Expression Constructs
N1-GFP vector was from David Cheresh, the University of California, San Diego. HA-tagged V14Rho and N19 Rho in pcDNA 3.1 were from Martin Schwartz, University of Virginia. pEF-BOS-HA/Csk-wt, pEF-BOS-HA/Csk-S364A and pEF-BOS-HA/Csk-S364C were from Kjetil Tasken, University of Oslo, Norway. Murine PKA catalytic subunit (PKAcat) and mutationally inactive PKA (dnPKA) cDNAs (from Susan Taylor, University of California, San Diego and G. Stanley McKnight, University of Washington, Seattle, respectively) were subcloned into topoTA-pcDNA 3.1V5/His (Invitrogen, Carlsbad, CA).

Transgene expression and analysis
Transgene expression was measured by blotting with anti-V5 (Invitrogen, Carlsbad, CA), anti-HA (Cell Signaling Technology, Inc., Beverly, MA), anti-myc (Cell Signaling Technology, Inc., Beverly, MA) or anti-GFP antibodies (Invitrogen, Inc., Carlsbad, CA). Transfection efficiency was 70-90%. Cells were also infected with CA10 retroviruses expressing SrcY529A, Src Δ251 or Csk, from Phil Soriano, University of Washington, Seattle. Cells were cultured for 48h prior to use. Statistical analyses were performed using Student's t-test.

Endothelial cell tube formation
300µl of growth factor reduced Matrigel were added to 6 wells of a 24-well plate and allowed to solidify at 37°C for 1h. 40,000 HUVECs were added to each well. After 3h, medium was removed and replaced with 800µl of endothelial growth medium, growth medium with 20µg/ml forskolin or vehicle control (2% DMSO). Plates were incubated at 37°C for 24h. Cells were fixed in 1% paraformaldehyde at 4°C. The number of branchpoints per field were counted and averaged. The data represents the mean of three separate experiments, two replicates per experiment. To count the number of cells per branchpoint, cells were stained with 1µM DAPI and eight branchpoints were imaged at 10x magnification. Number of DAPI stained nuclei were counted per branchpoint and averaged. The data shown is the mean of three separate experiments. The number of total nuclei per field was maintained the same in all treatment groups and only the number per branchpoint varied. Matrigel cultures were photographed using a Nikon Eclipse TE300 microscope, 5MHz Roper Scientific Camera (from Princeton Instruments), under illumination from a Xenon 75 Watt lamp with a Nikon Plan Fluor 10x/0.3 objective. Images were analyzed using Metamorph imaging software.

Video microscopy and data analysis
HUVECs cultured on 2.5-cm vitronectin coated glass coverslips 37°C, 5% CO_2, pH 7.4 with or without 20 µM forskolin/500 µM dibutyryl cAMP or vehicle control (0.4% DMSO) were viewed at 200X magnification in brightfield (Zeiss, Oberkochen, Germany). Images were recorded on a CCD camera using IP Lab software (Webster, NY) at 1-minute intervals for 60 min. Cell shape and migration distances were quantified using IMAQ Vision Builder software (National Instruments Corporation, Austin, TX). Measurements and calculations were made on eleven cells every ten minutes for each treatment and the mean +/- standard error of measurement was determined. Distance

migrated was measured as the average distance cellular nuclei moved over 10 minute intervals +/- S.E.M.

Cell polarity determination

The polarity of cells was determined by two methods: the Heywood Circularity Factor and the Ellipse ratio. The Heywood Circularity Factor is the ratio of the perimeter of a cell to the perimeter of a circle with the same area, according to the following formula: Particle perimeter divided by 2 (sq root of π X particle area). The closer the shape of a particle is to a circle, the closer the Heywood circularity factor is to 1. The Ellipse ratio is the ratio of the major axis of an ellipse divided by the minor axis of an ellipse. The more elongated an ellipse, the higher the ellipse ratio; the closer the ellipse comes to a circle, the closer the ellipse ratio is to 1. Cell shape was calculated using IMAQ Vision Builder software (National Instruments Corporation, Austin, TX).

