Supplemental Materials and Methods:

Mice

Conditional *Bax* mice (Knudson et al, 1995), *Bak* null mice (Lindsten et al, 2000), *Tie2-cre* mice (Kisanuki et al, 2001), *caspase 8$^{-/-}$* mice (Salmena et al, 2003) and *Mlkl$^{-/-}$* mice (Murphy et al, 2013) have all been described previously. Animals were maintained on an inbred C57BL/6 background. The day of birth was termed P0. Mice of both genders were used at all ages.

Immunohistochemical staining

Primary antibodies were rat anti-PECAM1 (5 µg/ml, clone MEC 13.3, BD Pharmingen 553370, lot #7449), goat anti-collagen IV (40 µg/ml, Millipore AB769, lot #2118904), rabbit anti-cleaved (activated) caspase 3 (used at 1:50, clone 5A1, Cell Signaling Technologies 9664, lot #19) and rabbit anti-FLI1 (1 µg/ml, Abcam AB15289, lot #GR44225-3). Secondary antibodies were anti-rat IgG DyLight 488 (1:400, Jackson ImmunoResearch 711-485-152, lot #87833), anti-rat IgG Cy3 (1:400, Jackson ImmunoResearch 712-165-150, lot #108743), anti-goat IgG Alexa-Fluor 647 (1:400, Jackson ImmunoResearch 705-605-147, lot #105631), anti-rabbit IgG Cy3 (1:400, Jackson ImmunoResearch 711-165-152, lot #106486) and anti-rabbit IgG DyLight 488 (1:400, Jackson ImmunoResearch 711-485-152, lot #86173) as appropriate.

Image analysis

Apoptotic EC were defined as cleaved caspase 3^{+}/PECAM1^{+} cells enclosed by collagen IV in the *x*, *y*, *z* axis from confocal z-stack images. Quantification of cleaved (activated) caspase 3-positive ECs was performed manually using FIJI distribution of ImageJ software (Schindelin et al, 2012) from confocal z stacks of entire superficial layer at P6 and P14. Apoptotic cells within the remodelling zone were defined as having arterial or venous localisation determined by measuring distance to the nearest artery or vein. All quantification at P6 was quantified on whole retinal vasculature unless otherwise indicated. At P8, blood vessel area and EC density were quantified within a zone 150 µm wide on either side of radial arteries and 100 µm of radial veins. P14 and adult retinal vascular density and EC counts were quantified on independent maximum intensity z-projection images of the superficial, intermediate and deep

layers around each major vessel then data from each layer was combined. Vessel surface area measurements of retinas were quantified based on the collagen IV signal at P6 for whole retina overview and combined PECAM1 and collagen IV signal at P6, P8, P14 and adult for analysis specific to arterial and venous regions in maximum intensity projection images generated from confocal z stacks. Vasculature at the optic nerve head and residual hyaloid vasculature were manually masked and excluded from vessel surface area quantification where appropriate. Quantification was performed using the thresholding function and included removal of non-specific background using the "despeckle" filter. Radial outgrowth at P6 was quantified as the mean of 4 measurements from the centre of the retina to the edge of the sprouting front in different directions. Vessel regression at P6 was quantified on 4 plexus region segments per retina. Vessel regression was determined as a ratio of $PECAM1^+$ vessel segment length to collagen IV^+ vessel segment length. Vessel regression analysis was performed as follows: binary masks of PECAM1 and collagen IV channels were produced through auto thresholding and the use of various image and morphological filters. A collagen IV^+ $PECAM1^-$ vessel segment mask (regressing vessels) was generated by subtracting the PECAM1 channel mask from the collagen IV channel mask. Collagen IV^+ $PECAM^-$ vessel segment mask and the collagen IV channel mask were then skeletonized and their total vessel lengths measured. These steps were all implemented in a custom FIJI macro allowing for full automation of the data set. Capillary EC density was quantified on $FLI1^+$ signal found enclosed within a combined PECAM1 and collagen IV mask. Capillary vessel area and cell density quantification was performed in a semi-automated fashion through custom written macros in the FIJI distribution of ImageJ software (Schindelin et al, 2012). In major arteries and veins ECs were quantified manually on $FLI1^+$ signal found enclosed within PECAM1 signal. Quantification of major vessel area was performed using Adobe Photoshop for manual tracing of $PECAM1^+$ signal followed by quantification of area using FIJI distribution of ImageJ software (NIH, Bethesda, MD, USA) from confocal z-stack projections. Veins were quantified on vessel segments 550 µm long, measured from the T-junction at the retina margin. Arteries were quantified on major vessel segments contained within an imaged region of at least 450 µm x 860 µm. Proliferating ECs were defined as $EdU^+/FLI1^+/PECAM1^+$ cells in maximum intensity projection images and quantified in an automated fashion using a custom written macro using FIJI software. Vessel side branches were defined as continuous

