that SoxF transcription factors are regulators of different stages of embryonic haematopoiesis. At E8.0, *Sox7* and *Sox18* are detected in the developing paired dorsal aortae, whereas expression of *Sox17* emerges in the anterior regions of the dorsal aorta at E8.5–8.75 (refs 15,16). Studies using mESCs have shown that Sox7 and Sox18 play a role in the regulation of primitive haematopoiesis[12,13]. Our observation that primitive haematopoiesis was not perturbed in

Sox17$^{-/-}$ cultures suggests that the function of Sox17 differs from that of Sox7 and Sox18. The differential function of these factors is supported by studies showing that the knockdown of *Sox7* and *Sox18* expression has no impact on the development of HSCs in zebrafish[28] suggesting that Sox17 is the sole SoxF member involved in definitive haematopoiesis. □

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

R.C. and G.K. designed experiments and wrote the manuscript. G.K. supervised the project. R.C. performed all mESC and transplantation experiments. A.Y. performed confocal imaging. Y.Y. performed JASPER analysis and luciferase assays. A.B. and C.B. generated the doxycycline-inducible mESC line.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Embryo generation. Animals were maintained and bred at the Animal Resource Center in the MaRS complex according to institutional guidelines. *Sox17*$^{GFP/+}$ and *Sox17*$^{fl/+}$ mice were a gift from S. Morrison (University of Texas Southwestern, USA). *VEC–Cre*$^{+}$ mice were a gift from N. Speck (University of Pennsylvania, USA). Embryos were generated through timed matings with the day of vaginal plug designated embryonic day 0.5. Pregnant dams were killed and the developmental stage of the embryos was determined by counting the number of somite pairs. Embryos with 36–40 somite pairs were used for confocal microscopy and embryos with 41–47 somite pairs were used for transplantation studies.

Embryo cell preparation and FACS sorting. Embryonic cells for flow cytometry and transplantation were acquired as follows. The AGM regions from *Sox17*$^{GFP/+}$ embryos were dissected and dissociated in 0.2% collagenase B (Roche) for 20 min at 37 °C. Cells were washed and Fc receptors were blocked by incubating with anti-CD16/32 antibody (1:20, eBioscience, clone 93, cat. no. 14-0161) for 20′. Cells were washed, stained with the indicated antibodies for flow cytometry or stained with anti-CD144 APC (1:200. eBioscience, clone BV13, cat. no. 17-1441) and sorted by FACS. Cells were sorted on a FACSAria (BD Biosciences).

Transplant analyses. Female B6.SJL-*Ptprc*^{a} (CD45.1) recipient mice were purchased from Taconic and subjected to a split dose of 900 cGy, 4 h apart. Each recipient received 3 embryo equivalents of the designated FACS-sorted fractions and 5×10^4 carrier spleen cells through intrafemoral injection. To analyse peripheral blood engraftment we used anti-CD45.1 PercpCy5.5 (1:400, BD, clone A20, cat. no. 560580), anti-CD45.2 AF780 (1:200, eBioscience, clone 104, cat. no. 47-0454), anti-GR1 FITC, anti-CD3 AF647 (1:100, BD, clone 17A2, cat. no. 557869), anti-CD4 PacificBlue (1:100, Invitrogen, cat. no. MCD0428), anti-CD8 FITC (1:100, BD, clone 53-6.7, cat. no. 553031), anti-IgM APC (1:100, BD, clone 11/41, cat. no. 550676), anti-CD19 PE-Cy7 (1:400, BD, Clone 1D3, cat. no. 552854) and anti-CD11b PE-Cy7 (1:400, BD, clone M1/79, cat. no. 552850). Cells were analysed on an LSR II flow cytometer (BD Biosciences) and analysed with FlowJo (Tree Star).

