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# Canonical and Noncanonical Vascular Endothelial Growth Factor Pathways

## New Developments in Biology and Signal Transduction

Courtney K. Domigan, Safiyah Ziyad, M. Luisa Iruela-Arispe

**Abstract**—The past 5 years have witnessed a significant expansion in our understanding of vascular endothelial growth factor (VEGF) signaling. In particular, the process of canonical activation of VEGF receptor tyrosine kinases by homodimeric VEGF molecules has now been broadened by the realization that heterodimeric ligands and receptors are also active participants in the signaling process. Although heterodimer receptors were described 2 decades ago, their impact, along with the effect of additional cell surface partners and novel autocrine VEGF signaling pathways, are only now starting to be clarified. Furthermore, ligand-independent signaling (noncanonical) has been identified through galectin and gremlin binding and upon rise of intracellular levels of reactive oxygen species. Activation of the VEGF receptors in the absence of ligand holds immediate implications for therapeutic approaches that exclusively target VEGF. The present review provides a concise summary of the recent developments in both canonical and noncanonical VEGF signaling and places these findings in perspective to their potential clinical and biological ramifications. (*Arterioscler Thromb Vasc Biol.* 2015;35:30-39. DOI: 10.1161/ATVBAHA.114.303215.)

**Key Word:** endothelial cells

The remarkable finding that genetic deletion of one vascular endothelial growth factor (VEGF) allele impairs endothelial differentiation and vascular morphogenesis<sup>1,2</sup> propelled a large number of laboratories to focus on uncovering the signaling mechanisms activated downstream receptor binding. Much has been clarified, and excellent reviews are available describing the most relevant second messengers and their relative contributions to endothelial cell migration, survival, and differentiation.<sup>3-5</sup> These effects result from VEGF ligand interacting with cell surface VEGF receptor tyrosine kinases (RTKs) on the endothelium and are considered to be canonical VEGF signaling. This mode of receptor activation deserves this classification not only because it was described first but also because it appears to be the prevalent form by which VEGF induces proliferation, migration, and vascular morphogenesis.

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Here, our objective is to highlight recent developments that uncover additional modulators of the VEGF–VEGF receptor (VEGFR) signaling axis. Specifically, we will discuss the

biological relevance of receptor/ligand heterodimers, signaling compartmentalization, contribution of cell surface proteins to downstream cellular functions, and autocrine signaling. Furthermore, we will discuss the data on ligand-independent receptor activation or noncanonical VEGF signaling. This more recent mode of receptor activation seems to occur either at the cell surface, through specific galectin or gremlin, or in an intracellular compartment via Src-mediated activation. Interestingly, Src activation was recently described for 2 of the 3 VEGF RTKs, and it seems to have distinct downstream consequences to ligand-mediated transphosphorylation in all cases.<sup>6,7</sup>

Although the contributions of alternative and noncanonical VEGF pathways have been technically difficult to ascertain, the biological effects of these pathways are on par with those of canonical VEGF and are critical to our understanding of VEGF effects in vivo. In addition to their impactful biological information, the findings can help explain differences in the clinical outcomes of therapies that specifically target either ligands or receptors.

### Refinement of Canonical Signaling

Recent publications have provided additional complexity to the process of VEGF–VEGFR2 canonical signaling without

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Nonstandard Abbreviations and Acronyms	
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
Gal1	galectin-1
Gal3	galectin-3
Nrp1	neuropilin-1
PIGF	placental growth factor
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
WT	wild type

altering its basic tenets. During the past 5 years, several studies have reinforced the contribution of receptor and ligand heterodimers and highlighted the effect of additional cell surface partners to the signaling process. These findings uncovered that small changes in the constellation of molecular players can provide a powerful impact to the signaling outcomes.

### Unconventional Heterodimers Contribute to VEGF Signaling

Canonical VEGF signaling is generally thought to be an interaction between homodimeric VEGF ligands and homodimeric VEGF RTKs (Figure 1, center). However, it has long been hypothesized that VEGFRs could heterodimerize under physiological conditions, and in fact computational models predicted that VEGFR1/2 heterodimers make up 10% to 50% of active signaling complexes in the endothelium.<sup>8</sup> In further support, heterodimerization has been well demonstrated for other RTKs. For example, in vascular smooth muscle cells, epidermal growth factor receptor (EGFR) becomes activated by platelet-derived growth factor stimulation through basal EGFR heterodimerization with platelet-derived growth factor  $\beta$ R.<sup>9</sup> Recently, experiments exploring heterodimerization of VEGF signaling components have uncovered interactions between heterodimeric ligands and heterodimeric receptors.

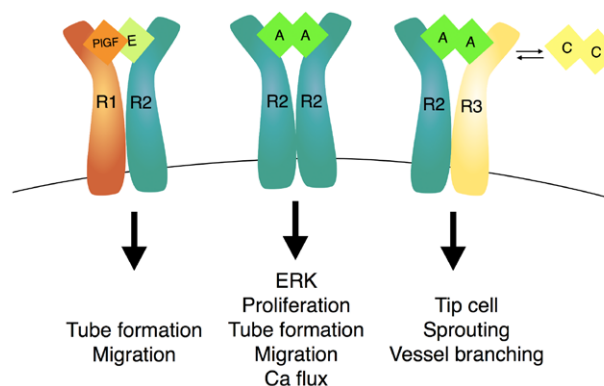
In early experiments, artificial systems were used in an attempt to clarify the possible effects of heterodimeric receptors on canonical VEGF signaling. Experiments on immortalized cells expressing high levels of both VEGFR1 and VEGFR2 showed that addition of VEGF increases VEGFR1/2 association by immunoprecipitation and produces distinct signaling outputs than homodimer receptors.<sup>10</sup> VEGFR3 is coexpressed with VEGFR2 in normal lymphatic endothelium, and dimerization in response to VEGF-C was observed in both primary cells and in 293 cells overexpressing both receptors.<sup>11</sup> Although the downstream cascade is not well understood, a unique pattern of phosphorylation was observed on kinase-dead VEGFR3 when in the presence of VEGFR2, strongly suggesting direct phosphorylation of VEGFR3 by VEGFR2 within the heterodimer.<sup>11</sup>

