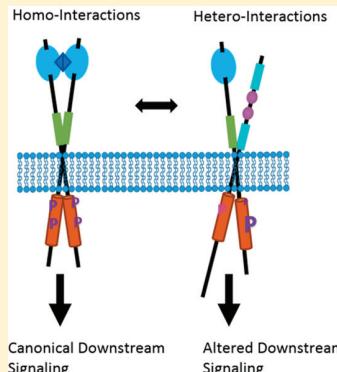


The RTK Interactome: Overview and Perspective on RTK Heterointeractions

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ABSTRACT: Receptor tyrosine kinases (RTKs) play important roles in cell growth, motility, differentiation, and survival. These single-pass membrane proteins are grouped into subfamilies based on the similarity of their extracellular domains. They are generally thought to be activated by ligand binding, which promotes homodimerization and then autophosphorylation in trans. However, RTK interactions are more complicated, as RTKs can interact in the absence of ligand and heterodimerize within and across subfamilies. Here, we review the known cross-subfamily RTK heterointeractions and their possible biological implications, as well as the methodologies which have been used to study them. Moreover, we demonstrate how thermodynamic models can be used to study RTKs and to explain many of the complicated biological effects which have been described in the literature. Finally, we discuss the concept of the RTK interactome: a putative, extensive network of interactions between the RTKs. This RTK interactome can produce unique signaling outputs; can amplify, inhibit, and modify signaling; and can allow for signaling backups. The existence of the RTK interactome could provide an explanation for the irreproducibility of experimental data from different studies and for the failure of some RTK inhibitors to produce the desired therapeutic effects. We argue that a deeper knowledge of RTK interactome thermodynamics can lead to a better understanding of fundamental RTK signaling processes in health and disease. We further argue that there is a need for quantitative, thermodynamic studies that probe the strengths of the interactions between RTKs and their ligands and between different RTKs.



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1. INTRODUCTION

Receptor tyrosine kinases (RTKs) are the second largest family of membrane receptors. There are 58 RTKs in humans, and as shown in Figure 1, they are grouped into 20 subfamilies based on the homology of their extracellular (EC) domains. Unlike G-protein coupled receptors (GPCRs), channels, and transporters, the RTKs have a single transmembrane (TM) helix which connects an N-terminal extracellular ligand-binding domain to a C-terminal intracellular (IC) kinase domain. Since a single TM helix is not efficient at transducing conformational changes across the plasma membrane, the receptors of this family rely on lateral interactions to become activated and initiate downstream signaling cascades. These signaling cascades, in turn, control many critically important biological processes, including cell growth, survival, and differentiation. Recent years have brought significant progress in our understanding of the physical interactions that regulate RTK function. RTKs are best known for forming signaling homodimers, but it is now clear that they are capable of engaging many interaction partners. While these interaction partners are from diverse classes of proteins, the focus of this review is the interactions between RTKs from different subfamilies.

2. OVERVIEW OF THE CURRENT PARADIGM OF RTK INTERACTIONS

2.1. Interactions between RTKs in the Plasma Membrane and Their Response to Ligands

The general structure of RTKs can be seen in Figure 1. Their N-terminal EC domains, which are usually several hundred amino acids long, bind activating ligands and contain characteristic arrays of structural domains.^{4–6} This is followed by a TM helix, a juxtamembrane (JM) segment, a kinase domain of approximately 275 amino acid residues, and in some cases a C-terminal tail that is up to 300 amino acid residues long. Contact between two kinase domains is needed to stimulate catalytic activity, which results in the cross-phosphorylation of receptor molecules and phosphorylation of cytoplasmic substrates, ultimately activating signaling cascades that control cell behavior.^{5,7–9}

After the discovery of RTKs in the 1970s,^{10–12} RTK research was governed by the canonical model of activation. This model postulates that RTKs are monomeric in the absence of ligand and only form dimers upon ligand binding to the EC domain. However, it has now been shown that RTKs can form dimers

even in the absence of ligand (Figure 2A).^{13–20} Different RTKs form unliganded dimers with different interaction energies, but their existence appears to be largely universal. Unliganded dimers are stabilized through lateral interactions between the kinase, JM, and TM domains, while the EC domains usually inhibit dimerization.^{21–25}

In accordance with the law of mass action, RTK expression levels control the relative distribution of monomers and dimers (Figure 2B). As interactions are required for RTK activity, the value of the two-dimensional dissociation constants and the expression levels exert control over activity. This means that, even in the absence of ligand, increased expression—which is common in many cancers^{26–28}—can shift the equilibrium from a predominantly monomeric to a predominantly dimeric population, triggering signaling cascades.

Even though RTK activity in the absence of ligand is possible, ligands are still important for normal function. The ligands are usually polypeptides, and they are commonly referred to as “growth factors.” They typically bind to the receptors with picomolar to nanomolar affinity,^{29–34} and they stabilize the dimers by directly interacting with two copies of the receptor and/or by causing conformational changes. Ligands have been shown to induce structural changes that arguably propagate along the entire length of the RTK, through the TM domain, and ultimately affect the kinase domain (Figure 2A). In general, the kinase domain can exist in both a catalytically active state and an inactive state, and ligand binding results in the kinase domain being converted from its inactive state into its active state.^{35,36}

Studies on endothelial growth factor receptor (EGFR) and other ErbB (erythroblastic oncogene B) subfamily members, fibroblast growth factor receptors (FGFRs), and vascular endothelial growth factor receptor 2 (VEGFR2) have indicated that ligand binding leads to a conformational switch in the TM helix, implying that the EC and TM domains are structurally coupled.^{13,21,37,38} A study by Sinclair et al. further demonstrated that the TM and JM domains of EGFR are coupled, as different ligands induce different TM and JM conformations.³⁹ Moreover, data by Bell et al. suggest that the TM and IC domains are also structurally coupled, and that the TM domain dimer structure controls kinase activity.⁴⁰ There is also evidence that the conformation of the kinase domain can be sensed by the EC domain, as different inhibitors binding to the EGFR kinase domain have different effects on EGF binding.⁴¹

However, others believe that ligand-induced structural changes in the EC domain are not propagated to the IC domain, because the linkers between the different regions are unstructured. For example, Springer et al. have argued that a single ligand-bound EGFR conformation can be coupled to multiple kinase domain arrangements.⁴² Furthermore, a single EGFR kinase domain arrangement can couple to two different EC states,⁴³ suggesting that the EC and IC domains of EGFR can change conformations independently of each other. One possible explanation is that the different ligands differentially stabilize RTK dimers, leading to different kinetic lifetimes and signaling.⁴⁴ It has also been proposed that ligand binding causes changes in the EC domain that alter the local cell membrane, and these alterations are sensed by the intracellular domain.⁴⁵

The debate over the physical effects of ligand binding, briefly overviewed above, highlights the fact that many of the most fundamental questions about RTK activity are still unanswered, despite intense research since the 1970s. There is remarkable consensus, however, that lateral interactions between the RTKs are absolutely critical for RTK activation. This has led to the

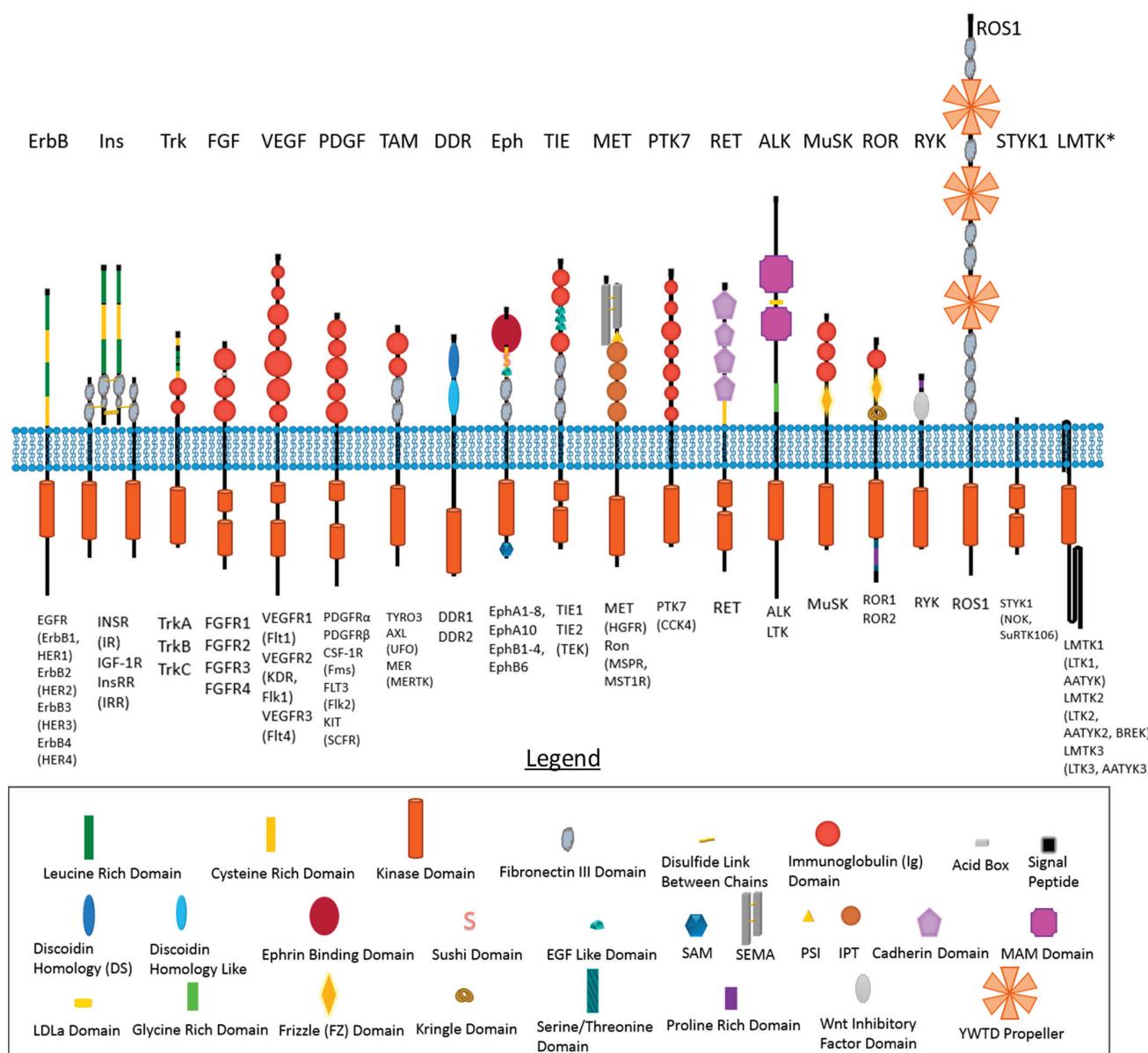


Figure 1. Cartoon representation of the 58 RTKs grouped into 20 RTK subfamilies. It has long been appreciated that heterointeractions can occur within a subfamily. This review focuses on the heterointeractions between subfamilies. Key RTK features and domains are depicted as distinct shapes as explained in the legend. The plasma membrane is shown in blue. The N-terminal extracellular domains are shown above the membrane. RTK subfamily names are listed above the receptor, while the names of the individual RTKs in the subfamily are listed below, with common alternative names listed in parentheses. In general, the structures of all members of a given subfamily are very similar, with minor differences in the size of the full-length proteins and of the individual domains. Notable exceptions are that (i) the fifth Ig domain (third closest to the membrane) of VEGFR3 is proteolytically cleaved and held together by disulfide bonds; (ii) LTK lacks both MAM groups and the LDLa domain, and it is accordingly much shorter than the depicted ALK; and (iii) STYK1 appears to lack a signal sequences and does not seem to localize to the plasma membrane despite having a putative transmembrane domain. *The LMTK proteins were predicted to be tyrosine kinases, but later experiments demonstrated that they only have serine/threonine kinase activity.^{1,2} If they are not counted as RTKs, there are only 55 total RTKs and 19 RTK subfamilies in humans.

development of models that explain how RTK homointeractions occur and regulate biological activity. Despite the fact that a wide range of heterointeractions have been described in the literature, these interactions are rarely taken into account when developing mechanistic models. We argue here for the importance of updating these models to account for the numerous heterointeractions which are known to occur and to affect RTK activity.

2.2. RTK Interactions Regulate RTK Function

In order for an RTK to become active, its kinase domain must be phosphorylated, and this occurs when the two kinases in a dimer cross-phosphorylate each other on select tyrosines. Accordingly, productive lateral interaction is required for RTK activity, and the unphosphorylated monomers are inactive. Once phosphorylated, the activity of the kinase domain is enhanced. As a result, the kinase domain can bind adaptor proteins^{9,46–50} and phosphorylate other molecules,^{51–53} and this causes activation

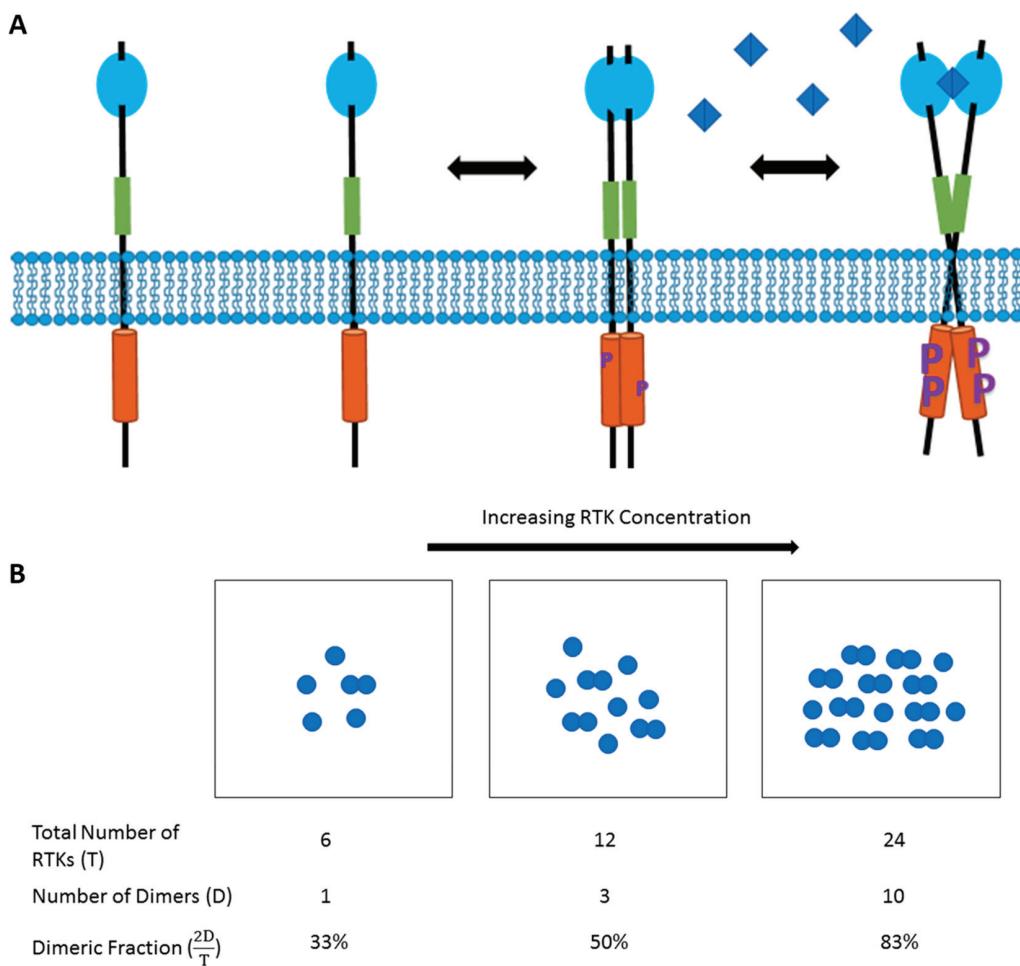


Figure 2. (A) Simplified model of RTK dimerization and activation for a generic RTK and ligand (blue diamond). Inactive monomers dimerize to form unliganded dimers with basal activity. Ligand binding induces a conformational change and enhances phosphorylation (purple “P”), leading to full signaling activity. The process can be fully described by thermodynamic cycles such as those in Figure 3. (B) Cartoon depicting the law of mass action for RTKs which exist in a monomer–dimer equilibrium. The single circles represent the RTK monomers, and the overlapping circles represent the RTK dimers (D). Three different RTK concentrations are depicted. As the total RTK concentration increases from left to right, so does the fraction of receptors which are dimeric. Dimeric fraction is defined as the fraction of total RTKs (T) which exist as a dimer: $\frac{2D}{T}$.

of downstream signaling pathways. Although RTKs have a diverse range of roles, the pathways they mediate often lead to cell growth and proliferation, and abnormally high phosphorylation is linked to many cancers.^{54–57} In fact, several RTKs were originally identified as products of oncogenes.

Recent work has shown that unliganded dimers are often phosphorylated on selected tyrosines,^{21,58,59} which is known as “basal activity,” and this appears to play an important role in pathogenesis. Many cancers exhibit increased expression of RTKs in the absence or even loss of ligand.^{55,56,60,61} Furthermore, there are pathogenic mutations that predominantly affect the basal phosphorylation of the receptor. The G380R mutation in FGFR3, which is the genetic cause of achondroplasia, the most common form of human dwarfism, increases FGFR3 dimerization and phosphorylation in the absence of ligand.⁶² Intriguingly, the formation of EphA2 unliganded dimers actually inhibits oncogenic signaling.⁵⁹ EphA2’s oncogenic activity is caused by the soluble kinase Akt phosphorylating serines on the EphA2 monomers. The formation of the unliganded dimers decreases serine phosphorylation, increases tyrosine phosphorylation, and decreases

EphA2-controlled cell migration, which generally correlates with metastasis and invasiveness.

Although unliganded dimers are important, ligands are a vital part of RTK signaling. RTKs often require ligand to initiate downstream signaling cascades. For example, FGFR2 has been shown to be a Grb2-stabilized dimer in the absence of ligand, and it is phosphorylated to a low degree.^{58,63,64} Ligand binding leads to Grb2 phosphorylation, which drives the dissociation of Grb2, allowing other proteins to bind, and this triggers downstream signaling by the receptor.

3. THERMODYNAMICS OF RTK INTERACTIONS

The use of thermodynamic cycles allows for rigorous analysis of RTK interactions. These cycles account for all possible receptor–receptor and receptor–ligand interactions, and for all of the possible pathways: from monomers, which are inactive, to liganded dimers, which are signaling-competent and active. These thermodynamic cycles can be used to interpret experimental data and predict the concentrations of the different types of dimers using measured equilibrium constants and the total concentrations of receptors and ligands.

