AIBP Limits Angiogenesis Through γ-Secretase-Mediated Upregulation of Notch Signaling

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**Rationale:** Angiogenesis improves perfusion to the ischemic tissue after acute vascular obstruction. Angiogenesis in pathophysiological settings reactsivate signaling pathways involved in developmental angiogenesis. We showed previously that AIBP (apolipoprotein A-I [apoA-I]–binding protein)–regulated cholesterol efflux in endothelial cells controls zebra fish embryonic angiogenesis.

**Objective:** This study is to determine whether loss of AIBP affects angiogenesis in mice during development and under pathological conditions and to explore the underlying molecular mechanism.

**Methods and Results:** In this article, we report the generation of AIBP knockout (Apoa1bp−/−) mice, which are characterized of accelerated postnatal retinal angiogenesis. Mechanistically, AIBP triggered relocalization of γ-secretase from lipid rafts to nonlipid rafts where it cleaved Notch. Consistently, AIBP treatment enhanced DLL4 (delta-like ligand 4)–stimulated Notch activation in human retinal endothelial cells. Increasing high-density lipoprotein levels in Apoa1bp−/− mice by crossing them with apoA-I transgenic mice rescued Notch activation and corrected dysregulated retinal angiogenesis. Notably, the retinal vessels in Apoa1bp−/− mice manifested normal pericyte coverage and vascular integrity. Similarly, in the subcutaneous Matrigel plug assay, which mimics ischemic/inflammatory neovascularization, angiogenesis was dramatically upregulated in Apoa1bp−/− mice and associated with a profound inhibition of Notch activation and reduced expression of downstream targets. Furthermore, loss of AIBP increased vascular density and facilitated the recovery of blood vessel perfusion function in a murine hindlimb ischemia model.

In addition, AIBP expression was significantly increased in human patients with ischemic cardiomyopathy.

**Conclusions:** Our data reveal a novel mechanistic connection between AIBP-mediated cholesterol metabolism and Notch signaling, implicating AIBP as a possible druggable target to modulate angiogenesis under pathological conditions. (Circ Res. 2017;120:1727-1739. DOI: 10.1161/CIRCRESAHA.116.309754.)

**Key Words:** AIBP ■ angiogenesis ■ cholesterol ■ cholesterol efflux ■ lipid rafts ■ Notch signaling ■ lipids and lipoprotein metabolism

Angiogenesis, the formation of new blood vessels from preexisting vasculature, mediates delivery of nutrients and oxygen to developing organs while removing metabolic waste products. Angiogenesis requires selection of endothelial tip cells by a gradient of VEGF (vascular endothelial growth factor) from the parent vessels, directional migration, and the establishment of connections between new sprouts. Two major signaling pathways, VEGFR and Notch, fine-tune angiogenic sprouting in a reciprocal manner.3-5

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In mammals, the Notch family comprises 2 Jagged-like ligands 1–2, 3 delta-like ligands (DLL1, 3, and 4), and 4 Notch receptors (Notch1–4).6-8 The role of DLL4 and Jagged-like ligands 1 during angiogenesis have been well-studied, with DLL4 inhibiting and Jagged-like ligands 1 promoting angiogenesis under most developmental or pathological conditions.9-12 DLL4 is abundant in filopodia-rich tip cells, which respond to surrounding molecular cues. The DLL4 expression, triggered by VEGF, activates Notch signaling and suppresses the tip cell phenotype in the neighboring stalk cells. Moreover, DLL4 expression is also induced by Notch, which may be a signal-relay mechanism that maintains the stalk cell fate. Notch activation involves a disintegrin and metalloprotease-mediated cleavage at the S2 site and γ-secretase-mediated cleavage at the S3 site of the Notch receptor,
What Is Known?

- Increased blood vessel formation after cardiac ischemia contributes to improved cardiac recovery.
- AIBP (apolipoprotein A-I–binding protein), a secreted protein, accelerates cholesterol efflux from endothelial cells, disrupts lipid rafts, and impairs raft-associated VEGFR2 (vascular endothelial growth factor receptor-2) signaling, thereby restricting angiogenesis.
- Notch signaling counteracts VEGFR2 signaling, inhibits angiogenesis, and stabilizes blood vessels.
- Notch activation entails its cleavage by γ-secretase, release of the intracellular domain, and activation of a transcriptional program.

What New Information Does This Article Contribute?

- AIBP-mediated disruption of lipid rafts relocates Notch1 and γ-secretase from lipid rafts to nonraft domains.
- Increased localization of Notch1 and γ-secretase in the nonraft domains facilitates Notch1 cleavage and its activation.
- AIBP deficiency in mice attenuates Notch signaling.
- AIBP knockout augments retinal and pathological angiogenesis in mice.
- Expression level of AIBP in the heart is increased in patients with ischemic cardiomyopathy.

Angiogenesis is essential for tissue remodeling and repair after cardiac ischemia. Treatments that improve blood vessel formation holds great promise for patients with coronary artery disease. We previously found that the secreted protein, AIBP, limits angiogenesis via modulation of cholesterol metabolism. In this article, we generated AIBP knockout mice and found that, consistent with our prior study, loss of AIBP increased developmental retinal angiogenesis and pathological angiogenesis in a murine hindlimb ischemia model. Furthermore, we showed that cardiac AIBP expression is increased in human ischemic cardiomyopathy. Our findings of AIBP-regulated angiogenesis may guide new treatment strategies that bolster angiogenesis in the pathological conditions.

Nonstandard Abbreviations and Acronyms

- ABC: ATP-binding cassette
- AIBP: apoA-I–binding protein
- ApoA-I: apolipoprotein A-I
- CD31: cluster of differentiation 31 protein
- DLL: delta-like ligand
- ECs: endothelial cells
- HDL: high-density lipoprotein
- ICM: ischemic cardiomyopathy
- MjCD: methyl-1-cyclodextrin
- NICD: Notch1 intracellular domain

AIBP, encoded by the Apo1bp gene in mice and APOA1BP gene in humans, is a secreted protein that physically associates with apoA-I29; AIBP is ubiquitously expressed and abundant in most human organs with secretory functions. Of note, AIBP is not expressed in the skeletal muscle but is highly expressed in vascular smooth muscle cells and in heart muscle,30 which implicates AIBP in cardiovascular functions. AIBP is evolutionarily conserved from Drosophila to human,29,31 and thus, we propose that the role of AIBP in angiogenesis is also conserved from zebra fish to mice to humans. Importantly, understanding the molecular mechanism for AIBP-regulated angiogenesis may guide the development of novel therapeutic strategies targeting angiogenesis in different pathological settings. To this end, we generated Apo1bp−/− mice, with which we discovered a new mechanistic underpinning of AIBP-regulated angiogenesis, which downregulates Notch in a γ-secretase-dependent fashion and restricts murine retinal and pathological angiogenesis. Importantly, loss of AIBP accelerates angiogenesis without affecting the integrity and functionality of blood vessels, suggesting that AIBP is a potential pharmacological target for treating cardiovascular disease.

