Mechanisms and regulation of endothelial VEGF receptor signalling

Michael Simons[1], Emma Gordon[2] and Lena Claesson-Welsh[2]

Abstract | Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are uniquely required to balance the formation of new blood vessels with the maintenance and remodelling of existing ones, during development and in adult tissues. Recent advances have greatly expanded our understanding of the tight and multi-level regulation of VEGFR2 signalling, which is the primary focus of this Review. Important insights have been gained into the regulatory roles of VEGFR-interacting proteins (such as neuropilins, proteoglycans, integrins and protein tyrosine phosphatases); the dynamics of VEGFR2 endocytosis, trafficking and signalling; and the crosstalk between VEGF-induced signalling and other endothelial signalling cascades. A clear understanding of this multifaceted signalling web is key to successful therapeutic suppression or stimulation of vascular growth.

Receptor tyrosine kinases
Cell surface receptors that, on binding ligands, dimerize and undergo a conformation change that activates intracellular tyrosine kinase, leading to tyrosine phosphorylation of the receptor's intracellular domain as well as signal transducers.

Vascular endothelial growth factors (VEGFs) include vertebrate VEGFs A–D, placenta growth factor (PlGF), parapoxvirus VEGFE and snake venom VEGFF[1]. Vertebrate VEGFs are broadly expressed, structurally related dimeric molecules that have crucial roles in the formation, function and maintenance of the vasculature. VEGFs also have important roles in other organ systems, including the central nervous system (CNS), kidney, lung and liver, where they directly influence organ function and development[1,2].

VEGFs bind with high affinity to the receptor tyrosine kinases (RTKs) VEGFR1–R3, and VEGFR2 is the main signalling VEGFR in blood vascular endothelial cells. VEGFR1–R3 show a similar overall structural organization, including the common features of seven extracellular immunoglobulin homology domain repeats, a transmembrane domain and a split tyrosine kinase domain. Still, the receptors display differences in their mode of activation, signalling and biological effects (BOX 1; TABLE 1).

VEGFs also bind with high affinity to the neuropilin (NRP) family members NRP1 and NRP2 and to heparan sulfate proteoglycans (HSPGs); these are denoted VEGF co-receptors. The ability of VEGFs to simultaneously bind to various types of transmembrane proteins initiates formation of multiprotein complexes that include, in addition to receptors and co-receptors, several non-VEGF-binding auxiliary proteins, such as integrins and ephrin B2 (REF. 3).

According to the consensus model for ligand-induced activation of RTKs[4], VEGF binds to a cognate VEGFR to induce receptor homodimerization or heterodimerization, leading to activation of the tyrosine kinase and autophosphorylation of tyrosine residues in the receptor intracellular domains. Phosphotyrosines and surrounding amino acid residues constitute binding sites for adapter molecules, which initiate various intracellular signalling pathways. These pathways mediate immediate responses, such as vascular permeability, and longer-term responses that require gene regulation, such as endothelial cell survival, migration and proliferation. Non-canonical VEGFR signalling is initiated by non-VEGF-dependent activation of VEGFRs. Examples include VEGFR2 phosphorylation by the shear-stress-activated cytoplasmic SRC tyrosine kinases or binding of non-VEGF ligands[3,5,6].

VEGFR signalling is tightly regulated at numerous different levels, including receptor expression levels, the availability and affinities for binding of its different ligands, the presence of VEGF-binding co-receptors, non-VEGF-binding auxiliary proteins and inactivating tyrosine phosphatases, the rate of receptor cellular uptake, extent of degradation and speed of recycling. VEGFR endocytosis and trafficking regulate the specificity as well as the duration and amplitude of the signalling output. Once they are in the cytoplasm, VEGFRs are either shuttled to lysosomes for degradation or recycled back to the membrane via fast or slow recycling pathways. In the case of VEGFR2, the activation of ERK1/2 signalling, which is essential to VEGFR2 biology, is dependent on the speed of the receptor's intracellular trafficking[7,8].

In addition, VEGFR signalling output is directly or indirectly regulated by crosstalk between VEGFRs as well as between VEGFR-dependent and VEGF-independent intracellular signalling pathways, thus

[1]Yale Cardiovascular Research Center, 300 George St, New Haven, Connecticut 06511, USA.
[2]Uppsala University, Department of Immunology, Genetics and Pathology, Rudbeck Laboratory and Science for Life Laboratory, Dag Hammarskjöldsv 20, 751 85 Uppsala, Sweden.
Correspondence to M.S. and L.C.-W.
michael.simons@yale.edu; lena.welsh@igp.uu.se

doi:10.1038/nrm.2016.87
Published online 27 July 2016

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

Neuropilins
(NRPs). Transmembrane glycoproteins that can bind to vascular endothelial growth factors (VEGFs) and semaphorins and act either as receptors or co-receptors (together with VEGF receptors and plexins) to modulate intracellular signalling.

Heparan sulfate proteoglycans
(HSPGs). Proteoglycans carrying heparan sulfate chains that can bind to various heparin-binding growth factors found on the plasma membrane or the extracellular matrix. They can function as vascular endothelial growth factor co-receptors or signal independently.

creating complex positive and negative feedback loops. Together, these regulatory mechanisms control the activation of specific pathways and the strength and duration of the signal.

Here, we review recent studies that shed new light on these mechanisms and their biological relevance, presenting our current understanding of the function and regulation of this important family of receptors. The primary focus of this Review is VEGFR2, which is the principal endothelial VEGF signalling receptor, although other VEGFRs are mentioned when appropriate.

VEGFR2 activation mechanisms

Several mechanisms function together to regulate the activation of VEGFR2, involving both canonical mediators (that is, the classical VEGF ligands) and non-canonical mediators (non-VEGF ligands or other types of stimuli).

Canonical VEGFR2 activation. The classical ligands for VEGFR2 include the alternatively spliced variants of the prototype VEGF, which is now denoted VEGFA, as well as the processed forms of the related VEGFC and VEGFD ligands. The mode of VEGFR2 activation is influenced by the different variants of VEGFA that differ in their ability to interact with NRPs and with HSPGs, in which the latter may be present on the cell surface or in the extracellular matrix (ECM). For example, VEGFA121 fails to bind to the ECM, whereas VEGFA165 contains basic amino acid motifs in exon 8 that confer ECM-binding ability; binding to the ECM is even stronger for VEGFA189 and VEGFA205, which contain additional ECM-binding sequences[9]. Moreover, VEGFA121, unlike other VEGFA isoforms (see below), does not bind to NRP1. This greatly increases the spatial range of action for VEGF121, as it is not tethered to the ECM nor to NRP1. The biological effects of VEGFA[10], VEGFC and VEGFD[11] are further modulated by proteolytic processing. The complex interactions of VEGF ligands with accessory molecules and co-receptors (FIG. 1) are thought to influence VEGF biology by stabilizing ligand–receptor complexes, leading to prolonged signalling duration. This may be true in particular for HSPGs[12]. However, the roles of these accessory molecules are often more intricate, as described below.

Box 1 | **Key features and functions of VEGFR1 and VEGFR3**

VEGFR1 as a negative regulator in endothelial biology

Vascular endothelial growth factor receptor 1 (VEGFR1; also denoted Fms-like tyrosine kinase (FLT1)) is widely expressed in various cell types, including monocytes and macrophages, vascular smooth muscle cells and neuronal cells[175]. It binds to VEGFA, VEGFB and placenta growth factor (PlGF) and exists as a homodimer or a heterodimer with VEGFR2, depending on the ligand[17]. A synthetic heterodimer-specific ligand fails to induce endothelial cell proliferation but stimulates cell migration and nitric oxide production[176]. Moreover, VEGFR1–VEGFR2 heterodimers block VEGFA-induced ERK1/2 activation and Ca^{2+} entry, suggesting that heterodimers act as a negative regulator of VEGFR2 homodimer activity[176].

Although VEGFR1 binds to VEGFA with a high affinity (10 pM), the induction of VEGFR1 phosphorylation is weak. VEGFR1 tyrosine phosphorylation sites have been determined[177] (TABLE 1), but downstream signalling remains largely unexplored. However, both PlGF and VEGFB promote signalling in sensory nerves through VEGFR1 (REF. 175), indicating that VEGFR1 can transduce signals in certain cell types, if not in endothelial cells. *Vegfr1*$^{-/-}$ mouse embryos die at embryonic day 8.5–9 from excessive endothelial cell proliferation and disorganized vasculature[117], owing to increased availability of VEGFA to bind to VEGFR2 and increased VEGFR2 signalling. Expression of a splice variant encoding a soluble VEGFR1 (sVEGFR1) reduces VEGFR2 signalling by acting as a VEGF trap[3]. Thus, both full-length and sVEGFR1 are largely viewed as decoys that control the amount of VEGFA available to bind to and activate VEGFR2. The regulation of endothelial cell surface VEGFR1 expression and sVEGFR1 secretion are of paramount importance. A clinically relevant example is the association of peripartum cardiomyopathy and pre-eclampsia with increased expression and secretion of sVEGFR1 (REFS 178,179).

Unlike VEGFA, VEGFR1 ligands PlGF and VEGFB are dispensable for vascular development. PlGF is crucial for inflammation-associated angiogenesis[180], whereas VEGFB is implicated in fatty acid uptake in endothelial cells, particularly in the heart[181,182]. The mechanism underlying the different biological repertoires of the VEGFR1 ligands remains to be clarified.

VEGFR1 has also been reported to bind to native low-density lipoprotein (LDL), which induces VEGFR1 autophosphorylation, clathrin-independent endocytosis and degradation[183]. The apolipoprotein B particle downregulates VEGFR1 expression[184], leading to reduced endothelial VEGFR1 expression in atherosclerotic lesions, which may promote plaque growth by increased VEGFA–VEGFR2 signalling.

VEGFR3 in blood and lymphatic vessel biology

VEGFR3 is expressed in several endothelial cell types, including capillary, venous and lymphatic endothelium and also in neuronal progenitors[185], macrophages and osteoblasts[186]. VEGFR3 is expressed by both blood and lymphatic endothelial cells during early development[187] and it is re-introduced in blood endothelial cells during angiogenesis in certain settings[2], such as during angiogenic sprouting in the retina[188].

VEGFR3 binds to unprocessed VEGFC and VEGFD, whereas proteolytically processed ligands bind to both VEGFR2 and VEGFR3 with high affinity, promoting VEGFR2–VEGFR3 heterodimerization[19,21]. Heterodimerized VEGFR2 fails to phosphorylate VEGFR3 on carboxy-terminal tyrosines, which has potential consequence for the binding of the adapter molecule SHC and the activation of the RAF–ERK1/2 pathway. In agreement with this, VEGFC-induced VEGFR2–VEGFR3 heterodimers activate AKT signalling, whereas VEGFR3 homodimers induce ERK1/2 activation[18]. VEGFR2–VEGFR3 heterodimers have been identified in intact tissues[20], but their *in vivo* role remains to be clarified.

Prospero homeobox protein 1 (PROX1)-positive lymphatic progenitors in the cardinal vein migrate and differentiate during embryogenesis to form lymphatic vessels in a VEGFC/VEGFR3-dependent process[189], and VEGFC-mediated AKT activation is required for embryonic and adult lymphangiogenesis[190]. VEGFC-induced AKT activation involves VEGFR3–VEGFR2–NRP1 (neuropilin 1) complex formation, whereas ERK1/2 activation is primarily driven by VEGFR3 homodimers without contribution from NRP1 or NRP2 (REF. 18). Downstream effectors that regulate lymphatic endothelial cell migration have been studied in zebrafish and include the SoxF transcription factors and the transcriptional modulator MafBa[191].

The involvement of protein tyrosine phosphatases (PTPs) in VEGFR3 signalling largely remains to be defined. A PTP non-receptor type 14 (PTPN14) has been implicated in lymphatic development, but its role in VEGFR3 signalling is not established[192]. Moreover, vascular endothelial PTP (VEPTP) has been shown to act as a VEGFR3 tyrosine phosphatase in lymphatic endothelium and to modulate VEGFC-induced ERK1/2 and AKT activation[18].

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

Table 1 | **VEGFR features in the endothelium**

	VEGFR1	VEGFR2	VEGFR3
Canonical ligands	VEGFA, VEGFB and PlGF	VEGFA, processed VEGFC and VEGFD	VEGFC and VEGFD
Non-canonical activation	Unknown	Shear stress, gremlins, galectins, lactate and LDLs	Shear stress
Dimerization forms	R1 homodimers and R1–R2 heterodimers	R2 homodimers, R1–R2 and R2–R3 heterodimers	R3 homodimers and R2–R3 heterodimers
Soluble forms	sVEGFR1 splice variant	sVEGFR2 processed and splice variants	Unknown
Expression pattern in the vasculature	Blood vascular endothelial cells*	Blood vascular and lymphatic endothelial cells	Blood vascular and lymphatic endothelial cells
VEGF-binding co-receptors	NRPs and HSPGs	NRPs and HSPGs	NRPs and HSPGs
Molecular partners	Unknown	Integrins, VE-cadherin, PTPs, TIE2 and ephrinB2	Integrins, VE-cadherin and PTPs
(Auto)phosphorylation sites (mouse sequence number)	JM: None Kinase insert: No Tyr (Y) in the sequence Kinase domain: Y914‡ C-terminal tail: Y1213,Y1242, Y1327 and Y1333	JM: Y799‡ Kinase insert: Y949 Kinase domain: Y1052 and Y1057 C-terminal tail: Y1173 and Y1212	JM: None Kinase insert: No Y in the sequence Kinase domain: Y1063 and Y1068‡ C-terminal tail: Y1230, Y1231, Y1265, Y1337 and Y1363
Key biology in endothelial cells	Negative regulation of VEGF signalling	Transduces all known effects of VEGFA	Transduces all known effect of VEGFC and VEGFD
Constitutive knockout phenotype	Embryonically lethal at embryonic day 9 from uncontrolled endothelial cell overgrowth	Embryonically lethal at embryonic day 8.5 owing to deficient endothelial cell lineage commitment	Embryonically lethal at embryonic day 9.5 due to vascular remodelling defects and pericardial fluid accumulation
Established therapeutic target	Under preclinical and early phase clinical development for inflammation-related diseases, such as cancer, obesity and type 2 diabetes	Neutralizing VEGFA antibodies in retinopathy and VEGFR2 kinase inhibitors in cancer	Under preclinical and early phase clinical development for lymphangiogenesis-related disease

*For complete information on VEGFR expression patterns, please refer to UniProt and other databases. ‡Certain phosphorylation sites have been suggested, for example, from the phenotype of mutants, but are not directly demonstrated in the full-length receptor. C-terminal, carboxy-terminal; HSPG, heparan sulfate proteoglycans; JM, juxtamembrane; LDL, low-density lipoprotein; NRP, neuropilin; PlGF, platelet growth factor; PTP, protein tyrosine phosphatase; sVEGFR, soluble vascular endothelial growth factor receptor; VE-cadherin, vascular endothelial cadherin.