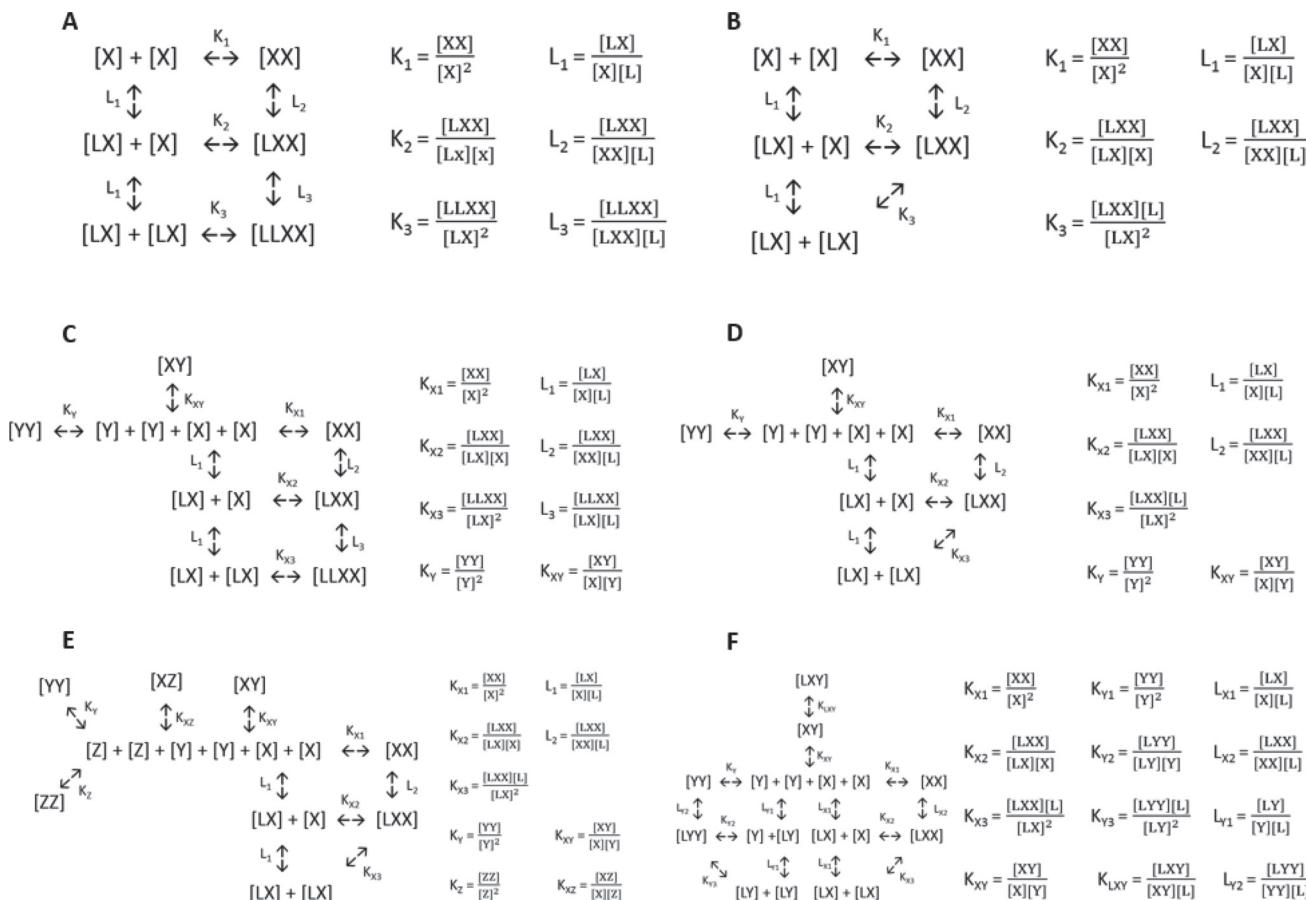


Figure 3. Thermodynamic cycles which allow for rigorous analysis of RTK interactions. The interactions are governed by the RTK dimerization constants, K_p and the ligand binding constants, L_i ; these constraints are defined to the right of the cycles. The concentrations of the receptors are in molecules per unit area, while the concentrations of the ligands are in molecules per unit volume. These constants are interdependent on each other, as paths along the cycle which share a beginning and ending state are thermodynamically equivalent. Once the dimerization and ligand binding constants are known, it is possible to predict the concentrations of monomers and dimers—and in particular, the concentration of the signaling-competent, liganded dimers—for any given receptor and ligand concentrations. (A) The “binding in an aggregating system” model,³ describing the homodimerization of a receptor (X) which binds monomeric ligand (L). The receptor can form homodimers (XX) and bind its ligand as either a monomer (LX), a dimer (LXX), or a liganded dimer (LLXX). (B) A model depicting the homodimerization of a receptor (X) which binds a dimeric ligand (L). The liganded monomer (LX) can interact with an unliganded monomer (X) to form the liganded dimer (LXX). Alternatively, two liganded monomers (LX) can interact to form the liganded dimer (LXX) while releasing a ligand (L) into solution. (C) A model describing the homo- and heterodimerizations of receptors X and Y (XX, YY, and XY), where X binds monomeric ligand (L), but Y does not bind ligand. (D) A model describing the homo- and heterodimerizations of receptors X and Y (XX, YY, and XY), where X binds dimeric ligand, but Y does not bind ligand. (E) A model describing the heterodimerization of X and Y (XY) and X and Z (XZ), where X binds dimeric ligand (L), but neither Y nor Z bind ligand, nor can they interact with each other; the three receptors also form homodimers (XX, YY, and ZZ). (F) A model describing the homo- and heterodimerizations of receptors X and Y (XX, YY, and XY), where both X and Y bind dimeric ligand (LX, LXX, LY, and LY), and the heterodimer does so as well (LXY).

One such thermodynamic cycle is shown in Figure 3A. This is the so-called “binding in an aggregating system” model, described in the classical text by Wyman and Gill,³ and it is applicable for monomeric ligands which bind to a receptor which homodimerizes. The interactions are governed by three dimerization constants— K_1 (dimerization of unliganded monomers), K_2 (dimerization of a liganded monomer with an unliganded monomer), and K_3 (dimerization of liganded monomers)—and three ligand binding constants— L_1 (ligand binding to a monomer), L_2 (ligand binding to an unliganded dimer), and L_3 (ligand binding to a dimer with one ligand already bound); these constants are fully defined to the right of the cycle in Figure 3A. All paths along the cycle which share a beginning and an ending state are thermodynamically equivalent, and therefore, the constants are interdependent on each other. For instance, $K_1 L_2 = L_1 K_2$ and $K_2 L_3 = L_1 K_3$.

The equilibrium constant K_1 can be determined by experiments conducted in the absence of ligand that report on the two-dimensional concentrations of monomers, $[X]$, and dimers, $[XX]$, in the membrane. Experiments can also be performed in the presence of ligand, and these can measure the two-dimensional concentration of the RTK in the membrane, $[X]_{\text{Total}}$, and the concentration of the ligand bound to the receptors in the membrane, $[L]_{\text{Bound}}$. These two measurable parameters depend on the equilibrium constants; on $[X]$; and on the free, soluble ligand concentration, $[L]$:

$$\begin{aligned} [L]_{\text{Bound}} &= [LX] + [LXX] + 2[LLXX] \\ &= [X][L](L_1 + L_2 K_1[X] + 2L_2 L_3 K_1[X][L]) \end{aligned} \quad (1)$$

$$\begin{aligned}
 [X]_{\text{Total}} &= [X] + [LX] + 2([XX] + [LXX] + [LLXX]) \\
 &= [X](1 + L_1[L]) + 2K_1[X]^2(1 + L_2[L] + L_2L_3[L]^2)
 \end{aligned} \tag{2}$$

When $[X]$ is determined from eq 2 and substituted into eq 1, eq 1 provides a connection between all the measurable parameters and the three unknowns: L_1 , L_2 , and L_3 . Thus, measurements of $[L]$ and $[X]_{\text{Total}}$, performed at four or more different ligand concentrations, allow for L_1 , L_2 , and L_3 to be determined using least-squares fitting procedures. The dimerization constants K_2 and K_3 can be then calculated from K_1 , L_1 , L_2 , and L_3 . It is important to note that the plasma membrane is best viewed as a two-dimensional structure; accordingly, the receptor concentrations are given in receptors per unit area (e.g., mol/ μm^2), not receptors per unit volume (e.g., mol/ μm^3). The unbound ligand is, however, in three dimensions, and hence some of the association constants have rather unusual units.

The utility of the thermodynamic cycles approach was first demonstrated in a comprehensive study of EGF binding to EGFR, in which both the concentration of the ligand and the concentration of the receptor were varied.⁶⁵ By fitting the model to a large experimental data set, the researchers were able to determine all the thermodynamic constants for homoassociation and ligand binding. This work demonstrates that the behavior of RTKs in cells can largely be explained by a relatively simple physical-chemical model. Once the dimerization and ligand binding constants are known, it is possible to predict the concentrations of monomers and dimers, and in particular, the concentration of the signaling-competent liganded dimers, for any given receptor and ligand concentration.

While EGF is monomeric, many RTKs are activated by dimeric ligands. For instance, VEGF is a disulfide linked dimer, and one VEGF ligand binds to and activates the VEGFR2 dimer.^{66,67} The relevant thermodynamic cycle in the case of a dimeric ligand is shown in Figure 3B. In this case, a liganded monomer can interact with an unliganded monomer to form the fully liganded dimer. Alternatively, two liganded monomers can interact to form the liganded dimer while releasing a ligand into solution. The equilibrium constraints are the same as in the monomeric ligand case, except that K_3 involves two liganded monomers releasing a bound ligand upon dimerization, and there is no L_3 . All the equilibrium constants can be determined in a manner similar to that of the monomeric ligand case.

Thermodynamic cycles can be also used to account for heterodimerization. Parts C and D of Figure 3 correspond to the cases of monomeric and dimeric ligands, respectively. In these models, we assume that there is a second RTK (Y), which can participate in both homodimerization (YY) and heterodimerization (XY), but it does not bind any ligand. Similar cycles can be created to describe more complex interaction models, such as the formation of higher order oligomers, the binding of multiple ligands to an RTK, the binding of a ligand to multiple RTKs, the occurrence of multiple heterointeractions (Figure 3E), and the binding of a ligand to both RTKs and the heterodimer (Figure 3F). Such thermodynamic cycles can explain how heterointeractions can decrease the concentration of liganded homodimers, how changes in the total amount of a receptor can change the relative amounts of monomers and dimers, and how drugs which decrease the stability of homodimers can increase the concentration of heterodimers. These models could guide our understanding of the effect of heterointeractions on biological

function, as discussed under Using Thermodynamic Models To Understand Heterointeractions.

For the thermodynamic cycles to provide accurate predictions of the concentration of the different types of dimers, the association constants describing the strengths of interaction need to be experimentally determined. There are many methods which can be used to study protein-protein interactions, but few of them work with membrane proteins. Still fewer are suitable for making detailed quantitative measurements of interaction constants. Below, we discuss methods that are used to study membrane protein interactions, and we highlight methods that can produce quantitative information.

4. METHODS TO STUDY PROTEIN-PROTEIN INTERACTIONS IN THE MEMBRANE

4.1. Affinity Capture

Many methods for determining protein-protein interactions (and other proteins-biomolecule interactions) can be described as affinity capture. The basic idea is that a protein of interest is modified such that it can be selectively purified in a way that preserves interactions with other proteins. In a related strategy, the protein of interest marks nearby proteins, and the marked proteins are selectively captured. Once the proteins are isolated, the identity of unknown proteins can be determined. One of the most common methods for determining the identity of the purified proteins is mass spectroscopy (MS). It is beyond the scope of this review to discuss the details of different types of MS experiments and analyses, but this has been extensively reviewed elsewhere.⁶⁸⁻⁷² Affinity capture techniques vary from describing the interactions between two proteins with a high degree of detail to high throughput methods that generate thousands of pairs of interactions that are analyzed with statistical machine learning methods and other big data analytic techniques. In general, these methods provide evidence of interactions and provide qualitative information on how these interactions change under different conditions, but they cannot provide quantitative information about the strength of interactions.

4.1.1. Coimmunoprecipitation. Coimmunoprecipitation involves isolating a protein from cell lysates.^{73,74} An antibody is bound to the protein of interest, and then the antibody-protein complex is precipitated with beads conjugated to antibody binding proteins (usually protein A or protein G). After washing off the rest of the cell lysate and separating the complex from the bead, the presence or absence of a specific interaction partner is determined, typically using western blotting. Of note, this is not a high throughput method, and it only provides binary or semiquantitative information (e.g., the amount of the binding partner detected via western blot increases in cells treated with ligand relative to untreated cells).

This is one of the few methods which does not need tagged versions of the proteins, and hence can be used to study proteins as they are naturally expressed by the cells (endogenous proteins), but it does require extracting the protein out of its native environment. It cannot distinguish if two proteins interact directly or if they are part of a larger complex. Weak interactions might not be detected, as the interactions have to be strong enough to persist through the cell lysing process and initial precipitation. This is a particular concern for membrane proteins, as interactions might be lost when moving from the hydrophobic membrane to the aqueous assay conditions. Cross-linking agents can be used to stabilize the interactions, although this raises concerns about spurious interactions appearing.

It is possible to obtain information about the oligomer size upon cross-linking by comparing how the precipitated complex runs on a gel in comparison to a molecular ladder of known weight (i.e., does the weight match a dimer, trimer, tetramer, etc.). However, this is not the most robust means of determining oligomer size, as the shape of the complex affects how it runs on the gel, and this process requires cross-linking to increase stability. Despite these limitations, coimmunoprecipitation can provide evidence of interactions, it is widely performed in many laboratories due to the lack of expensive equipment and use of commercially available reagents, and it has been the most commonly used technique to study RTK heterointeractions.

4.1.2. Two-Hybrid Screening. The first two-hybrid assay to study protein–protein interaction was developed in 1989 by Fields and Song using yeast.⁷⁵ Known as yeast two-hybrid (Y2H), the basic idea is that a reporter gene will only be transcribed if the protein of interest (bait) interacts with another protein (prey). This is accomplished by splitting the Gal4 transcriptional activator into its two domains, the DNA-binding domain and the activating domain, and attaching the bait to the DNA-binding domain and the prey to the activating domain. Transcription of the reporter gene—for example, a gene which encodes for an essential nutrient lacking from the media—will only occur if the DNA-binding domain and the activating domain are in close proximity (importantly, direct binding is unnecessary), which can only happen if the bait and prey interact with each other. By creating large cDNA libraries encoding for prey fused to the activation domain, Y2H can be used as a high throughput method to quickly determine thousands of protein interactions. Accordingly, Y2H and closely related variants have been used to create large scale interactome maps, including a map of human RTK–phosphatase interactions.^{76,77} The technical details of all the different two-hybrid systems are beyond the scope of this review, and they have been reviewed elsewhere.^{78,79}

It is important to note the limitations of Y2H. Fusing the proteins to the DNA-binding and activating domains could affect their ability to interact with other proteins. The proteins must still be able to fold and interact properly in an environment different from where the proteins are normally found, as the interactions are being studied in the nuclei of yeast cells; moreover, the normal associated proteins and post-translational modifications will be lacking. Studying membrane proteins in an aqueous environment is virtually impossible, as they are prone to misfold and aggregate. This aggregation contributes to Y2H having a high rate of false positives.

Not all of these issues can be eliminated, but advancements in the methodology have helped to reduce their effect, particularly in regard to membrane proteins. A variant known as membrane yeast two-hybrid (MYTH) solves the aqueous environment problem by allowing the interactions to be studied in the membrane.^{80,81} MYTH uses ubiquitin split into two stable moieties, and the bait and prey are tagged with these moieties. The tag on the prey is fused with a reporter molecule (the *E. coli* DNA-binding domain LexA connected to the herpes simplex virus VP16 transcriptional activation domain), and when the bait and the prey interact, the two moieties become in close contact, allowing for the formation of a pseudoubiquitin. This pseudoubiquitin is recognized by deubiquinating enzymes (DUBs), which release the reporter molecule, freeing it to enter the nucleus and activate the reporter gene. Since DUBs are only found in the cytosol, MYTH is only applicable to membrane proteins that contain a cytosolic portion which can

be tagged. A variant of this method for use in mammalian cells called mammalian-membrane two-hybrid (MaMTH) has been developed using a different reporter molecule and reporter gene.⁸²

MYTH and MaMTH allow membrane proteins to be studied in the membrane. Moreover, using mammalian cells allows for mammalian proteins to be studied in an environment where relevant adaptor proteins and post-translational modifications may be present. As with the original Y2H system, concerns related to the effect of tagging the proteins and using an overexpression system still apply. None of these methods can be used to obtain interaction strengths or other quantitative information, but they can screen thousands of potential interactions to reveal hits that can then be investigated using quantitative techniques.

4.1.3. BioID. A high throughput method for detecting potential protein–protein interactions is known as proximity-dependent biotin identification (BioID).⁸³ In this method, a biotin ligase is fused to the protein of interest, and then the modified protein is introduced into cells. By supplementing the culture media with biotin, proteins that are proximal to the protein of interest become biotinylated, and then can be isolated (e.g., using a streptavidin pull-down) and identified, typically using MS. The details of how to implement BioID and couple it with traditional affinity purification methods have been reviewed elsewhere,^{84,85} but a brief discussion of the method is given below.

BioID allows for protein interactions to be studied in live cells, and a large number of interactions can easily be identified. However, this method does not directly probe for interactions, but rather detects proteins which are near the target. It has been estimated that approximately 50% of detections occur within 20–30 nm of the target protein, but the exact resolution is unknown.⁸³ Moreover, the biotin ligase used in the development of BioID (BirA*) can only react with primary amines, meaning that interaction partners lacking these cannot be detected. There is some possibility of nonspecific binding, and larger proteins could be more likely to be identified by MS. All this means that BioID results should not be taken as proof of protein–protein interaction, but as positive hits in a screen which warrant further investigation.

As with any method involving a fusion protein, the effects of this modification need to be kept in mind. The biotin ligase is 35 kDa, which is a little larger than GFP, and whether or not this affects protein interaction or function must be assessed for each protein of interest. The biotinylation of the interacting proteins could alter the secondary modifications or the interactions of the proteins, and the addition of biotin to the media could alter biological activity. Since the fusion protein needs to be introduced into cells, typically by transient or stable transfection, BioID cannot study fully endogenous protein interactions, although the biotinylated interaction partners can be endogenous.

