Selective regulation of Notch ligands during angiogenesis is mediated by vimentin

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Notch signaling is a key regulator of angiogenesis, in which sprouting is regulated by an equilibrium between inhibitory Dll4-Notch signaling and promoting Jagged-Notch signaling. Whereas Fringe proteins modify Notch receptors and strengthen their activation by Dll4 ligands, other mechanisms balancing Jagged and Dll4 signaling are yet to be described. The intermediate filament protein vimentin, which has been previously shown to affect vascular integrity and regenerative signaling, is here shown to regulate ligand-specific Notch signaling. Vimentin interacts with Jagged, impedes basal recycling endocytosis of ligands, but is required for efficient receptor ligand transendocytosis and Notch activation upon receptor binding. Analyses of Notch signal activation by using chimeric ligands with swapped intracellular domains (ICDs), demonstrated that the Jagged ICD binds to vimentin and contributes to signaling strength. Vimentin also suppresses expression of Fringe proteins, whereas depletion of vimentin enhances Fringe levels to promote Dll4 signaling. In line with these data, the vasculature in vimentin knockout (VimKO) embryos and placental tissue is underdeveloped with reduced branching. Disrupted angiogenesis in aortic rings from VimKO mice and in endothelial 3D sprouting assays can be rescued by reactivating Notch signaling by recombinant Jagged ligands. Taken together, we reveal a function of vimentin and demonstrate that vimentin regulates Notch ligand signaling activities during angiogenesis.

vimentin | Notch | angiogenesis | Jagged

The intermediate filament (IF) proteins are a large protein family with tissue- and developmental stage-specific expression. Whereas IFs provide cells with mechanical stability, there is mounting evidence indicating that IFs are involved in a range of metabolic, signaling, and regulatory processes that are unrelated to mechanical functions (1–14). The vimentin IF has been shown to act as a scaffold for signaling proteins that regulate epithelial mesenchymal transition (EMT), cancer cell invasion, wound healing and tissue repair, tissue aging, as well as inflammatory signaling (3, 4, 6–8, 10). Vimentin anchors and organizes adhesion molecules as well as actomyosin complexes (15) to regulate cell adhesion and migration (4, 5). Vimentin also influences protein function through regulation of protein trafficking and is involved in the regulation of gene expression (7, 8, 10, 16–18). Vimentin, thus, has regulatory functions especially in dynamic cellular processes. Recently, vimentin has been linked to angiogenesis and vascular homeostasis, and lack of vimentin is associated with defects in vascular tuning, endothelial migration, adhesion, and sprouting, as well as flow-induced arterial remodeling (1, 2). Whereas the above-listed studies determine that vimentin-deficiency could compromise vascular integrity, it is unclear whether there is direct causal relationship between vimentin and vascular development, and molecular links to signaling pathways that regulate angiogenesis have not been identified.

The Notch signaling pathway is a key regulator of angiogenesis. Genetic removal of Notch components results in disorganized and nonfunctional tissue and embryonic lethality (19–25). Deregulation of ligands or alterations in Notch activity is associated with pathological angiogenesis and disturbances in vascular remodelling (19–21, 26–30). The Notch ligands Dll4 and Jagged 1 have opposing roles during angiogenesis (19, 25). Dll4 and Jagged 1 signaling regulates tip cell versus stalk cell selection in the branching endothelium, and the creation of new branching points (22, 31). VEGF-VEGFR2 signaling is a key driver of angiogenesis and induces Dll4 expression in the tip cell (32). Dll4 activation of Notch in neighboring cells reduces expression of VEGFR2 and inhibits tip cell selection. In contrast to Dll4, Jagged-Notch signaling promotes tip cell selection and sprouting by antagonizing Dll4-Notch signaling (31, 33–36). How the competition between the ligands is balanced and how ligand-receptor signaling specificity is achieved is under intense investigation (31, 33, 34, 37, 38). Modification of the Notch receptor by the Fringe family of glycosaminyltransferases has been shown to enhance Dll4-Notch signaling and to suppress sprouting (31). However, other mechanisms that balance the competition between Jagged and Dll4 signaling are still to be elucidated.

Notch signaling is elicited by ligand binding to the Notch receptor on neighboring cells, which leads to proteolytic processing of the receptor and release of the Notch intracellular domain (NICD), which translocates to the nucleus and regulates transcription of target genes (37, 39, 40). Notch signaling is critically regulated

Significance

Angiogenesis, the formation of new blood vessels from existing ones, is essential for embryonic development and necessary for tumor growth and invasion. The Notch signaling pathway regulates angiogenesis where the two Notch ligands, Dll4 and Jagged 1, exert opposite functions on sprouting. We found that the intermediate filament vimentin balances angiogenesis by binding specifically to the proangiogenic Jagged ligands. This binding provides a force-generating mechanism on the Jagged–Notch complex to ensure efficient transendocytosis and Notch activation. The interaction between vimentin and Jagged constitutes a mechanism behind selective regulation of Notch ligands during angiogenesis. The interaction may be amendable by therapeutic intervention and can facilitate strategies targeting a variety of diseases related to Jagged deregulation.

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by protein trafficking (41–43). In addition to controlling number of ligands and receptors on the cell surface, ligand endocytosis is required for receptor activation (41). Two separate models for the requirement of endocytosis have been suggested: the first model suggests that recycling is important for maturation of the ligands to become active, whereas according to the second model, transendocytosis of the extracellular domain of the Notch receptor (NECD) is needed to produce a strain on the receptor. This strain is termed the pulling force (41, 42, 44, 45) and is thought to reveal the cleavage site and initiate proteolytic processing of the receptor with subsequent release of the NICD.

Here we demonstrate a previously unexplored link between vimentin and Notch signaling in the vasculature. We show that vimentin not only interacts with Jagged, but also regulates Jagged-mediated receptor transendocytosis, expression of Fringe genes, and ligand-specific Notch activation. Our data suggest that vimentin is important for balancing Jagged and Dll4 signaling and in the absence of vimentin, Dll4-Notch signaling dominates to suppress angiogenesis.

Methods

For cell culture, quantitative PCR (qPCR), Western blotting, immunofluorescence, proximity ligation assay (PLA), Notch activity, and Jagged trafficking assays, please see *SI Methods*. Animal experiments were approved by and carried out in accordance with the National Animal Experiment Board of Finland and conformed to the regulations set by The Finnish Act on Animal Experimentation.

