The Cdh5-CreERT2 transgene causes conditional Shb gene deletion in hematopoietic cells with consequences for immune cell responses to tumors

Qi He1, Xiujuan Li1,2, Kailash Singh1, Zhengkang Luo3, Mariela Meija-Cordova3, Maria Jamalpour1, Björn Lindahl4, Vitezslav Kriz3, Reetta Vuolteenaho4, Maria Ulvmar5 & Michael Welsh1

The tamoxifen-responsive conditional Cdh5-CreERT2 is commonly used for endothelial cell specific conditional deletion of loxP-flanked gene sequences. To address the role of endothelial cell Shb gene for B16F10 melanoma immune responses, tamoxifen-injected Cdh5-CreERT2/WT and Cdh5-CreERT2/Shbflx/flx mice received subcutaneous tumor cell injections. We observed a decrease of tumor myeloid cell Shb mRNA in the tamoxifen treated Cdh5-CreERT2/Shbflx/flx mice, which was not present when the mice had undergone a preceding bone marrow transplantation using wild type bone marrow. Differences in CD4+/FoxP3+ Tregs were similarly abolished by a preceding bone marrow transplantation. In ROSA26-mTmG mice, Cdh5-CreERT2 caused detectable floxing in certain bone marrow populations and in spleen cells. Floxing in bone marrow could be detected two months after tamoxifen treatment. In the spleen, however, floxing was undetectable two months after tamoxifen treatment, suggesting that Cdh5-CreERT2 is operating in a non-renewable population of hematopoietic cells in this organ. These data suggest that conditional gene deletion in hematopoietic cells is a potential confounder in experiments attempting to assess the role of endothelial specific effects. A cautious approach to achieve an endothelial-specific phenotype would be to adopt a strategy that includes a preceding bone marrow transplantation.

The Shb gene1 plays a role in tumor biology in numerous settings2-4. Many of the findings point to an endothelial cell involvement4,15, but the Src-homology 2 domain protein B (SHB) also has an impact on immune or hematopoietic cell behaviour15-14. Shb is required for vascular endothelial growth factor-A (VEGFA) dependent angiogenesis and vascular leakage in endothelial cells9 and these effects appear to be mediated via regulation of focal adhesion kinase2.15. T-cell receptor activation12 also requires Shb and in the absence of Shb, T cells exhibit an augmented Th2-response14. Absence of Shb decreases hematopoietic stem cell proliferation causing a reduced ability of myeloid cells to repopulate after bone marrow replacement13. Our recent finding that CD8+ cell infiltration into B16F10 melanomas was influenced by the Shb gene raised the possibility that endothelial cells exert an influence on immune responses to tumors in a manner that could be of relevance to tumor expansion and metastasis4, and we decided to investigate this further by crossing the Cdh5-CreERT2(1Rha) transgene Cre-recombinase onto the Shbflx/flx background. This transgene is considered the gold standard for endothelial specific conditional deletion of loxP target genes16 and has not been reported to generate inactivation of hematopoietic cells in adult mice unlike the Tie2-Cre transgene which efficiently causes gene deletion in hematopoietic cells17 or the constitutive Cdh5-Cre transgene that causes recombination in embryonic hematopoietic cells18. One

1Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden. 2Cyrus Tang Hematology Center, Soochow University, Suzhou, China. 3Institute of Molecular Genetics of the CAS, Prague, Czech Republic. 4Transgenic Animals Facility, Oulu University, Oulu, Finland. 5Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden. Correspondence and requests for materials should be addressed to M.W. (email: Michael.welsh@mbc.uu.se)
Scientific RepoRts | (2019) 9:7548 | https://doi.org/10.1038/s41598-019-44039-z
www.nature.com/scientificreports/report suggests Cdh5-Cre-dependent recombination in hematopoietic cells in adult mice but that study used a different Cdh5 promoter fragment to drive Cre expression19. A third transgenic Cdh5-CreERT2 mouse was generated that perturbed angiogenesis but was not further investigated in detail with respect to its capacity to cause non-endothelial cell gene inactivation20. Herein we observed that the Cdh5-CreERT2 transgene16 causes conditional gene deletion in certain hematopoietic cells with functional consequences that can be avoided by implementing protocols utilizing a preceding bone marrow transplantation.