Methods

Preparation of Whole Mount Retinas

After euthanization, eyes of neonatal mice at the appropriate developmental stage were isolated, fixed in 4% paraformaldehyde (PFA), and dissected. Retinal cups were subjected to immunostaining with the indicated Abs or extraction of total RNA or proteins as described below.

In Situ Hybridization

Murine retinas were incubated sequentially in 15% sucrose-PBS, 30% sucrose-PBS, and optimal cutting temperature compound and then used to prepare 5-μm-thick sections. After fixation in 4% PFA and acetylation in 1% triethanolamine and 0.25% acetic anhydride, the slides were prehybridized and incubated overnight with hybridization solution containing the RNA probes at 65°C. Subsequently, the sections were washed and probed with AP-conjugated anti-DIG Ab or anti-fluorescein Ab. The sections were developed with the addition
of fast red or NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) substrate. For double-probe in situ hybridization, both digoxigenin-terminatedApoa1bp and fluorescein-terminatedBrn3 were hybridized with the same section, and the sections were then first incubated with AP-conjugated anti-digoxigenin antibody, for which NBT/BCIP was used as a chromogenic substrate. Subsequently, the sections were washed and post-fixed with 4% PFA and further incubated with AP-conjugated anti-fluorescein antibody and developed with fast red (Roche). The Brn3b plasmid for making the riboprobe was kindly provided by Dr Chenghua Gu (Harvard Medical School).

### Retinal Whole Mount Immunostaining
Retinas were PFA-fixed, permeabilized, and blocked in TBST (Tris-buffer saline with Tween 20) containing 1% BSA and 5% goat or donkey serum. After 3 washes with PB/T buffer (0.5% Triton X-100, 1 mMol/L CaCl₂, 1 mMol/L MgCl₂, and 1 mMol/L MnCl₂ in PBS, pH 6.8), the retinas were incubated overnight with primary antibodies diluted in the blocking solution at 4°C. The following primary Abs were used: rat anti-CD31 (cluster of differentiation 31 protein; 1:200; BD Biosciences), rabbit anti-Collagen IV (1:250; Millipore), and rabbit anti-NF2 (1:250; Millipore). The secondary Abs were Alexa Fluor-488 or -594 conjugated anti-rat or anti-rabbit IgGs (1:500; Jackson ImmunoResearch). After antibody staining, retinas were postfixed in 4% PFA for 15 minutes and then were flat-mounted under a dissecting microscope. Images were captured using Olympus Fluoview FV1000 Laser Scan Confocal Microscope. The acquired images were quantified for retinal vascular density, numbers of tip cells, and filopodia as previously described using Image J (National Institutes of Health).

### Hematoxylin and Eosin and Immunohistochemistry
The PFA-fixed Matrigel plug was washed in PBS and then embedded in paraffin. Five-micrometer sections were prepared and de-paraffinized by sequential incubation in xylene and ethanol. After rehydration, the slides were stained with hematoxylin and eosin following a standard protocol.

For immunohistochemistry staining of Matrigel plugs harvested from the mice, 5-µm-thick paraffin section were deparaffinized, hydrated, and boiled in 10 mMol/L sodium citrate buffer (pH 6.0) for 15 minutes to facilitate antigen retrieval. After a brief treatment with Background Buster Blocking Agent (Innovex Biosciences), the slides were incubated with the primary antibody for 45 minutes. After washing, the slides were incubated sequentially with STAT-Q secondary linking antibodies (Innovex Biosciences), HRP (horseradish peroxidase)-labeled streptavidin and Innovex substrate (DAB [3,3'-diaminobenzidine tetrahydrochloride]) for color development following the manufacturer’s instructions.

### DLL4-Induced NICD Production
Human retinal ECs were plated and kept in culture for 4 hours onto 6-well plates that were precoated with or without recombinant human DLL4 protein (0.5 µg/mL) in 0.1% BSA/PBS for 1 hour at room temperature. Cells were further incubated with AIBP (0.1 µg/mL), HDL (50 µg/mL), or their combination for 4 hours, with 20 µM DAPT (N-[3,5-difluorophenylacetyl]-L-alanyl-2-phenylglycine-1,1-dimethyl ethyl ester; γ-secretase inhibitor) for 24 hours, 10 mMol/L methyl-β-cyclodextrin (MgCD) for 10 minutes, or 10 µg/mL water-soluble cholesterol (Sigma) for 2 hours. After the various treatments, cells were washed with PBS and lysed. The NICD levels were assessed by Western blot using a γ-secretase cleavage-specific antibody Val1744 (Cell Signaling Technology).

### Results

#### Apoa1bp Expression in the Retinal Ganglion Cells
Apoa1bp-null mice were generated as described (Online Figure IA); polymerase chain reaction and Western blot analysis indicated the absence of AIBP (Online Figure IB through ID). AIBP-null mice are viable and fertile and have no apparent morphological defects compared with control mice under naïve conditions. Our study in zebra fish showed that Aibp functions noncell autonomously in angiogenesis and that the tissue localization of Aibp at 24 hours post-fertilization is similar to that of Sema 3a. To assess the mRNA localization of murine Apoa1bp, in situ hybridization was performed on adjacent sections through postnatal day 5 (P5) murine retinas for Apoa1bp and Brn3b, a retinal ganglion cell marker. As with our study in zebra fish, Apoa1bp mRNA was localized in the Brn3b-positive retinal ganglion cell layer of a murine retina (Online Figure II), which expresses Sema3E. As expected, the Apoa1bp mRNA signal was absent in the Apoa1bp knockout retina (Online Figure IIA and IIC). AIBP expression in retinal ganglion cell implies a paracrine role of AIBP in murine retinal angiogenesis.