Recently, strides have been made to detect VEGFR heterodimers in increasingly dynamic settings. VEGFR heterodimers have been successfully detected by immunoprecipitation and in situ proximity ligation assays, both of which rely on

close physical binding of 2 disparate proteins.<sup>12</sup> Using these techniques, it was found that endothelial cells frequently coexpress VEGFR2 and VEGFR3 and that heterodimers are common in developing endothelial cells actively engaged in angiogenesis, particularly at tip cell filopodia. Although VEGFA does not bind or activate VEGFR3, blockade of VEGFR3 decreases VEGFA-mediated sprouting, suggesting that VEGFR3 contributes to VEGFA response via VEGFR2/3 heterodimers<sup>12</sup> (Figure 1, right). Phosphorylation of VEGFR2 by the VEGFR1-specific ligand placental growth factor (PIGF) uncovered the presence of VEGFR1/2 heterodimers in vivo, which act to enhance angiogenic response.<sup>13</sup>

In addition to receptors, VEGF ligands heterodimerize in certain conditions. Because of their close protein homology, PIGF and VEGFA were predicted to form heterodimers when coexpressed in the same cell<sup>14</sup> and in fact have been observed in the conditioned media of several tumor cell lines.<sup>15</sup> Although some VEGFA/PIGF heterodimers induce mitosis and chemotaxis,<sup>15</sup> in vivo data show VEGFA heterodimerization with the specific PIGF isoform PIGF1 effectively antagonizes VEGFA signaling and angiogenesis.<sup>16</sup> VEGFA/PIGF heterodimers were exploited in a tumor model where overexpression of a dysfunctional PIGF mutant acted to sequester active VEGFA in heterodimers and therefore suppressed tumor angiogenesis.<sup>17</sup> These results demonstrate that although some VEGF heterodimers have proangiogenic signaling capacity, other heterodimers act to inhibit the angiogenic signaling output.

The ability of PIGF and VEGF to heterodimerize has been used as a tool to explore the physiological function of endogenous VEGFR1/VEGFR2 heterodimers.<sup>18</sup> In these experiments, a synthetic ligand specific to VEGFR1/2 heterodimers was created by coexpressing VEGFR2-specific ligand VEGF-E (a viral VEGF mimetic protein) and the VEGFR1-specific ligand



**Figure 1.** Heterodimeric vascular endothelial growth factor (VEGF) ligand and receptors alter canonical signaling outputs. Typically, VEGF signaling is thought to take place between 2 homodimers of VEGF which causes the dimerization of 2 homodimers of VEGFR2 (center), leading to extracellular regulated kinases (Mapk1; ERK) activation and downstream cellular effects. Artificial heterodimers of placental growth factor (PIGF) and VEGF-E have been used to study signaling outputs of VEGF receptor (VEGFR)1/VEGFR2 heterodimers because PIGF binds only VEGFR1 and VEGF-E binds only VEGFR2 (left). VEGFR1/VEGFR2 signaling is distinct from VEGFR2 homodimerization because it induces tube formation and cellular migration, but not other cellular effects. Heterodimers of VEGFR2/VEGFR3 can be induced by either VEGF-A or VEGF-C ligand, contribute to sprouting, and are found endogenously at tip cell filopodia (right).

PIGF. Application of this ligand to endothelial cells induced several angiogenic responses such as VEGFR2 phosphorylation, migration, and tube formation. However VEGFR1/2 activation did not induce proliferation, extracellular regulated kinases (Mapk1; ERK) signaling, and other VEGFR2 functions, suggesting that the heterodimer holds a unique signaling function<sup>18</sup> (Figure 1, left).

Direct and indirect detection of VEGFR and ligand heterodimers indicates that heterodimerization is a true physiological phenomenon. The experiments described here carefully target heterodimeric complexes for activation or blockade and taken together suggest that distinct signaling effects are easily overlooked when only homodimers are expected. Incorporation of VEGF ligand and receptor heterodimers into the canonical model may help explain otherwise unpredicted signaling and developmental effects.

### New Developments in VEGFR1 Signaling

The effects of VEGFR1 have been difficult to pin down in endothelial culture models because VEGFR1 has a 10-fold lower kinase activity than VEGFR2<sup>19</sup> and it induces little detectable downstream signaling.<sup>20</sup> VEGFR1 does, however, have a clear biological and signaling impact, particularly during development, as indicated by homologous recombination<sup>21</sup> and in pathological conditions such as diabetes mellitus. Diabetes mellitus is marked by progressive nephropathy caused by a disruption in osmotic pressure, which damages the specialized endothelium of the kidney glomeruli. Early progression of this disease is associated with abnormal VEGF-mediated angiogenesis.<sup>22</sup> Peptide inhibition of VEGFR1 in a mouse model increased symptoms of nephropathy, accompanied by glomerular cell death.<sup>23</sup> This blockade of VEGFR1 resulted in a suppression of diabetes mellitus–induced phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and phospho-Akt and upregulation of FoxO3a (Forkhead box O3A). The signaling cascade resulting from VEGFR1 blockade depressed phospho-nitric oxide synthase 3 (endothelial cell; eNOS), producing an increase of oxidative stress within the kidney. These results show that normal VEGFR1 signaling provides a protective effect in the kidney and in fact signals to stimulate nitric oxide production within endothelial cells.<sup>23</sup>

Although these experiments demonstrate clear effects driven by VEGFR1, it is unclear whether the primary signaling event required the kinase activity of VEGFR1. Over a decade ago, it was elegantly demonstrated that deletion of the intracellular kinase domain of VEGFR1 is fully compatible with normal angiogenesis and embryonic development,<sup>24</sup> unlike the inactivation of the full receptor.<sup>21</sup> Combined these and several other findings are consistent with a model in which VEGFR1 act as a decoy receptor, blocking VEGF access to VEGFR2 rather than producing an independent signaling cascade on its own. Recent experiments have helped develop our understanding of the cellular ramifications of VEGFR1 regulation on VEGFR2 signaling in conditions of stress.