Integrins
Transmembrane receptors that link the extracellular matrix to the cell and transmit signals to communicate the characteristics of the surrounding environment across the membrane.

Extracellular matrix
(ECM). The non-cellular component of tissues and organs comprising proteins and carbohydrates that provide structural and biochemical support for cellular structures.

Ligand binding is thought to induce VEGFR dimerization. However, pre-formed VEGFR2 dimers with a certain level of kinase activity have been shown to exist *in vitro*[13]. The dimer is stabilized upon ligand binding through several points of contact between the different receptor domains. Moreover, ligand binding induces a switch to a particular configuration of the transmembrane domains, which is accompanied by rotation of the dimers[4], of critical importance for full activation of kinase activity. Different ligands can influence the degree of rotation of the receptor molecules differently, and thereby the extent of receptor activation. For example, VEGFB has been shown to lack the ability to optimally rotate its receptor, VEGFR1, compared with PlGF[14] and so is a weaker activator of VEGFR1 signalling. In addition to classical VEGF ligands, the alternatively spliced VEGFxxxb forms contain a unique exon 8b conferring anti-angiogenic effects[15]. However, the VEGFxxxb variants are also weak VEGFR2 agonists[16], and it is at this point unclear how they exert their anti-angiogenic effects.

Signalling studies and computational modelling[17] suggest the existence of VEGFR1–VEGFR2 (REF. 18) and VEGFR2–VEGFR3 heterodimers[19,20]. VEGFR2–VEGFR3 heterodimers are induced in response to VEGFC and VEGFD on lymphatic endothelial cells both *in vitro* and *in vivo* and may promote a different pattern of signalling from VEGFR3 homodimers[19] (BOX 1). Furthermore, VEGFA, in particular, the VEGFA189 isoform, can induce VEGFR2–VEGFR3 heterodimers[21], indicating that receptor heterodimerization may not require the ligand to simultaneously bind to both receptors in the dimer.

Much less is known about VEGFR1–VEGFR2 heterodimers. Several factors are thought to control their formation, including relative distribution of the receptors on the apical versus basolateral plasma membrane and their levels of expression. There is a tenfold excess of VEGFR2 over VEGFR1 molecules on the surface of cultured endothelial cells[22], but the binding affinity of VEGFA to VEGFR1 is higher (10 pM) than to VEGFR2 (100 pM)[23]. Thus, it is hard to predict a priori whether most VEGFR1 proteins form R1–R1 homodimers or R1–R2 heterodimers. VEGFR1–VEGFR2 crosstalk can also occur through pre-sensitization of endothelial cells to VEGFA by transphosphorylation of VEGFR2 by the VEGFR1 kinase[24]. Whether this transphosphorylation requires heterodimerzation of receptors or involves other mechanisms is not clear.

Activation of the receptors leads to *trans*-phosphorylation of tyrosine residues on the receptor intracellular domain (that is, phosphorylation of one receptor molecule by the other molecule in the dimer).

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

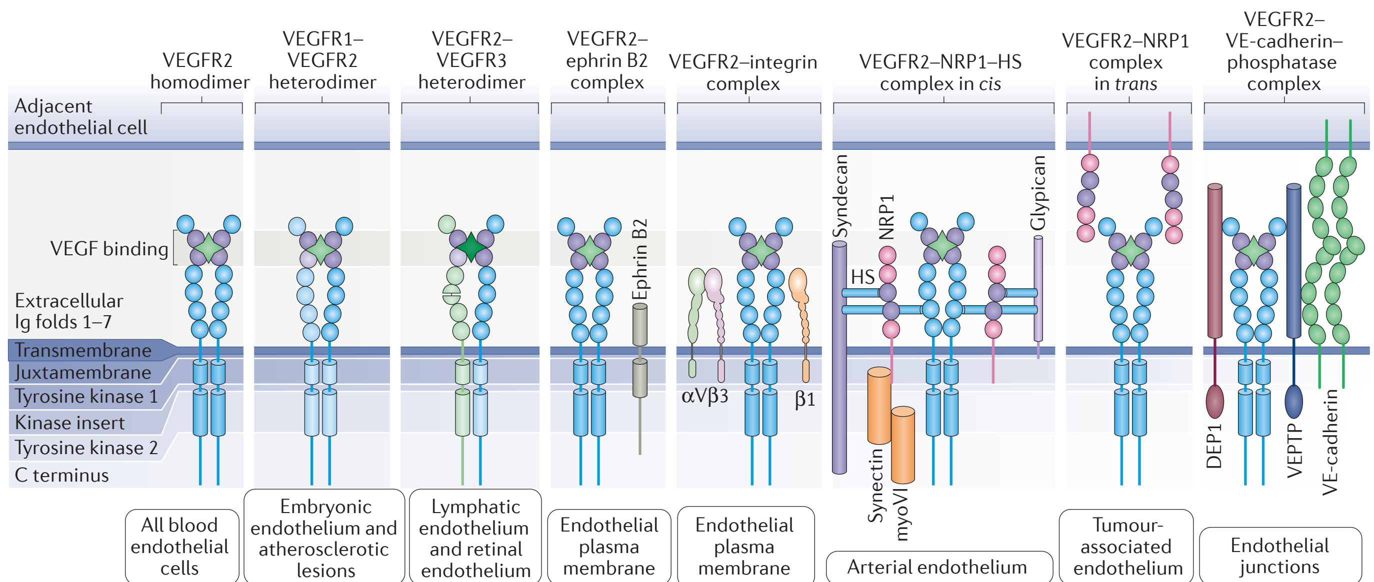

Figure 1 | **VEGFR2 structure and receptor signalling complexes.** Vascular endothelial growth factor (VEGF) ligands (VEGFA: light green; VEGFC or VEGFD: dark green) bind to the second and third extracellular immunoglobulin (Ig) loops (purple) of VEGFR2, inducing receptor dimerization. Note that the fifth Ig loop in VEGFR3 is not disulfide-bonded. Differential phosphorylation of tyrosine residues in the intracellular domain of VEGF receptor 2 (VEGFR2; consisting of a juxtamembrane domain, a split tyrosine kinase domain and carboxy-terminal tail) results in differential biological outcomes. VEGFR2 can form a homodimer or heterodimers with VEGFR1 or VEGFR3, which bind to VEGF ligands through their second Ig loop (purple) with contribution from the third (Ig) loop (light purple). VEGFR2 homodimer signalling is modulated by different VEGF-binding co-receptors, such as heparan sulfate (HS) proteoglycans (syndecan or glypican) and neuropilins (NRPs), as well as non-VEGF-binding auxiliary proteins, such as vascular endothelial cadherin (VE-cadherin), integrins, ephrin B2 and protein tyrosine phosphate (PTP), which form multiprotein complexes with unknown constellations (BOX 2). VEGFR2 may also form *trans*-complexes with NRP1 expressed on adjacent cells. Different receptor configurations influence VEGFR2 signalling output. The biological context in which each receptor complex is known to function is indicated. Note that, although it is well known that NRP1 binds to VEGF, it is shown as a monomer interacting indirectly with VEGF and VEGFR2. DEP1, density-enhanced phosphatase 1; myoVI, myosin VI; VEPTP, vascular endothelial protein tyrosine phosphatase.

Cytoplasmic tyrosine kinases, such as SRC, can also phosphorylate VEGFR2 (REF. 25) (TABLE 1 lists known VEGFR tyrosine phosphorylation sites). *In vitro*, VEGFR2 is also phosphorylated on serine and threonine residues in response to VEGFA, which is of importance for degradation of the receptor[26].

Modulation of canonical VEGFR activation by co-receptors. VEGF co-receptors can qualitatively and quantitatively modulate VEGFR signalling. In particular, the roles of the NRPs have received considerable attention. Both NRP1 and NRP2 bind to VEGFs as well to the axonal guidance molecules denoted class 3 semaphorins, which also bind to plexin receptors[27,28]. Specific motifs in VEGFA exon 7 and exon 8 bind to NRP1 and NRP2, and a 50-fold stronger affinity is shown for NRP1 compared with NRP2 (REF. 29). The exon 8-encoded C-terminal arginine is essential for the interaction of VEGFA with the L1 loop of NRP1, and additional contributions from exon 7-encoded electronegative residues contribute to the high affinity of this binding. However, in the case of NRP2, direct repulsion between its electronegative L1 loop (absent in NRP1) and the electronegative exon 7-encoded residues of the VEGFA heparin-binding domain lead to a lower affinity for VEGFA.

Semaphorins
Secreted or membrane-bound guidance proteins that control cell movement through multimeric cognate receptor complexes.

VEGFA simultaneously binds to NRPs and VEGFRs on endothelial cells, inducing the formation of NRP–VEGFR complexes[30,31]. However, it should be noted that the configuration of the NRP1–VEGFA–VEGFR2 complexes is not known — that is, which interactions are directly or indirectly mediated by, for example, HSPGs — and it is also unclear whether higher-order complexes are formed and how they are regulated (BOX 2). NRP1 has a key role in VEGFR2 intracellular trafficking by binding of its C-terminal PDZ-binding domain to the PDZ-domain-containing protein synectin (also known as GIPC1). NRP1-guided endosomal translocation of VEGFR2 is crucial for VEGFA-induced ERK1/2 activation[32,33]. Other VEGFA downstream pathways, such as the p38 MAPK, also require NRP1, possibly through endosomal signalling[34]. By contrast, when binding in *trans*, between adjacent cells, inclusion of NRP1 in the VEGFA–VEGFR2 complex retains the receptor on the cell surface and suppresses angiogenesis[35].

In addition to its role in regulating VEGFR2 trafficking, NRP1 affects VEGF signalling in several other ways. As it is capable of binding to VEGF without involvement of VEGFR2, NRP1 can sequester the ligand. In retinal neurons, VEGFA binds to NRP1 and VEGFR2 (REF. 36), but in hindbrain neurons, which do not express

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

Focal adhesions
Contact points between the cell and the extracellular matrix comprising integrins and actin filament bundles.

Tetraspanin
Transmembrane-4 glycoproteins that interact both with themselves and with other transmembrane receptors to regulate various cellular processes, such as fusion, receptor trafficking and motility.

VE-cadherin
Adherens junction type II cadherin expressed on endothelial cells and localized at cell–cell junctions. Involved in the regulation of vascular integrity and permeability.

VEGFRs, NRP1 sequesters VEGFA[30,37]. NRP1 may also signal independently of VEGF; VEGFA has been reported to induce ERK1 and AKT activation and to regulate neuronal survival independently of VEGFRs[38]. Whether VEGFR-independent VEGF–NRP1 signal transduction occurs in the endothelium has not been established.

Mice with homozygous disruption of *Nrp1* die *in utero* with extensive cardiovascular and CNS defects[39,40]. Surprisingly, mutations of the sequence in *Nrp1* that encodes the VEGFA-binding site (Tyr297 (Y297) or Ser320) is compatible with normal vascular development[30,41]. Mice carrying a knock-in of an *Nrp1* mutant that is devoid of its cytoplasmic domain also display normal vasculogenesis and angiogenesis[42]. However, these mice have a profound postnatal arteriogenic defect owing to decreased VEGFA-induced ERK1/2 signalling[32]. These findings may be explained, in part, by recent reports demonstrating that NRP1 functions in conjunction with the transforming growth factor-β (TGFβ) family of receptors, independently of VEGFR2, to control vascular sprouting[43,44].

Another very important VEGFR2 partner is the integrins, particularly integrin-β1 and integrin-β3. Note that sequence motifs for VEGF–integrin binding remain to be identified. VEGFA induces VEGFR2–integrin-β3 association, which is required for full VEGFR2 activation[45]. VEGFR2 activation in turn induces integrin-β3 tyrosine phosphorylation and directs interactions between the two[46]. The VEGFR2–integrin-β3 complex may contain additional partners, such as syndecan 1 and SRC, promoting both VEGFR2 and integrin-αvβ3 activation[47]. ECM-binding VEGFA isoforms also promote VEGFR2–integrin-β1 complex formation, thus shifting cell surface localization of VEGFR2 to focal adhesions, which is accompanied by prolonged receptor activation[9]. Tetraspanin CD63 is another member of the VEGFR2–integrin-β1 complex; CD63 binds to both VEGFR2 and integrin-β1 and loss of CD63 expression impairs VEGFR2 signalling[48].

Box 2 | **Outstanding questions in VEGFR signalling and biology**

- Protein tyrosine phosphatase (PTP) regulation of vascular endothelial growth factor receptor (VEGFR) downstream signalling pathways:
  - Is there PTP specificity for certain VEGFR phosphotyrosine sites and pathways?
  - Is there a temporal regulation of the VEGFR2–PTP interaction: would a transient VEGFR2–PTP contact (for example, PTP1B) favour a specific VEGFR2 phosphosite deactivation site, whereas a prolonged contact (for example, vascular endothelial PTP (VEPTP)) would lead to pan-phosphotyrosine dephosphorylation?
  - Is PTP activity regulated by VEGFR signalling, or is it merely a matter of proximity and level of expression?
- Uniqueness of VEGFR signalling pathways:
  - Why does VEGFR-induced signalling (for example, via ERK activation) result in a different biological effect than when the same pathway is induced by another growth factor (for example, fibroblast growth factor (FGF))?
  - Does it depend on the amplitude and/or longevity of the signal, or crosstalk with other pathways?
- Differences in homodimeric versus heterodimeric VEGFR signalling:
  - Do VEGFR1–VEGFR2 heterodimers exist *in vivo*? What is their biological activity?
  - When are VEGFR2–VEGFR3 heterodimers formed? What is the consequence?
- The role of spatial organization of VEGFR2 signalling complexes:
  - Does it localize signalling to specific subcellular compartments?
  - Does it influence signalling amplitude and time course by either preventing or directing VEGFR2 intracellular trafficking?
  - How are the various components in the signalling complex packaged together? Is the complex configuration stable or dynamic? If dynamic, how is this regulated?
- Plasma membrane versus cytosol signalling:
  - What determines activation of a specific signalling pathway on the plasma membrane versus the cytosol?
  - What is the implication of this specificity?
- Details of VEGFR2 signalling pathways:
  - How is receptor proximal activation of the p38 MAPK, SHB, STAT3 and small GTPases signalling regulated?
  - To what extent are VEGFR2 pathways modulated by flow? Does this regulation result in different outcomes than what is observed after static ligand-induced activation?