Aortic Ring Assay. Eight-week-old vimentin wild-type, heterozygous, or null Sv129/Pas mice were killed and aortae harvested. Rings were embedded into 1.5 mg/mL collagen type I and once the collagen had polymerized, fed with Opti-MEM containing 2.5% FBS and 40 ng/mL VEGF. Aortic ring assays were incubated at 37 °C with 5% CO_2, and after 4 d, media were replaced. After 6 total days of incubation, aortic ring assays were fixed with 4% paraformaldehyde in PBS for 20 min and rinsed two times with Tris-glycine.

Vimentin Null Aortic Rings with Notch Ligands. A total of 1 μg of Recombinant Delta-like ligand 1 (Dll1) (R&D Systems, 5026-DL-050), Delta-like ligand 4 (Dll4) (R&D Systems, 1506-D4-050), Jagged (R&D Systems, 599-JG-100), or IgG Fc control (Jackson Immunoresearch, 009–000-008) were coupled to 10 μL Protein A agarose beads (Roche, 11719408001), diluted in PBS to give a final volume of 100 μL. The bead–ligand mixtures were gently rotated for 30 min at room temperature (RT), and the entire 100 μL mixture was added to 900 μL collagen type I, on ice, for a final collagen concentration of 2 mg/mL. Aortae were harvested from vimentin null mice, as described above. A total of 70 μL of the collagen–bead mixture was added per well of a full area 96-well plate, three wells at a time, and 0.5-mm aortic rings were oriented so the lumen was visible. After the collagen polymerized, aortic rings were fed with 150 μL Opti-MEM containing 2.5% FBS and 40 ng/mL VEGF and incubated at 37 °C with 5% CO_2. After 4 d of incubation, the media were replaced. Rings were fixed after 7 d with 4% paraformaldehyde in PBS and rinsed twice with Tris-glycine.

Quantification of Aortic Ring Sprouting Responses. To quantify endothelial sprouting responses, four images were taken of sprouting structures originating from the upper edge of the ring using an Olympus CKX41 inverted microscope equipped with a Q color 3 Olympus camera. Average sprout length was quantified using ImagePro Analyzer software by measuring the distance from the edge of the ring to the tip of invading multicellular structures. The average number of endothelial sprouts per ring was quantified from the four images. Error bars represent SEM.

Immunostaining of Aortic Rings. Aortic rings stored in Tris-glycine were permeabilized for 30 min with 0.5% Triton X-100 in PBS, then blocked overnight at 4 °C with blocking buffer (0.1% Triton X-100, 1% BSA, and 1% goat serum in TBS). Rings were immunostained using primary antibodies directed against PECAM-1 (Santa Cruz, sc-1505R), VE-cadherin (Enzo Life Sciences, ALX-210-232-C100), or rabbit IgG as a control (Invitrogen, 02-6102), followed by incubation with Alexa-594 or Alexa-488 conjugated secondary antibodies (Invitrogen, A11012 and A11008) and FITC-conjugated phalloidin (Invitrogen, A12379) or cy3-conjugated alpha smooth muscle actin (Sigma, C6198), respectively. Cell nuclei were stained with 1.09 μM DAPI (Invitrogen, D1306). *Z* stacks of 1 μm step-size compressed images of aortic rings were taken using a Nikon TI A1R inverted confocal microscope at 40× magnification. (Scale bar, 100 μm.)

Receptor Binding and Ligand Transendocytosis. Cells were plated on 12-well plates, or coverslips for immunofluorescence, a few days in advance. rN1ECD (R&D Systems, cat. no. 1057-TK) (1 μg/mL) and Alexa-Fluor 488 goat anti-human IgG (Invitrogen, cat. no. A11013) (1:200) were diluted in sterile PBS and incubated on rotation in +4 °C for 1 h. Simultaneously, the cells were blocked with DMEM containing 10% goat serum and 1% BSA for 45 min in +37 °C. The rN1ECD–Alexa-488 solution was further diluted in blocking solution at a ratio of 1:5 and this solution was added to the cells, followed by incubation in 37 °C for 2 h. The cells were then detached, centrifuged (450 × g, 5 min) and quenched with 200 μg/mL Trypan blue in PBS for 5 min at RT. Then the cells were centrifuged again and excess Trypan blue was removed. The cells were resuspended in PBS and analyzed by FACS. For immunofluorescence, the incubation was followed by fixation and immunocytochemistry was conducted as stated above. To produce resistance to Jagged-mediated endocytosis and mimic force-dependent ligand-mediated receptor endocytosis and Notch activation, the N1ECD was coupled to fluorescent protein A agarose beads (N1ECDPrtA) (42).

Chorion Allantois Membrane Angiogenic Assay. Matrigel plugs (V = 150 μL) were mixed with H_2O, 50 ng/mL FGF, 0.2 mg/mL DAPT, 10 μM WFA, as well as with both DAPT and WFA, and allowed to solidify on top of precut 1.5 cm^2 pieces of 500-μm nylon mesh. Nylon mesh with Matrigel was placed on chorion allantois membrane (CAM) on top of major blood vessel and incubated for 5 d. After 5 d, the embryos were visualized live by a Canon 6D SLR, with a 24-105Lf4 lens at maximum focal length using a 25-mm extension tube. Angiogenic branches in Matrigel were quantified via optical visualization.

Results

Vimentin Interacts with Jagged. We have previously shown that loss of the astrocytic intermediate filament cytoskeleton, composed of GFAP, vimentin, and nestin, affects Jagged-mediated Notch signaling during neurogenesis (13). As genes that are coexpressed in certain tissues have a higher probability to share a functional relationship (46), we screened tissue expression data in the GeneSapiens database (47) and found a strong positive correlation between the expression of vimentin and Jagged ligands in several tissues, e.g., in blood, breast, leukocytes, central nervous system, heart, kidney, muscle, and testis (Fig. S1*A*). A significant correlation was also evident in several types of cancer (Fig. S1*B*). The data suggest the notion of a functional relationship between Jagged and vimentin.