Microscopy and imaging. Whole-mount immunohistochemistry was performed as described previously[29]. Rat anti-mouse CD31 (1:500, BD, clone MEC 13,3, cat. no. 557355) was used with goat anti-rat Alexa Flour 555 (1:1,000, Invitrogen, cat # A21434). Rabbit anti-mouse GFP (1:1,000, MBL, cat. no. 598) was used with goat anti-rabbit Alexa Flour 488 (1:1,000, Invitrogen, cat. no. A11304). Rat anti-mouse CD117 (1:250, eBioscience, clone 2B8, cat. no. 3 14-1171) was used with goat anti-rat Alexa Fluor 647 (1:1,000, Invitrogen, cat. no. A21247). Specimens were analysed with a Zeiss AxioObserver inverted microscope and images were acquired with Zeiss Zen software.

mESC maintenance and differentiation. *Sox17–mCh*erry fusion (Sox17–mC) ESCs were provided by H. Lickert, Institute of Stem Cell Research, Helmholtz Zentrum München, Germany. Sox17$^{-/-}$ were provided by S. Morrison. mESC lines were maintained in serum-free/feeder-free culture conditions (SF-ES) supplemented with 2i (refs 30,31).

mESCs were differentiated in a serum-free media protocol. mESCs were trypsinized using Trypsin-LE (Invitrogen) and cultured in serum-free differentiation (SF-D) media[31] in the absence of growth factors at a concentration of 2×10^5 ml^{-1} for 48 h to form embryoid bodies. At D2, embryoid bodies were dissociated with Trypsin-LE (Invitrogen) and reaggregated at a concentration of 2×10^5 ml^{-1} in SF-D media containing recombinant human (rh) VEGF (5 ng ml^{-1}, R&D, cat. no. 293-VE), rhBMP4 (8 ng ml^{-1}, R&D cat. no. 314-BP) and ActivinA (2 ng ml^{-1}, R&D, cat. no. 338-AC) for 30 h. At D3+6 h, embryoid bodies were dissociated and reaggregated at a concentration of 5×10^5 ml^{-1} in 24-well ultralow-attachment dishes (Corning) in SF-D containing ActA (3 ng ml^{-1}) for 24 h. At D4+6 a final volume of rhVEGF (5 ng ml^{-1}), rhBMP4 (10 ng ml^{-1}) and the TGFβ inhibitor SB431542 (6 μM, Sigma, cat. no. S-4317) was added to the cultures. At D5+6, embryoid bodies were dissociated and the Flk-1^{+} cells were isolated by FACS. Flk-1^{+} cells were plated down on Matrigel (VWR, cat. no. 354230)-coated 96-well plates at a concentration of 5×10^4 per 200 μl in SF-D containing KL (2% vol/vol)-conditioned medium, rhVEGF (5 ng ml^{-1}) and rhbFGF (10 ng ml^{-1}, R&D, cat. no. 233-FB) for 48 h to form haemogenic endothelium. At D7, monolayers were trypsinized using Trypsin-LE and VEC^{+}CD45^{-} haemogenic endothelial cells were isolated by FACS and reaggregated at a concentration of 2×10^5 per 200 μl in ultralow-attachment 96-well plates in SF-D containing KL (2% vol/vol)-conditioned medium, rhVEGF (20 ng ml^{-1}), recombinant mouse (rm) TPO (50 ng ml^{-1}, R&D, cat. no. 488-TO), rmIL6 (10 ng ml^{-1}, R&D, cat. no. 406-ML), rmFlt3L (10 ng ml^{-1}, R&D, cat. no. 427-FL) and rhBMP4 (30 ng ml^{-1}) to induce haematopoiesis.

Flow cytometry and cell sorting. Embryoid bodies generated during differentiations were dissociated using Trypsin-LE and then stained in FACS buffer (PBS+3%FCS+0.02%NaN3) with the following antibodies: anti-Flk-1 biotin (in house hybridoma), SA-APC (1:200, BD, cat. no. 554067), anti-CD41 FITC (1:100. BD, clone MWReg30, cat. no. 553848, 1B5), anti-CD144 PE (1:200, eBioscience, clone BV13, cat. no. 12-1441), anti-CD45 eF450 (1:200, eBioscience, clone 3-F11, cat. no. 48-0451), anti-Ly6A/E FITC (1:100, BD, clone E13-141.7, cat. no. 553335), anti-CD93 PercpCy5.5 (1:200, eBioscience, clone AA4.1, cat. no. 45-5892), anti-CD31 PE-Cy7 (1:200, eBioscience, clone 390, cat. no. 25-0311) and anti-CD184 APC (1:200, BD, clone 2B11, cat. no. 558644). Cells were analysed on an LSR II flow cytometer (BD Biosciences) and analysed with FlowJo (Tree Star). Cells were sorted on a FACSAria (BD Biosciences).