Non-canonical VEGFR2 activation. Non-VEGF ligands, as well as mechanical forces, such as shear stress, can induce tyrosine phosphorylation and activation of VEGFR2 (that is, non-canonical VEGFR2 activation)[25,49]. Mechanical stimuli activate VEGFR2 signalling at least in part through the formation of a mechanosensory complex that includes, in addition to VEGFR2, platelet endothelial cell adhesion molecule 1 (PECAM1, also known as CD31) and vascular endothelial cadherin (VE-cadherin)[5,49]. The purinergic receptor P2Y2 and G proteins Gq and G11 (G_q/G_{11}) have also been implicated in fluid shear-stress-induced endothelial responses by activation of SRC and AKT, leading to endothelial nitric oxide synthase (eNOS) activation and phosphorylation of PECAM1 and VEGFR2 (REF. 50). A common denominator in these responses is the cytoplasmic tyrosine kinase SRC, which phosphorylates VEGFR2, thus initiating downstream signalling[25].

More-recent studies have shown that VEGFR3 may also be a part of the mechanosensory complex[51,52] and that the transmembrane domain of VE-cadherin binds directly to the transmembrane domain of VEGFR2 and VEGFR3 (REF. 53). Shear stress induces PECAM1 phosphorylation, perhaps via the SRC-related cytoplasmic tyrosine kinase FYN[54], followed by VEGFR2 phosphorylation and PI3K activation, leading to a Krüppel-like factor 2-dependent increase in VEGFA[55] and arterial marker expression[56]. In addition, there is evidence for shear-stress-induced matrix metalloproteinase-dependent release of VEGFA from the ECM, promoting VEGFR2 activation[57].

Several non-VEGF ligands can activate VEGFR2, although the underlying mechanisms and functions in endothelial biology remain poorly understood. Among these is the bone morphogenetic protein (BMP) antagonist gremlin (GREM1), which can induce endothelial cell sprouting, migration and invasion[58,59] by directly binding to VEGFR2 with an affinity comparable to that of VEGFA, resulting in receptor activation[59]. GREM1 also promotes the formation of VEGFR2–integrin-αvβ3 complexes that have a role in endothelial cell polarization and basolateral localization of VEGFR2 (REF. 60).

Galectins, which are β-galactoside-binding proteins, can also induce VEGFR2 activation[61]. The main galectins involved in VEGF signalling are VEGFR2-binding

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

galectin 3 and NRP1-binding galectin 1. Galectin 3 is ubiquitously expressed and crosslinks various glycoproteins to form a cell surface molecular lattice. The glycosyltransferase β1,6-*N*-acetylglucosaminyl transferase 5 (MGAT5) promotes interactions between galectin 3 and VEGFR2 on the plasma membrane that leads to VEGFR2 phosphorylation. Reduced corneal neovascularization in both *Lgals3*-knockout (the gene encoding galectin 3) and *Mgat5*-knockout mice indicates that this pathway is physiologically relevant[62].

Lactate and low-density lipoproteins (LDLs) are other known non-canonical inducers of VEGFR2 activation. Endothelial cells exposed to lactate *in vitro* upregulate their expression of VEGF and VEGFR2 (REF. 63). Lactate also activates the PI3K–AKT pathway by upregulating ligands for VEGFR2 and the RTKs AXL (ligand denoted GAS6) and TIE2 (ligand denoted ANG1), but much remains to be learned about these events[64]. LDL-treated endothelial cells become less responsive to VEGFA and show reduced VEGFR2 expression that is secondary to increased endocytosis and degradation[65].

The biology of VEGFR2 proximal signalling

The canonical and non-canonical activation of VEGFR2 turns on intracellular signalling pathways that are crucial to endothelial biology. These include the phospholipase Cγ (PLCγ)–ERK1/2 pathway, which has a central role during vascular development and in adult arteriogenesis[3,66]; the PI3K–AKT–mTOR pathway, which is crucial for cell survival, regulation of vasomotion and regulation of barrier function[67]; and SRC and small GTPases, which are involved in cell shape, cell migration and polarization, as well as regulation of endothelial junctions and the vascular barrier function[68]. In addition to these extensively studied signalling events, VEGFR2 activates other pathways that are poorly understood. These include stress kinases, such as p38 MAPK, STATs and G protein-coupled receptor (GPCR)-dependent signalling.

The PLCγ–ERK1/2 pathway and Ca^{2+} signalling. VEGF-induced ERK1/2 signalling regulates endothelial cell proliferation, migration, arterial fate specification and homeostasis[66,69–71] (FIG. 2). Phosphorylation of VEGFR2 Y1173 in mice (which is Y1175 in the human protein) is crucial for ERK1/2 activation and its mutation to phenylalanine (Y1173F) has the same effect as *Vegfr2* gene inactivation: namely, early embryonic lethality owing to suppressed endothelial progenitor differentiation[72,73]. Phosphorylated Y1173 binds to and activates PLCγ, resulting in the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases Ca^{2+} from the endoplasmic reticulum, supporting DAG-induced activation of Ca^{2+}-dependent protein kinase Cβ2 (PKCβ2)[74,75], which then regulates the RAF1–MEK–ERK1/2 cascade. This pathway bypasses the more common RTK-induced RAS activation of the RAF1–MEK–ERK1/2 cascade[3]. In zebrafish, *plcg* knockdown leads to a complete loss of VEGF-induced ERK1/2 activation[76]. The *in vivo* role of PKCβ is less clear, as mice with global disruption of *Prkcb,* encoding both PKCβ1 and PKCβ2 isoforms, show no gross vascular defects[77]. This suggests either compensation by other PKCs or the presence of non-PKCβ-dependent pathways for VEGF-induced ERK1/2 activation.

The PLCγ–PKC pathway regulates many aspects of endothelial cell biology, including cell fate specification, proliferation and migration, in part by activating transcription factors of the E26 transformation-specific (ETS) family. ETS factors are essential in transcriptional regulation of many genes that are key for endothelial cell function[70,78–81]. The PLCγ–PKC pathway also mediates phosphorylation of histone deacetylase 7 (HDAC7), which activates genes involved in endothelial cell proliferation and migration[82]. The pY1173 site also binds to the adapters SH2 domain-containing adapter protein B (SHB) and SHC-transforming protein 2 (SHC2; also known as SCK)[5]. It remains to be determined how each of the different pY1173-interacting proteins PLCγ, SHB and SCK contributes to signalling downstream of pY1173 in VEGFR2.

Ca^{2+} signalling is vital in VEGF biology, not only for PLCγ-mediated activation of the ERK1/2 pathway but also for activation of the nuclear factor of activated T cell (NFAT) family of transcription factors. Calmodulin, which is a Ca^{2+} sensor, and calcineurin, which is a Ca^{2+}-dependent serine/threonine phosphatase, regulate nuclear translocation and transcriptional activation of NFAT proteins. NFAT reduces the expression levels of VEGFR1, resulting in increased signalling by VEGFR2, owing to greater availability of VEGFA[83] (BOX 1). However, NFAT activity represses VEGFA expression during early myocardial valve formation[84]. Moreover, the NFAT pathway balances VEGF signalling by an endogenous negative regulator of calcineurin activity, Down syndrome candidate region 1 (DSCR1)[84], with DSCR1 gene (*Rcan1*) inactivation, leading to hyperactive NFAT signalling and endothelial apoptosis[85].

The PI3K–AKT pathway and small GTPases. The AKT serine/threonine kinases AKT1–3 have a wide range of substrates and influence many biological processes, including cell survival, proliferation and apoptosis[86,87]. Activation of AKT requires binding of its plextrin homology (PH) domain to the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3) generated by PI3K (FIG. 2). AKT1 is the predominant isoform involved in the regulation of pathological and adult angiogenesis as well as vascular maturation and metabolism[88,89] by the mTOR complex 2 (REF. 67). By contrast, the *Akt2* knockout lacks an overt phenotype[90].

VEGFR2 lacks a binding site for the SH2-domain-containing p85 subunit of PI3K and activates PI3K indirectly, either by SRC and VE-cadherin[91] or by AXL[92]. Embryos with a kinase-dead p110α catalytic subunit of PI3K develop vascular defects owing to reduced activation of small GTPases and suppressed endothelial migration[93].

The small GTPases RHO, CDC42 and RAC1 affect many cellular processes in endothelial cells, including cytoskeletal organization, cell morphology, adhesion, migration and junctional integrity. VEGF-driven

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

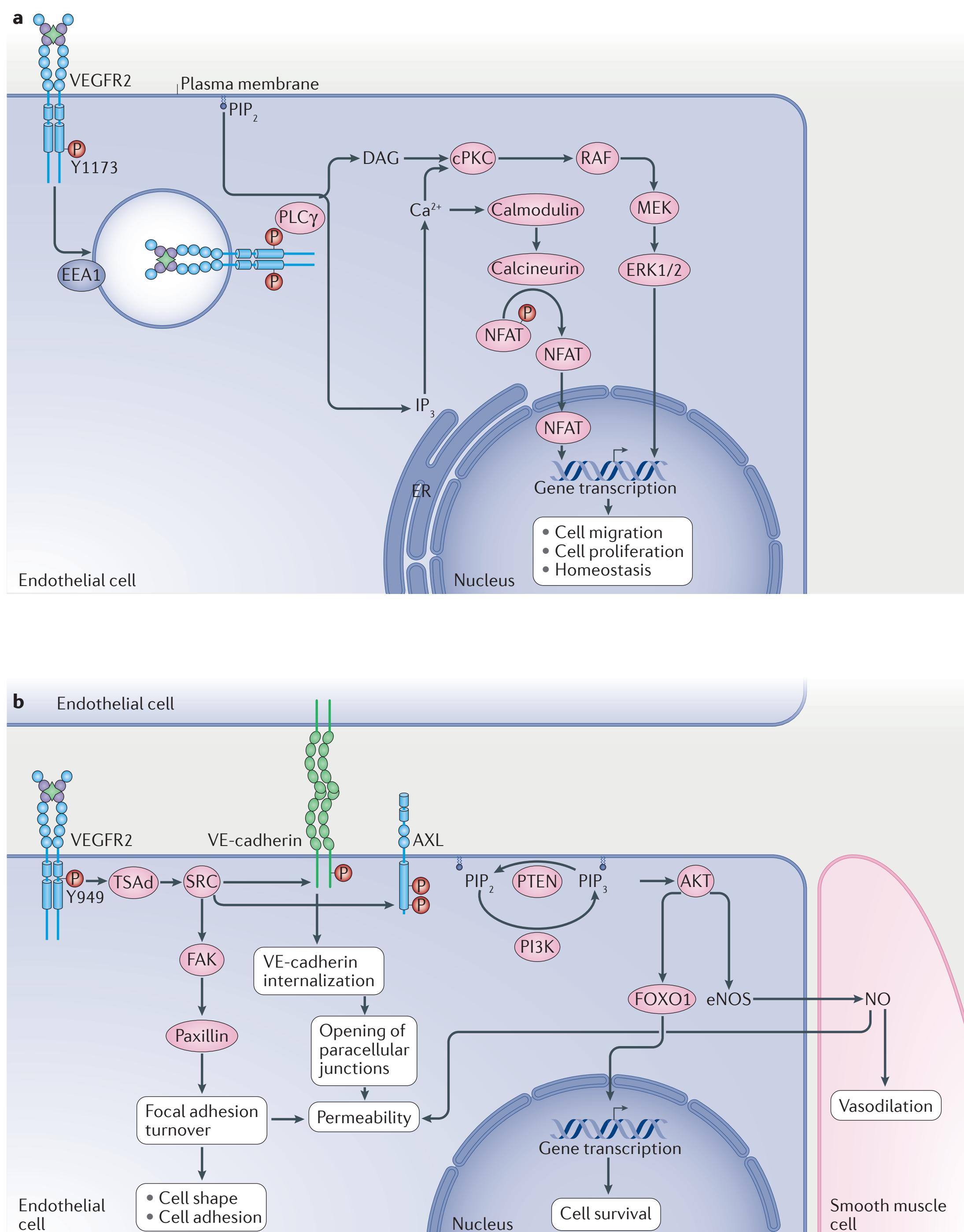

Figure 2 | **VEGFR2 signal transduction pathways. a** | Schematic representation of vascular endothelial growth factor (VEGF)-activated VEGF receptor 2 (VEGFR2)–Tyr1173 (Y1173) signalling. Phosphorylation of Y1173 in VEGFR2 induced by VEGF binding results in internalization of VEGFR2 into early endosome antigen 1 (EEA1)-positive endosomes and subsequent Ca^{2+}-dependent signalling in phospholipase Cγ (PLCγ) and nuclear factor of activated T cells (NFAT) pathways, leading to changes in gene transcription that affect biological processes, such as cell migration, proliferation and homeostasis. **b** | Schematic representation of VEGF-activated VEGFR2–SRC–AKT signalling. Phosphorylation of Y949 in VEGFR2 leads to the activation of SRC at cell–cell junctions and subsequent downstream signalling events that determine cell shape, survival and vessel permeability. In addition to signalling within endothelial cells, AKT-mediated activation of endothelial nitric oxide synthase (eNOS) induces nitric oxide (NO) signalling in adjacent smooth muscle cells, leading to vasodilation (described in detail in the main text). cPKC, conventional protein kinase C; DAG, diacylglycerol; ER, endoplasmic reticulum; FAK, focal adhesion kinase; FOXO1, forkhead box protein O1; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; TSAd, T cell-specific adapter; VE-cadherin, vascular endothelial cadherin.

