

Ligand bias underlies differential signaling of multiple FGFs via FGFR1

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Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 3

October 16, 2023 (this version)

Reviewed preprint version 2

August 10, 2023

Reviewed preprint version 1

June 6, 2023

Sent for peer review

April 6, 2023

Posted to bioRxiv

April 4, 2023

 https://en.wikipedia.org/wiki/Open_access

 Copyright information

Abstract

The mechanism of differential signaling of multiple FGF ligands through a single FGF receptor is poorly understood. Here, we use biophysical tools to quantify multiple aspects of FGFR1 signaling in response to FGF4, FGF8 and FGF9: potency, efficacy, bias, ligand-induced oligomerization and downregulation, and conformation of the active FGFR1 dimers. We find that the three ligands exhibit distinctly different potencies and efficacies for inducing responses in cells. We further discover qualitative differences in the actions of the three FGFs through FGFR1, as FGF8 preferentially activates some of the probed downstream responses (FRS2 phosphorylation and extracellular matrix loss), while FGF4 and FGF9 preferentially activate different probed responses (FGFR1 phosphorylation and cell growth arrest). Thus, FGF8 is a biased ligand, when compared to FGF4 and FGF9. Förster resonance energy transfer experiments reveal a correlation between biased signaling and the conformation of the FGFR1 transmembrane domain dimer. Our findings expand the mechanistic understanding of FGF signaling during development and bring the poorly understood concept of receptor tyrosine kinase ligand bias into the spotlight.

eLife assessment

This manuscript describes **useful** data on the mechanisms underlying the activation of the receptor tyrosine kinase FGFR1 and stimulation of intracellular signaling pathways in response to FGF4, FGF8, or FGF9 binding to the extracellular domain of FGFR1. **Solid** evidence for quantitative differences in the downstream responses induced by the three ligands is presented. This manuscript will be of interest to biochemists and cell biologists working on receptor tyrosine kinases and general cell signalling across membranes.

Fibroblast growth factor receptors (FGFRs) belong to the family of receptor tyrosine kinases (RTKs), which signal via lateral dimerization in the plasma membrane to control cell growth, differentiation, motility, and metabolism (1–3). The four known FGFRs (FGFR1–4) are single-pass membrane receptors, with an N-terminal ligand-binding extracellular (EC) region composed of three Ig-like domains, a transmembrane (TM) domain, and an intracellular (IC) region that contains a juxtamembrane (JM) domain, a kinase domain, and a cytoplasmic tail (4). The cross-phosphorylation of tyrosines in the activation loop of the FGFRs in the ligand-bound dimers is known to stimulate catalytic activity, resulting in the phosphorylation of secondary intracellular tyrosines, which recruit cytoplasmic effector proteins (5). These effector proteins, once phosphorylated by the FGFRs, trigger downstream signaling cascades that control cell behavior (6, 7).

The FGFRs signal in response to extracellular ligands with a beta trefoil fold, called fibroblast growth factors (FGFs) (8). As many as 18 different FGF ligands are known to bind to and trigger distinct cellular responses through a total of seven variants of four FGFRs (FGFR1–4) (9–11). The diversity in FGF-FGFR interactions far exceeds that of other RTK systems, reflecting the morphogen role that FGF signaling plays in the development of many tissues and organs. FGFR1 is well known for its critically important role in the development of the skeletal system, and has been implicated in many cancers (12–16). One specific example of a developmental process controlled by FGFR1 is limb bud outgrowth (17, 18). The limb bud forms early in the developing embryo and consists of mesenchymal cells sheathed in ectoderm. The process starts as the mesenchymal cells begin to proliferate until they create a bulge under the ectodermal cells above. This is followed by the formation of the apical ectodermal ridge (AER) by the cells from the ectoderm, which specifies the proximal-distal axis of the limb, ensuring limb outgrowth (17, 18). These roles are carried out by several FGF ligands, including FGF4, FGF8, and FGF9, which are produced and secreted by the ectodermal cells in the AER and have similar spatio-temporal expressions. The ligands diffuse into the mesenchymal region adjacent to the AER, and act upon the mesenchymal cells which express only the “c” variant of FGFR1 (FGFR1c). This leads to the activation of signaling pathways downstream of FGFR1c, promoting survival and proliferation of the mesenchymal cells.

Experiments involving genetic ablation of FGF4, FGF8 and FGF9 have led to distinct limb malformations (19). Thus, the actions of these ligands through FGFR1c are different, but the mechanism behind the differential response of FGFR1c to multiple ligands has not been investigated. More broadly, it is not known how a cell recognizes the identities of different FGF ligands, when bound to and signaling through the same FGFR. Here we sought to investigate the molecular mechanism behind differences in FGFR1c signaling in response to FGF4, FGF8, and FGF9.

Results

Differences in FGF-induced activation of the ERK pathway in cultured chondrocytes

To investigate whether FGF4, FGF8 and FGF9 induce differential signaling via FGFR1, we used rat chondrosarcoma (RCS) chondrocytes, an immortal chondrocyte cell line used to model proliferating chondrocytes in the developing limb (20). By western blot, the RCS cells express detectable amounts of both FGFR1 and FGFR2 (**Figure 1A**). While FGFR3 and FGFR4 cannot be detected by western blotting, transcripts for FGFR3 and FGFR4 can be found by qPCR. To create an RCS variant that expresses FGFR1 only, CRISPR/Cas9 was used to disrupt the endogenous FGFR2,

FGFR3 and FGFR4 genes in RCS cells (21 [↗](#)) to generate cells expressing only the endogenous FGFR1 (RCS^{Fgfr1}). As a control, we used CRISPR/Cas9 to disrupt all endogenous *FGFR* genes in RCS cells (**Fig. 1A** [↗](#); RCS Fgfr1-4 null). The RCS^{Fgfr1} cells were treated with FGF4, FGF8 and FGF9 for up to 1h, and activation of the RAS-ERK MAP kinase pathway, which represents the major downstream signaling FGFR module, was monitored by western blot (**Fig. 1B** [↗](#)). FGF4, FGF8 and FGF9 showed significant differences in FGFR1-mediated activation of ERK, with FGF4 inducing the strongest signal, compared to weaker signals detected in cells treated with FGF8 and FGF9. To investigate the dynamics of ERK activation in further detail, we used a genetic reporter of ERK activity. Briefly, the RCS^{Fgfr1} cells were transfected with the transcriptional reporter pKrox24 (22 [↗](#)), engineered to induce the expression of eGFP upon ERK pathway activation. The pKrox signal was monitored for 48 hours by automated widefield microscopy. Significant differences in pKrox24 transactivation were found, with FGF4 inducing the strongest signal, in both magnitude and duration, compared to FGF9 and FGF8. Significant differences were also observed between FGF8 and FGF9-induced ERK activation (**Fig. 1C** [↗](#)). These experiments showed that the three FGF ligands induce differential activation of ERK in RCS chondrocytes, consistent with the genetic ablation experiments (19 [↗](#)), and prompted studies into the mechanism behind these differences.

Differences in FGF-induced FGFR1 oligomerization

While most RTKs signal as dimers, it has been reported that under some conditions RTKs can form oligomers with different signaling capabilities (23 [↗](#), 24 [↗](#)). Therefore, we considered the possibility that FGF4, FGF8, and FGF9 promote the formation of different types of FGFR1 oligomers in the plasma membrane. We thus assessed the association state of FGFR1, labeled with YFP, using fluorescence intensity fluctuations (FIF) spectrometry. The fluorophores were attached to the C-terminus of FGFR1 via a GGS flexible linker; this attachment has been used before and has been shown to not affect function (25 [↗](#)). The FIF experiments utilized 293T cells stably transfected with FGFR1-YFP.

FIF calculates molecular brightness of the YFP-tagged receptors in small regions of the cell membrane (26 [↗](#)). The molecular brightness, defined as the ratio of the variance of the fluorescence intensity within a membrane region to the mean fluorescence intensity in this region, and is proportional to the oligomer size (27 [↗](#)). The molecular brightness distributions of monomeric (Linker for Activation of T-cells (LAT); gray) (28 [↗](#), 29 [↗](#)) and dimeric (TrkA in the presence of 130 nM nerve growth factor (NGF); black) (30 [↗](#)) controls in 293T cells are shown in **Figure 2A** [↗](#), along with the brightness distribution for FGFR1 in 293T cells stably expressing FGFR1-YFP (red). We found that FGFR1, in the absence of ligand (red line), exists in a monomer/dimer equilibrium, as its brightness distribution is between the distributions of LAT (monomer control) and TrkA in the presence of saturating concentration of NGF (dimer control). The FIF experiments also report on the concentration of the receptors at the cell surface, which we find to be in the range 100-200 FGFR1/ μm^2 in the stable cell line. This concentration is similar to previously reported FGFR expression levels of the order of $\sim 80,000$ receptors per cell, corresponding to ~ 80 -100 receptors/ μm^2 (31 [↗](#)).

Next, we performed FIF experiments in the presence of FGF4, FGF8, and FGF9. The FGFs were added at high concentrations (130 nM), which exceed the reported FGF binding constants (in the $\sim$ nM range) (32 [↗](#), 33 [↗](#)) such that all FGFR1 receptors are FGF-bound. The brightness distributions in the presence of the FGFs are shown in **Figure 2A** [↗](#). We see that the brightness distributions recorded in the presence of FGF8 (green) and FGF9 (blue) overlap with the distribution for the dimer control, while the distribution for FGF4 (orange) appears shifted to higher brightness. In fact, these data fall between the dimer control and the large oligomer control (EphrinA1+ephrinA1-Fc) (24 [↗](#), 34 [↗](#)-36 [↗](#)). This brightness distribution suggests that the average oligomer size may be increased beyond a dimer in the presence of FGF4. We analyzed the likelihood of this possibility using a statistical test. Since the distributions of molecular brightness are log-normal, we analyzed the corresponding log(brightness) distributions which are Gaussian

