Regulation of Vascular Endothelial Growth Factor Receptor Function in Angiogenesis by Numb and Numb-Like

Max van Lessen, Masanori Nakayama, Katsuhiko Kato, Jung Mo Kim, Kozo Kaibuchi, Ralf H. Adams

Objective—Vascular endothelial growth factor (VEGF) signaling is a major regulator of physiological and pathological angiogenesis. VEGF receptor activity is strongly controlled by endocytosis, which can terminate or enhance signal transduction in the angiogenic endothelium, but the exact molecular regulation of these processes remains incompletely understood. We have therefore examined the function of Numb family clathrin-associated sorting proteins in angiogenesis.

Approach and Results—We show that Numb proteins are expressed by endothelial cells during retinal angiogenesis in mice. Inducible inactivation of the Numb/Numbl genes in the postnatal endothelium led to impaired vessel growth, reduced endothelial proliferation and sprouting, and decreased VEGF receptor activation. Biochemistry and cell biology experiments established that Numb can interact with VEGFR2 and VEGFR3 and controls VEGF receptor activation in response to ligand stimulation. Experiments in cultured endothelial cells showed that Numb proteins counteract VEGF receptor degradation and promote VEGFR2 recycling back to the plasma membrane.

Conclusions—Numb proteins control VEGF receptor endocytosis, signaling, and recycling in endothelial cells, which promotes the angiogenic growth of blood vessels. (Arterioscler Thromb Vasc Biol. 2015;35:1815-1825. DOI: 10.1161/ATVBAHA.115.305473.)

Key Words: angiogenesis ■ endocytosis ■ endothelial cell ■ endothelial cell sprouting and proliferation ■ VEGF signaling ■ VEGFR2

The angiogenic growth of blood vessels is indispensable during fetal and postnatal development, but also plays important roles during regeneration or in disease processes in the adult organism. Vascular endothelial growth factor (VEGF) family growth factors and, in particular VEGF-A, have emerged as critical regulators of angiogenesis.1 Signaling of VEGF-A through its receptor vascular endothelial growth factor receptor 2 (VEGFR2) promotes endothelial cell (EC) proliferation and sprouting during blood vessel growth. The related growth factor VEGF-C, which signals predominantly through VEGFR3, can also stimulate angiogenesis but is primarily a regulator of lymphangiogenesis.1,2

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VEGF signaling is controlled at multiple levels, including the internalization of VEGFR2 and VEGFR3 from the plasma membrane (PM), which can positively or negatively influence the activity of these receptors.3 Receptor endocytosis can involve different routes, among which the clathrin pathway is most relevant and best understood. In this process, clathrin molecules are recruited to specific surface receptors either by direct binding to the AP2 adaptor complex (AP2) or through clathrin-associated sorting proteins (CLASPs). After the formation of clathrin-coated pits and the dynamin-dependent release of vesicles into the cytoplasm, vesicles fuse with early endosomes and, subsequently, undergo a series of sorting processes that can direct internalized receptors to lysosomal degradation or recycling back to the PM.4 The fate of internalized VEGF receptors strongly depends on function of different CLASPs. Cargo-specific adaptors of the Epsin family, namely Epsin 1 (Epn1) and Epsin 2 (Epn2), promote the degradation of VEGFR2 and VEGFR3.5,6 Although EC-specific Epsn1/Epsn2 double mutants developed normally, VEGFR2 signaling in tumor blood vessels was excessively enhanced, leading to deregulated, nonproductive angiogenesis.5,6 Similarly, excessive VEGFR3 activity, caused by defective internalization and degradation of this receptor, compromised lymphatic valve formation in Epsn1/Epsn2 mutants.7 Another CLASP, namely disabled homolog 2 (Dab2), also promotes the endocytosis of VEGFR2 and VEGFR3, which does not lead to the termination of signaling and, instead, promotes certain signal transduction processes downstream of these receptors.8 Other evidence also supports that VEGFR2 internalization and trafficking away from the PM is a prerequisite for triggering its full signaling activity.9-11 Neuropilin-1, a coreceptor of VEGFR2, and recruitment of the PDZ domain protein GIPC/synectin regulate VEGF-dependent artery formation and direct internalized VEGFR2 from the degradative...
Efficient EC-specific inactivation deletion of Numbl/Numbl was further corroborated by quantitative real-time PCR analysis of RNA extracted from P6 lungs, a tissue that was chosen because it contains a particularly high fraction of ECs. Numb transcript levels showed a 72% reduction in Numbl/NumblECKO mutants relative to control littermate samples. In contrast no significant change in the expression of Gapdh or Cdh5 (the gene encoding vascular endothelial cadherin [VE-cad]) was observed (Figure I in the online-only Data Supplement). Likewise, Western blot analysis indicated reduced Numbl and Numbl protein expression in P6 Numbl/NumblECKO lungs (Figure I in the online-only Data Supplement). Residual protein bands are likely to correspond to Numb expression by nonvascular cells, such as lung epithelium. Endogenous expression of Numb and Numbl was also confirmed in cultured human umbilical cord vein endothelial cells (HUVECs) with the same antibodies as used for mouse lung. Despite the high degree of sequence homology between Numb and Numbl, we found that expression of the human gene products can be efficiently and selectively disrupted by small interfering RNA (siRNA)–mediated knockdown (Figure I in the online-only Data Supplement).

**Numb and Numbl Promote Retinal Angiogenesis**

Next, we performed a detailed and quantitative analysis of vascular parameters in P6 Numbl/NumblECKO and control retinas (Figure 1A–1E). This showed that inactivation of the Numb and Numbl genes led to markedly reduced vascular outgrowth, less branching in the vascular plexus, and a smaller area covered by ECs relative to control littermates (Figure 1A and 1B). High-resolution imaging further revealed a significant reduction in the number of endothelial sprouts emerging from the mutant angiogenic front (Figure 1A and 1B). In contrast, the number of filopodia extending across equally long segments of the mutant and control angiogenic front was not different, and the length of filopodia was slightly but significantly increased in the Numbl/NumblECKO retinal endothelium (Figure 1B). Labeling of proliferating cells by intraperitoneal injection of 5-ethyl-2′-deoxyuridine of pups at 1.5 hours before analysis revealed a 25% reduction in EC proliferation in the Numbl/NumblECKO endothelium relative to littermate controls (Figure 1C and 1D).

Potential defects in vascular stability, which can arise from defects in EC migration, survival, or cell–cell junction formation, were assessed by whole-mount staining for isoclitin B4 and collagen IV. Regression of vessel branches and sprouts, which can be visualized as empty (collagen IV+ and isoclitin B4+) collagen sleeves, was significantly enhanced in the Numbl/NumblECKO retinal vasculature (Figure 1C and 1E). Numbl and Numbl have a reported role in the assembly of adherens junctions in epithelial cells by regulating the insertion of E-cadherin into junctional complexes. However, gross analysis of VE-cad, the main cadherin in ECs, was only mildly reduced in Numbl/NumblECKO retinal vessels (Figure II in the online-only Data Supplement). Immunostaining of the tight junction protein Claudin-5, which is positively regulated by VE-cad, was unaffected (Figure II in the online-only Data Supplement). Vascular permeability, measured in the brain cortex after perfusion with the low molecular weight tracer biotin, was also unaltered arguing against junctional defects.
and leakage in Numb/Numb<sup>IECKO</sup> mutants (Figure II in the online-only Data Supplement).

