A Snail1/Notch1 signalling axis controls embryonic vascular development

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Notch1-Delta-like 4 (Dll4) signalling controls vascular development by regulating endothelial cell (EC) targets that modulate vessel wall remodelling and arterial–venous specification. The molecular effectors that modulate Notch signalling during vascular development remain largely undefined. Here we demonstrate that the transcriptional repressor, Snail1, acts as a VEGF-induced regulator of Notch1 signalling and Dll4 expression. EC-specific Snail1 loss-of-function conditional knockout mice die in utero with defects in vessel wall remodelling in association with losses in mural cell investment and disruptions in arterial–venous specification. Snail1 loss-of-function conditional knockout embryos further display upregulated Notch1 signalling and Dll4 expression that is partially reversed by inhibiting γ-secretase activity in vivo with Dll4 identified as a direct target of Snail1-mediated transcriptional repression. These results document a Snail1-Dll4/Notch1 axis that controls embryonic vascular development.
The evolutionarily conserved Notch signalling pathway plays critical roles in controlling multiple aspects of vascular development and endothelial cell (EC) function, ranging from proliferation, motility and lumen formation to vessel stability and cell fate determination. In vertebrates, the Notch signalling pathway consists of four Notch family receptors (Notch1–Notch4) and four Notch ligands ((Jagged1, Jagged2, Delta-like (Dll) 1, Dll3 and Dll4)). Receptor-ligand interactions between neighbouring cells initiate the proteolytic cleavage of the Notch receptor extracellular domain, the γ-secretase-dependent release of the Notch intracellular domain, and its subsequent translocation to the nucleus where it associates with the DNA-binding protein, RBPJK/CBF1/Su(H), as well as a co-activator of the Mastermind-like family, thereby triggering the transcription of downstream target genes, including Hey/Hes family members. EC-specific deletion or overexpression of key components of the Notch signalling pathway, for example, Notch1, its transcriptional active intracellular form (N1ICD) or Dll4, impinges on the normal development of the embryonic vasculature and results in early embryonic lethality. Recent studies suggest that vascular endothelial growth factor (VEGF), ETS factors, Sox and Notch regulate Dll4 expression in complex cascades that may be further impacted by the canonical Wnt pathway. However, despite the fact that even subtle changes in Dll4 expression impaire vascular development, the development of the embryonic vasculature in vivo remain largely undefined.

The zinc-finger transcriptional repressor, Snail1, has been shown to play an essential role in the induction of epithelial–mesenchymal transition (EMT) and gastrulation in the developing mouse embryo. Mouse embryos homozygous for a Snail1-null mutation (Snail1–/– embryos) display defects in mesodermal formation and die shortly after embryonic day (E) 7.5. Recently, Gridley and colleagues reported that the epiblast-specific deletion of Snail1 at later stages in mouse embryonic development is permissive for normal gastrulation, but results in defective left–right asymmetry determination and cardiovascular development. However, as all embryonic tissues were rendered Snail1 deficient in these studies, the possibility that Snail1 functions in an EC-autonomous fashion in vivo remains unexplored.

Here we report that EC-specific Snail1 loss-of-function (LOF) conditional knockout (KO) mice display an early embryonic lethal phenotype with marked defects in vascular remodelling, morphogenesis and arterial–vein specification. Unexpectedly, the observed changes in vascular development phenocopy a subset of the defects commonly associated with upregulated Dll4/Notch1 signalling. Indeed, we now identify Snail1 as a VEGF-induced, cis-acting, negative feedback regulator of Dll4 and Notch1 expression in vitro and in vivo. These studies characterize a novel, EMT-independent function of Snail1, whereby the transcription factor as a required regulator of the Notch signalling cascade that controls vascular development during embryogenesis.

Results

EC-specific Snail1 deletion leads to embryonic lethality. To interrogate a potential role for Snail1 during embryonic vascular development, conditional Snail1fl/fl mice were crossed with a Tie2-Cre transgenic line, thereby targeting Cre recombinase in ECs as well as haematopoietic stem cells. Under these conditions, no viable pups expressing Snail1fl/fl;Tie2-Cre+/– (that is, Snail1 LOF mutants) were born (Fig. 1a and Table 1). In timed matings, Snail1 LOF embryos are morphologically indistinguishable from control embryos (that is, littermates expressing Snail1fl/fl;Tie2-Cre–/+ or Snail1fl/fl;Tie2-Cre–/–) up to E9.5 (Fig. 1a). However, by E10.5 and E11.5, Snail1 LOF mutant embryo size (crown-rump length) is decreased by 32 and 47%, respectively, as compared with their control littermates (Fig. 1b). Furthermore, ~50 and 100% of Snail1 LOF embryos die at E11.5 or 12.5, respectively (Table 1), demonstrating that the Tie2-Cre-driven deletion of Snail1 dramatically impacts early embryonic development.

To further specify the cell lineage that contributes to Snail1 LOF-induced embryonic lethality, conditional Snail1fl/fl mice were crossed with a Vav1-Cre line that drives Cre recombinase expression specifically in haematopoietic cells during definitive haematopoiesis. In marked contrast to the Tie2-Cre+/– mice, analysis of embryos at E14.5, E17.5 or postnatal day 1 (P1) demonstrated no differences in viability or gross morphology between Snail1fl/fl;Vav1-Cre+ mice and control littermates expressing Snail1fl/fl;Vav1-Cre– or Snail1fl/fl;Vav1-Cre– (Fig. 1c and Table 2), indicating that Snail1 deletion confined to haematopoietic cells alone does not phenocopy Snail1 LOF mutants. As yolk sac-derived blood islands serve as the sole source of primitive haematopoietic cell production up to E9.5 (a stage before Vav1-Cre is expressed), we further used haematopoietic colony-forming assays to assess the potential impact of Tie2-Cre-mediated Snail1 deletion on early stages of haematopoiesis. However, in vitro differentiation of yolk sac haematopoietic progenitor cells derived from E9.5 Snail1 LOF mutants and control littermates demonstrates comparable erythroid colony formation (CFU-E and BFU-E) (Fig. 1d,e). Taken together, these data support a model wherein the loss of Snail1 expression in the endothelial compartment is incompatible with early embryonic development.