Haematopoietic progenitor colony assay. Cells were plated in 1% methylcellulose (wt/vol, Sigma) containing 10% (vol/vol) plasma-derived serum (PDS; Antech), 5% (vol/vol) protein-free hybridoma medium (PFHM-II; Invitrogen), transferrin (300 μg ml, Roche), glutamine (2 mM), ascorbic acid (50 μg ml^{-1}), 1-thioglycerol (4.5×10^{-4} M) and the following cytokines: KL (2% vol/vol)-conditioned medium, rmTPO (50 ng ml^{-1}, R&D), erythropoietin (2 U ml^{-1}), rhIL11 (25 ng ml^{-1}, R&D, cat. no. 218-IL), IL3 (1% (vol/vol)-conditioned medium, GM-CSF (1% (vol/vol)-conditioned medium and rmIL6 (5 ng ml^{-1}). Cultures were maintained at 37 °C, 5% CO_2 for 8 days. Mean and standard errors of 3 independent experiments were calculated.

OP9-DL1 co-culture assay for T-lymphoid cells. Irradiated (gamma irradiation 30 gray) OP9-DL1 cells were seeded 1 day before use on gelatin (0.1% vol/vol) 96-well tissue culture treated plates (TC, Falcon). mESC-derived progenitors were then plated as single cells in αMEM (Invitrogen) with 20% (vol/vol) FCS containing rmFlt3L (10 ng ml^{-1}, R&D), rmIL7 (5 ng ml^{-1}, R&D, cat. no. 407-ML) and 2% (vol/vol) KL-conditioned medium for the first week. Then media were changed every 5 to 7 days to αMEM containing 20% (vol/vol) FCS, rmFlt3L (10 ng ml^{-1}) and rmIL7 (5 ng ml^{-1}). Cultures were examined after 21 days of culture by flow cytometry. For *in vitro* limiting dilution analyses, the progenitor frequency was calculated using ELDA software (WEHI Bioinformatics).

Quantitative real-time PCR. Total RNA was prepared with the RNAqueous-Micro Kit (Ambion) and treated with RNase-free DNase (Ambion). RNA (500 ng to 1 μg) was reverse transcribed into cDNA using random hexamers and Oligo(dT) with Superscript III Reverse Transcriptase (Invitrogen). qPCR was performed on a MasterCycler EP RealPlex (Eppendorf) using QuantiFast SYBR Green PCR Kit (Qiagen). Expression levels were normalized to the housekeeping gene β-actin (*Actβ*). Primer sequences are listed in Supplementary Table S1.

Luciferase assays. Approximately 660 bp of *Notch1* sequence upstream of the transcriptional start site was cloned into the pGL3-basic vector (Promega). Mutant BS1 and BS2 sites were generated by site-directed mutangenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). U2OS cells (2×10^4) were plated in triplicate and transfected with 50 ng of the indicated promoter/reporter constructs, 10 ng pRL-TK, 3–30 ng pCag-Sox17 or vector control, using FuGENE 6 (Promega). After stimulation, firefly substrate activity was measured using britelite plus (PerkinElmer) and *Renilla* substrate activity was measured using Stop & Glo (Promega). Firefly values were normalized to *Renilla* and then all normalized values set relative to the pGL3 control vector.

Statistical analysis. $N \geq 3$ for all experiments. Two-tailed Student's *t*-tests were performed for all analyses.