#### Loss of AIBP Increases Murine Retinal Angiogenesis
We assessed AIBP function in the retinal vasculature. The murine retina is avascular at birth; starting from postnatal day 1 (P1), a single superficial layer of blood vessels extends radially outwards from the center vessels at the optic nerve head toward the periphery until P7. Whole-mount immunofluorescent staining of the isolated retinas was performed with CD31 and Collagen IV on P5 retinas. AIBP-deficient retinas at P5 showed a profound increase in the radial extension of vascular plexus from the optic nerve to the periphery (Figure 1A and 1B) and in the total vascular area (Figure 1A and 1C). Next, we analyzed the branch points in Apoa1bp–/– and control littermates because extensive remodeling and sprouting is required to establish an interconnected vasculature network. The front and center areas analyzed in the retinas are illustrated in Figure 1D. Consistent with a role for AIBP in retinal angiogenesis, more branch points were observed in both front and central areas of the vascular plexus in the Apoa1bp–/– retinas compared with control littermate retinas (Figure 1E through 1G). Similar results were obtained in P3 neonates’ retinas (Online Figure IIIA). During angiogenesis, tip cells manifest exploratory and sprouting behavior by extending numerous filopodia. More endothelial tip cells were observed in AIBP null retinas than that in control littermates (Figure 2H and 2I), and the number of filopodia was significantly increased in Apoa1bp–/– retinas (Figure 1J and 1K). However, loss of AIBP had no effect on blood vessel formation at P7 and P9 (Online Figure IIIB and IIIC), which indicates that AIBP disruption accelerates normal retinal angiogenesis. The difference in angiogenesis may be caused by change in vessel stability. Thus, we stained for Collagen IV because regressed vessels generally leave empty sleeves of collagen IV–rich matrix deposits and which are used for the assessment of vessel stability. There was no apparent difference in vessel stability because similar numbers of empty sleeves (Col IV–CD31–) were found in the AIBP knockout and control mice (Online Figure IVA and IVB). Collectively, AIBP deficiency in mice results in significantly accelerated angiogenesis as evidenced by a greater radial extension length of vascular plexus, the development of a denser upper capillary layer, and a higher number of tip cells, as well as filopodia, in the retinas. This phenotype is, however, normalized by P7.

#### AIBP Controls Notch Signaling in Murine Retinas
Given that Notch signaling suppresses angiogenesis in most vascular beds, we determined whether accelerated retinal
angiogenesis in Apoa1bp−/− mice was because of impaired Notch activity. The activation of the Notch pathway was markedly attenuated as evidenced by diminished production of NICD levels in Apoa1bp−/− retinas (Figure 2A and 2B). Expression of Hey1, a transcriptional repressor of Notch that is selectively expressed in the blood vessels during retinal angiogenesis,6,39 Hey2, and

Figure 1. Retinal angiogenesis in Apoa1bp knockout mice. Retinas were isolated from postnatal day 5 (P5) Apoa1bp−/− mice and their control littermates and whole mount immunostaining performed for CD31 (cluster of differentiation 31 protein; green) and Collagen (Col) IV (red). Confocal images of retinas (A) and the quantification of vessel and retina diameter (B) and vessel area (C). D, The front and central areas of retinas analyzed are outlined in yellow and green. E, Enlarged areas of yellow and green squares in D. Quantification of branch points in the front (F) and the central areas (G). H and I, Tip cells and the quantification. Arrows show tip cells. J and K, Filopodia extension and the quantification. Mean±SD; n=3. **P<0.01. Scale bar: A, D, and E: 200 μm; H and J: 20 μm.
Hes1 were significantly decreased in P5 (Figure 2C) and P3 (Figure 2D) Apoa1bp−/− retinas. However, Notch receptors (Notch1 and Notch4), Notch ligand Dll4, and Notch pathway negative regulator Nrarp mRNA showed no significant change in P5 or P3 retinas (Online Figure VA and VB). Consistent with this finding, the Notch1 protein levels were comparable between Apoa1bp−/− and control retinas (Online Figure VC and VD). As expected, AIBP deficiency augmented VEGFR2 activation (Figure 2A; Online Figure VC and VD), but did not affect Vegfa gene expression (Online Figure VA and VB). These results indicate that loss of AIBP suppresses Notch signaling in postnatal murine retinas. Collectively, our results provide compelling support for the hypothesis that AIBP positively regulates Notch signaling, thereby, restricting angiogenesis.

We have previously shown that AIBP controls angiogenesis via a direct effect on VEGFR2 localization to cholesterol-rich lipid rafts.27 To distinguish the role of AIBP in VEGFR2 and Notch pathways, control and Apoa1bp−/− mice were treated with the Notch inhibitor DAPT, and retinal angiogenesis was assessed. DAPT inhibited retinal angiogenesis in Apoa1bp−/− mice to a significantly lesser degree than in control mice (Online Figure VI). These data indicate that AIBP regulates retinal angiogenesis through both Notch-dependent and independent pathways.

**AIBP Exerts Its Effect via γ-Secretase-Regulated Notch Signaling**

Binding of a Notch ligand to a Notch receptor initiates 2 cleavage steps that generate transcriptionally active NICD—the first step mediated by a disintegrin and metalloprotease and the second by γ-secretase.40 Because AIBP deficiency has no effect on Notch1 protein levels (Online Figure VC and VD), and our previous studies showed that AIBP-mediated depletion of cholesterol disrupts lipid rafts,27 we tested whether AIBP exerts its effect on γ-secretase-regulated Notch via alteration of lipid rafts. The γ-secretase complex, which includes the 4 proteins presenilin1, nicastrin, anterior pharynx defective 1, and presenilin enhancer 2,41–43 is localized to plasma membrane lipid rafts. Disruption of lipid rafts by modest cholesterol reduction induces γ-secretase translocation from lipid rafts to nonrafts, which augments the cleavage of its substrate, APP (amyloid precursor protein), in neurons.44 Therefore, we postulated that AIBP-mediated reduction of lipid raft abundance...
also enhances Notch cleavage via effects on γ-secretase distribution in ECs. We reasoned that AIBP may interact with cells to achieve an effect on lipid rafts. Thus, we verified the binding of AIBP to human retinal microvascular ECs (Online Figure VII). Next, we performed detergent-free, discontinuous gradient ultracentrifugation analysis of human retinal microvascular ECs treated with control media, AIBP, HDL₃, or their combination and found that coincubation with AIBP and HDL₃ translocated γ-secretase and Notch1 from lipid rafts to nonraft domains (Figure 3A).

