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Mini review

Probing phosphorylation events in biological membranes: The transducer function

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ABSTRACT

The extracellular environment is sensed by receptors in the plasma membrane. Some of these receptors initiate cytoplasmic signaling cascades involving phosphorylation: the addition of a phosphate group to a specific amino acid, such as tyrosine, in a protein. Receptor Tyrosine Kinases (RTKs) are one large class of membrane receptors that can directly initiate signaling cascades through their intracellular kinase domains, which both catalyze tyrosine phosphorylation and get phosphorylated. In the first step of signaling, the ligands stabilize phosphorylation-competent RTK dimers and oligomers, which leads to the phosphorylation of specific tyrosine residues in the activation loop of the kinases. Here we discuss quantitative measurements of tyrosine phosphorylation efficiencies for RTKs, described by the "transducer function". The transducer function links the phosphorylation (the response) and the binding of the activating ligand to the receptor (the stimulus). We overview a methodology that allows such measurements in direct response to ligand binding. We discuss experiments which demonstrate that EGF is a partial agonist, and that two tyrosines in the intracellular domain of EGFR, Y1068 and Y1173, are differentially phosphorylated in the EGF-bound EGFR dimers.

1. Introduction

The 58 human receptor tyrosine kinases (RTKs) regulate cell growth, migration, differentiation, and survival, playing crucial roles in development and in adult life [1–13]. Dysregulated RTK activity can lead to uncontrolled growth in cancer or hindered growth in developmental abnormalities [14–18]. Most of the 58 RTKs are implicated in many cancers or developmental syndromes, making them desirable drug targets [13,19–25].

All RTKs are composed of an extracellular (EC) domain, a single transmembrane (TM) domain, and an intracellular (IC) domain. The EC region contains the ligand-binding sites, while the IC region contains the kinase domain responsible for RTK activity. The term "RTK activity" describes the catalytic activity of the IC kinase domain, which facilitates the addition of phosphate groups to specific tyrosines of a neighboring RTK in a dimer or an oligomer [1,2]. First, the activation loop tyrosines are phosphorylated upon kinase-kinase contacts (Fig. 1). This event fully activates the kinases, leading to the phosphorylation of additional tyrosines in the IC domain (often in the unstructured cytoplasmic tail of the RTK) [5,26–30]. These tyrosines recruit cytoplasmic effector proteins, which are also phosphorylated by the RTK, to initiate signaling cascades that control cellular functions [1,2,31]. Some of the best-characterized signaling cascades initiated by RTKs are the MAPK, STAT, PI3K, and PKC cascades [1,2,12,32–37]. Signal transduction events have been studied for decades, but a quantitative picture of transduction across the plasma membrane in response to ligand binding has been lacking until recently.

The epidermal growth factor receptor (EGFR) is an RTK implicated in cell growth and differentiation in adult life and during embryonic development [38–41]. EGFR signaling is known to be critically important for the development of the epithelium and has been implicated in many cancers [38,42,43]. EGFR can be activated by seven different ligands, including EGF, the most widely studied ligand. Many tyrosines in the intracellular domain of EGFR are phosphorylated in response to ligand binding, including Y1068 and Y1173 in the long EGFR cytoplasmic tail.

It is known that signal propagation involves amplification, as well as additional modulation due to negative and positive feedback loops [2,12,44]. To decouple the two effects with the long-term goal of gaining a comprehensive understanding of signaling, we have measured

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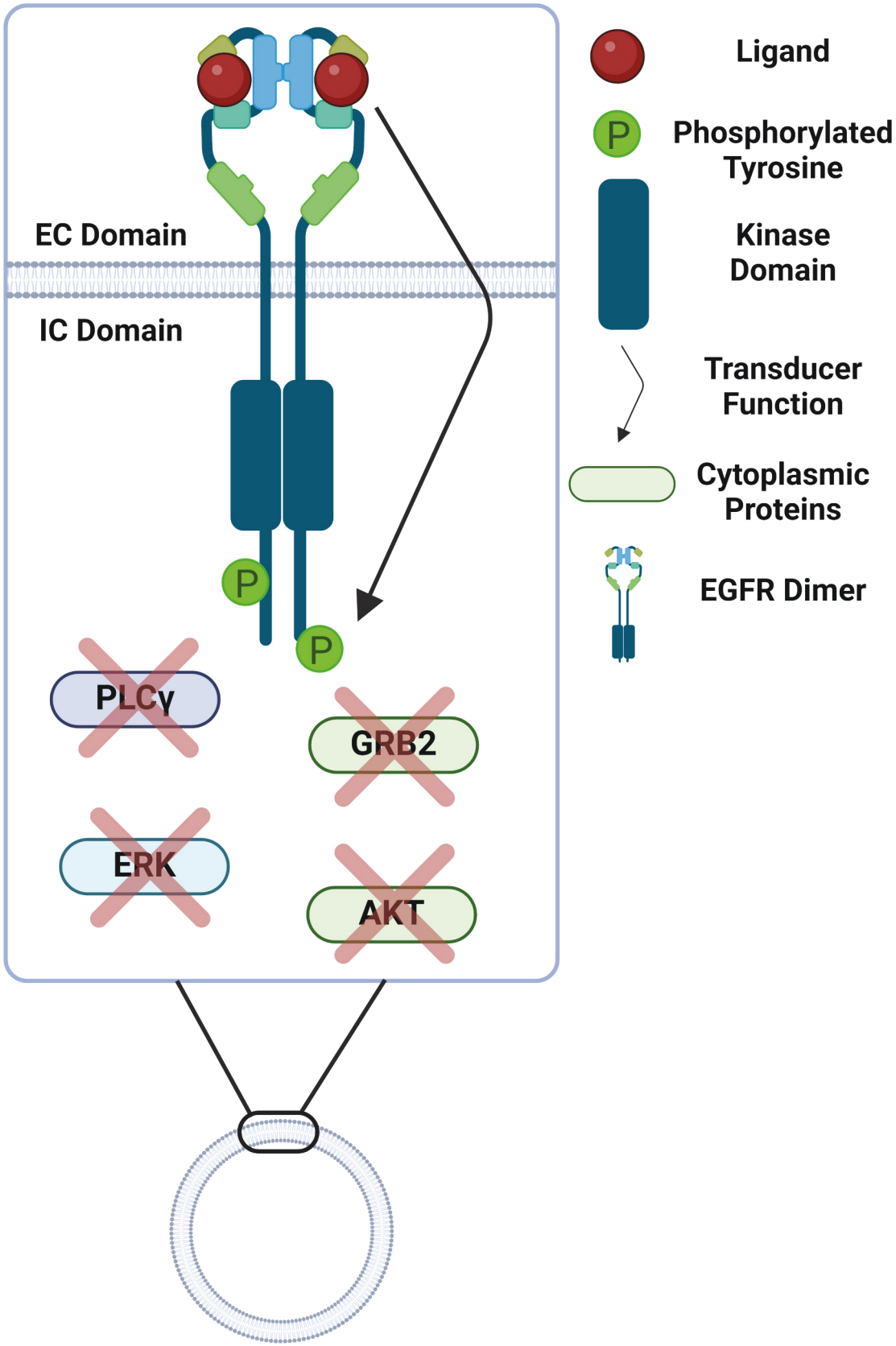