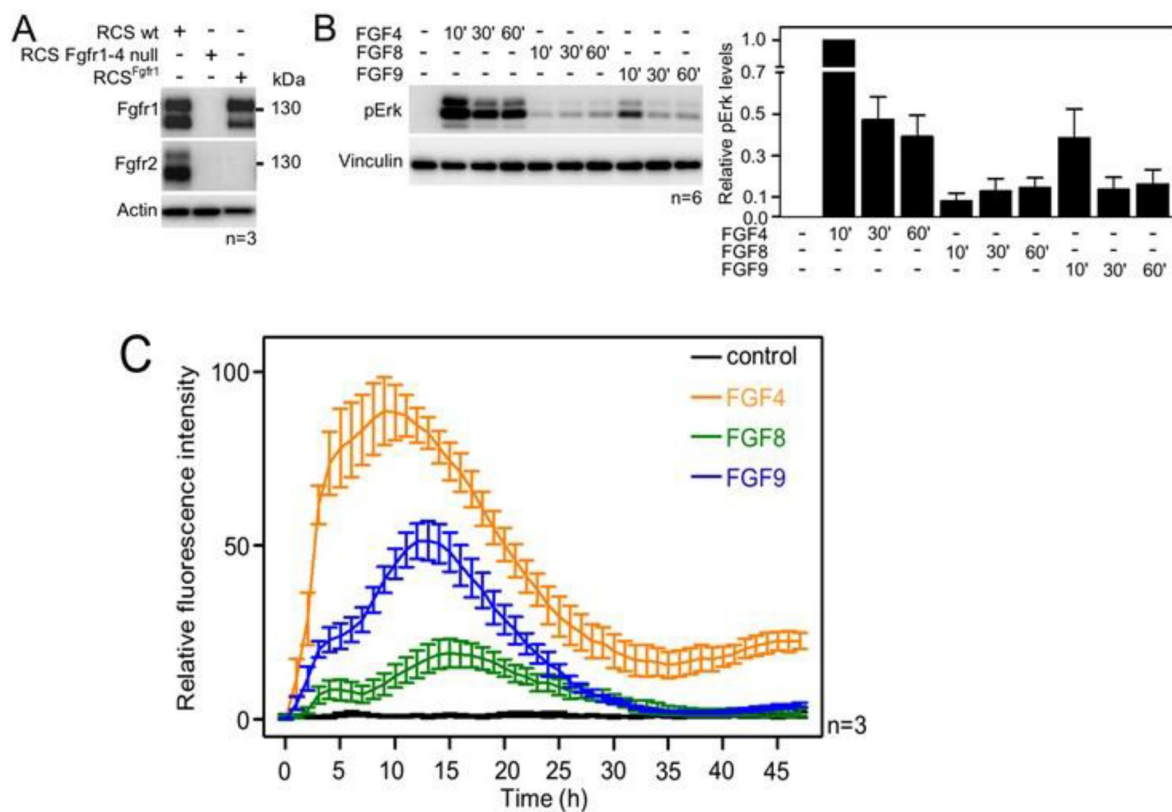


Figure 1.

(A) Expression of FGFR1 and FGFR2 in wildtype RCS cells, RCS *null* for FGFR1-4, and RCS cells expressing only endogenous FGFR1 (RCS^{Fgfr1}). Actin serves as a loading control; n, number of independent experiments. **(B)** RCS^{Fgfr1} cells were treated with FGF4, FGF8 and FGF9 for indicated times and ERK phosphorylation (pErk) was monitored by western blot. Vinculin serves as a loading control. pErk signal was quantified and graphed (right) as relative values compared to the 10' FGF4 stimulation; data show average and SEM of six independent experiments. **(C)** RCS^{Fgfr1} expressing the pKrox(MapERK)d1EGFP reporter were treated with FGF4, FGF8, and FGF9 and pKrox24 transactivation was monitored for 48 hours.

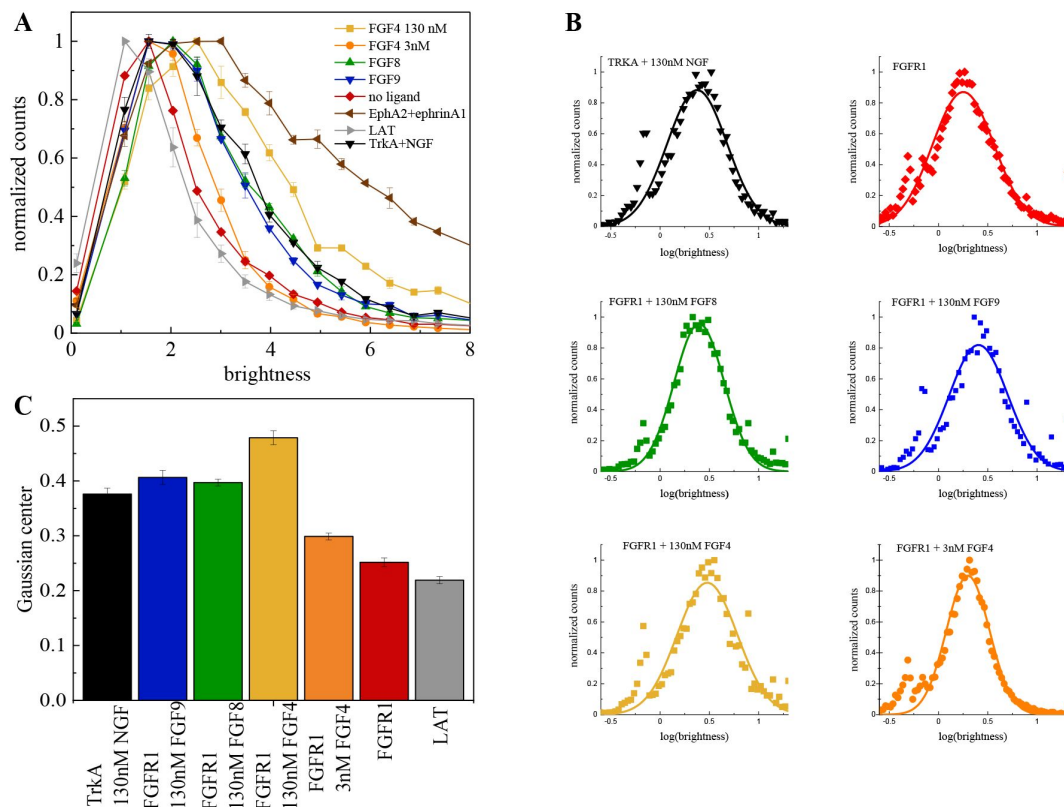


Figure 2

The oligomerization state of FGFR1, as measured by fluorescence intensity fluctuation (FIF) spectrometry. (A) Brightness distributions shown on the linear scale. Brightness scales with the oligomer size. LAT (gray) is a monomer control, TrkA+130 nM NGF (black) is a dimer control. EphA2 bound to ephrinA1-Fc (brown) is an oligomer control. All distributions are scaled to a maximum of 1. (B) Distributions of $\log(\text{brightness})$. Points represent the experimental FIF data, and the solid lines are the best fit Gaussians. (C) Means of the best-fit Gaussians and the standard errors of the mean.

(**Figure 2B** [↗](#)). The parameters of the Gaussian distributions and the standard errors were calculated and used in a Z-test. Results, shown in **Figure 2C** [↗](#), show that there are statistically significant differences ($Z > 2$, ([37](#) [↗](#))) between the FGFR1 brightness distribution in the FGF4 case and the distribution for the dimer control (TrkA+NGF). Likewise, there are statistically significant differences between the FGFR1+FGF4 brightness distribution, on one hand, and the FGFR1+FGF8 and FGFR1+FGF9 brightness distributions, on the other ($Z > 2$). Further, the distributions measured for FGFR1+FGF8 and FGFR1+FGF9 are the same as the dimer control distribution. This analysis suggests that while FGF8-bound and FGF9-bound FGFR1 forms dimers, FGF4 binding may promote the formation of higher order FGFR1 oligomers.

Differences in FGFR1 Phosphorylation Dose Response curves

Next, we studied FGFR1 signaling in response to FGF4, FGF8, and FGF9 using quantitative western blotting. In particular, we acquired FGFR1 dose response curves while varying the concentrations of FGF4, FGF8, or FGF9 over a broad range. As we sought mechanistic interpretation of the results, we used the same 293T cell line used in the FIF experiments, since the FGFR1 expression and the FGFR1 oligomer size in the cell line are known.

The cells were incubated with FGFs for 20 minutes at 37°C, after which the cells were lysed, and the lysates were subjected to SDS-PAGE while probing with antibodies recognizing specific phospho-tyrosine motifs. We assessed several responses: (i) phosphorylation of Y653/654, which are the two tyrosines in the activation loop of the FGFR1 kinase that are required for kinase activity ([4](#) [↗](#), [6](#) [↗](#), [38](#) [↗](#), [39](#) [↗](#)); (ii) phosphorylation of Y766 in the kinase tail of FGFR1, which serves as a binding site for PLC γ ([40](#) [↗](#)); (iii) phosphorylation of FRS2, which is an adaptor protein that is constitutively associated with FGFR1 through interactions that do not involve phosphorylated tyrosines ([41](#) [↗](#), [42](#) [↗](#)); and (iv) phosphorylation of PLC γ , which binds to Y766 ([43](#) [↗](#)). In addition, we blotted for the total expression of FGFR1, thus assaying for ligand-induced FGFR1 downregulation. Typical western blots are shown in **Figure 3A** [↗](#). The band intensities from at least three independent experiments were quantified and plotted for each response and each ligand concentration in **Figures 3C** [↗](#). The dose response curves in each panel were placed on a common scale by re-running some of the samples on a common gel, as shown in **Figure 3B** [↗](#). The protocol to place the dose response curves on a common scale is given in Supplementary Data.

The quantified results are shown in **Figure 3C** [↗](#), which reveals unexpected differences in the shape of the dose response curves. While the FGF8 and FGF9 dose response curves appear sigmoidal when plotted on a semi-log scale, as expected for a rectangular hyperbolic curve (**equation 1** [↗](#)), FGF4 exhibits an increase in phosphorylation up to 2.6 nM, followed by a marked decrease in phosphorylation for all studied responses with further increasing FGF4 concentration.

What could explain the very unusual FGF4 dose response curves in **Figure 3C** [↗](#)? We first considered the possibility that the shape of the FGF4 dose response is due to strong FGF4-induced FGFR1 degradation at high FGF4 concentrations. Data in **Figure 3C** [↗](#), however, do not support this view as the effect of FGF4 on FGFR1 downregulation is smaller when compared to the effects of FGF8 and FGF9. Thus, differential ligand-induced FGFR1 degradation cannot explain the shape of the dose response curves.