Results and Discussion
Mice (Cdh5-CreERT2/WT or Cdh5-CreERT2/Shbflx/flx pretreated with tamoxifen) with B16F10 melanomas were investigated for endothelial-dependent alterations in Treg immune cells as a consequence of Shb gene inactivation. Immune organs (thymus, inguinal lymph nodes, spleen, bone marrow and blood) were collected and subjected to immune profiling by FACS staining to detect CD4+/FoxP3+ Tregs. Absence of Cdh5-CreERT2 induced deletion of Shb in endothelial cells reduced a tumor-induced increase of CD4+/FoxP3+ Tregs in local lymph nodes and accentuated that cell population in bone marrow (Fig. 1a). Next, these analyses were supplemented with bone marrow transplantation experiments using wild type bone marrow to Cdh5-CreERT2/Shbflx/flx or Cdh5-CreERT2/WT mice. The shift in the CD4+/FoxP3+ population caused by absence of Shb disappeared (Fig. 1b) suggesting that the effects were cell autonomous to Tregs. Isolated endothelial (CD31+) and CD11b (Itgam) (S. Fig. 1B,C), respectively, excluding endothelial cell contamination as an explanation for the reduction in Shb mRNA in CD11b+ cells. The reduction in tumor CD11b+ Shb mRNA was also reverted by wild type bone marrow (Fig. 2b). This suggests conditional deletion of Shb in hematopoietic cells by the Cdh5-CreERT2 transgene as responsible for the effects.

We next investigated floxing of Shbflx/flx in bone marrow CD31+ and CD11b+ cells by genotyping and detected gene inactivation in both cell types at day 4 after the cessation of the tamoxifen injections in adult

Figure 1. Expression of CD4+/FoxP3+ Tregs in different immune organs in response to B16F10 melanoma growth. (a) Conditionally deleted Shb and corresponding wild type controls with or without tumors were sacrificed and immune organs collected. The percentages CD4+/FoxP3+ cells were determined in percent of parental populations (which did not differ between the experimental conditions). Means ± SEM are given for n = 4 (non-tumor) or n = 10 (tumor) of each genotype in three separate experiments. The experimental groups of each organ were subjected to one-way ANOVA to reject the null hypothesis (p < 0.01 for lymph node and p < 0.001 bone marrow) followed by Sidak’s multiple comparisons test to compare WT tumor with conditionally deleted Shb tumor. *Indicates p < 0.05 when compared with corresponding tumor wild type control. (b) CD4+/FoxP3+ cells in lymph nodes and bone marrow of tumor bearing mice that had received a wild type bone marrow transplantation 3 months prior to the tumor experiment. Means ± SEM for n = 5 of each genotype are given in one experiment. P values were 0.44 and 0.36, respectively.
mice (S. Fig. 2). Three single-cell RNAseq studies on hematopoietic bone marrow cells in adult mice have detected expression of VE-cadherin/Cdh5 mRNA, albeit at a low level/frequency21–23 and the following resources of single cell RNAseq in B16F10 tumors and mouse bone marrow have similarly demonstrated low Cdh5 expression in sparse non-endothelial cells (https://www.ebi.ac.uk/gxa/sc/experiments/E-EHCA-2/Results?geneId=ENSMUSG00000031871&k=16&clusterId=%5B1%5D; https://www.ebi.ac.uk/gxa/sc/experiments/E-GEOD-100426/Results?geneId=ENSMUSG00000031871).

