

RESEARCH ARTICLE

Crim1 maintains retinal vascular stability during development by regulating endothelial cell Vegfa autocrine signaling

Jieqing Fan^{1,2}, Virgilio G. Ponferrada¹, Tomohito Sato³, Shruti Vemaraju¹, Marcus Fruttiger⁴, Holger Gerhardt^{5,6}, Napoleone Ferrara⁷ and Richard A. Lang^{1,8,9,*}

ABSTRACT

Angiogenesis defines the process in which new vessels grow from existing vessels. Using the mouse retina as a model system, we show that cysteine-rich motor neuron 1 (Crim1), a type I transmembrane protein, is highly expressed in angiogenic endothelial cells. Conditional deletion of the *Crim1* gene in vascular endothelial cells (VECs) causes delayed vessel expansion and reduced vessel density. Based on known Vegfa binding by Crim1 and Crim1 expression in retinal vasculature, where angiogenesis is known to be Vegfa dependent, we tested the hypothesis that Crim1 is involved in the regulation of Vegfa signaling. Consistent with this hypothesis, we showed that VEC-specific conditional compound heterozygotes for *Crim1* and *Vegfa* exhibit a phenotype that is more severe than each single heterozygote and indistinguishable from that of the conditional homozygotes. We further showed that human CRIM1 knockdown in cultured VECs results in diminished phosphorylation of VEGFR2, but only when VECs are required to rely on an autocrine source of VEGFA. The effect of CRIM1 knockdown on reducing VEGFR2 phosphorylation was enhanced when VEGFA was also knocked down. Finally, an anti-VEGFA antibody did not enhance the effect of CRIM1 knockdown in reducing VEGFR2 phosphorylation caused by autocrine signaling, but VEGFR2 phosphorylation was completely suppressed by SU5416, a small-molecule VEGFR2 kinase inhibitor. These data are consistent with a model in which Crim1 enhances the autocrine signaling activity of Vegfa in VECs at least in part via VEGFR2.

KEY WORDS: Crim1, Vegfa, Endothelial cell, Angiogenesis

INTRODUCTION

Angiogenesis defines the process in which new vessels grow from existing vessels through branching morphogenesis (Phng and Gerhardt, 2009; Geudens and Gerhardt, 2011). During

angiogenesis, some vascular endothelial cells (VECs) in the originally quiescent vessels are induced to become tip cells. These cells are polarized and extend filopodia to probe microenvironmental cues, and migrate to lead the elongation of new vessel branches (Gerhardt et al., 2003). The VECs adjacent to tip cells become stalk cells, which proliferate and contribute to lumen formation (Gerhardt et al., 2003). Fusion of new sprouts in a process called anastomosis contributes to vascular network formation (Geudens and Gerhardt, 2011). This draft network then undergoes extensive remodeling to become functional (Potente et al., 2011). Dysregulated angiogenesis occurs in many pathological processes, including cancer (Gasparini et al., 2005), diabetic retinopathy (Crawford et al., 2009), retinopathy of prematurity (Flynn and Chan-Ling, 2006), and age-related macular degeneration (Jager et al., 2008), in which anti-angiogenic therapy can be of value (Gasparini et al., 2005).

The vascular endothelial growth factor (Vegf) family members are key regulators of vessel development and homeostasis. Vegfa is an indispensable pro-angiogenic factor in almost all non-pathological and pathological angiogenesis (Carmeliet and Jain, 2011). Vegfa signals via vascular endothelial growth factor receptor 2 (Vegfr2; Kdr – Mouse Genome Informatics), a conventional tyrosine kinase receptor. Vegfr1 (Flt1 – Mouse Genome Informatics) is an inhibitory receptor because it binds Vegfa avidly but does not signal with high activity (Shibuya, 2001). There is also a soluble isoform of Vegfr1 that inhibits Vegfa through sequestration (Shibuya, 2001). During angiogenesis, Vegfa signals to VECs to promote tip cell formation [by enhancing expression of Dll4 (Phng and Gerhardt, 2009; Geudens and Gerhardt, 2011)], tip cell migration (Gerhardt et al., 2003), stalk cell proliferation (Gerhardt et al., 2003) and VEC survival via Akt (Gerber et al., 1998). Vegfr3 (Flt4 – Mouse Genome Informatics), a receptor for Vegfc, is restricted to the lymphatic vessels in the adult, but is upregulated during developmental angiogenesis in tip cells and during pathological angiogenesis (Tammela et al., 2011). It has recently been shown that VECs themselves are a source of Vegfa and that the ‘private loop’ of signaling via Vegfr2 within the VECs is crucial (Lee et al., 2007; Segarra et al., 2012). Mice in which *Vegfa* was deleted specifically in VECs showed postnatal mortality associated with vascular degeneration (Lee et al., 2007), suggesting a role for autocrine Vegfa in vascular homeostasis. Although it has been shown that endothelial cells upregulate Vegfa production under stress conditions, such as hypoxia (Namiki et al., 1995; Lee et al., 2007), other molecules involved in regulation of the ligand and downstream effectors of this pathway are largely unknown.

Cysteine-rich motor neuron 1 (Crim1) is a type I transmembrane protein that has N-terminal homology to insulin-like growth factor binding protein (IGFBP) domain and six cysteine-rich von Willebrand factor C (vWC) repeats, which are similar to those of chordin, a BMP antagonist (Kolle et al., 2000). Crim1 is expressed in multiple tissues

¹Divisions of Pediatric Ophthalmology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA. ²Graduate Program of Molecular and Developmental Biology, University of Cincinnati, Cincinnati, OH 45267, USA.

³Department of Ophthalmology, National Defense Medical College, Saitama Prefecture 359-0042, Japan. ⁴UCL Institute of Ophthalmology, University College London, London EC1V 9EL, UK. ⁵Vascular Biology Laboratory, London Research Institute, Cancer Research UK, London WC2A 3LY, UK. ⁶Vascular Patterning Laboratory, Vesalius Research Center, Leuven 3000, Belgium. ⁷Department of Pathology and Moores Cancer Center, University of California San Diego, La Jolla, CA 92093, USA. ⁸Department of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA. ⁹Department of Ophthalmology, College of Medicine, University of Cincinnati, Cincinnati, OH 45229, USA.

*Author for correspondence (richard.lang@cchmc.org)

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and cell types, including the vertebrate CNS (Kolle et al., 2003; Pennisi et al., 2007), kidney (Wilkinson et al., 2007), eyes [including lens (Lovicu et al., 2000)] and the vascular system (Glienke et al., 2002; Pennisi et al., 2007; Wilkinson et al., 2007). It has been suggested that *Crim1* has a role in vascular tube formation *in vitro* (Glienke et al., 2002). It is localized in endoplasmic reticulum and accumulates at cell-cell contacts upon stimulation of endothelial cells (Glienke et al., 2002). Mice homozygous for a gene-trap mutant allele (*Crim1*^{KST264}) or germline mutants (Chiu et al., 2012) display perinatal lethality with defects in multiple organs, including hemorrhagic necrosis and enlarged glomerular capillary lumens (Wilkinson et al., 2007). The molecular function of *Crim1* has been somewhat enigmatic, but it is known to form complexes with N-cadherin and β -catenin (Ponferrada et al., 2012) and to bind growth factors including Vegfa (Wilkinson et al., 2007; Wilkinson et al., 2009). In the glomerulus of the kidney, *Crim1* on the cell surface of podocytes is proposed to regulate the delivery of Vegfa from podocytes to endothelial cells (Wilkinson et al., 2007; Wilkinson et al., 2009).

In the current study, based on the known Vegfa binding by *Crim1* (Wilkinson et al., 2007) and the expression of *Crim1* in retinal vasculature, we tested the hypothesis that *Crim1* is involved in the autocrine activity of Vegfa. Consistent with this, VEC-specific conditional compound heterozygotes for *Crim1* and *Vegfa* showed a phenotype more severe than each single heterozygote and

indistinguishable from that of the conditional homozygotes. Human *CRIM1* knockdown in cultured VECs resulted in diminished phosphorylation of VEGFR2, but only when VECs are required to rely on an autocrine source of VEGFA. VEGFA knockdown enhanced the effect of *CRIM1* knockdown on reducing VEGFR2 phosphorylation. An anti-VEGFA antibody did not enhance the effect of *CRIM1* knockdown in reducing VEGFR2 phosphorylation caused by autocrine signaling, but VEGFR2 phosphorylation was completely suppressed by SU5416, a small-molecule VEGFR2 kinase inhibitor. These data are consistent with a model in which *Crim1* enhances the autocrine signaling activity of Vegfa in VECs at least in part via Vegfr2.