We next considered the possibility that the shape of the FGF4 dose response curve is due to differential FGFR1 de-phosphorylation kinetics. In particular, we asked whether at high FGF concentration, a fast de-phosphorylation occurs for FGF4-bound FGFR1, while the de-phosphorylation of FGF8-bound and FGF9-bound FGFR1 is slower. As the western blot data were acquired after 20 minutes of stimulation, such differences in kinetics could explain the shape of the dose response. We thus measured Y653/654 phosphorylation via western blotting as a function of time, when 130 nM FGF was added to the cells for a duration of 0, 1, 5, 10, 20, and 60 minutes. The averages of 3 independent experiments are shown in **Figure 4A** [↗](#), along with the standard errors. We see a quick increase in phosphorylation over time, followed by a decrease which may

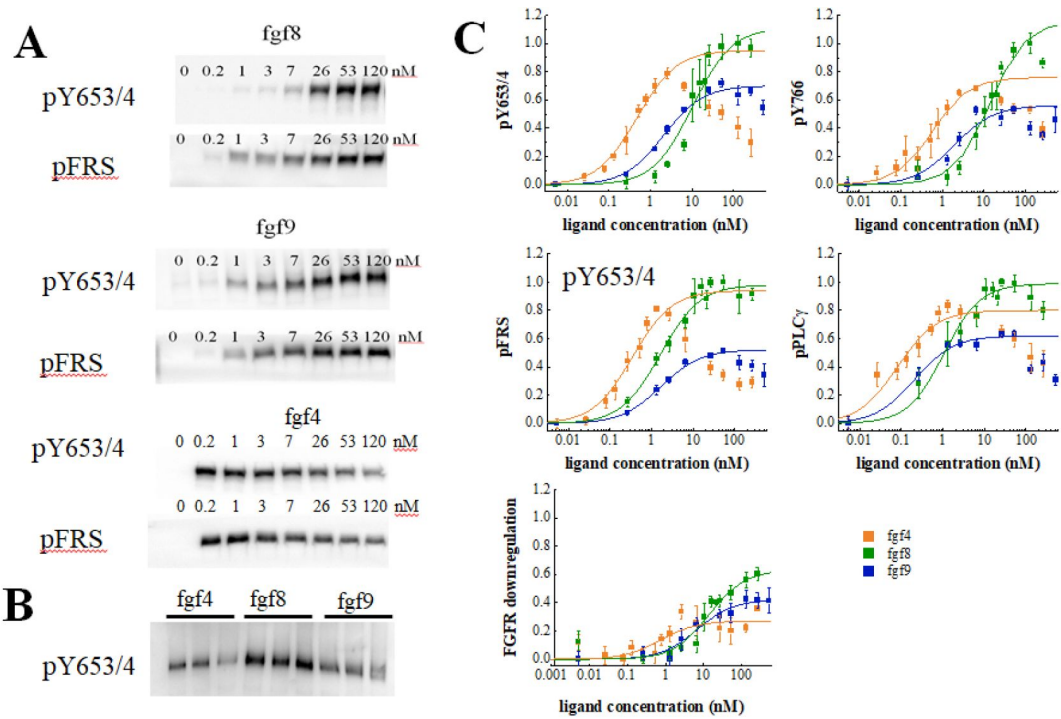


Figure 3

Phosphorylation of FGFR1 and downstream signaling substrates in HEK 293T cells. (A) Sample western blots for Y653/4 FGFR1 phosphorylation and FRS2 phosphorylation in response to FGF4, FGF8, and FGF9. (B) An example blot used for data scaling, where samples with maximum phosphorylation in response to FGF4, FGF8, and FGF9 are rerun on the same gel (C) Dose response curves from the Western blot experiments. The points represent the averaged data, while the solid lines are the best fit rectangular hyperbolic curves. Fit parameters are shown in [Table 1](#).

pFGFR			PLCg		
	EC50	Etop		EC50	Etop
FGF4	4.77E-10 ± 0.42E-10	0.95 ± 0.03	FGF4	7.98E-11 ± 2.15E-11	0.80 ± 0.04
FGF8	1.04E-8 ± 0.23E-8	1.11 ± 0.07	FGF8	1.05E-9 ± 0.23E-9	0.99 ± 0.03
FGF9	2.09E-9 ± 0.31E-9	0.70 ± 0.02	FGF9	2.05E-10 ± 0.40E-10	0.62 ± 0.02
p766			pFRS		
	EC50	Etop		EC50	Etop
FGF4	5.84E-10 ± 1.26E-10	0.76 ± 0.05	FGF4	3.42E-10 ± 0.70E-10	0.94 ± 0.06
FGF8	1.42E-8 ± 0.25E-8	1.16 ± 0.07	FGF8	1.62E-9 ± 0.29E-9	0.98 ± 0.03
FGF9	1.94E-9 ± 0.71E-9	0.56 ± 0.05	FGF9	1.65E-9 ± 0.15E-9	0.52 ± 0.01
Downregulation			Proliferation		
	EC50	Etop		EC50	Etop
FGF4	7.34E-10 ± 4.54E-10	0.27 ± 0.04	FGF4	2.59E-12 ± 1.1E-13	0.99 ± 0.00
FGF8	1.39E-8 ± 0.39E-8	0.63 ± 0.05	FGF8	1.60E-9 ± 2.3E-10	1.14 ± 0.04
FGF9	6.80E-9 ± 2.08E-9	0.42 ± 0.02	FGF9	5.89E-11 ± 8.5E-12	0.95 ± 0.00
Collagen Loss					
	EC50	Etop			
FGF4	1.24E-11 ± 2.79E-12	1.02 ± 0.01			
FGF8	8.51E-11 ± 2.86E-11	0.77 ± 0.03			
FGF9	5.67E-11 ± 2.89E-11	0.86 ± 0.06			

Table 1

Best fit parameters for dose response curves in Figures 3 [↗](#) and 5 [↗](#). EC50 is the potency of the ligand, and Etop is the efficacy (see equation 1 [↗](#))

be due to FGFR1 de-phosphorylation and/or FGFR1 downregulation. Notably, the phosphorylation decrease in response to FGF4 is very similar when compared to the decrease in the presence of FGF9, while the FGF8-induced phosphorylation decrease is smaller. Thus, the FGF4-induced de-phosphorylation kinetics are not fundamentally different when compared to the FGF9 results and cannot provide an explanation for the observed decrease in FGFR1 phosphorylation at high FGF4 concentration.

Another possible explanation for the shape difference in the dose response curves could be that the FGF4-stabilized FGFR1 oligomers, observed in the FIF experiments at high FGF4 concentration (**Figure 2**), are less active than the FGFR1 dimers. To gain further insights into FGF4-induced FGFR1 oligomerization, we performed FIF experiments in the presence of 2.6 nM FGF4, which corresponds to the peak of Y653/654 phosphorylation in the FGF4 dose response curve. The brightness distribution at 2.6 nM FGF4, shown in **Figure 2**, lies between the monomer control and the dimer control. The Z-test analysis shows that the 2.6 nM FGF4 brightness distribution is significantly different from both the monomer and dimer control distributions, as well as from the distribution observed in the presence of high FGF4 concentration. Thus, at low FGF4 concentration, FGFR1 exists primarily in monomeric and dimeric states, while higher FGF4 concentrations (>2.6 nM FGF4) induce the formation of FGFR1 oligomers. The increase in phosphorylation at low FGF4 concentration can therefore be associated with FGFR1 dimers, while the subsequent phosphorylation decrease could be correlated with the appearance of oligomers in addition to dimers. Thus, the assumption that FGFR1 oligomers are less active than FGFR1 dimers could provide an explanation for the observed shape of the FGF4 dose response curves.

Differences in Phosphorylation Potencies and Efficacies, and Demonstration of Ligand Bias

We next analyzed the dose response curves in **Figure 3C** to determine the potencies and the efficacies of FGF4, FGF8 and FGF9. These two parameters were determined as optimal fit parameters in the context of rectangular hyperbolic dose response curves (see **equation 1**), as done in the GPCR literature (44–46). The efficacy (E_{top} in **equation 1**) is the highest possible response that can be achieved for a ligand, typically at high ligand concentration. The potency (EC_{50} in **equation 1**), on the other hand, is the ligand concentration that produces 50% of the maximal possible response for a given ligand. A highly potent ligand will evoke a certain response at low concentrations, while a ligand of lower potency will evoke the same response at much higher concentration.

The fitting of the FGF4 dose response curves presented a particular challenge, as we could only fit the increasing portions of the dose response curves to a rectangular hyperbolic curve. As described in Materials and Methods, we truncated the data for the fit, including the data in the ascending portions of the curves and taking into account that the average errors in the western blots are 5-10%. In particular, we truncated all the acquired dose response curves at the highest ligand concentration that is within 10% of the maximum response value. Under the assumption that the decrease in the FGF4 dose response curve is due to oligomerization, the best-fit FGF4 dose response curves represent the response of the FGFR1 dimers to FGF4.

The best fits for all dose response curves are shown in **Figure 3C** with the solid lines. The best fit values of E_{top} and EC_{50} for all studied responses are shown in **Table 1**. FGF4 exhibits the highest potency, followed by either FGF8 or FGF9, dependent of the particular response. FGF8 exhibits the highest efficacy, followed by either FGF4 or FGF9, dependent of the particular response. In most cases, FGF8 is a full agonist, while FGF9 and FGF4 are partial agonists. However, FGF4 and FGF8 appear to be both full agonists when FRS2 phosphorylation is probed (see **Table 1** and **Figure 3C**).

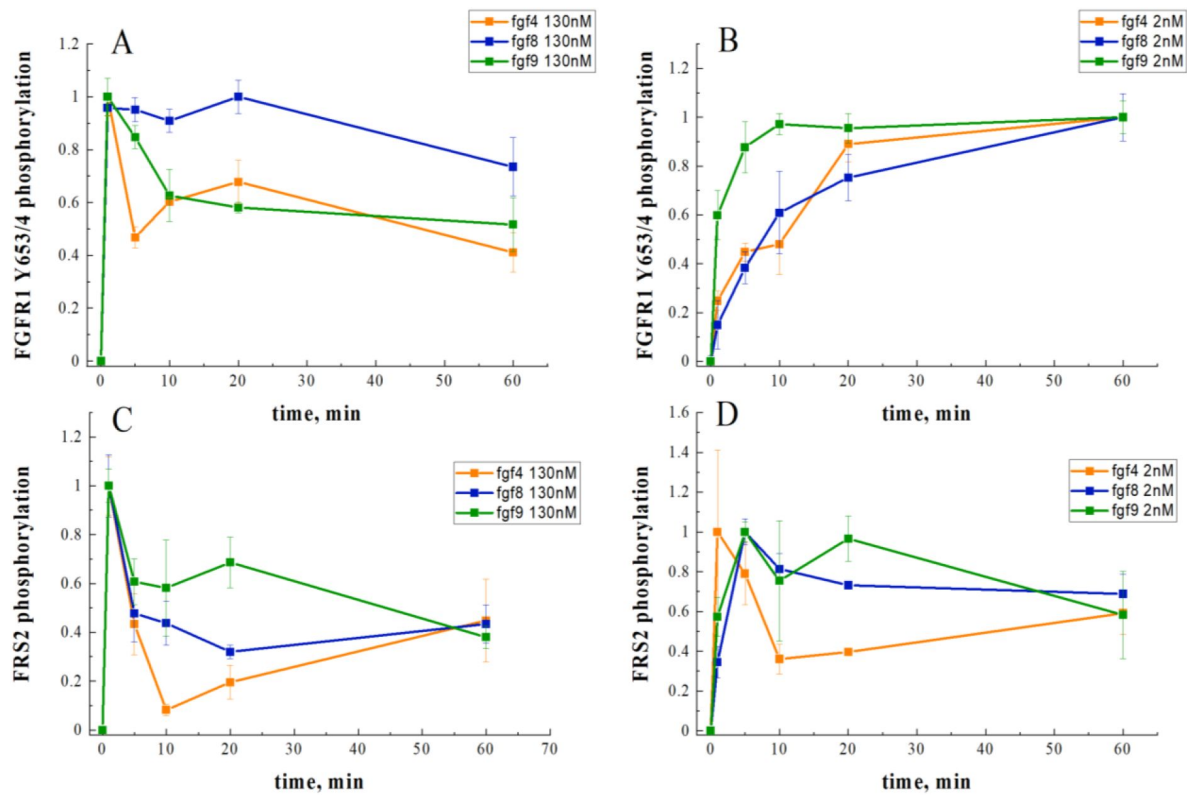


Figure 4

FGFR1 phosphorylation as a function of time after ligand addition. (A) Phosphorylation time course of Y653/654 at high ligand concentration (130 nM) (B) Phosphorylation time course of Y653/654 at low ligand concentration (2.6 nM) (C) Phosphorylation time course of FRS2 at high ligand concentration (130 nM) (D) Phosphorylation time course of FRS2 at low ligand concentration (2.6 nM)

The results in **Table 1** show that the rank-ordering of the different ligands is different when different responses are probed, which is indicative of ligand bias. “Biased agonism” or “ligand bias” is the ability of different ligands to differentially activate different signaling pathways downstream of the same receptor (47). Ligand bias reflects not just quantitative differences in downstream signaling, but a fundamental difference between the signaling responses to different ligands (48, 49). To determine if bias exists or not, we calculate bias coefficients using equation 2. Each bias coefficient, shown in **Table 2**, compares two responses and two ligands and reveals whether a response is preferentially engaged by one of the ligands ($\beta \neq 0$) or whether both response are activated similarly by both ligands ($\beta = 0$). We refer to the entire set of coefficients as a “bias map.”