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Fig. 1. The transducer function yields a quantitative link between ligand-receptor complex formation and the phosphorylation of critical tyrosines in the intracellular (IC) domain of the receptor. Shown is a schematic of a dimeric RTK (EGFR) in the plasma membrane. The binding of ligand (red) to the extracellular (EC) domain of the RTK stabilizes an RTK signaling oligomer, usually a dimer. Details about ligand binding to RTKs are known, as there are crystal structures of the isolated RTK domains in complex with ligands [2,94]. The bound ligands are believed to position the two kinase domains in the dimer in phosphorylation-competent orientation with respect to each other, but the exact mechanism is not understood. There are many high-resolution structures of isolated kinase domains, but details about their relative positioning in the dimer are unknown as there are no full-length RTK structures [95]. Each kinase phosphorylates multiple tyrosines in the IC domain of the neighboring receptor; in the case of EGFR the phosporylated tyrosines (green) are predominantly in the long unstructured post-kinase tails. Here we aim to quantify the transducer function (black arrow), which links the phosphorylation (the response) and the binding of the activating ligand to the receptor (the stimulus). Measurements are performed in plasma membrane derived vesicles, which do not retain cytoplasmic signaling molecules such as PLCγ, ERK, GRB2, and AKT. The latter can affect RTK phosphorylation via feedback mechanisms but are not present in the experiments.

the phosphorylation of EGFR in direct response to bound EGF, using a model system that lacks downstream signaling cascades that operate within the cell [45]. These measurements, which are overviewed below, showed that Y1068 and Y1173 in the EGFR kinase tail are differentially phosphorylated in response to EGF binding [45].

2. The transducer function

The transducer function is the quantitative link between the response and the stimulus which causes it [46]. In the case of RTK signal transduction across the plasma membrane, the response is the addition of a phosphate group to a tyrosine in the intracellular domain of the RTK, while the stimulus is the formation of ligand-bound RTK complexes. Both RTK phosphorylation [47,48] and ligand-binding curves [49], as a function of ligand concentration, have been measured previously, but in very different experimental contexts and have not been compared directly. To determine the transducer function, we measure both curves simultaneously in the same system so we can plot one versus the other, as described below.

It has long been known that signaling responses depend on the concentrations of activated receptors, via a hyperbolic functional dependence [50,51]. This hyperbolic relationship was initially deduced by Black and Leff based on fundamental principles [46]. It forms the basis of their "operational model," which applies to various receptor types including RTKs [52]. In this model, ligand-bound receptors serve as a stimulus to activate the response with a characteristic equilibrium dissociation constant, K_{resp} [53,54].

$$\text{response} = \text{transducer function}(\text{stimulus}) = \frac{\text{stimulus}*R_{max}}{\text{stimulus} + K_{resp}} \quad (1)$$

We designate the stimulus as the fraction of ligand-bound receptors, f_{bound}. We designate the response as the tyrosine phosphorylation per EGFR molecule, R_{phospo}. R_{max} is the maximum possible theoretical response (phosphorylation) that can be attained in the experiment [55,56]. Therefore:

$$R_{phospho} = \frac{f_{bound}*R_{max}}{f_{bound} + K_{resp}} \quad (2)$$

where K_{resp} is the ligand-bound receptor fraction that yields 50 % of R_{max}. Since R_{max} depends on experimental details, such as labeling yields for antibody batches, we can instead write:

$$\frac{R_{phospho}}{R_{max}} = \frac{f_{bound}}{f_{bound} + K_{resp}} \quad (3)$$

The ligand-bound receptor fraction f_{bound} is between 0 and 1. By setting $f_{bound} = 1$, we define:

$$\text{phosphorylation efficiency} = \frac{R_{phospho}(f_{bound} = 1)}{R_{max}} = \frac{1}{1 + K_{resp}} \quad (4)$$

This phosphorylation efficiency can be calculated if the transducer function is measured experimentally and R_{max} and K_{resp} are found from a two-parameter fit using Eq. (2). For this type of measurement, it is important that the ligand-bound receptor fraction, f_{bound}, is quantified directly. Ligand-binding to RTKs cannot be described by a simple binding reaction since ligand-binding and lateral RTK interactions are coupled [57]. Indeed, ligand-binding is strongly affected by the RTK association state, and RTK dimerization and oligomerization are affected by the presence of bound ligand [57]. Furthermore, the oligomerization state is challenging to measure in the membrane, and may vary with ligand concentration [58,59]. All complications in data interpretation due to complex RTK behaviors can be avoided through direct measurements of f_{bound}, along with phosphorylation measurements, $R_{phospho}$, in the same membrane, as a function of ligand concentration.

2.1. The RTK transducer functions cannot be measured using western blotting

For many years, the most widely used approach to quantify phosphorylation has been western blotting [60–62]. In these experiments, a large pool of cells expressing an RTK is lysed, after stimulation with the ligand. The proteins in the lysates are separated on SDS-PAGE gels, and then the phosphorylation of a particular tyrosine is probed using a highly specific anti-phosphoY antibody [47,63,64]. Thus, dose-response curves for RTK phosphorylation can be acquired if the ligand is varied over a broad concentration range, and the phosphorylation is measured using western blotting [47,48,65]. However, the transducer function measurements require that not only the response but the stimulus is known as well. This stimulus is f_{bound}, the fraction of ligand-bound receptors, which cannot be measured quantitatively in western blotting experiments. Furthermore, in western blotting experiments, the phosphorylation over many cells with different expressions of the RTKs is averaged, and the RTK expressions in the individual cells are unknown.

In addition, results in cells are affected by feedback loops, either negative or positive, which depend on the concentration of the expressed effector proteins [1,44]. These effects are sometimes referred to as "system bias" [66]. The concentrations of the cytoplasmic effectors vary among cell lines, leading to significant differences in reported results. It is thus important to decouple (i) effects that occur in direct response to ligand-binding and (ii) effects due to complex feedback mechanisms including receptor downregulation. It is also important to know the concentrations of all relevant signaling molecules that impact the measured response. Thus, western blotting has significant limitations for transducer function measurements.

2.2. Established model systems cannot be used for RTK transducer function measurements

There has been significant progress towards understanding signal transduction by G-protein coupled receptors (GPCRs) [51,67]. GPCRs are membrane proteins made of 7 TM helices, involved in the recognition of light, taste, odors, hormones, pain, and neurotransmitters, and thus involved in a myriad of biological processes [68–70]. In response to ligand-binding on the extracellular side, GPCRs bind to and activate heterotrimeric G proteins or arrestins on the cytoplasmic side [71,72]. While GPCRs have been reported to dimerize similarly to RTKs, it is now established that GPCRs signal predominantly as monomers, with dimerization likely fine-tuning their activity [67,73–75]. Researchers purify GPCRs and then reconstitute them in model lipidic systems such

as nanodisks [71,72]. In these model systems, the researchers can add a ligand that binds on one side of the receptor and simultaneously follow G-protein or arrestin binding to the other side.

Such a model system is not appropriate for studies of RTKs because it may put artificial constraints on the oligomer size of the RTK assemblies, which can be dimers or higher-order oligomers. Furthermore, while there are many reports where GPCRs have been purified and then reconstituted without losing activity, no such successes have been reported for RTKs thus far, to the best of our knowledge. It is thus preferable to use a model system that does not require RTK extraction out of the native plasma membrane and provides a contiguous membrane so the RTKs diffuse and interact with each other as they do in cells.