RESULTS

Crim1 is expressed in endothelial cells and pericytes

Crim1 is expressed in VECs *in vitro* and *in vivo* (Glienke et al., 2002). To examine the expression pattern of *Crim1* in angiogenic vasculature, we analyzed flat-mounted preparations of mouse embryonic hindbrain and postnatal retinas from a *Crim1*:GFP mouse line (MGI: 4846966). In the vasculature of both organs, GFP was expressed in VECs marked by Isolectin IB4 (Fig. 1A-I). Notably, in the center of the retinal vascular plexus, the GFP intensity was lower in VECs but also present in smooth muscle cells marked by NG2 (Cspg4 – Mouse Genome Informatics) labeling (Fig. 1E,F,

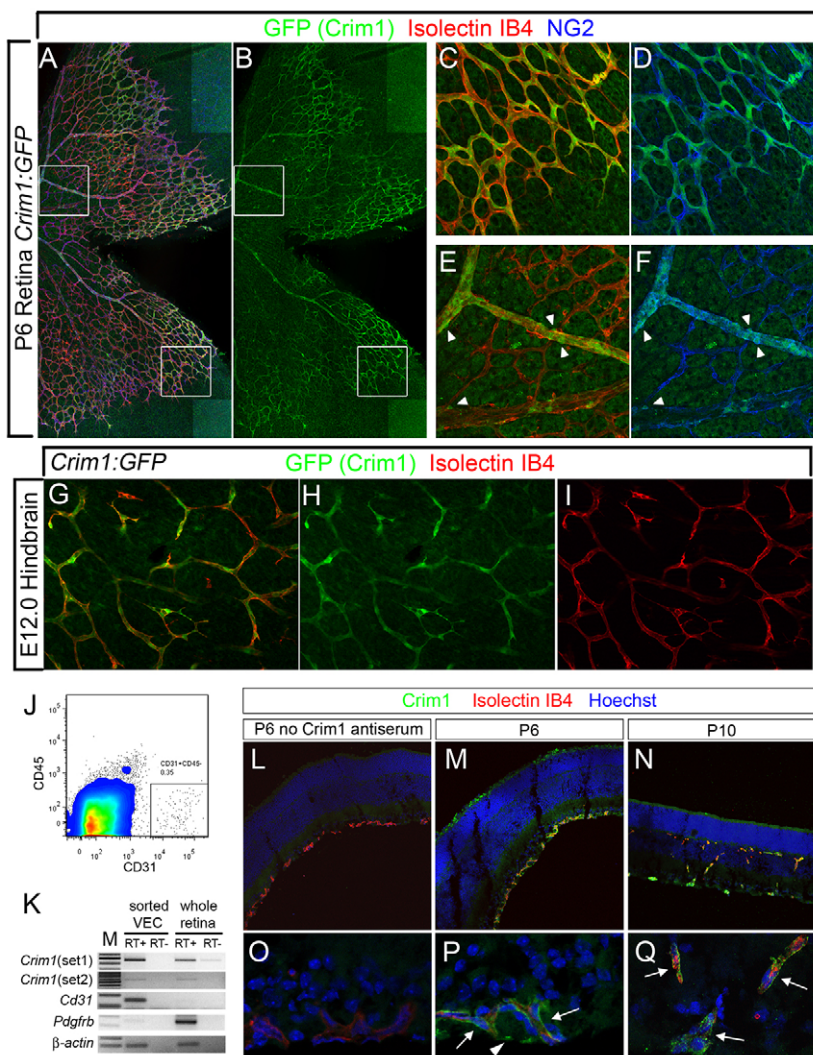


Fig. 1. *Crim1* is expressed in endothelial cells and pericytes in angiogenic vasculature. (A-F) Flat-mounted P6 *Crim1*:GFP mouse retina labeled with Isolectin IB4 and NG2 antibody. Enlarged images of the boxed regions (C-F) show colocalization of the GFP expression in Isolectin-labeled endothelial cells and NG2-labeled pericytes/smooth muscle cells (arrowheads). (G-I) GFP signal was also detected in hindbrain vasculature of the E12.0 reporter mouse. (J) Representative FACS chart showing the endothelial cell population sorted from retina. (K) End-point RT-PCR in sorted endothelial cells and whole retina. For each primer set, PCR products were amplified with similar amounts of cDNA and the same cycle number. +/- RT, with and without reverse transcription. M, DNA size marker. (L-Q) Transverse sections of P6 and P10 retina labeled with *Crim1* antiserum. No primary antibody was added in L, O. (O-Q) Higher magnification images. Arrows indicate *Crim1* expression in endothelial cells; arrowhead indicates *Crim1* expression in another cell type.

arrowheads). We also isolated CD31⁺ CD45⁻ VECs from wild-type P7 mouse retinas by FACS (Fig. 1J). We confirmed cell identity by end-point RT-PCR detecting the endothelial cell marker *Cd31* (*Pecam1* – Mouse Genome Informatics) and the pericyte marker *Pdgfrb* (Fig. 1K). *Crim1* transcripts were detected in retinal VECs using two different sets of primers (Fig. 1K). Crim1 protein was also labeled by immunofluorescence in P6 and P10 wild-type retinal sections using a newly developed antiserum. High immunoreactivity was observed in VECs labeled by Isolectin IB4 (Fig. 1M,N,P,Q), as well as in cells associated with the vasculature, which were probably pericytes (Fig. 1P, arrowheads). The expression of Crim1 in VECs indicated that it might have a role in vascular development.

Validation of the *Crim1*^{flox} allele

Crim1^{flox} mice were crossed with the germline-expressed *Elia-Cre* line to generate the *Crim1*^{flox} allele. Mice homozygous for this allele did not show lethality until embryonic day (E) 17.5 (supplementary material Fig. S2A), but postnatally we identified only three live pups from over 20 litters, indicating that homozygotes died perinatally. *Crim1*^{flox/flox} embryos exhibited anomalies that included peridermal blebbing, edema, hemorrhage (especially in the CNS), eye hypoplasia and syndactyly (supplementary material Fig. S2B-T). These changes phenocopy defects described in mice homozygous for the hypomorphic allele *Crim1*^{KST264} (Pennisi et al., 2007) and another germline null allele (Chiu et al., 2012) but occur with higher penetrance and severity (supplementary material Fig. S2B). These data validate *Crim1*^{flox} as a conditional loss-of-function allele.

Deletion of *Crim1* in VECs results in defective vascular development in the retina

We combined the *Pdgfb-iCreER* allele with *Crim1*^{flox} and used daily intraperitoneal injection of tamoxifen starting at P1 or P7 (supplementary material Fig. S1C) to elicit Cre recombinase activity. *Pdgfb-iCreER* mice, upon tamoxifen administration, exhibit specific Cre activity in VECs including tip cells (Claxton et al., 2008) (supplementary material Fig. S1D). The *Crim1*^{flox} allele was effectively recombined in retinal VECs as shown by genotyping PCR of DNA from FACS-sorted P7 endothelial cells (supplementary material Fig. S1E); the recombined *Crim1*^{flox} allele was detected in tail DNA and retina DNA only when mice had the *Pdgfb-iCreER* allele (supplementary material Fig. S1B,E).

In the first postnatal week, a primary vascular plexus grows within the ganglion cell layer of the mouse retina through sprouting and anastomosis, expanding from the optic stalk and reaching the periphery by ~P8 (Fruttiger, 2007). Tamoxifen-injected *Crim1*^{flox/flox}; *Pdgfb-iCreER* pups showed delayed radial expansion of vasculature from center to periphery over a P5-P7 timecourse as compared with Cre-negative control littermates (*Crim1*^{flox/flox} or *Crim1*^{flox/+}) (Fig. 2A-C). More distinctively, vessel density as quantified by branchpoint number was also reduced in both the angiogenic front and remodeling plexus area behind the front (Fig. 2D-F).

Starting at P7-P8, vessels sprout into the outer plexiform layer (OPL) and then turn, sprout and connect to form the deep vascular layer that resides between the OPL and the photoreceptors (Fruttiger, 2007). *Crim1*^{flox/flox}; *Pdgfb-iCreER* pups injected with tamoxifen from P7 to P10 exhibited a delayed invasion of vasculature into the deepest layer (supplementary material Fig. S3A-D), as shown by fewer vertical sprouts, reduced branchpoint number and reduced total vessel length (supplementary material Fig. S3E-G). Combined, these data showed that endothelial Crim1 plays a role throughout retinal vascular development.