**Analysis of Notch and Integrin Activity in Numb/Numbl Mutant Vessels**

Numb and Numbl are well known as negative regulators of Notch signaling in mammals.25,26 Yet, this role seems to be confined to specific morphogenetic processes, whereas other Notch-regulated activities are independent of Numb and Numbl.27 Notch is also a key regulator of sprouting angiogenesis, endothelial branch formation, and EC proliferation, all of which were reduced in the retina of Notch gain-of-function mice.28,29 However, endothelial expression of the Notch ligand Delta-like 4, which is positively regulated by Notch signaling, was comparable in control and in Numb/Numb<sup>IECKO</sup> retinas (Figure III in the online-only Data Supplement). Levels of the activated Notch intracellular domain fragment, which were assessed by Western blot analysis of whole lung lysates, were also comparable in mutants and littermate controls. Likewise, quantitative RT-PCR analysis of RNA extracted from control and Numb/Numb<sup>IECKO</sup> lungs did not reveal significant differences in the expression of Hey1, Dil4, and Flt4 (Figure...
III in the online-only Data Supplement), which are established Notch target genes in ECs. Sup3 Numb and Numbl are also known regulators of integrin endocytosis, suggesting that defective integrin function might be the cause of vascular defects in Numb/Numbl<sup>IECKO</sup> mutants. However, immunostaining of activated integrin β1, a subunit that is part of many integrin heterodimers and critical for angiogenesis, was comparable in the P6 control and Numb/Numbl<sup>IECKO</sup> retinal vasculature (Figure III in the online-only Data Supplement). Taken together, the results above did not indicate that defects in Numb/Numbl<sup>IECKO</sup> retinal angiogenesis were caused by alterations in Notch activity or integrin β1-mediated cell–matrix interactions.

**Numb and Numbl Interact With VEGF Receptors**

We have previously identified the phosphotyrosine-binding (PTB) domain containing protein Dab2 as an adaptor required for efficient VEGF receptor internalization and signaling. Numb and Numbl belong to the same protein family of PTB domain containing adaptors, and Numb and Dab2 have complementary and partially redundant functions in integrin endocytosis. Interestingly, the PTB domain of Numbl was shown to interact with the NPX(p)Y-related motif NSEY in VEGFR3. This prompted us to investigate a possible role of Numb and Numbl in VEGF receptor function. Incubation of mouse lung lysates with truncated recombinant human Numb
protein fragments fused to glutathione S-transferase revealed that VEGFR2 and VEGFR3 specifically interact with the N-terminal Numb-PTB fragment (aa 1–183; Figure 2A and 2B). VEGFR2, but not VEGFR3, also bound weakly to the C-terminal fragment (aa 184–651) containing the proline-rich region of Numb (Figure 2A and 2B). The specificity of the interaction between endogenous Numb and VEGFR2 was also confirmed in HUVECs by immunoprecipitation. After siRNA-mediated gene knockdown of KDR (VEGFR2) expression, the amount of VEGFR2 coimmunoprecipitated with Numb was drastically reduced (Figure 2C). VEGFR3 is only weakly expressed by HUVECs and was therefore not tested in this experiment, whereas we confirmed the interaction of both VEGFR2 and VEGFR3 with Numb in vivo by coimmunoprecipitation from whole mouse lung lysates (Figure 2D). Because the PTB-domains of Numb, Numbl, and Dab2 can interact with tyrosine motifs in cargo proteins independent of phosphorylation,37 we also investigated whether the interaction of Numb and VEGFR2 is affected by VEGF stimulation. VEGF treatment of HUVECs augmented the interaction of Numb and VEGFR2 in a time-course–dependent manner (Figure 2E). Peak phosphorylation of VEGFR2 at Tyr1175 after 5 minutes preceded the increase in interaction with Numb, which was observed after 10 and 15 minutes (Figure 2E). Similarly, the amount of VEGFR2 that was coprecipitated with the Numb-PTB fragment was increased after VEGF stimulation (Figure 2F).

Colocalization of Numb Proteins and VEGF Receptors

For characterization of the interaction between Numb and VEGFR2 in cultured HUVECs by immunostaining, we first validated the specificity of anti-Numb and anti-VEGFR2 antibodies by siRNA-mediated gene ablation (Figure IV in the online-only Data Supplement). In accordance with previous reports, Numb clustered in punctae at or near the PM, which were also positive for AP2, Clathrin, and Dynamin (Figure V in the online-only Data Supplement) and therefore presumably correspond to clathrin-coated pits.38 Unlike in epithelial cells,34 Numb immunostaining was not restricted to the basal substratum-facing surface but was also found near the apical membrane and VE-cad-containing cell–cell contacts (Figure VI in the online-only Data Supplement). Anti-VEGFR2 staining intensity in HUVECs was highly heterogeneous within different cells (data not shown). In cells with robust VEGFR2 immunoreactivity, VEGFR2 predominantly localized to intracellular vesicles, of which a large fraction was also positive for the early endosomal marker EEA1 (early endosome antigen 1; Figure VI in the online-only Data Supplement). Only punctate structures within membrane protrusions in the cell periphery were positive for both VEGFR2 and Numb, whereas Numb-containing vesicles near and around the nucleus were typically devoid of VEGFR2 (Figure VI in the online-only Data Supplement). Co-staining of Dab2 or the clathrin adaptor AP2 together with endogenous Numb or Green Fluorescent Protein (EGFP)–coupled Numb in HUVECs showed that almost all AP2-positive structures contained Numb and Dab2 (Figure VII in the online-only Data Supplement). Consistent with previous reports,22,39 GFP-Numb in HUVECs was also found Rab11-positive recycling endosomes (Figure VIII in the online-only Data Supplement). Taken together, these data indicate that AP2-positive clathrin-coated pits in HUVECs contain Numb proteins and Dab2, all of which can affect VEGF receptor activity.

Numb Proteins Control VEGF Receptor Levels and Activity

To examine how loss of Numb and Numbl affects VEGF receptor function, the levels of total protein and phosphorylated VEGFR2 and VEGFR3 were assessed in vivo. Whole-mount staining of the P6 Numbl/NumbliECKO retinal vasculature revealed reduced total VEGFR3 protein, whereas the amount of VEGFR2 appeared comparable (Figure 3A–3C). Western blot analysis, which is better suited for quantitative analysis, confirmed the reduction of VEGFR3 in Numbl/NumbliECKO lung lysate in comparison to littermate control (Figure 3D and 3E). This approach also revealed a small but significant reduction of VEGFR2 in 48% (12/25) of the mutant lung samples analyzed (Figure 3D and 3E). To enrich tyrosine-phosphorylated proteins, aliquots of lysates were also incubated with a pan antiphosphotyrosine antibody (clone 4G10) so that the detection of VEGFR2 and VEGFR3 in these immunoprecipitates reflects the amount of phosphorylated or phospho-protein–associated receptor. The amount of VEGFR2 precipitated by this approach was reduced by 25% in Numbl/NumbliECKO relative to control samples (Figure 3D–3F). Likewise, the amount of precipitated VEGFR3 was reduced in Numbl/NumbliECKO lungs, which correlated with substantially lower amounts of total VEGFR3 (Figure 3D–3F). VE-cad, which was used as standard for normalization in the analysis of the Western blot data, was not changed relative to Tubulin (Figure 3G). The decrease in VEGFR3 protein in Numbl/NumbliECKO lungs was not caused by reduced gene expression, as indicated by quantitative real-time PCR analysis (Figure 3G). Likewise, transcripts encoding VEGF1 (Flt1), VEGFR2 (Kdr), VEGF-A (Vegfa), and VE-cad (Cdh5) were comparable in mutant and control lung samples (Figure 3H).