Snail1 LOF mutant embryos display vascular deficits. At E10.5, Snail1 LOF embryos are smaller in size with associated defects in the patterning of several regions of the vascular tree despite similar somite numbers (35 ± 3 somites in wild type (WT) versus 33 ± 2 somites in LOF; n = 4) (Fig. 2a–f). Cranial blood vessels fail to undergo the remodelling events that normally lead to the generation of larger caliber vessels (compare Fig. 2a,d boxed areas). Vascularization of the tail is also impaired in Snail1 LOF embryos with the formation of truncated and disorganized networks (Fig. 2b,e,g). Intersomitic vessels (ISV), formed through angiogenic sprouting, are present in the Snail1 LOF mutant embryos, but are unable to organize or fully infiltrate the somites (compare Fig. 2c,f), displaying significant decreases in ISV length and branch points (Fig. 2h,i). Although Snail1 can exert anti-apoptotic effects in expressing cell populations, no differences in the number of TUNEL (TdT-mediated diUTP nick end labelling)-positive ECs within the dorsal aorta (DA) or surrounding vessels are observed between WT and Snail1 LOF embryos (Supplementary Fig. 1a–c).

Independent of vascular patterning defects, immunostaining for α-smooth muscle actin (α-SMA), a marker for vascular smooth muscle cells, demonstrates that although the DA of WT E10.5 embryos are surrounded by α-SMA-positive cells, arteries in Snail1 LOF embryos fail to develop well-developed, α-SMA-positive mural coats (Fig. 2j–q). Interestingly, using Snail1lacZ/wt mice, β-galactosidase reporter activity is not only detected inPECAM-1-positive ECs within the DA, velline artery and cardinal vein, but also in PECAM-1-negative, perivascular cells surrounding arterial beds (Fig. 2r and Supplementary Fig. 2a–j).

To determine whether perivascular Snail1 is also required during embryonic development, Snail1fl/fl mice were next crossed with a Dermo1-Cre transgenic line, thereby targeting Cre recombinase throughout the mesoderm, including the mural cell compartment.
as assessed in Dermo1-Cre<sup>+</sup>;Rosa26 crosses<sup>25,26</sup> (Fig. 2s). However, analyses of embryos at E14.5 or P1 demonstrated no differences in viability or gross morphology between Snail1<sup>fl/fl</sup>;Dermo1-Cre<sup>+</sup> mutants and control littermates expressing Snail1<sup>fl/fl</sup>;Dermo1-Cre<sup>−</sup> (Fig. 2t and Table 3), indicating that Snail1 expression in mural cell populations does not play a major role during embryonic vascular development.

Outside the embryo proper, yolk sacs in Snail1 LOF mutants at E10.5 display multiple vascular defects, including gross reductions in the number of perfused blood vessels in combination with marked tissue pallor (compare Fig. 3a,d). Strikingly, PECAM-1 whole-mount staining reveals the severely defective remodelling of the primary vascular plexus into both hierarchical and branched vessels in the Snail1 LOF yolk sacs (compare...
observed in vivo vascularization programme similar, if not identical, to that Snail1 may alter cardiac function with consequent (but indirect) Snail1 LOF embryos, we next considered the possibility that given the global defects in vascular organization observed in Snail1 directs vessel patterning independent of vascular flow interdigitate into the labyrinthine layer is reduced markedly mutants, the number of fetal blood vessels that invade and littermates (Supplementary Fig. 3a–f). Further, in Snail1 LOF arteries of WT yolk sacs are surrounded by vessels in Snail1 LOF yolk sacs fail to develop vessels that invade and trophoblast giant cells appear normal in structure and number, the labyrinthine layer is reduced in thickness and vascularization relative to WT littermates (Supplementary Fig. 3a–f). Further, in Snail1 LOF mutants, the number of fetal blood vessels that invade and interdigitate into the labyrinthine layer is reduced markedly (Supplementary Fig. 3a–f).

Snail1 directs vessel patterning independent of vascular flow. Given the global defects in vascular organization observed in Snail1 LOF embryos, we next considered the possibility that Snail1 may alter cardiac function with consequent (but indirect) effects on blood flow-induced vasculogenesis/angiogenesis. As such, vascular remodelling was assessed ex vivo by isolating the allantois from E8.25 WT and Snail1 LOF littersmates, and following their reorganization into vascular networks in explant cultures. In the ex vivo system, cultured allantoises engage a vascularization programme similar, if not identical, to that observed in vivo with no requirements for vascular flow29. At pre-culture, allantois explants derived from Snail1 LOF mutants exhibit an immature vascular pattern similar to that observed in WT littersmates (Fig. 3h,k). By contrast, at 24 h post culture, whereas WT explants readily undergo vascular morphogenesis while expressing Snail1 (as assessed in explants isolated from Snail1<sup>LacZ/WT</sup> reporter mice) (Fig. 3i,j and Supplementary Fig. 3g,h), Snail1 LOF explants display an almost complete defect in their ability to organize into vascular networks (Fig. 3i-n). Under these conditions, no significant differences in TUNEL-positive ECs were observed between WT and Snail1 LOF mutant explant cultures (Supplementary Fig. 3i), highlighting the importance of Snail1 in supporting neovessel formation under flow-independent conditions.

To further assess a vascular EC-specific role of Snail1 in controlling the angiogenic program in vitro, the ability of WT and Snail1 KO ECs to support neovessel formation in a transplant assay system was assessed30. To this end, ECs were isolated from β-actin-eGFP;Snail1<sup>LacZ/WT</sup> mice and transduced with either a control Adeno-βgal or an Adeno-Cre construct in vitro and cultured alone or stimulated with VEGF (Fig. 4a). As shown in Fig. 4b, VEGF-stimulated ECs markedly increase Snail1 protein levels in Adeno-βgal-infected cells, but not in Adeno-Cre-expressing cells. Furthermore, when green fluorescent protein (GFP)-expressing control ECs are seeded into poly-l-lactic acid scaffolds and transplanted into recipient immunocompetent hosts, neovessels are formed readily (Fig. 4c,d). As such, the ability of control and Snail1-deleted ECs to mount an angiogenic response in recipient mice was assessed in the absence or presence of exogenous VEGF. In the absence of VEGF, Adeno-βgal-transduced ECs form small networks of GFP<sup>+</sup>/PECAM<sup>+</sup> vascular structures (Fig. 4e,f). By contrast, Snail1-deleted ECs are unable to organize into vascular networks (Fig. 4g,h). Following VEGF supplementation, Adeno-βgal-transduced ECs dramatically enhance their angiogenic potential, while Snail1-deleted ECs are unable to mount morphogenic responses despite the fact that these 2-week-old implants contain larger numbers of single ECs with no increase in apoptosis (Fig. 4i and Supplementary Fig. 4). Taken together, these data support an EC-autonomous role for Snail1 in supporting the angiogenic programme.