Figure 3. AIBP (apolipoprotein A-I [apoA-I]–binding protein) regulates Notch signaling through its effect on γ-secretase localization. A, The distribution of γ-secretase and Notch1 in lipid rafts (fractions 4–6) and nonlipid rafts (fractions 9–11). Human retinal microvascular ECs (HRMECs) were incubated with 0.1 μg/mL AIBP, 50 μg/mL HDL₃, or their combination for 4 hours and were disrupted and separated into lipid raft and nonlipid raft fractions by ultracentrifugation. Immunoblots of presenilin1 (PS1) and Notch1 were shown. B, Methyl-β-cyclodextrin (MβCD) effect on Notch activation. HRMECs were seeded onto a 6-well plate precoated with or without 0.5 μg/mL recombinant delta-like ligand 4 (DLL4) and followed by treatment with 10 mmol/L MβCD for the indicated times. To ensure cells received equal DLL4 stimulation time, the treatment was started at different time so as to harvest the cells simultaneously for immunoblotting of Notch intracellular domain (NICD). C, Quantification of NICD protein levels in B (solid line) and measurements of free cholesterol content (dash line) removed from HRMECs with MβCD treatment. D, AIBP and HDL₃ effect on Notch activation. HRMECs were seeded as in B, followed by treatment with AIBP (0.1 μg/mL), HDL₃ (50 μg/mL), AIBP/HDL₃ combination for 4 hours, with 20 μM DAPT (N-[3,5-difluorophenyl][acetyl]-L-alanyl-2-phenylglycine-1,1-dimethyl ethyl ester; γ-secretase inhibitor) for 24 hours, with 10 mmol/L MβCD for 10 minutes or with 10 μg/mL water-soluble cholesterol for 2 hours. The different treatments were started at various time so as to lyse the cells simultaneously for Western blot of NICD. E, Quantification of NICD proteins in D. F, AIBP and HDL₃ effect on γ-secretase activity in HEK293 (human embryonic kidney) cells. HEK293 cells were transfected with NotchΔE or a control empty plasmid. After 48 hours, the resulting cells were treated as in D and used for immunoblots as indicated. NotchΔE was detected using anti-Myc Ab. G, Quantification of F. The relative values of NICD/NotchΔE/β-tubulin were normalized to that of control and the resulting numbers were used to generate the graph. Mean±SD; the experiments were done with 3 independent repeats. In all panels, **P<0.01; ***P<0.001.
Previous studies suggest that lipid rafts regulate γ-secretase. Thus, we examined the effect of cholesterol on DLL4-stimulated Notch activity by incubating human retinal microvascular ECs with MβCD, an efficient cholesterol sequestrant, for 0 to 60 minutes. MβCD treatment markedly increased NICD production, peaking at 10 to 15 minutes and returning to near basal levels at 60 minutes (Figure 3B and 3C). As expected, the NICD levels roughly correlated with the amount of cholesterol extracted from cells by MβCD (Figure 3C). We next examined the effect of AIBP on DLL4-induced Notch activity in human ECs. Human retinal microvascular ECs were incubated with AIBP, HDL3, or both. Coincubation with AIBP and HDL3, but not alone, significantly increased DLL4-stimulated NICD production (Figure 3D and 3E). In contrast, supplementation of cells with free cholesterol completely eliminated NICD generation (Figure 3D). As expected, the γ-secretase inhibitor DAPT abolished the production of NICD (Figure 3D and 3E). To further test the effect of lipid rafts on γ-secretase-mediated Notch signaling, a truncated version of Notch (NotchΔE) lacking the extracellular domain but retaining the γ-secretase cleavage site was transfected into HEK293T (human embryonic kidney) cells, followed by AIBP treatment in the presence or absence of HDL3. We found that AIBP and HDL3, in combination, but not alone, increased the γ-secretase-dependent generation of NICD from NotchΔE (Figure 3F and 3G). As in human ECs, treatment with MβCD or DAPT augmented or suppressed Notch activation, respectively (Figure 3F and 3G). Together, these results indicate that AIBP/HDL3 positively regulates Notch signaling by directing γ-secretase and Notch from lipid rafts to nonlipid domains, where enhanced γ-secretase-mediated Notch cleavage occurs.

Increasing HDL Levels Rescues Dysregulated Retinal Angiogenesis in AIBP Null Mice

We showed that AIBP-mediated cholesterol efflux increased Notch activity (Figure 3B through 3G), an effect achieved through reduction of lipid raft abundance. The apoA-I transgenic mice (Apoa1Tg), which have 2-fold higher levels of HDL cholesterol than controls, were used to correct the myeloproliferative disorder caused by increased lipid rafts in mice with a deficiency of the cholesterol transporters, ABCA1 and ABCG1. Therefore, we crossed Apoa1bp−/− mice with apoA-I transgenic mice to increase HDL levels in Apoa1bp−/− mice and determined the effect of increased HDL levels on dysregulated angiogenesis in Apoa1bp knockout retinas (Online Figure VIII A and VIII B). As expected, increased HDL levels in AIBP null retinas reversed the proangiogenic phenotype, including the radial extension of vascular plexus (Figure 4A through 4C), vascular branch points (Figure 4D through 4F), number of tip cells, and filopodia in the retinas (Figure 4G through 4J). Consistent with this, increased HDL levels reduced VEGFR2 activation in Apoa1bp−/− retinas (Online Figure VIII C). We observed no changes in vessel remodeling based on similar numbers of empty sleeves in the P5 retinas of Apoa1bp−/− and Apoa1bp−/−/Apoa1Tg mice (Online Figure IX). To assess whether Notch activity is enhanced in Apoa1Tg mice, NICD protein levels were probed in Apoa1bp−/− and Apoa1bp−/−/Apoa1Tg retinas. As illustrated in Figure 4K and 4L and Online Figure VIII A, NICD levels were elevated, and VEGFR2 phosphorylation was reduced in Apoa1bp null mice with apoA-I overexpression. In addition, quantitative real-time polymerase chain reaction analysis was performed to assess the expression of Notch receptors and ligands, as well as downstream target genes. Hey1, Hey2, and Hes1 mRNA levels were significantly increased at P5 in the retinas from Apoa1bp−/−/Apoa1Tg compared with that of Apoa1bp−/− (Figure 4M). Increased HDL levels had no significant effect on Notch1, Notch4, Dll4, Nrarp, or Vegfa gene expression (Online Figure VIII D).

Vascular Permeability and Pericyte Coverage in AIBP Knockout Mice

Our data indicate that the loss of AIBP accelerates developmental angiogenesis, which might be beneficial in patients with tissue ischemia. To explore the potential of AIBP for therapeutic purposes, we determined whether loss of AIBP affects perfusion and vasculature leakage. Adequate pericyte coverage is essential for the integrity of blood vessels. Therefore, mural cell coverage of ECs was investigated by whole mount immunofluorescence staining of NG2 and CD31 in P5 retinas. We found no difference in pericyte coverage in both front and central regions of P5 retinas between Apoa1bp−/− and control littermate neonatal mice (Figure 5A through 5D).

To further determine the effect of AIBP on vascular integrity in vivo, we performed fluorescein angiography on Apoa1bp−/− and control wild-type mice. Neither the Apoa1bp−/− mice nor the control wild-type littermates showed any retinal vascular leakage (Figure 5E). Collectively, our data suggest that loss of AIBP accelerates angiogenesis while retaining vascular integrity.