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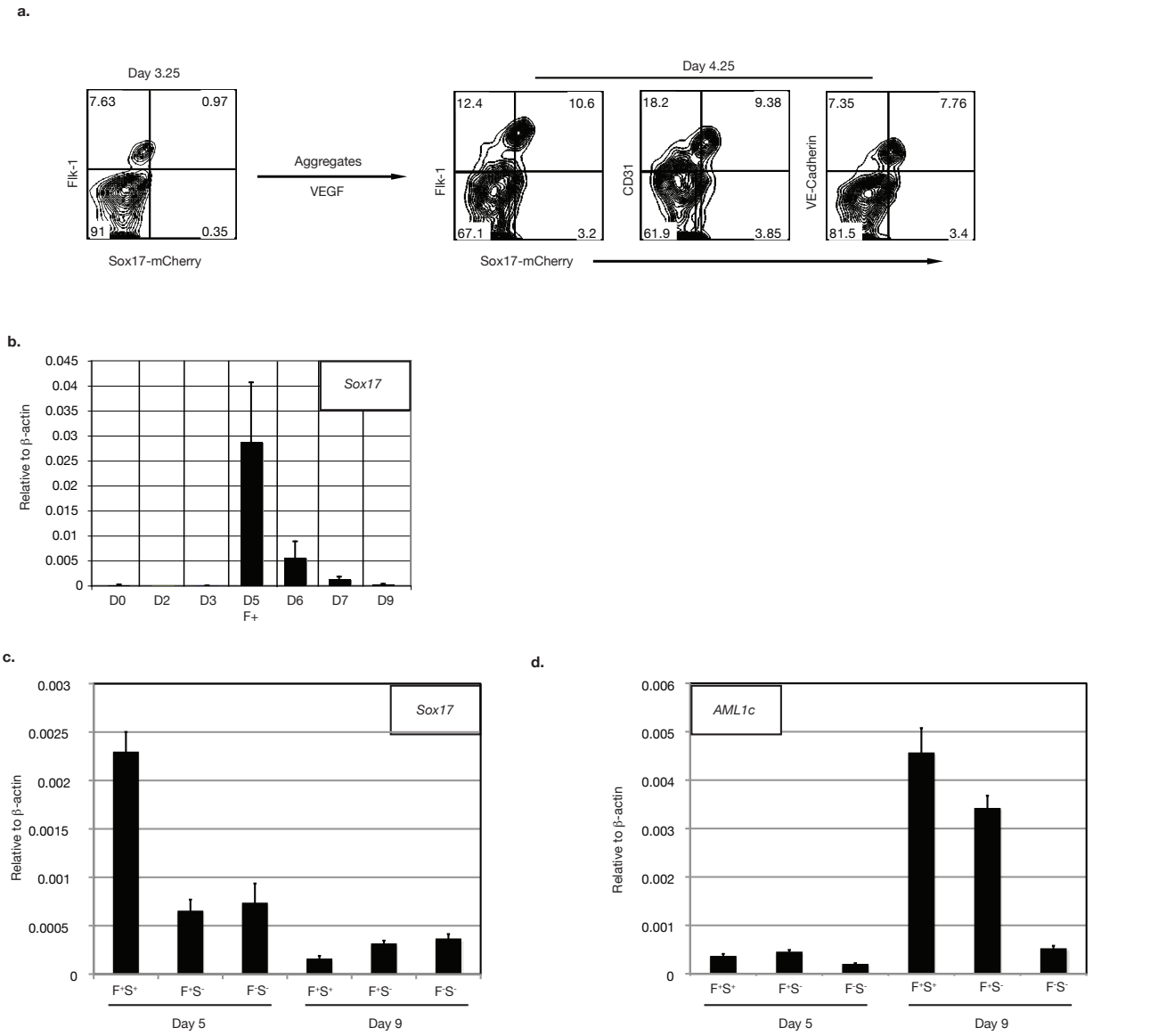

Figure S1 (a) Cells acquire the expression of *Sox17* during primitive hematopoiesis. D3.25 Flk-1$^+$ cells were isolated by FACS and aggregated in the presence of VEGF for 24 hours and analyzed by flow cytometry. (**b**) qRT-PCR analysis of the kinetics of *Sox17* transcript levels on the indicated days throughout the mESC differentiation protocol. *Sox17* expression peaks in D5.25 Flk-1$^+$ cells. (**c,d**) qRT-PCR analysis of *Sox17* and *AML1c* transcript levels from D5.25 and D9 Flk-1 and Sox17-mCherry sorted populations depicted in Fig 1c. Only fractions F$^+$Sox17-mC$^+$ and F$^+$Sox17-mC$^-$ that exhibited Sox17-mCherry expression began to express *AML1c* at D9. All data are presented as relative expression compared to *Actb*. Bars represent standard deviation of the mean of 3 independent experiments.