Loss of *Crim1* from endothelial cells caused abnormal vessel morphology and compromised VEC proliferation

The initial density of the sprouting vasculature can be affected by tip cell number (Hellström et al., 2007; Phng et al., 2009; Larrivée et al., 2012). The angiogenic front in *Crim1* VEC conditional mutants was hypocellular compared with that of wild-type littermates, as indicated by Erg1/2/3 antibody labeling of the VEC nuclei (Fig. 2G-I). In addition, a much higher occurrence of vessel segments of small caliber was observed in *Crim1* VEC conditional mutant mice (Fig. 2E,H).

Two-hour BrdU incorporation showed a reduced number of S-phase VECs in the conditional mutant pups (Fig. 2J-L). Tip cells of the *Crim1* VEC conditional mutant mice exhibited a mildly reduced density of filopodia, but these filopodia were still closely attached to the underlying astrocytes (Fig. 2M-Q). Expression of the Notch pathway target *Vegfr3* was normal in VEC conditional mutants (supplementary material Fig. S4), suggesting that loss of Crim1 from VECs was unlikely to disrupt the Notch signaling pathway.

Endothelial loss of *Crim1* results in defective cell adhesion molecule distribution and precocious vessel regression

Previous studies from our laboratory indicated that Crim1 has an active role in promoting neuroepithelium cell adhesion through regulation of cadherin-catenin complexes (Ponferrada et al., 2012). Other investigators also showed that Crim1 accumulates at endothelial cell-cell contacts under certain conditions (Glienke et al., 2002). Given the important role that the adhesion molecule VE-cadherin plays in initiating anastomosis (Hoelzle and Svitkina, 2012) and maintaining vessel stability (Dejana et al., 2009; Phng et al., 2009), we assessed the possibility of a cell adhesion anomaly in *Crim1*^{flox/flox}; *Pdgfb-iCreER* mouse retinal vasculature.

We observed an increase in vessel segments with discontinuous VE-cadherin labeling in *Crim1* conditional mutants (Fig. 3A-L) (one vessel segment is defined as an isolectin-labeled connection between two branchpoints). This change was more significant at the angiogenic front (Fig. 3M). VE-cadherin-negative segments were usually of reduced caliber. In addition, podocalyxin labeling, which marks the apical/luminal side of the vessels, was missing in VE-cadherin-negative regions (Fig. 3N-P,R-T, arrowheads). In summary, *Crim1* VEC conditional mutant mice had a higher percentage of thin, cadherin-deficient, non-lumenized vessel segments.

Normally, vessel pruning occurs mostly around arteries where the oxygen level is high (Adini et al., 2003; Phng et al., 2009). Regressed vessel segments appear as ‘vessel ghosts’, which are labeled for vascular basement membrane markers such as collagen IV but lack isolectin-positive VECs (Phng et al., 2009). In *Crim1*^{flox/flox}; *Pdgfb-iCreER* pups, there was a significant increase in vessel ghosts at the angiogenic front (Fig. 4A-D,M) but no significant increase within the remodeling plexus (Fig. 4E-H,M). We observed several cases of retracted sprouts (Fig. 4I,J) and vessel fragments completely disconnected from the vessel bed (Fig. 4K,L).

We also labeled active caspase 3, an early marker for apoptosis (Fig. 4N-Q). The VEC apoptosis index was significantly higher in *Crim1*^{flox/flox}; *Pdgfb-iCreER* retinas than in control retinas (Fig. 4N,O,R). We measured the distance of dying vessel fragments from the center of the retina and found that the increase in apoptosis in the VEC conditional mutant mice was most apparent within the region between 60% and 70% of the distance to the edge of the angiogenic front. This corresponds to the more angiogenically active vessel front (Fig. 4S). Mural cells recruited to newly formed capillaries are also crucial for vessel stability (Gerhardt and Betsholtz, 2003). In *Crim1*^{flox/flox}; *Pdgfb-iCreER*

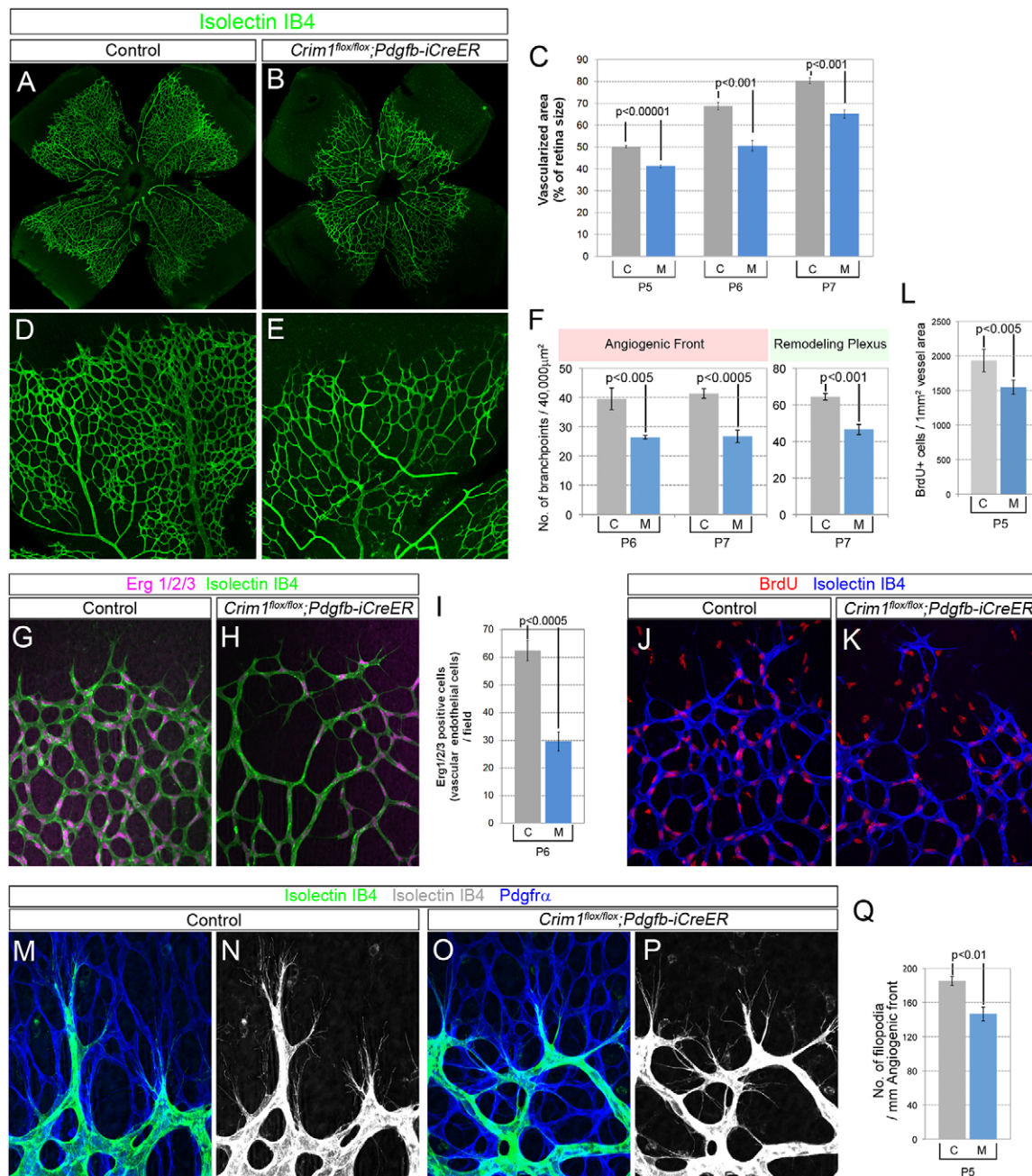


Fig. 2. Loss of *Crim1* from endothelial cells causes reduced vessel growth in the primary plexus of the retinal vasculature. (A,B,D,E) Flat-mounted P6 mouse retina labeled with isolectin IB4. *Crim1* VEC conditional mutant mice showed reduced vessel expansion to the periphery, as quantified in C. (F) Vessel density measured by counting branchpoint number in randomly selected 200 μm × 200 μm fields (five fields per retina). (G,H) High-magnification images showing anomalies in vascular morphology at the angiogenic front in P6 *Crim1* VEC mutant mice. (I) Quantification of Erg1/2/3 antibody-labeled VEC nuclei per field. (J-L) Two-hour BrdU incorporation experiment showed a reduced VEC proliferation profile in P5 *Crim1* VEC mutant pups. BrdU incorporation (cell number) was normalized to vessel-covered area, as labeled by Isolectin IB4. (M-P) Filopodia in *Crim1* VEC conditional mutant mice extend along a preformed astrocyte template labeled with *Pdgfra* antibody. Filopodia number was quantified and normalized to the outline of the angiogenic front. (C,F,I,L,Q) M, *Crim1*^{flox/flox}; *Pdgfb-iCreER* pups; C, control littermates. At least five retinas of each genotype were analyzed in every experiment. Error bars represent s.e.m.

mutant pups, the attachment of desmin⁺ (supplementary material Fig. S5) or NG2⁺ pericytes (data not shown) to VECs at the angiogenic front was not compromised, although smooth muscle actin (SMA) showed mildly reduced labeling around arteries (supplementary material Fig. S5E-H). This suggested that depletion of *Crim1* from VECs did not have a significant effect on mural cells. Combined, these data suggest that loss of *Crim1* from

VEC causes instability of newly formed vessels, resulting in vessel fragmentation and precocious regression.