4.2. Proximity Ligation Assay

The proximity ligation assay (PLA) is a method for detection of specific proteins and their interactions.^{86,87} It works by attaching DNA strands to the proteins of interest such that when the proteins come in close contact, a ligation reaction between the two DNA strands can occur. This DNA ligation product can be PCR amplified, allowing for sensitive detection of protein interactions. By attaching the DNA probes to primary or secondary antibodies against the proteins of interest, this assay

can be performed using commercially available kits for a wide variety of targets.^{88,89}

A modified version of PLA was developed to work *in situ*.^{88,89} The DNA probes are designed such that their linkage creates a circular DNA strand. This serves as a template for a rolling circle amplification (RCA) reaction, allowing for a large amount of rolling circle product (RCP) to be generated. In around an hour, DNA polymerase can form an RCP almost 1 μm in diameter, which is near the detection limit of conventional light microscopy. By hybridizing the RCP to fluorescently labeled oligonucleotides complementary to the detection probe, the RCP becomes labeled with hundreds of fluorophores, making for easy visualization.

PLA allows for the detection of protein–protein interaction in a relatively native environment, although the need for antibody-staining generally requires the cells to be fixed and permeabilized. The exact distance limit for detection varies based on the size of the antigen binding agent and the oligonucleotide sequence, but it has been roughly estimated that if the probes are within a few tens of nanometers, the interaction can be detected.⁸⁸ It is important to emphasize that PLA does not directly report on protein–protein interaction, but it indicates that two proteins of interest are within close proximity. Accordingly, it provides no information on the strength, geometry, or stoichiometry of interactions; furthermore, it cannot distinguish between proteins which directly interact with each other and those which are proximal due to mutual association with a third molecule or as part of a complex.

4.3. FRET

Förster resonance energy transfer (FRET) involves the nonradiative transfer of energy from one fluorophore (the donor) to another (the acceptor). This energy transfer decreases as a function of the distance between the two fluorophores to the sixth power, making it highly sensitive to small changes in distance.⁹⁰ It is accordingly commonly used as a conformational probe, since a change from an open or extended conformation to a closed or compact conformation can easily be seen as a change from low to high FRET (given appropriate labeling). FRET can be also used to study the association of proteins, both in solution and in the plasma membrane.

Furthermore, quantitative FRET methodologies exist which take into account the concentrations of the donor and acceptor that can be used to determine homo- and heterointeraction strengths. These methodologies work in both cell-derived and live-cell systems, and typically the proteins of interest are genetically tagged with the donor and acceptor fluorophores and introduced using stable or transient transfection. Transient transfection is advantageous in these experiments, as it allows a broad range of concentrations to be sampled. Indeed, concentration ranges spanning two to three orders of magnitude have been reported.^{91–93}

Quantitative imaging FRET (QI-FRET) is one such quantitative FRET method, and it can be performed using a traditional confocal microscope.^{94–96} The appropriately labeled sample is imaged three times: (i) a donor scan which gives the donor fluorescence when the donor is excited, (ii) a FRET scan which gives the acceptor fluorescence when the donor is excited, and (iii) an acceptor scan which gives the acceptor fluorescence when the acceptor is excited. These three scans impose a major constraint on the fluorophores which can be used, as the donor should not be excited by the acceptor excitation source, and there must be minimal bleed through between the two emission

channels. A common choice for QI-FRET is to use a member of the YFP family as the donor and mCherry as the acceptor.^{92,97} This fluorophore constraint is eliminated in the similar fully quantified spectral imaging FRET (FSI-FRET), which uses spectrally resolved two-photon imaging to acquire two scans—a FRET scan and an acceptor scan—and can use any two fluorophores which form a FRET pair.⁹⁸ The method has both high sensitivity and a high signal-to-noise ratio, but it requires a specialized microscope.⁹⁹

By measuring donor and acceptor concentrations and FRET efficiencies, association curves can be generated with the QI-FRET and the FSI-FRET methods, and these can be fit to different oligomerization models to determine the best fit model and the ΔG of interaction. In the case of membrane proteins, which are confined to the effectively two-dimensional plasma membrane, such fits require that the contribution of “proximity” or “stochastic” FRET is also taken into account.^{100–102} Detailed protocols to correct for this contribution are available in the literature.¹⁰²

Although to date quantitative FRET has mostly been used to study homointeractions, it is easily adapted to study heterointeractions. In the homointeraction case, a portion of the proteins of interest are labeled with the donor, and the rest are labeled with the acceptor. In the heterointeraction case, all of one protein is labeled with the donor, and all of the other is labeled with the acceptor. The only specific interactions which will result in FRET are the heterointeractions. Thus, for membrane proteins, specific heterointeractions will result in FRET that is higher than the proximity FRET. The calculation of heterodimerization constants requires that all homo- and heterointeractions are taken into account; detailed protocols can be found in the literature.^{97,103}

FRET has also been used to gain information about the size of the oligomer, by analyzing the dependence of FRET on the donor-to acceptor ratio.^{102,104,105} Although two-color, quantitative-FRET techniques are good at distinguishing between monomers (i.e., no interactions), dimers, and higher order oligomers, they struggle to precisely determine the sizes of oligomers larger than dimers.¹⁰² In some cases, oligomer size and geometry have been determined by histogramming the pixel-level apparent FRET values for a cell.^{106,107} This histogram is then fit to one or more Gaussians, and the fit for hundreds of cells which exhibit single peaks can be histogrammed into a “metahistogram.” By fitting the metahistogram to multiple Gaussians and comparing the number of peaks and the fit parameters to the theoretical FRET values for different donor and acceptor configurations, oligomer size and geometries are determined.

Overall, FRET has provided valuable information about the interactions of several RTKs. However, FRET requires labeling, and thus it cannot be used to study endogenous proteins. The fluorescent proteins are large (~27 kDa), and they may affect RTK interactions or function. Accordingly, the effect of the label must be assessed for each RTK of interest through control assays comparing RTK function with and without labels. FRET only assesses the interactions between the labeled proteins, and other interaction partners will be missed if they are unlabeled.

4.4. Statistical and Correlational Fluorescence Methods

There are several fluorescence methods which rely upon statistics and correlated movement or correlated fluorescence to provide information about the structure and interaction of molecules. These methods do not directly report on interactions

per se, but rather, they indicate the apparent size of or number of fluorophores in the labeled complex. This indirect nature precludes the methods from determining if the proteins are directly interacting or are part of a larger complex. Although these methods have generally been applied to homointeractions, they can be adapted to study heterointeractions. As with all techniques which require labeling the protein of interest, they cannot be used to study endogenous proteins, and it is possible that the modification affects normal protein function or interactions. Some of these methods are briefly discussed below, and ideally a combination of two or more methods should be used in order to get a reliable estimate of the oligomer size.

4.4.1. Fluorophore Localization Imaging with Photo-bleaching. Fluorophore localization imaging with photo-bleaching (FLImP) uses single particle tracking to determine the distance between two fluorophores.^{108,109} This is accomplished by using changes in the diffraction-limited image spots when one of them photobleaches and by fitting the point spread function. The distances between the fluorophores of thousands of traces are histogrammed, and the histogram is decomposed into different peaks. The peaks correspond to different distances between the fluorophores in the complexes, and this provides information about the oligomer size and geometry of the labeled proteins. FLImP is able to determine lateral distances between identical fluorophores that are within about 60 nm, although fixation is required to obtain resolution below 10 nm. There are, of course, limitations on how close two distances can be and still be resolved into two separate peaks, and the number of peaks cannot always be unambiguously determined. Although FLImP can be performed on commercially available microscopes, it is computationally intense, and the analysis is rather technical and requires care to ensure that accurate conclusions are reached. It is a highly statistical method, and errors—e.g., sample drift, autofluorescence, and the crowded cell environment—must carefully be taken into account. As FLImP uses single particle tracking, relatively low concentrations are required.

4.4.2. Number and Brightness. Number and brightness (N&B) is based on the fact that, although the same number of fluorophores will give the same average fluorescence intensity regardless of oligomer size, the variance in the intensity fluctuations will be different for different oligomer sizes.¹¹⁰ As an illustration of this concept, a dimer diffusing out of the imaging window will cause a larger fluctuation in fluorescence intensity than a monomer. There is also a two-color version which uses the cross-variance of the intensity fluctuations of the two fluorescence channels, and it is well-suited to study heterointeractions.¹¹¹ Experimentally, N&B works by rapidly taking a stack of images of the same region, and then computing the average fluorescence intensity and the variance across the stack for each pixel. This allows for the number of particles and the molecular brightness of each pixel to be determined. In theory, the brightness of a dimer is twice that of a monomer, and higher order oligomers scale linearly. However, in practice, issues with fluorophore maturation, quenching, and other complications can cause the brightness of a dimer to be less than double the brightness of a monomer. Accordingly, the most accurate results will be obtained when experimental results are compared to controls of known oligomer size rather than just scaling everything relative to the monomer. Care must also be taken to account for photobleaching and cell movement.

4.4.3. Spatial Intensity Distribution Analysis. Spatial intensity distribution analysis (SpIDA) is a spatial method which

works by fitting super Poisson distributions to intensity histograms.^{112,113} One or more regions of interest are analyzed from a single image, and oligomer size is determined by comparing the determined brightness to the brightness of a monomer control. As with N&B, it is possible that the brightness does not scale linearly with oligomer size, and best results are obtained when the experimental brightness is compared to controls of multiple oligomer sizes. SpIDA can be applied to both live cells and fixed samples, and thus endogenous proteins can be studied using immunofluorescent staining.

4.4.4. Pulsed Interleaved Excitation Fluorescence Cross-Correlation Spectroscopy. In general, fluorescence correlation spectroscopy (FCS) uses the temporal fluorescence intensity fluctuations through a small excitation volume coupled with correlational analysis to determine the diffusion coefficients and the concentration of a fluorophore.^{114–117} This is expanded in fluorescence cross-correlation spectroscopy (FCCS), which uses the autocorrelation and cross-correlation information on two different colors.^{118–120} If the fluctuations are occurring simultaneously in both channels, it means that the fluorophores must be moving together as part of a complex. For more detailed information about FCS and FCCS, please see one of the many extensive reviews on the subjects.^{121–125} A further expansion of this methodology is pulsed interleaved excitation FCCS (PIE-FCCS), which uses rapid alternation between multiple excitation sources such that the fluorescence emission generated from one excitation pulse is complete before the next excitation pulse arrives.^{126,127} Accordingly, spectral crosstalk can be eliminated, as the excitation source of each detected photon is known, and hence higher resolution is obtained. PIE-FCCS does not directly report on oligomer size or stoichiometries, but rather, it allows for a determination of the relative size of codiffusing species.

4.4.5. Raster Image Correlation Spectroscopy. Another fluorescence correlation method is raster image correlation spectroscopy (RICS).¹²⁸ This method involves repeated raster scanning over a viewing window to create an image stack. The two-dimensional spatial correlation of the fluorophore is then calculated using the image stack, and this yields diffusion coefficients and concentration. There is also a two-color, cross-correlation version which is more suitable for heterointeractions.¹²⁹ This can all be done on a commercial laser scanning microscope. Care needs to be taken to account for photo-bleaching and cell movement. Furthermore, as is the case with PIE-FCCS, RICS does not directly report on oligomer size or stoichiometries, but comparisons can be made using the size information that can be determined from the diffusion coefficients.

4.5. Multistep Photobleaching

A single molecule technique using total internal reflection fluorescence (TIRF) microscopy for determining oligomer size is known as multistep photobleaching.^{130,131} An area containing a small number (~50–200) of fluorescent spots is photobleached by repeated imaging, and for each spot, the number of bleaching steps is counted. If each subunit is fluorescently labeled, the number of bleaching steps corresponds to the oligomer size of the complex. Using single-color labeling, it is possible to obtain some information about heterointeractions by comparing both individually labeled cases to the dual-labeled case. It should be possible to study heterointeractions by using two-color labeling where each component is labeled with a

different color,¹³² although we are unaware of this having been applied to live cells.

Of note, in some cases, a smaller number of bleaching steps will be observed than the oligomer size due to a fluorophore being nonfluorescent, a subunit not being labeled, or a photobleaching event not occurring during the imaging time; all of this must be accounted for in the analysis. Multistep photobleaching does not directly report on interactions, but rather, every fluorophore which sequentially photobleaches is assumed to be part of the same complex, as it remained within a small volume during the image acquisition time. Furthermore, it struggles to distinguish discrete bleaching steps for complexes with more than five labeled subunits. As with all signal molecule techniques, it can only be used with low concentrations of fluorescent molecules.

4.6. Biomolecular Fluorescence Complementation

Biomolecular fluorescence complementation (BiFC) involves two nonfluorescent protein fragments coming together to form a fluorescent molecule.^{133,134} It is a type of protein-fragment complementation assay, in which the proteins of interest are fused to fragments of a third protein, and when those fragments combine, a detectable reaction occurs. There are many possible choices for the third protein which can be split into fragments—e.g., ubiquitin,¹³⁵ β -galactosidase,¹³⁶ luciferase,¹³⁷ and TEV¹³⁸—but we focus on BiFC due to its ability to directly visualize interactions and its applicability to living cells. (Of note, two-hybrid screening is also a type of complementation assay, but this was described earlier under *Affinity Capture* due to the way the results are typically analyzed.) To study homointeractions, a fraction of the protein of interest are labeled with one fluorophore fragment (A), and the rest with the other fluorophore fragment (B); to study heterointeractions, all copies of one protein of interest are labeled with one fluorophore fragment (A), and all copies of the other protein of interest is labeled with the other fluorophore fragment (B). In both cases, when the labeled proteins of interest interact, the two fluorophore fragments combine (AB) into a fluorescent protein. Multiple interactions can be studied simultaneously, as a multicolor BiFC variant exists where a protein of interest is labeled with a fluorophore fragment (C) which can combine with two different fluorophore fragments (D and E) such that these combinations (CD and CE) give different fluorescence,¹³⁹ and split fluorophores have been developed that have a wide array of colors.¹⁴⁰

BiFC has many strengths, including that it can be used to directly study interactions in live cells using conventional fluorescence microscopes without the need for specialized software. It can detect weak interactions, it has good spatial resolution, and the range of colors available allows for simultaneous visualization of multiple interactions. However, it still has many drawbacks. It requires modified proteins, so it cannot be used to study endogenous proteins, and the modifications can affect normal protein function or interactions. Once the two fragments have combined, the fluorophore may take almost an hour to mature (i.e., after formation, it may not be fluorescent until almost an hour later); this means that BiFC cannot be used to study short-lived interactions or protein dynamics in real time. Moreover, since the two fluorophore fragments combine irreversibly, the proteins effectively become permanently linked. Accordingly, interactions involving dynamic association and dissociation will be disrupted, and hence thermodynamic calculations cannot be made. Although BiFC

directly reports on interactions, it cannot determine whether two molecules are directly interacting with each other or whether the two molecules are part of a larger complex. Moreover, determining the concentration of the protein of interest is difficult, as doing so requires a secondary label.

5. INTERACTION DATABASES

There are several online databases that curate the literature and facilitate searches for biomolecular interactions. Depending on the database, these contain hundreds of thousands of protein–protein and protein–biomolecule interactions based on experiments, homology modeling, and computational predictions, as well as post-translational modifications. Although the databases are not specific for RTKs or membrane proteins, they can be useful starting points for trying to understand all the possible RTK interactions. Some of the larger databases are MIntAct¹⁴¹ (a merger of MINT¹⁴² and IntAct,¹⁴³ which also provides training to researchers to use the platform and emphasizes the adaption of standards), BioGRID¹⁴⁴ (Biological General Repository for Interaction Datasets, which includes protein, genetic, and chemical interactions for major model organisms), IID¹⁴⁵ (Integrated Interactions Database, which focuses on tissue specific protein interactions), DIP¹⁴⁶ (Database of Interacting Proteins, only experimentally determined protein interactions), HPRD¹⁴⁷ (Human Protein Reference Database, manually curated and only including human protein interactions), MIPS mammalian protein–protein interaction database¹⁴⁸ (mammalian protein–protein interactions focusing on individually performed experiments), and Reactome¹⁴⁹ (general biological pathways and reactions).

6. RTK HETEROINTERACTIONS WITHIN THE SAME SUBFAMILY

Most of the work in the field has focused on RTK homodimerization or homo-oligomerization. The studies on heterointeractions have generally investigated heterodimers between two members of the same RTK subfamily, as it has long been appreciated that these can form due to sharing a common ligand. Heterodimerization is viewed as a means to enhance diversity in signaling by a ligand which is capable of binding two or more related receptors. Indeed, it has been shown that ligand binding to RTK homodimers and heterodimers leads to the phosphorylation of different tyrosines, and to the recruitment of different adaptor proteins which mediate different biological responses (overviewed below).