A potential role of Numb proteins in VEGFR2 signaling was further supported by VEGF stimulation of control and siNUMB/siNUMBl knockdown HUVECs (Figure 4A). The amount of total phospho-VEGFR2 and, in particular, phosphorylation of tyrosine residue 1175 (Tyr 1175), which has been implicated in the activation of both PLCγ1/MAPK/extracellular signal-regulated kinase and phosphoinositide 3 kinase/AKT signaling,40,41 was significantly reduced at 15 minutes after VEGF administration (Figure 4A and 4B). At the same time point, total VEGFR2 was also reduced in siNUMB/siNUMBl relative to control HUVECs (Figure 4A and 4B). Consistent with the differences in VEGFR2 phosphorylation, VEGF–A–induced activation of PLCγ1 was attenuated in siNUMB/siNUMBl cells at 15 minutes, whereas the activation of extracellular signal-regulated kinases was not significantly altered (Figure 4A). In contrast, activation of the phosphoinositide 3 kinase pathway was significantly attenuated as indicated by reduced AKT phosphorylation of AKT at 10 and 15 minutes after VEGF–A stimulation (Figure 4A and 4B). VEGF-C is mainly a ligand for VEGFR3, but the fully processed form of the growth factor can also signal through VEGFR2 homodimers and VEGFR2/VEGFR3 heterodimers.42 siNUMB/siNUMBl knockdown led to reduced VEGF–C–induced phosphorylation of VEGFR2 and significantly lower VEGFR2/VEGFR3 heterodimerization
Moreover, the phosphorylation of extracellular signal–regulated kinases and AKT downstream of VEGF-C was strongly impaired in siNUMBl siNUMB HUVECs (Figure IX in the online-only Data Supplement). Thus, Numb proteins play important and ligand-dependent roles in VEGF receptor signaling.
Numb Controls VEGF Receptor Recycling

VEGFR2 levels in the growing vasculature depend on the balance between the synthesis, recycling, and degradation of this receptor. As levels of surface VEGFR2 (as determined by biotinylation) and total VEGFR2 were strongly reduced in siNUMB/siNUMBL HUVECs at 15 or 30 minutes after VEGF stimulation (Figure 4A and 5A), we next determined the relevance of VEGFR2 de novo synthesis and degradation in vivo and in cultured HUVECs. Analysis of lung lysates from mice treated for 2 hours with the protein synthesis inhibitor cycloheximide led to a nearly complete loss of VEGFR2 protein but not VEGFR3 or VE-cad (Figure 5B). High VEGFR2 turnover has also been suggested for HUVECs in vitro. Accordingly, VEGF stimulation of HUVECs in the presence of cycloheximide for 30 minutes led to a profound reduction of total and, as determined by biotinylation, surface VEGFR2 (Figure 5C). Removal (washout) of VEGF allowed the full recovery of total and surface VEGFR2 after 60 or 120 minutes to levels seen in unstimulated cells. Indicating that this recovery was mainly caused by new synthesis of VEGFR2, levels of the receptor were strongly reduced when cycloheximide was present during the recovery phase (Figure 5C). As the stimulation of siNUMB/siNUMBL HUVECs with VEGF-A in the presence of cycloheximide led to significantly faster reduction of VEGFR2 levels than in control cells (Figure 5D and 5E), we next investigated the role of Numb proteins in VEGFR2 degradation. It has been previously shown that inhibitors of lysosomal or proteasomal protein degradation can interfere with the destruction of internalized VEGFR2. Interestingly, differences in VEGFR2 levels seen between control and siNUMB/siNUMBL HUVECs at 30 minutes after VEGF stimulation were abrogated by the presence of Chloroquine, an inhibitor of lysosomal degradation, or MG132, which interferes with proteasome function and limits the availability of ubiquitin (Figure 5F and 5G). These findings indicate that Numb proteins antagonize the destruction of VEGFR2 after VEGF stimulation.

Finally, we conducted a series of recovery experiments to address the role of Numb/Numblike in VEGF receptor recycling (Figure 6A). Transient VEGF stimulation for 30 minutes followed by washout allowed the recovery of surface VEGFR2 in control HUVECs, which was slightly diminished after siRNA-mediated knockdown of NUMB/NUMBL or DAB2 (Figure 6B). Indicating partial functional redundancy of these highly related CLASPs, combined knockdown of NUMB/NUMBL and DAB2 expression in HUVECs prevented the recovery of surface VEGFR2 almost completely (Figure 6B). This was further corroborated by recovery experiments in the presence of cycloheximide, preventing de novo protein synthesis, and MitMAB,

Figure 4. Numb and Numblike regulate VEGFR2 signaling in vitro. A, HUVECs at 48 hours after transfection with siNUMB/siNUMBL or control siRNA (Scrambled) were stimulated with vascular endothelial growth factor (VEGF) for the indicated times, lysed, and subjected to Western blot with the indicated antibodies. In the top panel, samples were immunoprecipitated with a pan antiphosphotyrosine antibody (pTyr; 4G10) before Western blot. Note that total VEGFR2 tyrosine phosphorylation, phospho-Tyr1175, and phosphorylated PLCγ1 (p-PLCγ1) levels were not sustained in NUMB/NUMBL knockdown cells at 15 min after VEGF stimulation. Although activation of ERK (p-ERK) was unaffected, VEGF-induced AKT activation (p-AKT) was strongly attenuated. Note also the decreased total VEGFR2 after 15 min time point in siNUMB/siNUMBL cells. B, Densitometric quantification of the relative protein phosphorylation described in (A). Data are representative of at least 3 independent experiments and are presented as mean+SEM; n≥3. *P<0.05. ERK indicates extracellular signal-regulated kinase; HUVECs, human umbilical cord vein endothelial cells; PLC, phospholipase C; and VEGFR, vascular endothelial growth factor receptor.
which inhibits dynamin-dependent internalization of surface receptors. Recovery of surface VEGFR2, which is exclusively caused by the recycling of existing (internalized) receptor molecules back to the PM in this experiment, was strongly reduced after siNUMB/siNUMBL/siDAB2 triple knockdown relative to control HUVECs or cells treated with siNUMB/siNUMBL or siDAB2 siRNAs alone (Figure 6C). These findings argue that Numb proteins mediate VEGFR2 recycling, which is partially redundant with the function of Dab2. In line with this conclusion, siNUMB/siNUMBL/siDAB2 triple knockdown had the strongest effect on VEGF-induced sprouting from HUVEC spheroids in vitro. In comparison, siDAB2 or siNUMB/siNUMBL transfection alone led to a weaker but still significant reduction of HUVEC sprouting relative to scrambled control siRNA (Figure X in the online-only Data Supplement).