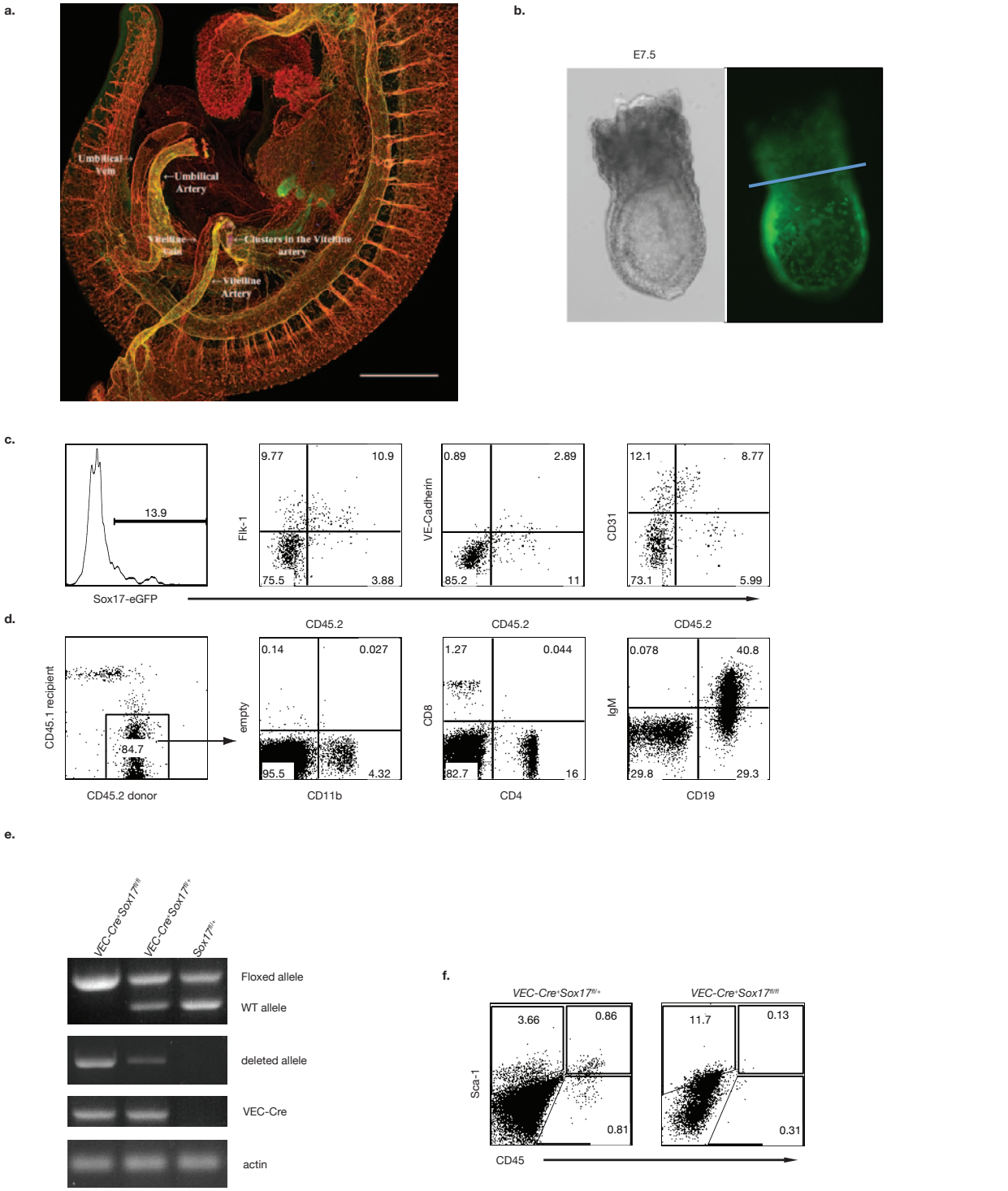

Figure S2 (a) *Sox17* is expressed in arterial, but not venous, endothelium. Whole-mount immunofluorescence of a 36-39 somite pair *Sox17*$^{GFP/+}$ embryo (10x) using antibodies recognizing GFP (Sox17), cKIT and CD31. Scale bars = 500μm. **(b)** *Sox17* is expressed in the yolk sac of E7.5 embryos. Fluorescence microscopy depicting Sox17-GFP (10x). Area above the blue line was isolated for flow cytometric analysis. **(c)** Sox17 is co-expressed with endothelial markers Flk-1, VEC and CD31 in the yolk sac of E7.5 embryos. **(d)** Representative recipient showing multilineage myeloid, T cell and B cell engraftment by donor derived *Sox17*$^{GFP/+}$ VEC^{+}Sox17^{+} (V^{+}S^{+}) HSCs. **(e)** Genotypes of the *VEC-Cre*$^{+}$*Sox17*$^{fl/fl}$ embryos were confirmed by PCR using primers that amplified the floxed versus wildtype allele, the VEC-Cre deleted allele and VEC-Cre. **(f)** Conditional deletion of *Sox17* by *VEC-Cre* results in a loss of hematopoietic progenitor populations as seen by Sca-1 and CD45 staining.

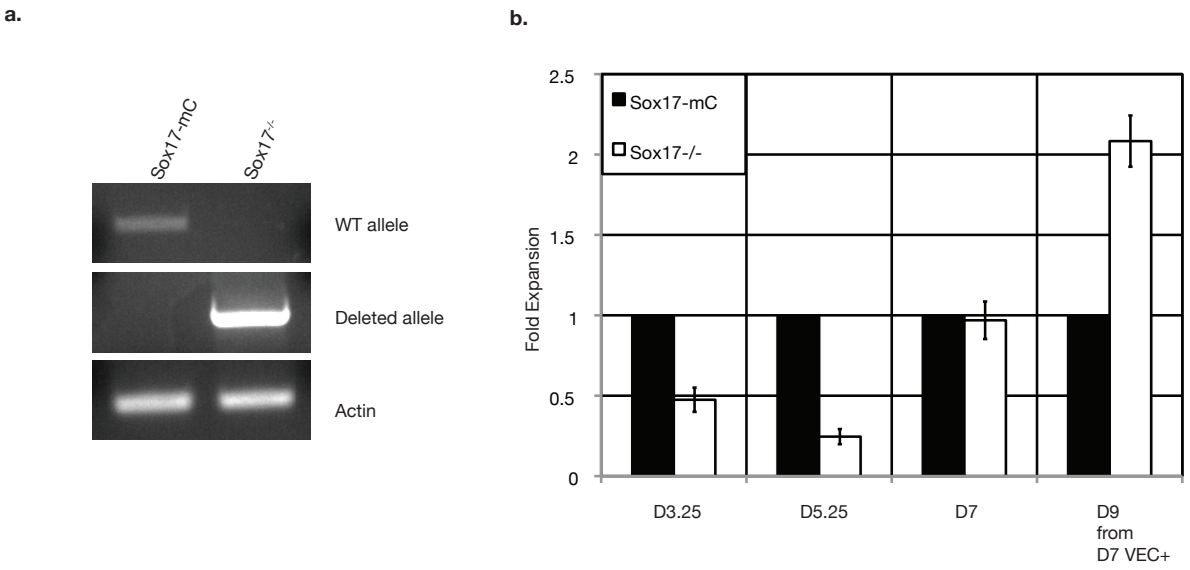

Figure S3 (**a**) PCR of genomic DNA isolated from Sox17-mC and Sox17$^{-/-}$ mESCs depicting deletion of the *Sox17* coding region. **(b)** Relative cell numbers at during differentiation in Sox17-mC or Sox17$^{-/-}$ cultures. Bars represent standard deviation of the mean of 3 independent experiments.

