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Computational Model of VEGFR2 pathway to ERK activation and modulation through receptor trafficking

Wan Hua Tan^a, Aleksander S. Popel^a, and Feilim Mac Gabhann^{b,*}

¹Department of Biomedical Engineering, Johns Hopkins University School of Medicine, 720 Rutland Ave., Baltimore, Maryland, 21205 USA

²Institute for Computational Medicine and Department of Biomedical Engineering, Johns Hopkins University, 3400 N. Charles St., Baltimore, Maryland, 21218 USA

Abstract

Vascular Endothelial Growth Factor (VEGF) signal transduction is central to angiogenesis in development and in pathological conditions such as cancer, retinopathy and ischemic diseases. We constructed and validated a computational model of VEGFR2 trafficking and signaling, to study the role of receptor trafficking kinetics in modulating ERK phosphorylation in VEGF-stimulated endothelial cells. Trafficking parameters were optimized and validated against four previously published *in vitro* experiments. Based on these parameters, model simulations demonstrated interesting behaviors that may be highly relevant to understanding VEGF signaling in endothelial cells. First, at moderate VEGF doses, VEGFR2 phosphorylation and ERK phosphorylation are related in a log-linear fashion, with a stable duration of ERK activation; but with higher VEGF stimulation, phosphoERK becomes saturated, and its duration increases. Second, a large endosomal fraction of VEGFR2 makes the ERK activation reaction network less sensitive to perturbations in VEGF dosage. Third, extracellular-matrix-bound VEGF binds and activates VEGFR2, but by internalizing at a slower rate, matrix-bound VEGF-induced intracellular ERK phosphorylation is predicted to be greater in magnitude and more sustained, in agreement with experimental evidence. Fourth, different endothelial cell types appear to have different trafficking rates, which result in different levels of endosomal receptor localization and different ERK response profiles.

Keywords

Angiogenesis; mathematical model; receptor tyrosine kinase; systems biology; endothelial signaling; trafficking

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*corresponding author, feilim@jhu.edu, 410-516-4723.

Author Contributions

WT, ASP, FMG designed the research approach. WT coded the model and ran the simulations. WT, ASP, FMG analyzed the data. WT created the figures and wrote the first draft of the manuscript. WT, ASP, FMG edited and approved the final version of the manuscript.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Introduction

The Vascular Endothelial Growth Factor (VEGF) signaling network is involved in angiogenic processes in physiological development and pathological conditions such as cancer, ocular diseases and ischemic diseases [1,2,3,4]. Approved anti-angiogenic drugs show promise in the treatment of cancer and age-related macular degeneration. These drugs include anti-VEGF monoclonal antibodies (e.g. bevacizumab, ranibizumab), tyrosine kinase inhibitors (e.g. sorafenib, sunitinib) and anti-VEGF aptamers (e.g. pegaptanib) [5]. More anti-angiogenesis drugs are in clinical trials and in pre-clinical development. While the putative drug targets and primary mechanisms of action are sometimes known, secondary targets and side-effects of these drugs are difficult to predict. Furthermore, drug efficacy is highly variable, patient-specific, and largely associated with innate and acquired resistance of multiple cancer cell types [1]. VEGF binds to and activates receptor tyrosine kinases (VEGFRs) on cell surfaces. Multiple signaling pathways downstream of VEGFRs are dynamic and heavily coupled; cross-talk interactions between them influence cell phenotypes. Computational models have the potential to describe, explain and predict VEGF signal transduction under various conditions, including quiescence, disease and drug interventions.

The VEGF-VEGFR family is complex. There are five genes encoding ligands (VEGF-A through -D and PlGF, placental growth factor) each with multiple splice isoforms, three receptor tyrosine kinases (VEGFR1, VEGFR2, VEGFR3), which actively signal as homo- and heterodimers in various combinations, and two non-signaling co-receptors (NRP-1 and NRP-2) [6]. Here we present a mass-action ordinary differential equation (ODE) model describing VEGF-A (hereafter referred to as VEGF) stimulation of VEGFR2 homodimers *in vitro*; this is the primary ligand-receptor pair responsible for initiating the main signaling pathways in endothelial cells (e.g. PI3K/Akt pathway, MAPK cascades). These signaling pathways lead to physiologically significant cellular processes including migration, proliferation, and survival, and emergent multicellular behaviors, including angiogenesis, vascular permeability, and vasodilation.

Computational models of VEGFR signaling using mass-action kinetics [7] have yet to reach the level of sophistication as those for the ErbB or EGFR (epidermal growth factor receptor) family [8,9,10,11,12,13], and those for platelet-derived growth factor receptors (PDGFRs) [14,15,16,17]. The above-mentioned receptors are also receptor tyrosine kinases and there are commonalities in their downstream effectors (e.g. PI3K/Akt pathway, MAPK cascade). However, they often appear in different cell types and the biological outcomes of these signal transduction pathways may differ between cell types. For example, EGFRs in epithelial cells are often associated with regulation of proliferation, PDGFRs in fibroblasts with wound healing and VEGFRs in endothelial cells with angiogenesis.

The bivalent VEGF ligand binds two VEGFR monomers to initiate signaling; this is similar to the PDGF system but different from the monovalent EGF ligands. There are two possible mechanisms: ligand-induced dimerization or ligand binding to pre-dimerized receptors [18]. Upon ligation, the cytoplasmic tails of the two VEGFR2 monomers phosphorylate each other on residues Y1175, Y1214, and other tyrosine sites [19]. These phosphotyrosine sites can then recruit adaptor molecules such as Shc and Grb2. Adaptor molecules lack enzymatic function and instead act as molecular bridges between multiple proteins. Scaffolding or docking proteins bind to adaptor proteins, joining the receptor complex. Scaffolding proteins consist of multiple binding domains and hence are generally larger than adaptor proteins [20]. Adaptor and scaffolding proteins generally contain Src homology-2 (SH2) domains that are responsible for their recruitment to the receptor at the phosphotyrosine sites [21].

The mitogen-activated protein kinase (MAPK) cascade is a highly conserved signaling pathway consisting of three tiers of kinases following ligand stimulation of RTKs. It is activated in response to VEGF stimulation of VEGFR2 and VEGFR3 [2]. For the purposes of this study, we focus on the modeling the extracellularly-regulated kinase (ERK1/2), which is experimentally the most well-studied MAPK. Downstream of VEGFR2, the ERK pathway leads to cell proliferation [2]. There is an observed variability in the duration and magnitude of ERK phosphorylation, and it has been demonstrated to be dependent on cell type, ligand type and concentration [22].

Initiation of the ERK cascade occurs through Ras, and two parallel pathways lead to its activation [12]: Shc-dependent and Shc-independent. Active Ras-GTP stimulates Raf kinase. Interestingly, hyperphosphorylation of Raf leads to its inactivation, while its monophosphorylated form is active [23]. As such, we refer to the activated Raf molecule as Raf_a; this in turn phosphorylates MEK at two sites. Doubly-phosphorylated MEK phosphorylates ERK at two sites, to yield ERK_{pp}, which can translocate to the nucleus to regulate cellular proteins and nuclear transcription factors [22]. In our analysis, the output of the computational model that we study most and compare to experimental data is ERK_{pp}.