2.3. Osmotically derived plasma membrane vesicles as a model system for transducer function measurements

We have leveraged the model system of osmotically-derived plasma membrane vesicles to study RTK phosphorylation in direct response to ligand binding [45]. These vesicles are highly permeable to hydrophilic proteins [77], and thus both the EC and IC domains of the receptor are experimentally accessible by the researcher. Thus, we can simultaneously observe ligand-binding and tyrosine phosphorylation (Fig. 2). We use fluorescent ligands to visualize ligand-binding to the receptors. We use fluorescently labeled anti-phosphoY antibodies that recognize only one specific phosphorylated tyrosine on a specific RTK, to probe activation. The receptors are also labeled and their membrane densities are quantified.

The osmotic stress vesiculation is carried out without the use of harsh chemicals [78]. The lipid composition of the osmotically-derived vesicles is practically the same as the lipid composition of the plasma membrane [77]. Both soluble and peripheral proteins leak out from the vesicles produced with the osmotic stress method [77]. Dextrans of molecular weight of 73,000 Da (hydrodynamic radius of 6.5 nm), when added externally, permeate the vesicles and equilibrate across the plasma membrane within 1 h [45]. Externally added antibodies (hydrodynamic radius of 5.4 nm) also enter the vesicles, such that the concentration inside and outside of the vesicles is identical [45]. Thus, phosphorylation of the RTK on the cytoplasmic side of the plasma membrane is detected through the use of fluorescent, highly specific anti-phosphoY antibodies. These antibodies are recruited to the vesicle membrane upon tyrosine phosphorylation in response to ligand stimulation.

3. Measurements of the EGFR transducer function

Here we overview measurements described in [45], shown schematically in Fig. 2. CHO cells were transfected with DNA encoding EGFR tagged with a fluorescent protein, mTurquoise (mTurq), attached via a flexible linker to the C-terminus [31]. 24 h later, the cells were subjected to osmotic vesiculation and the vesicles were incubated with Alexa488-labeled anti-phosphoY1068 EGFR antibody. To start the phosphorylation reaction, the ligand EGF was added together with a Magnesium/ATP kinase cocktail, and the solution was incubated for 1 h. The cocktail contained phosphatase inhibitors which inactivate the phosphatases. Thus, the phosphorylation of Y1068 was a direct consequence of ligand-binding and signal propagation across the length of EGFR. All other influences were minimized. Transmembrane phosphatases were inhibited. Proteins of molecular weight of up to at least 210 kDa leak out from all the osmotically-derived vesicles [77], and antibodies of molecular weight 150 kDa permeate the vesicles. Labeled signaling molecules, such as PLCγ-GFP, PLCδ-GFP, PKCθ-GFP, and GRB2-Venus [77], are not found in the vesicles, which gives confidence that endogenous cytoplasmic proteins that may be relevant to EGFR signaling are not present (Fig. 1). Noteworthily, critically important downstream molecules, such as RAF, ERK, AKT, PKC, etc. are rather small, with molecular weights below 100 kDa.

We utilized an EGF ligand from mouse which is commercially available and labeled at the N-terminus with rhodamine (Thermofisher, E3481). This ligand acts fundamentally the same way as human EGF [45]. Images of individual vesicles were captured using a confocal microscope in three channels to measure: (i) the fluorescence of rhodamine, attached to EGF, to quantify the ligand bound to the receptors in the plasma membrane in each vesicle, (ii) the fluorescence of Alexa488, linked to the anti-phosphoY antibody, to quantify phosphorylated EGFR in the membrane, and (iii) the fluorescence of mTurq, linked to the receptor, to quantify EGFR in the plasma membrane in each vesicle. Data were collected 1 h after adding the ligand and the ATP cocktail. This time point was selected based on the kinetic phosphorylation traces for

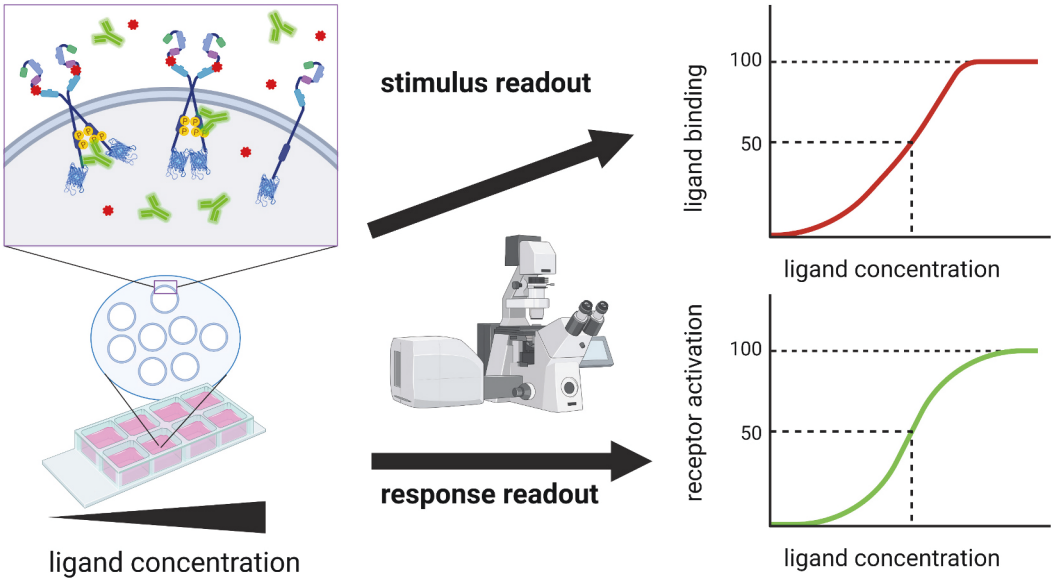

Fig. 2. Overview of experimental set up for transducer function measurements, as first described in [45]. Experiments are performed in single plasma membrane derived vesicles. The binding of a fluorescent ligand to the receptors, tagged with fluorophores, is quantified to obtain the ligand binding curve (the stimulus). The recruitment of fluorescent antibody to phosphorylated tyrosines is quantified to obtain the receptor activation curves (the response). The transducer function is the mathematical link between the response and the stimulus.

individual vesicles, which indicated that the signal stays constant after 30 min [45].

One vesicle, imaged in the three channels in the presence of 5 nM EGF, is presented in Fig. 3A. 3085 vesicles were analyzed after imaging in the three channels while varying the concentration of rho-mEGF. Fig. 3B shows the ligand binding data. The left side shows the ligand binding curves measured in the experiment where Y1068 phosphorylation was assayed simultaneously. The right side shows the ligand binding curves measured in the experiment where Y1173 phosphorylation was assayed simultaneously. The y-axis represents the ratio of: (i) the fluorescence of membrane-bound rhodamine-EGF for each vesicle and (ii) the fluorescence of EGFR-mTurq in the same vesicle. This ratio is proportional to the ligand-bound EGFR fraction, f_{bound}, and is scaled to 1. Specifically, the average bound fraction at high concentration is set to 1, and all the data are scaled accordingly. The x-axis in Fig. 3B represents the ligand concentration.

Fig. 3C displays the phosphorylation of Y1068 (left) and Y1173 (right) as a function of ligand concentration, representing the dose response curves. The y-axis shows the ratio of: (i) the fluorescence of AlexaF488, linked to the anti-pY antibody, and (ii) the fluorescence of EGFR-mTurq. These values are proportional to the extent of EGFR phosphorylation. All Y1068 data are on the same scale, and all Y1173 data are on the same scale.