Discussion

Previous work has established that VEGF receptor internalization can either promote the rapid termination of VEGF signaling or enhance the activity of this pathway and facilitate receptor recycling. Such surprisingly diverse outcomes have been linked to coreceptors of VEGFR2, such as Neuropilin-1 or ephrin-B2, and the distinct roles of different CLASPs.5,10,12,15,47 Given that these molecules strongly influence the signaling activity of the VEGF pathway and thereby control processes, such as developmental angiogenesis, artery formation, or tumor neovascularization, our understanding of the underlying regulation is still inadequate. Here, we have identified Numb and Numbl as novel regulators of VEGF receptor trafficking and signaling (Figure 6D). Inducible inactivation of the Numb and Numbl genes specifically in postnatal ECs led to a marked reduction in retinal angiogenesis and, in particular, endothelial proliferation and sprouting. Despite of the established role of Numb as a negative regulator of Notch signaling,25,26 no overt difference in endothelial Notch activation was detected in the retinal vasculature and lungs from control and Numb/NumblIECKO ECs mice. This finding is consistent with previous studies showing that Numb/Numbl influence Notch signaling in certain developmental processes but not others.14,48 Numb has also been implicated in the control of integrin β1 endocytosis during cell migration.33,34 However, inactivation of the gene encoding integrin β1 in ECs led to hypersprouting in the retinal vasculature,49 which is the opposite of the Numb/NumblIECKO phenotype. Numb/NumblIECKO mice also showed no overt differences in active integrin β1. Instead, we discovered that Numb and Numbl promote VEGF...
Figure 6. Numb, Numbl, and Dab2 promote VEGFR2 recycling. A, Schematic representation of internalization and recycling assays. B, C, Western blot of HUVECs transfected for 48 hours with siNUMB/siNUMBL, siDAB2, a mixture of all 3 siRNAs (siNUMB/siNUMBL/siDAB2) or control siRNA (Scrambled), as indicated. After vascular endothelial growth factor (VEGF) stimulation (30 min) and 60 min VEGF washout, the recovery of surface VEGFR2 was reduced in siNUMB/siNUMBL/siDAB2 cells and, to lower extent, in siNUMB/siNUMBL or siDAB2-treated HUVECs (B). The contribution of VEGFR2 recycling in absence of de novo synthesis (CHX) after VEGF stimulation (30 min) was assessed in the absence of protein synthesis (CHX inhibition) and presence of the dynamin inhibitor MitMAB (preventing repeated internalization) during the 30 min recovery phase after VEGF washout. Under these conditions, the level of surface (biotinylated) VEGFR2 was strongly diminished in siNUMB/siNUMBL/siDAB2 HUVECs (C). Molecular weight markers (kDa) and results of densitometric analysis are indicated. Data are representative of at least 3 independent experiments. D, Schematic representation of findings. Clathrin-associated sorting proteins (CLASPs) differentially regulate the fate of internalized VEGF receptor. Numb/Numbl together with Dab2 promote VEGF receptor recycling and enhance signal transduction, whereas Epsins have been shown to mediate the degradation of active receptor, which terminates signaling activity. VEGFR2 function is also sustained by high levels of de novo synthesis in mice and cultured HUVECs. CHX indicates cycloheximide; HUVECs, human umbilical cord vein endothelial cells; and VEGFR, vascular endothelial growth factor receptor.
receptor activation and signaling by preventing receptor degradation after internalization. VEGFR2 signaling has been shown to raise VEGFR3 expression both at the transcript and protein levels in Numb/Numbl^+/− mice. The absence of Numb and Numblike reduces VEGFR3 tyrosine phosphorylation and lower VEGFR3 proliferation during angiogenesis,50 extracellular signal-regulated kinases activation was only defective downstream of VEGF-C. These distinct effects might be caused by differences in ligand binding and the extent of receptor activation.

A role of heterodimers of VEGFR2 and VEGFR3, which only form in response to VEGF-C but not VEGF-A,52 is also conceivable. Indeed, reduced association of VEGFR2 to VEGFR3 was seen in siNUMB/siNUMBL HUVECs.

Our data also show that the turnover of VEGFR2 in vivo is extremely high in comparison to VEGFR3 or VE-cad. This means that factors controlling the de novo synthesis or the stability/lifetime of VEGFR2 will strongly affect its bioavailability and thereby signaling capacity. Increased VEGFR2 degradation after loss of Numb/Numbl might be therefore of particular importance in settings where VEGF receptor de novo synthesis is limited or inhibited.

In HUVECs, Numb was concentrated at the apical and basal surfaces, and colocalized with VEGFR2 and clathrin/AP2 in structures that presumably represent clathrin-coated pits. In contrast, Numb was only rarely found in early endosomes containing VEGFR2, hinting at a potentially transient interaction similar to what has been reported for Dab2 in the internalization of low-density lipoprotein receptor.51 Interestingly, we also observed a high degree of colocalization between Numb and Dab2 in clathrin/AP2-positive structures, suggesting that these molecules might be available simultaneously (Figure 6D) and might therefore have partially redundant functional roles. Given the great importance of the VEGF signaling in numerous physiological and pathological processes, our findings might prove beneficial for the development of new strategies aiming at the therapeutic modulation of the VEGF pathway.

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M. van Lessen, M. Nakayama, and R.H. Adams designed experiments and interpreted results. M. van Lessen and M. Nakayama generated and characterized mouse mutant lines. M. van Lessen and J.M. Kim conducted all in vitro experiments. K. Kato and K. Kaibuchi generated and provided anti-Numb antibody and plasmids encoding full-length human Numb (GenBank: AF171941.1) or Numb fragments in pEGFP (Clontech) and pGEX-4T-1 (GE Healthcare). M. van Lessen and R.H. Adams wrote the manuscript.

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Disclosures

None.

References


Significance

The vascular endothelial growth factor signaling pathway is a master regulator of angiogenic blood vessel growth. Vascular endothelial growth factor receptor function can be positively or negatively regulated by internalization from the cell surface. Here, we have identified Numb family clathrin-associated sorting proteins as novel positive regulators of vascular endothelial growth factor receptor signaling and stability. Our findings provide new insight into the vascular endothelial growth factor signaling pathway and might thereby aid the development of new therapeutic strategies for human diseases with deregulated blood vessel growth.
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Supplementary Figure I: Numb and Numblike are expressed in endothelial cells.

A. Whole-mount double immunofluorescence for isolectin B4 (IB4) and two different Numb antibodies in the P6 mouse retina. Numb/Numbl inactivation for 5 days led to strongly reduced anti-Numb/Numbl immunoreactivity in the retinal vasculature. Arrows in insets indicate enriched Numb staining at vessel borders and absence of signal in Numb/Numbl iECKO capillaries.

B. qRT PCR analysis of RNA extracted from lungs derived from P6 control or Numb/Numbl iECKO mice. Mutant mice showed about 72% reduction in Numb transcript levels but no significant change in Cdh5 (VE-cadherin) expression indicating similar EC content compared to control lungs.

C. Immunoblotting of lung lysate from Numb/Numbl iECKO mice revealed significantly reduced protein levels of Numb and Numbl relative to control lungs. Residual Numb/Numbl immunoreactivity (A), Numb transcripts (B) or Numb/Numbl protein (C) is likely to reflect expression in non-vascular cells. Molecular weight markers (kDa) are indicated.

D. Cultured human umbilical vein endothelial cells (HUVECs) 48 hours after transfection with siNUMB or siNUMBL siRNA. Immunoblotting revealed no apparent cross-reactivity of Numb and Numbl antibodies. Data are presented as mean + s.e.m.; n ≥ 3. *P < 0.05; NS, not significant.
Supplementary Figure II: Numb/Numbl inactivation reduces VE-cadherin staining without affecting junctional integrity.

**A.** Primary components of adherens junctions and tight junctions, namely VE-cadherin (VE-cad) and Claudin-5, were stained in P6 control and Numb/Numbl iECKO retinas at 5 days after Pdgfb-iCre-driven, tamoxifen-induced gene inactivation. VE-cadherin staining was reduced by 25% in Numb/Numbl iECKO mutants relative to control retinas (arrows in insets). Moreover, in Numb/Numbl iECKO mutants, VE-cadherin-positive vesicular structures were observed (arrowheads). These alterations did not overtly affect the formation of tight junctions, as indicated by comparable Claudin-5 staining intensities in mutant and control retinas (arrows in insets).

**B.** Quantitation of the staining intensities described in (A). Data are representative of at least three independent experiments and are presented as mean ± s.e.m.; n = 3. *, P < 0.05.

**C.** Biotin (443 Dalton) was perfused from the left cardiac ventricle of P15 mice at two weeks after gene deletion. In brain cortical vibratome sections the vessels of both control and Numb/Numbl iECKO were well perfused but did not show extravasation of biotin into brain parenchyma. Data are representative of two independent experiments.
Supplementary Figure III: Absence of overt differences in endothelial Notch signaling or integrin β1 activation after Numb/Numbl inactivation.