To directly demonstrate Cdh5-CreERT2 activity in hematopoietic cells, the ROSA26-mTmG reporter mouse was used together with the transgene. This reporter expresses under basal conditions tdTomato and begins expressing GFP after floxing. However, the tdTomato signal is stable and may remain for weeks after Cre recombinase24. The GFP/FITC signal was intermediate (10³–10⁴) in bone marrow cells (S. Figs 3, 4) and consequently there was no distinct difference between GFP-positive cells and autofluorescence. For that reason, Cre-negative controls were always used in parallel under identical conditions. In the bone marrow, lineage-positive cells lacked recombination that showed statistical significance seven days after tamoxifen injections (Fig. 3). In cKit⁺Sca1⁺Lineage⁻ (K⁺S⁻L⁻) stem and multi-potent progenitor cells a low but detectable degree of recombination occurred that was consistently higher than that of the Cdh5-CreERT2 negative cells using the identical gating for GFP⁺ (Fig. 3, S. Fig. 3). Another bone marrow population with detectable recombination was a hitherto uncharacterized cKit⁺Sca1⁺Lineage⁺ cells (Fig. 3, S. Fig. 4). We could not detect significant endothelial cell contamination in the K⁺S⁺L⁻ population by FACS analysis (S. Fig. 3) since there were no or very few GFPhi-cells (>10⁴) present in that population, which is in contrast to what was observed in lung endothelial cells in which the GFP/FITC FACS-signal is >10⁴ (S. Fig. 5) when analyzed under identical conditions. The low degree of endothelial cell contamination could be a consequence of bone marrow crushing without digestion during preparation and the gating for high cKit expression. Standard protocols for bone marrow endothelial cell isolation include enzymatic digestion25,26 and endothelial cell cKit expression is lower than that of cKit⁺Sca1⁺Lineage⁻ hematopoietic stem/multipotent progenitor cells22,27.

In spleen, myeloid cells (CD11b⁺/Gr1⁺), B cells (CD19⁺/B220⁺) and T cells (CD4⁺/CD8⁺) all exhibited recombination of the ROSA26-mTmG locus and the effect became more apparent by fixing the cells and staining for GFP (Fig. 3, S. Fig. 6). The expression of the ROSA26-mTmG reporter was significantly lower in hematopoietic (CD45⁺/CD31⁺) cells than in endothelial cell when flow cytometry was performed under identical conditions in the same FACS analysis (S. Fig. 5, results not shown). Lung CD45⁺CD31⁺-positive hematopoietic cells also showed detectable recombination (S. Fig. 5) comparable to that of spleen myeloid cells (S. Fig. 6). When ROSA26-mTmG recombination was analyzed two months after tamoxifen treatment, floxing was detected in the bone marrow populations, whereas floxing could not be detected in the spleen (Fig. 4). This suggests that floxing in spleen occurs in a non-renewable hematopoietic population in the spleen.

Figure 2. Shb mRNA in tumor CD31⁺ and CD11b⁺ cells from Cdh5-CreERT2/WT and Cdh5-CreERT2/Shbflx/flx mice. (a) Shb mRNA with or without conditional deletion of the Shb gene by qPCR. Relative values compared with corresponding wild type controls are given as means ± SEM for n = 10 in three separate experiments. *** Indicates p < 0.001 by Students’ t-test when compared with corresponding wild type control. (b) Shb mRNA after bone marrow transplantation. N = 5 of each genotype in one experiment and ** indicates p < 0.01 when compared with wild type control by a Students’ t-test.
Figure 3. (a) GFP+ percentage in different bone marrow and spleen hematopoietic cell populations. ROSA26-mTmG mice with or without the Cdh5-CreERT2 transgene were pre-treated with tamoxifen and maintained for seven days before sacrifice. Means ± SEM are given. Values in mice without Cdh5-CreERT2 reflect autofluorescence. ***Indicates p < 0.001 when compared with corresponding control without the Cdh5-CreERT2 transgene by a Students’ t-test. LIN+ = lineage positive bone marrow cells; K+S+L− = cKit positive, Sca1 positive and lineage negative (LSK) bone marrow cells; K+S+L+ = cKit positive, Sca1 positive and lineage positive bone marrow cells; Spleen myeloid = Gr1 positive/CD11b positive spleen cells; Spleen B cell = B220 positive/CD19 positive spleen cells; Spleen T cell = CD4 positive/CD8 positive spleen cells. N = 4 for the bone marrow and n = 5 for the spleen populations in three separate experiments. (b) Staining spleen cells for GFP using an anti-GFP-FITC antibody. After staining of spleen cells for lineage markers as in Fig. 3a, cells were stained for GFP prior to FACS. Values as percentage of parental cell population are means ± SEM. *Indicates p < 0.05 when comparing Cdh5-CreERT2 mice with wild type controls without Cdh5-CreERT2 (autofluorescence) by a Students’ t-test. N = 5 in three separate experiments. (c) Histogram showing Cdh5-
In summary, the Cdh5-CreERT2 transgene results in conditional deletion in hematopoietic cells. The effect may seem modest as suggested by the ROSA26-mTimG reporter data ranging between 3–20% but could play a significant role in certain settings. The data are based on two separate Cdh5-CreERT2 sublines excluding the possibility that unique drifting in phenotypic properties has occurred during local breeding. Firstly, differences between different hematopoietic cell types are noted. A partial explanation may lie in differences in Cdh5-CreERT2 expression but also in how susceptible the locus is to recombination in different cell types.28,29 In addition, different loci may show different susceptibility to floxing.30 Another issue is whether there is positive or negative selection for the hematopoietic cell with a gene inactivation causing altered cellular properties. Finally, the pathological condition at which the study is conducted may influence the efficacy of conditional deletion. Currently in tumor CD11b+ cells, 75% of these appear to have deleted their Shb gene based on mRNA content and this had phenotypic consequences since tumor-induced alterations in Tregs were observed.