Loss of AIBP in Mice Enhances Neovascularization in the Matrigel Plug Assay and Improves Functional Recovery of the Vasculature in the Murine Hindlimb Ischemia Model

To test the effect of AIBP absence on in vivo angiogenesis under conditions simulating a pathological process, we injected Matrigel into a subcutaneous location. The Matrigel hardens into a plug, which is invaded by immune cells and becomes vascularized. This is a model of neovascularization occurring with ischemia and inflammation. Five days after injection, the Matrigel plugs were retrieved, and hematoxylin and eosin and immunohistochemistry were performed to evaluate blood vessel formation and perfusion within the plugs. As expected, there were more blood vessels in Matrigel plugs from Apoa1bp−/− mice than that from control mice (Figure 6A through 6C). Quantitative real-time polymerase chain reaction analysis confirmed that expression of EC markers CD31 and VE (vascular endothelial)-cadherin was significantly increased in Matrigel plugs from Apoa1bp−/− mice (Figure 6D and 6E). These data indicate that AIBP inhibits inflammatory neovascularization in mice, which agrees with the results of accelerated postnatal retinal angiogenesis. Remarkably, the effect of AIBP ablation on Matrigel neovascularization was more robust than in retinal angiogenesis, suggesting an important role of AIBP in regulation of angiogenesis under pathological conditions.

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Figure 4. Rescue effect of increased high-density lipoprotein (HDL) levels on angiogenesis and Notch signaling in AIBP (apolipoprotein A-I [apoA-I]–binding protein) null retinas. Retinas were isolated from Apoa1bp−/−Apoa1Tg and control Apoa1bp−/− mice at postnatal day 5 (P5), and whole-mount immunostaining of CD31 (cluster of differentiation 31 protein; green) and Col IV (red) was performed. A, Confocal images of retinas. Quantification of vessel and retina diameter (B) and vessel area (C). Retinal vasculature (D) and the quantification of branch points in the front areas (E) and central areas (F). G and H, Tip cells and the quantification. I and J, Filopodia extension and the quantification. K, Notch intracellular domain (NICD), phospho-VEGFR2 (vascular endothelial growth factor receptor-2; pVEGFR2), and total VEGFR2 protein levels in P5 retinas from Apoa1bp−/−Apoa1Tg and control Apoa1bp−/− mice. L, Quantitative real-time polymerase chain reaction (RT-PCR) analysis of the Notch downstream Hey1, Hey1, and Hes2 gene expression in the retinas from Apoa1bp−/−Apoa1Tg and Apoa1bp−/− mice at P5. Mean±SD, n=3; *P<0.05; **P<0.01. Scale bar: A and D: 200 μm; G and I: 20 μm.
Next we examined whether enhanced pathological angiogenesis in Apoa1bp−/− mice was because of attenuated Notch activity. Western blot analysis revealed significantly reduced NICD levels in Matrigel plugs from Apoa1bp−/− mice (Figure 6F and 6G). Moreover, expression of Notch downstream target genes Hey1, Hey2, and Hes1 was drastically reduced in Matrigel plugs from Apoa1bp−/− mice (Figure 6H), whereas Notc1h and Notch4, Dll4, and Nrarp mRNA levels showed no significant change (Online Figure X). These results suggest that AIBP inhibits angiogenesis via positive regulation of Notch activity.

To further assess the role of AIBP in pathological angiogenesis, we used the murine hindlimb ischemia model. In unoperated animals, similar values of limb perfusion were observed in wild-type and AIBP knockout mice using laser Doppler imaging (Figure 7A). However, after induction of limb ischemia by femoral artery ligation, the increase in gastrocnemius capillary density and the recovery of limb perfusion was significantly enhanced in the AIBP knockout animals (Figure 7A through 7D). The results suggest that AIBP deficiency promotes functional blood vessel formation.

**Increased AIBP Expression in Human Ischemic Cardiomyopathy**

Augmented angiogenesis promotes cardiac recovery after ischemia. To extend our findings on AIBP to the context of human cardiomyopathy, we analyzed AIBP expression in normal hearts or in hearts from patients with ischemic cardiomyopathy (ICM). Interestingly, AIBP expression was markedly increased in the myocardial tissue from ICM patients by comparison to that from patients without ICM (Figure 8). The data imply a possible role of AIBP in ICM, where increased AIBP expression may contribute to the ischemic pathophysiology.

**Discussion**

Here we showed that AIBP suppresses angiogenesis via enhanced Notch activity. AIBP knockout in mice increased...
postnatal retinal angiogenesis, augmented neovascularization in subcutaneous Matrigel plugs, and enhanced the increase in capillary density and recovery of limb perfusion in the murine hindlimb ischemia model (Figures 1, 6, and 7). Moreover, we revealed that AIBP-triggered lipid raft content reduction enhances the codistribution of γ-secretase and Notch in nonlipid raft fraction and subsequently increases the γ-secretase-mediated cleavage of Notch1, thereby, augmenting Notch activity (Figure 3). Our study connects AIBP-regulated cholesterol content in the plasma membrane with Notch signaling, which extends our findings in zebra fish and implies the conserved role of AIBP in angiogenesis. More importantly, we showed that loss of AIBP does not influence vascular integrity and that accelerated angiogenesis in Apoa1bp−/− mice produces functional blood vessels (Figure 5). Interestingly, AIBP expression is substantially elevated in patients with ICM (Figure 8), suggesting that AIBP may be a novel determinant of tissue ischemia. Our findings indicate that AIBP may be a pharmacological target for cardiovascular therapeutics.

AIBP Effect on Developmental and Pathological Angiogenesis

The human APOA1BP gene is located at 1q21.2-1q22, which corresponds to the 1q21-q23 locus for familial combined hyperlipidemia, a common, multifactorial, and heterogeneous dyslipidemia predisposing individuals to premature coronary artery disease. Despite this observation, there are no studies linking AIBP polymorphism with dyslipidemia or risk of cardiovascular disease. Our study is the first to identify a non-cell autonomous role of AIBP in angiogenesis.

Pathological angiogenesis usually recapitulates certain features of developmental angiogenesis. In our Matrigel experiment, which simulates some aspects of pathological angiogenesis, AIBP exerted a striking effect on regulating Notch and angiogenesis (Figure 6). However, the effect of AIBP on Notch in postnatal retinal angiogenesis was modest (Figure 1), which may be because of the more acute nature of Matrigel-induced pathological angiogenesis that amplifies the function of AIBP in regulating Notch (Figure 6).
Lipid Rafts, γ-Secretase, and Notch