MAPK cascades are often ultra-sensitive [24], exhibiting high cooperativity and bistability depending on the type of input. In particular, efforts in computational studies have shown that with increasing positive feedback, multi-level cascades and multi-site phosphorylation, cooperativity increases [25]. However, with increasing negative feedback, competitive inhibition, enzyme saturation and presence of scaffolding proteins, MAPK cascades can exhibit bistability [25]. Recent efforts in computational modeling of MAPK cascades have included scaffolding proteins like KSR and MP-1 that have different specificity for different MAPKs [25]. Research groups have also begun to build spatial models of MAPK cascades which allow for mathematical descriptions of diffusion, reaction and receptor trafficking [26]. In this study, we focus our efforts on time-dependent models of MAPK signaling, using large systems of heavily-coupled, non-linear ordinary differential equations (ODEs). We neglect diffusion within the cell, but include receptor trafficking and compartmentalization of signaling (signal initiation from cell surface and endosomal receptors).

Conventionally, receptor trafficking processes such as internalization have been thought to downregulate extracellular signals, with active receptors targeted for endosomal sorting and degradation. However, more recent studies have shown that the endosomal pool of receptors actively signal, and that inhibition of the cell's internalization machinery can lead to a decrease in the phosphorylation of biomarkers such as ERK. Such observations have been shown reproducibly in EGFR [27], PDGFR [28] and VEGFR [29] systems, indicating a highly conserved mechanism for RTKs. For a comprehensive description of the impact of trafficking on VEGFR signaling, see the following recent reviews: [30,31,32,33]. The role of lipids in MAPK have been shown to be significant and scaffold proteins localize MAPK signaling to different intracellular compartments [34]. One of the aims in constructing this model is to understand the extent of the potential for downstream signaling activated from the endosomal compartment. Hence, our model of the VEGFR2 pathway to ERK1/2 activation includes an actively-signaling endosomal compartment that can lead to ERK phosphorylation in parallel with the cell-surface receptors.

Differences in signal transduction between the various receptor-ligand systems can be attributed, in part, to differences in receptor trafficking processes. These processes impact both free and scaffolded receptors, and are likely to be system-specific. This may then lead to differential activation of downstream pathways. Differential receptor trafficking kinetics have been observed and modeled mathematically [9]. For example, in the ErbB family of

receptors, ErbB1 heterodimers are internalized faster than ErbB2 heterodimers. Such differences in receptor trafficking rates have a significant impact on the magnitude and duration of downstream signaling molecules.

Differences between EGFR and VEGFR trafficking have been observed experimentally. In the absence of stimulation, EGFR1 resides largely at the cell surface and displays a very low rate of constitutive internalization and recycling; they are almost undetectable in the internal compartment [35]. However, approximately 40% of VEGFR2 exists in an intracellular endocytic compartment [36], and the internal pool of VEGFR2 is in rapid flux [35]. Under ligand stimulation, internalized EGFR tends to be degraded but VEGFR2 tends to be recycled at an increased rate [37]. The dynamic nature of VEGFR2 traffic renders it more difficult to study *in situ*. Hence, there is a motivation to model VEGFR2 trafficking kinetics *in silico*. Furthermore, accurate mathematical description of trafficking processes improves the biophysical detail of our VEGF-VEGFR signaling models, in turn improving predictive capabilities.

Differences in receptor trafficking can also result from different ligand presentation to the receptor. *In vivo*, ligands may be presented to the receptor in the soluble form within the interstitial fluid, or in an immobilized form, reversibly bound to the extracellular matrix (ECM). Most *in vitro* cell culture experiments use the ligand added to media in its soluble form. Recently some efforts to understand the effect of VEGF presentation to VEGFRs on endothelial cells have been published. When HUVECs are stimulated by fibronectin-bound VEGF, intracellular ERK phosphorylation is greater in magnitude and more sustained than stimulation by soluble VEGF [38,39]. In addition, the VEGF concentration threshold for detectable ERK phosphorylation is lower than its soluble VEGF counterpart [39]. This suggests that matrix-bound VEGF may be a more potent stimulus. Collagen-bound VEGF also showed sustained pERK stimulation and a larger pVEGFR2 signal, but downstream Akt phosphorylation was less sustained than for soluble-VEGF [40].

The mechanisms for these observed differences in ERK phosphorylation due to VEGF presentation are not well understood. There are several possibilities, which can all act in tandem to produce the observed changes in prolonged signaling. First, matrix-bound VEGF may induce the association of VEGFRs with integrins, possibly through an extracellular scaffolding mechanism. There is some controversy as to which integrins synergize with VEGFRs to a larger extent. These integrins may include $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins [39]. VEGFR2 synergy with β_1 integrins is hypothesized to promote association with focal adhesions. In addition, $\alpha_v\beta_3$ integrins have been hypothesized to synergize with VEGFR2 signaling through c-Src [41]. Second, matrix-bound VEGF results in slower internalization of VEGF-VEGFR2, due to the anchoring of VEGF by the ECM [42]. Additionally, matrix-bound VEGF has been shown to have a half-life three times as long as soluble VEGF, possibly due to the physicochemical interactions with the ECM [42]. In this model, we interpret these biomechanical changes as a slower internalization of VEGF-VEGFR2 complexes.

Computational models of protein kinase signal transduction have demonstrated that phosphatases impact signal rate and duration, due to their high catalytic efficiency and large rate constants [43]. In contrast, kinases primarily control signal amplitude [43]. However, phosphatases are often not modeled explicitly, perhaps due to difficulties in detection and hence a lack of experimental data. Protein Tyrosine Phosphatase-1B (PTP-1B) is one of the better-studied cytoplasmic phosphatases. Its dephosphorylation targets can be pro- or anti-signaling and therefore the overall role of PTP-1B is not clear. For example, it dephosphorylates RTKs, decreasing signal initiation; but also dephosphorylates the STAM2-

Hrs complex on endosomes, effectively preventing endosomal sorting for lysosomal degradation [44].

In VEGFR systems, PTP-1B may play a critical role in modulating signal transduction. In HUVECs, Nakamura *et al.* demonstrated that PTP-1B negatively activates ERK phosphorylation through *in vitro* PTP-1B siRNA studies [45]. Additionally, they found PTP-1B to be over-expressed and around 11 times more active in mouse hindlimb ischemia, compared to non-ischemic control mice. Lanahan *et al.* have also shown that PTP-1B knockdown increases pERK levels in Synectin $-/-$ mice, while knockdown of other phosphatases such as DEP-1, VE-PTP and R-PTP μ had a statistically insignificant effect on pERK [46]. These studies confirm the biological significance of PTP-1B in VEGFR signaling. Besides PTP-1B, other receptor phosphatases such as DEP-1 and VE-PTP have been studied extensively. These transmembrane phosphatases often cluster with VE-Cadherin, necessitating the detection and analysis of VE-cadherin-VEGFR2 complexes in these studies. DEP-1 has been shown to negatively regulate pERK [29] but positively regulate VEGF-mediated Src and Akt activation, as well as endothelial survival [47]. High throughput microarray studies and VE-PTP silencing have been used to demonstrate the important role of VE-PTP in regulation of VEGFR2 activity and angiogenesis [48]. Receptor phosphatases have been increasingly studied in VEGFR systems and their role in modulating downstream signaling can be difficult to predict. In this study, PTP-1B kinetic parameters are estimated. However, optimization algorithms tend to minimize the effect of PTP-1B, suggesting that the effects of signal down-regulation are largely accounted for by receptor degradation.