We assessed statistical significance of the differences in bias coefficients for each pair of responses using ANOVA as described in Materials and Methods. The results of the statistical analysis are shown in **Table 2**, where gray shading indicates statistical significance between either FGF4 or FGF9, on one hand, and the reference ligand FGF8, on the other. We see no statistical significance between FGF4 and FGF9 (Supplemental Table S2).

Based on the ANOVA analysis, we conclude that FGF8 is biased towards phosphorylation of FRS2, against phosphorylation of Y653/654 and Y766, and against PLC γ activation, as compared to FGF9. Furthermore, FGF8 is biased towards phosphorylation of FRS2 and against phosphorylation of Y653/654 and Y766, as compared to FGF4.

Phosphorylation time courses cannot explain the existence of ligand bias

Previously, it has been suggested that ligand bias may arise due to differences in the time courses of phosphorylation (50, 51). We therefore sought to compare phosphorylation of the activation loop tyrosines Y653/654 and FRS2 over time. We chose these two particular responses because FGF8 is biased towards FRS2 and against Y653/654 FGFR1 phosphorylation, when compared to FGF4 and FGF9 (**Table 2**). Thus, we complemented the Y653/654 phosphorylation time course in **Figure 4A**, acquired at high concentration (130 nM FGF), with kinetics measurements of Y653/654 phosphorylation at low (2.6 nM) FGF concentration, as well as FRS2 phosphorylation at low (2.6 nM) and high (130 nM ligand) FGF concentrations. Three independent time courses were acquired for each ligand-receptor pair, and the results were averaged. Data, scaled such that the maximal phosphorylation is set to 1, are shown in **Figure 4B-D**.

We observe differences in the time courses of Y653/654 and FRS2 phosphorylation. In the case of FRS2, we always observe fast accumulation of phosphorylated FRS2, which then decreases over time. Such a behavior has been observed for other RTKs and can be explained by the fact that auto-phosphorylation within the RTK dimers/oligomers occurs faster than phosphatase-mediated de-phosphorylation, after which a steady state is established (52, 53). An early maximum in phosphorylation (at 5 minutes) is seen for FRS2 at both low and high FGF concentration, and is very pronounced for high FGF concentration. An early phosphorylation maximum is also observed for Y653/654 FGFR at high FGF concentration, but not at low FGF concentration.

When comparing the time courses of FGFR1 phosphorylation, in every panel of **Figure 4**, we do not see discernable differences in the time course of FGF8-induced phosphorylation, as compared to FGF4 or FGF9. Thus, the time course of phosphorylation alone cannot identify FGF8 as a biased ligand, when compared to FGF4 or FGF9.

β		
	4v8	9v8
pY653/654 vs pY766	-0.07 $\pm$ 0.16	0.05 $\pm$ 0.15
pY653/654 vs pPLCγ	-0.24 $\pm$ 0.18	0.01 $\pm$ 0.14
pY653/654 vs pFRS2	-0.61 $\pm$ 0.16	-0.78 $\pm$ 0.12
pY766 vs pPLCγ	-0.18 $\pm$ 0.19	-0.04 $\pm$ 0.17
pY766 vs pFRS2	-0.54 $\pm$ 0.17	-0.83 $\pm$ 0.16
pPLCγ vs pFRS2	-0.37 $\pm$ 0.20	-0.79 $\pm$ 0.13
pY653/654 vs Downregulation	-0.36 $\pm$ 0.26	-0.36 $\pm$ 0.16
pY766 vs Downregulation	-0.29 $\pm$ 0.27	-0.41 $\pm$ 0.19
pPLCγ vs Downregulation	-0.12 $\pm$ 0.28	-0.37 $\pm$ 0.16
pFRS2 vs Downregulation	0.25 $\pm$ 0.27	0.42 $\pm$ 0.15
Collagen Loss vs Growth Arrest	-1.77 $\pm$ 0.20	-1.13 $\pm$ 0.18

Table 2

Calculated Bias Coefficients using [equation 2](#). Gray shading indicates statistical significance between either FGF4 or FGF9 and the reference ligand FGF8 (see Supplemental Table S2 for p-values).

Differences in cellular responses and demonstration of functional bias

Biased signaling manifests itself in different cellular responses, which can be cell-specific (48). We therefore investigated if cells expressing FGFR1 respond differently when stimulated with FGF8 and FGF9, as these two FGFs are biased and their effects at high concentrations can be directly compared as the phosphorylation responses come to a plateau (Figure 3). We compared FGFR1 clearance from the plasma membrane due to FGF-induced uptake, cell apoptosis, and cell viability for the 293T cells used in the western blotting experiments. These cellular responses have been previously reported to occur downstream of FGFR1 activation (7, 54, 55).

To study FGFR1 endocytosis, we measured the decrease in FGFR1-YFP concentration in the plasma membrane in response to a 2-minute treatment of 130 nM of FGF8 or FGF9. After fixation, the plasma membranes in contact with the substrate were imaged on a confocal microscope, and the fluorescence intensities for hundreds of cells stimulated with either FGF8, FGF9, or no ligand were recorded. The average fluorescence intensities for the three groups, from three independent experiments, are shown in Figure 5A. We observed a decrease in fluorescence, corresponding to a decrease in plasma membrane concentration of FGFR1-YFP in response to FGF8, as compared to FGF9 and no ligand. This effect was statistically significant by ANOVA ($p < 0.05$). On the other hand, there were no statistically significant differences in FGFR1-YFP membrane concentrations between the FGF9-treated and control groups. These results indicate that FGF8 is more efficient at inducing FGFR1 removal from the plasma membrane immediately after ligand addition, as compared to FGF9. Of note, this result is consistent with the data in Figure 3C, which show that the efficacy of FGF8-induced downregulation, measured in the western blot experiments 20 minutes after ligand addition, was higher than that of FGF9. However, the total FGFR1 expression was not affected by a 2-minute treatment with ligand (Figure S1), consistent with the expectation that the FGF8-induced decrease in FGFR1 membrane concentration in Figure 5A reflects enhanced internalization that precedes degradation.

We next sought to measure and compare the relative amounts of live FGFR1-expressing cells after stimulation with FGF and after six days of starvation, using a MTT assay. The read-out as a function of FGF concentration is shown in Figure 5B, revealing a slight decrease in cell viability with increasing concentration for both FGF8 and FGF9. Data were fitted to linear functions, and the slopes were compared using a t-test. Results show that there is a significant difference in cell viability when cells are stimulated with FGF8 or FGF9 ($p < 0.015$), and the effects are significant when compared to the case of no FGF.

To monitor the apoptosis of cells exposed to different FGF8 and FGF9 concentrations, we used a commercial kit that measures the combined activity of caspases 3 and 7 (Magic Red Caspase Kit) through an increase in caspase substrate fluorescence. Experiments were conducted at 0, 20, 40, and 100 nM FGF, under starvation conditions, and the results for each set of experiments were scaled such that caspase activity was 1.0 in the absence of FGF. Experiments were repeated at least five times. Data in Figure 5C show the scaled caspase activity as a function of FGF concentration. We see a significant increase in caspase activity in the case of FGF8, but not FGF9. Data were fitted to linear functions, and the best-fit slopes were compared using a t-test. The difference between the slopes was statistically significant ($p < 0.005$), indicating that FGF8 promotes apoptosis more efficiently than FGF9 in 293T cells. We thus observe that FGF8 is more efficient than FGF9 at promoting FGFR1 clearance from the plasma membrane, apoptosis, and cell viability under starvation conditions. This is a manifestation of the ligand bias seen upstream.

Since the FGF-induced effects on 293T cell responses were modest, and the dose response curves did not exhibit rectangular hyperbolic shapes to allow bias coefficient calculations, we quantified two well known functional responses of RCS cells to FGF treatment: growth arrest and loss of collagen type 2 expression (56, 57). We performed the experiments with the RCS^{Fgfr1} cells

used to acquire the data in [Figure 1](#). Collagen 2 amounts were measured using western blotting after 48 hours of treatment with FGFs at varying concentrations ([Figures 5D and E](#)). Similarly, the growth arrest of RCS^{Fgfr1} cells was determined after 72 hours of stimulation with the three FGFs ([Figure 5F](#)). The dose response curves in [Figures 5E](#) and [5F](#) are distinctly different from each other and all exhibit rectangular hyperbolic shapes. We thus see that FGF4, FGF8 and FGF9 have very different effects on the two functional responses that we quantified.

The dose response curves were fitted using [eqn 1](#) and the EC50 and Etop values are reported in [Table 1](#). The three FGFs exhibit different potencies, with FGF4 being the most potent inducer of both responses. The potencies of FGF8 and FGF9 for collagen 2 reduction are similar, but their effects on cell proliferation differ significantly. The efficacies of the three ligands in inducing collagen 2 reduction are different, with FGF4 behaving as full agonist and FGF8 and FGF9 as partial agonists. The efficacies in inducing growth arrest are similar for the three FGFs.

The bias coefficients are reported in [Table 2](#). FGF8 is strongly biased towards collagen loss and against growth arrest, when compared to FGF4 and FGF9. The effect is highly significant (Table S2).

Structural determinants behind FGFR1 biased signaling

We sought possible structural determinants behind the observed FGF bias. For GPCRs, it is now well established that different GPCR ligands stabilize different receptor conformations, where each of the conformations has a preference for a subset of downstream signaling molecules (either G proteins or arrestins) ([25](#), [58](#), [59](#)). We therefore asked if structural differences in the FGF4, FGF8, and FGF9-bound FGFR1 dimers may explain bias for FGF8, as compared to FGF4 and FGF9.