To elucidate the mechanisms and nature of the interrelationship between Jagged and vimentin, we first analyzed whether the proteins physically interacted. Proximity ligation assays demonstrated that vimentin interacts with Jagged 1. Interactions between vimentin and Dll4 were significantly less prominent (Fig. 1 *A* and *B*). Coimmunoprecipitation assays further confirmed the interaction between Jagged and vimentin (Fig. 1*C*, and Fig. S2*A*). Vimentin interacts with membrane-tethered proteins through PDZ motifs (48) and Jagged and Dll carry distinct PDZ sequences in their ICDs (49–51). Whereas the ECDs are important for receptor binding, the ICDs play important roles in signal activation through posttranslational modifications (37, 52) and interactions with endocytic regulators (41–43). To elucidate whether the ICDs of Dll4 and Jagged 1 influence vimentin binding and Notch signaling, we swapped the ICDs of Dll4 and Jagged 1 (53) to generate the chimeric variants: JaggedECDDll4ICD and Dll4ECDJaggedICD. Swapping the ICD of Jagged with that of Dll4 led to loss of vimentin interaction as demonstrated by loss of the PLA signal (Fig. 1*D*). We next expressed Dll4 and Jagged, and the chimeric ligands in mouse embryonic fibroblasts (MEFs) derived from WT (VimWT) and vimentin knockout (VimKO) mice and cocultured them with 293HEK reporter cells stably expressing full-length Notch 1 (FLN reporter cells) and then measured signal activation in FLN reporter cells stably expressing full-length Notch 1 using the CSL (CBF-1, suppressor of hairless, Lag-2)-based luciferase reporter system (FLN reporter cells) (13). Transfection efficiency and expression levels of the different proteins were confirmed by immunofluorescence and Western blot (WB). VimWT MEFs expressing Dll4 were significantly stronger signal activators than MEFs expressing Jagged (Fig. 1*E*). However,

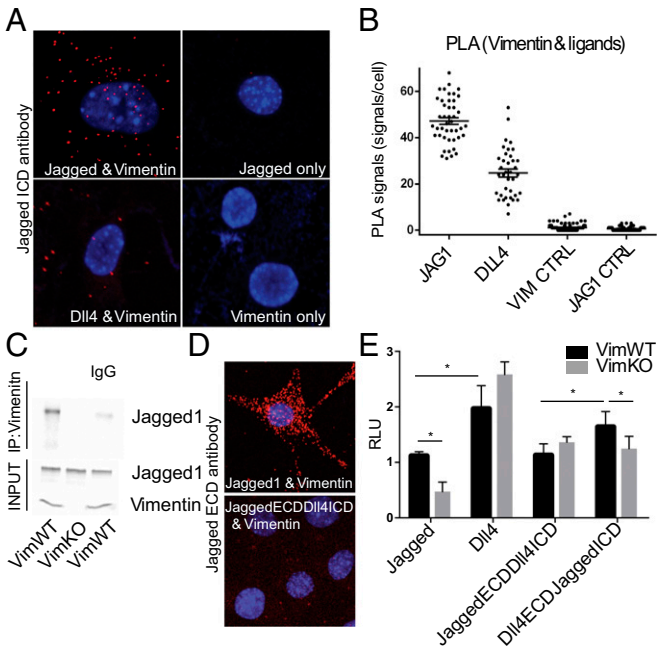

Fig. 1. Jagged interacts with vimentin and the intracellular domain of Jagged potentiates signal activation. (*A*) In situ PLA was used to demonstrate physical interaction between vimentin and Jagged versus Dll4. Vimentin or Jagged were used as negative controls. (*B*) Number of PLA signals per cell. Quantification of signals is shown from three separate experiments. *P* values are <0.0001 for all groups compared with Jagged. (*C*) Jagged coimmunoprecipitates with vimentin from VimWT MEFs. (*D*) PLA using an antibody against the Jagged 1 extracellular domain shows strong PLA interaction between Jagged and vimentin, but the chimeric ligand JaggedECDDll4ICD where the intracellular domain of Jagged is switched to Dll4 shows no visible interaction. (*E*) The signal sending potential of Dll4, Jagged, and chimeric ligands, JaggedECDDll4ICD and Dll4ECDJaggedICD, was measured by coculturing ligand expressing VimKO and VimWT MEF cells with 293HEK cells expressing the Notch 1 receptor (293HEK-FLN1) using a luciferase-based reporter system. Values represent means of three separate experiments including three experimental repetitions ± SEM. Statistical significance was determined using one-way ANOVA and Bonferroni post hoc test, $P < 0.05$. RLU, relative light unit.

whereas Dll4 signaling was unaffected by vimentin depletion, Jagged signaling was significantly reduced in VimKO MEFS (Fig. 1*E*). Further, signaling from the Dll4ECJaggedICD-expressing MEFs was significantly higher than from cells expressing JaggedECDDll4ICD. Signaling from JaggedECDDll4ICD was not significantly different in VimWT and VimKO cells. However, signaling from Dll4ECJaggedICD was significantly reduced in VimKO cells (Fig. 1*E*). The data suggest that Jagged ICD contributes to signaling strength and vimentin is required for efficient Jagged signaling.

Vimentin Regulates Jagged Recycling and Surface Levels. To gain further insight into a potential functional link between vimentin and Jagged, we analyzed Jagged protein levels in VimWT and VimKO MEFs. The protein levels of Jagged in MEFs were unaffected by the absence of vimentin (Fig. 2*A*), but were surprisingly enhanced in vimentin-negative SW13 cells (Fig. S2*B*), demonstrating a cell-type dependent effect. However, we observed no difference in proteasomal (Fig. S2*C*) nor lysosomal degradation of Jagged 1 in the absence of vimentin in SW13 cells (Fig. S2*D*), demonstrating that vimentin does not directly affect Jagged turnover.

Endocytosis of Notch ligands is important for Notch activity (41–43). As vimentin has been shown to regulate protein trafficking (3), we assessed whether vimentin affects Jagged routing. To this end, we tracked the ligand with fluorophore-conjugated recombinant peptides, mimicking the extracellular domain of Notch 1 ($N1ECD^F$). Flow cytometry demonstrated enhanced binding and internalization of $N1ECD^F$ in VimKO compared with VimWT cells (Fig. 2*B*). Confocal microscopy revealed that the $N1ECD^F$-positive vesicles were differently distributed in VimKO cells compared with VimWT MEFs (Fig. 2*C*).