A, B, Whole-mount double immunofluorescence for isolectin B4 (IB4, upper panel) and Delta-like 4 (Dll4, bottom) in P6 mouse retinas at 5 days after Pdgfb-iCre-driven, tamoxifen-induced gene inactivation. Dll4 immunoreactivity in the distal angiogenic plexus was similar in control and Numb/Numbl iECKO animals (A). Note the characteristic high Dll4 staining in sprouts at the angiogenic front (arrowheads) in comparison to the adjacent capillary plexus. Quantitation of Dll4 staining intensity (B). C, Similar NICD protein levels in P6 whole lung lysate of control and Numb/Numbl iECKO mice. Similar VE-cadherin and Tubulin levels reflect comparable EC and total protein content. D, RT-qPCR of RNA extracted from P6 control and Numb/Numbl iECKO lungs did not reveal overt differences after pairwise comparison of the Notch target genes Dll4, Hey1, Hes1 and Flt4. The Cdh5 and Gapdh transcript levels reflect similar endothelial cell and total RNA content. Data are presented as mean ± s.e.m.; n ≥ 3. *, P < 0.05; NS, not significant. E, Representative confocal images of isolectin B4 (IB4, upper panel) and activated integrin β1 (lower panel) stained P6 vasculature of control and Numb/Numbl iECKO retinas 5 days after Pdgfb-iCre-driven, tamoxifen-induced gene inactivation. Immunoreactivity of activated integrin β1 was similar in control and Numb/Numbl iECKO samples. Data represent two independent experiments.
Supplementary Figure IV: Validation of Numb and VEGFR2 immunostainings in HUVECs. 

A, B, HUVECs were transfected with control siRNA (Scrambled), siRNA against Numb and Numbl (siNUMB/siNUMBL) or VEGFR2 (siKDR) for 48 hours, fixed and stained with phalloidin and antibodies specific for Numb or VEGFR2. The ablation of NUMB and NUMBL (A) or KDR (B) strongly reduced the immunoreactivity relative to control-transfected cells. Data are representative of at least three independent experiments.
Supplementary Figure V: Numb localizes to AP2, Clathrin and Dynamin-positive structures.

A-C, HUVECs were fixed and co-stained with phalloidin (white fluorescence) and antibodies specific for Numb (red) and Clathrin (green) (A), AP2 (green, B) or Dynamin (green, C). With few exceptions (arrows in insets of A and B) all Numb punctae also stained positive for Clathrin and AP2. Analogously, only few AP2 punctae were not Numb-positive (arrowhead in B), while there was a fraction of Clathrin-positive but Numb-negative structures (arrowhead in A). Most Numb-positive punctae colocalized or were in close proximity with Dynamin (C). Nuclei, DAPI (blue). Data are representative of three independent experiments.
Supplementary Figure VI: Colocalization of Numb with VEGFR2 in the cell periphery.

A, HUVECs were fixed and stained with DAPI (blue) the antibodies against Numb (green). Optical single plane cross-sectioning (XY) revealed punctate Numb signal predominantly at or near the apical and basal plasma membrane, while Numb-positive punctae were rare in the cytoplasm. B, In confluent HUVECs, Numb-positive punctae (white/red) were distributed over the entire cell body and were partially enriched at VE-cadherin (VE-cad; white/blue) containing cell-cell contacts (insets). The channel for DAPI has been pseudocolored (blue -> green). Data are representative of at least three independent experiments. C, Immunostaining of Numb (green), VEGFR2 (red) and EEA1 (blue) in HUVECs. Nuclei, DAPI (white). In the cell periphery (inset 1), Numb and VEGFR2 colocalized in EEA-1 negative membrane punctae (insets 3-5). In the perinuclear region (inset 2), VEGFR2 was found in or near EEA1-positive endosomes, which were largely devoid of Numb staining (inset 2, 6-8). Data are representative of at least three independent experiments.
Supplementary Figure VII: Different CLASPs overlap in the same AP2-positive structures.

A, HUVECs were fixed and co-stained with the indicated antibodies. With few exceptions (arrows in insets), all Dab2-containing punctae were also positive for AP2. Similarly, only few AP2 punctae were not positive for Dab2 (arrowheads in insets). B, HUVECs were infected with lentivirus encoding EGFP-Numb and stained with Dab2 antibodies after 24 hours. With few exceptions (arrows in insets), all EGFP-Numb positive punctae colocalized with Dab2 (arrowheads). Data are representative of at least three independent experiments.
Supplementary Figure VIII: Colocalization of EGFP-Numb with Rab11.
HUVECs were infected with lentivirus encoding EGFP-Numb. 24 hours after transduction the cells were fixed and stained with Rab11 antibodies. Some Numb positive punctae were also positive for Rab11, which is highlighted by single plane cross-sectioning (XY) through individual EGFP-Numb and Rab11 double positive dots (arrow, yellow circle). Data are representative of two independent experiments.
Supplementary Figure IX: Numb/Numbl control VEGF-C signaling.

A, B, HUVECs transfected with siNUMB/siNUMBL or control siRNA (Scrambled) were treated with VEGF-C, lysed and subjected to immunoprecipitation with a pan anti-phosphotyrosine antibody (4G10) (A) or an antibody against VEGFR3 (B) before immunoblotting. NUMB proteins promote full VEGFR2 phosphorylation (A) and the formation of VEGFR2/VEGFR3 heterodimers (B).

C, D, Densitometric quantitation of immunoblots shown in A and B.

E, F, Immunoblotting (E) and densitometric quantitation (F) showing attenuated VEGF-C-induced phosphorylation of ERK at 5 and 10 minutes, and of AKT at 10 and 15 minutes in siNUMB/siNUMBL cells relative to control cells. Data are representative of at two (B) or at least three (A, E) independent experiments. Data are presented as mean of two experiments (D) or mean ± s.e.m.; n ≥ 3. *, P < 0.05.
**Supplementary Figure X: Numb/Numbl and Dab2 promote VEGF-induced sprouting in spheroids.**

**A**, 24 hours after transfection with siNUMB/siNUMBL, siDab2, siNUMB/siNUMBL/siDab2 or control siRNA (Scrambled) HUVECs were aggregated overnight in a hanging drop followed by embedding into collagen I type gels in co-culture with fibroblasts and in the presence or absence of VEGF, as indicated. Bright-field images were acquired 48 hours post embedding showing reduced sprouting after the ablation of siNUMB/siNUMBL or siDab2 relative to control. The most profound reduction of sprouting was seen in siNUMB/siNUMBL/siDab2 triple knockdown cells. **B**, quantitation of the sprouts per spheroid described in (A). Data are representative of two independent experiments and are presented as mean + s.e.m.; n = 2. *, P < 0.05.
Materials and Methods

Mouse genetics

*Cdh5-CreERT2* or *Pdgfb-iCre* transgenic mice were bred into a background of animals carrying loxP-flanked *Numb* and *Numblike* genes. All animals were on a mixed C57BL6/129 background. Tamoxifen-injected Cre-negative littermate controls were used for all in vivo experiments. Images shown in the figures represent data obtained with *Pdgfb-iCre* transgenes unless indicated otherwise. Cre activity in newborn mice was induced by three consecutive intraperitoneal tamoxifen (Sigma, T5648; 1 mg/ml in ethanol/peanut oil; generated from a 10x stock containing 25% ethanol and 75% peanut oil) injections of 50 µl given on P1, P2 and P3. The phenotype of mutant mice and wild type littermates was analyzed at P6 or P15, as indicated. PCR primers used for genotyping are listed in table I. All animal experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by local animal ethics committees.