Lipid rafts are implicated in the regulation of γ-secretase activity, which cleaves Notch, thereby, activating its signaling. γ-Secretase seems to manifest greater activity in lipid rafts in vitro studies and facilitates the cleavage of its substrate APP. On the other hand, lowering brain cholesterol levels in mice using statins increases amyloid production. Further, in human patients with a genetic mutation that causes cellular cholesterol accumulation, early onset of APP aggregation is also found. Notably, γ-secretase is located in nonlipid raft membranes during embryonic development, but in lipid rafts in adults. These 3 pieces of evidence suggest that reduction of lipid raft levels does not necessarily lead to an effect that is associated with decreased γ-secretase activity. Rather, in cultured neurons, a modest reduction of lipid raft abundance confers enhanced cleavage of APP by γ-secretase in nonraft fractions. In support of this, our results indicate that AIBP/HDL treatment relocates γ-secretase from the lipid raft fractions to the nonraft fractions; this precipitates codistribution of γ-secretase with Notch1 in the nonraft fractions and enhances Notch signaling (Figure 3). Thus, our study uncovered a new molecular mechanism by which AIBP regulates angiogenesis via its effect on γ-secretase-mediated Notch signaling. Our results suggest that AIBP acts primarily on Notch receptor proteolytic processing because quantitative real-time polymerase chain reaction and immunoblotting analysis indicated no substantial expression change of DLL4 and Notch1 in murine retinas (Online Figures V, VIIID, and X). Taken together, these results support a model in which AIBP regulates angiogenesis through its effect on γ-secretase translocation from lipid rafts to nonlipid rafts and ensuing Notch cleavage (Online Figure XI).

AIBP-Regulated Angiogenesis and Vascular Integrity

In addition to identifying the inhibitory role of AIBP in angiogenesis, we also assessed the effect of AIBP on vascular integrity and permeability. We showed that in contrast to excessive VEGF treatment or Epsin deficiency, which promotes non-functional angiogenesis and results in a leaky vasculature, loss of AIBP augments functional vasculature formation, as illustrated by normal extent of pericyte coverage and undetectable fluorescein leakage (Figure 5). Furthermore, we showed that AIBP deficiency facilitated blood vessel formation and improved the functional recovery of vasculature in a murine hindlimb ischemia model, which suggests the possibility of an AIBP-based therapy for angiogenesis-associated diseases. Given that stimulation of angiogenesis is considered beneficial for the treatment of myocardial infarction and that AIBP is markedly increased under the ICM condition (Figure 8), it will be interesting to determine whether targeting AIBP is salutary for ICM.
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Disclosures

None.

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AIBP Limits Angiogenesis Through γ-Secretase-Mediated Upregulation of Notch Signaling
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Mouse generation and breeding

All mouse experiments were approved by the HMRI Institutional Animal Care and Use Committee (IACUC).

To generate LoxP-Apoalbp mice, a vector including 4 Kb 5’ upstream, the whole 6 exons and 2 Kb 3’ downstream of Apoa1bp gene was constructed and used for targeting B6 ES cells. The ES cell clones that had legitimate LoxP-Apoalbp homologous recombination were further verified with chromosome counting, and two were selected and transplanted into the blastocyst to generate floxed Apoa1bp chimeras, which were crossed with B6 Albino to obtain germline-transmitted Loxp-Apoalbp mice. The floxed Apoa1bp mice were crossbred with CMV-Cre mice to produce global AIBP knockout mice, which were backcrossed with B6 mice to remove Cre. Apoa1bp<sup>B6</sup>ApoAI<sup>Tg</sup> mice were generated by crossing the Apoa1bp<sup>B6</sup> mice with ApoAI<sup>Tg</sup> mice (JAX).

Quantitative RT-PCR

Total RNA from the retina was extracted using a MiniPrep kit (Zymo Research) and reversely transcribed to cDNA (qScript; Quanta Biosciences) in a Miniopticon thermal cycler (Bio-Rad, C1000 Touch) following the manufacturer’s instructions. The qRT-PCR products were measured using SYBR green-based detection (Applied Biosystems). The relative expression levels of the different genes were calculated based on the Ct values obtained for the gene of interest and 18S rRNA (the internal control).

The primers were designed using Primer3 software (http://frodo.wi.mit.edu/) as following: mouse Hes1, sense: 5’-CCAGCCAGTGCTAACACGA-3’, anti-sense: 5’-AATGCCGGAGCTATCTTTCT-3’; mouse Hey1, sense: 5’-GCGCGGAGGAAATGGAAA-3’, anti-sense: 5’-TCAGGTGATCCACAGTCATCTG-3’; mouse Hey2, sense: 5’-AAGCGCCCTTGTGAGGAAAC-3’, anti-sense: 5’-GGTAGTGTGGTGATGGAGAC-3’; mouse Nrarp, sense: 5’-TTCAACGTAACGTGCTCCGGG-3’, anti-sense: 5’-TGCCGTCGTGACTGACTG-3’; mouse Dll1, sense: 5’-CAGGACCTTCTTCCGCGTATG-3’, anti-sense: 5’-AAGGGGAATCGGATGGGT-3’; mouse Dll4, sense: 5’-TTCCAGGCAACCTCTCCGA-3’, anti-sense: 5’-ACTGCCGCTATTCTTTGCCC-3’; mouse Notch1, sense: 5’-GATGGCCTCAATGGGTACAAG-3’, anti-sense: 5’-TCGTTGTTGATGTCAATGT-3’; mouse Notch4, sense: 5’-CTCTTGCCACATCTTCTCCT-3’, anti-sense: 5’-TGCAGTGGGATGATCCCTC-3’; mouse CD31, sense: 5’-ACGTGTTGTGTCCTATGCAAG-3’, anti-sense: 5’-TCAGTTGCTGCCATTCAAT-3’; mouse VE-Cadherin, sense: 5’-CAGTCTTCTTGGGAGCTTC-3’, anti-sense: 5’-GGGGCAAGGATCTTTTCT-3’; mouse RPS18, sense: 5’-AGTCCAGCAGCATTTTGGCAG-3’, anti-sense: 5’-TCATCCTCGGTAGTTCTC-3’.

ISH riboprobe Synthesis

Digoxigenin-labeled and fluorescein-incorporated riboprobes were generated as described 1,2. Briefly, a RNA synthesis system containing 1 μg of linearized template DNA, 2 μl 10× concentrated DIG-NTP or Fluorescein-NTP labeling mixture (Sigma Aldrich), 2 μl 10× concentrated transcription buffer, and 2 μl (40 U) T7 RNA polymerase (NEB) were prepared and incubated at 37°C for 2 hours. The DNA template was digested by addition of 2 μl (20 U) DNase, RNase-free (NEB) and incubation for a further 15 min at 37°C. The synthesized RNA was precipitated with 2.4 μl LiCl (4 M) in the presence of 75 μl prechilled ethanol. The precipitates were left for at least 30 min at -70°C or 2 hours at -20°C. The precipitates were spun down, washed with cold ethanol (70% [v/v]), air-dried and dissolved in RNase-free water.
**Western blot**
Immunoblot was done as previously described\(^3\). In brief, the murine retinal tissues were homogenized on ice using a Manual Disperser (PT 1200E) in the lysis buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol supplemented with complete protease inhibitor cocktail (Thermo Scientific). Cells were washed with ice-cold PBS (pH 7.4) and then lysed. The lysates were centrifuged at maximum speed for 15 min at 4°C. Fifty μg cell lysates were prepared for SDS-PAGE analysis (Bio-Rad). The proteins were subsequently transferred to PVDF membrane, which was blocked with 5% non-fat milk, and then incubated with primary antibodies overnight at 4°C, and further incubated with corresponding secondary antibodies, with 3 times of TBST wash after each antibody incubation. Blots were captured with a chemiluminescence detection system (ECL prime, GE Healthcare) in FluorChem M system (FM 0510, ProteinSimple).

