SUPPLEMENTAL MATERIAL

Mice

Mice homozygous for a conditional allele of synectin *(gipc1)* were generated by flanking exon 2 with *loxP* sites. Exon 2 contains the start codon for the synectin gene. A bacterial artificial chromosome (BAC) clone, containing the synectin genomic region, was obtained by screening the RPCl-22 129/SvEvTacBr BAC library (Genome Resource Facility at the Hospital for Sick Children). A 10kb *BstB I-Mfe I* fragment containing exon 2 was subcloned into pSMART LC Kan (Lucigen) plasmid vector for the construction of the targeting vector. A replacement targeting vector was constructed with the insertion of a loxP site into *BsrB I* restriction site upstream of exon 2, and the insertion of an Frt/loxP flanked neomycin cassette positioned 5' of exon 2, into the *PshAI* site downstream of exon2. The integrity of the construct was confirmed by restriction digestion and DNA sequencing. Using standard techniques previously described {Lathrop, 2010 #686}, targeted 129SvIMJ embryonic stem (ES) cells were generated and C57BL/6/129 SvIMJ mouse lines were developed from four positive ES cell clones. The presence of the targeted allele was identified by PCR and Southern blot. Three chimeras were born and bred to C57BL/6 mice, and F1 agouti offspring mice were genotyped by PCR to validate germline transmission.

The Frt-flanked neo gene was deleted *in vivo* by breeding gipc1$^{flox/+}$ with the FLPeR (flipper) mice {Farley, 2000 #687}. Offspring were backcrossed seven times into C57/Bl6 and genotyped by PCR. The *gipc1*^{flox} allele was genotyped with primer pair Flox-*BsrBI-Fwd*: 5'AAGCAAAGGACAGTGCCAGT3', *BsrBI Rev*: 5'GGACCCACATACCTAGACTGC3'. Exon 2 was deleted by crossing the *gipc1*$^{flox/flox}$ mice with the Sm-MHC-Cre {Xin, 2002 #294} , Tie2-Cre {Koni, 2001 #296}, Cdh5(PAC)-CreERT2 {Wang, 2010 #1227} and Pdgfbicre/ERT2{Claxton, 2008 #1247} mice. *Gipc1*$^{flox/flox}$ animals were crossed with Sm-MHC-Cre mouse line and the progeny was bred to produce mice without synectin expression in smooth muscle cells (Syn^{SMKO}). To produce mice with endothelial-specific knockout of synectin, homozygous floxed synectin mice were crossed with the constitutive active Cre mouse line, Tie2-Cre

(Syn^{ECKO}) and with the inducible Cre mouse lines (iEC-SynKO), *Cdh5-CreERT2* {Wang, 2010 #1227} and *Pdgf-icre/ERT2*{Claxton, 2008 #1247}. After established the different mouse lines, the genotyping was performed by Transnetyx (Cordova) using a quantitative real-time PCR-based (qPCR-based) system to detect the presence or absence of a target sequence within each sample. The animals were maintained in the Animal Research Center at Yale University. All animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University.

Tamoxifen injection

Induction of Cre expression in pups from was done by consecutive intragastric injections (P1-P3) of 100 ug of tamoxifen per pup. Induction of Cre expression in adult mice was done by 5 alternate day intra-gastric injections (P1-P9) and by other 5 alternate subcutaneous injections from (P11-P20) of 50ul/pup of tamoxifen (2mg/ml in corn oil).

Primary smooth muscle and endothelial cell isolation and culture

Primary smooth muscle cells (SMCs) were isolated as previously described {Ray, 2001 #257} with minor modifications. In summary, the dorsal aorta was removed from adult mice and submerged in sterile PBS containing penicilin-streptomycin (100 I.U./ml penicillin:100 ug/ml streptomycin) and amphotericin B (2.5 µg/mL). The vessels were incubated in enzymatic digestion solution (Collagenase - 1mg/mL, Elastase-0.5mg/mL in media without serum) for 10min at 37ºC. After the first enzymatic digestion the vessels were stripped of adventitia and placed into new enzymatic solution for 1h at 37ºC, centrifuged and plated in culture medium (DMEM with 20% Fetal bovine serum, nonessential amino acids, sodium pyruvate, L-glutamine, and antibiotics at standard concentrations]). After 7–10 days, the cells began to migrate out from the tissue sections, reaching confluence in 15-20 days. The cells were harvested and protein lysates extracted to undergo western blotting in order to check synectin protein levels. Primary endothelial cells were isolated from the heart and lung of adult mice using a previously described protocol {Chittenden, 2006 #66;Li, 2002 #698}. In summary, the tissues (heart and lung) of 4 mice/group were