Cell culture and migration assays

Human umbilical vein endothelial cells (HUVEC) were grown in Endothelial Growth Medium (containing 2% fetal bovine serum, bFGF and VEGF; Clonetics, San Diego, CA). Cell migration assays on ECM substrates were performed as previously described[19]. Haptotaxis type cell migration studies were performed using vitronectin coated Boyden chamber type 8 µm inserts. Endothelial cell migration was measured after 4 hours at 37ºC by crystal violet staining and quantification of the number of cells migrated to the underside of the insert per 100X microscopic field. Statistical significance was determined using Student's t-test. Each study was performed at least four times.

Evans Blue Vascular Permeability Assay

Anesthetized mice (n=10) received 100 µL of 2% Evans blue dye in PBS injected via the tail vein. Immediately thereafter, mice were injected in the left femoral vein with 100µM forskolin in a final volume of 100µL or an equal volume of diluent (2.0% DMSO) to achieve an initial serum concentration of 5 µM. After 20 minutes, the right ear was injected intradermally with 25 µL of saline solution containing 50ng VEGF, whereas the left ear was injected with saline only. Animals were euthanized after 10 minutes, and skin patches were removed and soaked in formamide (400 µL) overnight at 55°C. The levels of extracted Evans blue dye were measured by absorbance at 600 nm in a 96-well plate. Ears were also digitally imaged at 1X and 10X using a handheld personal digital camera. These studies were performed 3 times.

In vivo Silver Nitrate Staining

6 week old mice (n=6) were injected in the left femoral vein with 100µM forskolin in a final volume of 100µL or an equal volume of diluent (2% DMSO) to achieve an initial serum concentration of 5 µM. After 20 minutes, animals were sacrificed and the left ventricle of the heart was perfused sequentially with 5% dextrose, 0.25% AgNo3, 1% NH4Br, and 3% CoBr each for 1 min, followed by 10mL neutral buffered formalin. The thoracic aorta and the left common carotid arteries were excised and immersed in formalin 1 hour at 4 degrees. Aorta were incised longitudinally with iridectomy scissors and mounted flat on a glass slide with the endothelial surface up. Carotid arteries were embedded in OCT for cryosections. Analysis of silver nitrate localization was performed

by high-powered light microscopy using a Nikon Eclipse E600 microscope with Spot RT Color Camera (from Diagnostic Instruments) and Nikon PlanApo 20X/ and 100X/ objectives. These studies were performed 2 times.

In ovo chick chorioallantoic membrane model of angiogenesis
Angiogenesis assays were performed in ten day old embryonated chicken eggs as described (ref). Embryos were stimulated with filter paper disks saturated with 30 ng bFGF or VEGF. 24 hours later, 10µM PTHrP, CGRP or isoproterenol were applied to the saturated filter disc. bFGF-stimulated CAMs were also transfected by application of 4 µg N1–GFP or pcDNA3 dnPKA plasmid DNA. CAMs were harvested on the fourth day. Vessels were visualized with an Olympus SZH10 stereo microscope with DF PlanApo 1X objective and a SPOT RT Color Camera with SPOT image capture software (Diagnostic Instruments, Sterling Heights, MI). Vessel branch points were quantified within the 5 mm disc area. At least ten embryos are used per treatment group. Data is expressed as average number of branch points per treatment group.

Zebrafish vascular development
Transgenic Tg(fli1:EGFP) zebrafish embryos in which GFP is expressed in endothelial cells were purchased from The Zebrafish Model Organism Database (www.zfin.org) as reported by Lawson and Weinstein. Adult fish and embryos were maintained according to Zebrafish: A Practical Approach (Nusslein-Volhard and Dahm). Zebrafish maintenance and procedures were approved by the University of California at San Diego institutional animal care and use committee. Transgenic Tg(fli1:EGFP) zebrafish embryos were incubated without or with increasing concentrations of forskolin or the PKA inhibitor H89. One, five or ten µg/ml forskolin or 50 ng/ml H89 (final concentration) was added to optically transparent Tg(fli1:EGFP) zebrafish larvae at 18hpf (when intersegmental blood vessels start to form). Twenty-four hours later, trunk vascular was imaged at 200 and 600x magnification using a Nikon C1-si confocal microscope. Blood vessel morphology was recorded by fluorescence microscopy, images were 3D rendered and analyzed using Imaris software.