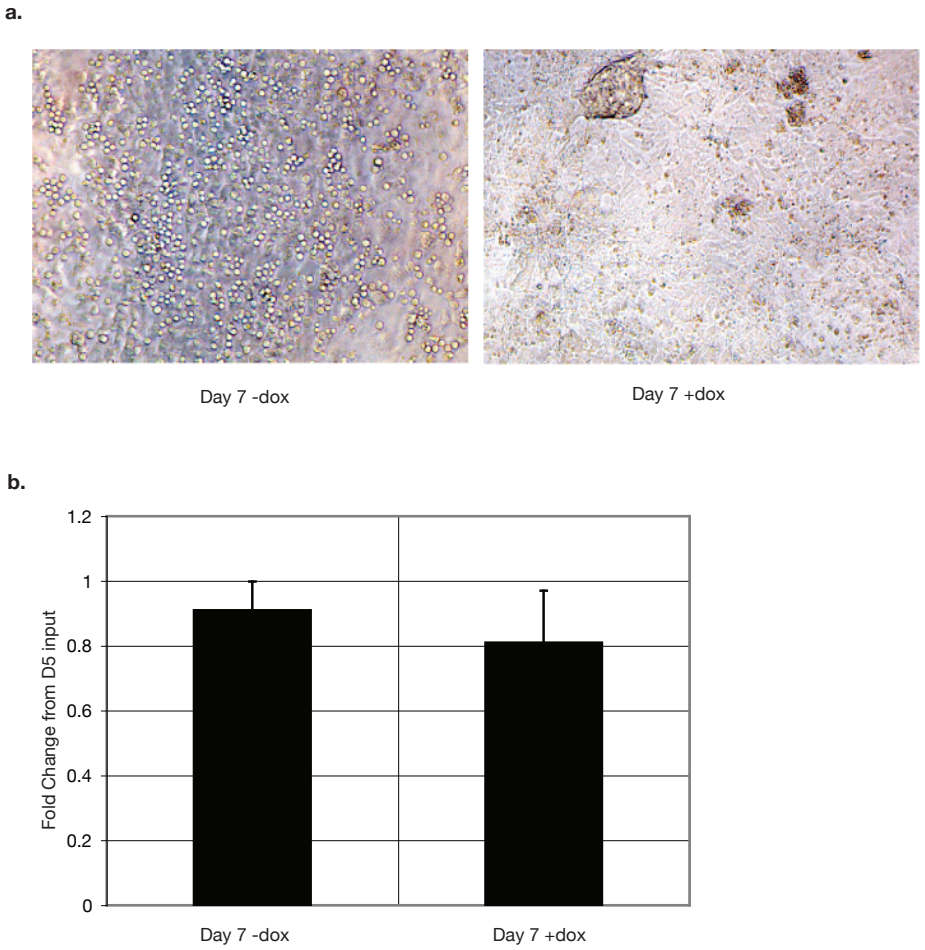

Figure S4 (**a**) Bright-field images (10x) showing that D5 Flk-1$^+$ cells cultured as monolayers for 2 days undergo the endothelial-to-hematopoietic transition to produce round hematopoietic cells (left). In the presence of *Sox17* enforced expression hematopoiesis is restrained and cells maintain an endothelial phenotype (right). (**b**) Cell numbers of D7 cells cultured in the presence or absence of doxycycline relative to input at D5.25. Enforced expression of *Sox17* does not significantly affect total cell numbers. Bars represent standard deviation of the mean of 3 independent experiments.