To further investigate intracellular routing of ligands, we tracked the movement of $N1ECD^F$ vesicles in VimWT and VimKO cells. In VimKO cells, the average linearity of the vesicle tracks was

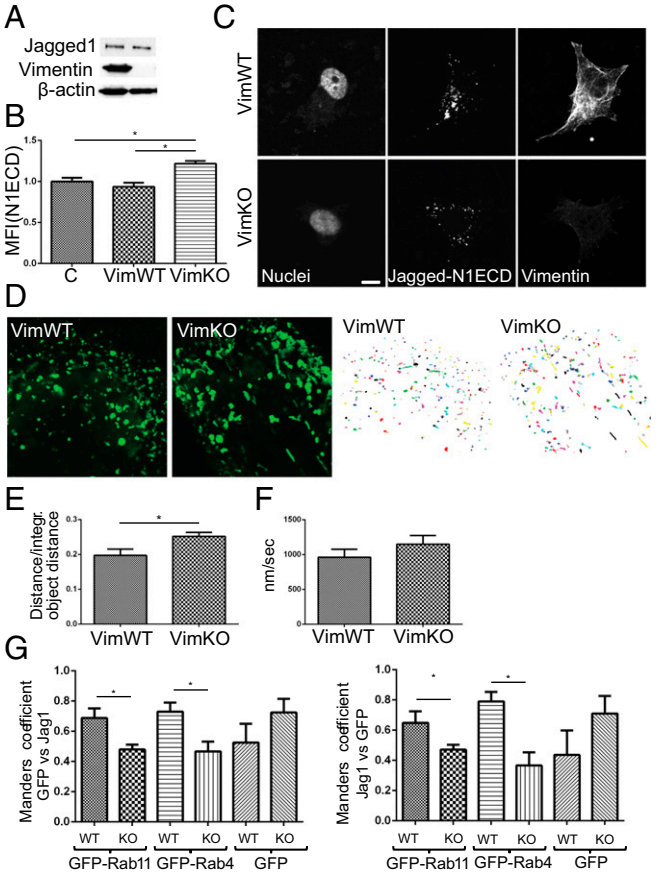

Fig. 2. Vimentin alters Jagged 1 trafficking. (*A*) Representative immunoblot shows Jagged 1 protein levels in VimWT and VimKO MEFs. (*B*) Quantification of ligand endocytosis VimWT and VimKO cells using fluorescently labeled recombinant peptides mimicking the Notch 1 extracellular domain conjugated to a fluorescent probe (N1ECD) to label the ligands. Values represent means ± SEM. Statistical significance was determined using one-way ANOVA and Bonferroni post hoc test, $*P < 0.05$. MFI, mean fluorescence intensity. (*C*) Representative images of N1ECD localization in VimWT and VimKO cells. (Scale bar, 10 μm.) (*D*) VimWT and VimKO cells were live labeled with N1ECD–Alexa-488 and vesicles with green fluorescence were followed for 1 min. Projection of N1ECD–Alexa-488 positive vesicle movement in VimWT and VimKO MEFs during 1 min of vesicle tracking. Vesicles were segmented and tracked with CellProfiler. Graphs were created using R from information produced with CellProfiler. One color represents one vesicle. (*E*) Analysis of N1ECD–Alexa-488 labeled vesicle track linearity in VimWT and VimKO cells. Values represent means ± SEM. Statistical significance was determined using Student's *t* test, $P < 0.05$. (*F*) Analysis of N1ECD–Alexa-488 labeled vesicle speed in VimWT and VimKO cells. (Scale bar, 10 μm.) (*G*) Ligand localization into different endosomal compartments was analyzed by evaluating the colocalization between Jagged 1-stained vesicles and Rab4- or Rab11-linked recycling endosomes. Jagged 1 colocalization with Rab4 and Rab11 was analyzed by Manders' colocalization coefficient. Confocal microscopy images of 30 cells from three experiments described above were quantified using Fiji ImageJ. $P < 0.05$.

significantly enhanced compared with WT cells, implying that vimentin decreases directional mobility of N1ECD^F vesicles (Fig. 2 *D* and *E*). No significant differences were detected in movement speed of the vesicles (Fig. 2 *D* and *F*). We next analyzed the occurrence of Jagged in different endosomal compartments. The colocalization of Jagged with Rab4 or Rab11, markers for fast and slow recycling endosomes, respectively (54), was reduced in VimKO cells compared with VimWT (Fig. 2*G*).

We then used a biotin cell surface labeling and stripping protocol to analyze recycling of Jagged 1 in VimWT and VimKO MEFs. The results indicated that fairly small amounts of Jagged were recycled at steady state in both VimWT and VimKO MEFs. Recycling of ligands appeared faster in VimKO cells (Fig. 3*A*), which is in agreement with the reduced presence of Jagged in recycling endosomes (Fig. 2*G*). We subsequently used a biotinylation assay to analyze Jagged surface levels and demonstrated that Jagged was accumulated at the cell membrane in VimKO MEFs (Fig. 3*B*). Confocal microscopy of cells immunolabeled for Jagged corroborated that the surface levels were enhanced (Fig. 3*C*). Importantly, surface levels were also enhanced in vimentin-negative SW13 cells, suggesting that surface accumulation is directly linked to vimentin depletion (Fig. S2 *E* and *F*) and not related to total Jagged levels (Fig. 2*A* and Fig. S2*B*).

We next assessed the ability of Jagged to bind the extracellular domain of Notch using a Jagged 1 fingerprint assay. VimWT and VimKO MEFS were cultured on coverslips coated with peptides corresponding to the extracellular domain of Notch (N1ECD). After protein cross-linking, we extracted the cells in a harsh detergent and the Jagged 1 cross-linked to N1ECD was detected by immunolabeling. More Jagged 1 was found bound to the coated N1ECD in VimKO cells compared with VimWT cells in line with enhanced surface accumulation of Jagged (Fig. 3*D*). The data demonstrated that the Jagged ligands on the surface of VimKO cells efficiently bound the Notch receptor. These data may explain the high binding and internalization of N1ECD^F demonstrated in Fig. 2*B*.