There are several reasons why Cdh5-CreERT2 activity in hematopoietic cells has remained hitherto undetected. The tdTomato signal is very stable so one must gate tdTomato/GFP double positive cells and due to the relatively weak GFP signal these are only moderately above background fluorescence as depicted by the Cre-negative cells. In addition, the population most active in the bone marrow is an uncharacterized lineage+ /Sca1+ /cKit+ population probably reflecting a progenitor subtype. Finally, conditional deletion is initially (after one week) readily detected in spleen and undetectable two months later.

The potential of conditional deletion in hematopoietic cells can easily be avoided by performing a preceding bone marrow transplantation but that has potential adverse effects as well. Besides the simple fact that the mice will be of older age since complete hematopoietic restitution takes three months and causes changes in the relative proportions of different hematopoietic subpopulations,30 the irradiation has direct adverse effects on endothelial cells.31

Conclusion

The Cdh5-CreERT2 transgene may conditionally delete target genes in certain hematopoietic cells and in order to avoid possibly confounding results, a strategy employing a preceding bone marrow transplantation can be implemented.

Materials and Methods

Mice. The conditional Shb<sup>in</sup> (Shb<sup>tm1a(EUCOMM)Hmgu</sup>) mouse on the C57Bl/6 strain was generated by the Intrafrontier I3 mouse project at the Biocenter Transgenic Core Facility, University of Oulu, Oulu, Finland. The C57BL/6N embryonic stem cell clone HEPD0613_4_G08-1 targeted with the "knock-in-first" construct was obtained from EUCOMM, see (http://www.mousephenotype.org/data/genes/MGI:98294)32. The tm1a allele contains an IRES: lacZ trapping cassette and a floxed promoter-driven neo cassette inserted into an intron of the Shb gene. The clone was characterized by chromosome, copy number and internal PCR analysis (http://www.mousephenotype.org/data/genes/MGI:98294) and Southern blot analysis. The ES cell clone HEPD0613_4_G08-1 was injected into laser-treated morulas (Zyrcos, Hamilton Thorne) that were transferred into pseudopregnant recipients. The chimeric mice were backcrossed with C57BL/6 mice, initially with C57Bl/6OlaHsd and after arrival to Uppsala heterozygous mice were bred with C57Bl/6J for four generations. The conditional construct sequence can be found at https://www.ncbi.nlm.nih.gov/nuccore/JN955283.1?report=genbank&to=37884 and the map of the vector at https://www.i-dcc.org/limits/targ_rep/alleles/19006/vector-image.

The Cdh5-CreERT2 transgenic mouse was kindly provided by Dr Ralf Adams, Max Planck Institute for Molecular Biomedicine, Münster, Germany and imported on two separate occasions to Uppsala University.33 One of the imports was used for the conditional deletion of Shb (Cdh5-CreERT2/Shb<sup>tm</sup>) (Shb<sup>tm1b(EUCOMM)Hmgu</sup>) and the other for crossing with ROSA26-mTimG (B6.129(Cg)-Gt(Rosa26)Sorim[ACTB-tdTomato,-EGFP]lin)34 (https://www.jax.org/strain/007676) to assess Cre recombination. All mice used for experimentation in this study were older than 8 weeks.