The purpose of this study is to evaluate the highly dynamic nature of VEGFR2 trafficking, which is difficult to study *in vitro*, but has significant effects on ERK phosphorylation downstream in the context of VEGF-stimulated endothelial cells. Since the MAPK cascade is very sensitive to input changes, small changes in trafficking rates may cause significant changes to downstream ERK signaling. In addition, we are interested in modeling the negative regulatory effect of PTP-1B on ERK phosphorylation. We hypothesized that trafficking parameters such as internalization, recycling and degradation tightly regulate VEGFR2 activation, and thus modulate ERK phosphorylation to a large extent.

Results

Model Parameterization

We assembled four experimental datasets with time-dependent quantification of VEGFR2 and/or ERK phosphorylation following VEGF stimulation of endothelial cells [47,49,50,51]. Due to the VEGFR-specific nature of the VEGFR trafficking parameters [36,37], and to the high sensitivity of the model outputs to these parameters (verified by analysis, see below), we used our computational model to compute 30–40 sets of estimates for the trafficking parameters (for each dataset) that fit the data (Supplemental Figure S1), based on multiple randomized initial guesses. Eight parameters were estimated in this way for this model: the internalization, recycling and degradation rates of free (Figure 2A, C, E) and VEGF-bound VEGFR2 (Figure 2B, D, F); the initial concentration of PTP-1B (Figure 2H); and the rate of association of PTP-1B with VEGFR2 (Figure 2G). These parameters were chosen because (1) the model outputs are very sensitive to them; and (2) they are highly likely to be RTK-specific, in contrast to other downstream parameters that are assumed to be similar to the values in EGFR or PDGFR systems. Each experimental dataset provided a multiple distinct set of estimates for each parameter, and different combinations of parameters were fitted for each dataset, limited by the number of time-points available in each dataset to avoid overfitting (Supplemental Table S5). The combinations were chosen to maximize the number of independent estimates for each parameter. For several of the parameters, the

values estimated based on different datasets were in good agreement with each other (Figure 2, right) and with estimates based on other signaling pathways [52]. This suggests that the ‘true’ values of those parameters are within these ranges.

In the MAPK cascade, the duration of ERK phosphorylation can vary; duration increases once ERK phosphorylation is saturated [25]. Model simulations fitted against three of the four datasets [47,49,50] gave ERK phosphorylation profiles where the half-maximum duration is about 15–20 minutes, while model simulations fitted to [51] gave ERK phosphorylation profiles where the corresponding duration is ~50 minutes (Figure S2A–H). By comparing the distributions of the fitted parameter sets (Figure S2B, D, F), a longer ERK activation duration appears to correlate with slower internalization rate and slower degradation rate on average (see also Figure 2B, 2F, ‘Zhang’).

Differences in the designs of the experiments that yielded these datasets may contribute to observed differences in phosphorylated ERK (pERK) duration. These factors include VEGF dosage and VEGFR2 density; the latter can vary cell-to-cell for the same endothelial cell type as well as between different endothelial cell types [53]. In this case, the cell type is different—microvascular endothelial cells for the *Zhang et al* data set vs. HUVECs—and this may also manifest through altered control of VEGFR2 trafficking. Our analysis suggests that VEGFR2 internalization and degradation can impact the dynamics of ERK phosphorylation. Perhaps surprisingly, our sensitivity analysis will suggest that small changes in trafficking kinetics can modulate both the magnitude and duration of ERK phosphorylation to an extent comparable to that of MAPK-cascade kinetic parameters (*described later*).

The estimated VEGFR trafficking parameters are an order of magnitude larger than those of ErbB family receptors [9,13] supporting the differences observed between EGFR and VEGFR trafficking *in vitro* [35]. The estimated trafficking parameters fall within the same range as those estimated based on the VEGFR2/PI3K/Akt model [52], despite the focus on different downstream signaling pathways. This increases our confidence in these parameter values and is promising for integrating the signaling modules in future.

Parameter fit estimates of PTP-1B initial concentrations and association rates with VEGFR2 varied more across different datasets (Figure 2G, H). The trust-region-reflective algorithm tended to return low values of PTP-1B across all datasets, such that the number of PTP-1B bound to VEGFR2 is less than 10 molecules per cell throughout the simulations. This suggests that in this model, PTP-1B is not critical, and other signal-downregulating mechanisms, such as VEGFR2 degradation, suffice in describing the system.

To cross-validate the base case model of transient ERK activation, we selected a specific set of parameter estimates using only the experimental data from *Lamallice et al* of pVEGFR2 [50], (Figure 2, indicated by red stars and the letter ‘T’ for transient), and compared the predictions of the model against independent data from [47] and [49] (Figure 3A–B). Note that while we can and do estimate parameter sets from these other sources (Figures 2A–F), in Figure 3A–B we used them as validation only for a specific parameter set. The model using this baseline parameter set reproduced the behavior of two key nodes in the network, pR2 and pERK. Note, as described in the methods, the aggregation of multiple molecular species containing phosphorylated VEGFR2, denoted ‘pR2’ (Figure 3A), and the aggregation of singly and doubly phosphorylated ERK ($ERK_p + ERK_{pp}$), denoted ‘pERK’ (Figure 3B). This aggregation allows us to compare the model output with experimental results, in which multiple phosphorylated forms of VEGFR are detected together and the phosphorylated ERK forms are detected together.

Given this analysis, we simulate two primary types of pERK phosphorylation time-course profiles using this model. First, a more transient response, where the half-maximum duration of pERK is 15–20 minutes and the magnitude is approximately 1% activation of all ERK molecules in the cell. Second, a more sustained response, where the half-maximum duration of pERK is 50 minutes or longer and the magnitude greater than 50% activation. For many of the figures in this study, we use baseline parameter sets that produce these responses (Table S4; indicated by stars in Figure 2); in other figures (e.g. Figure 3C–F), we show results for all parameter sets obtained from the four experimental datasets.

Relationships between VEGFR2 and ERK phosphorylation

Model simulations using all the sets of parameter estimates (obtained from Figure 2 and Figure S1) show that the magnitude of VEGFR2 phosphorylation and ERK phosphorylation are related in a log-linear fashion regardless of whether VEGFR2 is at the cell surface or internalized (Figure 3C). Once ERK phosphorylation is saturated, any increases in VEGFR2 phosphorylation increase the duration of ERK phosphorylation (Figure 3E). This is primarily illustrated by those simulations using parameters from the *Zhang et al* dataset [51] (Figure 3E, red points), where the time-dependent model outputs are shown in Supplemental Figure S1G–H.

In contrast to the magnitude, the duration of VEGFR2 phosphorylation affects ERK phosphorylation to a lesser extent. Linear trends are seen between pR2 duration and maximum pERK (Figure 3D) only with one dataset, that of *Chabot et al* [47]. With all other datasets, there is no obvious trend. Interestingly, the relationship between the durations of pR2 and pERK peaks are linear for all datasets (Figure 3F).