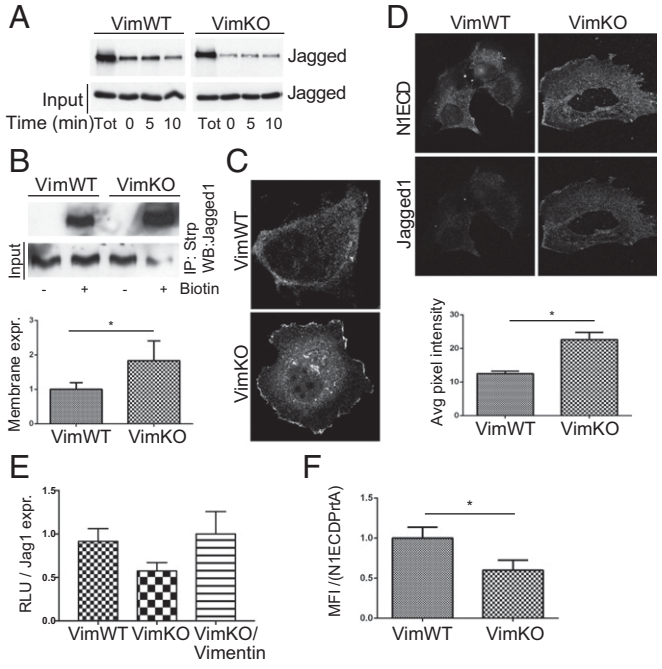

Fig. 3. Vimentin regulates Jagged recycling and Notch activation. (*A*) Analysis of Jagged 1 recycling in VimWT and VimKO cells using a biotin cell surface labeling and stripping protocol. (*B*) Cell surface proteins were labeled with biotin and immunoprecipitated using streptavidin agarose beads. Immunoblotting was performed with an antibody detecting Jagged 1. (*C*) Representative confocal microscopy images showing Jagged 1 immunofluorescence in VimWT and VimKO cells. (*D*) Representative confocal microscopy image shows Jagged 1 cell surface localization and N1ECD binding in VimWT and VimKO cells. (Scale bar, 10 μm.) The graph shows quantification of pixel intensity. Values represent means ± SEM. Statistical significance was determined using Student's *t* test, $P < 0.05$. (*E*) Jagged signal sending potential measured by coculturing VimWT and VimKO cells and VimKO cells reexpressing vimentin with 293HEK cells expressing the Notch 1 receptor (293HEK-FLN1) using a luciferase-based reporter system. Jagged signal sending potential as related to surface levels of Jagged. Values represent means ± SEM. Statistical significance was determined using Student's *t* test, $P < 0.05$. RLU, relative light unit. (*F*) The ability of VimKO and VimWT to internalize N1ECD–Alexa-488 coupled to protein A agarose beads (N1ECDPrtA). Internalization of N1ECDPrtA in VimKO and VimWT cells as related to surface levels of Jagged. Values represent means ± SEM. Statistical significance was determined using Student's *t* test, $P < 0.05$. MFI, mean fluorescence intensity.

Vimentin Potentiates Jagged-Mediated Force-Dependent Notch-Activating Endocytosis. Notch signaling is linear and tightly regulated by the levels of receptors and ligands present on the surface of the signal-receiving and signal-sending cell. However, our data suggest that vimentin depletion reduces Jagged-mediated Notch activation (Fig. 1*E*), despite enhancing Jagged surface levels (Fig. 3 *B* and *C*). We assessed the signal potential of VimKO and VimWT MEFs expressing endogenous levels of Jagged, by coculturing the cells with reporter cells. Despite elevated Jagged levels and enhanced receptor binding in VimKO MEFs, Notch activation in reporter cells was not enhanced. On the contrary, when signal activity was related to ligand levels, the Notch activation potential was significantly reduced in VimKO. This effect could be rescued by the reintroduction of vimentin (Fig. 3*E*). Vimentin-negative SW13 cells also demonstrated reduced signaling potential compared with vimentin-expressing SW13 cells (Fig. S2*G*). During active signaling, Notch transendocytosis has been proposed to create a force on the receptor, known as the "pulling force," which is thought to reveal the cleavage site and promote receptor activation (42). A recent publication demonstrated that ligand-mediated NECD transendocytosis during signal activation is mechanistically distinct from steady-state ligand endocytosis (55). We attached a fluorescently labeled Notch1ECD to Protein A beads (N1ECDPrtA) to resemble the mechanical strain raised during receptor transendocytosis (42) and assessed internalization of N1ECDPrtA by flow cytometry. N1ECPrtA internalization was reduced in VimKO cells (Fig. 3*F*), in contrast to the enhanced internalization of N1ECD^F (Fig. 2*B*). Internalization of N1ECPrtA was also decreased in vimentin-negative SW13 fibroblasts, a phenotype that was rescued by the reintroduction of vimentin (Fig. S3*A*). On the contrary, basal endocytosis of Jagged as measured by internalization of N1ECD^F was enhanced in vimentin-negative SW13 cells (Fig. S3*B*). Taken together, the data show that despite increased surface levels of Jagged and efficient receptor binding, the signal activation potential of Jagged is reduced in the absence of vimentin.

IFs are phosphoproteins and phosphorylation of vimentin regulates organization of the IF network and vimentin–protein interactions. Phamacological activation of the vimentin kinase PKC enhanced Notch activation in cocultured reporter cells. (Fig. S4*A*). We next tested whether phosphorylation on serines 4, 6, 7, 8, and 9, known PKC targeted sites in the N terminus of vimentin, influenced Jagged interactions and signaling using phosphomutant forms of vimentin. Immunoprecipitation assays demonstrated that Jagged interacted with both wild-type vimentin and phosphomutants, but the interaction with the phosphomimicking mutant was slightly enhanced (Fig. S4*B*). Furthermore, both the signal activation potential (Fig. S4*C*) and receptor transendocytosis (Fig. S4*D*) were enhanced by expression of the phosphomimicking mutant, indicating that vimentin-mediated Jagged regulation is dependent on the phosphorylation status of vimentin.

Deletion of Vimentin Disrupts Sprouting Angiogenesis and Attenuates Embryonic Angiogenesis. Our data indicate that vimentin binds to Jagged and potentiates Jagged-mediated receptor transendocytosis and Notch activation, but does not interact with Dll4. Dll4 and Jagged ligands have opposite functions during angiogenesis, which makes angiogenesis an interesting process for evaluating the physiological effects of ligand-specific regulation. Blocking Dll4 or inhibiting Notch signaling by γ-secretase inhibitors (GSIs) leads to hyperbranching, whereas Jagged inhibition reduces branching and attenuates angiogenesis (31, 38, 56). As vimentin is important for efficient Jagged signaling, loss of vimentin could disturb the equilibrium between proangiogenic Jagged and antiangiogenic Dll4 signaling.

This notion was supported by the disrupted angiogenesis observed in chorioallantoic membranes of fertilized chicken embryos observed upon treatment with the vimentin-targeting drug Withaferin A (WFA). WFA inhibited branching and microvessel formation (Fig. 4 *A* and *B*). The effect of WFA was counteracted by Notch inhibition by the GSI DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester) (Fig. 4 *A* and *B*) (35), indicating that the effect of WFA treatment was overridden by blockage of Dll4 activity.