PECAM1[+] vessels connected to the major artery being quantified. At P6, side branches were quantified on 4 arteries per retina, each 1000 µm along the artery from the optic nerve head to the periphery. At P8, side branches were quantified along the entire length of a major artery. Secondary sprouts at P8 were identified as vertical sprouts in confocal z-stack images. Capillary vessel width analysis was determined blinded to genotype by dividing total vessel area (thresholded on PECAM1 signal) by total vessel length (determined from skeletonized PECAM1 signal) in a semi-automated, custom written macro in the FIJI distribution of ImageJ software (Schindelin et al, 2012). Major vessel width was calculated as above in a manual fashion. Adult retina venous capillary vasculature was quantified in 3D using the 3D visualisation module of Imaris (Bitplane). The 3D surface was created using PECAM1 signal. All vessels greater than 7 µm in diameter were considered to be non-capillary vessels and excluded from analysis.

Supplemental References:

Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson, J. A. and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol*. **230**, 230-242.

Knudson, C. M., Tung, K. S., Tourtellotte, W. G., Brown, G. A. and Korsmeyer, S. J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*. **270**, 96-99.

Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K. et al. (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell*. **6**, 1389-1399.

Murphy, J. M., Czabotar, P. E., Hildebrand, J. M., Lucet, I. S., Zhang, J. G., Alvarez-Diaz, S., Lewis, R., Lalaoui, N., Metcalf, D., Webb, A. I. et al. (2013). The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity*. **39**, 443-453.