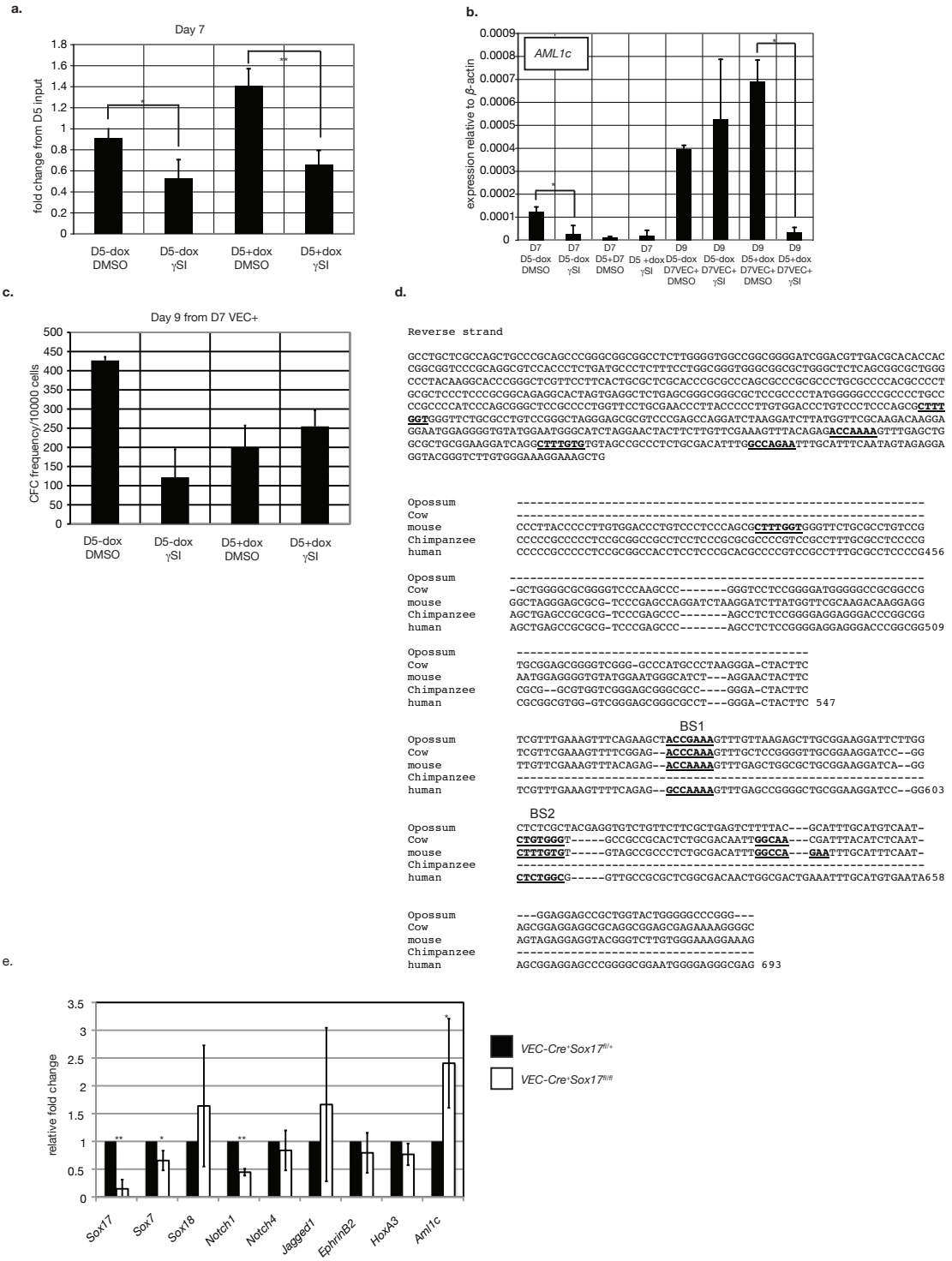

Figure S5 (**a**) Inhibition of Notch signaling with γ-secretase inhibitor (γSI) decreases cells numbers between D5 and D7 of culture with or without *Sox17* overexpression. Bars represent standard deviation of the mean of 3 independent experiments, * P=0.028, ** P=0.0036. (**b**) qRT-PCR analysis showing the relative expression of *Aml1c* normalized to *Actb* during differentiation of inducible Sox17-ESCs with γSI. Inhibition of Notch signaling in D5 Flk-1+ cells from D5.25 to D7 decreased *AML1c* expression and prevented the recovery of *Aml1c* expression in +dox-treated cultures at D9. Bars represent standard deviation of the mean of 3 independent experiments; D7 P=0.003, D9 P=0.001. (**c**) Hematopoietic progenitor potential in the D9 aggregates generated from the D7 VEC^{+}CD45^{-} monolayer population grown under the indicated conditions. Bars represent standard deviation of the mean of 3 independent experiments. **(d)** Alignment of *Notch1* genomic sequence. The four putative Sox17-binding motifs are indicated in underlined bold black font with the two evolutionarily conserved bindings sites BS1 and BS2 labeled. **(e)** qRT-PCR based analyses showing expression of indicate genes in AGM regions isolated from E11.5 *VEC-Cre^{+}Sox17$^{fl/+}$*and *VEC-Cre^{+}Sox17$^{fl/fl}$* embryos. Values shown are relative to *Act*β. For comparison of the 2 populations, the values for the expression levels in the *VEC-Cre^{+}Sox17$^{fl/+}$*cells are set to 1. Bars represent standard deviation of the mean of 3 independent mice. *Sox17* P=0.0016, *Sox7* P=0.03, *Notch1* P=0.0012.