harvested, minced finely with scissors and then digested in 25ml collagenase 0.2% (w/v) at 37^{o}C for 45 min. The cells were pelleted and resuspended in DMEM-20% FBS. The cell suspension was incubated, with rotation, with PECAM-1-coated beads (Invitrogen) at room temperature for 20min. Using a magnetic separator, the bead-bound cells were recovered, washed with DMEM-20%FBS, suspended in 10ml complete culture medium (same as described above used for smooth muscle cell culture but complemented with 100ug/ml heparin, 100ug/ml ECGF growth supplement [ECGF: Biomedical Technologies, Stoughton, MA]) , and finally plated in a gelatin-coated 10cm tissue culture dish.

Western blotting

Cells were washed with PBS and lysed in RIPA buffer with protease (Sigma) and phosphatase (Bioproducts) inhibitors. The cell lysate was used for western blot. Equal amounts of protein were loaded in 12% ReadyGels (Bio-Rad), separated and transferred at constant voltage.

Reagents and Antibodies

The following antibodies were used in the study: mouse monoclonal αSMA clone 1A4 (#A2547, Sigma), goat polyclonal anti-VE-cadherin (#SC6458,Santa Cruz), rabbit polyclonal anti-synectin (courtesy Dr. A. Horowitz), anti- phospho-VEGF Receptor 2 (Tyr1175 #2478, Cell Signaling), anti-total VEGFR-2 (#2479, Cell Signaling) anti-phospho p44/42 MAP Kinase (phospho-ERK, #9106, Cell Signaling), anti-p44/42 MAP Kinase (total ERK, #9102, Cell Signaling). The growth factors, PDGF-BB (220-BB) and VEGF-A165 (293-VE) were obtained from R&D Systems.

RNA isolation and RT-PCR

Total RNA was extracted from Syn^{SMKO} carotid artery and aortas and from iEC-SynKO lungs, using RNeasy plus Mini Kit (Qiagen), cDNA synthesis was performed with iScript cDNA Synthesis kit (BioRad) and PCR amplification (RT-PCR) was performed with synectin specific primers:

LSyn (1-2): 5'CAGGTCTCCCAGCCAGAGT3'

RSyn (1-2): 5'GTAGTCGGAAGGCCTCAGC 3'

Quantitative real time PCR (qRT-PCR) was used to measure synectin expression in iEC-SynKO lungs and in Syn^{ECKO} endothelial cells (data not shown). SYBR GREEN Mastermix (BioRAD) and Bio-Rad CFX94 detection system were used. Data were normalized to endogenous VE-Cadh primers shown in the table below and confirmed with commercial available primers from Qiagen (VE-Cadh #QT01052044).

List of primers used for qPCR:

*L(VeCadh)*q	ATTGAGACAGACCCCAAACG
*R(VeCadh)*q	TTCTGGTTTTCTGGCAGCTT
*Lsyn(ex1-2)*q	AGTTTCGAGAGGACCGAGCA
Rsyn(ex1-2)q	CCTCCTCATTTCCACCAGA
*LSyn (ex2)*q	GCAGCAGGAGAATCCCAGAT
*RSyn (ex2)*q	GCCGTACAGCTCCTTGACAT

Hindlimb Ischemia Model

Surgical hindlimb ischemia

This was done as previously described by our lab {Tirziu, 2005 #303;Chittenden, 2006 #66}. Briefly, surgical procedures were performed in mice under anesthesia and sterile conditions. A vertical longitudinal incision was made in the right hindlimb (10 mm long). The right femoral artery and its side branches were dissected and ligated with 6 – 0 silk sutures spaced 5 mm apart, and the arterial segment between the ligatures was excised.