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

migration requires RAC1 and RHOJ[94,95], whereas three-dimensional organization of endothelial cells *in vivo* depends on activation of RHOA[96]. Vessel lumen formation, which depends on dramatic cytoskeletal reorganization and junctional maturation, involves RAC1 and CDC42 and their exchange factors dedicator of cytokinesis protein 4 (DOCK4) and DOCK9, respectively[97]. Endothelium-specific loss of CDC42 results in disorganized cell–cell junctions, reduced focal adhesion and polarity[98]. Although the details on how VEGFR2 proximal signalling regulates CDC42 remain to be determined, recent data indicate that Slit homologue 1 protein (SLIT1) ligand acting through roundabout homologue 1 (ROBO1) and ROBO2 modulates binding of the adapters NCK1 and NCK2 to VEGFR2, thereby controlling downstream CDC42 activity[99]. Moreover, it is well established that shear stress induces the activation of RAC[100], yet the VEGFR2 proximal signalling that regulates the small GTPase activity during this process has not been deduced.

The SRC pathway. The endothelial SRC family of cytoplasmic tyrosine kinases includes the highly related SRC, YES and FYN. Activation of SRC by VEGFR2 is dependent on pY949 (mouse sequence; Y951 in the human sequence)[101] in the receptor kinase insert (FIG. 2; TABLE 1), which binds to the SH2 domain of T cell-specific adapter (TSAd)[102], which in turn binds to the SH3 domain of SRC. Shear stress is also a powerful and rapid inducer of SRC activity[103], but the exact mechanism remains unknown.

SRC substrates include major cytoskeletal components, such as actin and cell–cell adhesion components, regulating vascular permeability–vascular leakage, as well as cell–matrix components, regulating cell adhesion[104]. Cell adhesion occurs at focal contact points where ECM receptors, such as integrins, link to the cell cytoskeleton. Focal adhesions contain an array of specific signal transducers, notably focal adhesion kinase (FAK) and its substrate paxillin. Whereas SRC activation may occur downstream of FAK, SRC-dependent phosphorylation of FAK is a prerequisite for the orchestration of cell shape and adhesion[105] in response to VEGF or mechanical stimuli. VEGF-induced activation of FAK is also implicated in vascular leakage[106].

SRC regulates endothelial adherens junctions by phosphorylating VE-cadherin in response to VEGFA[107]. The phosphorylated VE-cadherin undergoes endocytosis leading to disruption of adherens junctions and increased vascular permeability[101].

Finally, the blood–brain barrier (BBB) may be resistant to VEGFR2–SRC signalling under normal conditions. However, pathological processes, such as those involved in glioblastoma, interrupt the BBB and vessels become leaky owing to excessive production of VEGF[108].

Stress kinases and STATs. In addition to the signalling pathways described above, VEGFR2 also activates the p38 MAPK (that is, stress-activated protein kinase 2), although the mechanism is poorly understood. This affects numerous endothelial cell functions, including shear-stress-induced angiogenesis[109], migration[110], permeability[111] and survival[112]. Although p38 MAPK activation requires NRP1 (REF. 34) and Ca^{2+} entry into cells, it is independent of PLCγ, as inhibition of PLCγ fully inhibits ERK1/2 but not p38 MAPK activation[113]. Ca^{2+} influx then activates protein tyrosine kinase 2β (PTK2β; also known as RAFTK or PYK2), which acts in combination with SRC to activate p38 MAPK. This sequence of events is confirmed by observation of decreased p38 MAPK activity following SRC inhibition or knockdown[113].

STATs form an SH2 domain-containing protein family, including STAT1 and STAT3, which have important roles in endothelial cell biology. VEGF mediates phosphorylation of STAT proteins in endothelial cells, leading to their nuclear translocation and regulation of transcription[114]. STATs are broadly implicated in regulation of the cell cycle and apoptosis and affect endothelial activation and vascular inflammation[115]. STAT3 transcriptionally activates VEGF gene expression and thereby promotes microvascular density[116].

Regulation of signalling strength and duration

VEGFR signalling is tightly regulated at numerous different levels, including the expression levels of its ligands and receptors. Indeed, a 50% reduction in the levels of VEGFA is embryonically lethal and various pathologies are associated with decreased VEGFA bioavailability[6]. Moreover, expression of a splice variant encoding a soluble VEGFR1 (sVEGFR1) reduces VEGFR2 signalling by acting as a VEGF trap[3] (BOX 1). Further fine tuning of VEGFR2 signalling includes regulation of the extent and duration of its dephosphorylation by protein tyrosine phosphatases (PTPs) and the rate of its endocytosis, as well as crosstalk with other signalling pathways[66].

VEGFA excess is equally detrimental to VEGFR2 signalling. Endothelial *Vegfr1* knockout results in early embryonic lethality owing to excessive exuberant angiogenesis[117]. This is purely owing to the ability of VEGFR1 to function as a VEGFA trap as knock-in mutants of VEGFR1 lacking its tyrosine kinase domain[118] or VEGFR1 mutants that lack the intracellular and transmembrane domains are viable, although diseases accompanied by inflammation, such as certain cancers, are affected[3].

Most RTKs induce very similar sets of signals to regulate cell proliferation, survival, migration, and so on. Yet the end results often differ dramatically for various receptors. For example, although VEGF-driven ERK1/2 activation is essential for the control of arterial fate specification, fibroblast growth factor (FGF)-dependent ERK1/2 activation does not have this effect. Differential regulation of signalling can be a means to attain biological specificity.

Moreover, VEGFR2 signalling is clearly influenced by its numerous partners that localize signalling to specific subcellular compartments, such as focal adhesions, endosomes and cell–cell junctions, in which potential substrates may be preferentially lost or enriched, and influence signalling amplitude and time course by either preventing or directing VEGFR2 intracellular trafficking, which may switch signalling from productive to abortive.

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

Thus, VEGFR2 signalling is hardly an event carried out by VEGF ligands that activate solitary VEGFR2 dimers. Yet the large number of known VEGFR2 partners raises an important question of structural organization of the signalling complex (BOX 2).

Crosstalk with receptors and intracellular signalling pathways. Signalling pathways are often depicted as linear cascades of interactions in which each pathway acts separately from others. The reality is more complex, as the pathways converge and are regulated in an intricate manner by positive and negative feedback loops[119]. Moreover, many proteins in the linear phosphorylation sequence described above receive additional signalling inputs that can profoundly affect the final outcome.

Early observations pointed to a crucial interplay and synergy between FGF and VEGF signalling as the combination of these two growth factors induced a much stronger *in vitro* angiogenic response than either by itself[120]. In agreement, in many experimental settings, FGF-driven angiogenesis is blocked by VEGF inhibition, implying that FGF controls angiogenesis upstream of VEGF[121,122]. This hierarchical regulation seems to play a similar part in lymphangiogenesis as FGF2-induced lymphatic growth is inhibited by the blockade of VEGFR3 signalling[123].

Several observations provide mechanistic insights into FGF-dependent regulation of VEGF signalling (FIG. 3a). FGF induces ERK1/2-dependent activation of ETS transcription factors, promoting binding to the FOX:ETS motif in the first intron enhancer of *Vegfr2*, thereby enhancing its transcription. In the absence of this interaction, VEGFR2 expression rapidly declines[70]. Another link is provided by the adapter protein FRS2α, which is required for FGF receptor (FGFR) signalling and has the ability to bind to several other RTKs, including VEGFRs[124]. VEGFRs phosphorylate FRS2α and its endothelial knockout leads to reduced activation of both VEGFA and VEGFC signalling, resulting in impaired angiogenesis, arteriogenesis and lymphangiogenesis[124]. Importantly, FRS2α phosphorylation by ERK1/2 activated by one RTK (for example, epidermal growth factor receptor (EGFR)) inhibits its ability to mediate signal transduction by another RTK (for example, FGFR)[125]. The functional impact of such crosstalk for FGF–VEGF signalling pathways is yet to be examined.

VEGF activation of ERK1/2, which is essential to many of its biological functions, is modulated by the crosstalk centred on the serine/threonine kinase RAF (FIG. 3a). RAF1 activation requires dephosphorylation of the inhibitory Ser259 site and phosphorylation of the activating Ser338. Ser259 is phosphorylated under normal conditions, thereby repressing MEK–ERK1/2 activation[126]. Several kinases, including protein kinase A (PKA), PKCα[127], AKT1 (REFS 69,128) and LATS1 (REF. 126) have been implicated in its phosphorylation, whereas protein serine/threonine phosphatase 1 and phosphatase 2A can dephosphorylate Ser259 (REF. 66). These regulatory

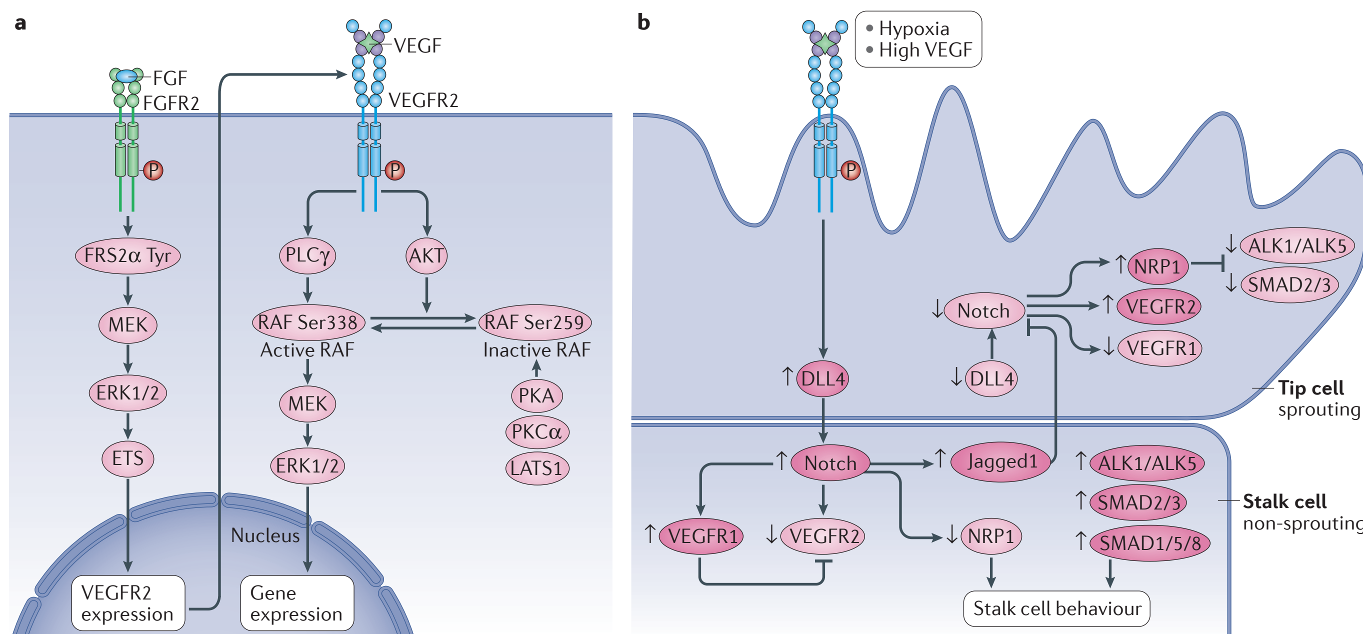

Figure 3 | **Regulation of VEGFR2 signalling by receptor crosstalk. a** | Fibroblast growth factor (FGF) signalling modulates the sensitivity of endothelial cells to vascular endothelial growth factor (VEGF) by inducing *Vegfr2* (which encodes VEGF receptor 2 (VEGFR2)) gene expression via the adapter protein FRS2α, in turn modulating the MEK–ERK1/2 pathway induced by VEGF–VEGFR2. In addition, ERK1/2 signalling is modulated by RAF and AKT crosstalk downstream of VEGFR activation. **b** | Notch signalling acts both upstream and downstream of VEGFR2. Upon ligand stimulation as a result of hypoxia, complex signalling networks regulate the expression of Delta-like protein 4 (DLL4; a Notch ligand) in highly migratory, proliferative tip cells, activating Notch in neighbouring stalk cells. In turn, Notch activity suppresses VEGFR2 expression and sensitivity to VEGF in stalk cells, thereby suppressing the tip cell phenotype and allowing formation of the stable, lumenized vasculature. Small vertical arrows indicate protein expression levels and consequent activity of receptors and downstream effectors. ALK, activin receptor-like kinase; ETS, E26 transformation-specific; FGFR2, FGF receptor 2; NRP1, neuropilin 1; PKA, protein kinase A; PLCγ, phospholipase Cγ.

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

loops allow crosstalk between VEGFR2 and signalling pathways such as PI3K–AKT[69,129] and LATS–Hippo[126] that can influence numerous processes, including vascular development, angiogenesis and arteriogenesis[130–132].

Notch signalling is involved in cell fate control during development, stem cell self-renewal and postnatal tissue differentiation[133]. Gene inactivation of either endothelial Notch1 or the Notch ligand Delta-like protein 4 (DLL4) results in embryonic lethality[134,135] as a consequence of loss of artery–vein specification and deregulated angiogenesis owing to a failure of specification of tip cells and their neighbouring stalk cells. In the tip cell, at the vascular front of the growing vessel, VEGF stimulation of both VEGFR2 and VEGFR3 promotes the expression of DLL4 (REFS 136–138) (FIG. 3b). DLL4 induces Notch signalling in adjacent stalk cells, resulting in higher levels of the Notch ligand Jagged1, which antagonizes DLL4–Notch signalling, allowing stalk cells to revert to tip cells in response to VEGF[139]. Although VEGF–VEGFR signalling lies upstream of Notch, the latter, in turn, provides signalling feedback, as inhibition of the DLL4–Notch pathway results in increased expression of VEGFR2 and VEGFR3 and decreased expression of sVEGFR1 (REFS 137,140,141). Thus, Notch controls the sensitivity of cells to VEGF.