For example, the members of the ErbB subfamily form same subfamily heterodimers depending on their expression levels and which ligands are present, and this allows for increased signaling complexity and an enhanced ability to respond to changing stimuli.^{35,150,151} There are four members of the ErbB subfamily—EGFR, ErbB2, ErbB3, and ErbB4—and they are important for cell division, survival, and migration; organ growth and development; and maintenance of adult tissue.^{152–157} ErbB overexpression and mutations are associated with many cancers,^{158–162} making the ErbBs major drug targets.^{163–166} The receptors and their ligands are associated with several other disorders, including decreased ErbB4 activity playing a role in schizophrenia^{167,168} and a link to psoriasis.^{169,170}

Different ligands bind to different ErbBs, and this can cause different dimer pairings to form. Endothelial growth factor (EGF) binds to EGFR, while Neu differentiation factor (NDF, which is a form of neuregulin-1) binds to ErbB3 and ErbB4; in

fibroblast cells, EGF promotes EGFR–ErbB2 heterodimers while NDF promotes ErbB2–ErbB3 and ErbB2–ErbB4 heterodimers.¹⁷¹ Different interactions cause different tryosines to become phosphorylated and different adaptor proteins to bind. For instance, EGFR homodimers bind c-Cbl, while EGFR–ErbB2 heterodimers do not.¹⁷² While EGFR homodimers and EGFR–ErbB4 heterodimers both bind Shc, only the homodimer binds Grb2.¹⁷¹ Intriguingly, ErbB2 has no known EGF-like ligand, and it appears to be largely dependent on heterodimerization for its activity, which may explain why ErbB2 is the preferred binding partner of the other three ErbBs.¹⁷³ ErbB3 is also highly dependent on heterodimerization for its signaling, as its kinase activity is impaired, and hence it requires heterodimerization to become phosphorylated.¹⁷⁴

This dependence on heterodimerization has been seen in numerous cell types. For instance, in hematopoietic cells, variants expressing only one ErbB could not be mitotically activated, but a variant with both ErbB2 and ErbB3 (and, to a lesser extent, one with EGFR and ErbB3) had strong lamellipodia activity in response to ligand.¹⁷⁵ A similar result has been seen in the neoplastic transformation of fibroblast cells, where ErbB2 or ErbB3 alone does not have a large effect, but in combination they are transformative, and this is associated with increased ErbB3 phosphorylation.¹⁷⁶ The presence of heterodimers can affect the time frame of signaling, as in myeloid cells, EGFR homodimers are quickly degraded, terminating the signal, but EGFR–ErbB2 and EGFR–ErbB3 heterodimers are recycled to the cell surface, prolonging the signal.¹⁷⁷

Another RTK subfamily where heterointeractions play a key role is the VEGFRs. There are three VEGFRs—VEGFR1, VEGFR2, and VEGFR3—and they are critical for angiogenesis,^{66,178,179} with VEGFR2 being of particular importance, while VEGFR3 is key for the development of the lymphatic system.^{180,181} Inhibiting the VEGFRs has been the focus of major clinical effort, because many cancers overexpress VEGFRs or VEGF ligands or have VEGFR mutations,^{182–186} and there are many such inhibitors now on the market.¹⁸⁷ Furthermore, aberrant angiogenesis is part of the pathology of numerous other diseases, including macular degeneration,^{188,189} diabetic retinopathy,^{190,191} and rheumatoid arthritis.^{192,193}

The role of VEGFR1 during development is somewhat nonintuitive, as mice which do not express VEGFR1 have abnormally organized vasculature and die in utero,¹⁹⁴ but mice with a truncated version which completely lacks the kinase domain appear to develop normally.¹⁹⁵ VEGFR1 is capable of binding the primary ligand of VEGFR2, VEGFA, as well as forming heterodimers with VEGFR2.¹⁹⁶ Accordingly, it is believed that the primary role of VEGFR1 is to serve as a negative regulator of VEGFR2 by tightly controlling the amount of free VEGFA and VEGFR2 homodimers.^{66,197,198} This mechanism of regulation is more complicated than just sequestration, as the heterodimers do have some activity. They have been found to induce migration and PI3 and PLC γ phosphorylation in response to VEGFA, although to a different degree than VEGFR2 homodimers.¹⁹⁹

Moreover, VEGFR1 can directly phosphorylate VEGFR2, as a kinase dead version (i.e., the kinase domain can be phosphorylated as normal, but it cannot phosphorylate another molecule) of VEGFR2 is phosphorylated by VEGFR1.²⁰⁰ A variant of VEGFA which can only bind VEGFR1 induces different activity than the VEGFR1 specific ligand placenta growth factor (PIGF). This VEGFA variant is unable to rescue *PIGF*^{−/−} mice, and it suppresses VEGFR2 phosphorylation

while PIGF enhances it; the two ligands cause different VEGFR1 tyrosines to become phosphorylated and induce different gene expression profiles. A ligand that binds specifically to the VEGFR1–VEGFR2 heterodimer (a dimer of PIGF and VEGFA, a VEGFR2 specific ligand) also has a unique effect: it causes VEGFR2 phosphorylation, but does not appear to affect heterodimer formation, and relative to VEGFA or VEGFE, it only weakly activates ERK1/2 and does not induce cell proliferation.²⁰¹ Of note, VEGFR1–VEGFR2 heterodimers can form in the absence of ligand, but both VEGFA and a VEGFA–PIGF dimer—which had previously been found in the media of several human tumor lines and has a mitogenic effect on cells, but significantly less than that of VEGFA^{202,203}—increase heterodimer formation.²⁰⁰

VEGFR2 is also capable of forming a heterodimer with VEGFR3. There are five tyrosines on the carboxy tail of VEGFR3 which are normally phosphorylated in homodimers, but only three of them are phosphorylated in VEGFR2–VEGFR3 heterodimers.²⁰⁴ In endothelial cells which naturally have both VEGFR2 and VEGFR3, VEGFR2–VEGFR3 heterodimers could not be detected by coimmunoprecipitation after addition of VEGFA, which only very weakly binds to VEGFR3, but could be detected after addition of VEGFC, which binds to both receptors.²⁰⁵ However, using PLA, a small number of VEGFR2–VEGFR3 heterodimers were observed in the absence of ligand, with a small increase occurring after VEGFA addition, and a large increase occurring after VEGFC addition. This emphasizes the importance of using multiple techniques to study heterointeractions, and it demonstrates how immunoprecipitation in particular is liable to miss weak interactions. The same study found that after VEGFA addition, there were approximately 8-fold more VEGFR2 homodimers than heterodimers, and after VEGFC addition, there were approximately 2-fold more VEGFR3 homodimers than heterodimers. In three-dimensional embryoid bodies, VEGFA, and to a lesser extent VEGFC, induces angiogenic sprouting. Heterodimers, as seen by PLA, were significantly more concentrated in these sprouts than the stalk. Since the degree of heterodimerization is dependent on the concentrations of the receptors and ligands, different distributions are likely to be seen during different stages of angiogenesis, and hence the formation of heterodimers allows for increased signal complexity and fine-tuning, and it provides a means for the same set of receptors to cause different functional outputs during different processes.

Several other RTK subfamilies have important heterointeractions, and we briefly discuss them here. (For more information on RTK subfamilies not described here, see their first appearance in [Overview of Known RTK Cross-Subfamily Heterointeractions](#).) PDGFR α and PDGFR β can dimerize to form an $\alpha\beta$ heterodimer, and this interaction results in a different tyrosine being phosphorylated than in the homodimer cases²⁰⁶ and unique downstream effects.²⁰⁷ In our lab, we have shown that truncated versions of the FGFRs containing the EC and TM domains can form heterodimers in the absence of ligand, and their stabilities are similar to the homodimer stabilities.⁹⁷ MET and Ron form heterodimers and directly phosphorylate each other, amplifying and sustaining the signaling of both pathways.^{208,209} Many heterointeractions are known to occur between members of the Eph subfamily. For example, EphB1 and EphB4 can cross-phosphorylate and activate EphB6;^{210,211} EphB2 coclusters with and phosphorylates EphA3, and the interactions appear to modulate cell retraction and segregation signaling;²¹² EphB6 suppresses

Table 1. A List of Known Interactions Between RTKs of Different Subfamilies

RTK subfamily	RTK	known cross-subfamily hetero-interactions
Eph	EphA2	EGFR, ^{253,254} ErbB2, ^{255,256}
	EphA4	FGFR1, ^{257–259} FGFR2, ^{257,259} FGFR3, ^{257,259} FGFR4, ^{257,259}
	EphA7	RYK ²⁶⁰
	EphB2	RYK ^{260,261}
	EphB3	RYK ^{260–262}
	EGFR	AXL, ^{263–265} EphA2, ^{253,254} FGFR1, ^{4,266} FGFR3, ^{4,267} IGF-1R, ^{268,269} MET, ^{270–278} PDGFR α , ^{279,280} PDGFR β , ^{281–283} Ron, ^{276,284–287} ROR1, ^{288,289} STYK1, ^{a,291} TrkB, ^{a,292–295}
	ErbB2	AXL, ^{263,296,297} EphA2, ^{255,256} FGFR1, ^{4,266} FGFR3, ^{4,298} IGF-1R, ^{299–301} MET, ^{273,302} TrkA, ³⁰³ TrkB ³⁰⁴
	ErbB3	FGFR1, ^{4,266,305} FGFR2, ^{4,306} FGFR3, ^{4,298} IGF-1R, ^{301,307,308} MET ¹ , ^{272,273} ROR1, ^{288,289,309}
FGF	ErbB4	AXL, ²⁶³
	FGFR1	EphA4, ^{257–259} EGFR, ^{4,266} ErbB2, ^{4,266} ErbB3, ^{4,266,305} IGF-1R, ^{a,305} MET, ^{a,266,305} PDGFR α , ^{266,305,310}
	FGFR2	EphA4, ^{257,259} ErbB3, ^{a,306}
	FGFR3	EGFR, ^{a,267} EphA4, ^{257,259} ErbB2, ^{a,298} ErbB3, ^{a,298} KIT ³¹¹
	FGFR4	EphA4, ^{257,259}
	IGF-1R	EGFR, ^{268,269} ErbB2, ^{299–301} ErbB3, ^{301,307,308} FGFR1, ^{a,305} MET, ^{a,312,313} Ron, ^{314,315}
	MET	AXL, ^{263,316} EGFR, ^{270–278} ErbB2, ^{273,302} ErbB3, ^{272,273} FGFR1, ^{a,266,305} IGF-1R, ^{a,312,313} RET, ²⁷³ ROR1, ^{289,317} VEGFR2, ³¹⁸
MuSK	Ron	EGFR, ^{276,284–287} IGF-1R, ^{314,43,15} PDGFR β , ³¹⁹
	MuSK	ROR1 ³²⁰
	KIT	FGFR3, ³¹¹
	PDGF	PDGFR α , ^{279,280} FGFR1, ^{266,305,310}
	PTK7	PDGFR β , ²⁶³ EGFR, ^{281–283} Ron, ³¹⁹ VEGFR2, ^{321,322}
	RET	ROR2, ^{323,324} VEGFR1, ³²⁵ , ^{326,327}
	ROR	MET, ²⁷³ TrkA, ^{a,328,329} TrkB, ^{a,330} VEGFR2, ^{331,332}
RYK	ROR2	EGFR, ^{268,289} ErbB3, ^{288,289,309} MET, ^{289,317} MuSK ³²⁰
	RYK	PTK7 ^{323,324}
	STYK1	EphA7, ²⁶⁰ EphB2, ^{260,261} EphB3, ^{260–262}
	TAM	EGFR, ^{a,290}
	TYRO3	EGFR, ^{263,296,297} ErbB4, ²⁶³ MET, ^{263,316} PDGFR β , ²⁶³ VEGFR2, ^{a,333}
	Trk	TrkA, ^{a,291} ErbB2, ³⁰³ RET ^{a,328,329}
	TrkB	EGFR, ^{a,292–295} ErbB2, ³⁰⁴ RET ^{a,330}
VEGFR	VEGFR1	PTK7 ³²⁵
	VEGFR2	AXL, ^{a,333} MET, ³¹⁸ PDGFR β , ^{321,322} PTK7, ^{326,327} RET ^{331,332}

^aThere is indirect evidence for this interaction, but no direct experimental demonstration that this specific interaction occurs.

EphA2 phosphorylation on serine 897 and antiapoptosis signaling;²¹³ and EphA4 interacts with EphB2 and enhances ephrin-B2 induced phosphorylation, and this interaction is important for regulating cell mitogenic activity and may play a role in the differential effect of the different ephrin ligands.²¹⁴ ROR1 and ROR2 form heterodimers, and it is believed that this interaction helps regulate Wnt-5a signaling, which is critical for the formation of synapses in hippocampal neurons.²¹⁵ TYRO3 and AXL heterodimerize, and the interaction appears to amplify the signaling of both receptors.²¹⁶ INSR and IGF-1R frequently heterodimerize in many tissues, and the heterodimers often form to a higher degree than expected by a simple expression level analysis, especially in cancer.^{217–220} The two TIE (tyrosine kinase with Ig and EGF homology domains) receptors, which are important for vasculature development and adult homeostasis, also form heterocomplexes.^{221,222} TIE1, which has no known activating ligand, appears to negatively regulate TIE2 by forming ligand-independent dimers with it, and these heterodimers decrease TIE2 phosphorylation and downstream signaling; different TIE2 ligands stabilize or destabilize these heterodimers to different degrees, allowing for fine control over TIE2 activity.^{223–226} It is clear that there is a wide range of different RTK heterointeractions within the same subfamilies. Many, but not all, are caused by ligands which bind to both receptors, and they are important for signaling regulation, amplification, and diversification.

7. LIGANDS BINDING TO MULTIPLE SUBFAMILIES

As evidenced by RTK heterointeractions from the same subfamily, heterodimers are often caused by a ligand which is capable of binding two different receptors. There are several known instances of a ligand associated with one RTK subfamily interacting with another subfamily, and we briefly overview some of these interactions here. The membrane bound ephrins typically interact with the Eph receptors, but they can also interact with several other RTKs. For instance, RET is necessary for proper ephrin-A growth signaling in neurons, and RET knockout mice have inhibited peroneal axon projections.²²⁷ Direct interaction between RET and ephrin-A2 and ephrin-A5 can be seen in neurons by coimmunoprecipitation and PLA. In neurons, addition of ephrin-A5 enhances neuronal branching and synaptic density induced by the TrkB ligand brain-derived neurotrophic factor (BDNF), and RNAi silencing of TrkB diminishes this effect.²²⁸ All three Trks coimmunoprecipitate with ephrin-A5 and ephrin-A7 when a receptor and ligand are exogenously expressed in CHO cells. Moreover, in a neuronal cell line, addition of the TrkA ligand nerve growth factor (NGF) induces interaction between TrkA and ephrin-A5, and the binding appears to enhance Akt signaling. Normally, ephrin-B1 causes dissociation of embryonic cells in *Xenopus* embryos, but addition of FGFs inhibits this process.²²⁹ Ephrin-B1 becomes phosphorylated after FGF addition as long as kinase active FGFR1 is present, and ephrin-B1 coimmunoprecipitates with phosphorylated FGFR1 and FGFR2. Furthermore, ephrin-B1 is phosphorylated after addition of platelet-derived growth factor (PDGF), and this appears to be due to an interaction with a platelet-derived growth factor receptor (PDGFR).²³⁰

PDGFR α and PDGFR β have also been found to interact with VEGFA. In MSCs that have the PDGFRs but not the VEGFRs, addition of VEGFA increases migration and proliferation, and inhibition or knockdown of the PDGFRs abolishes this effect; VEGFA and the PDGFRs were seen to interact via coimmunoprecipitation that was stabilized by cross-linking.²³¹

This interaction was also seen using an isotope labeled version of VEGFA, and VEGFA was found to competitively inhibit PDGFs from binding, although it was able to activate PDGFR α to some degree.²³²

Interestingly, there is some evidence for the existence of heteroligands composed of ligands associated with two different subfamilies. PDGF-BB was found to interact with FGF2 by surface plasmon resonance (SPR), and this interaction lead to the formation a PDGF-BB(FGF2)₂ trimer, as determined by both steady-state fluorescence and solid-phase immunoassay, with an estimated one-step dislocation constant in the pico- to femtomolar squared ($\text{pM}^2\text{--fM}^2$) range.²³³ Computational modeling indicates that a VEGF–EGF dimer could exist, and that it could bind to EGFR with normal affinity, but it would have impaired binding to a VEGFR.²³⁴ In experiments using a synthetic VEGF–EGF heteroligand purified from yeast, the heteroligand binds to EGFR with almost 10-fold higher affinity than EGF, and it binds to VEGFR2 with about the same affinity as VEGF, as determined by ELISA.²³⁵ Additionally, the heteroligand induces phosphorylation of both VEGFR2 and EGFR, and a version with a radio labeled cargo is successfully internalized by cells.

Such multiple subfamily binding ligands could result in cross-subfamily heterointeractions similar to the heterointeractions seen within a subfamily. Moreover, given that RTKs dimerize in the absence of ligand, it is not unreasonable to expect that RTKs that do not share a common ligand could physically interact with each other. Cross-subfamily interactions are made more plausible by the fact that the kinase domains across the RTK subfamilies are closely related,³⁶ in fact, drugs designed to inhibit RTK kinase domains often inhibit several RTKs.^{236,237} Accordingly, kinase–kinase interactions help stabilize homodimers in the absence of ligand,^{13,21} and the kinase domains of RTKs from different subfamilies are likely to also help stabilize heterointeractions. The TM domains may also be contributing to cross-subfamily heterointeractions. Below, we explore the literature on RTK heterointeractions from different subfamilies, and we discuss their possible biological significance.

8. OVERVIEW OF KNOWN RTK CROSS-SUBFAMILY HETEROINTERACTIONS

RTK cross-subfamily interactions have been reported for over a dozen different subfamilies, and these interactions involve around half of the RTKs. These interactions occur in a wide variety of circumstances, and their function is varied and often not well understood. However, it is clear that these interactions have important biological consequences, particularly with regard to cancer progression and its treatment, and hopefully future quantitative experiments will help clarify their nature and function. Table 1 provides a list of RTKs known to engage in cross-subfamily heterointeractions. Below is a detailed overview of the contents of Table 1. Readers primarily interested in an overview of cross-subfamily heterointeractions and a discussion of their possible effects may proceed to Using Thermodynamic Models To Understand Heterointeractions.

8.1. Eph Receptors Interact with Multiple RTKs

We begin our overview of known interactions between RTKs of different subfamilies with the Eph receptors. The Eph receptors (erythropoietin-producing human hepatocellular carcinoma) are the largest class of RTKs, having 14 members in humans. They are split into nine EphAs and six EphBs, based on their ability to bind the ephrin-A and ephrin-B ligands. Ephrin ligands

are membrane proteins located on adjacent cells, and the Eph–ephrin interaction causes bidirectional signaling: Eph receptor dimerization and higher order oligomerization followed by phosphorylation causes forward signaling, while the ephrins can dimerize and trigger reverse signaling in the adjacent cell.^{238–240} This complex signaling is important for many cellular processes,²⁴¹ such as neuronal development and axon guidance,^{242,243} migration and proliferation,^{214,244,245} inflammation,²⁴⁶ and cardiovascular development.²⁴⁷ Additionally, aberrant Eph signaling is associated with many medical conditions, including cancer^{248–250} bone and joint disorders,²⁵¹ and cardiovascular disease.²⁵²

8.1.1. EphB2 and EphB3 Interact with RYK. The first discovered cross-subfamily heterointeractions involving an Eph receptor are those of EphB2 and EphB3 interacting with RYK (related to tyrosine kinase). RYK is a kinase dead receptor which binds Wnts and Frizzled³³⁴ and is important for Wnt signaling.³³⁵ It is generally involved in planar cell polarity,^{336,337} axon guidance,³³⁸ neuronal differentiation,³³⁹ and stem cell maintenance.³⁴⁰ The interactions between RYK and EphB2 and RYK and EphB3 were discovered due to the similarity between the phenotype of RYK null mice and mice deficient in both EphB2 and EphB3.²⁶⁰ RYK null mice have craniofacial deformities consistent with a complete cleft of the secondary palate, they have shortened limbs, and most die on the day of birth. In transient transfection cell culture experiments, heterocomplexes of both RYK–EphB2 and RYK–EphB3 (mice RYK with human EphB2 and EphB3) coimmunoprecipitated from HEK 293T cells, and tyrosine phosphorylation of the kinase dead RYK was observed in both cases. (Of note, RYK–EphA7 heterocomplexes also coimmunoprecipitated, but no RYK phosphorylation was detected.) This cross-phosphorylation appears to be unidirectional, as coexpressing RYK with a kinase dead mutant of EphB3 did not cause detectable RYK phosphorylation. Importantly, RYK, EphB2, and EphB3 (along with EphA7) all overlap spatiotemporally in the developing palatal shelves and tongue, and hence RYK–EphB2 and RYK–EphB3 interactions appear critical for proper murine craniofacial development.