**HDL\(_3\) isolation**
HDL\(_3\) isolation was carried out as described\(^4\). Fresh plasma from healthy donors was obtained from the blood bank of HMRI. A plasma density of 1.12 g/mL was prepared with KBr and used for ultracentrifugation at 35,000 rpm for 2 days at 4°C (ThermoFisher Sorvall WX 80). The lower portion of the first spin containing HDL was used to prepare a KBr density of 1.21 g/mL and centrifuged at 40,000 rpm for 3 days at 4°C. The HDL\(_3\) fraction was collected and dialyzed against PBS and 2 mmol/L EDTA. The purity of HDL\(_3\) was verified with SDS-PAGE gel separation followed by Coomassie Blue staining. HDL\(_3\) preparations were tested for possible endotoxin contamination using a LAL kit (ThermoFisher). The HDL\(_3\) used for cell culture experiments contained endotoxin levels lower than 50 pg/mg proteins, corresponding to 2.5 pg/ml.

**Cell culture**
HEK-293 cells were obtained from ATCC and cultured in Dulbecco’s Modified Eagle’s medium (DMEM, Life Technology) containing 10% fetal bovine serum (FBS, Thermo Scientific), 100 units/ml penicillin and 100 μg/ml streptomycin (Lonza) in humidified incubator at 37°C with 5% CO\(_2\). HUVECs and HRMECs were purchased from Lonza and cultured in Endothelial Basal Medium (EBM\(_2\)) with 2% FBS and all supplements as well as growth factors that contained in the Bulletkit at 37°C with 5% CO\(_2\).

**AIBP binding to HRMECs**
AIBP was biotinylated as previously described\(^5\). Approximately 2×10^4 cells HRMECs were plated in a ZellkulTuR microplate 96-well plate (Greiner) and cultured for 2 days at 37°C humidified incubator, and the same amount of cells were plated in a transplant 96-well plate to ensure cell confluence when carrying out the assay. The biotinylated AIBP, starting from 100 μg/ml and serially diluted in EBM2-1%-BSA, were incubated with HRMECs for 2 hours on ice. The cells were then washed 3 times with PBS, fixed with 4% PFA for 15 min at room temperature, and were further incubated with neutrAvidin-alkaline phosphatase (Jackson ImmunoResearch) for 30 min. After 3 washes with PBS and 3 washes with deionized water, a lumiphos 530 (Lumigen, Southfield, MI) substrate were added and the results were measured using a Tecan Infinite M1000 pro. Data were expressed as relative light units counted per 100 ms, with triplicates for each concentration. The Kd of AIBP binding to HRMECs were calculated using a total and non-specific binding algorithm of the GraphPad Prism 5.0 software.

**Detergent-free isolation of lipid rafts**
Lipid rafts were isolated using a detergent-free, discontinuous gradient ultracentrifugation method as previously described\(^6\). Briefly, human ECs were washed twice with ice-cold PBS and cells were scraped from the plate in 0.5 M sodium carbonate buffer (pH 11.0) containing protease inhibitor cocktail (Sigma), homogenized and sonicated for 3 × 10 sec. A 90% sucrose (w/v) solution in MBS (25 mM Mes, 0.15 M NaCl, pH 6.5) was used to adjust samples to 45% sucrose (w/v) in ultracentrifugation tubes. The 35% and 5% sucrose solutions were added consecutively to the mixture. After ultracentrifugation at 35×10^3 rpm for
20 hours at 4°C in a SW-41 rotor (Beckman), a discontinuous sucrose density gradient of 5-35% was generated. Eleven 1 ml fractions were collected from the top to the bottom of each gradient. Equal volumes of each faction (adjust to the same protein concentration) were analyzed by SDS-PAGE with presenilin1 (Cell Signaling Technology) and Notch1 (BD Biosciences) Abs.

**Free cholesterol measurement**
Total lipids were extracted from the cells and free cholesterol levels were measured according to the manufacture’s protocol (BioVision).

**γ-secretase-mediated Notch cleavage in HEK293 cells**
We followed an established protocol for DLL4 treatment. HEK-293 cells seeded in 6-cm dishes were transiently transfected with NotchΔE construct which lacks extracellular domain of the protein and possesses only the γ-secretase cleavage site (Addgene). Experiments were performed in the presence of AIBP, HDL₃, or other chemicals as above mentioned. Western blot was used to detect γ-secretase-dependent generation of NICD.

**DAPT administration**
DAPT, an γ-Secretase inhibitor, was purchased from Cayman Chemicals and were dissolved in DMSO. At P4, DAPT or DMSO was injected s.c, above the eyes, with a dose of 50 mg/kg per mouse as described. Twenty-four hours later (P5), retinas were analyzed using CD31 or Isolectin B4 staining.

**Fluorescein angiograph**
Mice were anesthetized by intraperitoneal (IP) injection of a combination of ketamine (65–100 mg/kg), xylazine (10–20 mg/kg), and acepromazine (1–3 mg/kg). Pupils were dilated with 1% tropicamide (Bausch & Lomb, Incorporated, Rochester, NY, USA). Fluorescein sodium (AK-FLUOR, 10 mg/kg, Akorn, Inc.) was delivered into mice by tail vein injection. Immediately after injection, FA was recorded using Heidelberg Retina Angiograph (HRA)-OCT device (Spectralis) from Heidelberg Engineering (Heidelberg, Germany) as described previously.

**In vivo Matrigel angiogenesis assay**
A mixture containing 500μl of Matrigel (BD Biosciences), 50 ng/ml VEGF (R&D) and 30 U/ml heparin (Sigma) was subcutaneously injected into the dorsal surface of Apoalbp⁻/⁻ and control mice. Two Matrigel plugs were implanted per mouse. Five days later, the mice were sacrificed and the Matrigel plugs were retrieved, processed where appropriate, and analyzed accordingly.