EC-specific deletion of Snail1 upregulates Notch signalling. To assess the global impact of Snail1 expression on endothelial function in an unbiased fashion, primary ECs isolated from E10.5 Snail1<sup>LacZ/WT</sup> embryos were transduced with either a control Adeno-βgal or an Adeno-Cre construct in vitro, RNA isolated and subjected to transcriptional profiling. Remarkably, using a minimum of twofold change as a cutoff, Snail1 deletion alters the expression of ~500 unique transcripts with marked effects noted in gene ontologies associated with angiogenesis-associated functions (Fig. 5a,b). Unexpectedly, however, Snail1-KO ECs also display increased expression of multiple Notch targets, including Dll4, Notch1, Jag1, Hey1, guanylate cyclase 1 beta 3 (GUCY1B3), meningoial 1 (MN1), insulin growth factor (IGF2) and fibroblast growth factor 13 (FGF13)<sup>23</sup>. Furthermore, arterial markers, including ephrin-B2 and Dpp (also known as 8430408G22Rik<sup>32</sup>), are also upregulated in Snail1 KO ECs, suggesting that the deletion of Snail1 may interface with arterial specification/differentiation (Fig. 5c,d). Unexpectedly, however, Snail1-KO ECs also display increased expression of multiple Notch targets, including Dll4, Notch1, Jag1, Hey1, guanylate cyclase 1 beta 3 (GUCY1B3), meningoial 1 (MN1), insulin growth factor (IGF2) and fibroblast growth factor 13 (FGF13)<sup>23</sup>. Furthermore, arterial markers, including ephrin-B2 and Dpp (also known as 8430408G22Rik<sup>32</sup>), are also upregulated in Snail1 KO ECs, suggesting that the deletion of Snail1 may interface with arterial specification/differentiation (Fig. 5c,d). Unexpectedly, however, Snail1-KO ECs also display increased expression of multiple Notch targets, including Dll4, Notch1, Jag1, Hey1, guanylate cyclase 1 beta 3 (GUCY1B3), meningoial 1 (MN1), insulin growth factor (IGF2) and fibroblast growth factor 13 (FGF13)<sup>23</sup>. Furthermore, arterial markers, including ephrin-B2 and Dpp (also known as 8430408G22Rik<sup>32</sup>), are also upregulated in Snail1 KO ECs, suggesting that the deletion of Snail1 may interface with arterial specification/differentiation (Fig. 5c,d). Unexpectedly, however, Snail1-KO ECs also display increased expression of multiple Notch targets, including Dll4, Notch1, Jag1, Hey1, guanylate cyclase 1 beta 3 (GUCY1B3), meningoial 1 (MN1), insulin growth factor (IGF2) and fibroblast growth factor 13 (FGF13)<sup>23</sup>. Furthermore, arterial markers, including ephrin-B2 and Dpp (also known as 8430408G22Rik<sup>32</sup>), are also upregulated in Snail1 KO ECs, suggesting that the deletion of Snail1 may interface with arterial specification/differentiation (Fig. 5c,d). Unexpectedly, however, Snail1-KO ECs also display increased expression of multiple Notch targets, including Dll4, Notch1, Jag1, Hey1, guanylate cyclase 1 beta 3 (GUCY1B3), meningoial 1 (MN1), insulin growth factor (IGF2) and fibroblast growth factor 13 (FGF13)<sup>23</sup>. Furthermore, arterial markers, including ephrin-B2 and Dpp (also known as 8430408G22Rik<sup>32</sup>), are also upregulated in Snail1 KO ECs, suggesting that the deletion of Snail1 may interface with arterial specification/differentiation (Fig. 5c,d). Unexpectedly, however, Snail1-KO ECs also display increased expression of multiple Notch targets, including Dll4, Notch1, Jag1, Hey1, guanylate cyclase 1 beta 3 (GUCY1B3), meningoial 1 (MN1), insulin growth factor (IGF2) and fibroblast growth factor 13 (FGF13)<sup>23</sup>. Furthermore, arterial markers, including ephrin-B2 and Dpp (also known as 8430408G22Rik<sup>32</sup>), are also upregulated in Snail1 KO ECs, suggesting that the deletion of Snail1 may interface with arterial specification/differentiation (Fig. 5c,d). Unexpectedly, however, Snail1-KO ECs also display increased expression of multiple Notch targets, including Dll4, Notch1, Jag1, Hey1, guanylate cyclase 1 beta 3 (GUCY1B3), meningoial 1 (MN1), insulin growth factor (IGF2) and fibroblast growth factor 13 (FGF13)<sup>23</sup>. Furthermore, arterial markers, including ephrin-B2 and Dpp (also known as 8430408G22Rik<sup>32</sup>), are also upregulated in Snail1 KO ECs, suggesting that the deletion of Snail1 may interface with arterial specification/differentiation (Fig. 5c,d). Unexpectedly, however, Snail1-KO ECs also display increased expression of multiple Notch targets, including Dll4, Notch1, Jag1, Hey1, guanylate cyclase 1 beta 3 (GUCY1B3), meningoial 1 (MN1), insulin growth factor (IGF2) and fibroblast growth factor 13 (FGF13)<sup>23</sup>. Furthermore, arterial markers, including ephrin-B2 and Dpp (also known as 8430408G22Rik<sup>32</sup>), are also upregulated in Snail1 KO ECs, suggesting that the deletion of Snail1 may interface with arterial specification/differentiation (Fig. 5c,d).



| Table 1 | Viable progeny from Snail1<sup>LacZ/WT</sup> mice intercrossed with Snail1<sup>LacZ/WT;Tie2-Cre<sup>+</sup></sub> mice as a function of age. |
|--------|-----------------|
| Age    | Viable mutants/total |
| E9.5   | 10/10            |
| E10.5  | 20/20            |
| E11.5  | 8/17             |
| E12.5  | 0/14             |