***Crim1* and *Vegfa* function cooperatively in retinal vasculature development**

Previous studies showed that *Crim1*, via its cysteine-rich motif, is able to physically bind ligands bearing a cysteine-knot motif,

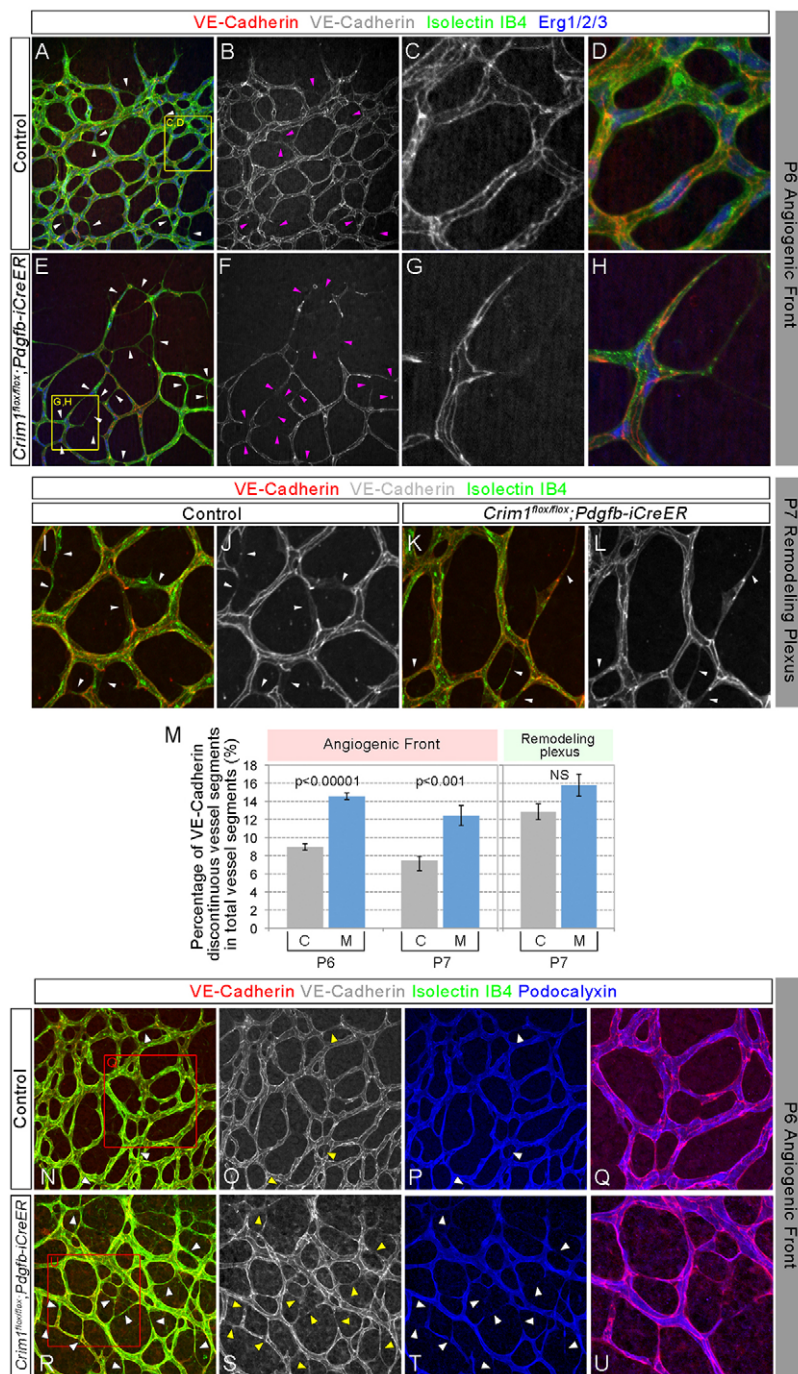


Fig. 3. Abnormal adhesion junction distribution in *Crim1* VEC conditional mutants. (A-L) Antibody labeling in flat-mounted retina preparations shows VE-cadherin distribution in the P6 angiogenic front (A-H) and P7 remodeling plexus (I-L) areas. White and pink arrowheads point to vessel segments without continuous VE-cadherin distribution. (M) Quantitation of vessel segments without continuous junctional VE-cadherin signal (normalized to total Isolectin IB4-labeled segments). M, *Crim1^{fllox/flox}; Pdgfb-iCreER* pups; C, control littermates. At least five retinas of each genotype were analyzed at P6 or P7. Error bars represent s.e.m. (N-U) VE-cadherin and podocalyxin colabeling showing that VE-cadherin-deficient vessel segments, which occurred more frequently in *Crim1^{fllox/flox}; Pdgfb-iCreER* pups, did not have a lumen (white and yellow arrowheads).

including several BMPs, PDGF and VEGFA, before their secretion (Wilkinson et al., 2003; Wilkinson et al., 2007). This, and the Vegfa dependence of retinal angiogenesis, raised the possibility that *Crim1* might regulate Vegfa activity. In the retina, in the first postnatal week, *Vegfa* transcript is found in astrocytes and neurons (Fruttiger, 2007; Rao et al., 2013). Vegfa protein becomes tethered to the extracellular matrix of astrocytes in avascularized regions ahead of the angiogenic front (Gerhardt et al., 2003; Stenzel et al., 2011). In addition to paracrine Vegfa, VEC-derived autocrine Vegfa signaling has been implicated in vessel homeostasis (Lee et al., 2007).

To examine whether autocrine signaling is also required for angiogenesis *in vivo*, we generated *Vegfa* VEC conditional knockout

mice by crossing the *Pdgfb-iCreER* line to the *Vegfa^{loxP}* line (Gerber et al., 1999) and induced Cre activity by tamoxifen injection. Compared with Cre-negative control littermates, conditional heterozygotes and homozygotes showed a reduced vascularized area (Fig. 5A-C) and a Vegfa dose-dependent reduction in vessel density (Fig. 5A,B,D). This phenotype has many similarities to the *Crim1* VEC conditional phenotype. In particular, *Vegfa* conditional deletion results in many more collagen IV⁺, isolectin-negative vessel ghosts (supplementary material Fig. S6A,B,E,F) and vessel segments that show discontinuous VE-cadherin labeling (supplementary material Fig. S6C,D,G,H). Furthermore, like the *Crim1* conditional mutant, in the *Vegfa* conditional mutant discontinuous VE-cadherin labeling correlated with the absence of podocalyxin, suggesting the absence