Both prolonged VEGF exposure and cellular stress result in an increased ratio of VEGFR1:VEGFR2 in the endothelium. Endothelial cells exposed to VEGF, for example in a squamous cell carcinoma setting, express complex

VEGFR1, whereas normal endothelium display higher levels of VEGFR2.<sup>25</sup> Prolonged VEGF exposure induces Akt/ERK survival pathways which inhibit degradation of VEGFR1. Concurrently, VEGF signaling through the c-Jun N-terminal kinase (Mapk8; JNK)/c-Jun pathway, leads to the endocytosis and degradation of VEGFR2, keeping the signaling pathway in check. VEGFR1 is required for the VEGF-induced survival advantage, most likely mediated by an increase in Bcl-2 expression.<sup>25</sup>

Under serum starvation conditions, normal endothelium first elevates VEGFR2 levels, an event that is followed by its downregulation 24 hours thereafter.<sup>26</sup> Meanwhile soluble VEGFR1 decreases during the early phase, and then increases to above normal levels after the 24-hour period. Although full-length VEGFR1 levels are not altered during this time, increased soluble VEGFR1 sequesters VEGF in the extracellular matrix (ECM), preventing it from accessing VEGFR2. Accordingly, the serum starvation response increases responsiveness to VEGF and prosurvival cues at early stages; but at late stages, the effect leads to a reduction in VEGF responsiveness and an increase in apoptosis.<sup>26</sup> Although the direct signaling output of VEGFR1 is unclear, it seems to be highly valued by the endothelium as a tool to control VEGFR2 function.

### Alteration of VEGF Signaling Outputs by Cell Surface Proteins

Integrins are a family of ECM-binding receptors, that on ligand engagement, induce angiogenic signaling and survival pathways within the endothelium.<sup>27</sup> Thus, it should not come as a surprise that activation of integrin receptors might be tightly associated with VEGFR2 responses to VEGF. Addition of VEGF induces physical association of VEGFR2 with integrin subunit  $\beta$ 3, and when integrin signaling is blocked, VEGFR2 cannot be fully phosphorylated.<sup>28</sup> Cross talk between these 2 receptor classes has been demonstrated in several biological platforms, where activation of either receptor stimulates binding and activation of the other.<sup>27</sup>

More recent experiments demonstrate that VEGF binding to ECM alters VEGFR2/integrin cross talk.<sup>29</sup> VEGF is spliced into  $\geq 9$  different isoforms, which vary in their ability to bind to the ECM or diffuse freely in a soluble form.<sup>30</sup> VEGF isoforms elicit unique vascular phenotypes, but only recently has this effect been characterized at the signaling level. The bound or soluble availability of VEGF robustly alters the kinetics of VEGFR2 signaling by manipulating its relationship to  $\beta$ 1 integrin.<sup>29</sup> Bound VEGF increases the association of VEGFR2 with  $\beta$ 1 integrin, which alters cell surface organization of VEGFR2 clusters and prolongs receptor activation. This provides a distinct signaling compartmentalization than the one offered by soluble ligand. Differences in signaling clusters at the cell surface translate to an extension of the downstream kinetics of the p38/MAPK (mitogen-activated protein kinase) pathway.<sup>29</sup> Together these results indicate that the ECM context of the endothelium affects not only direct activation of integrins but also modulates interactions between integrins and VEGFRs which has downstream signaling consequences.

Progressively complex endothelial receptor clusters are being uncovered which may fine-tune angiogenic responses

in different tissue beds. For example, CD63 is a transmembrane tetraspanin expressed by endothelial cells that, when silenced, results in abrogated angiogenic response to VEGF and other growth factors.<sup>31</sup> CD63 binds both VEGFR2 and  $\beta 1$  integrin, and ablation of CD63 was found to disrupt the VEGFR2- $\beta 1$  integrin complex formation and downstream signaling in response to VEGF.<sup>31</sup> Another novel VEGFR2 complex important for conveying VEGF signaling requires coordination by syndecan-1. Syndecan-1 organizes a complex of VEGFR2, vascular endothelial (VE)-cadherin, and  $\alpha v \beta 3$  integrin, without which the endothelium cannot respond to VEGF or VE-cadherin engagement.<sup>32</sup> Further experiments coupling cell surface complexes found *in vitro* with *in vivo* functional experiments may help unravel signaling disparities among different biological settings.

Interactions between receptors, ligands, ECM, and intracellular signaling machinery are further muddled by the fact that these complex interactions occur in a 3-dimensional environment. In a cancer setting, for instance, VEGFRs are often expressed both on neoangiogenic endothelium, as well as on the tumors themselves, and so have an opportunity to interact with ligand and each other in opposing cell types (referred to as trans interactions).

On VEGF stimulation, VEGFR2 and its coreceptor neuropilin-1 (Nrp1) were found to form complexes *in trans* at the cell-cell interface between cocultured cells expressing either single receptor.<sup>33</sup> These complexes produce distinct signaling cascades in endothelial cell, in part because of improper internalization of VEGFR2. In mouse tumor models, transexpression of Nrp1 suppressed angiogenesis and tumor growth by arresting VEGFR2 internalization and therefore downstream signaling.<sup>33</sup> These findings further expand the circumstances that must be taken into account when studying angiogenic signaling pathways. Realistically, a 2-dimensional monoculture can only reveal so much about the biology at work in a human patient.

### Expanding Biological and Signaling Effects of Autocrine VEGF

Canonical VEGF signaling occurs in a paracrine manner, where cell surface endothelial VEGFRs are activated by VEGF ligand originating from a secondary cell type. However, recent explorations have expanded our understanding of VEGF pathways to include autocrine signaling in the canonical pathway, meaning activation of receptor by ligand produced in the same cell. We also discuss the evidence for intracrine signaling, which is cell-autonomous autocrine signaling that occurs entirely within the cell in an intracellular compartment.

### Autocrine VEGF Signaling in the Endothelium

Endothelial cells constitutively express VEGFRs, and a subset of endothelial cells also coexpress VEGF in homeostatic conditions. Aortic endothelial cells in particular exhibit the highest levels of VEGF expression compared with other endothelial cell types and also showed phospho-VEGFR2.<sup>34</sup> Besides the aorta, arterial endothelium has been shown to express VEGF in a salt-and-pepper pattern in larger vessels where it is induced by shear stress.<sup>35</sup> Genetic experiments ultimately provide the best evidence that autocrine VEGF

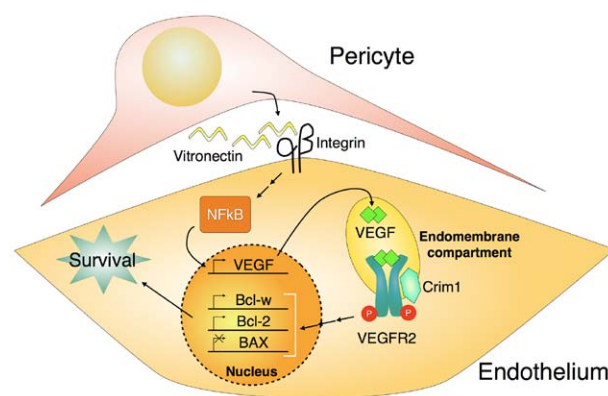
signaling occurs within the endothelial compartment itself. Genetic excision of VEGF from the endothelium uncovers a broad need for autocrine VEGF in the vasculature. Endothelial VEGF ablation results in degeneration of the endothelia in multiple vascular beds, resulting in a sudden death phenotype that is lethal in 55% of the mutant mice by 6 months of age.<sup>36</sup>

