Supplementary Methods

Cell cultures and reagents. HUVECs (human umbilical vein endothelial cell), HIAECs (human iliac artery endothelial cell) were obtained from Cambrex Clonetics Cell Systems. SFs (human skin fibroblast cells, Detroit 551) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Antibody specific for cleaved Notch1 (NICD) was used for detecting Notch activation (Cell Signaling Technology).

Generation of phage antibody. Synthetic phage antibody libraries were built on a single framework (humanized anti-ErbB2 antibody, 4D5) by introducing diversity within the complementarity-determining regions (CDRs) of heavy and light chains (Lee et al., 2004; Liang et al., 2006). Plate panning with naïve libraries was performed against His-tagged human Dll4 (amino acid 1-404) immobilized on maxisorp immunoplates. After four rounds of enrichment, clones were randomly picked and specific binders were identified using phage ELISA. The resulting hDll4 binding clones were further screened with His-tagged murine Dll4 protein to identify cross-species clones. For each positive phage clone, variable regions of heavy and light chains were subcloned into pRK expression vectors that were engineered to express full-length IgG chains. Heavy chain and light chain constructs were co-transfected into 293 or CHO cells, and the expressed antibodies were purified from serum-free medium using protein A affinity column. Purified antibodies were tested by ELISA for blocking the interaction between Dll4 and rat Notch1-Fc, and by FACS for binding to stable cell lines expressing either full-length human Dll4 or murine Dll4. For affinity maturation, phage libraries with three different combination of CDR loops (CDR-L3, -H1, and –H2) derived from the initial clone of interest were constructed by soft randomization strategy so that each selected position was mutated to a non-wild type residue or maintained as wild type at about 50:50

frequency (Liang et al., 2006). High affinity clones were then identified through four rounds of solution phase panning against both human and murine His-tagged Dll4 proteins with progressively increased stringency.

RNA interference. SMARTpool small interfering RNA (siRNA) duplexes targeting human Dll4 and Si Control Non-Target siRNA #2 were purchased from Dharmacon. Transfection of siRNR duplexes (50 nM) was done with HUVECs at 40% confluency using Optimem-1 and Lipfectamine 2000 (Invitrogen). FACS analysis was done 48 hr after siRNA transfection.

Notch ligand : Notch blocking ELISA. 96-well microtiter plates were coated with recombinant rat Notch1-Fc (rrNotch1-Fc, R&D Systems) at 0.5 μg/ml. Conditioned medium containing Dll4-AP (amino acid 1-404 of Dll4 fused to human placenta alkaline phosphatase) was used in the assay. To prepare conditioned medium, 293 cells were transiently transfected with plasmid expressing Dll4-AP with Fugen6 reagent (Roche Molecular Biochemicals). Five days posttransfection, the conditioned medium was harvested, filtered and stored at 4°C. Purified antibodies titrated from 0.15 to 25 μg/ml were preincubated for 1hr at room temperature with Dll4-AP conditioned medium at a dilution that conferred 50% maximally achievable binding to coated rrNotch1-Fc. The antibody/Dll4-AP mixture was then added to rrNotch1-Fc coated plate for 1hr at room temperature, after which plates were washed several times in PBS. The bound Dll4-AP was detected using 1-Step PNPP (Pierce) as substrate and OD 405 nm absorbance measurement. Identical assay was performed with Dll1-AP (human Dll1, amino acid 1-445). Similar assays were carried out with purified Dll4-His (C-terminal His-tagged human Dll4, amino acid 1-404) and Jag1-His (R& D system). The bound His-tagged ligands was detected with mouse anti-His mAb (1 μg/ml, Roche Molecular Biochemicals), biotinylated goat-anti-mouse (Jackson ImmunoResearch) and Streptavidin-AP (Jackson ImmunoResearch).