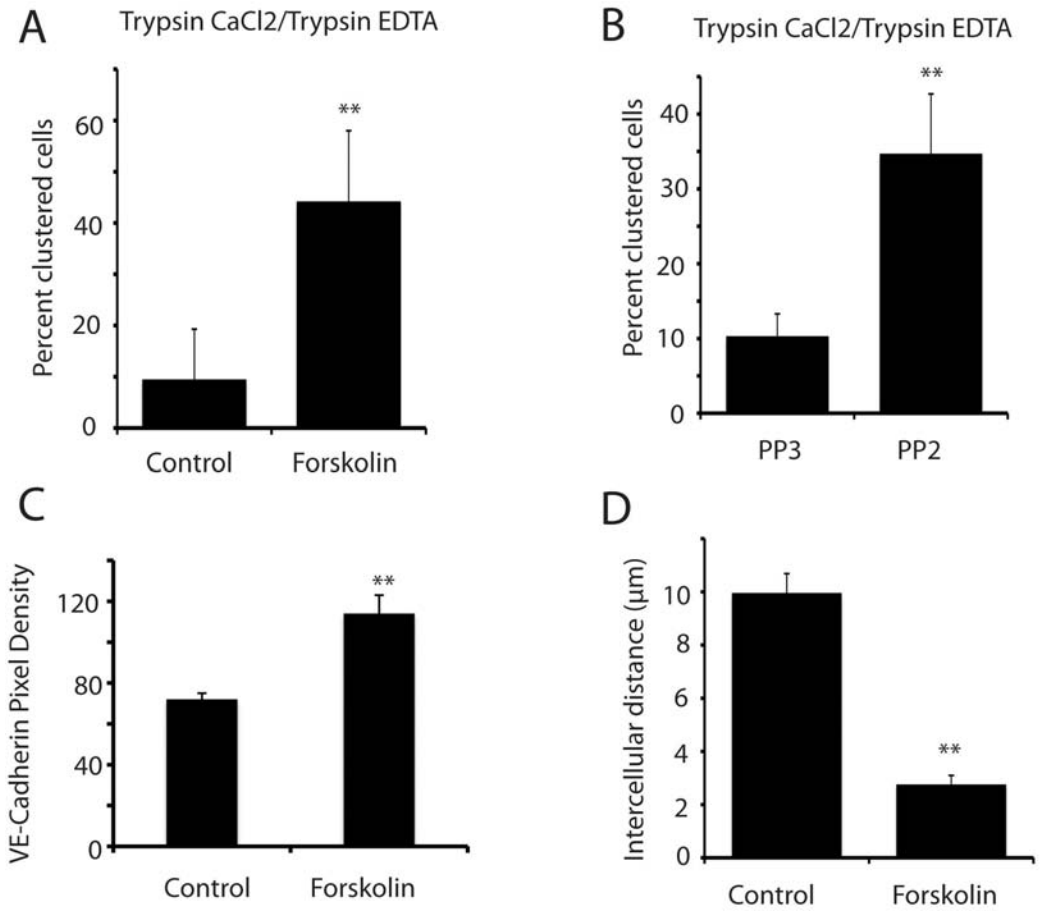

Figure S1. Intercellular Adhesion is stimulated by PKA activation
(A-B) Percent cell–cell adhesion in endothelial cells treated with forskolin (A), PP2 (B) or control. Endothelial cell monolayers were dissociated with trypsin in the presence of calcium or in the presence of EDTA and the ratio of clustered cells in the presence of CaCl2 to total cells in the absence of Ca2+ (in EDTA) was calculated. (C) Density of VE-Cadherin in cell junctions of control or forskolin treated cells. (D) Intercellular distances in endothelial cell cultures treated with or without forskolin were measured using digital calipers using Metamorph image processing software.