| Table 2 | Viable progeny from Snail1<sup>LacZ/WT</sup> mice intercrossed with Snail1<sup>LacZ/WT;Vav1-Cre<sup>+</sup></sub> mice as a function of age. |
|--------|-----------------|
| Age    | Viable mutants/total |
| E14.5  | 10/10            |
| E17.5  | 8/8              |
| P1     | 7/7              |
**Figure 2 | Endothelial-specific deletion of Snail1 derails vascular development.** Whole-mount PECAM-1 immunofluorescent staining of E10.5 WT (a–c) and Snail1 LOF (d–f) embryos. Defective remodelling and branching are highlighted in the cephalic (rectangled area in a and d), tail (rectangled area in b and e) and ISVs (area demarcated by dotted lines in c and f with ISV width of field marked by the double-headed arrows). Scale bar, 100 μm. (g–i) Quantification of relative vascular density in tail (PECAM-1-positive vessel area in rectangle box; g) vessels as well as vessel length (h) and branch points (defined as the junction point of three vessels; i) of ISV in WT and Snail1 LOF embryos (n = 4 in each group). Data are presented as mean ± s.e.m. **P < 0.01, Student’s t test. Cross-sections (j,k,n,o) and sagittal sections (l,m,p,q) obtained from E10.5 WT (j–m) and Snail1 LOF (n–q) embryos were stained with anti-PECAM and anti-α-SMA antibodies to detect ECs and vascular smooth muscle cells, respectively. Boxed areas in l and p are shown at higher magnification in m and q, respectively. The arteries in WT embryos are surrounded by α-SMA-positive cells, whereas arteries in LOF embryos recruited few α-SMA-positive cells. Scale bar, 50 μm (j,k,n,o); 100 μm (l,p); 10 μm (m,q). (r) Cross-sections from E10.5 Snail1LacZ/wt embryos were stained with X-Gal/lacZ followed by PECAM-1 immunohistochemical staining. Green or red arrows denote the Snail1-positive ECs and perivascular cells, respectively. Scale bar, 50 μm. (s) Cross-sections from E12.5 ROSA26;Dermo1-Cre+ embryos were stained with X-Gal/lacZ followed by α-SMA immunohistochemical staining. Red arrows mark dual-positive perivascular cells. Scale bar, 50 μm. (t) Gross examination of whole embryos from Dermo1-Cre crosses at E14.5. No differences were observed between Snail1fl/fl;Dermo1-Cre+ mutants and their control littermates in size, stage or overall appearance. Scale bar, 2 mm.
these observations to increases in protein expression levels in situ, tissue sections from E10.5 WT and Snail1 LOF mutants were immunostained for Dll4 and ephrin-B2. As predicted, both Dll4 and ephrin-B2 levels are markedly increased in the DA of Snail1 LOF mutant embryos (compare Fig. 5g,h,i,j,k and Fig. 5m,n,p,q). Furthermore, Snail1 LOF ECs misexpress Dll4 and ephrin-B2 in cardinal vein fields (Fig. 5h,i,k,l,n,o,q,r).

Given that the phenotype and gene expression profile of Snail1 LOF mutant mice are consistent with exaggerated Dll4-Notch1 signalling4–6, we next tested the ability of the Notch signalling inhibitor, DAPT, to potentially rescue a portion of the vascular defects observed in the mutant mice6,33. To this end, timed pregnant mice were treated with a non-toxic, low dose of DAPT in an effort to decrease, but not totally inhibit, Notch signalling6,33. Confirming the efficacy of this treatment protocol, cross-sections and whole cell lysates recovered from DAPT-treated WT embryos display a marked reduction in N1ICD expression as assessed by N1ICD immunofluorescent staining and western blot analysis (Supplementary Fig. 5a–g). However, whereas partial inhibition of Notch signalling does not affect vascular patterning in WT embryos, DAPT-treated Snail1 LOF embryos display improved levels of vascular remodelling/branching in both the cephalic region (Fig. 6a–d and Supplementary Fig. 6a,b) and yolk sac (Fig. 6e and

| Table 3 | Viable progeny from Snai1ľ/ľ mice intercrossed with Snai1ľ/wt;Dermo1-Creľ mice as a function of age. |
|---------|--------------------------------------------------------------------------------------------------|
| Age     | Viable mutants/total                          |
| E14.5   | 3/3                                         |
| P1      | 5/5                                         |

Figure 3 | EC Snai1ľ drives vascular remodelling in extra-embryonic tissues. Gross examination of yolk sacs dissected from E10.5 WT (a) and Snail1 LOF (d) embryos. Scale bar, 2 mm. Whole-mount PECAM-1 staining of yolk sac dissected from E10.5 WT (b) and Snail1 LOF (e) embryos. Insets, representative images of whole-mount α-SMA staining. Red lines mark continuous vessels with a diameter ≥ 20 μm. Scale bar, 100 μm. Gross examination of WT (c) and Snail1 LOF (f) embryos with yolk sac at E11.5. Note the absence of vascular structures in the LOF mutant yolk sacs. Scale bar, 2 mm. (g) Quantification of relative vessel (diameter ≥ 20 μm) length in yolk sacs from E10.5 WT (b) and Snail1 LOF (e) embryos (n = 4 each). Data are presented as mean ± s.e.m. **P < 0.01, Student’s t test. Whole-mount PECAM-1 staining of allantois explants dissected from E8.25 WT (h–j) and Snail1 LOF (k–m) embryos. At pre-culture (h,k), vascularization of allantois explants derived from WT and Snail1 LOF embryos were comparable, whereas at 24 h post culture, the Snail1 LOF embryo explants display marked defects in their ability to generate vascular structures (i,j,l,m). Cell nuclei were stained with TOTO3 (blue). Scale bar, 100 μm. (n) Quantification of relative vessel length in the WT and Snail1 LOF explant cultures (n = 5 in each group). Data are presented as mean ± s.e.m. **P < 0.01, Student’s t-test.
Supplementary Fig. 6c–h). Further, these changes occur in tandem with decreased Notch signalling \textit{in vivo} as assessed by reduced \textit{Hey1} and \textit{ephrin-B2} expression (Fig. 6f). Although DAPT-treated \textit{Snail1} LOF embryos displayed a trend towards larger size, in a fashion consistent with improved vascular remodelling, the differences did not reach statistical significance (Fig. 6g). Importantly, increased vascular remodelling in \textit{Snail1}-deleted cells is not confined to the \textit{in vivo} setting and can be confirmed \textit{ex vivo} in DAPT-treated explants of \textit{Snail1} LOF allantoises (Fig. 6h–l). Finally, to determine the impact of decreasing Notch signalling in ECs directly, WT or \textit{Snail1}-deleted ECs were cultured atop Matrigel hydrogels in the absence or presence of DAPT \textit{in vitro}. Following \textit{Snail1} excision in Adeno-Cre-transduced \textit{Snail1} \textit{f/f} ECs, sprouting behaviour is