Additionally, RYK, EphB2, and EphB3 (along with ephrin-B1 and ephrin-B2) all express in the cerebellum of mice and rat brains.²⁶² In a transient transfection experiment involving COS-7 cells, rat RYK coimmunoprecipitated with EphB3, and mutational studies indicated that the leucine-rich motifs of the extracellular domains of RYK are critical for interaction, while the kinase domain is not. When GFP-labeled RYK was overexpressed in embryonic cortical brain slices, cell migration was inhibited, but overexpression of GFP-labeled RYK without the leucine-rich domains did not have this effect. These data indicate that RYK may regulate cortical cell migration through its interactions with the Eph receptors and ligands.

Key differences have been observed between human RYK and the murine analogue. In a coimmunoprecipitation study also performed in HEK 293T cells, it was found that, although human RYK interacts with both EphB2 and EphB3, neither is able to phosphorylate RYK.²⁶¹ Murine RYK coimmunoprecipitates with AF-6²⁶⁰—a cell junction associated scaffold protein which is the target of activated Ras members³⁴¹ and can associate with EphB2 and EphB3³⁴²—while human RYK does not.²⁶¹ It is possible that these differences reflect a difference in the role of RYK in humans and mice, but it is also possible that the HEK 293T cells lack a coreceptor or a post-translational modification required for the interactions.

Although the exact role of the RYK–EphB2 and RYK–EphB3 interactions remains to be determined, it has been proposed that these interactions regulate the Eph receptor signaling that becomes distorted in human craniofrontonasal syndrome.³⁴³ This is an X-linked syndrome involving mutations in the gene that encodes for ephrin-B1 that results in severe craniofacial distortions, but it affects females significantly more than males.³⁴⁴ The idea is that RYK normally modulates EphB2 and EphB3 activity by altering EphB2 and EphB3 homodimerization potential, affinity for ephrin ligand, and/or the confirmation of the EphB2 and EphB3 dimer in a way which alters phosphorylation. In craniofrontonasal syndrome, the interactions between ephrin-B1 and EphB2 and EphB3 are altered. In heterozygous females, the mosaic pattern due to X-inactivation interferes with cell–cell interaction (ephrin-B1 is membrane bound and interacts with EphB2 and EphB3 from an adjacent cell), while homozygous males have uniformly altered signaling which is less deleterious. The role of RYK in this process has not been experimentally validated, but it emphasizes the need to study the role of heterocomplexes in RTK signaling. Furthermore, as this proposed disease mechanism involves alterations of normal interactions, it is well-suited to be studied using quantitative, thermodynamic approaches.

8.1.2. EphA4 Interacts with the FGFRs. EphA4 has been found to interact with the FGFRs. The FGFRs are important for proper musculoskeletal development,^{345–347} and their misfunction is associated with numerous growth and neurological disorders.^{238,348–351} A yeast two-hybrid screen using the juxtamembrane region of FGFR3 as bait revealed the intracellular domain of EphA4 as a potential interaction partner.²⁵⁷ Coexpressing EphA4 and FGFR1, -2, -3, or -4 in HEK 293 cells showed that all four heterointeractions occur in the absence of ligand via coimmunoprecipitation. Phosphorylation studies of a mutated version of EphA4 that is kinase dead with wild type FGFR1 or vice versa showed that increasing concentrations of the wild type receptor causes increasing phosphorylation of the kinase dead receptor. This indicates that there is bidirectional cross-phosphorylation between EphA4 and the FGFRs. In neuronal cells endogenously expressing FGFR1, -2, and -3 and EphA4, interactions could only be seen via coimmunoprecipitation in the presence of ephrin-A1; moreover, addition of ephrin-A1 potentiated the FGF induced phosphorylation of the FGFR adaptor protein FRS2 α and the downstream signaling molecule MAPK. Similar results have been seen in a glioblastoma line, where addition of EphA4 lacking the kinase domain inhibited FGFR1 phosphorylation and proliferation in response to FGF2.²⁵⁸ The same is true in neural stem/progenitor cells, where expressing EphA4 lacking the intracellular domain or FRS2 α lacking phosphorylation sites decreased the mitogenic effects of FGF2 and ephrin-A1.²⁵⁹ A study involving immunoprecipitation coupled with MS to identify the pull-down partners found that FGFR3 activity is correlated with EphA4 (and several other Eph receptors) being phosphorylated.³⁵²

8.1.3. EphA2 Interacts with EGFR and ErbB2. Interactions with EphA2 and EGFR and ErbB2 have also been observed. Addition of EGF increases EphA2 levels in both a human head and neck carcinoma cell line and in a human epidermoid carcinoma cell line that overexpresses EGFR.²⁵³ The receptors colocalize on the plasma membrane by immunofluorescence, and they coimmunoprecipitate in the absence of ligand, although addition of EGF increases the observable amount of interaction. (It is unclear if EGF increases

heterointeractions through direct binding to heterocomplexes, or if EGF-induced EphA2 upregulation results in more heterocomplexes through mass action.) In the absence of EGF, EphA2 is not phosphorylated, but it is phosphorylated 1 h after adding EGF. Addition of ephrin-A1 causes EphA2, but not EGFR, to be internalized, and it inhibits EGF induced migration. Moreover, EphA2 is commonly overexpressed in colorectal cancer and correlates with poor prognosis, and increased EphA2 levels correlate with a poor response to the EGFR inhibitor cetuximab.²⁵⁴ This suggests that EphA2 may be able to restore the activity EGFR when it is inhibited by cetuximab or that the heterointeraction inhibits cetuximab binding or activity.

The interaction between EphA2 and ErbB2 has been observed in the context of breast cancer. In mice models of ErbB2 positive breast cancer, the lack of EphA2 results in decreased tumorigenesis and metastasis, and the effect appears to be due to modulation of Ras/ERK signaling.²⁵⁵ EphA2 and ErbB2 coimmunoprecipitate in the absence of ligand, both in an exogenesis overexpression model and in primary mammary tumor cells that endogenously express both proteins. Inhibiting the ErbB2 kinase decreases EphA2 phosphorylation. In humans, high EphA2 levels in ErbB2 positive breast cancer correlates with poor patient prognosis, and in an EphA2 positive human breast cancer line, the exogenous expression of EphA2 is sufficient to confer resistance to the anti-ErbB2 drug trastuzumab.²⁵⁶ This effect appears to require EphA2 phosphorylation, as expressing a kinase dead version of EphA2 does not confer resistance. Moreover, the inhibition of the basal level EphA2 phosphorylation decreases proliferation and potentiates trastuzumab. Further evidence of cross-phosphorylation is that coexpression of EphA2 and ErbB2 is sufficient for EphA2 to be phosphorylated, and the phosphorylation is blocked by PP2 inhibition of Src. Accordingly, it appears that the EphA2–ErbB2 interaction has an oncogenic effect and can result in anti-ErbB2 drug resistance.

8.2. ROR1 Interacts with Multiple RTKs

RTK-like orphan receptor 1 (ROR1) is known to interact with members of several RTK subfamilies. It is part of the ROR subfamily consisting of ROR1 and ROR2; it is important during embryonic development for proper musculoskeletal, nervous system, and organ formation; and it is involved in the Wnt signaling pathway.^{353,353,354} Relatively recent research has shown that both ROR receptors are associated with numerous cancers, which has made them attractive therapeutic targets.^{355–359} Whether or not ROR1 has intrinsic kinase activity is unclear, as mutations in the kinase domain and an apparent inability to autophosphorylate or phosphorylate substrates generally leads to ROR1 being classified as kinase dead,^{309,360–362} but several groups have reported some kinase activity.^{288,363}

The interaction between ROR1 and MET was discovered by knocking down ROR1 in a large array of cancer lines.²⁸⁹ Only two lines exhibited greater than 50% growth inhibition, and although there was no correlation between ROR1 expression and inhibition, the inhibited lines were the only two that exhibited ROR1 phosphorylation. Both lines have MET amplification, and chemical inhibition or knockdown of MET abolishes ROR1 phosphorylation. In cell lines with high MET but negligible ROR1, exogenously expressing ROR1 results in ROR1 being phosphorylated. The transphosphorylation appears to be unidirectional, as downregulating ROR1 in cells which express both ROR1 and MET does not affect MET

phosphorylation. This appears to be a direct interaction, as ROR1 coimmunoprecipitates with MET. (ROR1 also coimmunoprecipitates with EGFR and ErbB2, but neither appears to be able to transphosphorylate ROR1.)

A follow-up study by the same lab investigated the physiological role of this ROR1–MET interaction, and concluded that it diversifies MET signaling.³¹⁷ MET lacking tyrosines that serve as docking sites for adaptor proteins could phosphorylate ROR1, but a kinase dead version could not. ROR1 has eight tyrosines predicted to be phosphorylatable—three in the kinase domain and five in the proline-rich domain of the postkinase tail—and deletion studies indicate that ROR1 phosphorylation is lost once the proline-rich domain is removed, although all truncated versions except the complete removal of the intracellular domain still immunoprecipitated with MET. However, mutating all five tyrosines in the proline-rich domain to phenylalanine (ROR1_{5F}) did not completely abolish MET induced phosphorylation, and the complete loss of phosphorylation requires the three tyrosines in the kinase domain to be mutated to phenylalanines as well. It appears that Src interacts with ROR1 (as well as MET), and this interaction requires the proline-rich domain to be present and results in the three tyrosines in the kinase domain becoming phosphorylated. Intriguingly, both ROR1 and a mutant where the three tyrosines in the kinase domain are mutated to phenylalanines (ROR1_{3F}) inhibit apoptosis and increase proliferation in cells with high MET levels, but ROR1_{5F} does not. Furthermore, ROR1 induces invasiveness, but neither ROR1_{5F} nor ROR1_{3F} has this effect. The ROR1–MET interaction accordingly is able to increase the signaling capacity of MET by separately allowing for increased survival and growth or invasiveness.

ROR1 is also able to interact with EGFR.²⁸⁸ Addition of EGF results in interactions between ROR1 and EGFR, as seen via coimmunoprecipitation, in both lung adenocarcinoma cells endogenously expressing the proteins and COS-7 cells exogenously expressing them. The interaction requires the cysteine-rich domain of the ROR1 extracellular domain, as deleting it eliminates the interaction, but not the kinase or proline-rich domains. Moreover, in lung adenocarcinomas, knocking down ROR1 results in significant growth inhibition, even in cell lines resistant to anti-EGFR drugs. Exogenous expression of ROR1, but not a fully kinase dead variant, enhances growth. This seems to be due in part to an effect on ErbB3, as ROR1 knockdown decreases ErbB3 phosphorylation and EGF-induced ErbB3–EGFR interaction, as seen via coimmunoprecipitation. The ErbB3 effect does not require Src activity, ROR1 kinase activity, or the presence of the ROR1 proline-rich domain. Accordingly, it appears that ROR1 can affect growth both through an interaction with EGFR and through an alteration of ErbB3 activity, as well as a separate Src-dependent mechanism.

A later study found a direct interaction between ROR1 and ErbB3 in triple-negative breast cancer (TNBC) cells.³⁰⁹ ROR1 is amplified in many TNBC patients, and levels correlate with poor patient prognosis. CRISPR-Cas9 was used to create ROR1 knockout versions of TNBC cells. These cells had reduced proliferation, migration, and invasiveness compared to the wild type; expressing wild type, but not a fully kinase dead variant, ROR1 restored the wild type phenotype to the knockout cells. Following neuregulin-1 stimulation in TNBC cells, ROR1 and ErbB3 coimmunoprecipitated, and ErbB3 was phosphorylated on a novel tyrosine, Tyr1307, and four tyrosines known to be phosphorylated by EGFR. Chemical inhibition of EGFR

removes phosphorylation of those four sites but not Tyr1307, while ROR1 knockdown abolishes Tyr1307 phosphorylation but not that of the other four tyrosines. Knocking down EGFR does not affect the ROR1–ErbB3 interaction, but overexpression of a fully kinase dead mutant of ROR1 abolishes Tyr1307 phosphorylation. By investigating downstream signaling molecules, the authors conclude that the ROR1–ErbB3 interaction triggers a signaling cascade that modulates the Hippo-YAP pathway, and this results in tumor cell proliferation and bone metastasis.

One last known interaction partner of ROR1 is muscle-specific kinase (MuSK).³²⁰ MuSK is found in skeletal muscles and in neurons, and it is critical for formation and maintenance of neuromuscular synapses.^{364–367} Not surprisingly, dysregulation of the MuSK signaling pathway is associated with several neuromuscular disorders.^{368,369} ROR1 and MuSK coimmunoprecipitate when exogenously expressed in Cos-7 cells.³²⁰ Moreover, ROR1 is phosphorylated, but only if Dok-7 (which binds to MuSK and activates it and also coimmunoprecipitates with ROR1) is also present. Of note, ROR1 coimmunoprecipitates with kinase dead MuSK but is not phosphorylated, indicating that either MuSK directly phosphorylates ROR1 or activated MuSK binds or phosphorylates a protein which does. Similar to what was seen with the ROR1–MET interaction,³¹⁷ deletion of the proline-rich domain does not affect coimmunoprecipitation, but the deletion does abolish ROR1 phosphorylation; however, in this case, the role of Src or other adaptor proteins has not been investigated. Although the effect of this ROR1–MuSK interaction is currently unknown, it appears to be physiologically important, as ROR1 and MuSK coimmunoprecipitate in mouse myogenic cells differentiated into myotubes that endogenously express ROR1, MuSK, and Dok-7.

8.3. PTK7 Interacts with Multiple RTKs

Another RTK with known heterointeractions is protein tyrosine kinase 7 (PTK7), which is a kinase dead RTK that is the only member of its family. It is important for a wide range of cell–cell communication and migration processes such as tissue homeostasis, morphogenesis, planar cell polarity, and wound healing, as well as being involved in the Wnt signaling pathway.^{370,371} PTK7 is overexpressed or mutated in many cancers,^{372,373} making it a therapeutic target^{374,375} and potential prognostic biomarker.^{376,377}

There is evidence that PTK7 interacts with the VEGFR family, but the exact nature of the interaction is still a source of debate. The Lee lab found that inhibiting PTK7 by using either the soluble extracellular domain as a decoy (i.e., a competitive inhibitor) or siRNA knockout results in human umbilical vein endothelial cells (HUVECS)—which endogenously express PTK7, VEGFR1, and VEGFR2—having decreased capillary-like tube formation, migration, and invasiveness in response to addition of the VEGF ligand.³²⁶ This lab later showed that, in HUVECs, inhibiting PTK7 reduces VEGF induced phosphorylation of VEGFR2 and its downstream signaling molecules, but not of VEGFR1.³²⁷ Moreover, PTK7 was found to coimmunoprecipitate with VEGFR2 but not VEGFR1 in both HUVECs and HEK 293 cells which were transfected with the proteins.

However, the Dana lab found the opposite results, as in several vascular endothelial cell lines, including HUVECs, PTK7 coimmunoprecipitated with VEGFR1, but not VEGFR2 or VEGFR3, and the amount of heterocomplex increased after VEGFA addition.³²⁵ Knocking down PTK7 via siRNA decreased VEGFA induced phosphorylation of VEGFR1 and

its downstream molecules; however, the knockdown did not affect VEGFR2 phosphorylation. It is possible that this difference in heterocomplex formation can be explained by differences in cell media affecting receptor expression levels or which ligands are present. The population of heterodimers can be highly dependent on expression levels (see Figure 4D for a thermodynamic explanation), and the interactions with ligand can magnify this effect. This emphasizes the importance of a thermodynamic understanding of RTK interactions, as well as the need for careful attention to conditions which can affect expression and interactions.

PTK7 is also known to interact with ROR2. Indeed, coimmunoprecipitation using transiently transfected MCF7³²³ or HEK 293T³²⁴ cells indicates that PTK7 interacts with ROR2. Furthermore, the HEK 293T study found no interaction with ROR1, and truncation experiments showed that the ROR2 interaction requires part of the PTK7 extracellular domain to be present, but none of the intracellular domain. This interaction was also seen by coimmunoprecipitation in mouse embryonic fibroblast (MEF) cells that endogenously express both proteins, and knockdown studies in developing *Xenopus* indicate a functional interaction between PTK7 and ROR2.³²⁴ In *Xenopus* neural crest cells, fluorescently labeled PTK7 and ROR2 colocalize, and addition of ROR2 could rescue migratory deficiencies caused by PTK7 knockout, but a kinase dead mutant of ROR2 could not.³²³ Although the role of the PTK7–ROR2 interaction is not well understood, it appears to have a functional role in development and is sometimes able to rescue impaired PTK7 function.

8.4. STYK1 and EGFR Crosstalk

The term “crosstalk” has generally been used in the literature to indicate the overlap of two separate signaling pathways. In this review, we use “crosstalk” to indicate evidence of a potential interaction which has not been directly verified. There is some indirect evidence that STYK1 (serine/threonine/tyrosine kinase 1) can interact with EGFR.²⁹⁰ STYK1 differs somewhat from the other RTKs, as it almost completely lacks an extracellular domain, and so it does not bind ligand. It also lacks a membrane signal sequence, so it behaves as a cytosolic protein despite having a canonical single pass “transmembrane” domain.³⁷⁸ Overexpression of STYK1 has been found to cause tumorigenesis and metastasis in mice models.³⁷⁹ It is overexpressed in many human tumors, and it may be a good biomarker in lung cancer.³⁸⁰

In transient transfection experiments, immunofluorescent staining revealed that STYK1 expression is cytosolic, and it produces small, dot-like fluorescence and larger, aggregate-like fluorescence.²⁹⁰ The aggregates largely colocalize with early endosomes, while the dot-like fluorescence does so to a smaller degree. Immunohistochemical staining comparing cervical and breast cancer tissue to healthy tissue shows that STYK1 levels are much higher in cancerous tissue and STYK1 is mostly aggregated, indicating that a similar behavior may occur in more native biological systems. Deleting the small extracellular domain had no effect, but deleting the transmembrane domain significantly reduced aggregation and abolished endosomal colocalization. Stimulation with EGF resulted in a high degree of colocalization with EGFR, although the colocalization decreased over time. This evidence is suggestive of the interaction between STYK1 and EGFR. Since little is known about the biological role of STYK1, the interaction between STYK1 and EGFR is a promising avenue of investigation.

8.5. IGF-1R Interacts with the ErbBs

The insulin/insulin-like growth factor receptors are preformed, disulfide linked dimers (each monomer is composed of an α chain and a β chain, and the dimer is a $\beta\alpha-\alpha\beta$ complex) which are important for regulating metabolism, proliferation, and differentiation.³⁸¹ Insulin receptor (INSR) binds insulin and is primarily involved in metabolic activity. Insulin-like growth factor 1 receptor (IGF-1R) binds insulin-like growth factor 1 and 2 (IGF-1 and IGF-2) and is important for cell proliferation and differentiation.³⁸¹ IGF-1R is overexpressed in many cancers, and the role that the interaction between it and the ErbBs plays in cancer has garnered a lot of attention.^{382,383} Moreover, general overlap between the signaling axes of IGF-1R and VEGFR, MET, PDGFR, and ALK has been observed in cancer.^{384,385} Evidence of direct interactions between the insulin receptors and ErbB receptors is one of the earliest examples of interactions between different RTK subfamilies, as chimeric studies in the early 1990s indicated potential interactions.³⁸⁶ Since then, numerous examples of direct interactions between the two subfamilies have been described.