The autocrine VEGF pathway in the endothelium is poorly understood, likely because it occurs through alternative pathways or at a low background level. However, some information about autocrine regulation has emerged. For example, heterotypic cell-cell interactions with pericytes have been shown to increase VEGF-mediated survival pathways in the endothelium.<sup>37</sup> In this case, pericytes secrete the ECM protein vitronectin which is a ligand for endothelial  $\alpha v$  integrin, and this induces VEGF upregulation and downstream survival factor Bcl-w expression (Figure 2). Another molecular player in this pathway is the transmembrane protein cysteine-rich transmembrane bone morphogenic protein regulator 1 (Crim1) that enhances autocrine VEGF signaling through VEGFR2 in an intracellular compartment.<sup>38</sup> In this case, autocrine VEGF potentiated proliferation and survival of endothelial cells that was associated with the expression of antiapoptosis factor Bcl-2<sup>38</sup> (Figure 2).

### Autocrine VEGF Signaling in Cancer

It is well documented that tumor cells evade apoptotic signals by co-opting proliferation and survival machinery, the VEGF pathway is no exception. Many nonendothelial tumors express VEGF as well as VEGFRs, and autocrine signaling has been identified in, to name a few, breast cancer,<sup>39</sup> colorectal cancer,<sup>40</sup> epidermal tumors,<sup>41</sup> and precursor lesions to esophageal cancer.<sup>42</sup>

In some instances, autocrine tumor signaling takes place via the major endothelial VEGFR (VEGFR2)<sup>41,43</sup>; however, the downstream signaling cascades may be distinct from classic angiogenic signaling. Glioblastoma multiform, a malignant brain tumor, expresses high levels of VEGF and VEGFR2 in



**Figure 2.** Autocrine vascular endothelial growth factor (VEGF) signaling in the endothelium. Pericytes secrete vitronectin which is a ligand for integrin  $\alpha v$ . Integrin signaling increases nuclear factor (NF)  $\kappa$ B activity, which activates expression of VEGF. Intracellular VEGF signaling via VEGFR2 in an endomembrane compartment is enhanced by the presence of the transmembrane protein cysteine-rich transmembrane bone morphogenic protein regulator 1 (Crim1). Autocrine VEGF signaling causes increased expression of pro-survival factors Bcl-w and Bcl-2 and decreased expression of proapoptotic BCL2-associated X protein (BAX), promoting cell survival.

glioma stem-like cells. In this CD133+ population, constitutively active phospho-VEGFR2 is found primarily in the tumor cytoplasm,<sup>44</sup> which is atypical of stimulated endothelium. However, the autocrine VEGF signaling cascade observed in precancerous Barrett esophagus cells is somewhat analogous to that observed in VEGF-stimulated endothelial cells.<sup>42</sup> In Barrett cells, autocrine VEGF signals through VEGFR2 inducing proliferation via phospholipase C (PLC)- $\gamma$ /protein kinase-C/ERK signaling.<sup>42</sup>

Increasingly it has been found that the VEGF coreceptor Nrp1 is highly correlated with tumor malignancy and is in fact a major player in autocrine VEGF pathways in cancer.<sup>45</sup> Even in glioblastoma stem cells, where proliferative signaling takes place through VEGFR2, the presence of Nrp1 modifies VEGFR2 recycling and boosts stores of intracellular VEGFR2 to high levels.<sup>44</sup> Autocrine VEGF signaling through Nrp1 is essential for maintenance of cancer stem cells in squamous tumors of the skin, but furthermore, deletion of Nrp1 from normal epidermis prevents tumor initiation entirely.<sup>46</sup> RhoA<sup>47</sup> and Rac-1<sup>48</sup> have been reported as effectors of Nrp1. In renal cell carcinoma, Nrp1 fell upstream of Ras activation and phosphorylation of ERK1/2 and Akt, even in the absence of other VEGFRs.<sup>49</sup> In human melanoma cells, Nrp1 also induces Akt activation, which is responsible for an invasive migratory cellular phenotype.<sup>50</sup>

In many tumor models, autocrine VEGF signaling relies on abnormal interactions between unrelated receptors that work synergistically to enhance survival and proliferation. Ablation of VEGF or VEGFR1 in epidermal tumor cells slows tumor proliferation, but this ablation in an EGFR-deficient background inhibits tumor formation entirely, suggesting a contribution of both EGF (epidermal growth factor) and VEGF pathways.<sup>41</sup>

The autocrine VEGF signaling pathway was dissected in great detail in triple-negative breast cancers. Here, it was found that VEGF/Nrp2 interaction stimulates  $\alpha 6 \beta 1$  integrin, which in turn activates focal adhesion kinase.<sup>51</sup> Focal adhesion kinase induces expression of the Hedgehog effector Gli1, although canonical Hedgehog signaling activation was not observed.<sup>51</sup> Instead, focal adhesion kinase activation of