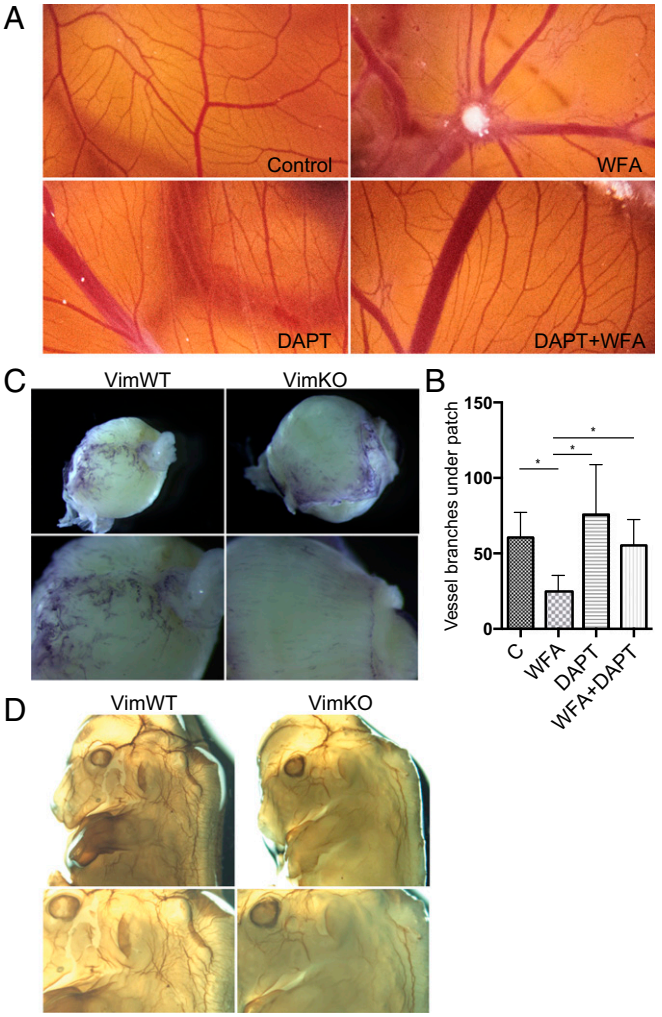

Fig. 4. Loss of vimentin disrupts angiogenesis. (*A*) Notch inhibition rescues the antiangiogenic effect of WFA. The effects of the vimentin-targeted drug WFA, the γ-secretase inhibitor DAPT, and the combination of DAPT and WFA were analyzed in the CAM angiogenesis model. (*B*) Number of vessel branches per field of view under the patch containing the drugs in one representative experiment. (*C*) Whole-mount PECAM-1 immunostaining of E11.5 placental tissue from VimWT and VimKO mice. (*D*) Whole-mount PECAM-1 immunostaining of VimWT and VimKO embryos at E12.5.

The influence of vimentin on angiogenesis was next assessed by analyses of the developing vasculature in the placental tissue and in embryos of VimWT and VimKO mice by whole-mount immunostaining for PECAM-1. The placental tissue of VimKO at embryonic day 11.5 (E11.5) displayed disturbed vascular patterning (Fig. 4*C*). By E12.5 the VimKO embryos showed a less developed and a less intricate vasculature with fewer blood vessels and less branching compared with WT embryos (Fig. 4*D*). The difference in vascular branching was particularly noticeable in the head region and the upper parts of the embryo (Fig. 4*D*).

We then assessed sprouting angiogenesis in VimKO and WT mice using an aortic ring assay. Aortic rings from VimKO and WT mice were excised and embedded in collagen containing growth factors to induce sprouting (57). The assay was optimized using different growth factor supplements to ensure appropriate conditions for endothelial outgrowth of endothelial-lined tubes (Fig. S5). Endothelial sprouting in response to VEGF stimulation was significantly reduced in VimKO aortic rings compared with WT (Fig. 5 *A* and *C*). Both the length of the sprouts and sprout number was affected (Fig. 5*B*). Endothelial outgrowths can be identified by V-Cad and PECAM. VimKO aortic rings did not produce these sprouts in the same manner as in VimWT (Fig. 5*C*). Aortic rings from heterozygous (VimHZ) mice displayed an intermediate phenotype, indicating that vimentin levels are critical in this system (Fig. 5 *A* and *B*).

Taken together the data above demonstrated that loss of vimentin disrupts sprouting angiogenesis and delays embryonic vascular development in line with disrupted balance between Jagged and Dll4 signaling.

Defective Sprouting of VimKO Endothelial Cells Can Be Rescued by Transactivation with Recombinant Jagged 1 Ligands. To further assess the effect of vimentin depletion on Notch signaling in endothelial cells, we down-regulated vimentin by shRNA (VimKD). Immunostaining of Dll4 and Jagged 1 (Fig. 6*A*) revealed that Dll4 and Jagged 1 were localized in distinct intracellular vesicles in endothelial cells, supporting the notion of selective regulation of Notch ligand endocytosis. Immunoblotting of control and VimKD cells revealed increased levels of the active form of Notch, NICD and, intriguingly increased levels of Fringe proteins in VimKD endothelial cells, whereas levels of Dll4 were unaffected (Fig. 6*B*). Increased levels of NICD and Fringe proteins were also observed in endothelial cells isolated from VimKO mice, whereas levels of Jagged 1 were unaffected (Fig. 6*C*). Fringe glycosyltransferases modify Notch receptors and enhance transactivation from Dll ligands. Increased expression of Fringe mRNA in vimentin-depleted cells was further verified by qPCR (Fig. 6*D*). In line with increased expression of Fringes, the expression of the Notch target gene Hes1 is increased in VimKO cells compared with VimWT cells upon activation of Notch by immobilized Dll ligands (Fig. S6*A*). No effects on surface levels of the Notch receptor (Fig. S6*B*) or NICD stability were observed (Fig. S6*C*).