In a screen of nine different non-small cell lung carcinoma (NSCLC) cell lines, it was found that the cell lines have differing sensitivity to the anti-EGFR drug erlotinib, and that the anti-IGF-1R drug AG1024 synergistically enhances erlotinib inhibition.²⁶⁸ A resistant derivative of one of the erlotinib-sensitive cell lines was created by culturing it with the drug, and it has significantly higher IGF-1R levels than the parent line. Notably, in both the parent and resistant lines, IGF-1R and EGFR coimmunoprecipitate, but more heterocomplexes are present in the resistant line, and IFGR-1R does not coimmunoprecipitate with either ErbB2 or ErbB3. Adding erlotinib increases the amount of IGF-1R–EGFR heterocomplexes in several cell lines (see Figure 4E for a possible thermodynamic explanation), as seen via coimmunoprecipitation, although heterocomplexes were not found in one of the highly sensitive lines in the presence or absence of erlotinib. This suggests that formation of an IGF-1R–EGFR heterocomplex confers resistance to erlotinib, because more resistant cell lines have more heterocomplexes and the drug induces heterocomplex formation in resistant lines. A possible mechanism of resistance is that the heterocomplex increases expression of the antiapoptotic protein survivin: the resistant cells, but not the sensitive ones, had an increase in survivin levels upon erlotinib addition.

Furthermore, IGF-1R–EGFR heterocomplexes have been linked to resistance to the anti-EGFR and anti-ERBB2 drug afatinib.²⁶⁹ The EGFR T790M mutation is the most common mutation that causes resistance to anti-EGFR therapy in NSCLC; afatinib binds tightly to EGFR T790M, but does not improve patient survival. An afatinib resistant NSCLC cell line which has the EGFR T790M mutation, as well as the common EGFR L858R mutation, was created. In both the parent and resistant lines, IGF-1R and EGFR coimmunoprecipitate and afatinib increases IGF-1R phosphorylation in a dose dependent manner. However, the resistant line has higher basal IGF-1R phosphorylation levels than the parent line. Moreover, although linsitinib inhibition of IGF-1R does not affect the parent line, it reduces growth of the resistant line, and shRNA knockdown of IGF-1R restores afatinib sensitivity in the resistant line. This suggests that the afatinib resistant cells are dependent on IGF-1R activity, possibly due to the IGF-1R–EGFR heterointeraction enabling EGFR activity.

Interactions between IGF-1R and ErbB2 have also been observed. In several breast cancer lines, downregulating IGF-1R decreases ErbB2 phosphorylation, and IGF-1R and ErbB2 coimmunoprecipitate and colocalize via immunofluorescence.²⁹⁹ In a breast cancer line which expresses both receptors but not their ligands, IGF-1R and ErbB2 only faintly coimmunoprecipitate, but addition of either IGF-1 or NDF substantially increases the amount of heterocomplexes. Complementary results were seen in a comparison between a breast cancer cell line that is sensitive to the anti-ERBB2 drug trastuzumab and the same line but with induced drug resistance.³⁰⁰ IGF-1R and ErbB2 coimmunoprecipitate in the resistant line, but not in the parent line. The addition of IGF-1 causes a small decrease in ErbB2 phosphorylation in the parent cells, but a significant increase in the resistant line. Interestingly, IGF-1 did not detectably affect heterointeraction in the resistant line, but it appears to cause a small degree of heterointeraction in the parent line. The IGF-1R inhibitor I-OMe-AG538 did not affect ErbB2 phosphorylation in the parent line, but in the resistant line, it did decrease ErbB2 phosphorylation and restore trastuzumab sensitivity. It is possible that IGF-1 induces heterocomplex formation by binding to both IGF-1R and ErbB2, but given that ErbB2 has no known activating ligands, it seems more probably that IGF-1R activation promotes the heterointeraction. This is similar to the manner in which ErbB2 heterodimerizes with other ErbBs (see **RTK Heterointeractions within the Same Subfamily** for more details).

Finally, interactions have also been seen between IGF-1R and ErbB3. In a breast cancer line that is sensitive to trastuzumab and the same line but with induced drug resistance, IGF-1R and ErbB3 coimmunoprecipitate and colocalize via fluorescence in the resistant line, but not in the parent line.³⁰¹ (Although trastuzumab is specific to ErbB2, ErbB3 is kinase dead and is largely dependent on heterointeractions, especially with ErbB2, for its activity.^{174,176}) ErbB2 and ErbB3 coimmunoprecipitated in both the parent and resistant lines, but significantly more in the resistant line, and shRNA knockdown of either IGF-1R or ErbB3 sensitized the resistant cells to trastuzumab. Loose evidence that heterotrimers form was seen in the fact that immunodepletion of IGF-1R inhibits ErbB2–ErbB3 interactions, and complexes corresponding by weight to a trimer were seen on a native PAGE. Additional evidence of interaction between IGF-1R and ErbB3 has been seen in other cancers. A comparison of an ovarian cancer cell line with resistance to trastuzumab to the nonresistant parent line found that the resistant line had increased proliferation but decreased EGFR and ErbB2 expression.³⁰⁷ Instead, IGF-1R and ErbB3 were significantly upregulated, and inhibiting either inhibited cell proliferation. Furthermore, in pancreatic cancer, IGF-1R, ErbB3, and their ligands are often overexpressed, and in cell lines, the bispecific antibody inhibitor of IGF-1R and ErbB3 istriratumab sensitizes the cells to traditional chemotherapeutics.³⁰⁸ Thus, IGF-1R interacts with EGFR, ErbB2, and ErbB3 under a wide range of conditions, and these heterocomplexes appear to confer resistance to several anti-ErbB cancer therapeutics.

8.6. PDGFRs Interact with EGFR and VEGFR

PDGFR α and PDGFR β have been found to interact with members of several different RTK families, including EGFR, VEGFR2, and FGFR1 (see **FGFRs Interact with Multiple RTKs in Cancer**). These receptors are two members of the PDGF subfamily, and they are important for the development of

mesenchymal cells in numerous organs during development, as well as wound healing in adults.³⁸⁷ Overexpression and mutations of both receptors and the PDGF ligands are associated with numerous cancers, and they have accordingly received a lot of interest as clinical targets.^{388–391}

Both PDGFR α and EGFR are sometimes overexpressed in glioblastomas (GBM). In patient-derived GBM sphere lines expressing both receptors, EGFR inhibition with gefitinib causes dephosphorylation of PDGFR α , and this is true even in lines with low EGFR expression.²⁸⁰ A later study by the same lab determined that surgically resected primary GBM tumor sphere lines have a higher degree of heterogeneity for EGFR and PDGFR α expression than the commercially available tumor lines, and this also occurs in the absence of receptor amplification.²⁷⁹ In these primary lines, EGF stimulation results in increased PDGFR α phosphorylation, and interaction with EGFR was seen via coimmunoprecipitation and PLA. Although the interaction could be seen in the absence of EGF, its addition increases heterointeraction. Moreover, EGF is required for PDGFR α phosphorylation in the heterocomplex; this phosphorylation is blocked by gefitinib. This heterointeraction appears to affect downstream signaling, as the heterogeneous receptor expression correlates with the phosphorylation of Akt and ERK and with cell proliferation. Increased PDGFR α correlates with decreased efficacy of EGFR inhibition. The PDGFR α –EGFR interaction appears to enable activated EGFR to phosphorylate PDGFR α and influences the signaling pathways in GBM.

PDGFR β is also known to interact with EGFR. In a fibroblast cell line which endogenously expresses both PDGFR β and EGFR, addition of EGF or EGFR overexpression increases PDGFR β phosphorylation, and the two receptors coimmunoprecipitate in the presence and absence of ligand.²⁸¹ Furthermore, PDGFR β phosphorylation increases with increasing EGFR activity, indicating that PDGFR β is phosphorylated by activated EGFR—either directly or through a larger complex—or EGFR upregulates PDGFR β or its ligand. Similar results were seen in rat aortic smooth muscle cells (VSMCs) which endogenously express both receptors.²⁸² EGFR phosphorylation significantly increases in response to PDGF-BB, and the receptors coimmunoprecipitate in the presence and absence of ligand. Notably, the PDGFR kinase inhibitor AG1295 did not prevent PDGF-BB induced activation of EGFR, but the EGFR inhibitor AG1478 did; moreover, inhibiting Src kinases with PP2 both decreases PDGF-BB induced EGFR phosphorylation and heterointeractions, indicating a role of the Src kinases in formation of the heterospecies. This interaction also appears to be important for ERK activation through metalloproteases, as in MEFs, PDGF-BB addition results in increased ERK and EGFR phosphorylation, but both can be blocked by chemical inhibition of metalloproteases or EGFR.²⁸³ These studies indicate that the PDGFR β –EGFR complex is functioning as part of a larger complex, but further studies are needed to confirm and clarify these interactions.

Whereas the PDGFR α – and PDGFR β –EGFR interactions have an activating effect, the PDGFR β interaction with VEGFR2 has an inhibitory effect.^{321,322} In both mice and chicken embryo models, separately adding either VEGFA or PDGF-BB promotes neovascularization, but adding both ligands together abolishes this effect; the same phenomenon was seen in primary human VSMCs with regard to proliferation and migration. This inhibitory effect was not seen in cell lines which only express VEGFR2 or PDGFR β , and inhibiting VEGFR2 but not

VEGFR1 eliminates it as well. Addition of VEGFA suppresses PDGF-BB induced phosphorylation of PDGFR β in VSMCs—although phosphorylation could be recovered by titrating in more PDGF-BB. Transfected cells that only endogenously express PDGFR β with VEGFR2 cause the same inhibition, although only minimally if a truncated version of VEGFR2 lacking the kinase domain is used. In the VSMCs, PDGFR β and VEGFR2 coimmunoprecipitate when both VEGFA and PDGF-BB are present—but not if only one or neither is—but this was not seen with PDGFR α . The same results were seen by transfecting HEK 293 cells with both receptors and using PLA, but heterointeractions were not observed if VEGFR2 lacking the kinase domain or kinase dead VEGFR2 was used. Comparable results were seen in human aorta-derived VSMCs, as transfection with heme oxygenase-1 (HO-1) increases VEGFR2 and VEGFA expression and decreases PDGF-BB induced migration and PDGFR β phosphorylation. After addition of both VEGFA and PDGF-BB, the receptors coimmunoprecipitate, and knockdown of VEGFR2 or VEGFA reduces the inhibitory effect of HO-1. The exact mechanism of this inhibition is unknown, but it is clear that it requires activated receptors, which is suggestive of the heterocomplex recruiting a phosphatase that deactivates one or both receptors. However, it is also possible that the heterocomplex can only form when both PDGFR β and VEGFR2 are phosphorylated, and the heterocomplex blocks binding of key adaptor proteins or results in nonproductive phosphorylation. Further experiments are required to elucidate the details of this interaction.

Although to the best of our knowledge no direct interactions between wild type PDGFR α and VEGFR2 have been reported, a chimeric fusion between a large portion of the VEGFR2 extracellular domain and the PDGFR α transmembrane and intracellular domains have been found in a surgical glioblastoma sample.³⁹² Transfection of NIH 3T3 cells with the fusion causes a tumorigenic phenotype. The fused receptor is constitutively active in the absence of ligand, and phosphorylation of downstream molecules is similar to that of PDGF-AA activated wild type PDGFR α . Immunoprecipitation studies indicate that the fused product can form homodimers, as well as heterodimers with both VEGFR2 and PDGFR α , and the heterodimerizing partners are phosphorylated.

8.7. RET and VEGFR2 Interaction

An interaction between RET and VEGFR2 has been observed. RET (rearranged during transfection) is important for neuronal and tissue development,^{393,394} and gain-of-function mutations are common in many cancers, leading to large efforts to develop inhibitors.³⁹⁵ In a ureteric bud cell line which endogenously expresses both VEGFR2 and RET, VEGFR2 phosphorylation increases in response to addition of the RET ligand glial cell-derived neurotrophic factor (GDNF), and RET phosphorylation increases in response to VEGFA.³³¹ A similar interaction was seen in response to ligand with respect to both proliferation and branching morphogenesis, and RET and VEGFR2 coimmunoprecipitate in the presence and absence of VEGFA. Furthermore, GDNF is necessary for VEGFA to increase vascular profusion in ischemic skeletal muscle,³³² and the efficacy of the anticancer drug sorafenib has been linked to its dual inhibition of RET and VEGFR2.³⁹⁶

8.8. Trks Interact with Multiple RTKs

Another subfamily of RTKs with multiple known heterointeractions is the tropomyosin-related kinases (Trks). This subfamily of RTKs is important for the development of the

primary and periphery nervous system; the survival, maintenance, and differentiation of neurons; and the transduction of sensory signals.^{397–399} They primarily bind neurotrophins—which are NGF, BDNF, neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) in mammals—but can also be activated by a wide range of other factors.⁴⁰⁰ Trk overexpression and mutations are associated with a wide variety of cancers, making them an attractive therapeutic target,^{401,402} as well as a wide range of neurological disorders,⁴⁰³ including Alzheimer's,^{404,405} depression,⁴⁰⁶ and schizophrenia.⁴⁰⁷

TrkA was found to coimmunoprecipitate with ErbB2 in a prostate cancer cell line after addition of NGF.³⁰³ Addition of NGF also increases ErbB2 phosphorylation, and dual inhibition with the anti-Trk drug lestaurtinib (CEP-701) and the anti-ErbB2 drug pertuzumab inhibits proliferation significantly more than either drug individually. There is also evidence of crosstalk between TrkA and EGFR.²⁹¹ In human monocytes, EGF increases NGF expression and phosphorylation of TrkA, and NGF increases the phosphorylation of EGFR. Moreover, chemical inhibition of either EGFR or TrkA decreases EGF and NGF induced activation of the cognate receptor. These results are indicative of bidirectional activation between TrkA and EGFR and ErbB2, although the evidence is currently tenuous.

TrkB has also been found to directly interact with ErbB2.³⁰⁴ Tumor samples of breast cancer brain metastasis (BBM) have much higher TrkB and ErbB2 phosphorylation than primary breast cancer samples, and immunofluorescence and cryo-electron microscopy with antibody labeling indicated colocalization of TrkB and ErbB2. Addition of BDNF increases ErbB2 phosphorylation and TrkB–ErbB2 colocalization. Of note, the brain microenvironment has high BDNF levels, and flow cytometry with antibodies showed that about half of the BBM cells have both TrkB and ErbB2. Accordingly, these interactions are likely to occur in the native biological system. In the presence and absence of both BDNF and NGF, TrkB and ErbB2 coimmunoprecipitate from BBM, and in silico modeling of the kinases of the receptors indicates that physical interaction between the two is plausible. Inhibiting TrkB with cyclotraxin and ErbB2 with lapatinib decreases the interaction between the two receptors and inhibits proliferation to a significantly greater degree than either drug individually. This suggests that the TrkB–ErbB2 interaction might increase the activity of ErbB2, and that the interaction could be oncogenic.

Although to the best of our knowledge there is no direct evidence of an interaction between TrkB and EGFR, there is significant evidence of a potential interaction. In NSCLC, TrkB expression correlates with metastasis and poor patient prognosis, and in an NSCLC cell line, addition of EGF causes an increase in TrkB phosphorylation.²⁹² Similar crosstalk was seen in a human ovarian cancer cell line, where addition of EGF increases TrkB phosphorylation, and addition of BDNF increases EGFR phosphorylation.²⁹³ Chemical inhibition of EGFR with PD153035 or TrkB with k25a inhibits both receptors' response to EGF and BDNF, as well as ligand induced Akt phosphorylation and proliferation. There is evidence that TrkB leads to anti-EGFR drug resistance in colorectal cancer. Indeed, in a colorectal cancer cell line, addition of BDNF blocks the antiproliferation effect of the anti-EGFR antibody cetuximab, and addition of k25a potentiates the effect of cetuximab.²⁹⁴ Under noncancerous conditions, primary cortical precursor cells harvested from mice brains have a low TrkB response to BDNF and NT3, but high phosphorylation in

response to EGF. The phosphorylation was not blocked by BDNF neutralizing antibodies, indicating that EGF was not simply upregulating BDNF.²⁹⁵ During development, high levels of TrkB are seen in the cerebral cortex before BDNF levels reach the levels seen in adult brains, and TrkB and EGFR are coexpressed in neuronal cells in the forebrain. It is clear that the interaction between TrkB and EGFR is important for nervous system development and cancer therapy. It remains to be determined whether or not there exists a direct, physical interaction, and quantitative studies of the interaction can help explain this complicated biology.

Crosstalk between the Trks and RET has also been seen. In neuroblastomas, the RET ligand GDNF increases TrkA expression.³²⁸ Furthermore, in mature sympathetic neurons, NGF increases RET phosphorylation, and this effect is blocked by inhibiting TrkA with k25a.³²⁹ An analogous crosstalk between TrkB and RET has also been observed. In neuroblastomas, BDNF increases RET phosphorylation and siRNA knockdown of TrkB blocks RA induced RET phosphorylation.³³⁰

8.9. FGFRs Interact with Multiple RTKs in Cancer

A large number of heterointeractions with the FGFRs have been identified as being involved with cancer. In general, FGFR mutations which cause gene amplification, increased activity, oncogenic fusions, increased ligand expression, and aberrant signaling activity have all been observed in cancers.^{57,408} This has led to an intense effort to develop FGFR based cancer treatments, but the clinical results have been mixed.⁴⁰⁹

One difficulty in targeting the FGFRs stems from the fact that even when treatment is initially effective, drug resistance often develops, and there is evidence that this resistance is often caused by the activity of other RTKs. An RTK phosphorylation assay of *FGFR1*-amplified lung cancer cell lines with FGFR inhibition resistance found that different cell lines have high phosphorylation of different RTKs.²⁶⁶ One cell line has high PDGFR α phosphorylation, and several have high phosphorylation of EGFR, ErbB2, ErbB3, and MET. Treating the cells with an FGFR inhibitor and an inhibitor for the coactivated RTK results in significantly increased apoptosis relative to either inhibitor individually, and only both drugs in combination cause suppressed phosphorylation of the FGFR1 signal mediating adaptor protein FRS2.