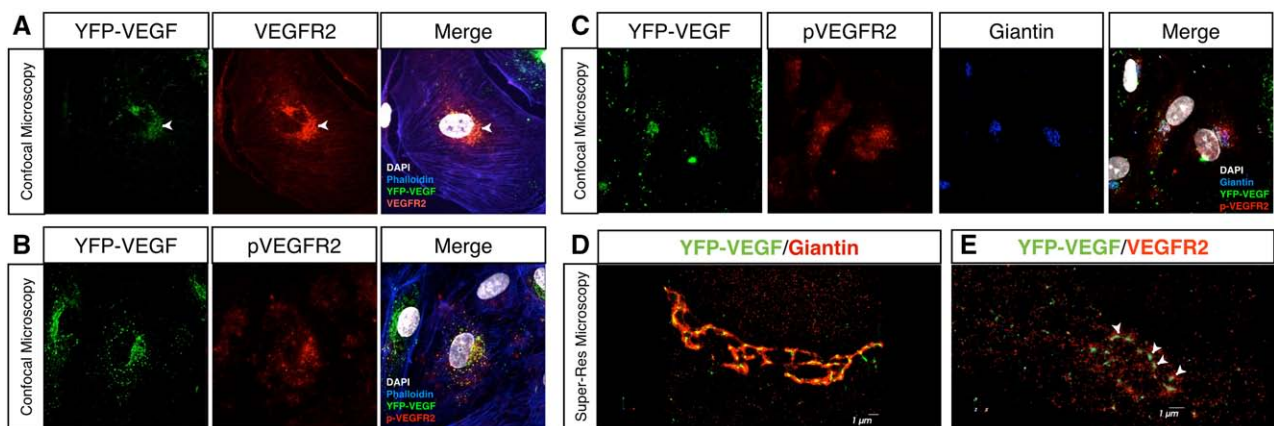
the Ras/MEK (mitogen activated protein kinase 7) pathway is required for induction of Gli1 expression, which in turn activates the stem cell factor BMI-1 (Bmi1 proto-oncogene, polycomb ring finger-1). A feedback loop is then enacted, where Gli1 enhances Nrp2 and VEGF expression, thus amplifying the signaling pathway and inducing an aggressive cancer stem cell phenotype.<sup>51</sup>

### Case for Intracrine VEGF Signaling

In theory, autocrine signaling may occur through secretion of VEGF which then interacts with cell surface receptors on the same cell type, if not the same exact cell. But several key experiments indicate that autocrine signaling can actually be intracrine in nature (ie, it occurs within an intracellular compartment and it does not require release of the ligand from the cell).

Studies on VEGFR2 have uncovered unusual RTK trafficking patterns that may be compatible with compartmentalized intracellular signaling. Early reports showed a large portion of total VEGFR2 resides within the cell in endosomal storage compartments which translocate to the cell surface on VEGF stimulation.<sup>52</sup> Later studies find the majority of endothelial VEGFR2 resides in the Golgi compartment at any one time,<sup>53</sup> suggesting endosomes and Golgi as potential sites of intracrine ligand/receptor interaction. Although we observe that a small number of endosome-like compartments contain VEGF, the majority of intracellular VEGF (visualized with a yellow fluorescent protein [YFP]-VEGF construct) colocalizes to a perinuclear compartment with VEGFR2 (Figure 3A and 3E), which is confirmed to be the Golgi compartment (Figure 3C). VEGF and VEGFR2 do seem to interact in this compartment, as intracellular phosphorylation of VEGFR2 can be observed within the Golgi when YFP-VEGF is expressed (Figure 3B and 3C). Super-resolution microscopy shows VEGF localization to the lumen of the Golgi (Figure 3D) and is clumped in tight association with VEGFR2 (Figure 3E).

Much of the compelling functional evidence for intracrine signaling relies on the comparison of extracellular antibody blockade of VEGF to intracellular VEGFR-inactivation with small molecule inhibitors. The autocrine signaling effects



**Figure 3.** Autocrine vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) 2 interactions in the Golgi. Confocal image shows yellow fluorescent protein (YFP)-VEGF colocalization with VEGFR2 (A) and phospho-Tyr1175 VEGFR2 (B) in a perinuclear region of the cell. C, Giantin staining confirms that YFP-VEGF and phosphorylated VEGFR colocalize in the Golgi compartment. D, Super-resolution images show YFP-VEGF localized to pockets within the lumen of the Golgi compartment (E) and in close proximity to intracellular VEGFR2 (arrowheads). See online-only Data Supplement for detailed experimental methods and video showing 3-dimensional view of D and E.

of VEGF in the endothelium can be inhibited by SU5416 (VEGFR2 inhibitor), but not anti-VEGF antibody,<sup>36</sup> which is also the case in the context of endothelial Crim1 deficiency.<sup>38</sup> Tumor stem cells<sup>44</sup> and hematopoietic stem cells<sup>54</sup> were shown to require VEGF intracellularly using a similar technique. Consistent with these experiments, meta-analysis of patients treated with RTK inhibitors found slightly more severe vascular side effects than those treated with extracellular VEGF-traps.<sup>55</sup>

One caveat of the external versus internal pharmaceutical approach is the promiscuity of small molecule RTK inhibitors. Although small molecule VEGFR inhibitors strongly suppress VEGFR phosphorylation in response to ligand, direct action of inhibitors on other non-VEGFRs has been reported. For instance, the VEGFR2 inhibitor SU5416 (which has gone to phase III clinical trials) is also a potent agonist of the aryl hydrocarbon receptor which regulates immune function.<sup>56</sup> Similarly, SU4312, another VEGFR2 inhibitor, directly inhibits neuronal nitrogen-oxygen synthase in cerebellar granule neurons.<sup>57</sup> Because several of the VEGFR inhibitors used in basic research have been approved for human trials, these molecules are now being closely scrutinized for other functions. Potentially, as study of these inhibitors becomes increasingly detailed, blockade of intracrine VEGF signaling by kinase inhibitors may be partially attributable to an off-target effect, particularly in animal models.

Another set of experiments that lend support to intracrine VEGF signaling involves the supplementation of VEGF-deficient cell lines with exogenous VEGF. Presumably, addition of extracellular VEGF mimics autocrine signaling that occurs at the plasma membrane, but in several cases this supplement fails to rescue a VEGF-deficient phenotype. This effect is observed in colorectal cancer,<sup>40</sup> as well as in the VEGF-deficient endothelial compartment.

It should be noted that several assumptions are made in VEGF supplementation experiments. A major assumption is that all VEGF is equal. If a tissue of interest secretes a specific VEGF isoform, but is supplemented with another (supplementation is almost universally done with recombinant VEGF164), then a one-to-one comparison of rescue cannot be made. Similarly, post-translational modifications to VEGF, such as glycosylation patterns, may be specific to each cell type and may interfere with signaling in ways we do not yet understand.

An experiment that partially addresses the issues raised by VEGF supplementation is to use VEGF derived directly from the compartment of interest. To determine if autocrine signaling is intracrine, cells from VEGF-deficient and wild-type (WT) populations can be cocultured so that the WT cells can supplement extracellular autocrine VEGF to neighboring mutant cells. If the VEGF-knockout phenotype persists in these conditions (these cells proliferate less or die more than their WT counterparts), then it can be concluded that this cell type undergoes intracrine signaling.