It is known that FRS2 binds to the FGFR1 JM domain in a ligand-independent manner ([42](#), [60](#)). It is also believed that the conformation of the JM domain of RTKs is influenced by the conformation of the TM domain dimer in response to ligand binding ([59](#), [61](#)). Further, the FGFR1 TM domain has already been shown to sense the identity of the bound FGF, either FGF1 or FGF2, and adopt a different TM domain dimer conformation ([25](#)). We therefore sought to investigate the possibility that the TM domains of FGFR1 dimerize differently when FGF8, as compared to FGF4 and FGF9, is bound to the FGFR1 EC domain.

A Förster resonance energy transfer (FRET)-based methodology has been instrumental in revealing differences in FGFR TM domain dimer conformations in response to FGF binding ([25](#)). In these experiments, we monitored differences in the intracellular distances and relative orientation of fluorescent protein reporters when different ligands bind to the EC domain. We used truncated FGFR1 constructs, which contain the entire EC and TM domains of FGFR1 followed by a flexible linker and either mCherry or YFP. The fluorescent proteins mCherry and YFP are a FRET pair and thus report on the separation between the C-termini of the TM domains in a FGFR1 dimer ([25](#)). Measurements were performed in plasma membrane derived vesicles, as previously described ([62](#)). High concentration of ligand (250 nM) was used, to ensure that all receptors are ligand-bound. We followed a quantitative protocol (termed QI-FRET) which yields (i) the FRET efficiency, (ii) the donor concentration, and (iii) the acceptor concentration in each vesicle ([63](#)). A few hundred vesicles, imaged in multiple independent experiments, were analyzed and the data were combined.

[Figure 6A](#), left, shows the FRET efficiencies as a function of total FGFR1 concentration in the presence of the three ligands, where each data point corresponds to one individual vesicle derived from one cell. The total FGFR1 concentration, on the x axis in [Figure 6A](#), left, is the sum of ECTM FGFR1-YFP and ECTM FGFR-mCherry concentrations. In [Figure 6A](#), right, we further show the

expressions of ECTM FGFR1-YFP and ECTM FGFR1-mCherry in the individual vesicles. Because transient expression levels vary from cell to cell, the vesicles produced in a single transfection experiment had a wide range of receptor concentrations.

We see that FRET efficiency in [Figure 6A](#) does not depend on the concentration, over a broad receptor expression range, indicative of constitutive FGFR1 association in the presence of FGF4, FGF8, and FGF9. To interpret the FRET data, we need to know the oligomer size of the ECTM FGFR1 construct. FIF experiments (not shown) demonstrated that the ECTM construct always forms dimers, even in the presence of high FGF4 concentration. Thus, the data in [Figure 6](#) correspond to ligand-bound ECTM FGFR1 dimers; in this case, FRET efficiency depends only on (i) the fraction of acceptor-labeled FGFR1, which is directly calculated from the data in [Figure 6A](#) and (ii) the so-called Intrinsic FRET, a structural parameter which depends on the positioning and dynamics of the two fluorophores ([64](#)). An Intrinsic FRET value was calculated for each vesicle from the data in [Figure 6A](#) using [equation 5](#). The values were binned to generate a histogram and are shown in [Figure 6B](#). These histograms were fitted with Gaussians, yielding Intrinsic FRET values of 0.54 ± 0.01 , 0.42 ± 0.01 and 0.52 ± 0.01 , for the cases of FGF4, FGF8, and FGF9, respectively. Thus, intrinsic FRET is lower for FGF8-bound ECTM FGFR1 dimers, as compared to FGF4 and FGF9-bound ECTM FGFR1 dimers. Since the fluorescent proteins were attached directly to the TM domains via flexible linkers, the measured differences in Intrinsic FRET reflect differences in the separation of the C-termini of the TM domains in the presence of FGF8, compared to FGF4 and FGF9. Thus, the TM domain dimer conformation is different in the FGF8-bound FGFR1 dimers as compared to FGF4 and FGF9-bound dimers.

The effective distances between the fluorescent proteins in the ECTM FGFR1 dimers are calculated using [equation 6](#), assuming random orientation of the fluorophores (justified because they were attached via flexible linkers). In the presence of FGF4 and FGF9, the effective distance between the fluorescent proteins is the same, $55 \pm 1 \text{ \AA}$ and $56 \pm 1 \text{ \AA}$ ($p > 0.05$). In the presence of FGF8, the effective distance between the fluorescent proteins is significantly higher, $60 \pm 1 \text{ \AA}$ ($p < 0.01$) as shown in [Figure 6C](#). These structural differences may underlie the observed signaling bias in response to FGF8, as compared to FGF4 and FGF9.

Discussion

The most significant discovery in this work is the existence of bias in FGFR1 signaling in response to three FGF ligands that are encountered during limb development. This concept of “ligand bias” is a relatively novel concept in RTK research ([68](#)). It describes the ability of ligands to differentially activate signaling pathways ([46](#), [47](#), [69](#)). Ligand bias has been studied primarily in the context of G-protein coupled receptors (GPCRs) and has transformed the fundamental understanding of GPCR signaling ([47](#)). Importantly, these investigations have produced optimized protocols to identify and quantify bias that are directly applicable to RTKs ([68](#)). Here we use such quantitative protocols to demonstrate that FGF8 but not FGF4 or FGF9 preferentially induces FRS2 phosphorylation, when compared to the phosphorylation of FGFR1 tyrosines. We also demonstrated that FGF8 preferentially induces collagen 2 loss over growth arrest, as compared to FGF4 and FGF9.

It must be noted that the term “RTK ligand bias” is often used in the literature to indicate any difference in signaling due to ligands. Here we use this term in the context of its classical definition, namely the differential activation of at least *two* different signaling responses ([46](#), [47](#), [69](#)). The term “bias” is distinctly different from the potencies and efficacies of a ligand for one particular response. For example, in [Figure 5E](#) we see that FGF8 has both lower potency and lower efficacy than FGF4, but these data cannot provide any information about bias. Bias can be assessed only if the data in [Figure 5E](#) are compared to the data in 5F, leading us to conclude that FGF8 is biased towards collagen 2 loss and against growth arrest, as compared to FGF4,

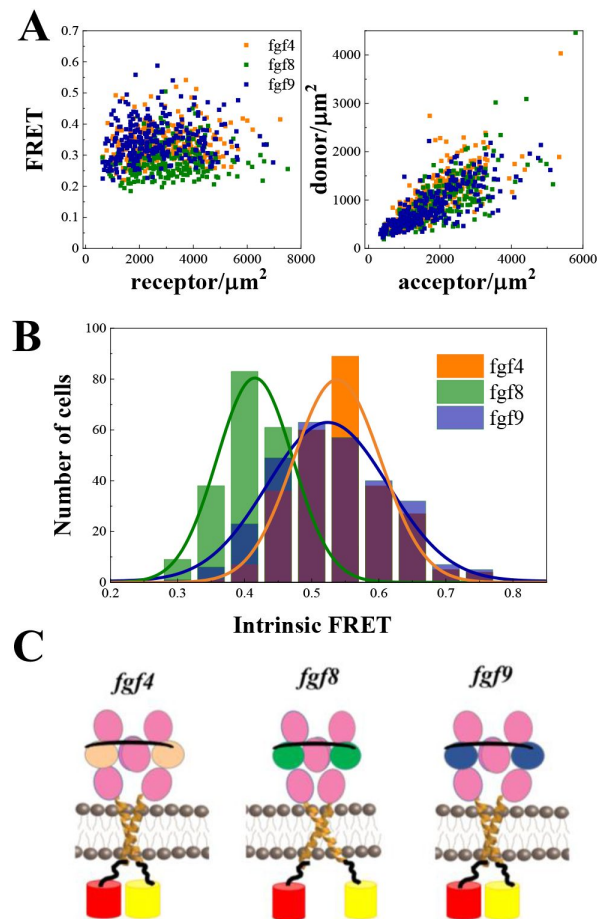


Figure 6

Differences in FGFR1 dimer conformations in response to FGF ligands. (A) and (B). FRET data for ECTM-FGFR1-YFP and ECTM-FGFR1-mCherry in the presence of saturating FGF4 (orange), FGF8 (green) or FGF9 (blue) concentrations. (A) Measured FRET efficiencies versus total receptor (ECTM-FGFR1-YFP + ECTM-FGFR1-mCherry) concentrations and measured donor (ECTM-FGFR1-YFP) concentrations versus acceptor (ECTM-FGFR1-mCherry) concentrations in single vesicles. (B) Histograms of single-vesicle intrinsic FRET values. Intrinsic FRET is a measure of the separation between the fluorescent proteins in the dimer. Different intrinsic FRET values were measured for FGF8 and FGF4/FGF9. (C) Graphical representation of experimental results showing that FGF8 induces a TM dimer conformation where the TM C-termini are positioned further apart from each other, as compared to the cases of FGF4 and FGF9.

despite the fact that FGF8 has the lowest potency and the lowest efficacy in inducing collagen loss. Potency, efficacy, and bias describe distinct aspects of RTK signaling, yet the differences in these characteristics have not been explicitly considered in prior studies of FGF signaling.

Ligand bias studies require the comparison of at least two responses and at least two ligands (46 [47](#), 69 [47](#)). Prior studies of the effects of different FGFs on FGFR signaling have utilized BAF/3 cells (9 [47](#)–11 [47](#)). In these cells, only a single response, cell proliferation, can be quantified and compared, and thus these cells cannot be used for bias studies. In HEK293T cells, the functional effects due to the FGF4, FGF8, and FGF9 are modest (**Figure 5A–C** [47](#)). However, here we were able to measure dose response curves for two functional responses in engineered RCS^{Fgfr1} cells (**Figure 5E, F** [47](#)). These cells allow us to model processes occurring in the developing mammalian limb, where the three FGF ligands (FGF4, FGF8, FGF9) released by the ectoderm at the surface of the limb bud signal to the underlying mesenchymal cell which expresses just one FGF receptor, FGFR1c (17 [47](#), 18 [47](#)).

In RCS cells, cellular phenotypes caused by FGF signaling can be quantified, and thus RCS cells have been used in studies exploring the mechanisms of FGF-FGFR signaling, including mechanisms behind FGF regulation of the cell cycle, cell proliferation, differentiation, premature senescence, loss of extracellular matrix, interplay between FGF and WNT signaling, cytokine and natriuretic peptide signaling, and others (56 [47](#), 57 [47](#), 70 [47](#)–75 [47](#)). In addition, treatments inhibiting pathological FGFR signaling, which are now either in human trials (RBM007, meclozine) or FDA-approved (vosoritide), were initially developed in RCS cells, benefiting from the well characterized molecular mechanisms of FGF signaling in these cells (21 [47](#), 76 [47](#), 77 [47](#)). Here we show that RCS cells can also be used to identify biased FGF ligands.