Due to these large variabilities in duration and magnitude of ERK phosphorylation, we will study multiple specific cases (i.e. parameter sets), rather than a single base case.

Hierarchical clustering of parameter estimates and model output features

As noted above, numerous sets of parameter estimates were computed based on randomized initial starting values and fitting different datasets. This ensured that a large physiologically relevant parameter space is explored, while maintaining fidelity to observed signaling dynamics. To identify relationships between these parameter sets and their features, we applied hierarchical clustering, an unsupervised learning technique, to the parameter values and to features associated with the model simulations including: the maximum of the pR2 profile; the maximum level and half-maximum duration of the pERK profile; and the residuals between the model simulations and experimental data-points. All features are standardized to a mean of zero and unit variance, and thus clusters derived from this analysis are based on similarities between distributions of the features. As there are multiple parameter sets that can give very similar model simulation outputs (Supplemental Figure S1), we explored which features work in tandem, if at all, to modulate model outputs.

Throughout all datasets, the maxima of pR2 and pERK profiles clustered closely together (Supplemental Figure S3), suggesting that their distributions are correlated, as seen in Figure 3C. In addition, the residuals (i.e. the error between the simulated and experimental data) clustered closely with pERK duration for all datasets. As the western-blot data are semi-quantitative, relative to the control densitometry, the optimization procedure is also semi-quantitative. As such, the shape of the profiles and their duration are important in determining the goodness-of-fit.

In fitting pR2 data from *Lamallice et al.* [50] (Figure S3A) and pERK data from *Zhang et al.* [51] (Figure S3D), *kdegb*, the parameter describing the degradation of VEGF-bound

VEGFR2 complexes, tends to form its own cluster, separate from other features. This may be because degradation is irreversible, unlike most other processes in the model. However, for the other two datasets (Figure S3B, C), the kinetic parameter estimates tend to form a group, separate from the model output features.

The size of the endosomal VEGFR2 pool affects the sensitivity of trafficking parameters and pERK

As mentioned in the Introduction, there has been increasing experimental evidence of active signal propagation in the early endosomes. For the VEGF system, studies with Synectin knockout mice have suggested that the main signaling component is in the early (EEA-1 positive) endosomes [46]. Synectin binds to myosin-VI and is an important protein in the endosome transport machinery. These experimental studies challenge previous understanding that most active signaling RTKs are localized on the cell surface. In the following simulations, we explore the significance of the size of the pool of receptors in early endosomes.

Surprisingly, among the multiple parameter fits found for long-duration ERK activation (Figure S1G), there were parameter sets that gave similar pERK profiles (sustained activation) but generated different distributions of pR2. We examine two here; these datasets are denoted 'L' (for Large endosomal pool of R2) and 'S' (for Small endosomal pool of R2) and the parameters are identified by those letters on Figure S2 and Table S4. For most of the significant signaling nodes of the ERK network, the two parameter sets give similar profiles (Figure 4A–G) except for the distribution of VEGFR2 between the cell surface and internalized compartment (Figure 4A–B). The main differences in the 2 parameter sets are: (1) the ratio of $k_{\text{intb}}/k_{\text{recb}}$ (internalization and recycling of VEGF-bound VEGFR2) is ~ 118 and ~ 0.18 for the large and small endosomal R2 pools, respectively (Figure 4H, S2B, D); and (2) the degradation rate of VEGFR2 for the large endosomal pool parameter set is ~ 0.15 times that of the small endosomal pool (Figure 4I, S2F).

With a larger, actively signaling endosomal pool, the reaction network is less sensitive to perturbations in VEGF dosage (Figure 4J, L) and to the rate of dissociation of VEGF from VEGFR2 (k_{dv}) (Figure 4K, M). This is because VEGF is predominantly extracellular, and internalization of VEGF-bound VEGFR2 complexes adds a time delay to the system, allowing for signal propagation to occur intracellularly.

The size of the endosomal pool changes the global sensitivity of the system markedly. We analyzed the sensitivity of three parameter sets: (1) The parameter set that produces a large endosomal pool and sustained half-peak pERK duration of ~ 50 minutes (Figure 4A–G, 'L'); (2) the parameter set that produces a small endosomal pool with a similar long-duration pERK profile (Figure 4A–G, 'S'); and (3) a parameter set from fits to data from *Lamallice et al.* (Figure S1A–B), where the endosomal pool is small and pERK half-peak duration is more transient (~ 20 minutes) (denoted 'T' in Figure 4H–I). The input in these eFAST analyses are the parameters estimated through the trust-region algorithm discussed in Methods. Input parameters were varied across 1 order of magnitude around the base case value, with a log-uniform distribution. 3619 combinations of the input parameters were generated for this series of sensitivity analyses.

It is clear that in the case with a large endosomal pool (Figure 5A), the parameters to which the MAPK cascade is sensitive are the same as those for iR2_p (internalized, phosphorylated VEGFR2 complexes). Conversely, with a small endosomal pool (Figure 5B–C), the sensitivity of the MAPK cascade follows that of R2_p (cell-surface phosphorylated VEGFR2). Since both cell-surface and internalized VEGFR2 activate the MAPK cascade, it is logical that the larger VEGFR2 compartment will more heavily influence the MAPK

cascade. In addition, from these eFAST indices, it is clear that the outputs are most sensitive to the internalization and degradation rates, out of all the parameters tested.

To summarize the results from this eFAST sensitivity analysis: (1) cell-surface VEGFR2 are more sensitive to receptor internalization than to degradation, (2) internalized VEGFR2 are more sensitive to receptor degradation than to internalization and (3) activation of the key kinases in the MAPK cascade is most sensitive to the same parameters as the dominant VEGFR2-complex compartment.

Effect of VEGF presentation on VEGFR2 trafficking and signaling

VEGF presentation (soluble vs. immobilized) can impact VEGFR2 trafficking rates. VEGFR2 associated with matrix-bound VEGF is internalized at a slower rate, because internalization requires unbinding from the extracellular matrix [42]. To understand the impact of matrix-bound VEGF on VEGFR2 signaling, we consider two parameter sets: one that produces a transient pERK response (Figure S1A); and one that produces a sustained pERK response (Figure S1G). The parameter sets were chosen that have the smallest residuals for their corresponding datasets.

Several VEGF concentrations within the range typically used in *in vitro* cell culture (5–500 ng/ml) were simulated for the transient pERK (dashed lines) and sustained pERK (solid lines) cases. In the transient cases, VEGFR2 (Figure 6A–C), Ras-GTP (Figure 6D) and Raf_a (Figure 6E) are activated and attenuated more quickly than in the sustained cases. However, the shorter duration of activated signaling molecules upstream results in a smaller magnitude of pMEK and pERK downstream (Figure 6F–G). The differences between the cases in response to VEGF are amplified as the signal propagates downstream. Comparing the dose dependency for the transient and sustained pERK cases (Figure 6G), a sustained pERK response is accompanied by a larger and faster pERK activation than a transient pERK response to the same VEGF concentration. In addition, the threshold for a large increase in pERK activation is lower for the sustained pERK case than for the transient case; these thresholds are clearer when plotting maximum pERK concentration vs. VEGF concentration (Figure 7A). These signaling characteristics are observed experimentally in pERK activation in HUVECs stimulated by fibronectin-bound VEGF or soluble VEGF [38,39]. The rate of internalization of VEGF-bound VEGFR2 estimated by the parameter fits tends to be smaller for the sustained pERK cases than for the transient pERK responses (Figure 2B, S2B); the slower internalization of VEGFR2 complexes may explain the prolonged and larger pERK activation by matrix-bound VEGF.