Figure 4 | \textit{Snail1} regulates angiogenic programme in a xenograft transplant model. (a) Representative image of isolated ECs from eGFP \textit{+};\textit{Snail1} \textit{f/f} mice. Scale bar, 50 \textmu m. (b) Adeno-\textit{β}-gal or Adeno-Cre-infected ECs were stimulated with vehicle or VEGF (100 ng ml\textsuperscript{-1}) for 12 h and subjected to western blot analysis. Results are representative of three or more experiments. (c,d) Whole-mount PECAM-1 (CD 31) staining of the implants after 14 d \textit{in vivo}. Scale bar, 50 \textmu m. (e-g,i,k) Gross view of implants. Scale bar, 2 mm. (h,j) Whole-mount PECAM-1 staining of the implants. Scale bar, 100 \textmu m. (m) Quantification of relative vessel length in the implants (\(n = 3\) in each group). Data are presented as mean \pm s.e.m. **, ##\textit{P} < 0.01, analysis of variance test.
Figure 5 | Upregulated Notch signalling in Snail1-deleted ECs. (a–c) Transcriptional profiling analysis of cultured ECs derived from E10.5 Snail1fl/fl embryos and transduced in vitro with Adeno-β-gal or Adeno-Cre to induce Snail1 KO recombination. Pie chart depicts the distribution of total transcripts changed in Snail1-deleted ECs as compared with control ECs (a). Gene ontology (GO) analysis of Snail1-deleted versus control ECs (b). Heat map representation of microarray data highlights the expression levels of key transcription factors, arterial–venous specification genes, EnMT-related genes, as well as Notch signalling target genes (c). (d) qRT-PCR analysis of cultured ECs from a. Snail1 KO ECs presented significantly higher levels of Dll4, Jag1, Notch1, Hey1, Hey2, and ephrin-B2. Data are presented as mean ± s.e.m. (n = 3). **P < 0.01, Student’s t-test. (e) Western blot analysis of cultured ECs from a. Dll4, Notch1, Hey1, and ephrin-B2 were upregulated in Snail1 KO ECs. Data are presented as mean ± s.e.m. **P < 0.01, Student’s t-test. (f) qRT-PCR analysis of ECs freshly isolated from E10.5 WT and Snail1 KO embryos. ECs isolated from Snail1 LOF embryos displayed significantly higher levels of Dll4, Jag1, Hey1, and ephrin-B2. Data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, Student’s t-test. (g–l) Cross-sections from E10.5 WT and Snail1 LOF mutant embryos were co-stained with anti-PECAM-1 and anti-Dll4 antibodies. Cell nuclei were stained with DAPI (blue). (m–r) Cross-sections from E10.5 WT and Snail1 LOF mutant embryos were co-stained with anti-PECAM-1 and anti-ephrin B2 antibodies. Cell nuclei were stained with DAPI (blue).
Figure 6 | Administration of DAPT partially reverses Snail1 deletion-induced vascular defects. Confocal analysis of PECAM-1 stained whole-mounted untreated (a,c) or DAPT-treated embryos (b,d). Rectangled area highlights improved vascular remodelling in DAPT-treated Snail1 LOF mutant embryos. Scale bar, 100 μm. (e) Quantification of relative vessel length in yolk sacs from E10.5 untreated and DAPT-treated Snail1 LOF embryos (n = 4 each). Data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, Student’s t-test. (f) qRT-PCR analysis of ECs freshly isolated from untreated or DAPT-treated E10.5 embryos (n = 4 each). Data are presented as mean ± s.e.m. **P < 0.01, Student’s t-test. (g) Quantification of embryo size in a–d above. Allantoises dissected from WT (h,i) and Snail1 LOF mutant (j,k) embryos were cultured in the presence of vehicle (h) or 8 μM DAPT (i,k) for 24 h and ECs visualized by whole-mount PECAM-1 staining. Scale bar, 100 μm. (l) Quantification of relative vessel length in allantoises (n = 4 each). Data are presented as a mean ± s.e.m. **P < 0.05, Student’s t-test.
Snail1 directs transcriptional repression of Dll4. Early embryonic lethality, defective cranial and ISV formation, dysregulated expression of arterial markers and failures in the yolk sac remodelling of yolk SAC are all hallmarks in gain-of-function activation of the Notch1 signalling pathway, particularly the overexpression of Dll4 (refs 4,5,34,35). Given that Dll4 protein expression is enhanced in both the arterial and venous beds of Snail1 LOF mice (Fig. 5), we next explored the possibility that Snail1 might regulate Dll4 expression directly. Snail1 exerts many of its transcriptional effects by binding to E-box elements (consensus sequence, CANNTG) within the promoter proximal region of target genes13. In this regard, initial searches identified multiple conserved E-box elements that are located within the proximal region of the murine Dll4 promoter (see below). Hence, 293T cells were transiently transfected with a reporter construct containing the luciferase gene under the control of full-length murine Dll4 promoter (that is, bp −3,631 to +76)6. Under these conditions, Dll4 promoter activity is efficiently repressed when co-transfected with a Snail1 expression vector relative to the control vector (Supplementary Fig. 7a,b). Similarly, overexpression of Snail1 in purified ECs significantly represses Dll4 promoter activity (Fig. 7a). To identify the minimal promoter element containing the essential transcriptional regions, systematically deleted mutants of the Dll4 promoter region were constructed and the transcriptional activities of the mutants tested in purified WT ECs (Fig. 7b). The deletion of bp −2,027 to +76, bp −1,156 to +76 or bp −481 to +76 results in a loss of 98.5%, 97.3% or 83.2% of Dll4 promoter activity, respectively, suggesting that the bp −481 to +76 fragment serves as the Dll4 minimal promoter (Fig. 7b). To further define the E-box elements (at positions +43 to +48, −395 to −390 and −432 to −427) within this region, ECs were electroporated with reporter constructs harbouring successive point mutations in the 3 E-boxes (CANNTG→AANNTA). Snail1 expression dramatically decreases luciferase activity in the WT minimal promoter sequence, but not in the mutant construct (Fig. 7c). Conversely, although Snail1 KO ECs display a significantly increased luciferase activity when transduced with the WT minimal promoter sequence relative to WT ECs, the upregulated activity is lost in the presence of the mutant construct (Fig. 7c,d). After chromatin immunoprecipitation (ChIP) of FLAG-Snail1 in transfected ECs followed by quantitative PCR (qPCR) of the Dll4 promoter and upstream region, FLAG-Snail1 ChIPs are enriched in the P1 and P2 regions (containing E-box-1 and E-box-2/-3, respectively) of the Dll4 promoter, but not in the upstream P3 region, suggesting that Snail1 represses the transcriptional activity of Dll4 by specifically binding to these three E-box elements (Fig. 7e). As the ability of Snail1 to repress transcriptional activity of target genes has been linked to the recruitment of histone-modifying cofactors36–38, ChIP analyses in WT and Snail1 KO ECs were performed using antibodies directed against di- or trimethylated H3K4 (H3K4me2 or H3K4me3) and acetylated H3K9 (H3K9Ac), histone markers associated with increased transcriptional activity36–38. As shown in Fig. 7f, the levels of H3K4me2, H3K4me3 and H3K9Ac are significantly increased at the Dll4 promoter in Snail1 KO ECs relative to WT ECs.