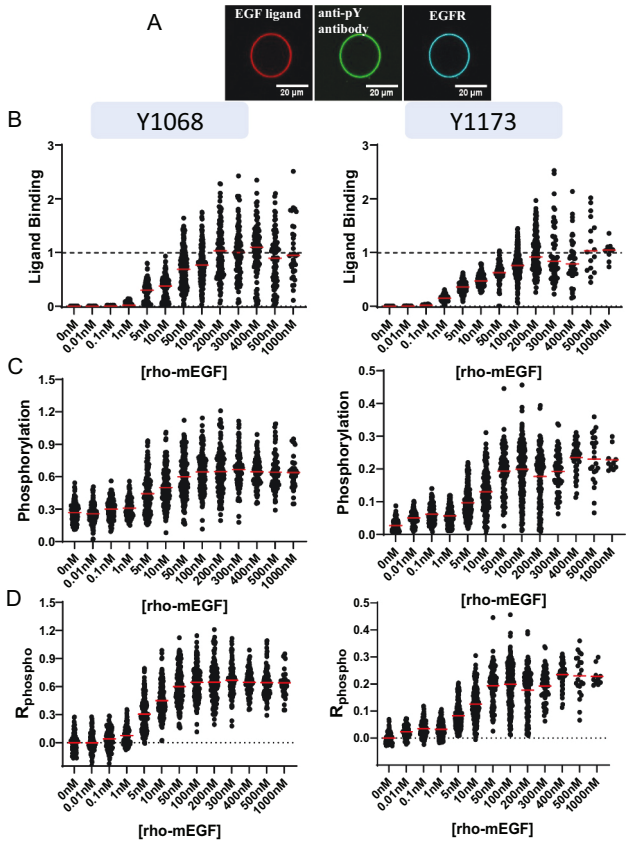

Fig. 3. Quantitation of rho-EGF binding and EGFR phosphorylation. (A) One vesicle imaged in three different scans in the presence of 5 nM rho-mEGF. (B) Binding curves in experiments set up to simultaneously probe the phosphorylation of one tyrosine, either Y1068 or Y1173. Shown is the ratio of rhodamine-EGF to EGFR-mTurq, scaled to 1. Each data point is derived from one vesicle. (C) Phosphorylation of EGFR, measured through recruitment of AlexaFluor488 anti-pY1068 or pY1173 EGFR antibody to EGFR on the membrane. Each point represents one individual vesicle, and is given by the ratio of either anti-pY1068 fluorescence or anti-pY1173 fluorescence and EGFR-mTurq fluorescence, without scaling. (D) Phosphorylation in response to ligand binding. Data are corrected to remove the contribution of constitutive EGFR activation in the unliganded EGFR dimers. Correction protocol is in [45]. Data are replotted from [45].

We see that the phosphorylation at zero ligand in Fig. 3C is different from zero. This occurs because EGFR can be auto-phosphorylated at zero ligand, but cannot activate efficiently downstream signaling [79,80]. We corrected the dose-responses for the contribution of the unliganded EGFR dimers using a thermodynamic model of EGFR association and previously measured EGFR-EGF and EGFR-EGFR binding constants [49]. Details of the correction are given in ref. (45). The dose-response curves after correction are shown in Fig. 3D.

The transducer function is obtained by plotting the corrected dose-responses in Fig. 3D versus the stimulus (f_{bound} in Fig. 3B), and is shown in Fig. 4A. Each data point in Fig. 4A corresponds to one individual vesicle. The two unknowns, R_{max} and K_{resp}, are determined by fitting the individual vesicle data in Fig. 4A using Eq. (2). Fig. 4B shows the fits and the binned data. For Y1068 phosphorylation, K_{resp} (the ligand-bound receptor fraction that yields 50 % of R_{max}) was determined to be 0.40 ± 0.03. R_{max} (the maximum possible phosphorylation) was determined as 0.95 ± 0.03. For Y1173 phoshorylation K_{resp} was determined to be 0.86 ± 0.08 and R_{max} to be 0.39 ± 0.02.

As the fluorescent properties of the anti-Y1068 and anti-Y1173 antibodies differ, the two R_{max} values differ as well. This fact is taken into account by plotting the normalized transducer function, $R_{phospho}/R_{max}$ given by Eq. (4), in Fig. 4C. The Y1068 and Y1173 phosphorylation efficiencies are calculated as the normalized transducer function values at f_{bound} to 1. They are 0.71 ± 0.02 and 0.54 ± 0.02 for Y1068 and Y1173, respectively. These efficiencies are the maximum phosphorylation fractions achieved when all receptors are ligand-bound.

4. Conclusions and implications

RTKs are crucial for human health, development, and disease, and have thus been the topic of biomedical research studies for decades. Biophysical research in the RTK literature thus far has focused primarily on ligand-RTK binding [49,57,81,82] and on the interactions between RTKs in the presence of ligand and at zero ligand [28,29,83–85]. These studies have provided very important insights about the behavior of the RTKs, but it is not clear yet how these insights relate to RTK function. To obtain a comprehensive picture of signal transduction, we need to start quantifying the function response, and understand how different aspects of the interaction network underlie this function. Here we present one method to measure the phosphorylation of the receptors in direct response to ligand binding. We are looking forward to the development of additional methodologies to quantify RTK function, as we continue our quest to understand RTK behaviors.

The discussed methodology was developed to quantify EGFR phosphorylation, but can be used to study many different RTKs. The major limitation is the availability of specific anti-pY antibodies that can recognize their target tyrosine in the context of the folded kinase domain of the RTK in the plasma membrane. Tyrosines Y1068 and Y1173 are located in the unstructured long tail of EGFR, such that antibody binding to them is not obstructed.

The plasma membrane vesicles are derived from cells, and are thus better mimics of the plasma membrane than other models systems such as nanodisks. However, many aspects of the structure of the plasma derived vesicles are not fully understood. Lipid asymmetry is difficult to probe, as molecules which bind specific lipid components can access both leaflets. The lateral organization of the vesicles has not been characterized. We are looking forward to future studies that will bring clarity on the nature of the vesicles. In addition, we are looking forward to investigations which enrich or deplete lipid components of the vesicles to uncover if the lipid composition affects the transducer function.

The method and the model system overviewed here represent an expansion of the biophysics toolkit to study signaling. The described approach can provide mechanistic insights about different aspects of RTK activation. For instance, the methodology is well suited to uncover