Table I: Genotyping of mice

<table>
<thead>
<tr>
<th>Allele/Locus</th>
<th>Annealing</th>
<th>Primer Sequence</th>
<th>Product size</th>
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<tbody>
<tr>
<td><em>Cdh5-CreERT2</em></td>
<td>70 °C</td>
<td>5'-GGCTGCATTACCGTGATGCAACGA-3' 5'-GTGGAGATGGCCGCGAACCATT-3'</td>
<td>tg 720 bp</td>
</tr>
<tr>
<td><em>Pdgfb-iCreER</em></td>
<td>57 °C</td>
<td>5'-GGCGCCGGGATCCTCTAG-3' 5'-CCAGGCCTCGCGAACCATT-3'</td>
<td>tg 450 bp</td>
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<tr>
<td><em>Numb</em> loxed</td>
<td>65 °C</td>
<td>5'-GAAGGAGCCTCCAATACTGATTC-3' 5'-AGGCTTCTCGAAAATCTCTAC-3'</td>
<td>Numb&lt;sup&gt;1&lt;/sup&gt; 400 bp</td>
</tr>
<tr>
<td><em>Numblike</em> loxed</td>
<td>65 °C</td>
<td>5'-CTGTTCCTCGCTTCTGCTTA-3' 5'-GCAGGAGCAGGAAAAACCATTCTCTC-3'</td>
<td>Numb&lt;sup&gt;1&lt;/sup&gt; 320 bp Numb&lt;sup&gt;1&lt;/sup&gt; 280 bp</td>
</tr>
</tbody>
</table>

Abbreviations: transgenic (tg), wild type (+)

Immunohistochemistry

Histological analyses of retinal whole mounts including proliferation and vessel regression analysis as well as the analysis of the brain vasculature was performed as described previously.

For retinal whole mounts, whole animal eyes were fixed before dissection at 4 °C in 4% paraformaldehyde (PFA) either (1) overnight (visualization of filopodia and other fine morphological aspects of the retinal vasculature) or (2) 90 minutes at 4 °C, or 10 minutes at 4 °C with subsequent dissection and further fixation in methanol overnight at -20 °C (detection of extracellular epitopes and low abundance proteins). Next, eyes were blocked and permeabilized for 1-2 hours in blocking buffer (1% BSA, Sigma, A4378; 0.3% Triton X-100, Sigma, T8787) at room temperature. For staining, retinas were incubated overnight at 4°C with biotinylated isoelectin B4 (1:50) (Vector Laboratories, B-1205) and primary antibodies as indicated in Table II diluted in blocking buffer or, in case of overnight fixation, in Pblec (1% Triton X-100, 1 mM CaCl₂, 1 mM MgCl₂ and 0.1 mM MnCl₂ in PBS, pH 6.8). Following 5 washes for 20 minutes with wash buffer (0.5% BSA; 0.15% Triton X-100) the retinas were incubated with Alexa-Fluor-strepavidin conjugated secondary antibodies (Molecular Probes, 1:500 in wash buffer) for 2 hours at room temperature. Finally, retinas were washed 3 times in wash buffer for 20 minutes and 2 times in PBS prior to flat
mounting on microscope glass slides with Fluoromount-G (SouthernBiotech, 0100-01).

Table II: Antibodies used for mouse retina stainings

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Order number</th>
</tr>
</thead>
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<tr>
<td>Claudin-5</td>
<td>1:100</td>
<td>Life Technologies</td>
<td>341600</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>1:250</td>
<td>Chemicon</td>
<td>AB756P</td>
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<td>DLL4</td>
<td>1:100</td>
<td>R&amp;D Systems</td>
<td>MAB1389</td>
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<tr>
<td>Integrin b1</td>
<td>1:100</td>
<td>Becton Dickinson</td>
<td>553715</td>
</tr>
<tr>
<td>Numb (antibody #1; cross-reacts with Numblike)</td>
<td>1:100</td>
<td>Kozo Kaibuchi, Japan</td>
<td>Reference 8</td>
</tr>
<tr>
<td>Numb (antibody #2)</td>
<td>1:100</td>
<td>Cell Signaling</td>
<td>2756</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>1:100</td>
<td>BD Biosciences</td>
<td>555289</td>
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<tr>
<td>VEGFR2</td>
<td>1:100</td>
<td>BD Pharmingen</td>
<td>555307</td>
</tr>
<tr>
<td>VEGFR3</td>
<td>1:100</td>
<td>R&amp;D Systems</td>
<td>AF743</td>
</tr>
</tbody>
</table>

For assessment of EC proliferation, P6 pups were injected IP with 50 ug EdU (5-ethyl-2'-deoxyuridine) (Life Technologies, A10044) per gram of body weight. After an EdU pulse of 90 minutes, pups were sacrificed and retinas were prepared for immunofluorescence. After staining the retinal vasculature with IB4, Edu incorporated into dividing cells was detected with the Click-iT EdU Alexa Fluor647 Imaging Kit (life technologies, C10340) according to the manufacturer’s instructions. For brain sections, P15 old Mice were anesthetized and perfused from the left cardiac ventricle with 2 mg/mL EZ-Link Sulfo-NHS-Biotin (Thermo Scientific, 10509863) dissolved in PBS containing 1 mM CaCl₂, 1 mM MgCl₂ at 1 mL/minute and with 1 mL/gram body weight using a peristaltic Pump P-1 (GE Healthcare, 18-1110-91) and a needle (30G ½” 0,3 x 13 mM). After 10 min of incubation, brains were collected, cut in an anterior to posterior fashion into ~2 mM thick slices and fixed overnight at 4 °C in 4% PFA. After 2 washes in PBS, brains were embedded in 6.5 % low melting agarose (Serva, 11408) dissolved in PBS. Tissue blocks were left overnight at 4 °C and cut into 100 µm sections using a vibratome (LEICA VT 12005). For immunostaining, sections were blocked and permeabilized with blocking buffer (10% Donkey Serum, 0.3% Triton X-100 in PBS) for 1 hour at room temperature. In order to visualize EZ-Link Sulfo-NHS-Biotin, the sections were incubated Alexa-Fluor-streptavidin conjugated secondary antibodies (Molecular Probes, 1:500 in wash buffer) for 2 hours at room temperature. All incubation steps were performed with gentle agitation.

Cell culture

Human umbilical vein endothelial cells (HUVECs, life technologies, C-035-5C) were seeded into Corning tissue-culture treated culture dishes (Sigma) coated with Gelatin Solution (Sigma, G1393) in Medium 200 (life technologies, M-200-500) supplemented with LSGS (life technologies, S-003-10) in a humidified incubator at 37 °C and 5 % CO₂. For the analysis of spheroid sprouting HUVECs were cultured in EGM-2™ (Lonza, CC-4176).
For stimulation experiments, HUVECs were seeded at a density of 1.2 x 10^4 cells per cm^2 and incubated at 37 °C and 5 % CO_2 for 72 hours. Following 4 hours starvation in serum-free Medium 200, cells were incubated with pre-warmed Medium 200 containing 50 ng/mL of Recombinant Human VEGF (RELIATech, 300-076-L) or 50 ng/mL Recombinant Human VEGF-C (PeproTech, 100-20C) at 37 °C for the indicated time points. After that, the cells were either lysed directly or put back on ice and processed as indicated.

For RNA interference experiments, Ambion Silencer Select Pre-designed small interfering RNA (siRNA) was purchased from life technologies (Table III).

Table III: siRNAs used for knockdown in HUVECs

<table>
<thead>
<tr>
<th>Target</th>
<th>siRNA ID</th>
<th>Sequence</th>
<th>[nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dab2</td>
<td>s3896</td>
<td>5'-CGUCAUUUUUGGUACAGUt-3'</td>
<td>sense antisense</td>
</tr>
<tr>
<td>Kdr (VEGFR2)</td>
<td>s7823</td>
<td>5'-CCACGUGAUCCAGAt-3'</td>
<td>sense antisense</td>
</tr>
<tr>
<td>Numb</td>
<td>s16466</td>
<td>5'-CUAGGCACCUCUAGUUGAt-3'</td>
<td>sense antisense</td>
</tr>
<tr>
<td>Numblike</td>
<td>s17694</td>
<td>5'-AAAGGAACCUUUCGAlGgt-3'</td>
<td>sense antisense</td>
</tr>
<tr>
<td>Negative Control</td>
<td>AM4611</td>
<td>Not available</td>
<td>-</td>
</tr>
</tbody>
</table>

HUVECs were seeded at a density of 1.2 x 10^4 per cm^2 and incubated at 37 °C and 5 % CO_2 for 16-24 hours. Next, siRNA was transfected with Opti-Mem® (life technologies, 31985-047) and Oligofectamine Transfection™ Reagent (life technologies, 12252011) according to the manufacturer's instructions for 3 hours and at the concentrations indicated (Table III). Negative control siRNA was used at the same concentration as the RNA-specific siRNAs. The cells were analyzed 48 hours days post-transfection.