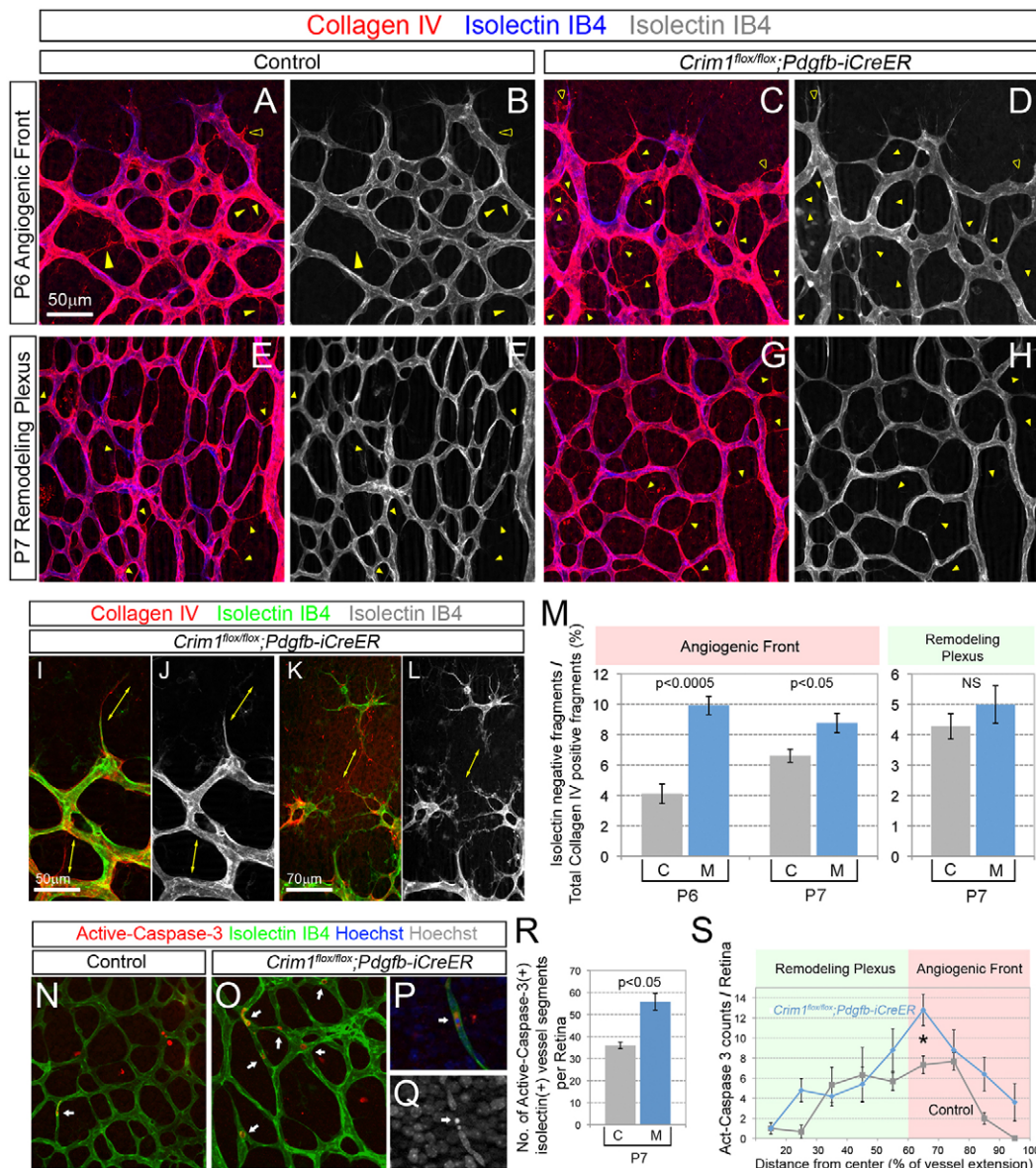


Fig. 4. Depletion of *Crim1* from endothelial cells causes precocious vessel regression. (A-H) Flat-mounted retinas labeled with Isolectin IB4 and collagen IV antibody. Collagen IV-positive and Isolectin IB4 labeling-negative vessel segments represented regressed capillaries, e.g. vessel ghosts (filled arrowheads) and retracted sprouts (empty arrowheads). The increase in vessel ghosts was more obvious at the angiogenic front (A-D) than in the remodeling plexus (E-H). Several cases of long retracted sprouts (I,J) or endothelial cells detaching from the vessel bed (K,L) were observed. (M) Quantification of vessel ghosts. (N-Q) Active caspase 3 labeling of retinas. Vessel segments with active caspase 3-labeled endothelial cells increase in *Crim1* conditional mutant pups (O, arrows). Active caspase 3 labeling coincided with DNA fragmentation (P,Q, arrows). (R) Quantification of vessel segments with active caspase 3-labeled endothelial cells. (S) Occurrence of active caspase 3-positive vessel segments at different distances from the center of the retina (relative to vessel expansion); * $P<0.05$. (M,R,S) M, *Crim1^{fllox/flox}; Pdgfb-iCreER* pups; C, control littermates. At least five retinas of each genotype were analyzed in every experiment. Error bars represent s.e.m.

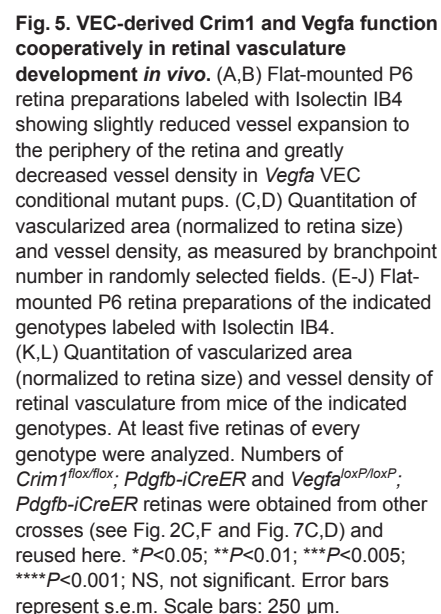
of a lumen. Combined, these data confirm that VECs are a biologically important source of Vegfa (Lee et al., 2007) and indicate that the *Crim1* and *Vegfa* VEC loss-of-function phenotypes are very similar.

One means to assess whether *Crim1* might regulate Vegfa function *in vivo* is to generate compound conditional mutants and perform a quantitative assessment of the phenotype. In this way, it is possible to determine whether two molecules contribute to the same biological process. Thus, we combined the *Vegfa^{loxP}*, *Crim1^{fllox}* and *Pdgfb-iCreER* alleles and found that both *Vegfa^{loxP/+}; Pdgfb-iCreER* and *Crim1^{fllox/+}; Pdgfb-iCreER* heterozygotes showed mildly reduced expansion of the vasculature as well as vessel density

(Fig. 5H,I compared with 5E,F). Interestingly, the *Vegfa^{loxP/+}; Crim1^{fllox/+}; Pdgfb-iCreER* double heterozygotes exhibited much more severely defective angiogenesis than either of the single heterozygotes (Fig. 5H-J) and recapitulated the retinal vascular phenotypes of *Crim1* or *Vegfa* VEC-specific knockouts (Fig. 5K,L). This synthetic phenotype suggests that *Crim1* and *Vegfa* function in the same pathway.

CRIM1 promotes autocrine VEGFA-VEGFR2 signaling within endothelial cells

In order to examine directly the possibility that *Crim1* regulates Vegfa signaling, we established lentiviral shRNA-mediated



VEGFR2 phosphorylation (supplementary material Fig. S8B). Interestingly, however, when we cultured HUVECs in VEGFA-deficient medium for 24 hours and added Na_3VO_4 to inhibit phosphatase activity, we recorded a significant reduction of VEGFR2 phosphorylation in the CRIM1 knockdown cells. This response was quantified by ELISA (Fig. 6C) but was also observed by immunoblot (Fig. 6D). These data suggested that CRIM1 modulates the autocrine signaling response to VEGFA.

To examine this possibility further, we assessed the relative impact and the combined impact of VEGFA and CRIM1 knockdown on the growth of HUVECs and on VEGFR2 signaling. In complete medium with a low supplemental level of VEGFA (~2 ng/ml), VEGFA or CRIM1 knockdown HUVECs exhibited slower growth kinetics (Fig. 7A), as shown previously (Fig. 6A). In addition, CRIM1/VEGFA double-knockdown cells showed a more