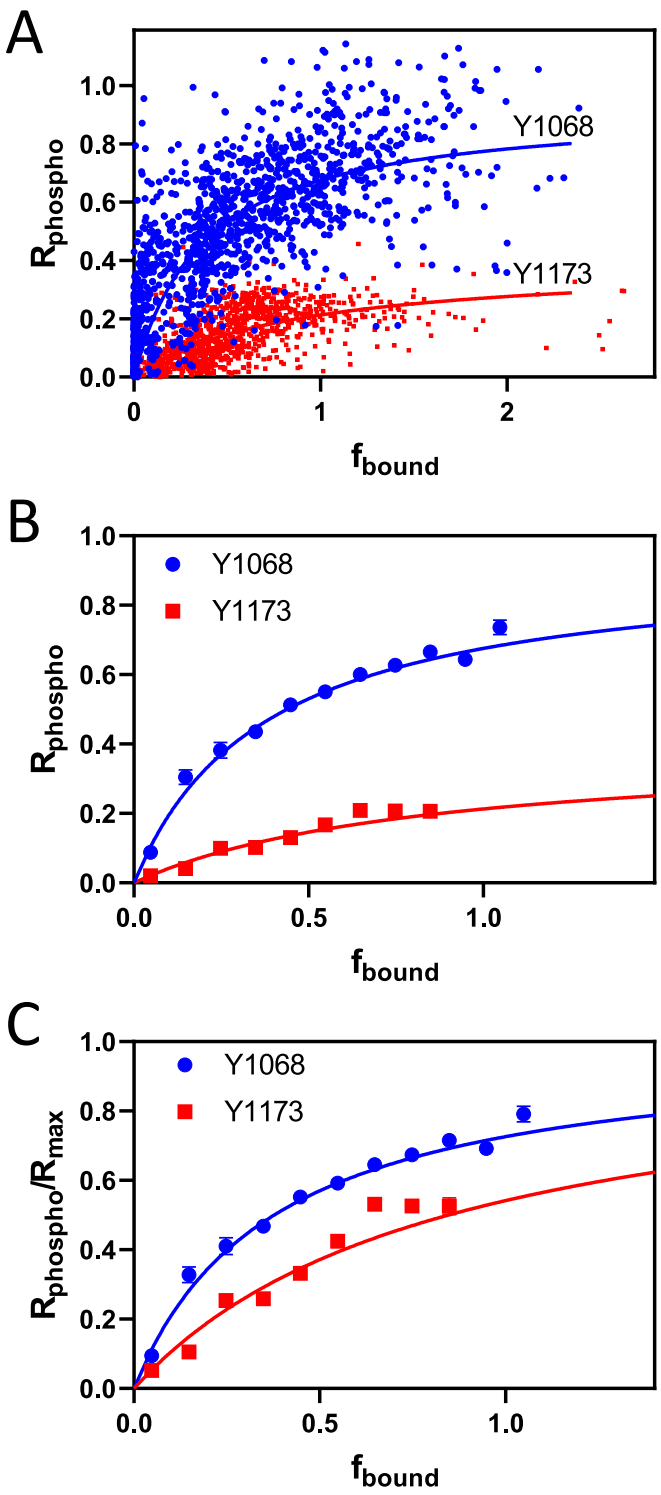

Fig. 4. The plot for the rho-mEGF EGFR transducer function. (A) Phosphorylation response in Fig. 3D vs ligand bound EGFR fraction (binding in Fig. 3B). The solid lines represent the best-fit transducer function that was obtained from all the single vesicles data using (Eq. (2)) (B) Binned single-vesicle data, shown along with the fits to all the data. The standard errors are smaller than the symbols. (C) The normalized transducer function, as given by Eq. (3). Data are replotted from [45].

cooperative effects in the phosphorylation of the different EGFR tyrosines. Noteworthy, there are many tyrosines in the tail of EGFR which can be phosphorylated, in addition to Y1068 and Y1173. The transducer functions for the different tyrosines can be measured, provided that suitable antibodies are raised. Through mutagenesis of selected tyrosines, we could gain mechanistic insights into the degree of cooperativity of tyrosine phosphorylation.

The phosphorylation of Y1068 and Y1173, assayed here, has profound importance for EGFR signaling. Y1068 phosphorylation results in Grb2 and Gab1 recruitment and the initiation of AKT and STAT3/5 signaling pathways [86–88]. Y1173 phosphorylation leads to Shc recruitment of and the initiation of the MAPK/ERK signaling cascade [86]. Here we find differences in the phosphorylation of these two tyrosines, manifested in different values of K_{resp} and the phosphorylation efficiencies. K_{resp} is the ligand-bound receptor fraction that yields 50 % of R_{max}. A smaller K_{resp} indicates more efficient phosphorylation. The value $K_{resp} \rightarrow 0$ denotes a true full agonist, and corresponds to a phosphorylation efficiency of $\rightarrow 1$. Since the phosphorylation efficiencies of Y1068 and Y1173 for EGF are smaller than 1, we conclude that EGF is a partial agonist for both responses.

K_{resp} for Y1068 phosphorylation was determined to be the smaller of the two (0.40 ± 0.03 versus 0.86 ± 0.08), indicating that EGF-mediated Y1068 phosphorylation is more efficient than Y1173 phosphorylation. This difference will lead to differential activation of the signaling cascades originating at these tyrosines. The manifestation of these differences in different cell lines will be likely different, due to differences in the identities and abundance of downstream signaling effectors [66]. Measurements of transducer functions in plasma membrane derived vesicles therefore can help separate effects that occur in the membrane in direct response to ligand binding from effects due to signal amplification and feedback loops in different cellular contexts. This will bring more complete understanding of the differential effects of RTK ligands and of the effects of RTK pathogenic mutations on discrete signal transduction steps [45].

It has been shown that different ligand and pathogenic mutations alter the preferences for phosphorylation of different tyrosines [45]. The transducer function reports directly on such preferences, through the values of K_{resp}. Thus, different EGFR pathogenic mutants can be characterized using the technique described here, and their transducer functions can be compared. A notable example of high impact studies benefiting from transducer function measurements will be the comparisons of different EGFR mutations causing non-small cell lung carcinoma (NSCLC). NSCLC represents over 85 % of all lung cancers and is associated with high mortality [89,90]. This cancer is due to EGFR mutations in approximately 10–15 % of Caucasian patients and in up to 50 % of Asian patients. Of the single amino acid mutations, the L834R mutation is the most common one, accounting for about 40–45 % of the cases where EGFR is mutated. Known uncommon NSCLC EGFR mutations include (i) the G893X mutation, where X can be C, A, S, or D, (ii) the L837Q mutation, and (iii) the S744I mutation [91–93]. These uncommon mutations account for about 10 % of the NSCLC cases with mutated EGFR. It is known that patients with these mutations exhibit 30 % lower progression-free survival when treated with tyrosine kinase inhibitors used in patients with the L834R mutation, implying functional differences. This suggests that the signaling of these EGFR mutants may be fundamentally different from the common L834R mutant, and from the wild-type. It can be hypothesized that the transducer functions are different for the wild-type and for the different mutants. This hypothesis can be tested with the methodology described here. Further, the methodology can be used to screen for molecules that bind the mutant EGFRs and alter the transducer function so it is similar to the transducer function of the wild-type. Such novel biased inhibitors will not block signaling, just alter it, and will therefore be less likely to induce resistance.

CRediT authorship contribution statement

Daniel Wirth: Writing – review & editing, Investigation, Conceptualization. **Ece Özdemir:** Writing – review & editing, Formal analysis. **Kalina Hristova:** Writing – original draft, Funding acquisition,

Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

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References

[1] J. Schlessinger, Receptor tyrosine kinases: legacy of the first two decades, Cold Spring Harb. Perspect. Biol. (2014) 6.
[2] M.A. Lemmon, J. Schlessinger, Cell signaling by receptor tyrosine kinases, Cell 141 (2010) 1117–1134.
[3] L. He, K. Hristova, Physical-chemical principles underlying RTK activation, and their implications for human disease, Biochim. Biophys. Acta 1818 (2012) 995–1005.
[4] E. Li, K. Hristova, Role of receptor tyrosine kinase transmembrane domains in cell signaling and human pathologies, Biochemistry 45 (2006) 6241–6251.
[5] M.D. Paul, K. Hristova, The transition model of RTK activation: a quantitative framework for understanding RTK signaling and RTK modulator activity, Cytokine Growth Factor Rev. 49 (2019) 23–31.
[6] R. Ke, X. Yang, C. Tianyi, M. Ge, J. Lei, X. Mu, The C342R mutation in FGFR2 causes Crouzon syndrome with elbow deformity, J. Craniofac. Surg. 26 (2015) 584–586.
[7] Z. Vajo, C.A. Francomano, D.J. Wilkin, The molecular and genetic basis of fibroblast growth factor receptor 3 disorders: the achondroplasia family of skeletal dysplasias, Muenke craniosynostosis, and Crouzon syndrome with acanthosis nigricans, Endocr. Rev. 21 (2000) 23–39.
[8] T. Iwata, L. Chen, C.I. Li, D.A. Ovchinnikov, R.R. Behringer, C.A. Francomano, C. X. Deng, A neonatal lethal mutation in FGFR3 uncouples proliferation and differentiation of growth plate chondrocytes in embryos, Hum. Mol. Genet. 9 (2000) 1603–1613.
[9] A.A. Qutub, F. Mac Gabhann, E.D. Karagiannis, P. Vempati, A.S. Popel, Multiscale models of angiogenesis, IEEE Eng. Med. Biol. Mag. 28 (2009) 14–31.
[10] M.A. Olayioye, R.M. Neve, H.A. Lane, N.E. Hynes, The ErbB signaling network: receptor heterodimerization in development and cancer, EMBO J. 19 (2000) 3159–3167.
[11] W.J. Fantl, D.E. Johnson, L.T. Williams, Signaling by receptor tyrosine kinases, Annu. Rev. Biochem. 62 (1993) 453–481.
[12] J. Schlessinger, Cell signaling by receptor tyrosine kinases, Cell 103 (2000) 211–225.
[13] M.D. Paul, K. Hristova, The RTK interactome: overview and perspective on RTK heterointeractions, Chem. Rev. 119 (2019) 5881–5921.
[14] K. Sakai, H. Yokote, K. Murakami-Murofushi, T. Tamura, N. Saijo, K. Nishio, Pertuzumab, a novel HER dimerization inhibitor, inhibits the growth of human lung cancer cells mediated by the HER3 signaling pathway, Cancer Sci. 98 (2007) 1498–1503.
[15] B.C. Browne, N. O'Brien, M.J. Duffy, J. Crown, N. O'Donovan, HER-2 signaling and inhibition in breast cancer, Curr. Cancer Drug Targets 9 (2009) 419–438.
[16] S. Foldynova-Trantirkova, W.R. Wilcox, P. Krejci, Sixteen years and counting: the current understanding of fibroblast growth factor receptor 3 (FGFR3) signaling in skeletal dysplasias, Hum. Mutat. 33 (2012) 29–41.
[17] W.A. Horton, J.G. Hall, J.T. Hecht, Achondroplasia, Lancet 370 (2007) 162–172.
[18] M.L. Cunningham, M.L. Seto, C. Ratisoontorn, C.L. Heike, A.V. Hing, Syndromic craniosynostosis: from history to hydrogen bonds, Orthod. Craniofac. Res. 10 (2007) 67–81.
[19] M.K. Webster, D.J. Donoghue, FGFR activation in skeletal disorders: too much of a good thing, Trends Genet. 13 (1997) 178–182.
[20] S.C. Robertson, J.A. Tynan, D.J. Donoghue, RTK mutations and human syndromes - when good receptors turn bad, Trends Genet. 16 (2000) 265–271.
[21] P.W. Janes, C.I. Slape, R.H. Farnsworth, L. Atapattu, A.M. Scott, M.E. Vail, EphA3 biology and cancer, Growth Factors 32 (2014) 176–189.
[22] M.E. Vail, C. Murone, A. Tan, L. Hii, D. Abebe, P.W. Janes, F.T. Lee, M. Baer, V. Palath, C. Bebbington, G. Yarranton, C. Llerena, S. Garic, D. Abramson, G. Cartwright, A.M. Scott, M. Lackmann, Targeting EphA3 inhibits cancer growth by disrupting the tumor stromal microenvironment, Cancer Res. 74 (2014) 4470–4481.
[23] J.E. Phay, M.H. Shah, Targeting RET receptor tyrosine kinase activation in cancer, Clin. Cancer Res. 16 (2010) 5936–5941.
[24] E. Boye, M. Jinnin, B.R. Olsen, Infantile hemangioma: challenges, new insights, and therapeutic promise, J. Craniofac. Surg. 20 (Suppl. 1) (2009) 678–684.
[25] A. Nessa, S.A. Latif, N.I. Siddiqui, M.A. Hussain, M.R. Bhuiyan, M.A. Hossain, A. Akther, M. Rahman, Angiogenesis-a novel therapeutic approach for ischemic heart disease, Mymensingh. Med. J. 18 (2009) 264–272.
[26] J.M. Kavran, J.M. McCabe, P.O. Byrne, M.K. Connacher, Z.H. Wang, A. Ramek, S. Sarabipour, Y.B. Shan, D.E. Shaw, K. Hristova, P.A. Cole, D.J. Leahy, How IGF-1 activates its receptor, Elife 3 (2014).
[27] L. Chen, W.M. Marsiglia, H. Chen, J. Katigbak, H. Erdjument-Bromage, D. J. Kemble, L. Fu, J. Ma, G. Sun, Y. Zhang, G. Liang, T.A. Neubert, X. Li, N. J. Traaseth, M. Mohammadi, Molecular basis for receptor tyrosine kinase A-loop tyrosine transphosphorylation, Nat. Chem. Biol. 16 (2020) 267–277.
[28] S. Sarabipour, K. Hristova, Mechanism of FGF receptor dimerization and activation, Nat. Commun. 7 (2016) 10262.
[29] S. Sarabipour, K. Ballmer-Hofer, K. Hristova, VEGFR-2 conformational switch in response to ligand binding, Elife 5 (2016).