Taken together, our data indicate that in the absence of vimentin, Jagged function is compromised and cells express enhanced levels of Fringe proteins. This may shift the balance between Jagged and Dll4 signaling toward Dll4 signaling, giving rise to the antiangiogenic phenotype. To explore this hypothesis, we reactivated Jagged-mediated Notch signaling in aortic rings from VimKO mice and in an in vitro 3D Matrigel sprouting assay using recombinant ligands. Activation of Notch signaling using immobilized Jagged 1 (58) significantly enhanced the sprouting from VimKO aortic rings (Fig. 6 *E* and *G*). By contrast, sprout length was not affected by activation of Notch signaling by Jagged 1 (Fig. 6*F*). An in vitro 3D angiogenesis assay with human umbilical vein endothelial cells (HUVECs) showed similar results.

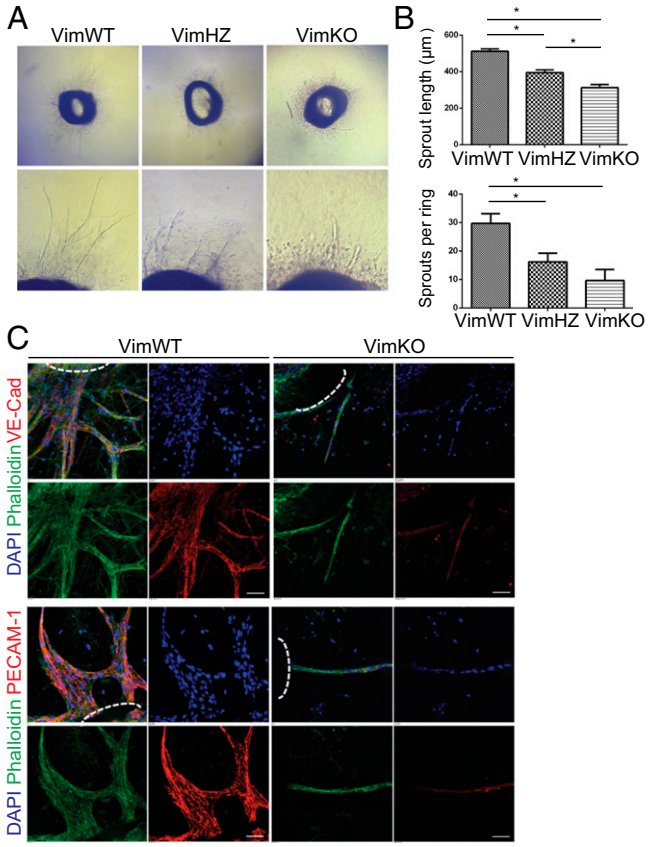

Fig. 5. Decreased sprouting from aortic rings from vimentin knockout mice. (*A*) Representative images showing endothelial sprouting in aortic rings from VimWT, VimHZ, and VimKO mice. (*B*) Quantification of endothelial sprout length and sprout number. Sprout length was quantified as the distance (in micrometers) from the edge of the ring to the tip of invading endothelial structure. Data represent the average number of endothelial sprouts per ring in a single plane. Error bars represent SEM. Statistical significance was determined using one-way ANOVA and Bonferroni post hoc test, $P < 0.05$. (*C*) Immunofluorescence staining was performed to determine whether sprouting structures contained endothelial and smooth muscle cells. Aortic ring assays using VimWT and VimKO aortic rings were stained with VE-cadherin and phalloidin-FITC (*Upper*) or PECAM-1 and phalloidin-FITC (*Lower*). In all panels, overlap and DAPI images are shown in *Upper Left* and *Right* images, respectively. *Z* stacks of 1-μm step-size compressed images of aortic rings were taken using a Nikon TI A1R inverted confocal microscope at 40× magnification. (Scale bar, 100 μm.) Dotted white line represents edge of aortic ring where outgrowth initiates.

Vimentin knockdown in HUVECs prevented sprouting in 3D, an effect rescued by Jagged 1 (Fig. 6*H*).

Discussion

We show that VimKO mice display disturbed Notch signaling and vascular patterning during embryogenesis. Depletion of vimentin restricts Jagged-mediated transactivation and enhances expression of Fringe proteins and disrupts sprouting angiogenesis. Aortic rings from mice lacking vimentin produce fewer sprouts compared with WT mice and sprouting is rescued by reactivating Jagged-mediated Notch signaling by recombinant ligands immobilized within the collagen matrix in which the aortic rings are embedded. These results add to the current view of the effect of these ligands on angiogenesis (31, 59). Jagged has been suggested to counteract Dll4 signaling through *cis*-inhibition by direct binding to the Notch receptors and inhibition of Dll4-mediated activation in the signal receiving cell (35, 60, 61). By contrast, our data show that the phenotype can be rescued by externally presented recombinant Jagged ligands, which support the existence of transsignaling mechanisms in the proangiogenic functions of Jagged.

There is mounting evidence that the vimentin filaments form a dynamic protein scaffold to integrate cellular processes during stages when tissues are undergoing development or remodeling. We have previously shown that vimentin promotes endothelial cell invasion by reinforcing a complex between RACK1 and focal adhesion kinase (FAK) to control FAK activity (62). Further, vimentin promotes endothelial invasion of 3D collagen matrices by complexing with and promoting membrane translocation of the metalloproteinase MT1-MMP, which is required for successful sprouting responses (2). Like Notch receptors, MT1-MMP is a transmembrane receptor that can be internalized to the endolysosomal