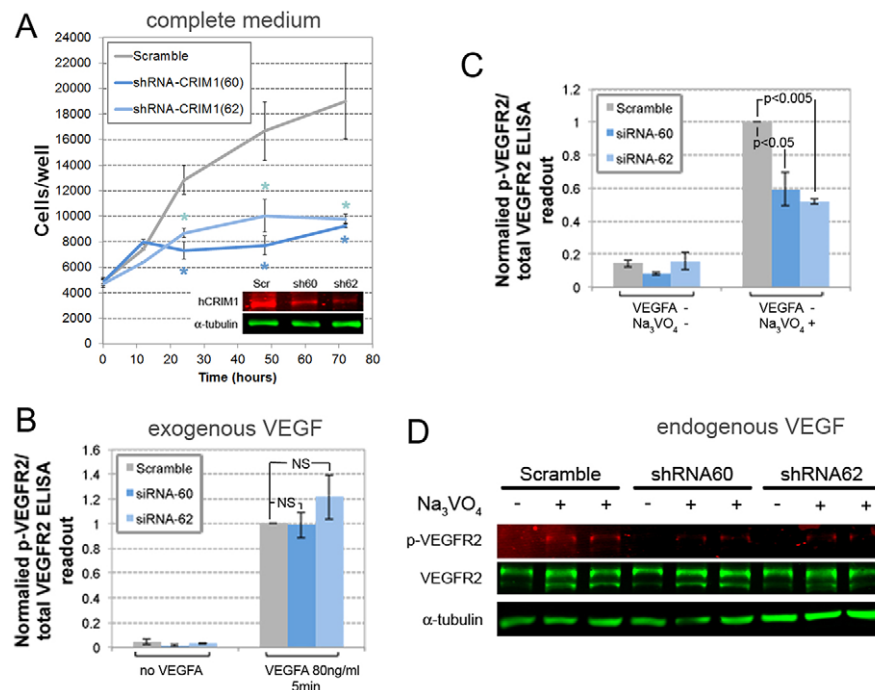


Fig. 6. Crim1 promotes VEGFA-VEGFR2 autocrine signaling in endothelial cells. (A) Growth curve of HUVECs infected by lentiviral particle expressing scramble shRNA and shRNAs specific for *CRIM1*. 5000 cells were plated into each well of fibronectin-coated 96-well plates at time zero. Efficiency of knocking down *CRIM1* protein with shRNAs was shown by immunoblot (inset). * $P < 0.01$, three independent experiments. (B) VEGFR2 phosphorylation in response to VEGFA stimulation. Control and *CRIM1*-deficient cells were starved for 24 hours and stimulated with 80 ng/ml recombinant human VEGFA for 5 minutes. Cell lysates were collected and analyzed using phospho-VEGFR2 and VEGFR2 ELISA kits and the ratio of phospho-VEGFR2 and total VEGFR2 readouts calculated. Measurements were normalized to that of control HUVECs stimulated with recombinant human VEGF. Error bars represent the s.e.m. of three independent experiments. (C) VEGFR2 phosphorylation without exogenous VEGFA. Control and *CRIM1* knockdown HUVECs were cultured in serum-free and VEGFA-depleted culture medium with or without 0.1 mg/ml Na_3VO_4 (to inhibit phosphatase activity) for 24 hours. Cell lysates were collected and analyzed using phospho-VEGFR2 and VEGFR2 ELISA kits and the ratio of the two calculated. All readouts were normalized to that of control HUVECs. Error bars represent s.e.m. of three independent experiments. (D) Immunoblot of whole cell lysates of control and *CRIM1*-deficient HUVECs cultured in VEGFA-depleted medium with or without addition of 0.1 mg/ml Na_3VO_4 .

severe and statistically distinct effect than either single knockdown (Fig. 7A,B, green trace). In similar experiments assessing cell number after 48 hours, we found that the effects of single and combined knockdowns were not compensated by additional exogenous recombinant VEGFA (Fig. 7B). When we assessed growth kinetics for *CRIM1* knockdown HUVECs in serum-free, VEGF-deficient medium, we found that the cell number actually decreased (supplementary material Fig. S8A), indicating that some cells die, as might be expected with limited VEGFA and limited alternative survival stimuli. A comparison of the separate or combined effects of VEGFA and *CRIM1* knockdown showed that under these conditions, as well, the double knockdown produced the most severe effect (Fig. 7C). An assessment by quantitative PCR of the expression levels of *BCL2*, a known anti-apoptosis factor, and of *BAX*, a pro-apoptosis factor, showed that the former exhibited reduced expression whereas the latter exhibited an increase with VEGFA, *CRIM1* and combined VEGFA/*CRIM1* knockdown (Fig. 7D). This establishes that VEGFA and *CRIM1* affect the same target genes in a survival pathway that functions within VECs.

The differential effects of an anti-VEGFA antibody (Avastin) and a VEGFR2 kinase activity inhibitor have been used to show that there is an exclusively autocrine signaling pathway in VECs (Lee et al., 2007). Using VEGFR2 phosphorylation in the presence of orthovanadate in minimal medium as a readout for autocrine signaling activity, we assessed the relative consequences of VEGFA

or *CRIM1* knockdown and determined whether Avastin or the VEGFR2 kinase activity inhibitor SU5416 could modulate signaling. As already shown, VEGFA or *CRIM1* knockdown had very similar consequences for VEGFR2 phosphorylation (Fig. 7E). In addition, we found that Avastin had a very limited ability to suppress VEGFR2 phosphorylation, whereas SU5416 abrogated this completely. Quantitatively, Avastin suppressed VEGFR2 phosphorylation by ~25%. This means that 75% of the signaling activity can be attributed to an Avastin-resistant, autocrine signaling pathway. Since *CRIM1* knockdown can suppress VEGFR2 phosphorylation by significantly more than this, there is a strong argument that, like VEGFA knockdown, *CRIM1* knockdown also suppresses autocrine signaling by endogenous VEGFA. This notion is reinforced by the observation that, when *CRIM1* and VEGFA knockdown are combined under the same conditions, there is a greater effect than either single knockdown on VEGFR2 phosphorylation (Fig. 7F). Together, these data suggest that *CRIM1* regulates the activity of VEGFA within the autocrine pathway known to be active within VECs.

DISCUSSION

We have investigated the function of *Crim1* in microvascular development *in vivo*. Using conditional deletion of a *Crim1*^{fllox} allele, we identified a crucial function for *Crim1* in the VECs of the developing retinal vasculature. We also provide evidence that, in VECs, *Crim1* regulates autocrine signaling by Vegfa.

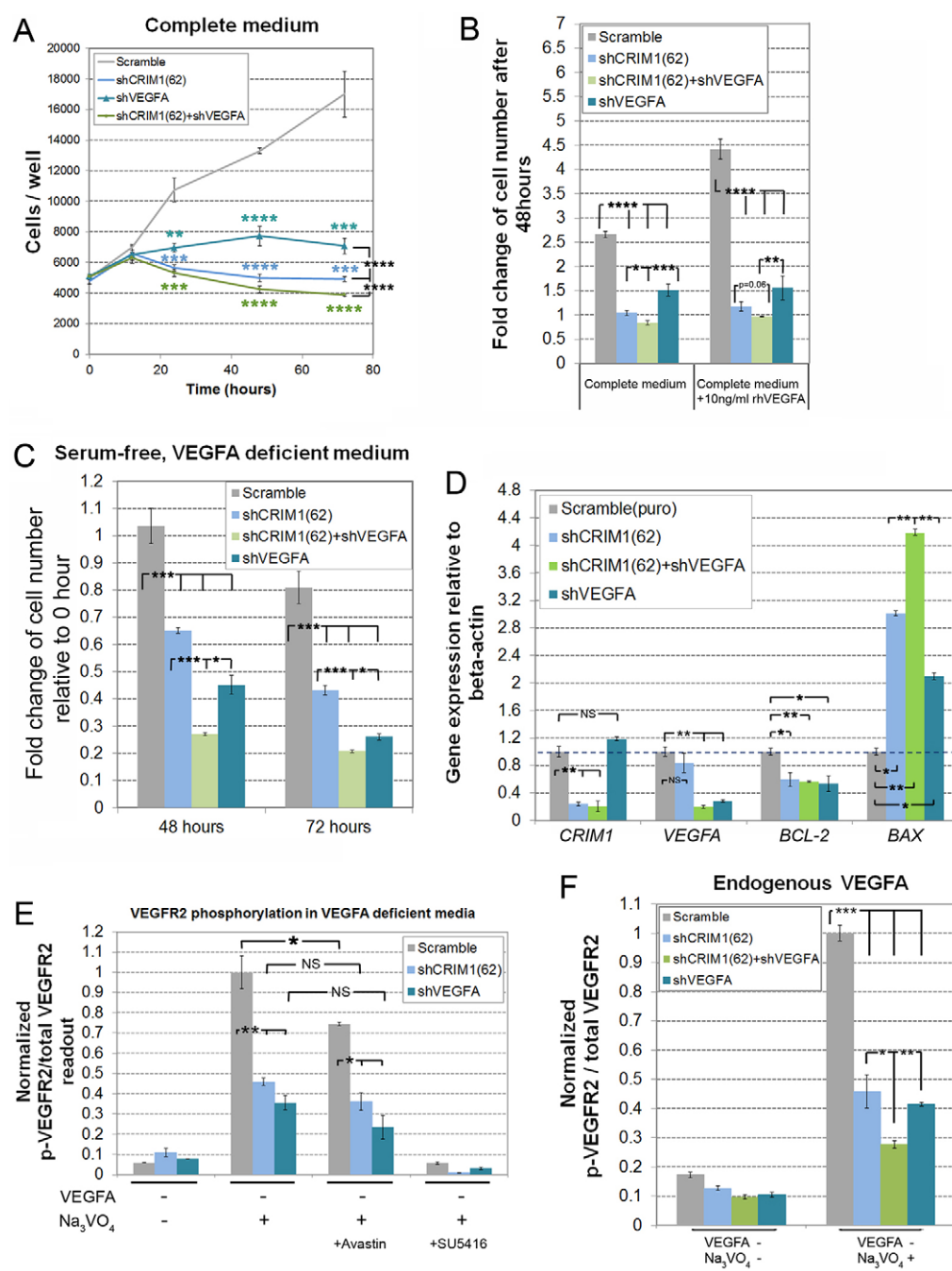


Fig. 7. Relative impacts and combined impact of VEGFA and CRIM1 knockdown on VEGFR2 signaling. (A) Growth curve of HUVECs expressing scramble shRNA and shRNAs targeting *CRIM1*, *VEGFA* or a combination of both. Colored asterisks indicate statistical significance between control and respective experimental groups. (B) Fold change in cell number after 48 hours compared with time zero of cells incubated in complete medium (EGM-2) or complete medium with addition of 10 ng/ml recombinant human (rh) VEGFA. (A,B) 5000 cells were plated into each well of 96-well plates at time zero.