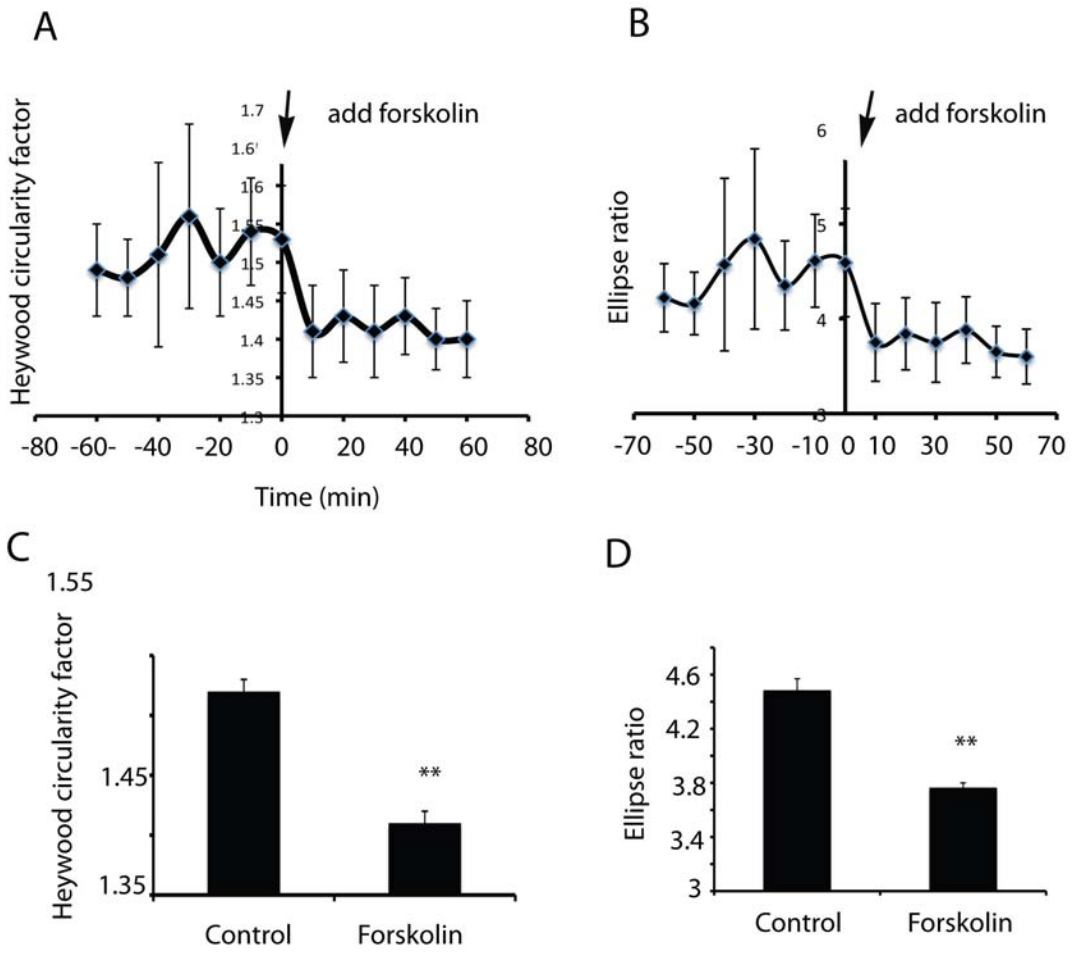

Figure S2. Quantification of the effect of PKA activation on cell polarity
The polarity of cells was determined by two methods: the Heywood Circularity Factor (A,C) and the Ellipse ratio (B,D). (A,B) Time courses of Heywood Circularity factor and Ellipse ratio. (C,D) Mean Heywood and Ellipse ratio over a one hour period. The Heywood Circularity Factor is the ratio of the perimeter of a cell to the perimeter of a circle with the same area, according to the following formula: Particle perimeter divided by [2 (sq root of π X particle area)]. The closer the shape of a particle is to a circle, the closer the Heywood circularity factor is to 1. The Ellipse ratio is the ratio of the major axis of an ellipse divided by the minor axis of an ellipse. The more elongated an ellipse, the higher the ellipse ratio; the closer the ellipse comes to a circle, the closer the ellipse ratio is to 1. Cell shape was calculated using IMAQ Vision Builder software (National Instruments Corporation, Austin, TX).