All animal experiments were approved by the Regional State Administrative Agency for Southern Finland and by the local animal ethics committee at the Uppsala County Court. EU regulations and guidelines for housing and care of laboratory animals were followed (DIRECTIVE 2010/63/EU on the protection of animals used for scientific purposes).

Genotyping. The primers used for genotyping were: ShbF2: 5’ GCC ACT TCT GGA GAG CAG TAG A 3’; ShbR3: 5’ ACA GTG TGA GCC GAT CTC TGG 3’; Construct primer: 5’ TCG TGG TAT CGT TAT CGG CC 3’ with annealing at 60°C. The mutant generates a band of 436 bp whereas the wild type a band of 741 bp. For demonstrating conditional deletion, the primers used were 5’-AAT AAT AAC CGG GCA GGG GG and 5’-GCC ATG GCT TGA ATG TGC TC at an annealing temperature of 65°C amplifying a 498 base pair product upon amplification of the floxed gene. This PCR reaction does not detect non-floxed Shb<sup>in</sup> due to the large size of the anticipated product (>3 kb).

Bone marrow transplantation. Bone marrow cells were collected from 8–10 weeks old wild type donor mice and transplanted to lethally irradiated (two times 4.5 Gy two hours apart) recipients (10 weeks old) of...
**Cdh5-CreERT2/WT** (wild type) or **Cdh5-CreERT2/Shbflx/flx** (5 mice each) as described. The mice were maintained for 3 months before further experimentation.

**Tumor studies.** B16F10 melanoma cells (2 × 10⁵) were injected subcutaneously as described in mice of 8–12 weeks of age that were injected for five consecutive days with 2 mg tamoxifen dissolved in peanut oil ten days after the last tamoxifen injection. Alternatively, mice subjected to a bone marrow transplantation three months prior with wild type bone marrow were used. When tumors reached a size of 0.5–1 cm³ mice were sacrificed and relevant organs collected.

**Immune cell profiling.** Thymi, spleens, inguinal lymph nodes, bone marrows and blood were collected and cells isolated that were stained for CD4 and FoxP3 and analyzed by fluorescence activated cell sorting (FACS) as described.

**Cell isolation by magnetic beads.** Tumors were excised and digested as described. Alternatively, iliac and femur bones were crushed and cells were collected by washing with PBS. Spleens were disrupted with a 1 ml syringe and cells collected in PBS. Blood was drawn immediately before sacrifice. Bone marrow, spleen and blood was subjected to red blood cell lysis by incubation for 15 minutes on ice in red blood cell lysis buffer (150 mM NH₄Cl, 10 mM NaHCO₃, 1 mM EDTA). Collected non-lysed cells (10⁶) in 180 μl were incubated with 10 μl CD11b microbeads (130-049-601, MACS Miltenyi, Germany) for 15 minutes at 4°C. After washing the cells were applied to a MS column (130-042-201, MACS Miltenyi), washed again and eluted. The original flow-through upon column application was collected, incubated with 4 μl biotin-anti-CD31 (102504, Biolegend) plus 20 μl anti-biotin microbeads (130-090-485, MACS Miltenyi) and further column purified as above. RNA from eluted cells was isolated using the RNeasy mini-kit (Qiagen, Hilden, Germany). Lung endothelial cells were isolated as described by collagenase digestion and purification on CD31+ beads.
Real-time reverse transcriptase PCR (qPCR). Gene expression was determined using the QuantiTec™ SYBRGreen real-time RT-PCR kit (Qiagen) as described15. For Shb, 5′-TTT GAT GCC AGC GAC CTG and 5′-GAG TCT GAG TCC AGC CTC TG primers were used. For bone marrow VE-cadherin/Cdh5 mRNA, the following primers were used: 5′-TTG GCC GGC TGA AGA AGT AGG AC; 5′-ACT GCC CAT ACT TGA CC; 5′-GCC TGT TTC TCT CGG TCC AA. Beta-actin values were subtracted and relative expression was determined according the formula 2^−ΔΔCt compared with relevant control.