Sensitivity to VEGFR2 density and trafficking: Implications for cell-type or cell-cell variation

This model is constructed based on data from western-blot assays using cell lysates. As such, the model simulates the average of a population of cells. However, VEGFR2 density may vary quite widely within a single population, on a cell-by-cell basis, as demonstrated through flow-cytometry receptor quantification studies [53]. In addition, distributions of VEGFR2 receptor density may vary between different cell types; for example, HUVECs typically have a lower VEGFR2 density than human microvascular endothelial cells (MECs), particularly following VEGF stimulation [53]. As expected, the magnitude of the VEGFR2 phosphorylation profiles scale linearly with VEGFR2 density (Figure 8A–C). This trend is also observed for early signaling molecules of the network, Ras-GTP (Figure 8D) and Raf_a (Figure 8E). However, at the level of MEK and ERK phosphorylation, increases in VEGFR2 density increase the duration of signaling, once saturation is reached (Figure 8F–G). The transient pERK response case tends to produce a faster response with a shorter duration in the earlier nodes of the signaling network (Figure 8A–F); for the same VEGFR2

density, MEK and ERK phosphorylation is of a shorter half-maximum duration for the trafficking parameter set that produces a more transient pERK response (Figure 8G–H, 7B).

Modular Sensitivity Analysis

Protein concentrations can vary from cell-to-cell, as well as across different cell types. The global sensitivities to the initial concentrations of the proteins in the model are presented here (Figure 9A). The various receptor and signaling complexes are sensitive to the initial concentration of their constituent proteins. For example, the sensitivity indices of VEGFR2 and VEGF are largest for the VEGFR2 complexes and the sensitivity indices for VEGFR2 and VEGF decrease for downstream protein complexes as signals propagate down the signaling cascade. However, proteins and complexes in the MAPK cascade are still appreciably sensitive to VEGF and VEGFR2, although no physical interactions or reactions between the two are described in the computational model. In comparison, complexes in the MAPK cascade are not sensitive to scaffolding proteins that associate with VEGFR2, such as Sos and Grb2, that have no direct interaction with the MAPK components.

Initial concentrations of phosphatases in the MAPK cascade affect their direct target kinases, as well as kinases and complexes in subsequent levels of the cascade. The phosphatase for Raf, denoted Pase1, affects kinases at all levels of the cascade. The phosphatase for MEK, denoted Pase2, affects MEK phosphorylation as well as ERK phosphorylation in the next level, although there is no direct interaction between the two.

Interestingly, Ras-GTP has small First FAST indices but the largest Total FAST indices. This indicates that Ras-GTP concentration is sensitive to combinations of initial concentrations of multiple proteins but not any single protein concentration on its own. As described in *Methods*, the total FAST indices for each output sums the variances for each parameter (initial concentration, in this case) and the co-variances of any higher order interactions with other parameters. Ras-GTP formation is sensitive to combinations of Raf, Pase1, and MEK (Figure 9A). This may seem counter-intuitive, because they are downstream of Ras, but there are feedback control mechanisms for Ras-GTP formation (Table S1, Reactions 27–30).

To compare the effect of trafficking kinetics and the biochemical reactions that directly modify or phosphorylate kinases in the MAPK cascade, eFAST analysis was conducted for each of the following reaction modules; Ras activation (Table S3, Parameters 9–16), Raf activation (Parameters 41–47), MEK phosphorylation (Parameters 48–56) and ERK phosphorylation (Parameters 57–66). The parameters from each of these four modules to which the system is most sensitive ($k_{aRasGDP}$, k_{dpRaf} , $k_{aMEKRaf}$, $k_{aERKMEK}$) were then analyzed alongside the VEGFR2 trafficking parameters (Figure 9B). As expected, VEGFR2 trafficking kinetic parameters have a greater effect on VEGFR2 complexes than the kinetics of the MAPK cascade. Starting from Raf_a and moving downstream, the MAPK cascade becomes more sensitive to the kinetic signaling parameters than to the trafficking kinetics. However, it is important to note that throughout the system, the FAST indices of the trafficking parameters are non-negligible, even when compared with the most sensitive parameters governing the biochemical reactions directly involving Raf, MEK and ERK.

Discussion

Coherent sets of parameter estimates studied together

In this study, we demonstrate that signaling downstream of VEGFR2 is sensitive to VEGFR2 trafficking kinetics, and that these kinetics can modulate both the duration and magnitude of ERK phosphorylation. In total, 145 parameter sets were fitted, using different

randomized initial starting values, for four different datasets from independent sources. Through hierarchical clustering (Figure S3), it was demonstrated that the magnitudes of pR2 and pERK clustered closely in simulations based on all four datasets. However, there was no consistent trend for the clustering of parameter estimates across the datasets. In two datasets (Figure S3A, D), the degradation kinetics were different from most other features and formed their own cluster; in the other two datasets, degradation kinetics were clustered with other trafficking kinetics such as internalization and recycling. This suggests that parameter estimates need to be studied in combination, not in isolation. Different parameter combinations give rise to similar pR2 profiles, which in turn determines the behavior of the system. Given that combinations of parameters can be important in determining the pR2 profile, how robust are these combinations of parameter estimates? Must the parameter sets be preserved, or can the parameters be “mixed-and-matched” and still fit the data reasonably well? A simulation was run using the median parameter estimates (marked in red, Figure 2) for each parameter and compared against each dataset. Qualitatively, the median parameter estimates fitted three of four datasets well (Figure S4A–F), comparable to the fitted parameter sets (Figure S1A–F).

In the dataset with a sustained pERK response, the half-maximum duration of pERK given the median parameter estimates is longer than that of the experimental dataset (Figure S4H). This may suggest that small changes to parameter values, after pERK saturation is reached, can impact its duration significantly, in agreement with other results throughout the study. From an input-output perspective (Figure 3C, E), increase in pR2 concentration increases pERK duration once the pERK saturating concentration is reached. Increases in VEGFR2 density beyond a threshold (~1000 R2-dimers/cell) increases pERK duration at saturation (Figure 8G, 7B) as well.

Which parameters the system is most sensitive to depends on the baseline values of the parameters

eFAST analysis is useful in obtaining a high-level understanding of how signals propagate within the network. Since the indices are computed based on time-integrated concentration profiles, delays in activation without changes in the magnitude of the concentration will not be taken into account in this analysis. It is useful in detecting changes in the sensitivity properties of the system within small ranges of parameter values (Figure 5); but the ranking of parameters, from most sensitive to least, can change for different baseline values of those parameters. This is demonstrated with the sustained pERK responses produced from small and large endosomal pools (Figure 5A, B). The large endosomal pool of VEGFR2 is created due to a slow receptor recycling rate. As a result, outputs are more sensitive to degradation than to internalization rates (Figure 5A). However, when a faster receptor recycling rate produces a smaller endosomal pool of VEGFR2, the internalization rate becomes more important than degradation.