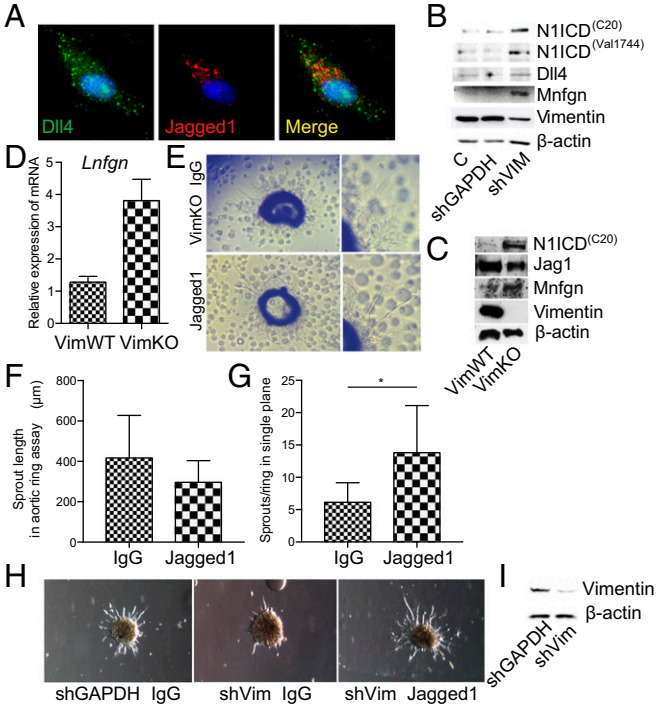

Fig. 6. Immobilized Notch Jagged 1 rescues sprouting. (*A*) Confocal image showing Dll4 and Jagged 1 immunolabeling in HUVECs. (*B*) Expression of Notch receptors, ligands, and regulators in control or vimentin knockdown HUVECs as determined by immunoblotting. The immunoblot shows expression of NICD by two different antibodies: C20 and Val1744, and Dll4, Manic Fringe (Mnfgn), and vimentin. β-Actin was used as a loading control. (*C*) Expression of Notch receptors, ligands, and regulators in endothelial cells isolated from VimWT or VimKO mice as determined by immunoblotting. Images from *B* and *C* are composites, and the bands were taken from several gels run from the same samples with equal loading. (*D*) The influence of vimentin on the expression of Lunatic Fringe determined by qPCR. (*E*) To determine whether exogenous addition of Notch ligands rescued vimentin null sprouting responses, Notch ligands or IgG control were coupled to protein A agarose beads and mixed with collagen before embedding vimentin null aortic rings. Representative images show endothelial sprouting in aortic rings from VimKO mice in the presence of immobilized Notch ligands Dll4 and Jagged1. (*F*) Quantification of endothelial sprout length in aortic rings from VimKO mice in the presence of IgG or immobilized Notch ligands Dll4 and Jagged 1. Sprout length was quantified as the distance (in micrometers) from the edge of the ring to the tip of the invading endothelial structure. (*G*) Quantification of the number of endothelial sprouts per ring in a single plane. Values represent means ± SEM. Statistical significance was determined using Student's *t* test, $P < 0.05$. (*H*) Representative images of an in vitro 3D angiogenesis assay with HUVECs transfected with shRNA against vimentin. Endothelial sprouting was analyzed in the presence of IgG or immobilized Notch ligands Dll4 and Jagged 1. (*I*) Expression of vimentin in control or vimentin knockdown HUVECs as determined by immunoblotting.

compartment (63), raising the possibility that loss of vimentin also negatively regulates MT1-MMP stability and endothelial sprouting through enhanced lysosomal degradation. Here, we demonstrate an important role of vimentin in balancing Notch signaling in sprouting angiogenesis, indicating that vimentin performs several vital functions to promote the various steps of angiogenesis.

The signal sending capacity of Jagged is impaired in the absence of vimentin. Ligand-mediated transendocytosis of the receptor produce a strain on the receptor, a process that requires assembly of actin filaments at the membrane (42). Vimentin binds actin (64), while also regulating cytoskeletal tension (65) and actin filament contractility at the membrane (60). Hence, vimentin might link Jagged to the actin cytoskeleton. In this respect, it is attractive to speculate that the interaction between Notch and vimentin would serve as a hub to coordinate cytoskeletal and developmental signaling in the regulation of angiogenesis. Vimentin is required for efficient Jagged-Notch signaling and provides dynamic control of the strength of signal activity as cellular effects of Notch signaling are highly dose sensitive (36, 53, 66). Such control is important, because Jagged recycling and surface accumulation is enhanced, but Jagged surface levels are decoupled from signaling strength in vimentin-depleted cells. The data imply that the maturation model and the pulling force model might not be mutually exclusive but rather coexist, maybe to produce different Notch activity levels with different cellular outcomes.

Our data demonstrate that vimentin interacts with Jagged. Intriguingly swapping the ICD and ECD of Jagged and Dll4 indicates that the Jagged ICD is a more potent signal activator, and vimentin enhances signaling. The ECD of Dll4 has a high affinity for Notch receptors (67) and may be a more important determinant of Dll4 signaling strength. Ligand-specific control of signaling strength may be obtained by posttranslational modifications (68), protein clustering, and localization (69). It was recently demonstrated that different force requirements are needed to activate Notch signaling by different ligands and the authors suggest that this may be a mechanism for Notch-expressing cells to tune their sensitivity to discriminate between different ligands (70). It is possible that a binding between Jagged and vimentin acts as a force-generating mechanism to ensure efficient Jagged-Notch activation. Taken together, our data reveal that Dll4 and Jagged ligands are differently regulated and vimentin contributes to Jagged signaling strength. The molecular basis requires further investigation but might involve specific sets of interacting proteins. Although the Jagged and Dll interactomes remain to be elucidated, the different ligands may have distinct links to the cytoskeleton (51, 71). Vimentin interacts with membrane tethered proteins through PDZ motifs (48) and Jagged and Dll ligands carry distinct PDZ sequences in their intracellular domains (49–51). Clathrin-mediated endocytosis (CME) of Dll depends on Epsin 1 and 2, together with the alternative adaptor protein clathrin assembly lymphoid myeloid leukemia protein (CALM), rather than AP2, which is central to CME of most proteins (42). Vimentin interacts with the AP adaptor complexes to regulate endocytosis (18, 72) and therefore might not affect Dll signaling. The view that vimentin potentiates Jagged function is corroborated by the data showing that Jagged expression correlates with vimentin expression in several tissues and in cancer, in particular. Elevated expression of both vimentin and Jagged has been implicated in tumor progression (6, 8, 10, 16, 73–76). Furthermore, observations in vimentin knockout mice links vimentin to defects in wound healing, fibrosis, inflammation, epidermal aging, and epithelial mesenchymal transition in cancer (4, 7, 8). These are all processes critically regulated by Jagged (77).

Vimentin may also specify ligand-specific signaling by modulating expression of Fringe proteins (31), as vimentin depletion increased Fringe gene expression. At present, we do not know the exact mechanisms through which vimentin regulates Fringe transcription. However, vimentin has been shown to affect gene expression (16), and increasing evidence points to a role for vimentin as a signaling hub (10, 74) and thus loss of vimentin may affect specific signaling pathways regulating expression.

Taken together, our data show that vimentin balances Notch signaling activities and they implicate a complex but distinct role for Jagged in angiogenesis, which relates to the control of signaling strength. Vimentin selectively promotes Jagged activity. Therefore, understanding the regulation of the vimentin–Jagged axis in angiogenesis may unravel new strategies to target the Notch pathway in angiogenesis, but also it has implications for the importance of this pathway in other cell systems known to coexpress these molecules, including differentiating stem cells and cancer.

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