Flow cytometry. The transgenic mice were injected for five consecutive days with tamoxifen (2 mg/mouse) and maintained for seven days before sacrifice. Bone marrow cells were stained with purified antibodies recognizing lineage markers (CD3, CD4, CD8, Gr1, CD11b, B220, CD19 and Ter119) followed by an anti-rat secondary antibody (PerCP-Cy5.5, Biolegend). Subsequently, cells were stained for cKit (APC-Cy7, ebioscience) and Sca1 (APC, ebioscience). Spleen and blood cells were stained for lineage (Gr1 and CD11b; B220 and CD19; CD4 and CD8) followed by anti-rat secondary antibody. Stained cells were subjected to flow cytometry by FACS essentially as described15. Spleen cells were fixed in 4% paraformaldehyde subsequently to initial flow cytometry and stained for Alexa488 anti-GFP prior to a second FACS. Antibodies used are listed in Supplemental Table 1 and FACs data were analyzed in Flowjo after flow cytometry on a BD Fortessa.

Statistics. Means ± SEM for the number of observations are given where one mouse is one n. The number of experiments, i.e. occasions at which a number of mice (of both genotypes) were analysed, are also given. Comparisons of chance differences were done by Students’ t-test when compared with corresponding control or by ANOVA followed by Sidak’s or Dunnett’s multiple comparisons test as indicated using Prism 8 software.