Processes modulating activated receptor density control the signaling system

The importance of VEGFR2 trafficking in modulating ERK signaling, increasingly recognized in the experimental literature [30,31,32,33], is clear from this study. Receptor degradation determines the total number of VEGFR2 in the system, which is the initial concentration parameter to which the outputs are most sensitive (Figure 9A). Internalization and recycling processes directly control the number of VEGFR2 in the cell-surface and endosomal compartments, and hence the receptors' propensity to degrade. In computational models at the tissue and whole-body scales, the sensitivity of VEGFR signaling to VEGFR density is also significant [54], suggesting that it is important to faithfully represent processes that control receptor density in mathematical modeling of the system.

The model simulations demonstrate two distinct pERK responses: sub-saturation and saturation. Many changes in the system can push the system from one phenotype to the other; for example, the level of phosphorylated VEGFR2 is important in doing so (Figure 3C, E). Increases in pR2 may be due to a larger VEGFR2 density, such as in human microvascular endothelial cells (Figure 8G, 7B) or differences in VEGFR2 trafficking (Figure 9B). This is supported by the available experimental data, where the three datasets from *Lamallice et al.*, *Chabot et al.* and *Bruns et al.* [47,49,50] are from HUVECs while that of *Zhang et al.* [51], which has longer pERK response duration, is from microvascular endothelial cells. We also demonstrate that the two distinct shapes in pERK response are not due to variations in VEGF dosage (Figure 6G, 7A); changes in VEGF concentration are predicted to alter the magnitude of pERK but not its time-course, and the datasets studied here consist of *in vitro* cell culture experiments using 5–80ng/ml of VEGF as stimulus. Other contributing factors beyond the scope of this model may also play a role in causing the different time course profiles of the experimental data. Through eFAST analysis, we demonstrated that outputs can be more sensitive to receptor trafficking kinetics than to the kinetics of the MAPK cascade, such as at the Ras-activation stage (Figure 9B). Even when MAPK kinetics are most important, receptor trafficking and receptor density are non-negligible factors, and in any case are more likely to be dynamically controlled and variable between cell types, cell locations or cell states.

Tyrosine Kinase Inhibitors impact VEGFR2 localization in the cell

VEGFR2-targeting TKIs may also have an impact on VEGFR2 trafficking. Semaxanib (SU5416), a selective VEGFR2 tyrosine kinase inhibitor, decreases VEGFR2 activation, but also VEGFR2 trafficking, resulting in endosomal accumulation of VEGFR2 [55]. This suggests that the intrinsic tyrosine kinase activity of VEGFR2 is required for its ubiquitination and sorting machinery. The Ubiquitin-Proteasome system, represented by VEGFR2 degradation in this model, may be a potential target for antiangiogenic cancer therapy [56].

Active signaling from the early endosomes can affect a reaction network's overall robustness to external stimulation. In our previous analysis of the PI3K/Akt pathway [52] we assumed that VEGFR2 in early endosomes did not activate the pathway, due to the lack of supporting experimental evidence for such a mechanism. In that case, the eFAST indices for $iR2_p$ were identical to those of their cell-surface counterparts ($R2_p$). Here, for the ERK pathway, we see that the indices are different for the internalized and cell surface receptors, and ERK signaling from endosomes has been experimentally confirmed [29,46].

Methods

Formulation of biochemical reactions

In order to simulate *in vitro* cell culture experiments, specifically VEGF stimulation of endothelial cells, we developed a deterministic mass-action ordinary differential equation (ODE) model, with computational implementation via the MATLAB Simbiology toolbox. The system is solved using *ode15s*, the MATLAB stiff ODE solver. We assume that molecular species activated in response to VEGF stimulation have set initial steady-state concentrations at zero time before the addition of VEGF. The adaptor and scaffolding proteins associated with ErbB receptors are similar to those of VEGFRs as demonstrated through immunoprecipitation assays [57,58] in HUVECs, and are used as the basis for this model.

All reactions, except VEGF binding to VEGFR2, occur inside the cell, either at the plasma membrane or on an endosome, with 105 reactions involving 14 distinct proteins and 59

kinetic parameters (Table S1, S2 & S3). Trafficking occurs between the three simulated compartments of the cell: the cell surface; internalized endosomes; and lysosomes, in which proteins are degraded. As MAPK kinase cascades can be activated for long durations, depending on the nature of the stimulus, we run simulations to produce concentration profiles of intracellular signaling molecules from 0 to 300 minutes (5 hours) following the addition of VEGF. Estimates for many signaling parameters were obtained from previously published mathematical models describing different receptor-ligand systems, specifically VEGFR2 [59] and ErbB receptors [9,11,13], as noted in supplemental Table S3.

Molecular interactions

Like the closely related PDGF ligands, VEGF ligands are covalently pre-dimerized. In our models, we assume that VEGFR2 are also pre-dimerized (Table S1, Reaction1). There are multiple possible mechanisms in receptor dimerization [18], but this is not the focus in this study. In our model, we approximate ligand-dependent receptor activation as a single step, resulting in autophosphorylation of the VEGFR2 cytoplasmic domains. Receptor phosphorylation creates docking sites for SH2-domain-containing adaptor proteins Shc and Grb2. We do not explicitly distinguish between autophosphorylation at the various tyrosine residues such as Y1175 and Y1214, as there is limited relevant training data; however for ERK signaling the upstream phosphotyrosine site is most likely pY1175.

All receptor complexes can undergo trafficking. Trafficking is represented by three kinetic parameters for each receptor complex—internalization, recycling and degradation with different rates for ligated and unligated receptors. Internalization and recycling are represented as reversible first-order reactions (Supplemental Table S1, reactions 47–57); degradation of the internalized molecules is modeled as irreversible first-order reactions (Table S1, reactions 58–68).

The biochemical reactions in the model are written as first or second order reactions, and the model is set up such that integration of additional cross-talk reactions is straightforward when more experimental evidence in VEGF-stimulated systems becomes available. The biochemical reactions (Table S1) can be grouped into six modules; (1) early receptor activation events such as receptor-ligand binding and GTPase-activating protein (GAP) association, (2) Shc-independent pathway to Ras-GTP activation, (3) Shc-dependent pathway to Ras-GTP activation, (4) MAPK cascade consisting of Raf, MEK and ERK, (5) receptor trafficking (internalization, recycling and degradation) and (6) VEGF dissociation from VEGFR2 complexes. Modules (1), (2) and (3) occur both for cell-surface receptors (Table S1, reactions 1–21) and endosomal receptors (Table S1, reactions 69–88) for reasons discussed in the Introduction.

ERK is activated through the canonical MAPK cascade [25], where scaffolded and phosphorylated VEGFR2 activates Ras-GTP, which in turn activates Raf. Activated Raf acts as an enzyme in phosphorylating MEK at two different sites, denoted as MEK_{pp} . MEK_{pp} then phosphorylates ERK at two sites, yielding ERK_{pp} . At each level of the cascade, phosphatases (denoted as $P'ase1$, $P'ase2$ and $P'ase3$) can act to dephosphorylate kinases, regulating the extent of activation in the system.