(C) Fold change in cell number after 48 hours incubation in serum-free EGM-2 without addition of supplemental VEGFA. 20,000 cells were plated in each well at time zero. (D) Gene expression measured by quantitative RT-PCR. Cells were cultured in fresh complete medium for 24 hours. Level of mRNA expression was normalized to that of β -actin. All error bars represent s.e.m. of three experiments. (E) VEGFR2 phosphorylation without exogenous VEGFA. Cells were incubated for 24 hours in serum-free EGM-2 without addition of supplemental VEGFA and with 0.1 mg/ml Na_3VO_4 . In one set of experiments, the medium was pre-incubated with 10 $\mu\text{g}/\text{ml}$ Avastin for 2 hours and then applied to the cells. In another set of experiments, medium contained 0.5 μM SU5416. (F) VEGFR2 phosphorylation without exogenous VEGFA. HUVECs infected by lentiviral particles expressing scramble shRNA and shRNAs targeting *CRIM1*, *VEGFA* or a combination of the two were incubated in serum-free, VEGFA-depleted medium containing 0.1 mg/ml Na_3VO_4 for 24 hours before cell lysates were analyzed by phospho-VEGFR2 and VEGFR2 ELISA and the ratio of the two readouts calculated. All error bars represent s.e.m. of three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$; NS, not significant.

Crim1 stabilizes nascent vessel connections

Angiogenic sprouting forms a draft vessel network that subsequently undergoes remodeling to become functional (Potente et al., 2011). Endothelial cell death (Lobov et al., 2005) and retraction of endothelial cells (Chen et al., 2012) can both contribute to vessel regression. In the mouse retina, some segment regression is likely to be triggered by the lack of oxygen demand close to large vessels (Sun et al., 2005), but, in addition, there is sporadic segment regression throughout the forming network. Vessel ghosts, which indicate a regressing segment, can be found even at the advancing front of an angiogenic network and reveal that remodeling is not completely restricted spatially. Disruption of certain molecular pathways, including the Notch pathway (Phng et al., 2009), will cause excessive vessel regression. The current analysis suggests that the transmembrane protein Crim1 has a role in stabilizing newly

formed vessel segments; when conditionally mutated in VECs, the consequence is many more vessel ghosts and overall compromise of the forming vascular network.

Does Crim1 regulate cadherin adhesion in the retinal vasculature?

There is evidence that Crim1 can regulate cell-cell adhesion. Crim1 is expressed in adherent cells, especially in those making new cell-cell contacts (Glienke et al., 2002). Crim1 can also form complexes with β -catenin and N-cadherin and, consistent with this, in *Xenopus* Crim1 stabilizes adhesion junctions and maintains the integrity of neuroepithelium during morphogenesis (Ponferrada et al., 2012). These data suggest that some functions of Crim1 could result from the formation of complexes with VE-cadherin, a cadherin family member known to be crucial for vascular development (Dejana et

al., 2009; Strilić et al., 2009). Several features of the *Crim1* VEC-specific conditional knockouts also suggested a consequence for adhesion, including: (1) an increase in non-perfusible, VE-cadherin-negative vessel segments; (2) an increase in the bifurcation of tip cells that mimics the behavior of the VE-cadherin knockdown tip cells (Montero-Balaguer et al., 2009); and (3) VECs separated from extending vessels at the angiogenic front, a feature that is also observed in VE-cadherin endothelial conditional mutants (Gaengel et al., 2012), suggesting compromise of stable junctions.

However, there is also evidence countering the suggestion that *Crim1* regulates VE-cadherin function. First, when VE-cadherin is depleted from endothelial cells, vessels show excessive branching and overgrowth (Gaengel et al., 2012). This contrasted with *Crim1* VEC conditional mutants in which vessel connections with discontinuous VE-cadherin labeling are thin and usually lacking a lining of endothelial cell nuclei (Fig. 3E-H). Second, although we could identify α -catenin and β -catenin in immunoprecipitates of VE-cadherin from cultured confluent endothelial cells, we were not able to identify *Crim1*, even when we used an epitope-tagged *Crim1* to increase detection efficiency (data not shown). These findings suggest that the endothelial cell-cell contact and vessel lumen defects in *Crim1* conditional mutants might be an indirect consequence of *Crim1* loss of function.

Crim1 regulates autocrine Vegfa signaling

Previous studies on *Crim1* in various organisms have suggested that it might regulate the bioavailability of growth factors and morphogens. *In vitro* studies demonstrate that *Crim1* regulates the rate of processing and delivery of Bmp4 and Bmp7 to the cell surface (Wilkinson et al., 2003), probably in the Golgi compartment. Another study has provided evidence that *Crim1* can physically bind signaling ligands such as Vegfa, Pgf or Pdgf before their secretion (Wilkinson et al., 2007). In the kidney, *Crim1* sequesters Vegfa at the surface of podocytes, so that loss of *Crim1* causes excessive Vegfa-Vegfr2 signaling and overgrowth of the glomerular vasculature (Wilkinson et al., 2007). There are also invertebrate homologs of *Crim1*. The *C. elegans* homolog of *Crim1* (CRM-1) controls body size through regulation of BMP signaling (Fung et al., 2007). *Drosophila crimpy*, which has sequence homology to *Crim1*, inhibits Glass bottom boat (a BMP family ligand) in motoneurons during neuromuscular junction development (James and Broihier, 2011). However, *in ovo* knockdown of *CRIM1* in *Xenopus* and Zebrafish and germline loss of function of *Crim1* in mouse do not produce defects in the early patterning of the body axis (Kinna et al., 2006; Chiu et al., 2012; Ponferrada et al., 2012), as would be expected if *Crim1* were a key universal regulator of BMP signaling.

Our current findings are consistent with previous analysis of the phenotype after germline deletion of a *Crim1* conditional allele (Chiu et al., 2012). When a germline loss-of-function homozygous mutant was produced by recombining our loxP-flanked *Crim1* allele with *EIIA-Cre*, we found an assemblage of changes (including hemorrhage, edema and syndactyly) very similar to the phenotype of the previously characterized insertional (Pennisi et al., 2007) and germline conditional (Chiu et al., 2012) mutants. Although *Crim1* mutant mice show hemorrhage, and *Crim1* has been implicated in binding Vegfa (Wilkinson et al., 2007), germline *Crim1* mutant mice do not show a major failure in early vascular patterning as would be anticipated if *Crim1* were a crucial regulator of Vegfa signaling (Carmeliet et al., 1996; Ferrara et al., 1996). Combined, these findings suggest that *Crim1* regulates signaling ligands in a temporally and spatially restricted manner.

It has been shown that VECs can produce Vegfa and that this autocrine source of Vegfa is indispensable for blood vessel homeostasis (Lee et al., 2007). The current model suggests that autocrine Vegfa is crucial for stimulating VEC survival in response to stress signals such as hypoxia, irradiation and reactive oxygen species, factors that would be ongoing challenges for mature vessels. The current analysis, in which we have deleted *Vegfa* in VECs during development, also indicates that autocrine Vegfa is important at developmental stages.

One consequence of VEC-specific *Crim1* loss of function is deficient angiogenesis in the retina. Prompted by data showing that *Crim1* can regulate Vegfa (Wilkinson et al., 2007) and that Vegfa is the major stimulus for angiogenesis in the retina (Carmeliet and Jain, 2011), we investigated the possibility that the *Crim1* phenotype resulted from a Vegfa signaling deficiency. We showed that, in the developing retinal vasculature, *Vegfa* or *Crim1* conditional deletion in VECs gave very similar phenotypes characterized by an increased number of vessel ghosts, discontinuous VE-cadherin labeling and an increase in the number of segments that showed the absence of a vessel lumen. In addition, conditional compound heterozygotes had a phenotype more severe than each single heterozygous conditional mutant and one that was indistinguishable from the homozygous conditional phenotype. The severe phenotype of the double heterozygotes is surprising, and this additive effect is consistent with the hypothesis that this is a synthetic phenotype resulting from the function of *Crim1* and *Vegfa* in the same pathway.