For lentivirus production, 293T/17 cells (ATCC, CRL-11268) were transfected with PLL3.7-EGFP-Numb together with the helper plasmids psPAX2 (packaging) (Addgene, plasmid 12260) and pMD.G (envelope) (constructed by Daniel Orey) using FuGENE 6 Transfection Reagent (E2691, Promega) based transfection according to manufacturer’s instructions. PLL3.7-EGFP-Numb-(wt) was constructed from pEGFP-Numb-wt and LentiLox 3.7 (pLL3.7; Addgene, plasmid 11795) using standard molecular cloning techniques.

After 72 hours, the virus supernatant was filter-sterilized, aliquoted and stored at -80 °C. For infection, HUVECs were plated on glass cover slips and infected the next day with viral supernatant (at least 10 µl/cm² cell growth area) and incubated at 37 °C and 5 % CO_2 for 12-16 hours. Finally, the cells were fixed directly in the dish with 4% PFA-PBS.

**Spheroid sprouting assay**

The spheroid sprouting assay was performed as described previously. In brief, HUVECs (Lonza) at passage 3 – 5, cultured in EGM-2™, were transfected with the indicated siRNAs (Table III). 24 hours post transfection, 200 HUVECs of each condition were aggregated overnight at 37 °C in a hanging drop in EGM-2 medium containing methylcellulose (25 volume% of a 1.2% solution of methylcellulose 4000
cP) (Sigma). Formed Spheroids were embedded in 2.5mg/mL type I Collagen (Corning, 354249) gel in a 12 well Corning tissue culture treated dish. To support endothelial sprouting, 1x10⁴ C3H10T½ fibroblasts (ATCC) were seeded in growth media supplemented with or without VEGF (50ng/ml) on top of the gels (VEGF usually included in EGM-2™ was omitted). 36 hours post embedding, at least 21 spheroids divided over six wells per knockdown and treatment group were analyzed in two independent experiments. Images were recorded with a Zeiss Axio Observer equipped with CCD-chip camera (Hamamatsu) and Volocity software (Improvision, Perkin Elmer).

**Immunocytochemistry**

HUVECs were plated on glass cover slips coated with Gelatin Solution (Sigma, G1393) at a density of 1.2 x 10⁴ per cm² and incubated at 37 °C and 5 % CO₂ for at least 12 hours. For immunostaining the cells were fixed in pre-warmed 4 % PFA-PBS for 10 minutes added directly to the medium, rinsed carefully for 3 times with PBS⁺⁻ (pH 7.4, 1 mM Mg²⁺, 0.1 mM Ca²⁺) and once with PBS⁺⁻ containing 100 mM Glycine. Next, the cells were permeabilized for 5 minutes with PBS containing 0.05 % Triton X-100 and blocked with blocking buffer (PBS with 1 % BSA) for 1 hour. Primary antibodies were diluted as indicated (Table IV) in Pierce Immunostain Enhancer (Thermo Scientific, 46644) and were layered onto the coverslips and incubated for 1 hour. Next, the cells were carefully rinsed in PBS for 12 times and incubated with suitable species-specific Alexa- Fluor-conjugated secondary antibodies (Molecular Probes, 1:1000 in Pierce Immunostain Enhancer) and, if applicable, Alexa Fluor-conjugated anti-phalloidin (1:500) for 1 hour. Finally, the cells were counterstained with DAPI (1:1000 in PBS, Sigma, D9542) for 5 – 10 minutes, rinsed 12 times in PBS and were then flat mounted on microscope glass slides with Fluoromount-G (SouthernBiotech, 0100-01). All steps were performed at room temperature. Images were recorded with a Leica SP5 microscope and Volocity software (Improvision, Perkin Elmer).

**Table IV: Antibodies used for immunofluorescence in cultured cells**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Order Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha Adaptin</td>
<td>1:100</td>
<td>Abcam</td>
<td>ab2730</td>
</tr>
<tr>
<td>Clathrin heavy chain</td>
<td>1:300</td>
<td>Calbiochem</td>
<td>CP45</td>
</tr>
<tr>
<td>Clathrin heavy chain (C-20)</td>
<td>1:300</td>
<td>Santa Cruz</td>
<td>sc-6579</td>
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<td>Dab2</td>
<td>1:200</td>
<td>Cell Signaling</td>
<td>12906</td>
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<tr>
<td>EEA1</td>
<td>1:300</td>
<td>BD Transduction Lab</td>
<td>610457</td>
</tr>
<tr>
<td>Numb (antibody #2)</td>
<td>1:300</td>
<td>Cell Signaling</td>
<td>2756</td>
</tr>
<tr>
<td>Rab11</td>
<td>1:100</td>
<td>Life Technologies</td>
<td>71-5300</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>1:300</td>
<td>R&amp;D</td>
<td>MAB938</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>1:200</td>
<td>R&amp;D Systems</td>
<td>AF357</td>
</tr>
</tbody>
</table>
Proteins were separated according to their size, shape and electrical charge using denaturing Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were transferred to Polyvinylidene fluoride (PVDF) Immobilon-P Transfer Membranes (IPVH00010, Millipore). Proteins were transferred in a XCell II Blot module (life technologies, EI9051) filled with NuPAGE Transfer Buffer (life technologies, NP0006-1) at 25 V for 1 hour at room temperature. Membranes were incubated in blocking buffer (0.3 % non-fat dry milk in TTBS (0.1% Tween in Tris-Buffered Saline)) for 30 minutes and with primary antibodies (Table V) and suitable species-specific secondary antibody conjugated to horseradish peroxidase (HRP; GE Healthcare) in blocking buffer with in-between washes for 1 hour each at room temperature. For detection, the membranes were incubated with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, RPN2232). Western blots were quantitated with ImageJ software.

### Table V: Antibodies used for western blotting (WB) and immunoprecipitation (IP)

<table>
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<th>Antibody</th>
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<td>Akt</td>
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<td>Cell Signaling</td>
<td>4691</td>
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<tr>
<td>Dab2</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>12906</td>
</tr>
<tr>
<td>NICD (Notch-1 cleaved)</td>
<td>1:200</td>
<td>Cell Signaling</td>
<td>2421</td>
</tr>
<tr>
<td>Numb (antibody #2)</td>
<td>1:1000</td>
<td>Cell Signaling</td>
<td>2756</td>
</tr>
<tr>
<td>Numblike</td>
<td>1:1000</td>
<td>Protein Tech Group</td>
<td>10111-1-AP</td>
</tr>
<tr>
<td>p44/42 MAPK antibody</td>
<td>1:3000</td>
<td>Cell Signaling</td>
<td>4695</td>
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<tr>
<td>phospho PLCγ (Tyr783)</td>
<td>1:1000</td>
<td>Cell Signaling</td>
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<td>phospho-AKT</td>
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<td>phospho-VEGFR2 (Tyr1175)</td>
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<td>Phosphotyrosine (IP: 5ug)</td>
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<tr>
<td>VEGFR3 (IP: 3ug)</td>
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<td>Santa Cruz</td>
<td>sc-321</td>
</tr>
</tbody>
</table>