Crosstalk between Notch and VEGFR is further complicated by the fact that Notch inhibition results in the upregulation of NRP1, which regulates tip and stalk cell selection in a VEGFR2-independent manner[44]. NRP1 promotes tip cell behaviour by suppressing stalk-cell-promoting SMAD2 and SMAD3 activation downstream of ALK1 and ALK5, as activation of ALK receptors cooperates with Notch to enhance HES and HEY expression, which regulate tissue-specific transcription factors[44,142]. Thus, there is a finely tuned feedback loop between VEGFRs, ALK receptors and Notch that is essential for proper patterning of the vasculature.

GPCRs modulate angiogenesis in certain cases by positively modulating VEGFR2 activation. P2Y purine nucleotide receptors transactivate VEGFR2 *in vitro*[143] and the downstream G_q/G_{11} proteins are required for VEGFR2 phosphorylation and activation of the ERK1/2 pathway[144]. A specific role for G_q/G_{11} in endothelial cell mechanotransduction and regulation of blood pressure involves phosphorylation of VEGFR2, possibly by SRC[50]. However, the sphingosine 1 phosphate GPCR S1PR1 restricts VEGFR2 signalling at least in part by stabilizing VE-cadherin and junctional quiescence[145], which brings VEGFR2 in close proximity to phosphatases, such as VEPTP and density-enhanced phosphatase 1 (DEP1) (see below).

Regulation by PTPs. Many PTPs have been implicated in the regulation of VEGFR2 signalling. VEPTP (also known as RPTPβ), which is a highly conserved receptor-type phosphotyrosine phosphatase, is of particular interest as it is strictly required for endothelial development[146]. Its knockout results in embryonic lethality at embryonic day 8.5 (E8.5) owing to endothelial hyperplasia and lack of vascular organization. VEPTP dephosphorylates several substrates, including TIE2, VE-cadherin and VEGFR2, that are important for the maintenance of endothelial barrier function[147,148]. VEPTP dephosphorylates junctionally localized VEGFR2 without selectivity for a particular tyrosine residue in a manner that is dependent on complex formation with TIE2 (REF. 149).

Unlike the transmembrane VEPTP, PTP1B is a ubiquitously expressed intracellular PTP involved in several metabolic processes[150]. PTP1B is anchored to the endoplasmic reticulum by a C-terminal 35-amino acid hydrophobic domain with its catalytic domain exposed to the cytoplasm. Given its unique location, PTP1B can regulate VEGFR2 activity in three distinct settings: when PTP1B-containing segments of the endoplasmic reticulum come into contact with the plasma membrane; when VEGFR2-containing endosomes become positioned in close proximity to the endoplasmic reticulum; and when newly synthesized VEGFR2 is present in the endoplasmic reticulum together with PTP1B[151].

PTP1B interactions with plasma membrane-localized VEGFR2 have not been clearly defined, but — like what has been observed with other RTKs — they probably occur at defined 'patches' near the cell surface that represent sites of endoplasmic reticulum–plasma membrane contact[151]. Once endocytosed, VEGFR2–PTP1B contacts exhibit a strong preference for early endosome antigen 1 (EEA1)-positive endosomes that tend to be more abundant in the sub-plasma cell membrane compartment[32,33]. Unlike VEPTP, PTP1B predominantly dephosphorylates Y1173 in VEGFR2, thereby selectively regulating RAF–MEK–ERK pathway activation[152].

An important and poorly understood issue in PTP-dependent regulation of VEGF signalling is the specificity of this control. Recent studies indicate that for some phosphatases, there seems to be specificity for particular VEGFR phosphotyrosines[153]. This raises several important questions on how specificity is governed: for example, by spatial constraints or specific recognition motifs to direct the PTP effect (BOX 2).

Receptor endocytosis and trafficking. Although plasma membrane residence is a prerequisite for activation of many RTKs, including VEGFRs, their ultimate signalling output depends on their interaction with various partner proteins, which is affected by the residence time of the activated VEGFR2 in the membrane, the rate of its endocytosis and intracellular trafficking and its degradation (FIG. 4).

There are different subcellular pools of VEGFRs, including receptors in various intracellular compartments[154], receptors diffusely distributed in the plasma membrane (or in different regions thereof, such as the apical or basolateral side of the cell[155]), lipid rafts, focal adhesions and at cell–cell junctions[149,156]. These specialized regions in the plasma membrane have been described as hotspots for signalling and may be distinctly selected for specialized signalling involving, for example, FAK, paxillin and integrin-linked kinase in focal adhesions. Importantly, signalling by VEGFRs is probably dependent on these different subcellular localizations; however, this information is still largely lacking. The VEGFR pool in the plasma membrane is expected to be freely diffusible and able to enter clathrin-enriched pits,

Tip cells
Highly migratory endothelial cells with polarized filopodial extensions at the leading position of the growing angiogenic sprout.

Stalk cells
Highly proliferative endothelial cells that follow tip cells and contribute to the elongation, lumenization and stabilization of the nascent sprout.

Lipid rafts
Specialized, dynamically assembled regions of the membrane enriched in certain proteins and lipids.

Clathrin-enriched pits
Invaginations in the plasma membrane assembled by the growth of a clathrin lattice that are involved in receptor endocytosis.

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

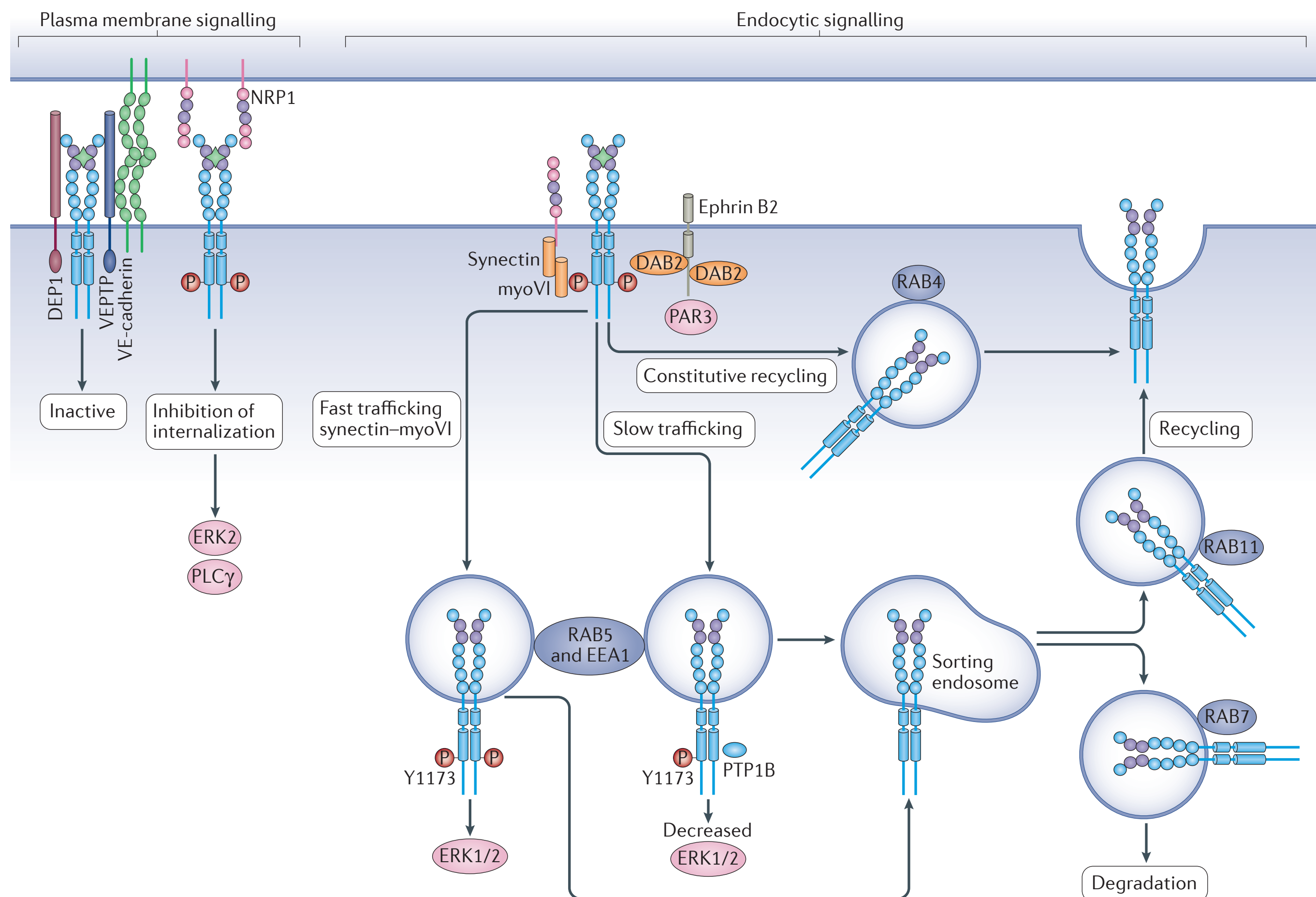

Figure 4 | **Plasma membrane versus endocytic VEGFR2 signalling.** VEGFR2 signalling can occur both at the plasma membrane and within endosomes. When in complex with VE-cadherin–VEPTP–DEP1 at endothelial cell junctions, VEGFR2 is maintained at the cell surface in a dephosphorylated state and signalling is inactive. Furthermore, VEGFR2 internalization is inhibited by NRP1 binding in *trans*, which leads to the activation of ERK2 and PLCγ rather than alternative downstream signalling occurring within endosomes. This results in decreased angiogenic signalling. When VEGFR2 forms a complex with NRP1 in *cis*, VEGFR2 is internalized and trafficked to RAB5-positive and EEA1-positive endosomes by a NRP1–synectin–myoVI complex. Internalization of the activated VEGFR2 is further mediated by an ephrin B2–DAB2–PAR3 complex, which promotes movement of VEGFR2 from the plasma membrane into the cell. Signalling continues within endosomes until VEGFR2 is dephosphorylated by PTP1B, which is localized on the endoplasmic reticulum but clustered close to endosomes. VEGFR2 can then be degraded by RAB7 endosome shuttling or recycled back to the cell surface via RAB11 endosomes. VEGFR2 may also undergo ligand-independent, constitutive recycling via RAB4 endosomes. DAB2, disabled homologue 2; DEP1, density-enhanced phosphatase 1; EEA1, early endosome antigen 1; myoVI, myosin VI; NRP1, neuropilin 1; PAR3, partitioning defective 3 homologue; PLCγ, phospholipase Cγ; PTP1B, protein tyrosine phosphatase 1B; VE-cadherin, vascular endothelial cadherin; VEGFR2, vascular endothelial growth factor receptor 2; VEPTP, vascular endothelial protein tyrosine phosphatase; Y1173, Tyr1173.

lipid rafts and focal adhesions when activated by VEGF. VEGFR trafficking to and from other subcellular sites has not been studied. The functional importance of a Golgi-associated VEGFR2 pool has not been fully investigated, but available evidence suggests its importance to VEGF signalling[157,158].

A paradigm shift in our thinking of RTK signalling came with the insight that, although VEGFRs as a rule are activated when residing on the cell surface, they also need to be internalized to transduce many, if not all, signalling pathways, such as the ERK1/2 pathway. However, the many events involved in VEGFR2 internalization are still unclear. For example, we do not fully understand which aspects of VEGFR2 signalling occur on the plasma membrane versus the cytoplasm and the reasons for this specificity. Understanding these processes is crucial to our ability to specifically target selective aspects of VEGFR2 signalling machinery.

VEGFR2 endocytosis proceeds in a clathrin-dependent manner. Clathrin does not directly bind to the receptor but is recruited by binding to various adapters, such as adapter protein 2 (AP2) or clathrin-associated sorting proteins (CLASPs), including AP180 and epsins, leading to the formation of clathrin-coated pits[159] and subsequent clathrin-coated vesicles. The clathrin-coated vesicles fuse with early endosomes and then proceed through a series of steps that can either direct their recycling back to the plasma cell membrane via the fast

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

(RAB4) or slow (RAB11) recycling pathways[160] or target them for degradation into lysosomes via the RAB7 pathway. In addition to ligand-initiated endocytosis, VEGFR2 also undergoes constitutive endocytosis. The details of this process are not well understood but the endocytosed receptor pool seems to undergo rapid recycling via the RAB4 pathway back to the plasma membrane[161].

An important aspect of VEGFR signalling is its entry into cells, which is a process that is still poorly understood. The deletion of ephrin B2, which interacts with both VEGFR2 and VEGFR3, leads to a complete lack of VEGFR2 endocytosis, following VEGFA binding in blood vascular endothelial cells[162] or VEGFR3 uptake after VEGFC binding in lymphatic endothelial cells[163]. As a result, there is a profound disruption of both developmental and postnatal angiogenesis and lymphangiogenesis. At the molecular level, ephrin B2 regulates the movement of VEGFR2 from the plasma membrane into endothelial cells. This is accomplished by a complex with disabled homologue 2 (DAB2) and the cell polarity regulator partitioning defective 3 homologue (PAR3) and can be inhibited by an atypical PKC phosphorylation of DAB2 (REF. 7).

VE-cadherin is another VEGFR2-interacting protein that controls its endocytosis. It is associated with VEGFR2 at cell–cell junctions[164], and this association maintains VEGFR2 in an inactive state, in part by exposure to VE-cadherin–VEPTP or VE-cadherin–DEP1 complexes. Knockout of the gene encoding VE-cadherin (*Cdh5*), leads to enhanced VEGFR2 endocytosis, prolonged receptor residence in early endosomes and activation of ERK signalling[165].

Recently, epsins — which are highly conserved membrane proteins that are involved in membrane curvature regulations — have been reported to affect VEGFR2 and VEGFR3 endocytosis. Endothelium-specific deletion of both epsins leads to enhanced VEGFR2 signalling and excessive non-productive angiogenesis[166], which can be corrected by reduction of VEGFR2 expression[167]. Similarly, lymphatic endothelial-cell-specific deletion of both epsins results in overactive VEGFR3 signalling and abnormal lymphangiogenesis, which can also be corrected by deletion of a single VEGFR3 allele[168].

The PTB domain-binding proteins NUMB and NUMB-like (NUMBL) interact and colocalize with VEGFR2 and VEGFR3 in AP2-positive clathrin-coated pits in endothelial cells[169]. The NUMB proteins prolong VEGFR2 signalling after internalization by preventing its degradation. A combined deletion of NUMB and NUMBL leads to a profound decrease in VEGFR2 recycling back to the membrane, which is a phenotype further augmented by DAB2 knockdown[169].