Thus far, differential effects in the signaling of one FGFR in response to different FGF ligands have been attributed to differences in ligand binding. It can be reasoned, however, that differences in ligand binding strengths, alone, cannot explain differential signaling. Indeed, if the differences between ligands are only in the binding strength, then a strongly binding ligand, at low concentration, will act identically to weakly binding ligand at high concentration. Here we discovered, using tools that are novel for the RTK field, that there are also qualitative differences in the actions of the ligands. FGF8 preferentially activates some of the probed downstream responses (FRS2 phosphorylation and collagen loss), while FGF4 and FGF9 preferentially activate different probed responses (FGFR1 phosphorylation and growth arrest). These effects occur in addition to previously measured differences in ligand binding coefficients (87 [47](#)).

Having established the existence of bias in FGFR1 signaling, we sought the mechanism that allows for the recognition of FGF8 binding to the FGFR1 EC domain, as compared to FGF4 and FGF9. We measured FRET efficiency occurring in FGFR1 dimers with fluorescent proteins attached to the C-termini of the TM helices via flexible linkers. We showed that FRET efficiency is different when FGF8 is bound to the EC domain, as compared to the cases of bound FGF4 and FGF9. These results suggest that the C-termini of the TM helices are spaced further apart in the FGF8-bound FGFR1 dimers as compared to FGF4 and FGF9-bound dimers. Thus, FGFR1 TM domains must sense the identity of the ligand that is bound to the EC domain. FRS2 binds to the JM domain, which immediately follows the TM domain (42 [47](#), 60 [47](#), 78 [47](#)). It is easy to envision that the conformation of the TM domain affects FRS2 behavior, as the FRS2 binding site is just 32 amino acids from the TM domain C-terminus. The larger TM domain separation in the FGF8-bound FGFR1 dimers may facilitate the preferential phosphorylation of FRS2 over other sites. Alternatively, the smaller TM domain separation upon binding of FGF4 and FGF9 may disfavor FRS2 phosphorylation. Further, the differential phosphorylation of FRS2 may be due to a different mode of FRS2 binding to FGFR1, differences in the accessibility of FRS2 by the active site of the FGFR1 kinase, or different accessibility of FRS2 by phosphatases.

The experimental data in **Figure 6** [↗](#) hint at the possibility that ligand bias arises due to differences in FGFR1 dimer conformations. If this is so, then conformational differences in the signaling complex in the plasma membrane underlie biased signaling for both RTKs and GPCRs, the two largest receptor families in the human genome. It is important to note, however, that there are researchers who disagree that structural information can be propagated along the length of the RTK, because the linkers between the RTK domains are flexible ([50](#) [↗](#), [83](#) [↗](#), [84](#) [↗](#)). If so, how do the kinases sense which ligand is bound to the EC domain? Some have proposed that differential downstream signaling occurs as a result of different kinetics of receptor phosphorylation/de-phosphorylation in response to different ligands ([50](#) [↗](#)). There is a report that tyrosine phosphorylation of EGFR is more sustained in response to epigen and epiregulin than to EGF ([50](#) [↗](#)). Specifically, it was found that EGF-induced EGFR phosphorylation of Y845, Y1086, and Y1173 returned to baseline much faster, as compared to phosphorylation in response to epigen and epiregulin ([50](#) [↗](#)). The authors argued that differences in kinetics do not depend on the specific ligand concentration used, and they attributed the characteristic kinetics of the response to the identity of the bound ligand. In other studies with other RTKs, however, the concentration of the ligand strongly influenced the kinetics of the response, questioning the applicability of this model to all RTKs ([85](#) [↗](#), [86](#) [↗](#)). Here we show that FGFR1 kinetics of phosphorylation also depend strongly on FGF concentration. Furthermore, we do not observe a discernable correlation between kinetics of FGFR1 phosphorylation and ligand bias, which provides further support for the importance of the FGFR1 dimer conformations as determinants of FGFR1 ligand bias.

We further see marked differences in the potencies of the three FGFs. Indeed, 50% of maximum phosphorylation in 293T cells is reached for ~0.5 nM FGF4, ~2 nM FGF9, and ~10 nM FGF8. Thus, the same level of phosphorylation of a specific tyrosine can be achieved for much lower concentrations of FGF4, as compared to FGF8 or FGF9. The potencies of FGF4 in inducing functional responses are also the highest in RCS cells. We also observe differences in the efficacies of the three FGF ligands. The highest phosphorylation in **Figure 3** [↗](#) is achieved in response to high concentrations of FGF8. Thus, FGF8 is a full agonist while FGF4 and FGF9 are partial agonists for most of the responses (with the exception of FRS2 phosphorylation). Along with differences in potencies and efficacies, we observe a significant difference in the very shape of the dose response curves. In particular, the FGF4 dose response does not follow the anticipated rectangular hyperbolic shape, unlike the cases of FGF8 and FGF9. We see that the FGF4-induced phosphorylation of FGFR1 and the effector molecules increases up to ~3 nM FGF4, and then gradually decreases as FGF4 concentration is further increased. The mechanism behind the decrease in activation at high FGF4 concentration is unknown, but our data suggest that it cannot be explained with differential kinetics of de-phosphorylation/downregulation. Furthermore, there is no correlation between the unusual shape of the FGF4 dose response and ligand bias, as FGF4 is not biased when compared to FGF9. Instead, we see an intriguing correlation between the decrease in FGFR1 phosphorylation and the appearance of FGFR1 oligomers that are larger than dimers. Indeed, the FIF data in **Figure 2** [↗](#) suggests that high FGF4 concentration promotes oligomerization of FGFR1. Oligomers are observed in full-length FGFR when bound to FGF4, but not when the kinase domains are deleted. This finding suggests that, at least in part, the oligomers are stabilized by kinase-kinase contacts.

We can explain the shape of the FGF4 dose response curves if we assume that phosphorylation is less efficient in FGFR1 oligomers as compared to FGFR1 dimers. It is important to note that another RTK, EGFR, has also been proposed to form oligomers in response to EGF, in addition to dimers ([88](#) [↗](#), [89](#) [↗](#)). EGFR dimers and oligomers have been proposed to have different activities ([88](#) [↗](#), [89](#) [↗](#)). It has been suggested that in the EGFR oligomer, each EGFR kinase may be able to phosphorylate and be phosphorylated by multiple neighboring kinases, leading to higher overall activation. Such a view, however, is not consistent with the FGFR1 data presented here. Perhaps, the functional role of FGFR1 oligomers is very different from that of the EGFR oligomers, as oligomerization seems to inhibit FGFR1 signaling.

In conclusion, we initiated this study in search of a possible difference in the response of FGFR1 to three FGF ligands. We found not one, but many differences. We observed quantitative differences in most aspects of the FGFR1 signaling response: ligand-induced oligomerization, potency, efficacy, and conformations of FGFR1 TM domain dimer. We also discovered the existence of ligand bias in FGFR1 signaling. The quantitative tools that we used were instrumental in revealing these differences, based on the analysis of FGFR1 dose response curves for the three ligands. Of note, such dose response curves are typically collected for GPCRs ([48](#), [90](#)-[92](#)), but rarely for RTKs. Future use of such quantitative tools for other RTKs may similarly reveal unexpected differences in response to different ligands and may uncover exciting new biology.

All 58 RTKs have been implicated in many growth disorders and cancers ([1](#), [93](#)-[95](#)), and have been recognized as important drug targets. Inhibitors that specifically target the RTKs have been under development for decades now. Recent years have seen significant improvements in RTK-targeted molecular therapies, but these therapies have not yet fundamentally altered the survival and the quality of life of patients ([96](#), [97](#)). This limited progress may be because RTK signaling is much more complex than currently appreciated, as demonstrated here for FGFR1. Furthermore, it is possible that ligand bias plays an important role in RTK-linked pathologies. The practice of evaluating bias for novel RTK-targeting therapeutics may empower the discovery of a new generation of biased RTK inhibitors that selectively target pathogenic signaling pathways, and thus exhibit high specificity and low toxicity.

Materials and Methods

Cell Culture

Human embryonic kidney cells (HEK293T), stably transfected with FGFR1-YFP, and RCS cells were grown in Dulbecco's modified eagle medium (DMEM; Thermofisher, PN 31600034) with 10% fetal bovine serum supplemented with 3.5 grams of glucose and 1.5 grams of sodium bicarbonate at 37°C and 5% CO₂. Chinese Hamster Ovarian (CHO) cells were grown in DMEM with 10% fetal bovine serum supplemented with 0.8 grams of glucose, 1.5 grams of sodium bicarbonate, and 1% NEAA at 37°C and 5% CO₂. The RCS *Fgfr1-4 null* cells were prepared by Crispr/Cas9-mediated inactivation of *Fgfr1*, *Fgfr2*, *Fgfr3*, and *Fgfr4* loci ([21](#)). RCS^{Fgfr1} cells were prepared by Crispr/Cas9-mediated inactivation of *Fgfr2* in *Fgfr3/4* double knock-out RCS cells (a gift from Carmine Settembre).

Western blots

HEK 293T cells stably expressing FGFR1 were seeded in equal volumes into 12 well plates and allowed to grow to ~70-90% confluency, while changing the media after 48 hours. The full media was aspirated and replaced with Dulbecco's modified eagle medium without FBS. Varying amounts of ligands (R&D systems; FGF4, #235-F4-025; FGF8, #423-F8-025, and FGF9, # 273-F9-025) were added to each well to create a ligand concentration gradient. Cells were incubated with the ligands at 37°C and 5% CO₂ for 20 minutes and then immediately placed on ice. Media was aspirated and cells were immediately lysed with 2x Laemmli Buffer (BioRad 1610737) + 5% BME. Lysates were moved to clean Eppendorf tubes and vortexed for 10 second intervals 6 times over the course of 5-10 minutes, staying on ice in the interim. Lysates were centrifuged, boiled at 98°C for 10 min, and then immediately placed on ice until cool. Lysates were then centrifuged again and stored at -20°C for later use. Lysates were thawed on ice and vortexed immediately prior to loading onto gel. Gels were run on ice at 140V for 3 hours using Bio-Rad Mini-Protean TGX precast gels in 1X Tris/Gly/SDS running buffer (BioRad #1610732). Gels were then equilibrated with transfer buffer (BioRad #1610734) supplemented with 20% methanol for 10 min. Western transfer was performed using the Iblot 2 Gel Transfer Device (Thermofisher #IB21001) and nitrocellulose packs (Thermofisher IB23001). Nitrocellulose membranes were removed and replaced with PVDF membranes (BioRad #1620177) that had been activated in 100% methanol, all

other steps were as prescribed by Thermofisher. Transfers were done at 25V for 7 minutes. Following transfer, membranes were immediately trimmed and placed in either 5% non-fat milk or 5% BSA in 1X TBS supplemented with 1% Tween (Thermofisher #1706475) (TBST) depending on the primary antibody used. Blocking was accomplished on a rocker for 1-2 hours at room temperature. Membranes were rinsed 2X with TBST and then primary antibody was added at a 1:1000 dilution. Primary antibodies (Cell Signaling: anti-Y653/654 #3471S, anti-FGFR1 #9740S, anti-pY766 FGFR1, #2544, anti-Actin #3700, anti-pERK1/2 #9101, anti-pFRS2 #3861S, anti-pPLCγ #2822, anti-PLCγ #2821, anti-Vinculin #13901; SantaCruz: anti-FGFR2 #sc122; Cedarlane: anti-collagen 2 #CL50241AP; Invitrogene: anti-V5 #46-0705) were incubated with the membrane overnight at 4°C on a rocker. The primary antibody was removed and the membrane was washed with TBST 3 times for 5-15 min. The secondary antibody (Promega: anti-rabbit #W4011; Sigma: anti-mouse #A6782) was added at a dilution of 1:10000 and allowed to incubate on a rocker at room temperature for 1-2 hours. The secondary antibody was removed and the membrane was washed with TBST 3x for 5-15 min. The membrane was incubated with chemiluminescent solution (Thermofisher Scientific West Femto Supersignal, #1706435) and imaged on a Bio-Rad Gel-Doc XRS+.