For the analysis of receptor degradation, HUVECs were serum starved for 4 hours in serum-free Medium 200 and then incubated for 10 minutes with cycloheximide (CHX, 10uM) (AppliChem, A0879,000) at 37 °C. For stimulation, 1 dish was left untreated (control) while the remaining dishes were stimulated with Medium 200 containing VEGF and CHX, in the presence or absence of Chloroquine diphosphate salt (100uM) (Jena Bioscience; CPP-A03) or MG132 (10µM) (Millipore; #474790) to inhibit lysosomal and proteasomal degradation, respectively. Finally, the cells were collected by direct lysis of in 1x SDS sample buffer. The samples were subjected to SDS-PAGE and probed with the indicated antibodies (Table V).
For the biotinylation of surface receptors, HUVECs were serum starved for 4 hours and then stimulated with VEGF as indicated (see stimulation with cytokines). For the assessment of receptor recovery to the cell surface and receptor recycling, cells were additionally washed 3x with warm PBS\textsuperscript{+} (pH 7.4, 1 mM Mg\textsuperscript{2+}, 0.1 mM Ca\textsuperscript{2+}) and incubated for 30 minutes in in serum-free Medium 200 in the presence or absence of Cycloheximide (10\mu M) and MitMAB (20\mu M) as indicated. Next, the cells were put on ice, washed with ice-cold PBS\textsuperscript{+} and then incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-Biotin (Thermo Scientific, 10509863) dissolved in PBS\textsuperscript{+} for 1 hour to biotinylate surface receptors. The reaction was stopped with ice-cold PBS\textsuperscript{+} containing 100 mM Glycine. Finally, the cells were collected in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40 (Sigma, CA-630) and protease inhibitor cocktail (Sigma, P2714, 1:100)). After centrifugation, the supernatant was incubated with Strepavidin Agarose beads (Life Technologies, S951) for 1 hour on a rotator at 4 °C to precipitate biotinylated proteins. The beads were washed with lysis buffer and biotinylated proteins were eluted in 100 \mu l of 1.5 x SDS sample buffer, boiled for 5 minutes and subjected to immunoblotting and probed with the indicated antibodies (Table V).

For immunoprecipitation (IP) experiments, confluent HUVECs or whole lungs of P6 old mice, were lysed in ice-cold lysis buffer. Lungs were homogenized with a pestle and a Dounce All-Glass Tissue Homogenizer (Kontes). After lysis and centrifugation, the supernatant was pre-cleared with uncoupled protein A or G Sepharose beads (GE Healthcare, 17-0780-01 or 17-0618-01) and then incubated with antibody (Table V) for 1-2 hours at 4 °C on a rotator. Immunocomplexes were captured by incubation with protein A or G for 1 hour at 4 °C on a rotator. The beads were washed with lysis buffer and bound immunocomplexes were eluted with in 30-80 \mu l of 1.5 x SDS sample buffer, boiled for 5 minutes and subjected to immunoblotting and probed with the indicated antibodies (Table V). For the IP of Numb (0.5% Chaps), VEGFR3 (1% NP-40) and tyrosine phosphorylated proteins, the lysis buffer (% NP-40, 0.1 % SDS, 0.1 % Deoxycholate) the lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA-NaOH (pH 8.0), 1 mM DTT, 1 mM Na\textsubscript{3}VO\textsubscript{4}, phosphatase inhibitor cocktail (Calbiochem\textsuperscript{®}, 524629, 1:50) and protease inhibitor cocktail (Sigma, P2714, 1:100)) was supplemented with the above indicated detergents.

**Purification of GST-fusion proteins and pulldown experiments**

Glutathione S-transferase (GST) fusion proteins of the phosphotyrosine-binding (PTB, amino acids 1-183) domain or the proline-rich region (PRR, amino acids 184-592) domain of human Numb were produced Rosetta 2 (DE3) (Calbiochem, 71397-3) and purified on glutathione-Sepharose 4B beads (GE Healthcare, 17094401) as described previously.\textsuperscript{10} GST pull-down assays were performed as previously described.\textsuperscript{11} In brief, 1 nmol of GST-fusion protein was immobilized on Glutathione Sepharose 4B (GE Healthcare, 17094401) beads. Confluent HUVECs or whole lungs of P6 old mice were lysed in ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA-NaOH (pH 8.0), 1 mM DTT, 1% NP-40, 0.1 % SDS, 0.1 % Deoxycholate, 1 mM Na\textsubscript{3}VO\textsubscript{4}, phosphatase inhibitor cocktail (Calbiochem\textsuperscript{®}, 524629, 1:50) and protease inhibitor cocktail (Sigma, P2714, 1:100)). Lungs were homogenized with a pestle and a Dounce All-Glass Tissue Homogenizer (Kontes). After lysis and centrifugation, the supernatant was pre-cleared with uncoupled protein glutathione-Sepharose 4B beads and then incubated with beads coated with GST-fusion proteins for 1 hour at 4 °C on a rotator. The beads were washed with lysis buffer and bound immunocomplexes
were eluted with in 30 – 80 µL of 1.5 x SDS sample buffer, boiled for 5 minutes and subjected to immunoblotting and probed with the indicated antibodies (Table V).

Inhibition of protein synthesis in vivo
P6 wild-type mice were injected intraperitoneally (IP) with Cycloheximide (CHX, 50ug per gram body weight in 6% ETOH and 0.14 M NaCl in PBS) (AppliChem, A0879,000). After 2 hours, mice were sacrificed and lungs were collected and washed in ice-cold PBS. Next, lungs were lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1 % SDS, 0.1 % Deoxycholate and protease inhibitor cocktail (Sigma, P2714, 1:100)) and homogenized by 30 strokes with a pestle and a Dounce All-Glass Tissue Homogenizer (Kontes®). The lysates were incubated for 20 minutes on a rotator (Stuart) at 4 °C with subsequent centrifugation for 20 minutes at 14,680 rpm at 4 °C. The supernatant was re-constituted with 3x SDS sample buffer (187.5 mM Tris-HCl (pH 6.8), 9% SDS, 15% glycerol, 6% 2-Mercaptoethanol, 0.006 % Bromophenol blue) in a 2:1 ratio and boiled for 5 minutes. Samples were subjected to immunoblotting and probed with the indicated antibodies (Table V).

RNA isolation and quantitative real-time PCR analysis
Mouse RNA was isolated from the left lung lobe of P6 old mice. Before RNA isolation, the lungs were snap frozen in liquid nitrogen and stored at -80 °C until further processing. RNA isolation was performed using the RNeasy Mini kit (Qiagen, 74106) with the additional help of a rotator stator homogenizer (IKA® T25 digital ULTRA-TURRAX) to disrupt and homogenize the tissue. Total RNA was quantified using a NanoDrop 8000 Spectrophotometer (Thermo Scientific). Prior to quantitative Real-Time (qRT) PCR analysis cDNA was synthesized from 500 ng total RNA by using the iScript cDNA Synthesis Kit (Bio-Rad, 170-8891) following the manufacturer’s guidelines. Next, TaqMan Gene Expression Assay and cDNA were combined with TaqMan Gene Expression Master Mix (Applied Biosystems, 4369016) and analyzed with a 7900HT Fast Real-time PCR System (Applied Biosystems). The murine TaqMan Gene Expression Assay used are listed (Table VI).

Table VI: TaqMan® Gene Expression Assays

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<th>Gene</th>
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Statistical analysis, quantitation and image processing
Results are presented as mean± s.e.m. of at least three independent experiments unless indicated otherwise. Significant differences in pairwise comparisons were evaluated by Student’s t-test. For multiple comparison testing One-way ANOVA followed by Dunnett’s (comparison of every mean to a control mean) or Tukey’s (comparison of every mean with every other mean) multiple comparisons test was performed using GraphPad Prism version 6.0b for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com. P<0.05 was considered statistically significant. Volocity (Improvision, Perkin Elmer), Photoshop CS and Illustrator CS (Adobe) were used for image processing.

References