Culture experiments showed directly that *CRIM1* regulates autocrine VEGFA signaling. First, we showed, as expected, that proliferation of HUVECs in culture is enhanced by VEGFA supplementation. In addition, we showed that HUVEC expansion is almost absent if we used shRNA targeting *CRIM1* even with an exogenous source of VEGFA. Starvation-stimulation experiments with exogenous VEGFA in *CRIM1* knockdown HUVECs did not, however, reveal any change in the level of VEGFR2 phosphorylation. This suggested that *CRIM1* is required for proliferation and survival in HUVECs, but not because it regulates the activity of exogenous VEGFA. By contrast, when HUVECs were denied exogenous VEGFA and required to rely on autocrine signaling, the outcome for VEGFR2 activation was different. In the absence of exogenous VEGFA, control HUVECs in serum-free medium did not proliferate but survived for 2 days or more. *CRIM1* knockdown resulted in a rapid reduction in cell numbers over this timecourse. Accompanying this elevated level of cell death was a reduced proportion of VEGFR2 in its active, phosphorylated state. Evidence that the documented VEGFR2 phosphorylation was a consequence of autocrine signaling came from a comparison of the effects of an anti-VEGFA antibody, which had a very limited effect, and that of a small-molecule VEGFR2 inhibitor, which completely abrogated phosphorylation. Combined, these data support a model in which *CRIM1* regulates VEGFA autocrine activity in the previously defined 'private loop' (Lee et al., 2007) and is consistent with the observation that *Crim1* can form physical complexes with Vegfa (Wilkinson et al., 2007).

MATERIALS AND METHODS

Generation of the *Crim1* conditional allele

We generated a conditional loss-of-function allele, *Crim1^{lox}*, using conventional gene targeting. With the very high GC content surrounding exon 1 (5' UTR and start codon), we targeted exons 3 and 4 (supplementary material Fig. S1A). This design left open the possibility that exons 1 and 2 might produce a functional truncated protein. Incorporation of sequences from the C-terminus of ornithine decarboxylase (cODC) as a signal for rapid

degradation (Matsuzawa et al., 2005) was designed to eliminate any expressed CRIM1 N-terminal sequence. The allele design incorporated *loxP* sites into an artificial exon that is initially in reverse orientation. Through two steps of recombination mediated by a pair of wild-type and a pair of variant *loxP* sequences that are incompatible, the ODC degradation signal was spliced into the mRNA, simultaneously deleting exons 3 and 4. This allele design strategy has been used previously (Schnütgen et al., 2003).

Mice

Pdgfb-iCreER (Claxton et al., 2008), *Elia-Cre* (JAX 003724), *Tg(CAG-Bgeo/GFP)* (*Z/EG*, JAX 003920) and the *Vegfa^{loxP}* line (Gerber et al., 1999) have been described previously. To induce endothelial cell gene deletion in *Pdgfb-iCreER* mouse pups, peanut oil-dissolved tamoxifen (Sigma) was injected intraperitoneally daily at 20 µg/g body weight. Tamoxifen-injected *Pdgfb-iCreER*-negative littermates were used as controls. Birth was defined as postnatal day (P) 1.

Whole-mount immunofluorescence of retinas

Antibody and isolectin labeling of retinas was performed as previously described (Stefater et al., 2011) with the following: Alexa 488-Isolectin IB4 (Life Technologies, I21411; 1:1000), Crim1 (homemade rabbit antiserum), collagen IV (rabbit; Abcam, ab19808; 1:500), BrdU (mouse; Dako, M0744; 1:100), VE-cadherin (rat; BD Biosciences, 555289; 1:100), VE-cadherin (goat; Santa Cruz, sc-6458; 1:200), CD31 (rat; BD Biosciences, 550274; 1:100), active caspase 3 (rabbit; R&D Systems, AF835; 1:100), podocalyxin (goat; R&D Systems, AF1556; 1:200), PDGFRα (goat; R&D Systems, AF1062; 1:100), desmin (rabbit; Abcam, ab15200; 1:500) and smooth muscle actin (mouse; Sigma, C6198; 1:200).

Cell culture

Pooled HUVECs (Lonza, CC-2519) were cultured on 0.1% gelatin-coated culture dishes in EGM-2 BulletKit medium (Lonza, CC-3162). Cells before passage five were used in all experiments. Lentiviral particles containing shRNA targeting *CRIM1* mRNA were acquired from TRC Mission Library (Invitrogen). Cells at 90% confluence were incubated with diluted lentiviral particles together with 8 µg/ml polybrene for 16 hours and then treated with 2 µg/ml puromycin for 2 days to enrich the infected cell population. Twenty-four hours after puromycin removal, cells were used in subsequent experiments. We tested four different shRNAs targeting *CRIM1* and chose the two that worked most effectively: TRCN0000063860 [shCRIM1(60)] and TRCN0000063862 [shCRIM1(62)]. Similarly, VEGFA was knocked down using TRCN0000003343, the effectiveness of which has been validated before (Segarra et al., 2012).

Growth curve of HUVECs

HUVECs (5000 cells) expressing scramble shRNA or shRNA targeting *CRIM1* or *VEGFA* were plated in each well of 96-well plates coated with 10 µg/cm² human fibronectin (Sigma, F2006) and cultured in EGM-2 medium. To monitor cell survival in VEGFA-deficient medium, 20,000 cells (confluent) were plated per well and cultured in EGM-2 without addition of serum or VEGFA.

VEGFR2 activation in cultured HUVECs

To detect the response to exogenous VEGFA, HUVECs expressing shRNAs were starved in EBM-2 (Lonza, CC-2156) containing only antibiotics and heparin for 24 hours and stimulated with recombinant human VEGFA protein (R&D Systems, 293-VE) with 2 mg/ml Na₃VO₄ for 5 minutes. To detect VEGFR2 activation by autocrine VEGFA, HUVECs expressing shRNAs were cultured in the same medium for 24 hours with or without 0.1 mg/ml Na₃VO₄. For control experiments, cells were cultured in medium either pre-incubated with 10 µg/ml Avastin (Genentech) or with 0.6 µM SU5416 (Sigma) added. Cell lysates were collected in RIPA buffer containing 2 mg/ml Na₃VO₄ and the phosphorylated VEGFR2 level detected either by ELISA (Cell Signaling, 7335 and 7340) or immunoblot using antibodies to phospho-VEGFR2 (Y1175, rabbit; Cell Signaling, 2478; 1:500), VEGFR2 (mouse; Santa Cruz, sc-6251; 1:400), CRIM1 (mouse;

Sigma, WH0051232M1; 1:500) or α-tubulin (rabbit; Abcam, ab89984; 1:4000).

Quantitative RT-PCR

RNA was extracted using the RNeasy Micro Kit (Qiagen) and cDNA was prepared using Superscript III reverse transcriptase (Invitrogen). Quantitative PCR was performed with iQ SYBR Green Supermix (Bio-Rad). Signals were detected using the Bio-Rad CFX1000 system and gene expression levels were calculated using the standard curve method.

Fluorescence-activated cell sorting (FACS)

Freshly isolated mouse retinas were incubated in DMEM (Gibco) containing 1 mg/ml collagenase A (Roche) and 3 U/ml DNase I on a shaker at 37°C for 30 minutes with gentle pipetting every 10 minutes. Cells were passed through cell strainers (BD Biosciences) and centrifuged at 500 *g* for 5 minutes. After washes in PBS containing 2 mM EDTA and reconstitution in the same buffer, cells were labeled with PE-CD31 and APC-CD45 (Ptpcr) antibodies (BD Biosciences) at 4°C for 20 minutes. After washes, cells were reconstituted in PBS containing 2 mM EDTA and passed through a cell strainer again before FACS analysis. PE (CD31)-positive and APC (CD45)-negative populations were sorted and collected as VECs.

Statistical analysis

Statistical analysis was performed using Student's *t*-test.

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.F., V.G.P., T.S., S.V. and R.A.L. designed the research, performed experiments and analyzed data. J.F. and R.A.L. wrote the paper. M.F., H.G. and N.F. contributed vital reagents.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.097949/-/DC1>

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