[30] D.R. Singh, F. Ahmed, M.D. Paul, M. Gedam, E.B. Pasquale, K. Hristova, The SAM domain inhibits EphA2 interactions in the plasma membrane, Biochim. Biophys. Acta 1864 (2016) 31–38.
[31] N. Del Piccolo, K. Hristova, Quantifying the interaction between EGFR dimers and Grb2 in live cells, Biophys. J. 113 (2017) 1353–1364.
[32] Z. Timsah, Z. Ahmed, C.C. Lin, F.A. Melo, L.J. Stagg, P.G. Leonard, P. Jeyabal, J. Berrout, R.G. O'Neil, M. Bogdanov, J.E. Ladbury, Competition between Grb2 and Plc gamma 1 for FGFR2 regulates basal phospholipase activity and invasion, Nat. Struct. Mol. Biol. 21 (2014) 180+.
[33] C.C. Lin, F.A. Melo, R. Ghosh, K.M. Suen, L.J. Stagg, J. Kirkpatrick, S.T. Arold, Z. Ahmed, J.E. Ladbury, Inhibition of basal FGF receptor signaling by dimeric Grb2, Cell 149 (2012) 1514–1524.
[34] J. Schlessinger, Autoinhibition control, Science 300 (2003) 750–752.
[35] J. Schlessinger, M.A. Lemmon, SH2 and PTB domains in tyrosine kinase signaling, in: Science's STKE : Signal Transduction Knowledge Environment 2003, 2003, p. RE12.
[36] E.B. Pasquale, Eph receptors and ephrins in cancer: bidirectional signalling and beyond, Nat. Rev. Cancer 10 (2010) 165–180.
[37] R. Trenker, N. Jura, Receptor tyrosine kinase activation: from the ligand perspective, Curr. Opin. Cell Biol. 63 (2020) 174–185.
[38] A. Wells, EGF receptor, Int. J. Biochem. Cell Biol. 31 (1999) 637–643.
[39] J.C. Lee, I. Vivanco, R. Beroukhim, J.H.Y. Huang, W.L. Feng, R.M. DeBiasi, K. Yoshimoto, J.C. King, P. Nghiemphu, Y. Yuza, Q. Xu, H. Greulich, R.K. Thomas, J.G. Paez, T.C. Peck, D.J. Linhart, K.A. Glatt, G. Getz, R. Onofrio, L. Ziaugra, R. L. Levine, S. Gabriel, T. Kawaguchi, K. O'Neill, H. Khan, L.M. Liau, S.F. Nelson, P. N. Rao, P. Mischel, R.O. Pieper, T. Cloughesy, D.J. Leahy, W.R. Sellers, C. L. Sawyers, M. Meyerson, I.K. Mellinghoff, Epidermal growth factor receptor activation in glioblastoma through novel missense mutations in the extracellular domain, PLoS Med. 3 (2006) 2264–2273.
[40] F.G. Barker, M.L. Simmons, S.M. Chang, M.D. Prados, D.A. Larson, P.K. Sneed, W. M. Wara, M.S. Berger, P.C. Chen, M.A. Israel, K.D. Aldape, EGFR overexpression and radiation response in glioblastoma multiforme, Int. J. Radiat. Oncol. Biol. Phys. 51 (2001) 410–418.
[41] G. Carpenter, S. Cohen, Epidermal growth-factor, Annu. Rev. Biochem. 48 (1979) 193–216.
[42] K.J. Wilson, J.L. Gilmore, J. Foley, M.A. Lemmon, D.J. Riese, Functional selectivity of EGF family peptide growth factors: implications for cancer, Pharmacol. Ther. 122 (2009) 1–8.
[43] D. Grausporta, R.R. Beerli, J.M. Daly, N.E. Hynes, ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling, EMBO J. 16 (1997) 1647–1655.
[44] J. Schlessinger, Common and distinct elements in cellular signaling via EGF and FGF receptors, Science 306 (2004) 1506–1507.
[45] D. Wirth, E. Ozdemir, K. Hristova, Quantification of ligand and mutation-induced bias in EGFR phosphorylation in direct response to ligand binding, Nat. Commun. 14 (2023) 7579.
[46] J.W. Black, P. Leff, Operational models of pharmacological agonism, Proc. R. Soc. Lond. B Biol. Sci. 220 (1983) 141–162.
[47] M. Gomez-Soler, M. Petersen Gehring, B.C. Lechtenberg, E. Zapata-Mercado, K. Hristova, E.B. Pasquale, Engineering nanomolar peptide ligands that differentially modulate EphA2 receptor signaling, J. Biol. Chem. 294 (2019) 8791–8805.
[48] C. Hu, C.A. Leche 2nd, A. Kiyatkin, Z. Yu, S.E. Stayrook, K.M. Ferguson, M. A. Lemmon, Glioblastoma mutations alter EGFR dimer structure to prevent ligand bias, Nature 602 (2022) 518–522.
[49] J.L. Macdonald, L.J. Pike, Heterogeneity in EGF-binding affinities arises from negative cooperativity in an aggregating system, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 112–117.
[50] T. Kenakin, G protein coupled receptors as allosteric proteins and the role of allosteric modulators, J. Recept. Signal Transduct. Res. 30 (2010) 313–321.
[51] T. Kenakin, Signaling bias in drug discovery, Expert Opin. Drug Discovery 12 (2017) 321–333.
[52] K. Karl, M.D. Paul, E.B. Pasquale, K. Hristova, Ligand bias in receptor tyrosine kinase signaling, J. Biol. Chem. 295 (2020) 18494–18507.
[53] J. Gundry, R. Glenn, P. Alagesan, S. Rajagopal, A practical guide to approaching biased agonism at G protein coupled receptors, Front. Neurosci. 11 (2017) 17.
[54] T. Kenakin, Measurement of receptor signaling Bias, Curr. Protoc. Pharmacol. 74 (2016) (2 15 11-12 15 15).
[55] F.J. Ehlert, Analysis of biased agonism, Prog. Mol. Biol. Transl. Sci. 160 (2018) 63–104.
[56] T. Kenakin, A scale of agonism and allosteric modulation for assessment of selectivity, bias, and receptor mutation, Mol. Pharmacol. 92 (2017) 414–424.
[57] C. King, K. Hristova, Direct measurements of VEGF-VEGFR2 binding affinities reveal the coupling between ligand binding and receptor dimerization, J. Biol. Chem. 294 (2019) 9064–9075.
[58] S.R. Needham, S.K. Roberts, A. Arkhipov, V.P. Mysore, C.J. Tynan, L.C. Zanetti-Domingues, E.T. Kim, V. Losasso, D. Korovesis, M. Hirsch, D.J. Rolfe, D.T. Clarke, M.D. Winn, A. Lajevardipour, A.H. Clayton, L.J. Pike, M. Perani, P.J. Parker, Y. Shan, D.E. Shaw, M.L. Martin-Fernandez, EGFR oligomerization organizes