References
1. Welsh, M., Jamalpour, M., Zang, G. & Akerblom, B. The role of the Src Homology-2 domain containing protein B (SHB) in beta cells. J Mol Endocrinol 56, R21–31, https://doi.org/10.1530/JME-15-0228 (2016).
2. Funa, N. S. et al. Dysfunctional microcirculation as a consequence of shb gene inactivation causes impaired tumor growth. Cancer research 69, 2141–2148, https://doi.org/10.1158/0008-5472.CAN-08-3797 (2009).
3. Akerblom, B. et al. Heterogeneity among KIP-1ag2 insulinosomas allows vascular endothelial growth factor-A independent tumor expansion as revealed by studies in Shb mutant mice: implications for tumor angiogenesis. Molecular oncology 6, 333–346, https://doi.org/10.1016/j.molonc.2012.01.006 (2012).
4. Zang, G. et al. Vascular dysfunction and increased metastasis of B16F10 melanomas in Shb deficient mice as compared with their wild type counterparts. BMC Cancer 15, 234, https://doi.org/10.1186/s12885-015-1269-y (2015).
5. Li, X. et al. Pro-tumoral immune cell alterations in wild type and Shb-deficient mice in response to 4T1 breast carcinomas. Oncotarget 9, 18720–18733, https://doi.org/10.18632/oncotarget.24643 (2018).
6. Gustafsson, K., Jamalpour, M., Trinh, C., Kharas, M. G. & Welsh, M. The Src homology-2 protein Shb modulates focal adhesion kinase signaling in a BCR-ABL myeloproliferative disorder causing accelerated progression of disease. Journal of hematology & oncology 7, 45, https://doi.org/10.1186/s11609-015-722-7-45 (2014).
7. Jamalpour, M. et al. Tumor SHB gene expression affects disease characteristics in human acute myeloid leukemia. Tumour Biol 39, 101028317720643, https://doi.org/10.1007/s13277-017-2064-3 (2017).
8. Jamalpour, M., Li, X., Gustafsson, K., Tyner, J. W. & Welsh, M. Disparate effects of Shb gene deficiency on disease characteristics in murine models of myeloid, B-cell, and T-cell leukemia. Tumour Biol 40, 1010428318771472, https://doi.org/10.1007/s13277-018-2714-z (2018).
9. Christoffersson, G. et al. Vascular adaptation to a dysfunctional endothelium as a consequence of Shb deficiency. Angiogenesis 15, 469–480, https://doi.org/10.1007/s10456-012-9275-z (2012).
10. Nikpour, M. et al. Shb deficiency in endothelial but not in leucocytes is responsible for impaired vascular performance during hindlimb ischaemia. Acta physiologica 214, 200–209, https://doi.org/10.1111/apha.12448 (2015).
11. Anneren, C., Lindholm, C. K., Kriz, V. & Welsh, M. The FRK/RAK-SHB signaling cascade: a versatile signal-transduction pathway that regulates cell survival, differentiation and proliferation. Current molecular medicine 3, 313–324 (2003).
12. Gustafsson, K. et al. Shb deficient mice display an augmented TH2 response in peripheral CD4+ T cells. BMC immunology 12, 3, https://doi.org/10.1186/1471-2172-12-3 (2011).
13. Gustafsson, K. et al. The Src homology 2 protein Shb promotes cell cycle progression in murine hematopoietic stem cells by regulation of focal adhesion kinase activity. Experimental cell research 319, 1852–1864, https://doi.org/10.1016/j.yexcr.2013.03.020 (2013).
14. Gustafsson, K., Willebrand, E. & Welsh, M. Absence of the adaptor protein Shb potentiates the T helper type 2 response in a mouse model of atopic dermatitis. Immunology 143, 33–41, https://doi.org/10.1111/imn.12286 (2014).
15. Zang, G. et al. aberrant association between vascular endothelial growth factor receptor-2 and VE-cadherin in response to vascular endothelial growth factor-a in Shb-deficient lung endothelial cells. Current molecular biology 25, 85–92, https://doi.org/10.1007/s10456-012-9275-z (2013).
16. Sorensen, L., Adams, R. H. & Gossler, A. DLL1-mediated Notch activation regulates endothelial identity in mouse fetal arteries. Blood 113, 5680–5688, https://doi.org/10.1182/blood-2008-08-174508 (2009).
17. Tang, Y., Harrington, A., Yang, X., Friesel, R. E. & Liaw, L. The contribution of the Tie2+ lineage to primitive and definitive hematopoietic cells. Genesis 48, 563–567, https://doi.org/10.1002/dvg.20654 (2010).
18. Chen, M. J., Yokomizo, T., Zeiger, B. M., Dzierzak, E. & Speck, N. A. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature 457, 887–891, https://doi.org/10.1038/nature07619 (2009).
19. Monvoisin, A. et al. VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium. Developmental dynamics: an official publication of the American Association of Anatomists 235, 3413–3422, https://doi.org/10.1002/dvdy.20982 (2006).
20. Okabe, K. et al. Neurons limit angiogenesis by titrating VEGF in retina. Cell 159, 584–596, https://doi.org/10.1016/j.cell.2014.09.025 (2014).
21. Giladi, A. et al. Single-cell characterization of haematopoietic progenitors and their trajectories in homeostasis and perturbed haematopoiesis. Nat Cell Biol 20, 836–846, https://doi.org/10.1038/ncb3156 (2014).
22. Kowalczyk, M. S. et al. Single-cell RNA-seq reveals changes in cell cycle and differentiation programs upon aging of hematopoietic stem cells. Genome Res 25, 1860–1872, https://doi.org/10.1101/gr.192237.115 (2015).
23. Olsson, A. et al. Single-cell analysis of mixed-lineage states leading to a binary cell fate choice. Nature 537, 698–702, https://doi.org/10.1038/nature19348 (2016).
24. Ulvmar, M. H., Martinez-Corral, I., Stanczuk, L. K. & Makinen, T. Pdgfrb-Cre targets lymphatic endothelial cells of both venous and non-venous origins. Genesis 54, 350–358, https://doi.org/10.1002/dvg.22939 (2016).
Acknowledgements
We are most grateful to Drs Taija Mäkinen, Uppsala University for suggestions and comments, Dr Karin Gustafsson, Department of Stem and regenerative Biology, Harvard University, Dr Raija Soininen, Transgenic Animals Facility, Oulu University, Finland for comments and generating the Shbflox mouse and Dr Ralf Adams, Max Planck Institute for Molecular Biomedicine, Münster, Germany for constructive critique. The Shb flox/flox mouse was generated by the support of the Intrafrontier I3 project under the EU contract Grant Agreement Number 312325 of the EC FP7 capacities specific programme. Additional support was provided by the Swedish Research Council (2016-01085), The Swedish Cancer Foundation (150880), Exodiab and the Family Ernfors fund.

Author Contributions
Q.H., X.L., K.S., Z.L., M.M., M.J., B.L. and M.W. did experimental work. V.K. and R.V. aided in generating the Shbflox mouse. H.Q., X.L., M.U. and M.W. designed the experiments. M.W. wrote the manuscript. All authors read and provided comments to the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-44039-z.

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019