The mechanism of this resistance was directly probed by investigating the effects of an FGFR inhibitor on six *FGFR1*-amplified lung cancer cell lines, and in all cases, the FGFR inhibitor by itself only had a minimal effect.³⁰⁵ HCC95 cells have high ErbB3 activity, and coinhibition of both FGFR and ErbB3 causes long-term inhibition of ERK and AKT phosphorylation. Other cell lines require FGFR and IGF-1R or FGFR and MET inhibition to significantly inhibit ERK phosphorylation. A similar effect involving FGFR1 and PDGFR α was observed in NCI-H170 cells, as inhibiting both causes long-term suppression of ERK phosphorylation, and, intriguingly, inhibition of either FGFR1 or PDGFR α increases phosphorylation of the other. Unlike the indirect nature of the above interactions, there is evidence that the FGFR1–PDGFR α interaction is direct, as FGFR1 and PDGFR α coimmunoprecipitate from these cells. Of note, FGFR1 and PDGFR α were previously found to interact in a noncancerous, human endothelial cell line, where it was noted that PDGF-BB inhibits the effects of FGF2, and both ligands being present appears to increase heterointeraction.³¹⁰

Moreover, RTK phosphorylation crosstalk has also been seen with FGFR2 using an RTK phosphorylation assay of *FGFR2*-amplified cancers.³⁰⁶ In all cases, a significant decrease in phosphorylation of ErbB3 and FGFR3 was seen in response to FGFR2 inhibition. Notably, the authors found that FGFR2 overexpression activates PI3K in a manner which requires ErbB3 transphosphorylation, presumably by FGFR2. Under the right conditions, it appears that many RTKs can rescue impaired FGFR1 or FGFR2 function.

This ability of other RTKs to compensate for an inhibited FGFR has also been observed in the case of FGFR3. A high throughput parallel siRNA screen was performed against all known protein kinases and phosphatases in 11 *FGFR*-mutated cancer lines.²⁶⁷ Although the *FGFR1* and *FGFR2* cell lines were inhibited by *FGFR* siRNA, the *FGFR3* lines did not exhibit any negative growth effects. In all the *FGFR3*-mutated cell lines, of all the tested siRNAs, knockdown of EGFR resulted in the greatest sensitivity to the *FGFR* inhibitor PD173074. The anti-EGFR drug gefitinib blocked the ERK phosphorylation restoration that was seen when PD173074 was used alone. In another study, an *FGFR3* mutant cancer line was made resistant to the *FGFR* inhibitor BGJ398 or the general kinase inhibitor ponatinib by incubating the cells with a stepwise increasing concentration of the drug.²⁹⁸ Relative to the parent cells, the resistant ones have a more metastatic phenotype and increased ErbB2 and ErbB3 phosphorylation (detected by pan RTK phosphorylation assays) and expression. Additionally, the parent cells are insensitive to the ErbB inhibitors AZD8931 and lapatinib, while the resistant cells are sensitive to both. The anti-*FGFR* drug resistance and metastatic phenotype are reduced by inhibiting either ErbB2 or ErbB3 by shRNA and are abolished by growing the cells in the absence of the drug for two to four weeks. Although the exact mechanism remains to be determined, it is clear that the ErbBs are able to compensate for inhibited *FGFR3* function in some cancers.

It has also been found that *FGFRs* are the cause of anti-RTK drug resistance in some cases. NSCLC often overexpress *FGFRs* and their ligands, and in an NSCLC cell line, *FGFR2* and *FGFR3* mRNA levels increased after treatment with the EGFR inhibitor gefitinib.⁴¹⁰ A similar study found that treatment of a panel of NSCLC cell lines with gefitinib to create resistant lines results in several cell lines having increased mRNA levels of *FGFR1*.⁴¹¹ In both these cells and in the *FGFR2* and *FGFR3* overexpressing cells, ERK phosphorylation and anchorage-independent growth could only be reduced using an *FGFR* inhibitor. A different study using a chronic myeloid leukemia (CML) cell line found that the *FGFR* ligand FGF2 confers resistance to the general tyrosine kinase inhibitor imatinib.⁴¹² This resistance is abolished with anti-*FGFR* inhibitors, and siRNA knockdowns indicates that it is specifically *FGFR3* which is responsible for the drug resistance. In a comparison between a gastrointestinal stromal tumor cell line normally sensitive to imatinib due to mutated KIT (a member of the PDGF family) and an insensitive variant, an siRNA screen revealed knockdown of *FGFR3* to have the largest inhibitory effect.³¹¹ Inhibiting either KIT or *FGFR3* reduces phosphorylation of the other, and KIT and *FGFR3* coimmunoprecipitate when both are ectopically expressed in HEK 293 cells. Addition of FGF2 reduces the efficacy of imatinib in the sensitive line, but not if KIT or *FGFR3* is knocked down, and inhibiting *FGFR3* restores imatinib sensitivity in the resistant line.

Most of the above studies do not directly investigate heterointeractions. In some cases, coimmunoprecipitation did

not show any interactions, but it is important to note that, especially with membrane proteins, weak interactions are often missed due to the washes and non-native conditions. Moreover, other studies have found heterointeractions between RTKs in cancer cells using coimmunoprecipitation only after the cells have become fully drug resistant.^{272,301} This highlights the need for quantitative studies that directly probe *FGFR* heterointeractions.

8.10. MET Interacts with Multiple RTKs in Cancer

Another RTK that has many heterointeractions associated with cancer is MET (for the interaction between MET and ROR1, see *ROR1 Interacts with Multiple RTKs*). MET (named because its gene was discovered after a human sarcoma line was further transformed by *N*-methyl-*N'*-nitroso-guanidine) plays important roles in epithelial cell proliferation and migration, embryogenesis, and angiogenesis.^{413–417} It has long been known that increased levels of MET and its ligand hepatocyte growth factor (HGF) are associated with many cancers.^{418,419} In more recent years, there has been interest in targeting MET in cancer therapy,^{61,420–423} with a particularly large clinical effort to treat NSCLC using MET and HGF inhibitors.⁴²⁴ However, these drugs have only been moderately successful.

Both EGFR and MET are often overexpressed in NSCLC, and in cell culture, adding EGF increases MET phosphorylation, while inhibiting EGFR decreases it.^{270,271} Furthermore, there is evidence that MET can confer anti-EGFR drug resistance to NSCLCs. A gefitinib resistant NSCLC cell line was created by prolonged incubation with the drug, and unlike in the parent cells, both EGFR and ErbB3 are phosphorylated in this cell line in the presence of gefitinib.²⁷² MET is amplified but not mutated, and although the MET inhibitor PHA-665752 has little effect on its own, in combination with gefitinib, it causes a significant reduction in cell proliferation and ErbB3 phosphorylation and an increase in apoptosis. Overexpression of MET is sufficient to confer resistance to gefitinib in the parent cells, and in the resistant but not the parent cells, MET and ErbB3 coimmunoprecipitate (see Figure 4D for how a change in expression levels can increase heterodimers). Across multiple NSCLC lines, combinations of MET, EGFR, ErbB2, ErbB3, and RET were found to be highly phosphorylated.²⁷³ PHA-665752 inhibition of MET reduces phosphorylation of all these receptors, increases apoptosis, and decreases cell proliferation, but gefitinib, lapatinib (anti-EGFR and -ERBB2), and vandetanib (anti-EGFR, -VEGFR2, and -RET) all have little effect. This indicates that MET causes the phosphorylation of the other receptors, and in these cell lines, EGFR, ErbB2, ErbB3, and RET all coimmunoprecipitate with MET. In these cancer cells, the evidence indicates that MET is able to directly phosphorylate inhibited ErbBs (and RET) to rescue inhibited function.

Similar MET heterointeractions occur in many other cancers, and the interactions often appear to cause MET activation. It has been found that EGFR and MET are often co-overexpressed in laryngeal cancer, and MET levels can serve as a predictor of patient outcome.⁴²⁵ In healthy human hepatocyte cell lines, increased levels of the EGFR ligand TGF α lead to increased MET phosphorylation, and in hepatoma cell lines, EGFR is highly expressed and endogenous levels of TGF α are sufficient to cause high MET phosphorylation in the absence of HGF; additionally, EGFR inhibition decreases MET phosphorylation.²⁷⁴ The reverse is not true, as HGF does not lead to the

phosphorylation of EGFR, indicating that the effect of this heterointeraction is at least somewhat unidirectional. Notably, in the cancer cell lines, EGFR and MET coimmunoprecipitate regardless of which ligands are present, but they do not coimmunoprecipitate in normal cells. It is difficult to say whether these differences are caused by differences in receptor expression, different ligand expression, or the presence of an unknown adaptor protein, but quantitative studies of protein–protein interactions could help create a model which can explain them.

Analogous results were seen in anaplastic thyroid carcinoma cells, as inhibiting EGFR leads to a decrease in MET phosphorylation.²⁷⁵ In canine osteosarcoma cells, MET and EGFR coimmunoprecipitate and TGF α reduces MET inhibition by crizotinib and increases MET phosphorylation.²⁷⁶ Furthermore, both MET and ErbB2 are expressed in many breast cancers, and increased MET and HGF levels in tumors correlate with resistance to the anti-ErbB2 drug trastuzumab.³⁰² Breast cancer cell lines that express both receptors exhibit increased proliferation and trastuzumab resistance in response to HGF, and MET inhibition increased trastuzumab sensitivity. In a pancreatic cancer cell line, crosstalk between MET and IGF-1R was observed, as addition of HGF and IGF-1 synergistically increases cell migration and invasiveness, and downregulating MET abolishes the effects of IGF-1 addition.³¹² A comparable result was seen in a prostate cancer cell line, as IGF-1 leads to MET phosphorylation, although slower than that of IGF-1R phosphorylation, and IGF-1R or MET knockdown abolishes IGF-1 induced MET activation.³¹³ These results demonstrate the complicated and widespread interactions that occur between MET and other RTKs, as MET can activate and be activated by numerous RTKs, and these interactions can be important for drug resistance and disease prognosis.

In glioblastomas, EGFR amplification or mutations are seen in 40–50% of cases, and the most common mutated form of EGFR is EGFRvIII, which cannot bind ligand but is constitutively active. By sorting GBM cells by EGFRvIII expression level using fluorescence-activated cell sorting (FACS) and then using MS to determine phosphorylation sites, it was found that MET phosphorylation significantly increases with increasing EGFRvIII levels.²⁷⁷ Inhibiting EGFR results in decreased MET phosphorylation, and inhibiting MET reduces EGFRvIII induced drug resistance. Inducing increased EGFRvIII expression with tetracycline in an engineered GBM cell line increases MET phosphorylation, but addition of EGF decreases it and restores sensitivity to Temozolomide without affecting EGFRvIII.²⁷⁸ In the absence of EGF, MET and EGFRvIII coimmunoprecipitate, but this interaction is lost upon EGF addition if wild type EGFR is also present. The authors propose that there is a conformational change in the EGFRvIII–EGFRwt heterodimers which prevents MET interaction. This may indicate that anti-EGFR treatment could increase MET activity, as the treatment could decrease EGFRvIII–EGFRwt heterodimers, and this in turn increases MET–EGFRvIII heterodimers (see Figure 4E for a possible thermodynamic explanation).

Intriguingly, the oncogenic effect of MET appears to be regulated by VEGFR2 in some cases.³¹⁸ In an array of mouse astrocytoma cell lines with varying amounts of VEGF, increasing VEGF levels correlates with decreasing invasiveness and MET phosphorylation, despite the fact that MET levels remain constant. Inhibiting VEGFR2, but not VEGFR1, abolishes this effect, and the addition of HGF does not affect VEGFR2

phosphorylation. In human GBM samples, MET and VEGFR2 coimmunoprecipitate, and the interaction can also be seen by PLA. This interaction requires the kinase domain of VEGFR2, as cells transfected with a truncated version of VEGFR2 lacking the intracellular domain do not exhibit an interaction with MET by coimmunoprecipitation or PLA. Neither VEGF nor HGF is required for the formation of a heterocomplex, but HGF is needed to observe MET phosphorylation within the heterocomplex. Addition of the general tyrosine phosphate inhibitor sodium orthovanadate removes the VEGF effect on MET phosphorylation, as does inhibiting the nonreceptor protein tyrosine phosphatase 1B (PTP1B). Coimmunoprecipitation studies indicate that VEGF enables recruitment of PTP1B to the MET–VEGFR2 complex, and then PTP1B dephosphorylates MET, regulating its oncogenic activity.

8.11. Ron Interacts with Multiple RTKs

The other RTK in the same subfamily as MET is Recepteur d'Origine Nantais (Ron). It shares a high degree of structural similarity with MET, and it is also known to have multiple heterointeractions. Ron is canonically activated by binding to its ligand, macrophage stimulating protein (MSP, also known as hepatocyte growth factor-like or HGFL), and it is important for embryonic and physiological development.⁴¹⁷ Overexpression and mutations of Ron are associated with a wide variety of cancers, which has resulted in a growing interest in using it as a therapeutic target.^{426–428} Ron and MET can heterodimerize and transphosphorylate each other, triggering downstream signaling cascades.^{208,209}

Ron has been found to interact with EGFR in a variety of systems. Using the mouse fibroblast cell line NIH 3T3, a phenotypic analysis done by overexpressing Ron and modulating the activity of endogenous EGFR indicated a functional link between Ron and EGFR, and these two receptors coimmunoprecipitate in the presence and absence of MSP and EGF.²⁸⁴ This Ron–EGFR link has also been found in dog osteosarcoma cells²⁷⁶ and head and neck squamous cell carcinomas (HNSCCs).²⁸⁵ The majority of HNSCC tumor samples tested overexpress Ron, and increasing Ron levels correlates with increasing EGFR. In HNSCC cell lines, MSP stimulation causes increased EGFR phosphorylation. EGFR and Ron coimmunoprecipitate in the presence of MSP, but only weakly in its absence. This interaction has also been seen in human bladder cancer cell lines, where inhibiting either EGFR or Ron decreases the phosphorylation of the other, and they coimmunoprecipitate.²⁸⁶ As determined by immunofluorescence, serum starvation results in the majority of the plasma membrane Ron being translocated to the nucleus.²⁸⁷ The nuclear fraction is not phosphorylated, but it does colocalize with EGFR, and siRNA knockdown of Ron decreases nuclear EGFR expression. A ChIP-chip analysis indicated that Ron and EGFR are both associating with stress response pathways, suggesting that, under cancerous conditions, the Ron–EGFR axis is acting as a transcription factor to promote survival. In addition to enhancing signaling through bidirectional cross-phosphorylation, the Ron–EGFR interactions appear to affect signaling by altering the spatial distribution of EGFR, which leads to altered gene expression.

A link between Ron and IGF-1R was first seen when a screen of childhood sarcoma lines revealed variable sensitivity to the IGF-1R inhibitor BMS-536924.³¹⁴ Experiments using an siRNA library against tyrosine kinases demonstrated that knocking down Ron results in the greatest sensitivity to BMS-536924 in resistant cell lines. This connection has also been seen in

pancreatic cancer cell lines, where Ron and IGF-1R were seen to interact in the presence and absence of MSP and IGF-1 via coimmunoprecipitation and PLA.³¹⁵ The Ron inhibitor BMS-777607 decreases heterointeraction. MSP does not result in IGF-1R phosphorylation, but IGF-1 does cause Ron phosphorylation. Altogether, these results suggest that the Ron–IGF-1R interaction is able to contribute to the pathology and potentially the drug resistance of several cancers.

Additionally, Ron has also been found to interact with PDGFR β .³¹⁹ In human mesangial cells (HMCs), Ron phosphorylation increases with addition of PDGF-BB, although the phosphorylation kinetics observed by western blotting are different than that of PDGFR β . Inhibiting PDGFR β with imatinib reduces Ron phosphorylation, and Ron and PDGFR β coimmunoprecipitate and colocalize on the plasma membrane via immunocytochemistry. Similar to the Ron–EGFR interaction seen in bladder cancer, Ron can localize to the nucleus, but unlike that case, it is the phosphorylated Ron which primarily does so. Moreover, the Ron–PDGFR β interaction is at least partly cell dependent, as in both human epidermal keratinocytes and peripheral blood-derived adherent monocytes expressing both PDGFR β and Ron, addition of PDGF-BB does not result in increased Ron phosphorylation.

8.12. AXL Interacts with Multiple RTKs

One final RTK with known cross-subfamily heterointeractions is AXL. AXL is a member of the TAM subfamily (named for the three members which compose it, TYRO3, AXL, and MER), and unlike most RTKs, it is not primarily involved in development, but rather is involved in maintaining adult tissue.⁴²⁹ It is important for processes such as the nervous system maintenance,⁴³⁰ inhibition of the innate immune system,^{431–433} and phagocytosis of apoptotic cells.^{434,435} Unsurprisingly, dysfunction of AXL is linked to many diseases, including cancer,^{436,437} autoimmune diseases,^{438–440} and infectious diseases.^{441,442}

An interaction between AXL and EGFR appears to play a role in drug resistance in several cancers. AXL expression was identified as a strong predictor of ErbB inhibitor resistance in cancer by applying a machine learning algorithm to publicly available cancer databases.²⁶³ The interaction was investigated using TNBC cells which endogenously express both AXL and EGFR. Inhibiting EGFR with erlotinib does not inhibit cell viability, but addition of the AXL inhibitor R428 does. Both MET and AXL have increased phosphorylation upon EGF addition, although the AXL ligand Gas6 does not activate EGFR, and siRNA silencing of AXL decreases EGF, TGF α , and HGF induced phosphorylation of downstream signaling molecules. A cross-linking coimmunoprecipitation assay indicated that EGFR, ErbB2, ErbB4, MET, and PDGFR β all interact with AXL.

This AXL–EGFR interaction has been seen in several other cancers and drug resistances. In a GBM cell line, EGF increases AXL phosphorylation on the same time scale as EGFR phosphorylation increases, and inhibiting AXL with BGB324 does not affect EGF induced phosphorylation, but inhibiting EGFR with gefitinib does.²⁶⁴ AXL and EGFR interact in the absence of ligand as seen by both coimmunoprecipitation and BiFC, and inhibiting AXL blocks EGF induced invasiveness. Across a range of squamous cell carcinoma cell lines, increasing AXL expression correlates with increasing resistance to the PI3K α inhibitor BYL719.²⁶⁵ The resistant cells exhibit more EGFR–AXL interaction relative to the sensitive ones as seen by

coimmunoprecipitation and PLA. Moreover, overexpressing AXL induces resistance in sensitive lines, but a kinase dead version of AXL does not, and inhibiting EGFR restores sensitivity regardless of AXL levels. This indicates that there exists an interaction between AXL and EGFR which confers resistance, and it requires the kinase domain of AXL to be functional. Specifically, inhibiting AXL decreases phosphorylation of the EGFR tyrosine 1173, which is important for the PLC γ /PKC signaling axis. Several studies have indicated that AXL expression can cause resistance to multiple anti-EGFR drugs in NSCLC cell lines, and AXL inhibition can restore drug sensitivity.^{443–445}

In addition to the AXL–EGFR interaction, an AXL interaction with ErbB2 has also been implicated in cancer drug resistance and poor patient prognosis. A study investigating the effects of increasing ErbB2 phosphorylation found that the phosphorylation of AXL increases with increasing ErbB2 phosphorylation.⁴⁴⁶ In breast cancer cells that express ErbB2, lapatinib resistant cell lines have increased AXL expression compared to sensitive lines, and inhibiting or silencing AXL restores sensitivity.²⁹⁶ Furthermore, AXL expression correlates with poor patient outcome in ErbB2 positive breast cancer. An AXL–ErbB2 interaction has been seen in both breast cancer cells (which exogenously express AXL and endogenously express ErbB2) and tumor samples (which endogenously express both receptors) via coimmunoprecipitation and PLA.²⁹⁷ Intriguingly, the AXL–ErbB2 interaction increases the concentration of AXL at the cell surface, possibly due to a decrease in endosomal degradation, an increase in recycling to the cell surface, or an increase in complex stability. This same effect has been observed with the EGFR–ErbB2 interaction, which causes an increase in the concentration of EGFR on the cell surface.¹⁷⁷ ErbB2 is able to phosphorylate kinase dead AXL, and this is inhibited by lapatinib, but AXL is unable to phosphorylate ErbB2. Coincubation of inhibitors for both AXL and ErbB2 causes increased inhibition of cell invasiveness, and a mice model indicated that AXL increases metastasis and intravasation, but tumors could still grow without it.