These types of experiments have been conducted in several model systems. Fluorescent-labeled WT and VEGF-deficient tumor cells were mixed in equal number and injected into a mouse xenograft model.<sup>49</sup> In this context, it was found that VEGF-deficient cells had a growth deficiency despite close contact with WT cells.<sup>49</sup> Similarly, coculture experiments were performed which allowed labeled WT and VEGF-knockout

endothelial cells to intermingle. In this case, the endothelial VEGF produced by neighboring cells was unable to rescue VEGF-knockout cell death phenotype.<sup>36</sup> These results have been questioned however, namely because autocrine VEGFR2 phosphorylation is lower in sparse HUVECs (human umbilical vein endothelial cells) and higher in confluent monolayers, which is interpreted to indicate that crowded cells have more access to VEGF secreted from neighboring cells.<sup>35</sup>

As the biological ramifications of autocrine VEGF signaling become increasingly understood, the underlying intracellular mechanisms require additional investigation. Most studies assume that autocrine VEGF signaling occurs through VEGFR2 activation, and some direct evidence of VEGFR2 phosphorylation has been observed in the presence of phosphatase inhibitors.<sup>36,38</sup> However, the downstream signaling cascades triggered by this phosphorylation event have not been investigated in-depth or the involvement of other VEGFRs.

Biologically, in the endothelium, autocrine VEGF signaling supports homeostasis. This is in contrast to extracellular VEGF that promotes endothelial activation: proliferation, migration, and angiogenesis. It is well known that receptors at the plasma membrane encounter a distinct lipid composition and pH environment from those in intracellular compartments.<sup>58</sup> These compartments, therefore, provide distinct chemical environments that may themselves alter signaling kinetics but also allow interaction with a unique set of spatially restricted partners. The RTK EGFR, for example, has been shown to localize to the mitochondria on ligand stimulation.<sup>59</sup> Here, EGFR interacts with a unique set of mitochondrial proteins to regulate bioenergetics and cell death, distinct from the players involved in canonical EGFR signal transduction.<sup>59</sup> Because discoveries in spatially isolated signaling are ongoing as a result of technical and paradigm advances, major differences in autocrine VEGF signaling from paracrine VEGF signaling are anticipated.

### Noncanonical Signaling: VEGF-Independent Activation of VEGF Receptors

One of the major recent advances in our understanding of VEGF signaling has been the realization that VEGF RTKs can be activated in a VEGF-independent manner, which we refer to as noncanonical VEGF signaling. This can occur through alternative ligands outside of the VEGF family or intracellularly by Src kinases.

#### Alternative Ligands: Galectin and Gremlin Binding to VEGF Receptors

VEGFRs, and nearly all cell surface proteins, must be glycosylated for proper function on endothelial cells.<sup>60</sup> Once dismissed as a simple chaperone for protein-folding, glycosylation is an often underestimated post-translational modification that in fact regulates a wide variety of biological functions once proteins are secreted. Glycosylation alters the biological function of proteins in 3 major ways: (1) stabilization of protein folds and ECM interactions, (2) direct modulation of protein function, and (3) provision of binding sites for glycan-binding proteins.<sup>61</sup> It is this third function that is most relevant to VEGF signaling, where glycan-binding proteins, called galectins, can act as alternative ligands to glycosylated RTKs.

Galectin-1 (Gal1) binds the VEGF coreceptor Nrp1 via its carbohydrate-binding domain in tumor-associated endothelial cells.<sup>62,63</sup> In the absence of VEGF, Gal1–Nrp1 interactions directly contribute to endothelial adhesion, and through the coactivation of VEGFR2, Gal1–Nrp1 induces JNK-mediated cellular migration<sup>62</sup> (Figure 4A). Vascular permeability, a cellular effect highly regulated by VEGF, can be induced by Gal1 signaling through VEGFRs.<sup>63</sup> In this case, phosphorylation of VEGFR1 triggers a PI3K/Akt/RhoA signaling cascade that reduced the amount of VE-cadherin at cell–cell junctions (Figure 4B). Both Nrp1 and VEGFR1 are required for this permeability effect, but VEGFR2 was found to be unnecessary.<sup>63</sup>

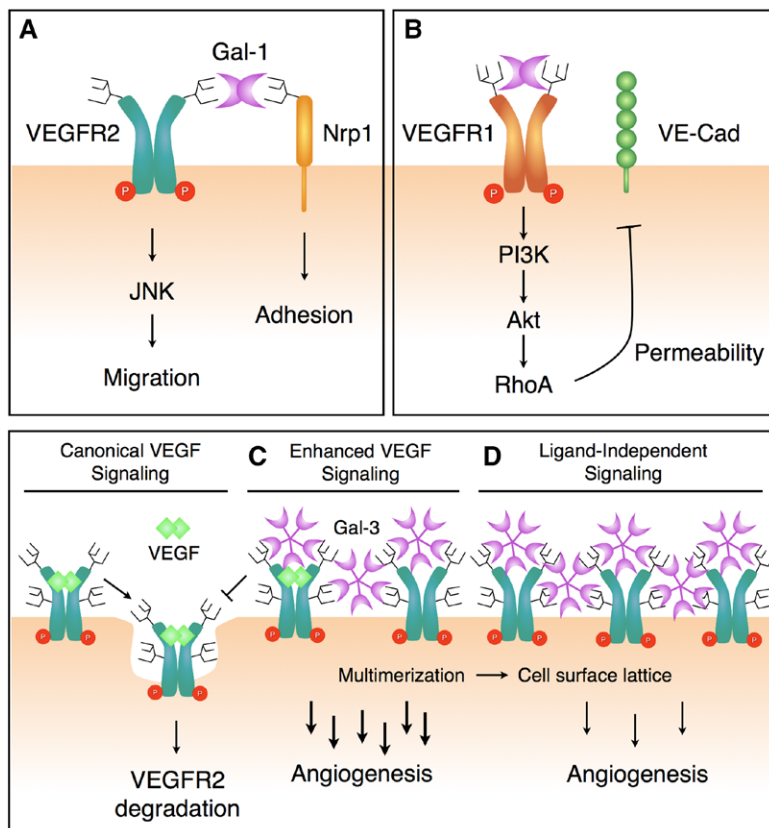
VEGFR2 is susceptible to binding by galectin-3 (Gal3),<sup>64</sup> a galectin capable of multimerizing and forming cell surface lattices with glycoprotein-binding targets.<sup>65</sup> Again, in the absence of VEGF, Gal3 binding induces a VEGF-like signaling at the cell surface but also amplifies the response of VEGFR2 to canonical VEGF ligand<sup>64</sup> (Figure 4C and 4D). Gal3-mediated alteration to VEGFR2 signaling is likely because of the incorporation of these proteins in plasma membrane lattice structures, which increases VEGFR2 retention at the cell surface<sup>64</sup> (Figure 4C and 4D). Previously, it was demonstrated that VEGF-induced internalization of VEGFR2 plays an important role in potentiation of ERK signaling by spatially separating the receptor from dephosphorylation by the phosphatase Ptp1b at the plasma membrane.<sup>66</sup> Although internalization seems to be relevant for potentiation of canonical signaling, alternative ligands, such as galectins, do not seem to require this internalization process.