Parameter fitting

Kinetic parameters (Table S3) associated with signaling molecules common to RTKs (eg, Gab1, Shc, Akt, ERK, etc) were taken from previously published models [9,11,13,52,59]. These signaling modules are expected to be similar between receptor types and cell types, and we assume that the kinetics of their reactions are largely conserved. In contrast, the kinetics of reactions involving VEGFR2 itself are more likely to be receptor-specific. These

include ligand binding and receptor trafficking. The VEGF-VEGFR2 binding and dissociation rates used in our model are derived from labeling experiments and Scatchard plot analysis, and have been used in previously published models [18,52]. VEGF receptor densities in primary endothelial cells are approximately one order of magnitude less than ErbB receptor densities in epithelial cell lines. In this model, the initial VEGFR2 density is based on quantification experiments from our group [53], and variation in that density is simulated to estimate sensitivity to receptor density. The average ligand concentration for each cell is adjusted to a cell volume basis, and estimated using a 1 pL cell volume and 10mm cell culture medium depth. VEGFR2-specific trafficking parameters were estimated as described in the next section.

The optimization routine was implemented using the trust-region-reflective algorithm (*lsqnonlin* in MATLAB), and the acceptable range of values for the six trafficking parameters and two dephosphorylation parameters was constrained within physiologically relevant bounds, typically three orders of magnitude wide (10^{-1} to 10^{-4} sec $^{-1}$ for trafficking parameters). Since the number of parameters that can be fitted was constrained by the number of datapoints, several assumptions were made regarding the internalization, recycling and degradation rates. Specifically, the receptor complexes were grouped into 3 categories. It was assumed that unligated and ligated receptor complexes have different internalization, recycling and degradation rates. For example, ligated receptors have been observed to be internalized at a faster rate than unligated receptors *in vitro* [60].

The parameters estimated are the receptor trafficking kinetics for both free and VEGF-bound receptors (internalization, recycling and degradation rates), PTP-1B initial concentration and association rates with VEGFR2. The experimental data consists of time-course phosphorylation profiles of VEGFR2 [47,50] and ERK [49,51] on western blots. Several experiments have shown VEGFR to have different endosomal sorting characteristics from EGFR [37]. Since the experimental data provide relative and not absolute concentrations, our fitting methodology minimized least squares error between the *normalized* simulation profiles and experimental datasets (Supplemental Figure S1B, D, F, H). All concentration profiles were normalized to the maximum concentration observed in the experimental data (Supplemental Figure S1A, C, E, G). The western-blot data available was semi-quantitative, and inherently relative to the control densitometry (no VEGF stimulation). The model, in contrast, simulates absolute values of receptor densities and signaling protein concentration profiles; therefore for the purpose of comparison between the simulations and the experiments, we assumed that the maximum concentration measured in experimental data was a close approximation to the actual maximum in the concentration profile. This is a fair assumption given that the experimental data points were collected at sufficiently frequent time intervals. Each of the four relevant datasets was used to generate at least 30 distinct sets of fitted trafficking parameters, each time with different random initial values for each of the parameters within the prescribed bounds. The outcome of each run is a different set of trafficking parameters, and the resulting values for each parameter were plotted, by experimental dataset (Figure 2A–F) and with all experimental datasets aggregated ('All'). An example of a complete set of trafficking parameters found in these best fits, using the *Lamallice et al* dataset [50] is indicated with red stars and the letter 'T' (Figure 2A–F). Examining the range of values obtained for each parameter set, we note that the range of estimated values for most parameters is consistent between experimental datasets (Figure 2A–H), and that the mean and median values of these estimates is similar for each parameter (Figure 2A–H, right). These results suggest that there is a high likelihood that the true value of these parameters are close to the mean/median.

From these four datasets, the parameter estimates gave model simulations of two distinct pERK profiles. The parameter fits on data from *Lamallice et al.*, *Chabot et al.* and *Bruns et*

al. [47,49,50] yielded a more transient response where the half-maximum duration was approximately 15–20 minutes. On the other hand, the parameter fits on data from Zhang *et al.* [51] gave a more sustained and saturated pERK response where the half-maximum duration was about 50 minutes. Hence, in this study, we consider some specific cases. One parameter set is chosen to represent the transient pERK response, while two parameter sets are chosen to represent the sustained pERK response, one with a large pool of endosomal VEGFR2 and one with a small pool. The parameter sets chosen had lowest sum of least squares error.

Model Outputs

The model outputs are the concentrations of the various signaling complexes (Figure 1) over time. To match these with experimental data, it becomes important to aggregate some complexes together. For example, due to the scaffolding and adaptor proteins, multiple molecular species in the model contain phosphorylated VEGFR2. The sum of all these is denoted 'pR2' (e.g. Figure 3A) and represents the population of actively signaling receptor dimers. This includes both membrane-associated receptors and internalized receptors. As noted earlier, in this model, the internalized receptors represent receptors in early endosomes while degraded receptors represent those sorted into late endosomes/lysosomes and marked for degradation; we assume that only the receptors at the surface and in early endosomes are signaling. A second example of aggregation is denoted 'pERK' (e.g. Figure 3B), which is the sum of singly and doubly phosphorylated ERK ($ERK_p + ERK_{pp}$) as both species are detected in an immunoblot.

Sensitivity Analysis

To perform a sensitivity analysis of the model, we used the eFAST method as described by Saltelli et al [61,62] and as implemented by Kirschner and colleagues [63]. We used Simlab 2.2 from Econometrics and Applied Statistics Unit (EAS) at the Joint Research Centre (JRC) of the European Commission (Ispra, VA, Italy; <http://simlab.jrc.ec.europa.eu/>). The eFAST analysis method is described in more detail in the Supplemental Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We build a model of the ERK signaling pathway downstream of VEGF-VEGFR2 activation
- We use this model and experimental data to estimate values for key parameters
- We identify which parameters have the most impact on the ERK pathway
- Altered trafficking due to matrix-binding of VEGF alters signaling patterns
- Different trafficking on endothelial cell types can explain signaling differences

Figure 1. Reaction Schematic

A, Complete schematic of VEGFR2 signaling to the ERK MAPK cascade. The MAPK cascade is controlled through the activation of Ras. Ras activation occurs through Shc-independent and Shc-dependent pathways. **B**, All reactions involving R2 complexes have a counterpart in the endosomal compartment. All receptors or receptor complexes are internalized, recycled and degraded at different rates for ligated and unligated receptors. These parameters are estimated based on optimization of model outputs against experimental data, and determine the localization of VEGFR2. VEGF may dissociate from all VEGFR2 complexes, resulting in a disintegration of the complex. 'iR2' and 'dR2' refers to internalized and degraded receptors respectively. 'X' refers to any molecular species bound to VEGFR2.