**Murine hindlimb ischemia model**
The hindlimb ischemia was performed in 9-month old male control or AIBP KO mice as previously reported with slight modification. Briefly, unilateral hindlimb ischemia was induced by ligating the femoral artery and its major branches. Afterwards, the mice were housed individually and blood flow in the affected and control limbs was imaged every 3 days. Total capillary density of the ischemic hindlimb cross sections was determined by staining the slides with rabbit anti-mouse CD31 (BD Biosciences), followed by horseradish peroxidase–conjugated secondary antibody.

**Laser Doppler measurement of vascular perfusion**
Blood flow to the ischemic or normal (nonischemic) hindlimb was examined with the use of a PeriScan PIM3 laser Doppler system (Perimed AB, Sweden), as described previously. Animals were prewarmed to a core temperature of 37.5°C, and heart rate was closely monitored for signs of stress from overheat. Hindlimb blood flow was measured preoperatively and postoperatively on the selected days (0, 3, 7, 10 and 14). The level of perfusion in the ischemic and normal hindlimbs was quantified with the use of the mean pixel value within the region of interest, and the relative changes in hindlimb blood flow...
were expressed as the ratio of the left (ischemic) over right (normal) Laser Doppler-detected blood perfusion.

**Human heart tissue collection**
Fresh cardiac tissues were obtained from the surgeon under a Houston Methodist IRB-approved study during Left Ventricular Assist Device insertion for patients. A small piece of scar-free left ventricle apex samples were obtained and PFA-fixed immediately, dissected and paraffin-embedded for histological analysis. Normal hearts were from donors that were not optimal for transplantation. The 3 patients were 36 years old female, 52 years old female and 43 years old male.

**Statistical analysis**
Statistical analysis was carried out using Prism or Microsoft Excel. Statistical significance, otherwise specified, was determined by unpaired two-sided t tests. In all figures: #, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.
Online Figures

**Online Figure I. Generation of Apoa1bp knockout mice.** (A) The construct used to make an Apoa1bp<sup>fl/fl</sup> mouse. E: exon. (B) PCR genotyping of genomic DNA from positive embryonic stem cells (ESC), Apoa1bp<sup>+/−</sup> mice and Apoa1bp<sup>−/−</sup> mice. (C) Total RNA was extracted from white blood cells of Apoa1bp<sup>fl/fl</sup>, Apoa1bp<sup>+/−</sup> and Apoa1bp<sup>−/−</sup> mice, and mRNA level of Apoa1bp was examined by qRT-PCR. (D) Western blot analysis of white blood cell lysates from Apoa1bp<sup>−/−</sup> mice and control animals.
Online Figure II. AIBP localization in the murine retinas.
ISH on frozen sections of P5 retinas. Representative ISH images of two adjacent sections for **Apoa1bp** (A) and **Brn3** (B). (C) ISH of **Apoa1bp** in an AIBP knockout retina. (D and E) Enlarged images of the dash line rectangles marked in A and B. (F) ISH for both **Apoa1bp** (blue) and **Brn3** (red) on the same section. In all the panels, arrow heads depict positive ISH signal in the RGC layer. Scale bar: A–C: 500 μm; D–F: 100 μm.
Online Figure III. Analysis of retinal angiogenesis.
Images of whole mount immunostaining of P3 retinas (A), P7 retinas (B) and P9 retinas (C) from Apoa1bp<sup>−/−</sup> and control littermates using CD31 (green) with or without Col IV (red) antibodies. Scale bar: 200 μm; Mean ± SD; n=3.
Online Figure IV. Normal vascular remodeling in AIBP KO mice. (A) Images of whole mount immunostaining of P5 retinas from $Apoalbp^{−/+}$ and control littermates using CD31 (green) and Col IV (red) antibodies. (B) CD31$^+$ vs. Col IV$^+$ area were compared. No significant difference of vascular remodeling between control and AIBP KO retinas were found. Scale bar, 20 μm. Mean ± SD; n=3.
Online Figure V. Characterization of Notch pathway in the retinas of P5 AIBP knockout neonatal mice and their control littermates. Quantitative RT-PCR analysis of the Notch downstream mediator gene Nrarp, the Notch ligands Dll4, the Notch receptor Notch1, Notch4 as well as Vegfa mRNA in P5 (A) and P3 (B) retinas from Apoa1bp⁻/⁻ mice or control littermates. (C) Western blot of Notch1, PS1, p-VEGFR2 (Tyr1175) and VEGFR2 expression. Mean ± SD; n=3. **, p<0.01.
Online Figure VI. AIBP regulates Notch-dependent and -independent retinal angiogenesis. Control or AIBP KO mice at P4 were treated with DAPT and retinal angiogenesis was analyzed at P5. Scale bar, 200 μm. Mean ± SD; n=3. *, p<0.05; **, p<0.01.
Online Figure VII. Binding of biotinylated AIBP (b-AIBP) to HRMECs. Confluent HRMECs were incubated with the indicated concentration of b-AIBP or inactive (heat-inactivated) AIBP for 2 hours at 4°C, and binding was assessed. A non-linear regression fit was applied to calculating the Kd (2.1±0.3×10⁻⁶ M). The data are representative of three independent repeats.
Online Figure VIII. Notch activity in the AIBP knockout retinas in the presence or absence of apoA-I overexpression. NICD protein expression (A), HDL-cholesterol concentration (B), VEGFR2 activation (C) and qRT-PCR analysis of Nrarp, Dll4, Notch1, Notch4, and Vegfa mRNA levels (D) in the retinas of Apoa1bp−/−ApoalTg and control Apoa1bp−/− mice at P5. Mean ± SD; n=3. **, p<0.01.
Online Figure IX. Similar vascular remodeling in Apoa1bp^{−/−} and Apoa1bp^{−/−} × Apoa1Tg mice.

(A) Images of whole mount immunostaining of P5 retinas from Apoa1bp^{−/−} and control littermates using CD31 (green) and Col IV (red) antibodies. (B) CD31^{+} vs. Col IV^{+} area were compared. No obvious difference of vascular remodeling between Apoa1bp^{−/−} and Apoa1bp^{−/−} × Apoa1Tg retinas were found. Scale bar, 20 μm. Mean ± SD; n=3.
Online Figure X. AIBP effect on Notch signaling during neovascularization in the Matrigel plugs. Quantitative RT-PCR analysis of the Notch target gene *Nrarp*, *Dll4*, *Notch1*, and *Notch4* mRNAs in the Matrigel plugs from *Apoa1bp*^+/−^ or control mice. Mean ± SD; n=3.
Online Figure XI. The model illustrates AIBP regulation of Notch signaling.
AIBP treatment accelerates cholesterol efflux from ECs to HDL, which disrupts lipid rafts and relocates Notch1 and γ-secretase from lipid rafts to non-lipid rafts, where increased co-distribution of the Notch receptor with γ-secretase will occur thus increasing Notch cleavage to generate NICD and subsequent signaling.
Online References