One unusual aspect of NUMB–NUMBL function is their differential effect on VEGFA-induced and VEGFC-induced PI3K versus ERK1/2 activation. In the case of VEGFA, the absence of NUMB and NUMBL results in decreased ERK1/2 and AKT activation, whereas for VEGFC, only PI3K activation is reduced[169]. One likely explanation is the role of VEGFR2–VEGFR3 heterodimers[19] in VEGFC-dependent activation of AKT but not ERK1/2 (REF. 18).

A hallmark of VEGFR2 signalling is the dependence on ERK1/2 activation for receptor endocytosis. Upon cell entry, VEGFR2 is found in RAB5-positive early endosomes[33,165]. These RAB5-positive endosomes are in close contact with the endoplasmic reticulum-resident PTP1B, which specifically dephosphorylates the VEGFR2 Y1173 site[32]. Subsequently, VEGFR2 is found in EEA1-positive subset of RAB5-positive early endosomes, in a manner that is dependent on the interaction of VEGFR2 via NRP1 with a synectin–myosin VI complex that is responsible for this trafficking step. A delay in the trafficking reduces VEGFR2-dependent ERK activation owing to prolonged contact of VEGFR2-containing endosomes with PTP1B[32,33,152].

Ubiquitylation has an important role in VEGFR2 clearance. Its activation leads to phosphorylation of the ubiquitin ligase CBL, which in turn polyubiquitylates the receptor[170]. Despite being a ubiquitin ligase, CBL activity is dispensable for VEGFR2 ubiquitylation, suggesting that another ligase is also involved. One candidate is the F-box-containing E3 ubiquitin ligase βTRCP1, which is recruited to VEGFR2 following phosphorylation of its cytoplasmic PEST domain at Ser1188–Ser1191 (REF. 26) in agreement with the known structural requirement for F-box ubiquitin ligases. The functional importance of VEGFR2 ubiquitylation has been demonstrated in numerous settings, including regulation of angiogenesis and tumour growth[171–173]. Furthermore, hyperglycaemia in mice has been shown to increase VEGFR2 ubiquitylation and reduce its expression, whereas glycaemic control reversed these abnormalities[174].

Conclusions

The past 5 years have seen key advances in our understanding of VEGF signalling, mainly by VEGFR2, and the consequential biological function. Some of the most important findings include: VEGFR2 endocytosis being a key regulator of signalling; the characterization of permeability-related and arteriogenesis-related signalling events; and the importance of PTPs.

A better understanding of the mechanisms that regulate VEGFR signalling should facilitate further exploitation of therapeutics to suppress or increase specific aspects of vessel formation or function. Several such key mechanisms still need to be unravelled. We now have a fairly detailed, if not fully complete, understanding of certain VEGFR2 signalling pathways (for example, VEGFR2 pY1173–PLCγ–ERK1/2 and VEGFR2 pY949–TSAd–SRC) and their roles in different VEGFR2 biology. However, there are many other signal transducers (such as p38 MAPK, SHB, STAT3 and small GTPases) that are implicated in VEGFR2 signalling and that are much less well characterized. There is still a lack of insight into receptor proximal initiation and activation of these pathways, as well as their contributions to endothelial biology. To verify or to exclude the participation of such signal transducers calls for ambitious *in vivo* studies. Another very interesting question is to what extent VEGFR2 pathways are modulated by flow, which may result in entirely different outcomes than what is observed under comparable static conditions.

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

Although we regard VEGFR2 homodimers as being the essential VEGF mediator in endothelial cells, there are probably dynamic fluctuations in the VEGFR dimeric constellations over time. The relative abundance of the cell surface expression of different VEGFRs and their affinities for the VEGF ligands will guide the initial set of dimeric constellations. Ligand-induced internalization of one particular type of VEGFR will in turn shift the relative VEGFR abundance and promote a new set of dimers. If the heterodimers convey different signals compared with homodimers, such fluctuations could considerably affect the biological output over time. Studying these aspects of VEGFR signalling will undoubtedly provide crucial insight into vascular biology and open new avenues for potential therapeutic applications.

1. Chung, A. S. & Ferrara, N. Developmental and pathological angiogenesis. *Annu. Rev. Cell Dev. Biol.* **27**, 563–584 (2011).
2. Potente, M., Gerhardt, H. & Carmeliet, P. Basic and therapeutic aspects of angiogenesis. *Cell* **146**, 873–887 (2011).
3. Shibuya, M. VEGFR and type-V RTK activation and signaling. *Cold Spring Harb. Perspect. Biol.* **5**, a009092 (2013).
4. Lemmon, M. A. & Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117–1134 (2010).
5. Koch, S., Tugues, S., Li, X., Gualandi, L. & Claesson-Welsh, L. Signal transduction by vascular endothelial growth factor receptors. *Biochem. J.* **437**, 169–183 (2011).
6. Shibuya, M. Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. *J. Biochem.* **153**, 13–19 (2013).
7. Nakayama, M. *et al.* Spatial regulation of VEGF receptor endocytosis in angiogenesis. *Nat. Cell Biol.* **15**, 249–260 (2013).
This paper shows how local VEGFR endocytosis and the activity of cell polarity proteins dynamically control angiogenesis *in vivo*.
8. Simons, M. An inside view: VEGF receptor trafficking and signaling. *Physiology* **27**, 213–222 (2012).
9. Chen, T. T. *et al.* Anchorage of VEGF to the extracellular matrix conveys differential signaling responses to endothelial cells. *J. Cell Biol.* **188**, 595–609 (2010).
10. Ferrara, N. Binding to the extracellular matrix and proteolytic processing: two key mechanisms regulating vascular endothelial growth factor action. *Mol. Biol. Cell* **21**, 687–690 (2010).
11. McColl, B. K. *et al.* Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. *J. Exp. Med.* **198**, 863–868 (2003).
12. Grunewald, F. S., Prota, A. E., Giese, A. & Ballmer-Hofer, K. Structure–function analysis of VEGF receptor activation and the role of coreceptors in angiogenic signaling. *Biochim. Biophys. Acta* **1804**, 567–580 (2010).
13. Sarabipour, S., Ballmer-Hofer, K. & Hristova, K. VEGFR-2 conformational switch in response to ligand binding. *eLife* **5**, e13876 (2016).
14. Anisimov, A. *et al.* The basis for the distinct biological activities of vascular endothelial growth factor receptor-1 ligands. *Sci. Signal.* **6**, ra52 (2013).
This study provides a mechanistic understanding for the inefficient activation of VEGFR1 by VEGFB, which, unlike PlGF, fails to bind to the IgG D3 loop in the receptor.
15. Bates, D. O. *et al.* VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res.* **62**, 4123–4131 (2002).
16. Kawamura, H., Li, X., Harper, S. J., Bates, D. O. & Claesson-Welsh, L. Vascular endothelial growth factor (VEGF)-A165b is a weak *in vitro* agonist for VEGF receptor-2 due to lack of coreceptor binding and deficient regulation of kinase activity. *Cancer Res.* **68**, 4683–4692 (2008).
17. Mac Gabhann, F. & Popel, A. S. Dimerization of VEGF receptors and implications for signal transduction: a computational study. *Biophys. Chem.* **128**, 125–139 (2007).
18. Deng, Y., Zhang, X. & Simons, M. Molecular controls of lymphatic VEGFR3 signaling. *Arterioscler. Thromb. Vasc. Biol.* **35**, 421–429 (2015).
19. Dixelius, J. *et al.* Ligand-induced vascular endothelial growth factor receptor-3 (VEGFR-3) heterodimerization with VEGFR-2 in primary lymphatic endothelial cells regulates tyrosine phosphorylation sites. *J. Biol. Chem.* **278**, 40973–40979 (2003).
20. Yao, L. C. *et al.* Pulmonary lymphangiectasia resulting from vascular endothelial growth factor-C overexpression during a critical period. *Circ. Res.* **114**, 806–822 (2014).
21. Nilsson, I. *et al.* VEGF receptor 2/-3 heterodimers detected *in situ* by proximity ligation on angiogenic sprouts. *EMBO J.* **29**, 1377–1388 (2010).
22. Imoukhuede, P. I. & Popel, A. S. Quantification and cell-to-cell variation of vascular endothelial growth factor receptors. *Exp. Cell Res.* **317**, 955–965 (2011).
23. Papadopoulos, N. *et al.* Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF trap, ranibizumab and bevacizumab. *Angiogenesis* **15**, 171–185 (2012).
24. Autiero, M. *et al.* Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat. Med.* **9**, 936–943 (2003).
25. Jin, Z. G. *et al.* Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. *Circ. Res.* **93**, 354–363 (2003).
26. Meyer, R. D. *et al.* PEST motif serine and tyrosine phosphorylation controls vascular endothelial growth factor receptor 2 stability and downregulation. *Mol. Cell. Biol.* **31**, 2010–2025 (2011).
27. van der Zwaag, B. *et al.* PLEXIN-D1, a novel plexin family member, is expressed in vascular endothelium and the central nervous system during mouse embryogenesis. *Dev. Dyn.* **225**, 336–343 (2002).
28. Ochsenbein, A. M. *et al.* Endothelial cell-derived semaphorin 3A inhibits filopodia formation by blood vascular tip cells. *Development* **143**, 589–594 (2016).
29. Parker, M. W., Xu, P., Li, X. & Vander Kooi, C. W. Structural basis for selective vascular endothelial growth factor-A (VEGF-A) binding to neuropilin-1. *J. Biol. Chem.* **287**, 11082–11089 (2012).
30. Fantin, A. *et al.* Neuropilin 1 (NRP1) hypomorphism combined with defective VEGF-A binding reveals novel roles for NRP1 in developmental and pathological angiogenesis. *Development* **141**, 556–562 (2014).
31. Plein, A., Fantin, A. & Ruhrberg, C. Neuropilin regulation of angiogenesis, arteriogenesis, and vascular permeability. *Microcirculation* **21**, 315–323 (2014).
32. Lanahan, A. *et al.* The neuropilin 1 cytoplasmic domain is required for VEGF-A-dependent arteriogenesis. *Dev. Cell* **25**, 156–168 (2013).
33. Lanahan, A. A. *et al.* VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. *Dev. Cell* **18**, 713–724 (2010).
34. Kawamura, H. *et al.* Neuropilin-1 in regulation of VEGF-induced activation of p38MAPK and endothelial cell organization. *Blood* **112**, 3638–3649 (2008).
35. Koch, S. *et al.* NRP1 presented in *trans* to the endothelium arrests VEGFR2 endocytosis, preventing angiogenic signaling and tumor initiation. *Dev. Cell* **28**, 633–646 (2014).
This paper describes how VEGF–VEGFR2 on an endothelial cell can bind to NRP1 on an adjacent cell (that is, *trans*-binding), regulating VEGFR2 endocytosis, tumour initiation and tumour angiogenesis.
36. Okabe, K. *et al.* Neurons limit angiogenesis by titrating VEGF in retina. *Cell* **159**, 584–596 (2014).
VEGFR2 expressed in neurons serves to regulate VEGF availability and angiogenesis.
37. Tillo, M. *et al.* VEGF189 binds NRP1 and is sufficient for VEGF/NRP1-dependent neuronal patterning in the developing brain. *Development* **142**, 314–319 (2015).
38. Cariboni, A. *et al.* VEGF signalling controls GnRH neuron survival via NRP1 independently of KDR and blood vessels. *Development* **138**, 3723–3733 (2011).
39. Kawasaki, T. *et al.* A requirement for neuropilin-1 in embryonic vessel formation. *Development* **126**, 4895–4902 (1999).
40. Jones, E. A., Yuan, L., Breant, C., Watts, R. J. & Eichmann, A. Separating genetic and hemodynamic defects in neuropilin 1 knockout embryos. *Development* **135**, 2479–2488 (2008).
41. Gelfand, M. V. *et al.* Neuropilin-1 functions as a VEGFR2 co-receptor to guide developmental angiogenesis independent of ligand binding. *eLife* **3**, e03720 (2014).
42. Fantin, A. *et al.* The cytoplasmic domain of neuropilin 1 is dispensable for angiogenesis, but promotes the spatial separation of retinal arteries and veins. *Development* **138**, 4185–4191 (2011).
43. Hirota, S. *et al.* Neuropilin-1 balances β8 integrin-activated TGFβ signaling to control sprouting angiogenesis in the brain. *Development* **142**, 4363–4373 (2015).
44. Aspalter, I. M. *et al.* Alk1 and Alk5 inhibition by Nrp1 controls vascular sprouting downstream of Notch. *Nat. Commun.* **6**, 7264 (2015).
45. Byzova, T. V. *et al.* A mechanism for modulation of cellular responses to VEGF: activation of the integrins. *Mol. Cell* **6**, 851–860 (2000).
46. West, X. Z. *et al.* Integrin β3 crosstalk with VEGFR accommodating tyrosine phosphorylation as a regulatory switch. *PLoS ONE* **7**, e31071 (2012).
47. Rapraeger, A. C. *et al.* Vascular endothelial-cadherin stimulates syndecan-1-coupled insulin-like growth factor-1 receptor and cross-talk between αVβ3 integrin and vascular endothelial growth factor receptor 2 at the onset of endothelial cell dissemination during angiogenesis. *FEBS J.* **280**, 2194–2206 (2013).
48. Tugues, S. *et al.* Tetraspanin CD63 promotes vascular endothelial growth factor receptor 2–β1 integrin complex formation, thereby regulating activation and downstream signaling in endothelial cells *in vitro* and *in vivo*. *J. Biol. Chem.* **288**, 19060–19071 (2013).
49. Tzima, E. *et al.* A mechanosensory complex that mediates the endothelial cell response to fluid shear stress. *Nature* **437**, 426–431 (2005).
50. Wang, S. *et al.* $P2Y_2$ and G_q/G_{11} control blood pressure by mediating endothelial mechanotransduction. *J. Clin. Invest.* **125**, 3077–3086 (2015).
51. Planas-Paz, L. *et al.* Mechanoinduction of lymph vessel expansion. *EMBO J.* **31**, 788–804 (2012).
This paper demonstrates mechanosensory activation of VEGFR3 and its regulation of lymph vessel function *in vivo*.
52. Baeyens, N. *et al.* Vascular remodeling is governed by a VEGFR3-dependent fluid shear stress set point. *eLife* **4**, e04645 (2015).
53. Coon, B. G. *et al.* Intramembrane binding of VE-cadherin to VEGFR2 and VEGFR3 assembles the endothelial mechanosensory complex. *J. Cell Biol.* **208**, 975–986 (2015).
This paper identifies the direct interaction between the transmembrane domains of VE-cadherin, VEGFR2 and VEGFR3, forming a junctional mechanosensory complex.
54. Chiu, Y. J., McBeath, E. & Fujiwara, K. Mechanotransduction in an extracted cell model: Fyn drives stretch- and flow-elicited PECAM-1 phosphorylation. *J. Cell Biol.* **182**, 753–763 (2008).
55. dela Paz, N. G., Walshe, T. E., Leach, L. L., Saint-Geniez, M. & D'Amore, P. A. Role of shear-stress-induced VEGF expression in endothelial cell survival. *J. Cell Sci.* **125**, 831–843 (2012).
56. Masumura, T., Yamamoto, K., Shimizu, N., Obi, S. & Ando, J. Shear stress increases expression of the arterial endothelial marker ephrinB2 in murine ES cells via the VEGF–Notch signaling pathways. *Arterioscler. Thromb. Vasc. Biol.* **29**, 2125–2131 (2009).
57. dela Paz, N. G., Melchior, B. & Frangos, J. A. Early VEGFR2 activation in response to flow is VEGF-dependent and mediated by MMP activity. *Biochem. Biophys. Res. Commun.* **434**, 641–646 (2013).