Blot images were stored digitally. The intensity of each band was quantified using imageJ. Intensities were scaled following a protocol in Supplemental Information.

Phosphorylation dose response curves

The band intensities from at least 3 and up to 5 independent samples were quantified. The intensities were averaged and plotted as a function of ligand concentration for each response. The averaged dose response curves for the three ligands were scaled to each other. This was accomplished by re-running the samples that yielded the highest phosphorylation band intensities for a specific response on the same blot (**Figure 3B**). Intensities for each ligand on the common blot were averaged and the averages for the different ligands were scaled by setting the maximum value to 1. The scaled dose response curves were fitted to a rectangular hyperbolic (Hill equation with n=1) given by:

$$response = \frac{[x] * E_{top}}{[x] + EC_{50}} \quad \text{eqn 1}$$

Here [x] represents the concentration of ligand, while E_{top} corresponds to the plateau at high ligand concentrations and EC_{50} corresponds to the ligand concentration value at which 50% of E_{top} is achieved.

To fit the data to **eqn 1**, we truncated each dose response curve at the highest ligand concentration that is within 10% of the maximum y value. This took into account that the average y value error is between 5-10%. The data was fit in Mathematica with the Nonlinear Model fit function using the Levenberg-Marquardt minimization method, while allowing for a maximum of 100,000 iterations. The errors of the fit were weighted as the inverse of the square of the error.

Calculation of bias coefficients

To calculate bias coefficients, FGF8 was chosen as the reference ligand. Bias coefficients, β , for FGF4 and FGF9 were calculated using **eqn 2**.

$$\beta_{4,9} = Log \left(\left(\frac{E_{top,1}}{EC_{50,1}} * \frac{EC_{50,2}}{E_{top,2}} \right)_{4,9} * \left(\frac{EC_{50,1}}{E_{top,1}} * \frac{E_{top,2}}{EC_{50,2}} \right)_8 \right) \quad \text{eqn 2}$$

Standard errors of β are calculated using the standard errors of EC_{50} and E_{top} , as determined from the fit in **eqn 1**, using the functional approach for multi-variable functions (98).

To compare bias coefficients for the three ligands and determine statistical significance in a three-way comparison, we follow the protocol in (68 [↗](#)), re-writing eqn 2 [↗](#) as:

$$\beta_{4,9} = \beta'_{4,9} - \beta'_8 \quad \text{eqn 3}$$

Where β' is calculated in equation 4 [↗](#).

$$\beta'_{4,8,9} = \text{Log} \left(\frac{E_{top,1}}{EC_{50,1}} * \frac{EC_{50,2}}{E_{top,2}} \right)_{4,8,9} \quad \text{eqn 4}$$

Standard errors of β' were calculated using the standard errors of EC_{50} and E_{top} using the functional approach for multi-variable functions (98 [↗](#)). The β' values and their corresponding errors are reported in Table S1.

Statistical significance of the differences between β' values were calculated with a one-way ANOVA using the multi-variable analysis option in Prism. The data that were inputted were the mean, SEM, and n, the number of points contributing to the fit. n=9 for FGF4, n=10 for FGF8 and n=7 for FGF9. The calculated p-values are shown in Table S2.

FRET measurements

CHO cells were seeded into tissue culture-treated 6-well plates at a density of 2×10^4 cells/well. 24 hours later, the cells were co-transfected with FGFR1-ECTM-eYFP and FGFR1-ECTM-mCherry using Eugene HD (Promega # E2311) according to the manufacturer's instructions. ECTM constructs included the entire extracellular domain and transmembrane domain up to residue 402, followed by a (GGG)₅ flexible linker and the fluorophore (25 [↗](#)). The amount of DNA as well as the donor to acceptor ratio of the DNA added was varied to achieve a wide range of donor and acceptor expressions (62 [↗](#)).

Cells were vesiculated ~24 hours after transfection as described previously (99 [↗](#)). Briefly, the cells were rinsed 2x with 30% PBS and then incubated for 13 hours at 37°C and 5% CO₂ in a chloride salt buffer. Vesicles were then transferred to 4-well glass-bottomed chamber slides for imaging on a Nikon confocal microscope with a 60x objective to capture images of the equatorial cross-sections of the vesicles in 3 channels: donor (eYFP), acceptor (mCherry), and FRET (58 [↗](#)). FRET was measured following the QI-FRET protocol (62 [↗](#), 64 [↗](#)), which yields the donor concentration, the acceptor concentration, and the FRET efficiency in each vesicle. The microscope was calibrated using solutions of fluorescent protein of known concentration, so that the fluorescence intensity could be directly correlated to fluorophore concentration.

In the case of constitutive dimers at high ligand concentration, when FRET does not depend on receptor concentration, the measured FRET and the Intrinsic FRET are connected as follows (63 [↗](#)):

$$\tilde{E} = \frac{E}{x_A} \quad \text{eqn 5}$$

Here x_A is the acceptor fraction, which is measured in each vesicle, along with the FRET efficiency, E . Equation 5 [↗](#) allows us to directly determine the value of the intrinsic FRET, $\tilde{E}$, in each vesicle. The dependence of the intrinsic FRET, $\tilde{E}$, on the distance between the fluorescent proteins in the dimer, d , is given by equation (6) [↗](#).

$$\tilde{E} = \frac{1}{1 + \left(\frac{d}{R_0}\right)^6} \quad \text{eqn 6}$$

Here R_0 is the Förster radius of the FRET pair. For eYFP and mCherry, R_0 is 53.1 Å (64 [↗](#), 100 [↗](#)). This equation assumes free rotation of the fluorescent proteins. This assumption can be justified because the fluorescent proteins are attached via flexible linkers.

Fluorescence Intensity Fluctuations (FIF) spectrometry

HEK 293T cells, stably transfected with FGFR1-YFP, were seeded on collagen-coated, glass bottom petri dishes (MatTek, P35GCOL-1.5-14-C) and allowed to grow to ~70% confluency at 37°C and 5% CO₂. Cells were grown in Dulbecco's modified eagle medium with 10% fetal bovine serum, supplemented with 3.5 grams of glucose and 1.5 grams of sodium bicarbonate. Cells were rinsed with 70% swelling media (1:9 serum-free media, diH₂O, 25 mM HEPES), and then swelled in 70% swelling media plus ligand for ~5 min before imaging. This treatment minimizes the ruffles in the plasma membrane and prevents endocytosis (101 [↗](#), 102 [↗](#)).

The plasma membranes facing the support were imaged on a TCS SP8 confocal microscope using a photon counting detector. Images were analyzed using the FIF software (26 [↗](#)). Briefly, the membrane was divided into 15 x 15 pixel regions of interest and the molecular brightness, ϵ , of each region was calculated as:

$$\epsilon = \frac{\sigma^2}{\langle I \rangle} - 1 \quad \text{eqn 7}$$

where I is the center of the Gaussian distribution and σ^2 is the variance in each segment. The brightness values from thousands of regions of interest were binned and histogrammed. The histograms were then normalized to 1.

Since the molecular brightness distribution is log-normal, the values of log(brightness) were histogrammed and fit to a Gaussian function:

$$\text{normalized counts} = a * e^{\frac{-(\theta-m)^2}{2*s^2}} \quad \text{eqn 8}$$

Here θ represents the log of the brightness, m is the mean of the Gaussian, s is the standard deviation of the Gaussian, and a is a constant. The best fit Gaussian parameters are shown in Table S3.

In order to determine whether the distributions were the same or different, a Z-statistics analysis was used, where the Z value is given by:

$$Z = \frac{m_1 - m_2}{\sqrt{q_1^2 + q_2^2}} \quad \text{eqn 9}$$

Here m_1 and m_2 represent the two means of the Gaussians being compared, and q_1 and q_2 are the standard deviations for each Gaussian divided by the square root of the number of cells analyzed. A minimum of 100 cells were analyzed for each data set.

A Z value of less than 2 means that the data sets are within 2 standard deviations of the mean and are therefore the same, while a Z value greater than 2 means that the two data sets are different (37 [↗](#)). Calculated Z values can be found in Table S3.

Ligand-induced FGFR1 removal from the plasma membrane

FGFR1 downregulation in the plasma membrane was assayed by measuring the FGFR1 membrane concentration before and 2 minutes after ligand addition. FGFR1-YFP in the plasma membrane in contact with the substrate was imaged in a TCS SP8 confocal microscope, and FGFR1-YFP fluorescence per unit membrane area was quantified. HEK 293T cells, stably transfected with FGFR1-YFP, were seeded on collagen-coated, glass bottom petri dishes (MatTek, P35GCOL-1.5-14-C) and allowed to grow to ~70% confluency at 37°C and 5% CO₂. Cells were rinsed with serum-free media and exposed to 130 nM of either FGF8 or FGF9 or no ligand and incubated at 37°C and 5% CO₂ for 2 minutes. The cells were fixed in a solution of 4% formaldehyde in PBS for 20 min at

room temperature. Samples were stored at 4°C prior to imaging. A minimum of 100 cells per condition were imaged, in three independent experiments. The receptor concentration in the membrane of each cell was quantified using the FIF software ([26](#)), and results for all analyzed cells per condition were averaged.

Apoptosis Assays

Apoptosis was probed using the Bio-Rad Magic Red Caspase 3-7 kit (Bio-Rad, ICT 935). HEK 293T cells stably expressing FGFR1-YFP were seeded in 96 well plates and allowed to grow to ~70% confluency. Media was aspirated and replaced with serum-free media with the Magic Red staining solution and varying concentrations of ligand. After ligand treatment the cells were placed in the incubator at 37°C and 5% CO₂ for 6 days. The fluorescence of the cleaved substrate (Cresyl Violet) was measured on a Synergy H4 plate reader. The excitation wavelength was 592 nm. Emission was measured at 628 nm.