To address the ability of endogenously derived Snail1 to repress Dll4 expression and modulate Notch signalling, control or Snail1-deleted ECs were treated with VEGF, a potent agonist of Dll4 expression7,8,39. Under these conditions, VEGF-stimulated ECs increase Snail1 protein levels in tandem with increases in Dll4 and N1ICD expression (Fig. 7g). By contrast, in Snail1 KO ECs, Dll4 expression at both the mRNA and protein levels is enhanced in the absence of the endogenous Snail1-dependent feedback inhibition loop, resulting in increased levels of N1ICD (Fig. 7h). Recent studies have documented the ability of VEGF to trigger Snail1 expression in carcinoma cell40,41, but the mechanisms by which VEGF regulates Snail1 in ECs have not been characterized previously. VEGF-stimulated ECs are known to activate a series of signalling cascades, including ERK, phosphatidylinositol 3-kinase/ Akt and nuclear factor-κB (NF-κB)10,42,43, that have each been implicated previously in regulating either Snail1 mRNA or protein expression17,44–46. Indeed, VEGF-dependent induction of Snail1, Dll4 and N1ICD, but not Snail2, in ECs occurs in tandem with the activating phosphorylation of ERK1/2 and Akt (Fig. 7i). Increased activation of ERK1/2 and Akt, in turn, triggers the Ser9 phosphorylation/ inactivation of glycogen synthase kinase 3β (GSK3β), the primary kinase responsible for Snail1 phosphorylation events that target the transcription factor for proteosomal destruction17,38,45 (Fig. 7i). Similar, if not identical, results were observed with basic fibroblast growth factor (bFGF), a second pro-angiogenic growth factor that likewise increases Dll4

Figure 7 | EC Snail1 regulates Notch activity through direct transcriptional repression of Dll4. (a) ECs derived from E10.5 Snail1/− embryos were electroporated with a mock or Snail expression vector (0.5 or 2.0µg) in combination with 0.5µg of a mouse Dll4 promoter reporter construct and luciferase activity determined. (mean ± s.e.m.; n = 3). *P<0.05, **P<0.01, Student’s t-test. (b) ECs from a were electroporated with full-length or deleted Dll4 promoter reporter constructs and subjected to luciferase assay (results are of three experiments performed). (c) Diagram depicts the mutations in the three E-boxes located within the proximal region of the mouse Dll4 promoter. Cultured ECs from a were electroporated with mock or human Snail1 expression vectors in combination with WT or mutated (MUT) Dll4 promoter reporter constructs. Luciferase assays and western blot analysis are shown. (mean ± s.e.m.; n = 3). **P<0.01, Student’s t-test. (d) Cultured control or Snail1-deleted ECs were electroporated with WT or MUT Dll4 promoter reporter constructs for luciferase assay (left) and western blot analysis (right). (mean ± s.e.m.; n = 3). **P<0.01, Student’s t-test. (e) Snail1-Dll4 promoter interactions in ECs expressing a Snail1 expression vector were assessed within the indicated regions (P1–P3) by ChIP/qPCR. (mean ± s.e.m.; n = 3). **P<0.01, Student’s t-test. (f) Lysates from cultured control or Snail1-deleted ECs were subjected to ChIP analysis using antibodies directed against H3K4me2, H3K4me3 or H3K9Ac, and Dll4 occupancy determined by qPCR. (mean ± s.e.m.; n = 3). **P<0.01, Student’s t-test. Control and Snail1-deleted ECs were treated with vehicle or VEGF for 12 h and subjected to western blotting (g) or qRT–PCR analysis (h), respectively. Western blotting results are representative of three experiments with qRT–PCR results presented as mean ± s.e.m. (n = 3). **P<0.01, analysis of variance (ANOVA). (i) WT ECs were stimulated with vehicle, VEGF (100 ng ml−1) or bFGF (20 ng ml−1) for 12 h and subjected to western blot analysis. Results are representative of three or more experiments. (j) WT ECs were pretreated with U0126 (20 µM), LY294002 (10 µM) or sanguinarine (2.5 µM) for 1 h followed by stimulation with VEGF or bFGF for 12 h. Cell lysates were prepared and subjected to western blotting (j) and qRT–PCR (k) analysis, respectively. Western blotting results representative of three experiments with qRT–PCR results presented as mean ± s.e.m. (n = 3). **P<0.01, ANOVA. (l) WT ECs were pretreated with vehicle or DAPT (8 µM) for 1 h followed by stimulation with VEGF (100 ng ml−1) for 12 h. Cell lysates were prepared and subjected to western blot analysis. Results are representative of three experiments.
a) Rel. Dll4 promoter activity
- Mock
- Snail1
- 0.5 µg
- 2.0 µg

b) Luc activity
- WT: 100
- KO: 1.5
- Mock: 2.7

E-box
- MUT XX
- WT: CANNTG
- KO: AAGGT

DII4-WT +DIH-WT
- Mock
- Snail1

β-Actin
- WT
- KO

Snail1 Snail2

β-Actin

p-ERK1/2 (Y202/Y204)

p-AKT (S473)

p-GSK3β (S9)

GSK3β

β-Actin

VEGF

Sanguinarine

DAPT

LY294002

Sanguinarine - –– +

VEGF + + +

bFGF ++

VEGF + + +

β-Actin

Snail1

p-GSK3β

β-Actin

Snail1

p-GSK3β

β-Actin

Snail1

p-GSK3β

β-Actin
expression in ECs47 (Fig. 7i). Finally, the central role played by GSK3β in Snail1 expression in ECs is confirmed by the ability of the synthetic GSK3 inhibitor, CHIR99021 (ref. 48), to directly increase EC Snail1 to near-maximal levels in the absence of either VEGF or bFGF (Supplementary Fig. 7c).