Salmena, L., Lemmers, B., Hakem, A., Matysiak-Zablocki, E., Murakami, K., Au, P. Y., Berry, D. M., Tamblyn, L., Shehabeldin, A., Migon, E. et al. (2003). Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity. *Genes Dev*. **17**, 883-895.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B. et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods*. **9**, 676-682.
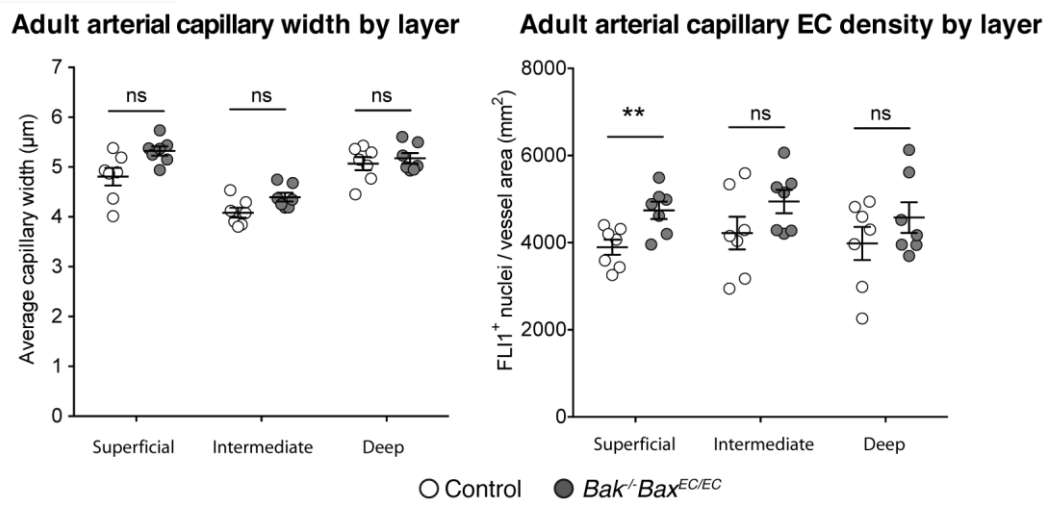
Supplemental Figures
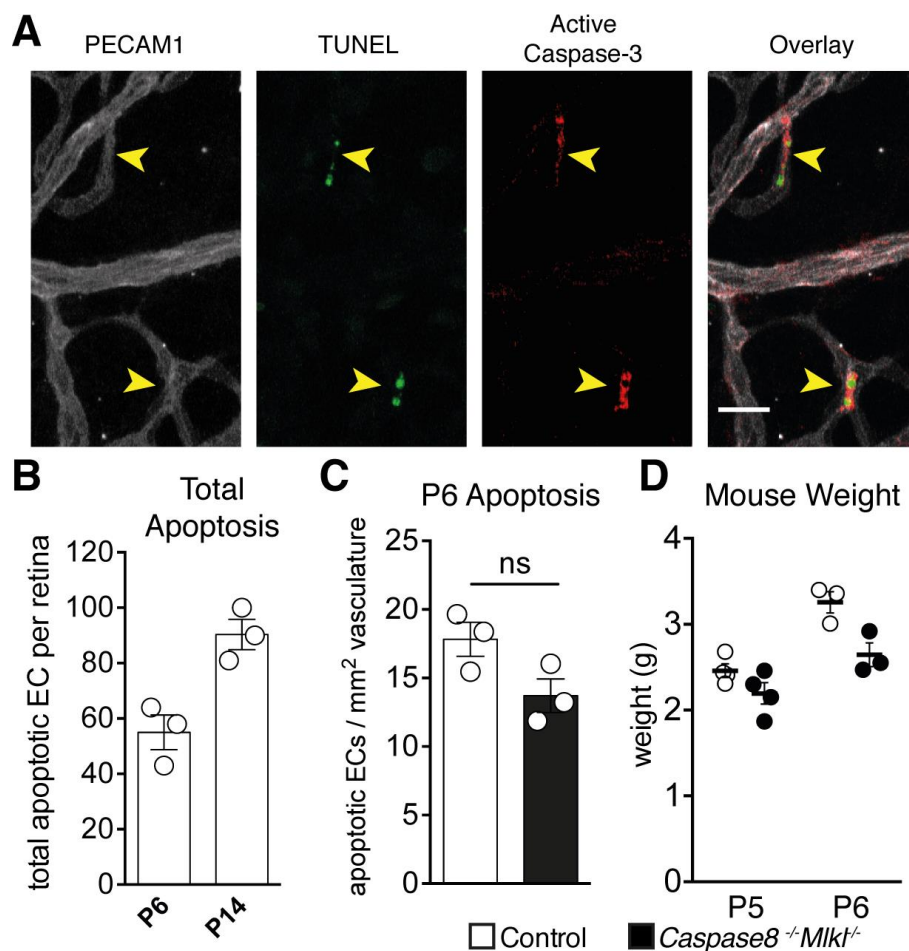
Supplemental Figure 1: Apoptosis quantification in neonatal retina.