Combined treatment of Gal1 and Gal3 enhances angiogenic tube formation at the cellular level, which is not observed on

addition of either single galectin.<sup>67</sup> Although the addition of single (or combined) galectins induced equivalent phospho-VEGFR2 levels, only combined Gal1 and Gal3 treatment uniquely induces VEGFR1 phosphorylation.<sup>67</sup> Similar to the effects of Gal3 on VEGFR2 trafficking, combined treatment decreased endocytosis of VEGF1, trapping it at the plasma membrane. The downstream effects of this were the induction of Hsp27 and amplification of ERK phosphorylation.<sup>67</sup>

Because the enzymes responsible for protein glycosylation vary from cell to cell and are highly reactive to cellular stimuli, the ability of Gal1 to bind to VEGFRs is context dependent. It was found that endothelial cells cultured in a tumor-like environment (mimicked in vitro with hypoxia and immunosuppressive cytokines) produced a high level of Gal1 glycan-epitopes.<sup>68</sup> In this setting, VEGFR2 is highly decorated with Gal1 epitopes, and direct VEGFR2–Gal1 binding is enhanced, even in the absence of VEGF ligand. Gal1 induces VEGFR2 autophosphorylation and triggers an Akt–Erk1/2 signaling cascade that closely resembles canonical VEGF signaling, although with altered VEGFR2 internalization kinetics.<sup>68</sup> This interaction is not exclusive to the tumor environment. Gal1–VEGFR2 signaling occurs in other specialized contexts, such as vessel growth in the developing placenta and maternal spiral artery remodeling.<sup>69</sup> Further investigation into the cellular glycosylation signatures of specific organs and pathological conditions may unveil differences in the angiogenic response because of galectin/VEGFR interactions.

Another alternative ligand that has been shown to induce VEGFR2 signaling in the absence of canonical VEGF is the protein gremlin, which belongs to the same cystein-knot superfamily as VEGF. Addition of purified gremlin, which is a BMP



**Figure 4.** Noncanonical ligand-independent signaling by galectins. **A**, In the absence of vascular endothelial growth factor (VEGF) ligand, galectin-1 binds neuropilin-1 (Nrp1), inducing endothelial adhesion and signaling through VEGF receptor (VEGFR) 2 which activates a migration cascade. **B**, Galectin (Gal) 1 binding to VEGFR1 produces a signaling cascade that degrades vascular endothelial (VE)-cadherin increasing vascular permeability. **C**, On canonical VEGF ligand binding, a short burst of angiogenic signaling occurs and VEGFR2 is quickly degraded. **D**, Gal3 multimerizes to form a cell surface lattice of VEGFR2 which resists degradation, allowing prolonged angiogenic signaling. **E**, Gal3 also induces VEGFR2 signaling in the complete absence of ligand.



(bone morphogenic protein) antagonist, was unexpectedly found to induce sprouting, migration, and invasion of endothelial cells in angiogenic assays.<sup>70</sup> Gremlin directly binds VEGFR2, induces VEGFR2 autophosphorylation on stimulation,<sup>71</sup> and further induces a complex of  $\alpha v \beta 3$  integrin with VEGFR2.<sup>72</sup> These functions are highly analogous to the effects of canonical VEGF ligand, suggesting a mode of action as a VEGF mimic.

### Ligand-Independent VEGF Receptor Signaling

VEGFRs can participate in noncanonical ligand-independent signaling cascades in specific circumstances. For example, shear stress has been shown to promote VEGF-independent, Src-mediated VEGFR2 activation leading to regulation of Akt and eNOS function *in vitro*.<sup>73</sup> However, demonstration of ligand-independent VEGFR2 phosphorylation *in vivo* was lagging until recently.

Diabetes mellitus is characterized by endothelial dysfunction that is, in part, because of hyperglycemia-induced reactive oxygen species (ROS).<sup>74</sup> Low levels of ROS are a necessary contributor in normal signaling cascades.<sup>75</sup> Excessive ROS, however, induces aberrant phosphorylation of VEGFR2, even in the absence of autocrine or paracrine VEGF ligand.<sup>7</sup> On ROS-induced phosphorylation, VEGFR2 does not undergo autophosphorylation observed in canonical signaling. Rather, similar to shear stress conditions, VEGFR2 is phosphorylated by the Src family of kinases, which induces downstream activation of PLC- $\gamma$ , but not p38.<sup>7</sup> ROS-induced signaling is intracrine and occurs in the Golgi compartment, after which VEGFR2 is degraded and subsequent response to VEGF addition is lessened because of lack of receptor.<sup>7</sup> For this reason, diabetic animals exhibit decreased VEGFR2 phosphorylation in response to VEGF, an effect that is strikingly rescued by blockade of ROS production by NAC (N-acetyl cysteine) treatment.<sup>7</sup>

In addition to VEGFR2, VEGFR1 undergoes ligand-independent signaling in macrophages in the context of atherosclerosis.<sup>76</sup> VEGFR1 can be expressed at high levels in macrophages, and addition of low-density lipoprotein induced VEGFR1 endocytosis in complex with low-density lipoprotein receptor. VEGFR1 autophosphorylation was observed in response to low-density lipoprotein treatment and triggered a similar downstream pathway to that found in VEGF-stimulated endothelial cells.<sup>76</sup> This signaling pathway regulated macrophage migration in response to low-density lipoprotein and may take part in macrophage recruitment and activation in atherosclerotic plaques.<sup>76</sup>

The role of Nrp1 in VEGF signaling has been difficult to understand, but a point of concordance is that Nrp1 acts as a coreceptor that binds and presents VEGF ligand to VEGFR2 to enhance VEGFR2 signaling effects.<sup>77</sup> However, Nrp1 has been reported to have some capacity as an independent signaling entity. Nrp1's 3 C-terminal (cytoplasmic) amino acids are required for ligand-dependent migration and survival, independent of the presence of VEGFR2.<sup>47,78</sup> New work shows Nrp1 also contributes to angiogenesis in an alternative, ligand-independent fashion. Stimulation of endothelial cells with the integrin ligand fibronectin induces Nrp1-dependent phosphorylation of paxillin.<sup>79</sup> It was found that the presence of Nrp1 mediates formation of a complex between integrins and Abl1

responsible for cytoskeletal remodeling and angiogenesis on fibronectin,<sup>79</sup> entirely in the absence of a specific Nrp1 ligand.

