Supporting Information

Ju et al. 10.1073/pnas.1001075107

SI Materials and Methods

Immunofluorescence and BrdU Double-Staining. Cells were grown on glass coverslips in six-well plates. Twenty-four to 48 h after transfection, cells were labeled with 10 μM BrdU for 1 h and then washed with PBS and fixed with 3.7% (vol/vol) formaldehyde. After permeabilization with 0.25% Triton in PBS, cells were blocked with 1% BSA, followed by staining with anti-HA or anti-FLAG antibodies and the corresponding secondary antibodies. Before staining with rat anti-BrdU antibody and anti-rat secondary antibody, cells were fixed again and treated with 4 N HCl/1% Triton X-100 to denature DNA and extract histones. For anti-Glu-tubulin staining, cells were fixed with methanol for 10 min, washed with PBS, and then blocked and stained as described above. All images were collected using a Bio-Rad 1024 laser-scanning confocal microscope. Orientation of Glu-tubulin was scored as being predominantly perinuclear or as displaying a perinuclear-to-lamellipodia orientation. The percentages of these cells with this orientation over the total number of cells were reported. Photoshop (Adobe) and ImageJ 1.42q (NIH) were used to adjust contrast and brightness only in parallel with control images.

PCR and Plasmid Construction. Primers designed to amplify NDAAM1, DAD, and CDAAM1 as well as full-length DAAM1 are described in Table S1. The NDAAM1, CDAAM1, and DAD fragments were amplified with primers NDAAM1F and NDAMBAM, CDAMNHE and CDAMNHE, and DMDADN5 and DMDADC3, respectively. The amplified PCR products were cloned into a gateway entry vector, pENTR/SD/D (Invitrogen), according to the manufacturer's instructions. The inserts on the entry vector, except the DAD fragment, were transferred to an expression vector, $pCS2^{+}$, which has been modified to make it Gateway-compatible by using the Gateway Vector Conversion Reagent System (Invitrogen). The DAD fragment was inserted into pEGFPC1 between EcoRI and BamHI.

Migration Assays and Tube/Network Formation Assay on Matrigel. Migration assays were performed in 24-well plates, BD Biocoat Control Inserts (catalog no. 354578; BD Biosciences), with 2 ng/mL VEGF for 6 h (1, 2). MPE cells were harvested, diluted to 5×10^4 cells/mL, and suspended in serum-free media. Cells (0.5 mL) were then added to each well in the upper chamber. Serum-free medium (0.75 mL) supplemented with VEGF (0.2 μg/mL; Sigma) was added to the lower chamber. The assembled chemotaxis chamber was incubated for 6 h at 37 °C with 5% CO_2 to allow cells to migrate through the filter. Nonmigrated cells on the upper surface of the filter were removed by gentle scraping with a PBS-doused sterile cotton-tipped applicator. After the removal of nonmigrated cells, cells were viewed directly under a fluorescent microscope [100× field of view (FOV), 5 FOVs per well, $n = 3$ wells per test condition]. Matrigel assays were performed as described in the product literature (BD BioSciences) (3).

1. Yamaguchi N, et al. (1999) Endostatin inhibits VEGF-induced endothelial cell migration and tumor growth independently of zinc binding. *EMBO J* 18:4414–4423.
2. Rousseau S, et al. (2000) Vascular endothelial growth factor (VEGF)-driven actin-based motility is mediated by VEGFR2 and requires concerted activation of stress-activated protein kinase 2 (SAPK2/p38) and geldanamycin-sensitive phosphorylation of focal adhesion kinase. *J Biol Chem* 275:10661–10672.
3. Nicosia RF, Ottinetti A (1990) Modulation of microvascular growth and morphogenesis by reconstituted basement membrane gel in three-dimensional cultures of rat aorta: A comparative study of angiogenesis in matrigel, collagen, fibrin, and plasma clot. *In Vitro Cell Dev Biol* 26:119–128.

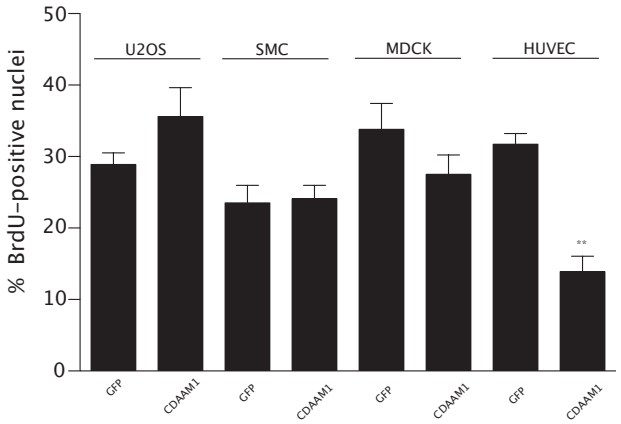

Fig. S1. CDAAM1 expression specifically inhibited endothelial cell proliferation. U2OS cells, rat SMCs, MDCK cells, and HUVECs were adenovirally transduced to express either GFP or CDAAM1. SMC and MDCK represent cell lines that form contiguous anisotropic tissues in vivo similar to endothelial cells. The antiproliferative effects of CDAAM1 are therefore very narrowly applicable to endothelial cells (**$P < 0.01$ by the Student's t test).