To next determine the potential roles of ERK and/or Akt activation in controlling the GSK3β-dependent regulation of Snail1 protein levels, ECs were pretreated with the MEK/ERK inhibitor, U0126, or the phosphatidylinositol 3-kinase/Akt inhibitor, LY294002 (ref. 17). In the presence of either inhibitor, the VEGF- or bFGF-induced suppression of GSK3β activity is prevented, thereby blocking increases in Snail1 protein levels (Fig. 7j and Supplementary Fig. 7d). Furthermore, consistent with the ability of NF-κB to directly trigger Snail1 mRNA expression44, the specific NF-κB inhibitor, sanguinarine46, attenuates the VEGF- and bFGF-mediated upregulation of Snail1 mRNA and protein expression without affecting the VEGF-mediated suppression of GSK3β activity (Fig. 7k and Supplementary Fig. 7d). Although previous studies have reported the ability of Notch1 signalling itself to affect Snail1 expression49,50, Snail1 protein levels in VEGF-stimulated ECs are unaffected by DAPT despite an almost complete inhibition of N1ICD formation (Fig. 7l). Together, these observations describe a regulatory axis wherein VEGF triggers increased Snail1 mRNA expression in an NF-κB-dependent manner while stabilizing Snail1 protein via the ERK/Akt-dependent phosphorylation/inactivation of GSK3β (Fig. 8).

**Discussion**

Snail1 is most frequently characterized as a zinc-finger transcriptional repressor that triggers the EMT programmes critical to early embryogenesis13. Although the epiblast-specific conditional deletion of Snail1 leads to a series of complex cardiovascular network defects15,16, we now report that the conditional deletion of Snail1 in ECs leads to early embryonic lethality in association with profound alterations in vascular development. Although only a subset of ECs in the Snail1LacZ/wt expressed β-galactosidase activity at the time points selected, these determinations do not allow for the tracking of Snail1 expression in a dynamic manner, as staining only reflects the steady state concentration of the enzyme at the time of assay. Alternatively, as observed during endothelial tip cell formation, changes in gene expression limited to small subsets of the developing vasculature can exert lethal effects during embryogenesis13. Future studies using Snail1 promoter-Cre transgenic lines and ROSA indicator strains should allow for a more rigorous lineage tracking of Snail1-expressing ECs during early development.

Transcriptional profiling of WT versus Snail1-deleted ECs uncovered a surprisingly complex range of Snail1-regulated targets, but our attention was drawn to the significant increases in Notch1 signalling and Dll4 expression in combination with increased levels of downstream target gene4,5,31,35,39. Importantly, similar changes in the Notch-Dll4 signalling axis were confirmed both in vivo, in ECs isolated directly from Snail1 LOF mutant embryos and following Snail1 excision from floxed ECs in vitro. Furthermore, many of the vascular defects in vessel branching and remodelling observed in our Snail1 LOF mutants, including defects in the embryonic and extraembryonic vasculature, resemble those described in mouse embryos engineered to express constitutively active Notch1 or Notch4 as well as increased levels of N1ICD or Dll4 (refs 4,5,35,51,52). Given the fact that the range of Snail1 targets within the endothelium extends far beyond the Notch1/Dll4 signalling axis, it is unlikely that all of the Snail1 LOF mutant vascular phenotypes would be expected to duplicate precisely those observed in more targeted interventions designed to specifically upregulate Notch signalling. For example, whereas Dll4 or N1ICD overexpression frequently, but not always, increases artery diameter4,5,35,47,52, we did not note significant effects on artery size in our model. It should be stressed, however, that experimental systems designed to overexpress Notch/Notch ligands are far different from the model described herein where endogenous Snail1 was deleted, leading to more ‘physiologic’ compensatory increase in Notch signalling. Nevertheless, many of the vascular defects observed in Snail1 LOF mutant embryos were partially reversed following DAPT treatment in vivo, in allantois explants ex vivo and in isolated ECs cultured in vitro, underlining the importance of Notch1 signalling-related targets. Interestingly, the degree of rescue observed following treatment of Snail1 LOF mutant embryos with DAPT parallel closely with those observed in an EC-specific, β-catenin gain-of-function mutant mouse model that similarly displays enhanced Dll4 expression and an embryonic lethal phenotype6.

Increases in Notch signalling in Snail1 LOF mutant embryos as well as isolated ECs are likely to be byproducts of the observed increases in both Notch1 and Dll4 expression. However, as increased levels of the Notch1 receptor alone (that is, in the absence of increased ligand expression or ligand-independent activation) are not predicted to drive, in and of themselves, robust N1ICD-dependent Notch signalling3,34, our attention turned to the effects of Snail1 on Dll4 expression as a potentially key effector of enhanced Notch signalling. Indeed, the Dll4 promoter was found to contain a series of E-boxes that serve as binding sites critical to Snail1 transcriptional repressor activity. Hence, in tandem with the VEGF-induced increase in Dll4 expression, VEGF also triggers Snail1 transcription and the post-translational stabilization of the protein via both GSK3β-
Snail1 deletion did not have an impact on vascular development to the degree observed with EC-specific targeting. As such, Snail1 is more likely to control mural cell recruitment in trans through paracrine signalling pathways (for example, platelet-derived growth factor, transforming growth factor-β, Ang1/Tie2 or Notch signalling)39. Although Snail1-dependent EnMT programmes are unlikely engaged to generate mural cells during development, EC-like endocardial cells are known to express Snail1 and undergo a distinct phenotypic switch to mesenchymal-like cells in the developing cardiac cushions23,24,58. Nevertheless, the role of Snail1 in this process remains controversial with earlier reports debating its potential importance relative to that of Snail2/Slug as the critical EnMT effector50,58,59. Although prior analyses of Tie2-Cre-targeted Snail1 heterozygote mice revealed only subtle defects in cardiac cushion cellularization58, we have found that Snail1 LOF mutant mice are unable to activate the associated EnMT programme (Supplementary Fig. 9). These findings serve to highlight the broader roles played by Snail1 in regulating endothelial as well as endocardial function in the developing vasculature and cardiac cushions, respectively, and support a model wherein the embryonic lethal phenotype of Snail1 LOF mice arises as a consequence of multiple defects in cardiovascular development. Broader roles for the Snail1-dependent regulation of EC function may extend into the postnatal setting as well. In preliminary studies, we have attempted to delete Snail1 postnatally to determine its role in retinal development (Supplementary Fig. 10). Despite deleting Snail1 by ~70%, defects in retinal angiogenesis were not detected (Supplementary Fig. 10) and additional studies are underway to delete Snail1 with higher efficiency to rule out the rescue of vascular responses by ECs that escaped Cre-mediated excision. This caveat aside, we find that Snail1-deleted ECs are unable to support the wound-like angiogenic responses that occur following transplantation into adult mice. Indeed, Snail1 has been detected in the neovascularisation surrounding human carcinomas, raising the possibility that Snail1–Dll4 interactions directly regulate vascular remodelling, and possibly lymphangiogenesis, in neoplastic states61–64. As adult ECs can also inappropriately re-activate embryonic programmes during pathologic fibrotic states to generate tissue fibroblasts65, a potential role for Snail1 in these postnatal events will be the subject of future studies.