Figure 2. Estimation of trafficking parameters and model validation using independent experimental data

Eight VEGFR2 trafficking parameters were estimated by fitting simulation results to independent time-course western blots of protein phosphorylation: Phosphorylated VEGFR2 data [47,50] and phosphorylated ERK data [49,51]. The number of fitted parameters for each dataset is limited by the number of time-points in the dataset. For each experimental dataset, the fits were performed multiple times (Supplemental Figure S1) starting with different initial values for the parameters. The outcome is a range of estimated values for each parameter. We show these parameter estimates in two ways: on the left, all of the parameter estimates are shown in a dot plot, separated by experimental dataset, as well as aggregated ('All'); on the right, the estimates are binned to show the high degree of clustering of parameter estimates (plus the mean and median values). Examples of complete parameter sets are indicated by letter: 'T' = Transient pERK response, small VEGFR2 endosomal pool; 'L' = L sustained pERK response, Large VEGFR2 endosomal pool; 'S' = S sustained pERK response, Small VEGFR2 endosomal pool; red, blue and green stars respectively. The distribution of estimated values for each parameter is consistent between datasets. The trafficking parameters (rate constants) estimated using this method are: **A**, k_{intf} , internalization of free receptors; **B**, k_{intb} , internalization of bound (VEGF-ligated) receptors; **C**, k_{recf} , recycling of free receptors; **D**, k_{recb} , recycling of bound receptors; **E**, k_{degf} , degradation of free receptors fit to dataset from [50]; **F**, k_{degb} , degradation of bound receptors; **G**, PTP-1B association rate with pR2; **H**, PTP-1B Initial Concentration.

Figure 3. Validation of parameter estimates and relationship between VEGFR and ERK phosphorylation

A–B, Simulations using one of the parameter sets based on phosphorylated VEGFR2 time-course data from [50] (indicated with red stars and the letter ‘T’ in Figure 2) recapitulate independent experimental observations of VEGFR2 and ERK phosphorylation profiles. Western-blot time-course data was normalized to the maximum concentration computed by the model; no other fitting is done. The molecules compared are: **A,** All membrane-associated and internalized phosphorylated VEGFR2 complexes [47]; **B,** Sum of singly and doubly phosphorylated ERK [49]. **C–F,** Input-output relationships between pR2 and pERK from simulations using parameter estimates show that magnitude of pR2 determines the output of the system **C,** Maximum of pERK response against maximum pR2 shows a log-linear relationship before saturation, for all datasets. **D,** Maximum of pERK response against half-maximum duration of pR2 show different trends for each dataset. **E,** Half-maximum pERK duration against maximum pR2 show linear trend after saturation. **F,** Half-maximum pERK duration against half-maximum pR2 duration show linear trends of varying gradients between different datasets.

Figure 4. Different localization of VEGFR2 can produce similar pERK responses but different sensitivity characteristics

A–G, Two simulations with different trafficking parameter sets derived from fitting to data from [51], each resulting in a sustained ERK response, and either Large or Small endosomal pools of VEGFR, denoted ‘L’ and ‘S’. **A**, Sum of cell surface and endosomal phosphorylated VEGFR2 complexes. **B**, Sum of endosomal phosphorylated VEGFR2. **C**, degraded VEGFR2. **D**, Ras-GTP. **E**, activated Raf (Raf_a). **F**, Sum of singly and doubly phosphorylated MEK (pMEK). **G**, Sum of singly and doubly phosphorylated ERK (pERK). **H**, Percentage of total pR2 in endosomes against ratio of kintb/krecb for each optimized parameter set and the chosen parameter sets discussed (‘L’ and ‘S’ as described above; ‘T’ = Transient pERK response with a small VEGFR2 endosomal pool). **I**, Percentage of total pR2 in endosomes against ratio of kintb/kdegb for each optimized parameter set and the chosen parameter sets discussed (squares). **J–M**, A large pR2 endosomal pool decreases sensitivity to VEGF concentrations and VEGF dissociation rates from VEGFR2 complexes. **J**, pERK response to a large endosomal pR2 pool and varying VEGF concentrations. **K**, pERK response to a large endosomal pR2 pool and varying VEGF dissociation rates from VEGFR2 complexes concentrations. **L**, pERK response to a small endosomal pR2 pool and varying VEGF concentrations. **M**, pERK response to a small endosomal pR2 pool and varying VEGF dissociation rates from VEGFR2 complexes concentrations.

Figure 5. eFAST analysis demonstrates that kinases in the MAPK cascade have sensitivity properties similar to VEGFR2 complexes in the larger compartment
 eFAST (extended Fourier Amplitude Sensitivity Analysis [61]) indices for cell-surface and internalized (endosomal) Ras-containing VEGFR2 complexes and select complexes in the MAPK cascade. **A**, Case 'L': Large endosomal pool of VEGFR2, producing a sustained pERK response, where degradation of bound receptors is more sensitive than internalization for most outputs. **B**, Case 'S': Small endosomal pool of VEGFR2, producing a sustained pERK response, where internalization of bound receptors is more sensitive than degradation for most outputs. **C**, Case 'T': small endosomal pool of VEGFR2, producing a Transient pERK response, where degradation of bound receptors is more sensitive than internalization for most outputs.

Figure 6. Different VEGFR2 trafficking alters pERK duration and sensitivity to VEGF concentration

Transient (dashed lines) and sustained (solid lines) pERK responses are similar to pERK responses to soluble and matrix-bound VEGF respectively [38,39], and have different VEGF dose dependencies. **A**, Sum of cell surface and endosomal phosphorylated VEGFR2 complexes. **B**, Sum of endosomal phosphorylated VEGFR2. **C**, degraded VEGFR2. **D**, Ras-GTP. **E**, activated Raf (Raf_a). **F**, Sum of singly and doubly phosphorylated MEK (pMEK). **G**, Sum of singly and doubly phosphorylated ERK (pERK).

Figure 7. Overview of the impact of VEGF concentration and VEGFR2 density on phosphoERK maximum and duration

A, Maximum of pERK profile in Fig. 6G, plotted against VEGF concentration applied. **B**, Half-maximum duration of pERK profile in Fig. 8G, plotted against VEGFR2 density.

Figure 8. Different VEGFR2 trafficking alters pERK duration and sensitivity to VEGFR2 density

Transient (dashed lines) and sustained (solid lines) pERK responses differ in how they are affected by varying VEGFR2 densities, modeling cell-cell or cell-type variation. **A**, Sum of cell surface and endosomal phosphorylated VEGFR2 complexes. **B**, Sum of endosomal phosphorylated VEGFR2. **C**, degraded VEGFR2. **D**, Ras-GTP. **E**, activated Raf (Raf_a). **F**, Sum of singly and doubly phosphorylated MEK (pMEK). **G**, Sum of singly and doubly phosphorylated ERK (pERK).

Figure 9. Modular eFAST analysis of the transient pERK response produced from a small VEGFR2 endosomal pool

A, Sensitivity of cell-surface and internalized (endosomal) Ras-containing VEGFR2 complexes and select complexes in the MAPK cascade, to initial concentration parameters. Activation of the MAPK cascade is still affected by VEGFR2 density although there is no physical interaction. **B**, eFAST indices for 4 most sensitive kinetic parameters in the MAPK cascade as well as VEGFR2 trafficking parameters. VEGFR2 trafficking parameters are non-negligible throughout.