(A) Representative image of apoptosis in P6 retinal vasculature prepared by trypsin digest method. Yellow arrowhead indicates apoptotic EC. PECAM1 (grey), TUNEL (green) and active caspase 3 (red). Scale bar=20 µm. (B) Absolute number of apoptotic EC per retina at P6 (n=3) and P14 (superficial vascular layer only, n=3) in WT mice. (C) Quantification of total apoptotic EC relative to vessel area in P6 *Caspase 8$^{-/-}$Mlkl$^{-/-}$* (n=3) and littermate controls (*Caspase 8$^{+/-}$Mlkl$^{-/-}$*, n=3). (D) Whole animal weight of control (*Caspase 8$^{+/-}$Mlkl$^{-/-}$*, white circles) and *Caspase 8$^{-/-}$Mlkl$^{-/-}$* (black circles) littermates analysed at P5 and P6. ns, p>0.05, two-tailed Student's *t*-test. All data are presented as mean ± SEM.
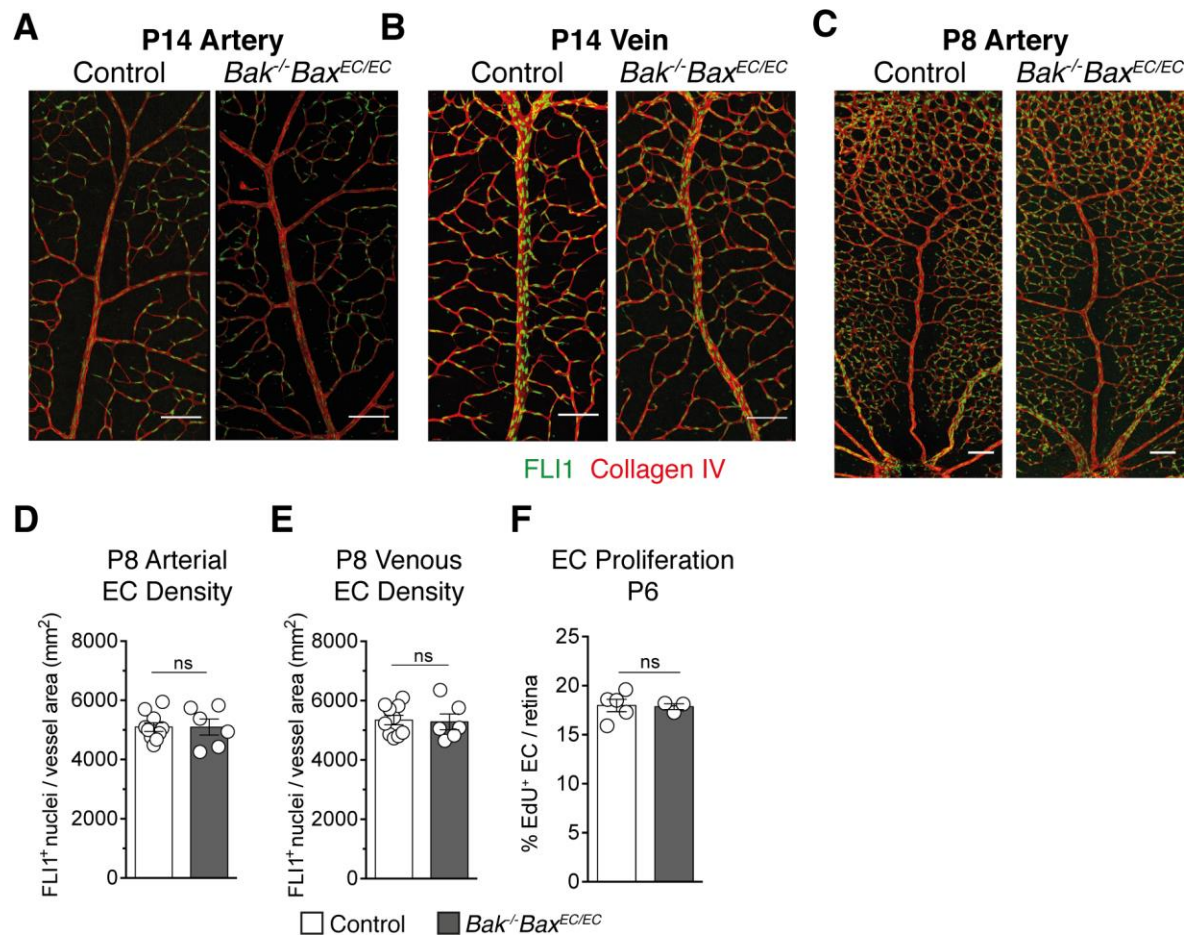
Supplemental Figure 2: Arterial retinal vasculature

(A) Representative images of EC density of P14 arterial region and (B) P14 vein region in control (left panel) and $Bak^{-/-}Bax^{EC/EC}$ (right panel) retinas. Collagen IV (red) and FLI1 (green). Scale bar=100 µm. (C) Representative images of P8 arterial regions from control (left panel) and $Bak^{-/-}Bax^{EC/EC}$ (right panel) retinas. Collagen IV (red) and FLI1 (green). Scale bar=100 µm. (D) EC density in arterial region and (E) venous region at P8 quantified on FLI1 and combined collagen IV and PECAM1 signal for control (n=10) and $Bak^{-/-}Bax^{EC/EC}$ (n=6). (F) EC proliferation quantified on whole P6 retina for control (n=5) and $Bak^{-/-}Bax^{EC/EC}$ (n=3). All data are presented as mean ± SEM. ns: $p>0.05$, two-tailed Student's t-test.

Supplemental Figure 3: Capillary width in the arterial region.

Quantification of adult arterial capillary width and EC density in the superficial, intermediate and deep layers. Data were collected at 7 weeks (control, n=7 and $Bak^{-/-}Bax^{EC/EC}$, n=7). All data are presented as mean ± SEM. **p<0.01; two-tailed Student's *t*-test with Holm-Sidak correction for multiple tests.