kinase-active dimers into competent signalling platforms, Nat. Commun. 7 (2016) 13307.
[59] Y. Huang, S. Bharill, D. Karandur, S.M. Peterson, M. Marita, X. Shi, M. J. Kaliszewski, A.W. Smith, E.Y. Isacoff, J. Kuriyan, Molecular basis for multimerization in the activation of the epidermal growth factor receptor, Elife 5 (2016).
[60] G.A. Bellus, E.B. Spector, P.W. Speiser, C.A. Weaver, A.T. Garber, C.R. Bryke, J. Israel, S.S. Rosengren, M.K. Webster, D.J. Donoghue, C.A. Francomano, Distinct missense mutations of the FCFR3 Lys650 codon modulate receptor kinase activation and the severity of the skeletal dysplasia phenotype, Am. J. Hum. Genet. 67 (2000) 1411–1421.
[61] L. He, K. Hristova, Pathogenic activation of receptor tyrosine kinases in mammalian membranes, J. Mol. Biol. 384 (2008) 1130–1142.
[62] N.Y. Yang, C. Fernandez, M. Richter, Z. Xiao, F. Valencia, D.A. Tice, E.B. Pasquale, Crosstalk of the EphA2 receptor with a serine/threonine phosphatase suppresses the Akt-mTORC1 pathway in cancer cells, Cell. Signal. 23 (2011) 201–212.
[63] H. Chen, C.F. Xu, J. Ma, A.V. Eliseenkova, W. Li, P.M. Pollock, N. Pitteloud, W. T. Miller, T.A. Neubert, M. Mohammadi, A crystallographic snapshot of tyrosine trans-phosphorylation in action, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 19660–19665.
[64] J.H. Bae, T.J. Boggon, F. Tome, V. Mandiyan, I. Lax, J. Schlessinger, Asymmetric receptor contact is required for tyrosine autophosphorylation of fibroblast growth factor receptor in living cells, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 2866–2871.
[65] M. Gomez-Soler, M.P. Gehring, B.C. Lechtenberg, E. Zapata-Mercado, A. Ruelos, M. W. Matsumoto, K. Hristova, E.B. Pasquale, Ligands with different dimeric configurations potently activate the EphA2 receptor and reveal its potential for biased signaling, iScience 25 (2022) 103870.
[66] P. Kolb, T. Kenakin, S.P.H. Alexander, M. Bermudez, L.M. Bohn, C.S. Breinholt, M. Bouvier, S.J. Hill, E. Kostenis, K.A. Martemyanov, R.R. Neubig, H.O. Onaran, S. Rajagopal, B.L. Roth, J. Selent, A.K. Shukla, M.E. Sommer, D.E. Gloriam, Community guidelines for GPCR ligand bias: IUPHAR review 32, Br. J. Pharmacol. 179 (2022) 3651–3674.
[67] T. Kenakin, Biased receptor signaling in drug discovery, Pharmacol. Rev. 71 (2019) 267–315.
[68] R.L. Rawls, G-proteins: research unravels their role in cell communication, in: Chemical & Engineering News, 1987.
[69] D.E. Clapham, The G-protein nanomachine, Nature 379 (1996) 297–299.
[70] I. Bohme, A.G. Beck-Sickinger, Illuminating the life of GPCRs, Cell Commun. Signal 7 (2009) 16.
[71] M. Choi, D.P. Staus, L.M. Wingler, S. Ahn, B. Pani, W.D. Capel, R.J. Lefkowitz, G protein-coupled receptor kinases (GRKs) orchestrate biased agonism at the beta2-adrenergic receptor, Sci. Signal. 11 (2018).
[72] X.E. Zhou, K. Melcher, H.E. Xu, Understanding the GPCR biased signaling through G protein and arrestin complex structures, Curr. Opin. Struct. Biol. 45 (2017) 150–159.
[73] L.M. Luttrell, T.P. Kenakin, Refining efficacy: allosterism and bias in G protein-coupled receptor signaling, Methods Mol. Biol. 756 (2011) 3–35.
[74] J.R. James, M.I. Oliveira, A.M. Carmo, A. Iaboni, S.J. Davis, A rigorous experimental framework for detecting protein oligomerization using bioluminescence resonance energy transfer, Nat. Methods 3 (2006) 1001–1006.
[75] S. Angers, A. Salahpour, E. Joly, S. Hilairet, D. Chelsky, M. Dennis, M. Bouvier, Detection of beta(2)-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET), Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 3684–3689.
[77] S. Sarabipour, R.B. Chan, B. Zhou, G. Di Paolo, K. Hristova, Analytical characterization of plasma membrane-derived vesicles produced via osmotic and chemical vesiculation, Biochim. Biophys. Acta 1848 (2015) 1591–1598.
[78] N. Del Piccolo, J. Placone, L. He, S.C. Agudelo, K. Hristova, Production of plasma membrane vesicles with chloride salts and their utility as a cell membrane mimetic for biophysical characterization of membrane protein interactions, Anal. Chem. 84 (2012) 8650–8655.
[79] P.O. Byrne, K. Hristova, D.J. Leahy, EGFR forms ligand-independent oligomers that are distinct from the active state, J. Biol. Chem. 295 (2020) 13353–13362.
[80] S.I. Liang, B. van Lengerich, K. Eichel, M. Cha, D.M. Patterson, T.Y. Yoon, M. von Zastrow, N. Jura, Z.J. Gartner, Phosphorylated EGFR dimers are not sufficient to activate Ras, Cell Rep. 22 (2018) 2593–2600.
[81] D. Alvarado, D.E. Klein, M.A. Lemmon, Structural basis for negative cooperativity in growth factor binding to an EGF receptor, Cell 142 (2010) 568–579.
[82] M.A. Lemmon, Z.M. Bu, J.E. Ladbury, M. Zhou, D. Pinchasi, I. Lax, D.M. Engelman, J. Schlessinger, Two EGF molecules contribute additively to stabilization of the EGFR dimer, EMBO J. 16 (1997) 281–294.
[83] I. Chung, R. Akita, R. Vandlen, D. Toomre, J. Schlessinger, I. Mellman, Spatial control of EGF receptor activation by reversible dimerization on living cells, Nature 464 (2010) 783–U163.
[84] D.H. Kim, S. Park, D.K. Kim, M.G. Jeong, J. Noh, Y. Kwon, K. Zhou, N.K. Lee, S. H. Ryu, Direct visualization of single-molecule membrane protein interactions in living cells, PLoS Biol. 16 (2018) e2006660.
[85] M.J. Kaliszewski, X. Shi, Y. Hou, R. Lingerak, S. Kim, P. Mallory, A.W. Smith, Quantifying membrane protein oligomerization with fluorescence cross-correlation spectroscopy, Methods 140-141 (2018) 40–51.
[86] R.N. Jorissen, F. Walker, N. Pouliot, T.P. Garrett, C.W. Ward, A.W. Burgess, Epidermal growth factor receptor: mechanisms of activation and signalling, Exp. Cell Res. 284 (2003) 31–53.
[87] S. Abdelhamed, K. Ogura, S. Yokoyama, I. Saiki, Y. Hayakawa, AKT-STAT3 pathway as a downstream target of EGFR signaling to regulate PD-L1 expression on NSCLC cells, J. Cancer 7 (2016) 1579–1586.
[88] R. Sordella, D.W. Bell, D.A. Haber, J. Settleman, Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways, Science 305 (2004) 1163–1167.
[89] H. Shigematsu, A.F. Gazdar, Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers, Int. J. Cancer 118 (2006) 257–262.
[90] M.F. Tsai, T.H. Chang, S.G. Wu, H.Y. Yang, Y.C. Hsu, P.C. Yang, J.Y. Shih, EGFR-L858R mutant enhances lung adenocarcinoma cell invasive ability and promotes malignant pleural effusion formation through activation of the CXCL12-CXCR4 pathway, Sci. Rep. 5 (2015) 13574.
[91] T. Zhang, B. Wan, Y. Zhao, C. Li, H. Liu, T. Lv, P. Zhan, Y. Song, Treatment of uncommon EGFR mutations in non-small cell lung cancer: new evidence and treatment, Transl. Lung Cancer Res. 8 (2019) 302–316.
[92] H.Y. Tu, E.E. Ke, J.J. Yang, Y.L. Sun, H.H. Yan, M.Y. Zheng, X.Y. Bai, Z. Wang, J. Su, Z.H. Chen, X.C. Zhang, Z.Y. Dong, S.P. Wu, B.Y. Jiang, H.J. Chen, B.C. Wang, C.R. Xu, Q. Zhou, P. Mei, D.L. Luo, W.Z. Zhong, X.N. Yang, Y.L. Wu, A comprehensive review of uncommon EGFR mutations in patients with non-small cell lung cancer, Lung Cancer 114 (2017) 96–102.
[93] G.M. O'Kane, P.A. Bradbury, R. Feld, N.B. Leighl, G. Liu, K.M. Pisters, S. Kamel-Reid, M.S. Tsao, F.A. Shepherd, Uncommon EGFR mutations in advanced non-small cell lung cancer, Lung Cancer 109 (2017) 137–144.
[94] A.A. Belov, M. Mohammadi, Molecular mechanisms of fibroblast growth factor signaling in physiology and pathology, Cold Spring Harb. Perspect. Biol. 5 (2013) a015958.
[95] K. Karl, K. Hristova, Pondering the mechanism of receptor tyrosine kinase activation: the case for ligand-specific dimer microstate ensembles, Curr. Opin. Struct. Biol. 71 (2021) 193–199.