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

58. Stabile, H. *et al.* Bone morphogenic protein antagonist Drm/gremlin is a novel proangiogenic factor. *Blood* **109**, 1834–1840 (2007).
59. Mitola, S. *et al.* Gremlin is a novel agonist of the major proangiogenic receptor VEGFR2. *Blood* **116**, 3677–3680 (2010).
60. Ravelli, C. *et al.* β3 integrin promotes long-lasting activation and polarization of vascular endothelial growth factor receptor 2 by immobilized ligand. *Arterioscler. Thromb. Vasc. Biol.* **35**, 2161–2171 (2015).
61. Dalziel, M., Crispin, M., Scanlan, C. N., Zitzmann, N. & Dwek, R. A. Emerging principles for the therapeutic exploitation of glycosylation. *Science* **343**, 1235681 (2014).
62. Markowska, A. I., Jefferies, K. C. & Panjwani, N. Galectin-3 protein modulates cell surface expression and activation of vascular endothelial growth factor receptor 2 in human endothelial cells. *J. Biol. Chem.* **286**, 29913–29921 (2011).
63. Kumar, V. B., Viji, R. I., Kiran, M. S. & Sudhakaran, P. R. Endothelial cell response to lactate: implication of PAR modification of VEGF. *J. Cell. Physiol.* **211**, 477–485 (2007).
64. Ruan, G. X. & Kazlauskas, A. Lactate engages receptor tyrosine kinases Axl, Tie2, and vascular endothelial growth factor receptor 2 to activate phosphoinositide 3-kinase/Akt and promote angiogenesis. *J. Biol. Chem.* **288**, 21161–21172 (2013).
65. Jin, F. *et al.* LDL attenuates VEGF-induced angiogenesis via mechanisms involving VEGFR2 internalization and degradation following endosome–*trans*-Golgi network trafficking. *Angiogenesis* **16**, 625–637 (2013).
66. Simons, M. & Eichmann, A. Molecular controls of arterial morphogenesis. *Circ. Res.* **116**, 1712–1724 (2015).
67. Zhuang, G. *et al.* Phosphoproteomic analysis implicates the mTORC2–FoxO1 axis in VEGF signaling and feedback activation of receptor tyrosine kinases. *Sci. Signal.* **6**, ra25 (2013).
68. Rodrigues, S. F. & Granger, D. N. Blood cells and endothelial barrier function. *Tissue Barriers* **3**, e978720 (2015).
69. Hong, C. C., Peterson, Q. P., Hong, J. Y. & Peterson, R. T. Artery/vein specification is governed by opposing phosphatidylinositol-3 kinase and MAP kinase/ERK signaling. *Curr. Biol.* **16**, 1366–1372 (2006).
70. Murakami, M. *et al.* FGF-dependent regulation of VEGF receptor 2 expression in mice. *J. Clin. Invest.* **121**, 2668–2678 (2011).
71. Deng, Y., Atri, D., Eichmann, A. & Simons, M. Endothelial ERK signaling controls lymphatic fate specification. *J. Clin. Invest.* **123**, 1202–1215 (2013).
72. Sakurai, Y., Ohgimoto, K., Kataoka, Y., Yoshida, N. & Shibuya, M. Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. *Proc. Natl Acad. Sci. USA* **102**, 1076–1081 (2005).
73. Takahashi, T., Yamaguchi, S., Chida, K. & Shibuya, M. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-γ and DNA synthesis in vascular endothelial cells. *EMBO J.* **20**, 2768–2778 (2001).
74. Takahashi, T., Ueno, H. & Shibuya, M. VEGF activates protein kinase C-dependent, but Ras-independent Raf–MEK–MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene* **18**, 2221–2230 (1999).
75. Xia, P. *et al.* Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. *J. Clin. Invest.* **98**, 2018–2026 (1996).
76. Lawson, N. D., Mugford, J. W., Diamond, B. A. & Weinstein, B. M. Phospholipase C gamma-1 is required downstream of vascular endothelial growth factor during arterial development. *Genes Dev.* **17**, 1346–1351 (2003).
77. Leitges, M. *et al.* Immunodeficiency in protein kinase cβ-deficient mice. *Science* **273**, 788–791 (1996).
78. De Val, S. *et al.* Combinatorial regulation of endothelial gene expression by Ets and forkhead transcription factors. *Cell* **135**, 1053–1064 (2008).
79. Wei, G. *et al.* Ets1 and Ets2 are required for endothelial cell survival during embryonic angiogenesis. *Blood* **114**, 1123–1130 (2009).
80. Lammerts van Bueren, K. & Black, B. L. Regulation of endothelial and hematopoietic development by the ETS transcription factor Etv2. *Curr. Opin. Hematol.* **19**, 199–205 (2012).
81. Wythe, J. D. *et al.* ETS factors regulate Vegf-dependent arterial specification. *Dev. Cell* **26**, 45–58 (2013).
82. Wang, S. *et al.* Control of endothelial cell proliferation and migration by VEGF signaling to histone deacetylase 7. *Proc. Natl Acad. Sci. USA* **105**, 7738–7743 (2008).
83. Jinnin, M. *et al.* Suppressed NFAT-dependent VEGFR1 expression and constitutive VEGFR2 signaling in infantile hemangioma. *Nat. Med.* **14**, 1236–1246 (2008).
84. Chang, C. P. *et al.* A field of myocardial–endocardial NFAT signaling underlies heart valve morphogenesis. *Cell* **118**, 649–663 (2004).
85. Ryeom, S. *et al.* Targeted deletion of the calcineurin inhibitor DSCR1 suppresses tumor growth. *Cancer Cell* **13**, 420–431 (2008).
86. Shiojima, I. & Walsh, K. Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ. Res.* **90**, 1243–1250 (2002).
87. Ju, R. *et al.* Angiopoietin-2 secretion by endothelial cell exosomes: regulation by the phosphatidylinositol 3-kinase (PI3K)/Akt/endothelial nitric oxide synthase (eNOS) and syndecan-4/syntenin pathways. *J. Biol. Chem.* **289**, 510–519 (2014).
88. Chen, J. *et al.* Akt1 regulates pathological angiogenesis, vascular maturation and permeability *in vivo*. *Nat. Med.* **11**, 1188–1196 (2005).
89. Lee, M. Y. *et al.* Endothelial Akt1 mediates angiogenesis by phosphorylating multiple angiogenic substrates. *Proc. Natl Acad. Sci. USA* **111**, 12865–12870 (2014).
90. Ackah, E. *et al.* Akt1/protein kinase Bα is critical for ischemic and VEGF-mediated angiogenesis. *J. Clin. Invest.* **115**, 2119–2127 (2005).
91. Carmeliet, P. *et al.* Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* **98**, 147–157 (1999).
92. Ruan, G. X. & Kazlauskas, A. Axl is essential for VEGF-A-dependent activation of PI3K/Akt. *EMBO J.* **31**, 1692–1703 (2012).
93. Graupera, M. *et al.* Angiogenesis selectively requires the p110α isoform of PI3K to control endothelial cell migration. *Nature* **453**, 662–666 (2008).
94. Tan, W. *et al.* An essential role for Rac1 in endothelial cell function and vascular development. *FASEB J.* **22**, 1829–1838 (2008).
95. Kaur, S. *et al.* RhoJ/TCL regulates endothelial motility and tube formation and modulates actomyosin contractility and focal adhesion numbers. *Arterioscler. Thromb. Vasc. Biol.* **31**, 657–664 (2011).
96. Hoang, M. V., Whelan, M. C. & Senger, D. R. Rho activity critically and selectively regulates endothelial cell organization during angiogenesis. *Proc. Natl Acad. Sci. USA* **101**, 1874–1879 (2004).
97. Abraham, S. *et al.* A Rac/Cdc42 exchange factor complex promotes formation of lateral filopodia and blood vessel lumen morphogenesis. *Nat. Commun.* **6**, 7286 (2015).
98. Barry, D. M. *et al.* Cdc42 is required for cytoskeletal support of endothelial cell adhesion during blood vessel formation in mice. *Development* **142**, 3058–3070 (2015).
99. Dubrac, A. *et al.* Targeting NCK-mediated endothelial cell front-rear polarity inhibits neovascularization. *Circulation* **133**, 409–421 (2016).
100. Collins, C. & Tzima, E. Rac[e] to the pole: setting up polarity in endothelial cells. *Small GTPases* **5**, e28650 (2014).
101. Li, X. *et al.* VEGFR2 pY949 signalling regulates adherens junction integrity and metastatic spread. *Nat. Commun.* **7**, 11017 (2016).
This study identifies the signal transduction pathway that is initiated by VEGFR2 phosphosite Y949, leading to transient opening of vascular junctions *in vivo*.
102. Sun, Z. *et al.* VEGFR2 induces c-Src signaling and vascular permeability *in vivo* via the adaptor protein TSAd. *J. Exp. Med.* **209**, 1363–1377 (2012).
103. Jalali, S. *et al.* Shear stress activates p60src–Ras–MAPK signaling pathways in vascular endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **18**, 227–234 (1998).
104. Ferrando, I. M. *et al.* Identification of targets of c-Src tyrosine kinase by chemical complementation and phosphoproteomics. *Mol. Cell. Proteomics* **11**, 355–369 (2012).
105. Westhoff, M. A., Serrels, B., Fincham, V. J., Frame, M. C. & Carragher, N. O. SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Mol. Cell. Biol.* **24**, 8113–8133 (2004).
106. Chen, X. L. *et al.* VEGF-induced vascular permeability is mediated by FAK. *Dev. Cell* **22**, 146–157 (2012).
107. Weis, S., Cui, J., Barnes, L. & Cheresh, D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. *J. Cell Biol.* **167**, 223–229 (2004).
108. Tornavaca, O. *et al.* ZO-1 controls endothelial adherens junctions, cell–cell tension, angiogenesis, and barrier formation. *J. Cell Biol.* **208**, 821–838 (2015).
109. Gee, E., Milkiewicz, M. & Haas, T. L. p38 MAPK activity is stimulated by vascular endothelial growth factor receptor 2 activation and is essential for shear stress-induced angiogenesis. *J. Cell. Physiol.* **222**, 120–126 (2010).
110. McMullen, M. E., Bryant, P. W., Glembotski, C. C., Vincent, P. A. & Pumiglia, K. M. Activation of p38 has opposing effects on the proliferation and migration of endothelial cells. *J. Biol. Chem.* **280**, 20995–21003 (2005).
111. Issbrucker, K. *et al.* p38 MAP kinase — a molecular switch between VEGF-induced angiogenesis and vascular hyperpermeability. *FASEB J.* **17**, 262–264 (2003).
112. Yilmaz, A., Kliche, S., Mayr-Beyrle, U., Fellbrich, G. & Waltenberger, J. p38 MAPK inhibition is critically involved in VEGFR-2-mediated endothelial cell survival. *Biochem. Biophys. Res. Commun.* **306**, 730–736 (2003).
113. McMullen, M., Keller, R., Sussman, M. & Pumiglia, K. Vascular endothelial growth factor-mediated activation of p38 is dependent upon Src and RAFTK/Pyk2. *Oncogene* **23**, 1275–1282 (2004).
114. Bartoli, M. *et al.* Vascular endothelial growth factor activates STAT proteins in aortic endothelial cells. *J. Biol. Chem.* **275**, 33189–33192 (2000).
115. Bromberg, J. & Darnell, J. E. Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* **19**, 2468–2473 (2000).
116. Kostromina, E. *et al.* Glucose intolerance and impaired insulin secretion in pancreas-specific signal transducer and activator of transcription-3 knockout mice are associated with microvascular alterations in the pancreas. *Endocrinology* **151**, 2050–2059 (2010).
117. Fong, G. H., Rossant, J., Gertsenstein, M. & Breitman, M. L. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376**, 66–70 (1995).
118. Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. & Shibuya, M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl Acad. Sci. USA* **95**, 9349–9354 (1998).
119. Fey, D., Croucher, D. R., Kolch, W. & Kholodenko, B. N. Crosstalk and signaling switches in mitogen-activated protein kinase cascades. *Front. Physiol.* **3**, 355 (2012).
120. Pepper, M. S., Ferrara, N., Orci, L. & Montesano, R. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis *in vitro*. *Biochem. Biophys. Res. Commun.* **189**, 824–831 (1992).
121. Presta, M. *et al.* Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine Growth Factor Rev.* **16**, 159–178 (2005).
122. Murakami, M. & Simons, M. Fibroblast growth factor regulation of neovascularization. *Curr. Opin. Hematol.* **15**, 215–220 (2008).
123. Kubo, H. *et al.* Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea. *Proc. Natl Acad. Sci. USA* **99**, 8868–8873 (2002).
124. Chen, P. Y. *et al.* The docking protein FRS2α is a critical regulator of VEGF receptors signaling. *Proc. Natl Acad. Sci. USA* **111**, 5514–5519 (2014).
125. Lax, I. *et al.* The docking protein FRS2α controls a MAP kinase-mediated negative feedback mechanism for signaling by FGF receptors. *Mol. Cell* **10**, 709–719 (2002).
126. Romano, D. *et al.* Protein interaction switches coordinate Raf-1 and MST2/Hippo signalling. *Nat. Cell Biol.* **16**, 673–684 (2014).
Crosstalk between signalling pathways can result in a switch from one biological response to another, downstream of one common stimulus.
127. Wellbrock, C., Karasarides, M. & Marais, R. The RAF proteins take centre stage. *Nat. Rev. Mol. Cell Biol.* **5**, 875–885 (2004).
128. Zhou, X. *et al.* Relaxin inhibits cardiac fibrosis and endothelial–mesenchymal transition via the Notch pathway. *Drug Des. Devel. Ther.* **9**, 4599–4611 (2015).