Methods

Mice. Mice were housed under standard condition and protocols approved by the University Committee on Use and Care of Animals. Mice carrying Snail1 alleles17 and mice with Snail1-LacZ knock-in alleles32 were generated and maintained in our laboratory. Tie2-Cre, Vav1-Cre, Dermo1-Cre and B2-actin-GFP transgenic mice were obtained from Jackson Laboratory. VE-cadherin-Cre ERT2 transgenic mice were provided by ML Iruela-Arispe (University of California, Los Angeles)66. Littermate controls of both sexes were used in all experiments. All mouse strains were backcrossed into the C57BL/6J background for at least seven generations. To inhibit Notch signalling in vivo, timed pregnant mice were injected subcutaneously with 100 mg kg−1 DAPT (Tocris Bioscience) dissolved in 10% ethanol and 90% corn oil at E7.5, E8.5 and E9.5 with the embryos dissected at E10.5 (ref. 6). Gene inactivation in Snail1LacZ/fl;bCreERT2-bExpressing embryos was targeted by intraperitoneal injections of 150 μl of tamoxifen solution (10 mg ml−1, dissolved in 1:10 ethanol/corn oil; Sigma) into pregnant females at E11.5 and E13.5. Gene inactivation in pups was triggered by intraperitoneal injections of 50 μl tamoxifen solution (10 mg ml−1) at P1 and P3. Eyes were retrieved from pups at P6 and fixed in 4% paraformaldehyde–PBS overnight at 4 °C. Retinas were dissected and subjected to whole-mount PECA1.

Haematopoietic colony-forming assay. For haematopoietic colony-forming assays, yolk sacs were isolated and incubated with 0.1% Collagenase D (Roche) in Hank’s PBS containing 10% FCS and Pen/Strep for 1 h at 37 °C with occasional agitation to aid dispersion of the tissue37. The cells were then washed and counted. For colony-forming unit (CFU) assays, 1.5 × 106 cells were seeded into 1 ml of methylcellulose media supplemented with interleukin 3, interleukin 6 and stem cell factor (M3434, Stem Cell Technologies). Clones (~30 cells) were scored as myeloid (CFU-C) or erythroid (BFU-E) at day 7 of culture.
**Allantois explant culture.** Allantoises were dissected from E8.25 mouse embryos and placed individually on gelatin-coated chamber wells as described24. Explants were cultured in DMEM medium containing 15% fibronectin-depleted fetal bovine serum for 24 h. In selected experiments, allantois explants were cultured *in vitro* in the presence of 8 μM DAPT. Pre-cultured or post-cultured explants were fixed in 4% paraformaldehyde–PBS and subjected to anti-PECAM-1 immunofluorescence or TUNEL assay.

**Immunofluorescence staining.** Embryos or yolk sacs were fixed in 4% paraformaldehyde–PBS for 2 h, permeabilized in 100% methanol for 30 min at −20°C and incubated in blocking buffer (5% goat serum in PBS) containing anti-PECAM-1 primary antibody (1:500, BD Biosciences) overnight at 4°C, followed by incubation with secondary antibodies (1:400, Alexa Fluor 488–labelled, Invitrogen) in blocking buffer for 2 h. For frozen sections, allantois explant cultures or ECs, tissues were fixed in 4% paraformaldehyde–PBS, permeabilized in PBST (PBS plus 0.3% Triton X-100) and incubated in blocking buffer (5% goat or donkey serum plus 2% BSA in PBS) with primary antibodies directed against PECAM-1 (1:500), z-SMA (1:500, Abcam), Dll4 or ephrin-B2 (1:200, R&D Systems), followed by incubation with secondary antibodies (1:400, Invitrogen). All images were processed using Image J and Photoshop CS5. Relative vascular density, vessel length and branch points were determined in multiple fixed areas of view in three or more WT versus LOF embryos, yolk sacs, allantois explants or ECs xenografts as described25,26.

**EC isolation and culture.** EC isolation was performed as described previously with modifications27. Briefly, E10.5 Snail1fl/fl embryos (of either male or female sex) or adult lungs (isolated from female mice) were dissected in ice-cold PBS and digested in a mixture of collagenase type I (Worthington), DNase I (Sigma–Aldrich) and dispase (Invitrogen) for 30 min at 37°C. ECs were then separated using Dynabeads (Invitrogen) coated with anti-PECAM-1 antibody and cultured in DMEM medium supplemented with 20% fetal bovine serum (Invitrogen) and EC growth supplement (BD Biosciences). Confluent ECs were trypsinized and separated using Dynabeads coated with anti-ICAM1 antibody (BD Biosciences). Following trypsinization, the purity of ECs is ~90% and the cells were used within two passages of their initial isolation. To obtain Snail1 WT and KO ECs, cells were infected with adenoviral-FLAG-Snail1-expressing vector using Amaxa Basic Nucleofector kit (Lonza) and subjected to ChIP assay as described68,69.

**Luciferase reporter assay.** A 3.7-kb fragment of the mouse Dll4 promoter (−3,631 to +76) subcloned in the pGL3 basic vector was kindly provided by C. Kune (Northwestern University, Chicago, IL). The minimal Dll4 promoter (−481 to +76) was amplified from the 3.7-kb fragment of Dll4 promoter and subcloned in the pGL3 basic vector (Promega). The Dll4-MUT was generated by mutating the Snail1 binding sites (E-boxes) CANNCTG to AANNNTA using the QuickChange Site-Directed Mutagenesis kit (Stratagene). A 2-kb fragment of the mouse Dll4 promoter (−1,039 to +1,82) was obtained from GenOposea, Inc. Dual-luciferase reporter assays were performed according to the manufacturer’s instructions (Promega).

**Statistical analysis.** Statistical analysis was performed with the Student’s t-test or by analysis of variance. *P < 0.05, **P < 0.01, ***P < 0.001. All experiments were repeated three or more times.

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Author contributions

Z.Q.W. designed and performed all experiments, analysed all data and wrote paper. R.G.R. generated Snail1fl/fl transgenic mice. K.C.L. performed hematopoietic colony-xenotransplantation assays. I.M. analysed data and wrote paper. S.J.W. analysed all data, designed experimental strategies and wrote paper.

Additional information

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