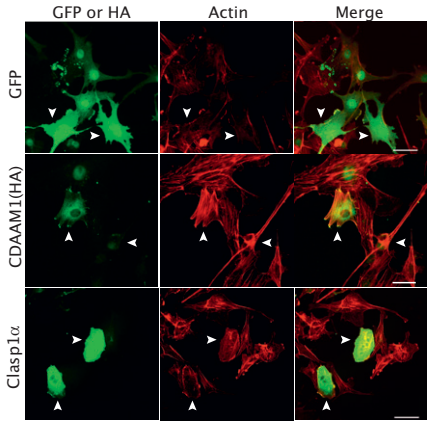

Fig. S2. CLASP1α expression did not induce actin polymerization in MPE cells. MPE cells were transfected with GFP, CDAAM1, or GFP-CLASP1α for 24 h and were then stained for actin with phalloidin, GFP, and GFP-CLASP1α with an anti-GFP antibody. (Scale bars = 50 μm.)

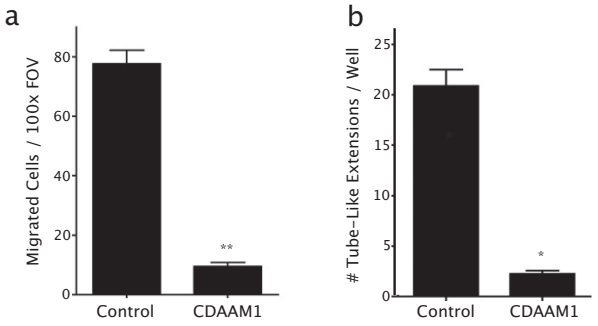

Fig. S3. CDAAM1 inhibited MPE cell migration and capacity to form coordinated network structures on Matrigel. (*A*) CDAAM1 inhibited MPE cell migration. MPE cells were transfected with or without CDAAM1 for 24 h. Cells were then subcultured into BD Biocoat Control Inserts (24-well plate format), and the migration assay was performed as described in *Materials and Methods*. (*B*) CDAAM1 inhibits the capacity of MPE to form tubes. MPE cells were transfected with CDAAM1 and used to perform network formation assays as described in *Materials and Methods*. Error bars represent SEM, with $n = 8$ per group (*$P < 0.05$; **$P < 0.01$ by the Student's t test).

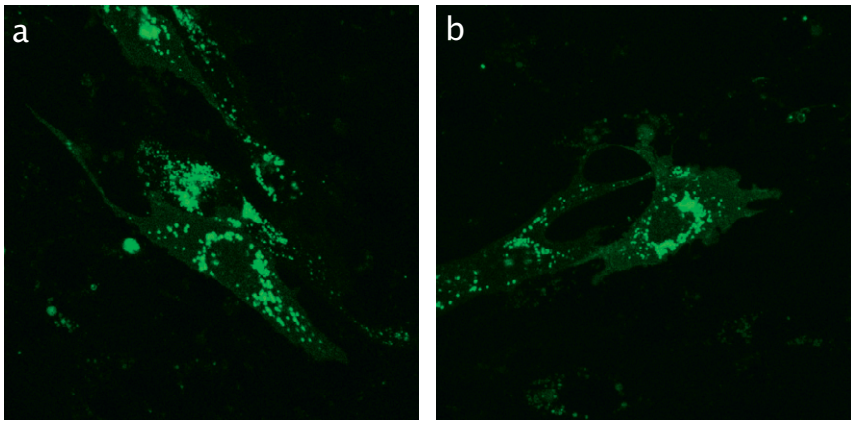

Fig. S4. Fz4-GFP in endothelial cells. (*A*) Mouse MPE cells transfected with Fz4-GFP. (*B*) Fzd4-GFP MPE cells after a 2-h treatment with Wnt5a-conditioned media. No obvious change in Fzd4 distribution was noted after treatment with Wnt5a.

Table S1. PCR sequences used for the generation of DAAM1 mutants

Primer	Sequence
NDAAM1F	5′-CACCATGGATTACAAGGATGACGACGATAAGGCCCCAAGAAAGAGAGGTGG-3′
NDAMBAM	5′-CCGCCATCCTTAAGCGTAGTCTGGGACGTCGTATGGGTAAACCAGGCACACGGCGCCCAA-3′
CDAMNHE	5′-CACCGCTAGCGCCACCATAAAGAGAAACTTGAAAAGGAG-3′
CDAMBAM	5′-CCG GGATCCTTAAGCGTAGTCTGGGACGTCGTATGGGTAGAAATTAAGTTTTGTGATTGG-3′
DMDADN5	5′-CACCGAATTCTATGCTAGCAGAAGCTAAAGACCTG-3′
DMDADC3	5′-CCTGGATCCTTACTTATCGTCGTCATCCTTGTAATCCATGAAATTAAGTTTTGTGATTGGT-3′