HUVEC growth inhibition. 12-well tissue culture plates (Falcon #353225) were coated with purified Dll4-His (C-terminal His-tagged human Dll4, amino acid 1-404, 0.5 ml/well, 5 ug/ml) in coating buffer (50 mM carbonate, pH9.6). Wells were washed in PBS before 4X 10^3 cells/well were seeded in triplicate. Cell proliferation was measured with Dojindo Cell Counting reagent (Dojindo, Kit-8 #CK04) on day 4. In some experiments, 0.08 μM DBZ was used to block Notch activation.

RNA extraction and Real-time quantitative RT-PCR. Extraction of total RNA from HUVECs in 2-D culture was done using RNeasy Mini Kit (Qiagen) as per instructions of the manufacturer. To extract total RNA from HUVECs growing in fibrin gels, fibrin gels were treated with 10X trypsin-EDTA (Gibco) for 5 min to remove the top layer fibroblasts, followed by neutralization with 10% FBS in PBS. The gel clots were then removed from tissue culture wells and subjected to centrifugation (10K for 5 min) in microtubes to remove excessive fluid. The resulting gel "pellets" were lysed with lysis buffer (RNeasy Mini Kit), and further processed as with HUVECs in 2-D culture. The quality of RNA was assessed using RNA 6000 Nano Chips and the Agilent 2100 Bioanalyzer (Agilent Technologies). Real-time quantitative RT-PCR reactions were done in triplicate using 7500 Real Time PCR System (Applied Biosystems). Human GAPDH was used as reference gene for normalization. The expression levels are expressed as the mean (±SEM) fold mRNA changes relative to control from 3 separate determinations. The primer and probe sequences for VEGFR2 and GAPDH are available upon request.

Mouse neonatal retina study Neonatal CD1 mice from the same litters were injected i.p. with PBS or YW152F (10 mg/kg) on P1 and P3. Eyes were collected on P5, and fixed with 4% PFA in PBS overnight. The dissected retinas were blocked with 10% goat serum in PBST for 3 hrs, then incubated overnight with primary antibodies. The primary cocktail included biotinylated isolectin B4 (25 μg/ml, Bandeiraea simplicifolia; Sigma), and one of the following: rabbit anti-mouse Ki67 (1:1, ready-to-use, clone Sp6, Lab

Vision), or mouse Cy3-conjugated anti-alpha SMA (1:2000, Sigma-Aldrich), with 10% serum in PBLEC (1% Triton X-100, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM $MnCl_2$, in PBS pH6.8). Retinas were then washed in PBST, and incubated overnight with secondary antibody combination of Alexa 488 streptavidin (1:200; Molecular Probes) and Cy3-anti-rabbit IgG (1:200; Jackson ImmunoResearch). After staining was completed, retinas were post fixed with 4% PFA in PBS. All overnight incubations were done at 4°C. Images of flat mounted retinas were captured by confocal fluorescence microscopy..

Tumor models. Beige nude female mice of 8- to 10-week-old were used. To obtain subcutaneous tumors, mice were injected with 0.1 ml cell suspension containing 50% matrigel (BD Bioscience) into the right posterior flank. $5X10^6$ human colon cancer HM7 cells, $10X10^6$ human colon carcinoma Colo205 cells, $10X10^6$ human lung carcinoma Calu6 cells, $10X10^6$ human lung carcinoma MV-522 cells, $10X10^6$ mouse leukemia WEHI-3 cells, or $10X10^6$ mouse lymphoma EL4 cells were injected into each mouse. For human melanoma MDA-MB-435 model, mice were injected into the mammary fat pad with 0.1 ml cell ($5X10^6$) suspension containing 50% matrigel. Anti-Dll4 was administered via i.p. (5 mg/kg body weight, twice weekly). In some studies, tumor-bearing mice were treated with anti-VEGF (clone B20-4.1, 5 mg/kg, twice weekly) alone or in combination with anti-Dll4. The tumor growth was quantitated by caliper measurements. Tumor volume (mm^3) was determined by measuring the length (l) and width (w) and calculating the volume ($V = lw^2/2$). 10 to 15 animals were included in each group.

Image quantification Images of fluorescent staining were analyzed using National Institutes of Health (NIH) Image J.

Statistical analysis Group differences were evaluated by Student's *t* test. P values less than 0.05 were considered significant.