VEGFR3, the canonical receptor for VEGFC, also undergoes ligand-independent signaling in nonpathological conditions. VEGFR3 associates with  $\beta 1$  integrin and becomes activated on addition of  $\beta 1$  ECM ligands, even in the absence of VEGFC.<sup>80</sup> Somewhat similar to ROS-mediated VEGFR2 signaling, ligand-independent phosphorylation of VEGFR3 does not occur by autophosphorylation but instead is mediated by c-Src in complex with VEGFR3 and  $\beta 1$  integrin.<sup>81</sup> c-Src phosphorylation allows recruitment of the adaptor proteins CRKI/II (v-crck avian sarcoma virus CT10 oncogene homolog) and SHC (src homology 2 domain containing transforming protein) and induces downstream JNK phosphorylation.<sup>81</sup>

Treatment with VEGFC is proangiogenic, and antibody blockade of VEGFR3–VEGFC interactions decrease angiogenesis, suggesting proangiogenic canonical signaling by VEGFR3 receptors. It was, therefore, unexpected to find that ablation of VEGFR3 in endothelial cells results in excessive angiogenic sprouting.<sup>6</sup> This result and others suggests that VEGFR3 mediates bimodal signaling: active proangiogenic signaling in response to ligand and passive antiangiogenic signaling in the absence of ligand. *In vivo*, passive ligand-independent signaling is responsible for inducing Notch1 transcriptional targets, maintaining a nonangiogenic stalk-cell phenotype. This ligand-independent signaling pathway takes place by way of PI3K and deregulation of transcription factor FoxC2.<sup>6</sup>

### Perspective

Decades of studies on VEGF signaling have converged on a straightforward model: paracrine VEGF interacts with specific RTKs on the surface of endothelial cells inducing angiogenesis. Importantly, targeting canonical VEGF signaling has produced successful therapeutic strategies against diseases where VEGF-mediated vessel outgrowth is the major contributor to the pathology, such as in macular degeneration and some forms of cancer.

Hidden in the strength of the canonical model are wealth of alternative modes of VEGF signaling. These include partner receptors and ligand-independent activation that have proven to alter, enhance, and convey signaling to subsets of downstream effectors not as obviously impacted by canonical signaling. The papers outlined in this review have produced compelling data to indicate that the VEGF field is moving beyond the straight forward paracrine model.

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None.

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## Significance

Although over 40 years of vascular research have created a model of canonical paracrine vascular endothelial growth factor (VEGF) signaling, recent advances in the field continue to uncover important additional nuances in VEGF biology. Highlighted in this review are alternative modes of VEGF signaling including heterodimeric ligands and receptors, cross-activation of VEGFR's by partner receptors, and autocrine/intracrine VEGF signaling. Also explored is noncanonical signaling, which occurs in the absence of VEGF ligand altogether. These results modify the VEGF signaling paradigm and provide a broader picture of VEGF biology.

# Arteriosclerosis, Thrombosis, and Vascular Biology



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## Canonical and Noncanonical Vascular Endothelial Growth Factor Pathways: New Developments in Biology and Signal Transduction

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### **Immunofluorescent Staining**

YFP-VEGF overexpressing HUVECs were grown to confluency on 22 x 22 glass cover slips (12-548-B, Fisher Scientific) and then serum starved overnight. Before fixation, cells were incubated for 5 minutes in 200uM sodium orthovanadate diluted in serum free media. Fixation was achieved using 1% PFA for 10 minutes. Cells were then permeabilized using 0.1% Triton PBS for 2 min. 10% normal serum in 0.01% Triton PBS was used to block the cells for 1 hour. Primary followed by fluorophore-conjugated secondary antibodies were diluted in 3% normal serum in 0.01% Triton PBS and incubated with cells for 1 hour: mouse monoclonal giantin (Clone 9B6, ab37266, Abcam, Boston, MA), rabbit polyclonal giantin (ab24586, Abcam), phospho- tyrosine 1175 VEGFR2 (Clone 19A10, #2478, Cell Signaling Technologies, Danvers, MA), total VEGFR2 (55B11, #2479, Cell Signaling Technologies), anti-GFP (632381, Clontech, Mountain View, CA), anti-mouse CF 568 (20105, Biotium, Hayward, CA), anti-rabbit Alexa Fluor 488 (A-11034, Invitrogen, Grand Island, NY), anti-mouse Cy3b (from Vutura), and anti-rabbit 647 (from Vutura). A Zeiss LSM710 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY) was used to image the subcellular localization.

### **3D Single Molecule Localization (SML) Super-Resolution Imaging**

Superresolution images were recorded on a Vutara SR 200 (Vutara, Inc., Salt Lake City, UT) commercial microscope based on the Single Molecule Localization (SML) biplane FPALM technology <sup>1,2</sup> with a 60X PLANAPO water immersion objective NA 1.2 and Photometrics Evolve 512 EMCCD camera with gain set at 50, and frame rate at 50 Hz. Samples were imaged using 647 nm and 561 nm excitation lasers, respectively, and

405 nm activation laser in photoswitching buffer comprising of 20 mM cysteamine, 1% betamercaptoethanol and oxygen scavengers (glucose oxidase and catalase) in 50mM Tris+10 mM NaCl buffer at pH 8.0. Maximal powers of 647 nm, 561 nm and 405 lasers was set at 10, 20, and 0.05 kW/cm<sup>2</sup> respectively. Data was analyzed by the Vutara SRX software (version 5.12). Single molecules were identified by their brightness frame by frame after removing the background. Identified particles were then localized in three dimensions by fitting the raw data in a customizable region of interest (typically 16x16 pixels) centered around each particle in each plane with a 3D model function which was obtained from recorded bead data sets. Fit results were stored as data lists for further analysis. The image resolution capable of experimentally being achieved is 20 nm laterally (x and y) and 50 nm axially (in z).

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