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.

129. Ren, B. *et al.* ERK1/2–Akt1 crosstalk regulates arteriogenesis in mice and zebrafish. *J. Clin. Invest.* **120**, 1217–1228 (2010).
This paper demonstrates the important role of PI3K–ERK1/2 crosstalk in the regulation of arterial growth.
130. Dai, X. *et al.* Phosphorylation of angiomotin by Lats1/2 kinases inhibits F-actin binding, cell migration, and angiogenesis. *J. Biol. Chem.* **288**, 34041–34051 (2013).
131. Nejigane, S., Takahashi, S., Haramoto, Y., Michiue, T. & Asashima, M. Hippo signaling components, Mst1 and Mst2, act as a switch between self-renewal and differentiation in *Xenopus* hematopoietic and endothelial progenitors. *Int. J. Dev. Biol.* **57**, 407–414 (2013).
132. Zhou, J. An emerging role for Hippo–YAP signaling in cardiovascular development. *J. Biomed. Res.* **28**, 251–254 (2014).
133. Herbert, S. P. & Stainier, D. Y. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nat. Rev. Mol. Cell Biol.* **12**, 551–564 (2011).
134. Gale, N. W. *et al.* Haploinsufficiency of δ-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc. Natl Acad. Sci. USA* **101**, 15949–15954 (2004).
135. Limbourg, F. P. *et al.* Essential role of endothelial Notch1 in angiogenesis. *Circulation* **111**, 1826–1832 (2005).
136. Tammela, T. *et al.* VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. *Nat. Cell Biol.* **13**, 1202–1213 (2011).
This paper provides evidence for an important role for VEGFR3 expressed in tip cells to reinforce Notch signalling, thereby regulating branching morphogenesis in the blood vasculature.
137. Suchting, S. *et al.* The Notch ligand δ-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc. Natl Acad. Sci. USA* **104**, 3225–3230 (2007).
138. Hellstrom, M. *et al.* Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* **445**, 776–780 (2007).
139. Benedito, R. *et al.* The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* **137**, 1124–1135 (2009).
140. Tammela, T. *et al.* Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature* **454**, 656–660 (2008).
141. Jakobsson, L. *et al.* Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nat. Cell Biol.* **12**, 943–953 (2010).
This is the first evidence of the VEGF-dependent dynamic shuffling of endothelial cells along the growing angiogenic sprout.
142. Larrivee, B. *et al.* ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. *Dev. Cell* **22**, 489–500 (2012).
143. Rumjahn, S. M., Yokdang, N., Baldwin, K. A., Thai, J. & Buxton, I. L. Purinergic regulation of vascular endothelial growth factor signaling in angiogenesis. *Br. J. Cancer* **100**, 1465–1470 (2009).
144. Zeng, H., Zhao, D. & Mukhopadhyay, D. KDR stimulates endothelial cell migration through heterotrimeric G protein $G_q/_{11}$-mediated activation of a small GTPase RhoA. *J. Biol. Chem.* **277**, 46791–46798 (2002).
145. Gaengel, K. *et al.* The sphingosine-1-phosphate receptor S1PR1 restricts sprouting angiogenesis by regulating the interplay between VE-cadherin and VEGFR2. *Dev. Cell* **23**, 587–599 (2012).
This study elegantly demonstrates how S1PR1 signalling decreases endothelial hyper-sprouting through stabilization of junctional VE-cadherin, thereby suppressing VEGFR2 phosphorylation and downstream signalling.
146. Baumer, S. *et al.* Vascular endothelial cell-specific phosphotyrosine phosphatase (VE-PTP) activity is required for blood vessel development. *Blood* **107**, 4754–4762 (2006).
147. Kuppers, V., Vockel, M., Nottebaum, A. F. & Vestweber, D. Phosphatases and kinases as regulators of the endothelial barrier function. *Cell Tissue Res.* **355**, 577–586 (2014).
148. Frye, M. *et al.* Interfering with VE-PTP stabilizes endothelial junctions *in vivo* via Tie-2 in the absence of VE-cadherin. *J. Exp. Med.* **212**, 2267–2287 (2015).
149. Hayashi, M. *et al.* VE-PTP regulates VEGFR2 activity in stalk cells to establish endothelial cell polarity and lumen formation. *Nat. Commun.* **4**, 1672 (2013).
150. Stuible, M. & Tremblay, M. L. In control at the ER: PTP1B and the down-regulation of RTKs by dephosphorylation and endocytosis. *Trends Cell Biol.* **20**, 672–679 (2010).
151. Anderie, I., Schulz, I. & Schmid, A. Direct interaction between ER membrane-bound PTP1B and its plasma membrane-anchored targets. *Cell. Signall.* **19**, 582–592 (2007).
152. Lanahan, A. A. *et al.* PTP1b is a physiologic regulator of vascular endothelial growth factor signaling in endothelial cells. *Circulation* **130**, 902–909 (2014).
This paper demonstrates the important role of PTP1B in the regulation of VEGFR2 pY1175 signalling and ERK1/2 pathway activation.
153. Mattila, E., Auvinen, K., Salmi, M. & Ivaska, J. The protein tyrosine phosphatase TCPTP controls VEGFR2 signalling. *J. Cell Sci.* **121**, 3570–3580 (2008).
154. Gampel, A. *et al.* VEGF regulates the mobilization of VEGFR2/KDR from an intracellular endothelial storage compartment. *Blood* **108**, 2624–2631 (2006).
155. Hudson, N. *et al.* Differential apicobasal VEGF signaling at vascular blood–neural barriers. *Dev. Cell* **30**, 541–552 (2014).
This is the first identification of distinct polarity of VEGFR1 and VEGFR2 expression in the CNS endothelium.
156. Saharinen, P. *et al.* Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell–cell and cell–matrix contacts. *Nat. Cell Biol.* **10**, 527–537 (2008).
157. Manickam, V. *et al.* Regulation of vascular endothelial growth factor receptor 2 trafficking and angiogenesis by Golgi localized t-SNARE syntaxin 6. *Blood* **117**, 1425–1435 (2011).
158. Tiwari, A., Jung, J. J., Inamdar, S. M., Nihalani, D. & Choudhury, A. The myosin motor Myo1c is required for VEGFR2 delivery to the cell surface and for angiogenic signaling. *Am J Physiol. Heart Circ. Physiol.* **304**, H687–H696 (2013).
159. McMahon, H. T. & Boucrot, E. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat. Rev. Mol. Cell Biol.* **12**, 517–533 (2011).
160. Ballmer-Hofer, K., Andersson, A. E., Ratcliffe, L. E. & Berger, P. Neuropilin-1 promotes VEGFR-2 trafficking through Rab11 vesicles thereby specifying signal output. *Blood* **118**, 816–826 (2011).
161. Zhang, X. & Simons, M. Receptor tyrosine kinase endocytosis in endothelium: biology and signaling. *Arterioscler. Thromb. Vasc. Biol* **34**, 1831–1837 (2014).
162. Sawamiphak, S. *et al.* Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. *Nature* **465**, 487–491 (2010).
163. Wang, Y. *et al.* Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature* **465**, 483–486 (2010).
164. Lampugnani, G. M. *et al.* Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, β-catenin, and the phosphatase DEP-1/CD148. *J. Cell Biol.* **161**, 793–804 (2003).
165. Lampugnani, M. G., Orsenigo, F., Gagliani, M. C., Tacchetti, C. & Dejana, E. Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. *J. Cell Biol.* **174**, 593–604 (2006).
166. Pasula, S. *et al.* Endothelial epsin deficiency decreases tumor growth by enhancing VEGF signaling. *J. Clin. Invest.* **122**, 4424–4438 (2012).
167. Tessneer, K. L. *et al.* Genetic reduction of vascular endothelial growth factor receptor 2 rescues aberrant angiogenesis caused by epsin deficiency. *Arterioscler. Thromb. Vasc. Biol.* **34**, 331–337 (2014).
168. Liu, X. *et al.* Temporal and spatial regulation of epsin abundance and VEGFR3 signaling are required for lymphatic valve formation and function. *Sci. Signal.* **7**, ra97 (2014).
169. van Lessen, M. *et al.* Regulation of vascular endothelial growth factor receptor function in angiogenesis by numb and numb-like. *Arterioscler. Thromb. Vasc. Biol.* **35**, 1815–1825 (2015).
170. Duval, M., Bedard-Goulet, S., Delisle, C. & Gratton, J. P. Vascular endothelial growth factor-dependent down-regulation of Flk-1/KDR involves Cbl-mediated ubiquitination. Consequences on nitric oxide production from endothelial cells. *J. Biol. Chem.* **278**, 20091–20097 (2003).
171. Husain, D. *et al.* Role of c-Cbl-dependent regulation of phospholipase Cγ1 activation in experimental choroidal neovascularization. *Invest. Ophthalmol. Vis. Sci.* **51**, 6803–6809 (2010).
172. Meyer, R. D., Husain, D. & Rahimi, N. c-Cbl inhibits angiogenesis and tumor growth by suppressing activation of PLCγ1. *Oncogene* **30**, 2198–2206 (2011).
173. Shaik, S. *et al.* SCF(β-TRCP) suppresses angiogenesis and thyroid cancer cell migration by promoting ubiquitination and destruction of VEGF receptor 2. *J. Exp. Med.* **209**, 1289–1307 (2012).
174. Dokun, A. O., Chen, L., Lanjewar, S. S., Lye, R. J. & Annex, B. H. Glycaemic control improves perfusion recovery and VEGFR2 protein expression in diabetic mice following experimental PAD. *Cardiovasc. Res.* **101**, 364–372 (2014).
175. Selvaraj, D. *et al.* A functional role for VEGFR1 expressed in peripheral sensory neurons in cancer pain. *Cancer Cell* **27**, 780–796 (2015).
176. Cudmore, M. J. *et al.* The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis. *Nat. Commun.* **3**, 972 (2012).
177. Ito, N., Wernstedt, C., Engstrom, U. & Claesson-Welsh, L. Identification of vascular endothelial growth factor receptor-1 tyrosine phosphorylation sites and binding of SH2 domain-containing molecules. *J. Biol. Chem.* **273**, 23410–23418 (1998).
178. Patten, I. S. *et al.* Cardiac angiogenic imbalance leads to peripartum cardiomyopathy. *Nature* **485**, 333–338 (2012).
The physiological and pathological role of sVEGFR1 is demonstrated in the negative regulation of VEGFA bioactivity.
179. Zeisler, H. *et al.* Predictive value of the sFlt-1:PlGF ratio in women with suspected preeclampsia. *N. Engl. J. Med.* **374**, 13–22 (2016).
180. Fischer, C. *et al.* Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* **131**, 463–475 (2007).
181. Aase, K. *et al.* Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect. *Circulation* **104**, 358–364 (2001).
182. Bellomo, D. *et al.* Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. *Circ. Res.* **86**, E29–E35 (2000).
183. Usui, R., Shibuya, M., Ishibashi, S. & Maru, Y. Ligand-independent activation of vascular endothelial growth factor receptor 1 by low-density lipoprotein. *EMBO Rep.* **8**, 1155–1161 (2007).
184. Avraham-Davidi, I. *et al.* ApoB-containing lipoproteins regulate angiogenesis by modulating expression of VEGF receptor 1. *Nat. Med.* **18**, 967–973 (2012).
185. Han, J. *et al.* Vascular endothelial growth factor receptor 3 controls neural stem cell activation in mice and humans. *Cell Rep.* **10**, 1158–1172 (2015).
186. Cha, Y. R. *et al.* Chemokine signaling directs trunk lymphatic network formation along the preexisting blood vasculature. *Dev. Cell* **22**, 824–836 (2012).
187. Alitalo, K. The lymphatic vasculature in disease. *Nat. Med.* **17**, 1371–1380 (2011).
188. Benedito, R. *et al.* Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF–VEGFR2 signalling. *Nature* **484**, 110–114 (2012).
189. Karkkainen, M. J. *et al.* Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat. Immunol.* **5**, 74–80 (2004).
190. Zhou, F. *et al.* Akt/protein kinase B is required for lymphatic network formation, remodeling, and valve development. *Am. J. Pathol.* **177**, 2124–2133 (2010).
191. Koltowska, K. *et al.* Mafba is a downstream transcriptional effector of Vegfc signaling essential for embryonic lymphangiogenesis in zebrafish. *Genes Dev.* **29**, 1618–1630 (2015).
192. Au, A. C. *et al.* Protein tyrosine phosphatase PTPN14 is a regulator of lymphatic function and choanal development in humans. *Am. J. Hum. Genet.* **87**, 436–444 (2010).

Acknowledgements
The authors gratefully acknowledge the following agencies for support of their work: grants from the Swedish Science Council, Swedish Cancer Foundation, World-wide Cancer Research and the Knut and Alice Wallenberg Foundation (L.C.-W.); US National Institutes of Health (NIH) grants R01 HL053793, HL084619 and P01 HL107205 (M.S.); and a Wenner–Gren foundation grant (E.G.). The authors also thank their colleagues at Yale Cardiovascular Research Center (YCVRC) and Uppsala University, as well as close colleagues around the world, for productive discussions and advice.

Competing interests statement
The authors declare no competing interests.

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.