Assessment of Blood Perfusion by Laser-Doppler flow-Imaging (LDI)

Measurement of blood flow was done by scanning both rear paws with a LDI analyzer (Moor Infrared Laser Doppler Imager Instrument, Wilmington, Delaware) before and after the surgical procedure (days 0, 3, 7, and 14). The animal was kept under 1% isoflurane anesthesia and its body temperature was strictly maintained between 36.5°C- 37.5°C as previously described {Tirziu, 2005 #303;Chittenden, 2006 #66;Zhuang, 2011 #1251}. Low or no perfusion is displayed as dark blue, whereas the highest degree of

perfusion is displayed as red. The images obtained were quantitatively converted into histograms with Moor LDI processing software V3.09. Data were reported as the ratio of flow in the right/left (R/L) hindlimb and calf regions (not shown). Measurement of blood flow was done before and after the surgical procedure (days 0, 3, 7, and 14).

For Syn^{SMKO} and Syn^{ECKO} mouse lines, 10 mutant and 9 control 10-12 week old females were used. For iEC-SynKO mouse line, 6 mutant and 6 control 10 week old males were used.

Micro-CT Angiography

For microcomputed tomography (mCT) of the cardiac, renal and hindlimb vasculature, euthanized mice were injected with 0.7ml solution (bismuth contrast solution) in the descending aorta. The mice were immediately chilled in ice and immersion fixed in 2% paraformaldehyde overnight. The vasculature was imaged and quantified as described previously {Chittenden, 2006 #66;Tirziu, 2005 #303} and in detail as follows: 2D mCT scans were acquired with a *GE eXplore* Micro-CT System (GE Healthcare) **,** using a 400 cone-beam filtered back projection algorithm, set to an 8-27-µm micron slice thickness. Micro-CT quantification was done as previously described {Simons, 2008 #607}.In brief, data were acquired in an axial mode, covering a volume of 2.0 cm in the z direction with a 1.04-cm field of view. During post-processing, a 40 000 gray-scale value was set as a threshold to eliminate noise (air, water, and bone signals) with minimal sacrifice of vessel visualization. The mCT data were processed using real time 3D volume rendering software (version 3.1, Vital Images, Inc. Plymouth, MN) and microview (version 1.15, GE medical system) software to reconstruct three 2D maximum-intensity projection images (x, y,and z axes) from raw data. Quantification was performed using a modified Image ProPlus 5.0 algorithm (Media Cybernatics). The data are expressed as vessel number, representing total number of vessels, of specified diameter counted in 200 z sections from thigh and kidney or in 350-400 z sections from heart images. For analysis of heart and kidney, and hindlimb vasculature, 4-5 mutant mice and 4 gender and age matched controls were used.

Harvest and staining of whole mount tissues

Spinotrapezius staining and analysis

Mouse spinotrapezius tissues were extracted as described previously {Bailey, 2008 #621;Mac Gabhann, 2010 #625}. Briefly, the dorsal skin was opened and the dorsal shoulder fat pad bisected to expose the spinotrapezius muscle. The superficial muscle was separated from the deeper-lying latissimus dorsi, stripped of fascia and cut at the cranial and medial edges for removal. Tissues were washed in PBS, permeabilized in blocking solution (1% BSA, 0.5% TWEEN-20 in PBS) for 4hours at room temperature (RT) and labeled with smooth muscle α-actin antibody (1A4-Cy3, 1:200) and lectin (IB4-Alexa488, 1:100) at RT for one hour and overnight at 4°C. The samples were washed in PBS and whole-mounted on slides for imaging.

Pseudocoloring of arterioles was performed using intensity filters, and confirmed by inspection. Complete maps of the spinotrapezius arterial networks were acquired at micron-resolution by stitching together 20-25 images (each image with 1.85 um/pixel), acquired by fluorescent microscopy (Nikon 80i Microscope). Quantification of vessel diameters was done using ImageJ software {Schneider, 2012 #1257}. Diameters were measured at (a) the points where the feed arteries entered the spinotrapezius muscle ("input arterioles") and (b) the set points of narrowest diameter ("bottlenecks"- not shown) in the parallel collateral arteriole pathways between the input arterioles. The data are expressed as the diameters of the input arterioles and the number of collateral arcades in these muscles.