Viability Assays

Cell viability was monitored using a MTT Cell Proliferation Assay Kit (Cell BioLabs, #CBS-252). HEK 293T cells, stably transfected with FGFR1-YFP, were seeded in 96 well plates and allowed to grow to ~70% confluency. Media was aspirated and replaced with serum-free media and with varying concentrations of ligand. After ligand addition, cells were kept at 37°C and 5% CO₂ for 6 days. Viability was measured according to the manufacturer's protocol. Briefly, CytoSelect MTT Cell Proliferation Assay Reagent was added directly to the cell media. After three hours of incubation at 37°C and 5% CO₂, the cells were lysed with detergent and kept at room temperature for 2 hours. The absorbance was measured on a Synergy H4 plate reader at 555 nm.

ERK activation, cell count and collagen expression experiments in RCS^{Fgfr1} cells

The pKrox(MapERK)d1EGFP reporter was stably expressed in RCS^{Fgfr1} cells using the piggyBac transposase. Activation of the pKrox(MapERK)d1EGFP reporter in RCS^{Fgfr1} cells was measured using an automated incubation microscope BioStation CT (Nikon, Tokyo, Japan). Phase contrast and fluorescence signal images were automatically acquired every 1 hour during a 48 hr time period. Fluorescence of the reporter was then processed and analyzed in Nikon BioStation CT software. For growth-arrest assay, the RCS cells were treated with 1 µg/ml heparin and fgf ligands for three days. Cell numbers were determined by counting (Beckman-Coulter). For collagen type 2 expression, the RCS cells were treated with 1 µg/ml heparin and FGF ligands for 48 hours and collagen type 2 expression was measured by western blotting.

Interface predictions with ClusPro and PyRosetta

The monomeric kinase domain structures were docked using the ClusPro 2.0 software ([65](#), [66](#)). The top ten dimer structures were selected based on ClusPro 'VdW+Elec' (Van der Waals and electrostatic) energy calculations. Each dimer interface was further optimized by introducing randomized structural perturbations to generate two thousand structural decoys using a custom-written code that employs the PyRosetta modeling suite ([67](#)). The resulting 20,000 dimer structures generated were scored using PyRosetta interface scoring functions ([103](#)). The RMSDs were calculated relative to a pre-optimized ClusPro structure. The dimer structures with the lowest interface score for each set of decoys were selected as the optimized dimer structures.

Acknowledgements

We thank Marie Tesarova for excellent technical assistance and Carmine Settembre, Telethon Institute of Genetics and Medicine, Italy, for providing *Fgf3/4* double knock-out RCS cells. Supported by NIH GM068619 and NSF MCB 2106031. PK is supported by the Czech Science Foundation (GA19-20123S, GF21-26400K); Ministry of Education, Youth and Sports of the Czech Republic (INTER-ACTION LUAUS23295); Grant Agency of Masaryk University (MUNI/G/1771/2020); National Institute for Cancer Research (Programme EXCELES, ID Project No. LX22NPO5102) - Funded by the European Union - Next Generation EU. BF is supported by the Agency for Healthcare Research of the Czech Republic (NU21-06-00512).

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Joint Public Review:

In this manuscript, Karl et al. explore mechanisms underlying the activation of the receptor tyrosine kinase FGFR1 and stimulation of intracellular signaling pathways in response to FGF4, FGF8, or FGF9 binding to the extracellular domain of FGFR1. The manuscript demonstrates that FGF4, FGF8, and FGF9 exhibit distinct binding modes towards FGFRs. It is also proposed that FGF8 exhibits "biased ligand" characteristics that is manifested via binding and activation FGFR1 mediated by unproven and speculative "structural differences in the FGF-FGFR1 dimers, which impact the interactions of the FGFR1 trans membrane helices, leading to differential recruitment and activation of the downstream signaling adapter FRS2".

Author Response

The following is the authors' response to the current reviews.

Comment. "The manuscript demonstrates that FGF4, FGF8, and FGF9 exhibit distinct binding modes towards FGFRs"

No, this paper is not about ligand binding, and there are NO binding data in the manuscript. This paper is about ligand-dependent functional bias. Previously, differential effects of ligands on the signaling of one FGFR have been attributed to differences in ligand binding, but that paradigm is incomplete, if not incorrect. This manuscript is the first demonstration that three FGF ligands induce bias in FGFR1 signaling. FGF8 preferentially activates some of the probed downstream responses (FRS2 phosphorylation and extracellular matrix loss), while FGF4 and FGF9 preferentially activate different probed responses (FGFR1 phosphorylation and growth arrest). The bias we report here cannot be the result of differences in ligand binding. Indeed, if the differences between ligands are only in the binding strength, then a strongly binding ligand at low concentration will act identically to weakly binding ligand at high concentration. Our article thus changes the current paradigm about how FGF ligands activate FGFR signaling.

Comment. It is also proposed that FGF8 exhibits "biased ligand" characteristics.

We do not "propose" the existence of ligand bias, we demonstrate it in the manuscript by following the latest IUPHAR community guidelines on bias identification and quantification (Kolb et al, 2022). We calculate bias coefficients, and we analyze the results using statistical tools.

Comment. ..."Unproven and speculative structural differences in the FGF-FGFR1 dimers".

This statement is not correct, as it is directly contradicted by the differences reported in Figure 6. This Figure presents the results of a quantitative FRET assay performed at high ligand concentration, which ensures that there are no monomeric receptors. Under these conditions, the measured FRET efficiency depends only on the dimer conformation. The measured differences in FRET efficiencies reveal distinct differences in the FGFR1 TM domain dimer conformations when FGF8 is bound to the extracellular domain of FGFR1, as compared to FGF4 and FGF9. The difference can be observed in the raw FRET data in Figure 6A. While these data do not reveal the exact molecular origin of the structural differences, they unequivocally prove that there are structural differences when different ligands are bound.

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Kolb P, Kenakin T, Alexander SPH, Bermudez M, et al. Community guidelines for GPCR ligand bias: IUPHAR review 32. *Br J Pharmacol*. 2022;179, 3651-3674.

The following is the authors' response to the previous reviews.

eLife assessment. This manuscript describes useful data on the mechanisms underlying the activation of the receptor tyrosine kinase FGFR1 and stimulation of intracellular signaling pathways in response to FGF4, FGF8, or FGF9 binding to the extracellular domain of FGFR1. Solid quantitative binding experiments are presented to demonstrate that FGF4, FGF8, and FGF9 exhibit distinct binding affinities towards FGFRs.

No, this paper is not about binding, and there is NO binding data in the manuscript. This paper is about function. This is the first demonstration that three FGF ligands induce bias in FGFR1 signaling. Thus far, differential effects in the signaling of one FGFR have been

attributed to differences in ligand binding, but this current paradigm is incomplete/incorrect. Our article changes the current paradigm in how FGF activate downstream FGFR signaling.

We have clarified this point by adding the following text in the Discussion.

"Thus far, differential effects in the signaling of one FGFR in response to different FGF ligands have been attributed to differences in ligand binding. It can be reasoned, however, that differences in ligand binding strengths, alone, cannot explain differential signaling. Indeed, if the differences between ligands are only in the binding strength, then a strongly binding ligand at low concentration will act identically to weakly binding ligand at high concentration. Here we discovered, using tools that are novel for the RTK field, that there are qualitative differences in the actions of the ligands. FGF8 preferentially activates some of the probed downstream responses (FRS2 phosphorylation and collagen loss), while FGF4 and FGF9 preferentially activate different probed responses (FGFR1 phosphorylation and growth arrest). These effects occur in addition to previously measured differences in ligand binding coefficients (87)."

We have also re-written the abstract.

"Abstract

"The mechanism of differential signaling of multiple FGF ligands through a single FGF receptor is poorly understood. Here, we use biophysical tools to quantify multiple aspects of FGFR1 signaling in response to FGF4, FGF8 and FGF9: potency, efficacy, bias, ligand-induced oligomerization and downregulation, and conformation of the active FGFR1 dimers. We find that the three ligands exhibit distinctly different potencies and efficacies for inducing responses in cells. We further discover qualitative differences in the actions of the three FGFs through FGFR1, as FGF8 preferentially activates some of the probed downstream responses (FRS2 phosphorylation and extracellular matrix loss), while FGF4 and FGF9 preferentially activate different probed responses (FGFR1 phosphorylation and cell growth arrest). Thus, FGF8 is a biased ligand, when compared to FGF4 and FGF9. Förster resonance energy transfer experiments reveal a correlation between biased signaling and the conformation of the FGFR1 transmembrane domain dimer. Our findings expand the mechanistic understanding of FGF signaling during development and bring the poorly understood concept of receptor tyrosine kinase ligand bias into the spotlight."

Reviewer #1 (Public Review):

Comment. Quantitative binding experiments presented in the manuscript demonstrate that FGF4, FGF8, and FGF9 exhibit distinct binding affinities towards FGFRs.

This paper is not about binding, and there is NO binding data in the manuscript. This paper is about function. Please see our response to the Elife assessment.

Comment. It is also proposed that FGF8 exhibits "biased ligand" characteristics that is manifested via binding and activation FGFR1 mediated by "structural differences in the FGF- FGFR1 dimers, which impact the interactions of the FGFR1 transmembrane helices, leading to differential recruitment and activation of the downstream signaling adapter FRS2".

We do not "propose" the existence of ligand bias, we demonstrate it in the manuscript by following the latest IUPHAR community guidelines on bias identification and quantification (Kolb et al, 2022). Specifically, we construct bias plots, we calculate bias coefficients, and we analyze the results using statistical tools.

Also, please note that ligand bias has no direct connection to binding strength, so the statement that biased ligand characteristics “is manifested via binding” is not correct.

Comment. In the absence of any structural experimental data of different forms of FGFR dimers stimulated by FGF ligands the model presents in the manuscript is speculative and misleading.

Figure 6 presents the “structural experimental data”. A quantitative FRET assay is performed at high ligand concentration, which ensures that there are no monomeric receptors. Under these conditions, the measured FRET efficiency depends only on the dimer conformation. The measured FRET efficiencies reveal distinct differences in the FGFR1 TM domain dimer conformations when the ligand FGF8 is bound to the extracellular domain of FGFR1, as compared to the cases of FGF4 and FGF8.

Because the Rosetta modeling of the kinase domains in the previous version of the paper is not based on experimental data, we have removed the modeling from the Results, and we have removed all references to it in the Discussion. Thus, all that is shown and discussed in the revised paper is based on experimental data.

We have substituted two paragraphs in the discussion with the following two sentences:

“The experimental data in Figure 6 hint at the possibility that ligand bias arises due to differences in FGFR1 dimer conformations. If this is so, then conformational differences in the signaling complex in the plasma membrane underlie biased signaling for both RTKs and GPCRs, the two largest receptor families in the human genome”.

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Kolb P, Kenakin T, Alexander SPH, Bermudez M, et al. Community guidelines for GPCR ligand bias: IUPHAR review 32. *Br J Pharmacol.* 2022;179, 3651-3674.