Retina Staining

The eyes were removed from neonates at postnatal day 5 (P5) and 17 (P17) and prefixed in 4% paraformaldehyde (4%PFA) for 15min at room temperature. The transgenic mouse line Pdgfb-icre/ERT2-IRES-EGFP {Claxton, 2008 #1247} allows detection of Cre recombination activity by assessing GFP expression. Therefore, dissected retinas from Pdgf-icre/ERT2;Syn^{FF} neonates were immediately inspected for GFP expression and iEC-SynKO were screened from the control littermates.

The dissected retinas were blocked overnight at 4° C in TNBT (0.1M Tris-HCl, 150 mM NaCl, 0.2% blocking reagent [PerkinElmer] supplemented with 0.5% TritonX-100). After washing, the retinas were incubated with IsolectinB4, Alexa Fluor® 488 Conjugate (Molecular Probes CatN#I21411) in Pblec (1 mM MgCl2, 1 mM CaCl2, 0.1 mM MnCl2, 1% Triton X-100 in PBS) for 2 hours at RT, P17 retinas were also stained with smooth muscle α-actin (SMA, IA4-Cy3 #C6198, Sigma). The retinas were washed 6 times, for 10min in PBS, fixed briefly for 5min in 4%PFA, washed twice in PBS and mounted in fluorescent mounting medium (DAKO, Carpinteria, CA, USA). Three mutant iEC-SynKO (*Pdgf-icre/ERT2)* mice and 3 control littermates as well as 4 mutant Syn^{ECKO} (*Tie2-cre)* mice and 4 control littermates were used for phenotypic analysis under a fluorescent microscope. Low and high magnification images were acquired using fluorescent (Nikon 80i Nikon Ti-E Eclipse inverted microscope) and confocal (ZEISS LSM710 laser scanning confocal) microscopes. Quantification was performed in iEC-SynKO neonates; 20-25 images per group were acquired and Biological CMM Analyzer software{Jones, 2008 #1259} was used to quantify vascular area and number of vessel branch points per image.

Adult angiogenesis models

In vivo matrigel assay

The growth factor reduced Matrigel (BD Bioscience) was thawed on ice, one day before procedure. The matrigel was pre-mixed with heparin (5U) with or without VEGF-A 165 (100ng/ml) and injected into subcutaneous tissues of 12-week old male mice from iEC-SynKO mouse line (*Cdh5-CreERT2*). After 7 days from injection, matrigel plugs were recovered from the sacrificed mice, embedded in OCT and cryosectioned in 10um sections.

To identify infiltrating endothelial cells, the sections were stained with anti-CD31 antibody (BD pharmingen TM) and random images from each plug were acquired by fluorescent microscopy. CD31-positive vessels were quantified using ImageJ v1.47g software and expressed as a read out of angiogenesis. Three mutant mice and 3 control littermates were used for the analysis.

Wound healing model

Wound healing assays were performed by the back punch model. In this model a wound is created in the back skin (6-mm punch) and the healing process is analyzed over time. The rate of wound closure is dependent on angiogenesis {Tonnesen, 2000 #1258}. Before surgery, mice were anaesthetized by intraperitoneal injection of ketamine (100mg/kg)/xylazine (10mg/Kg) solution. The fur was removed from the surgical site and the skin was cleaned with ethanol. Full thickness wounds were made with a sterile 6mm biopsy punch in the back skin (Miltex, # 33-36), keeping the underlying muscle intact. Images of the wounds were acquired with a LEICA M125 microscope with a HC80 HD camera (Leica Germany) and ImageJ v1.47g software was used to quantify wound sizes. Measure of the wound diameter was done at 0, 1, 3, 5 and 7 days after procedure and is shown as a percentage of the original wound. Four mutant mice and 4 control littermates were used for the analysis (12-14 wks, males).

Statistical analysis

Data are presented as mean ±SEM. Comparisons between 2 independent groups were performed with a 2-sample t-test. Differences were considered statistically significant if $p \leq 0.05$. Differences between multiple groups were assessed with 2-way ANOVA followed by the post-hoc Tukey's HSD multiple comparisons test.