There is also direct evidence of an interaction between AXL and MET, as in hypothalamic gonadotropin-releasing hormone neurons, AXL and MET coimmunoprecipitate (as do TYRO3 and MET, but not MER).³¹⁶ Overexpression of AXL induces MET phosphorylation, and overexpression of a kinase dead AXL variant or silencing AXL decreases MET phosphorylation and cell migration in response to HGF. Additionally, there is crosstalk between AXL and VEGFR2, although a direct interaction has not been observed.³³³ In several endothelial cell lines, VEGFA promotes AXL phosphorylation, and inhibiting AXL with R428 blocks VEGFA induced activation of Akt. Furthermore, silencing AXL abolishes VEGFA induced migration, permeability, and tube formation. Src family kinases (SFK) appear to be mediating the interaction by phosphorylating the AXL juxtamembrane region after being activated by activated VEGFR2.

9. USING THERMODYNAMIC MODELS TO UNDERSTAND HETEROINTERACTIONS

The use of thermodynamic cycles that incorporate RTK heterointeractions can help explain many of the complicated biological effects which have been described in the literature and are discussed above. For instance, the predictions in Figure 4 can explain how the presence of heterodimers can alter the liganded homodimer concentrations. In Figure 4A, we model the case of a

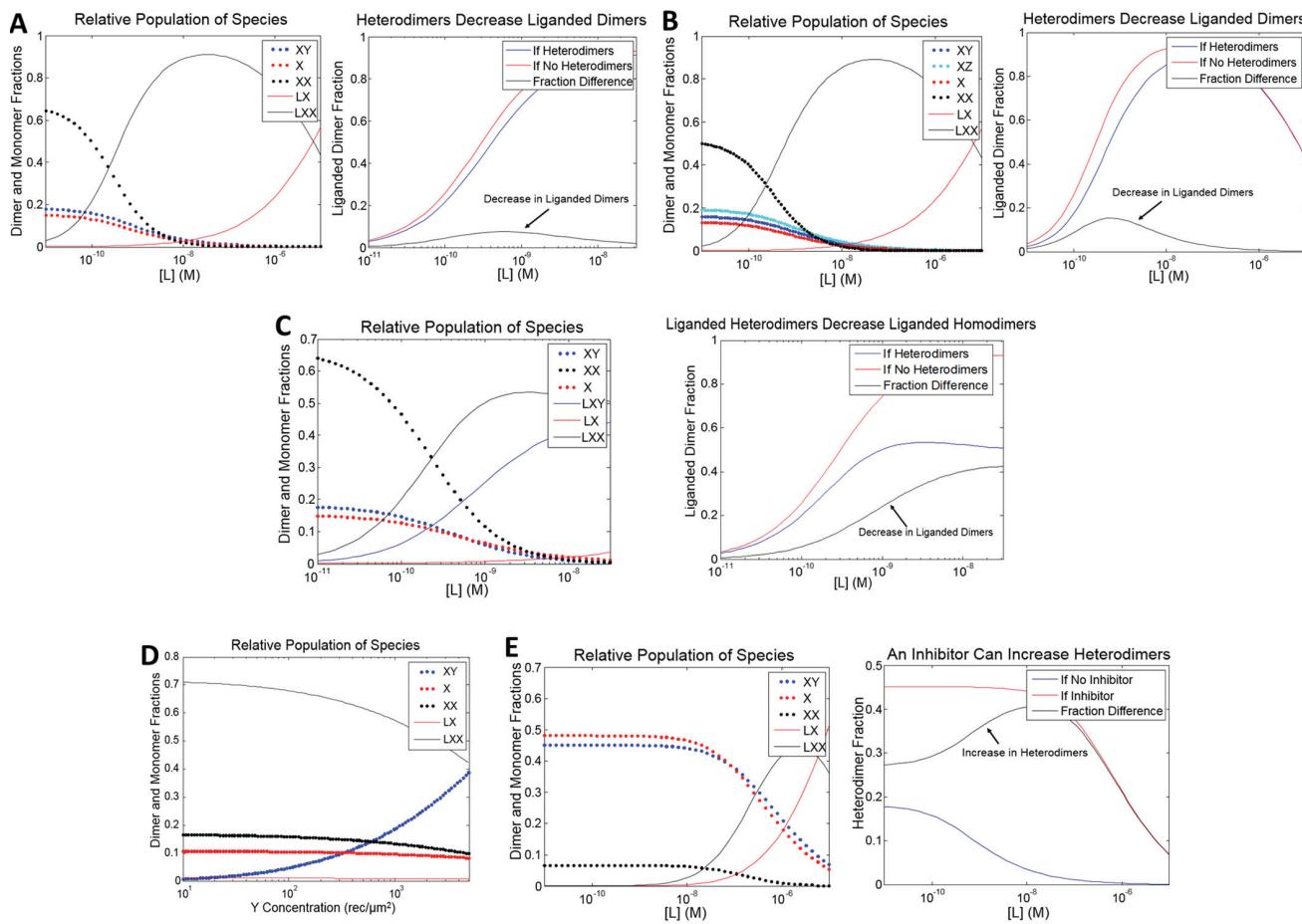


Figure 4. Predictions based on the thermodynamic cycles in Figure 3, produced with MATLAB. These predictions can help explain many of the complicated biological effects which have been described in the literature (see [Overview of Known RTK Cross-Subfamily Heterointeractions](#)). All receptors are assumed to have concentrations of 500 receptors/ μm^2 unless otherwise stated. Values of the constants used in the predictions are estimates for VEGFR2 dimerization and ligand binding; EGFR dimerization; and in the case of 4B, EphA2 dimerization. The K 's (receptor–receptor interactions) and L 's (ligand–receptor interactions) are association constants in units of $\mu\text{m}^2/\text{rec}$ and M^{-1} , respectively. (A) Prediction based on the thermodynamic cycle in Figure 3D: $K_X = 0.029 \mu\text{m}^2/\text{rec}$, $K_Y = 0.0088 \mu\text{m}^2/\text{rec}$, $K_{XY} = 0.0094 \mu\text{m}^2/\text{rec}$, $L_1 = 10 \times 10^7 \text{ M}^{-1}$, and $L_2 = 4 \times 10^9 \text{ M}^{-1}$. The left plot shows the different dimeric and monomeric fractions as a function of ligand concentration. The right plot compares the fraction of X receptors which exist as liganded dimers, for the case modeled in the left plot (blue), and for the case where X and Y cannot heterodimerize (red), as a function of ligand concentration. The black curve is the difference between the red and the blue curves, and it depicts the decrease in liganded dimers due to the presence of heterodimers. (B) Prediction based on the thermodynamic cycle in Figure 3E: $K_X = 0.029 \mu\text{m}^2/\text{rec}$, $K_Y = 0.0088 \mu\text{m}^2/\text{rec}$, $K_Z = 0.0049 \mu\text{m}^2/\text{rec}$, $K_{XY} = 0.0094 \mu\text{m}^2/\text{rec}$, $K_{XZ} = 0.0092 \mu\text{m}^2/\text{rec}$, $L_1 = 10 \times 10^7 \text{ M}^{-1}$, and $L_2 = 4 \times 10^9 \text{ M}^{-1}$. The left plot shows the different dimeric and monomeric fractions as a function of ligand concentration. The right plot compares the fraction of X receptors which exist as liganded dimers, for the case modeled in the left plot (blue), and for the case where the heterodimers XY and XZ cannot form (red), as a function of ligand concentration. The black curve is the difference between the red and the blue curves, and it depicts the decrease in liganded dimers due to the presence of heterodimers. (C) Prediction based on the thermodynamic cycle in Figure 3F: $K_X = 0.029 \mu\text{m}^2/\text{rec}$, $K_Y = 0.0088 \mu\text{m}^2/\text{rec}$, $K_{XY} = 0.0094 \mu\text{m}^2/\text{rec}$, $L_{X1} = 10 \times 10^7 \text{ M}^{-1}$, $L_{X2} = 4 \times 10^9 \text{ M}^{-1}$, $L_{Y1} = 10 \times 10^6 \text{ M}^{-1}$, $L_{Y2} = 4 \times 10^8 \text{ M}^{-1}$, and $L_{XY} = 4 \times 10^9 \text{ M}^{-1}$. The left plot shows the different dimeric and monomeric fractions as a function of ligand concentration. The right plot compares the fraction of X receptors which exist as liganded dimers, for the case modeled in the left plot (blue), and for the case where X and Y do not form heterodimers (red), as a function of ligand concentration. The black curve is the difference between the red and the blue curves, and it depicts the decrease in liganded dimers due to the presence of heterodimers. (D) Prediction based on the thermodynamic cycle in Figure 3D, which shows the different dimeric and monomeric fractions as a function of Y concentration for a fixed X concentration: the concentration of X is 250 $\text{rec}/\mu\text{m}^2$, the concentration of L is 1 nM, $K_X = 0.029 \mu\text{m}^2/\text{rec}$, $K_Y = 0.0088 \mu\text{m}^2/\text{rec}$, $K_{XY} = 0.0094 \mu\text{m}^2/\text{rec}$, $L_1 = 10 \times 10^7 \text{ M}^{-1}$, and $L_2 = 4 \times 10^9 \text{ M}^{-1}$. The concentration of liganded homodimers decreases and the concentration of the heterodimers increases as the concentration of Y increases. (E) Prediction based on the thermodynamic cycle in Figure 3D, which models the effect of an inhibitor of homodimerization by assuming that the homodimerization constant of the RTK which binds ligand, X, and the ligand binding constants are reduced 100-fold: $K_X = 0.00029 \mu\text{m}^2/\text{rec}$, $K_Y = 0.0088 \mu\text{m}^2/\text{rec}$, $K_{XY} = 0.0094 \mu\text{m}^2/\text{rec}$, $L_1 = 10 \times 10^5 \text{ M}^{-1}$, and $L_2 = 4 \times 10^7 \text{ M}^{-1}$. These decreases mimic the effect of a targeted inhibitor which decreases the ability of an RTK to form homodimers and to bind ligand. The left plot shows the different dimeric and monomeric fractions as a function of ligand concentration. The right plot shows the difference between the fraction of X receptors in heterodimers in the case of "no inhibitor," depicted in blue (identical conditions to Figure 4A) and in the case when the inhibitor is present (modeled on the left), depicted in red. The black curve is the difference between the red and the blue curves, and it shows a large increase in heterodimers in the inhibitor case.

dimeric ligand binding to an RTK which can heterodimerize (see Figure 3D for the schematic of this cycle). In other words,

we are assuming that an RTK, X, can bind a dimeric ligand, L; can form homodimers; and can form heterodimers with another

RTK, Y. The second RTK, Y, can also homodimerize, but it does not bind to the ligand.

The left plot in [Figure 4A](#) shows the distribution of the X receptors into different types of dimers and monomers as a function of ligand concentration. There are five molecular species containing X: XY heterodimers, blue dotted line; X monomers, red dotted line; XX homodimers, black dotted line; LX liganded monomers, red solid line; and LXX liganded dimers, black solid line. Under these conditions, almost 20% of the receptors exist in heterodimers when the ligand concentration is low, and the heterodimers slowly disappear as the ligand concentration increases. As expected, at low ligand concentrations, there are unliganded dimers and monomers, and as the ligand concentration increases, liganded dimers become the predominate species. Note, however, that at unphysiologically high ligand concentrations, liganded monomers have a substantial presence. Importantly, in this and all other plots on [Figure 4](#), the magnitude of the results are strongly dependent on the values of the receptor dimerization and ligand binding constants, but the general trends are the same over a wide range of values.

In [Figure 4A](#), we focus on the prediction for the liganded homodimers, as these are widely believed to be the most important, signaling-competent species. As seen in the right panel, the presence of heterodimers decreases the concentration of liganded homodimers. All assumptions and values for these graphs are the same as for the plot on the left. The red curve is the fraction of X receptors which exist as liganded dimers, shown as a function of ligand concentration in the absence of the Y receptors, and the blue curve is the fraction of X receptors which exist as liganded dimers in the presence of Y. The black curve is the difference between the red and blue curves, representing the decrease of the liganded homodimers due to heterodimerization. Importantly, the maximum value of the percent difference is around the expected physiological concentration of ligand.^{447,448} Although the effect on liganded dimers appears modest in this case, it can become much larger under some conditions, as discussed below. Furthermore, even small changes in the concentration of liganded dimers can have a large effect on signaling, as the signal is amplified through downstream signaling cascades.

A factor which furthers the decrease in liganded dimers is the presence of a second heterodimerization partner, as shown in [Figure 4B](#). All assumptions in [Figure 4B](#) are the same as in [Figure 4A](#), except that there exists a third RTK, Z, which can homodimerize and heterodimerize with X, but does not interact with Y or the ligand (see [Figure 3E](#) for the schematic of this cycle). The plot on the left shows the distribution of molecular species as a function of ligand concentration. It is similar to the left panel of [Figure 4A](#), except that there is a second heterodimer, XZ (the cyan dotted line). On the right is a plot showing the effect of both receptors on the concentration of liganded dimers. As seen in the prediction describing the decrease in liganded dimeric fraction (black line), the decrease is roughly twice the decrease seen when only one heterodimerizing partner is present. This effect increases when the number of heterodimerizing partners is increased, and many cell types simultaneously express several RTKs. All these potential heterodimerizing RTKs could synergize to substantially decrease the concentration of liganded homodimers.

Another factor which leads to a large decrease in liganded dimers is the ability of the ligand to bind to both RTKs and the heterodimer, as seen in [Figure 4C](#) (for known examples, see

RTK Heterointeractions within the Same Subfamily and Ligands Binding to Multiple Subfamilies). In this situation, in addition to the molecular species seen in [Figure 4A](#), there exist LY liganded monomers, LYY liganded dimers, and LXY liganded heterodimers. The ligand is assumed to bind to Y an order of magnitude weaker than to its cognate receptor X, and all other assumptions are the same as in [Figure 4A](#) (see [Figure 3F](#) for the schematic of this cycle). In the left panel, we show the predicted levels of the different types of dimers and monomers. This plot of the molecular species distributions as a function of ligand looks similar to that seen in [Figure 4A](#), except that there is a liganded heterodimer (the solid blue line).

In this case, the liganded heterodimer fraction increases with increasing ligand concentration until it is above 30% and is close to the fraction of liganded homodimers. This model can explain why many heterointeractions appear to increase after ligand addition. Again, the right panel shows the effect of heterodimers on liganded dimers, and in this case, the effect is fairly large, with the decrease being over 30% at certain ligand concentrations.

There are many other, subtler factors which can have a large effect on heterodimerization and, therefore, signaling. One example is seen in [Figure 4D](#), which shows how increasing the concentration of one RTK can result in an increase in heterodimers involving another RTK. The model and assumptions used here are exactly the same as in [Figure 4A](#), except that the concentration of the ligand binding RTK, X, is fixed while the concentration of the nonligand binding RTK, Y, is allowed to vary, and the concentration of ligand is also fixed. The prediction is that an increase in the concentration of Y leads to an increase in the percent of X receptors within XY heterodimers (dotted blue line), and a concomitant decrease in liganded homodimers (solid black line). This prediction applies to cases where factors such as drugs, disease states, or environmental stresses cause the upregulation of the expression of one RTK, and it explains how such conditions can increase heterointeractions.

A particularly interesting observation is that sometimes heterointeractions increase after the addition of an RTK inhibitor.²⁶⁸ This can potentially be explained by the prediction in [Figure 4E](#), which is similar to [Figure 4A](#), except that the homodimerization association constant of the ligand binding RTK, X, and the ligand binding constants are reduced 100-fold. These decreases mimic the effect of a targeted inhibitor which decreases the ability of the RTK, X, to form homodimers and to bind ligand. The prediction in the left panel shows that, at all but unphysiologically high ligand concentrations, almost all the RTK molecules exist as either monomers or heterodimers, with roughly equal amounts of each. The right panel shows the difference between the fraction of X receptors which are in heterodimers in the “no inhibitor case,” depicted in blue (identical conditions to [Figure 4A](#)) and in the case when the inhibitor is present, depicted in red. The black line indicates the difference (red line minus blue line), showing a large increase in heterodimers in the case where the inhibitor is present.

The discussed predictions based on thermodynamic cycles can help bring insight into a wide variety of biological systems and can be used to understand the effects of changing physiological conditions. However, the predictions are most meaningful when they are based on experimentally determined thermodynamic constants, which are rarely found in the literature. This is especially true in the case of heterointeractions, where there are only a few measurements. Accordingly, we argue

that there is a strong need for quantitative measurements of heterointeraction strengths.

10. THE RTK INTERACTOME

Above, we described many examples of RTK heterointeractions. It is possible that there exist many more RTK heterointeractions that have not yet been identified. Based on the current knowledge, we cannot exclude the possibility that any two RTKs, even if they belong to different subfamilies, can engage in heterointeractions under the right conditions. Accordingly, RTK heterointeractions may be ubiquitous, and thus each RTK may participate in an extensive network of RTK interactions, which we call the *RTK interactome*. Accordingly, current models of RTK activation are likely incomplete, as they are strongly biased toward homodimer-driven signaling.

Our understanding of the scope of the various RTK interactions is presently rudimentary, and we have only a partial view of the RTK interactome. No systematic investigation of all RTK–RTK interaction partners has been undertaken, and hence many interactions are probably unknown. Furthermore, the physical mechanisms underlying RTK cross-subfamily heterointeractions is largely unknown. It is possible that interactions between the kinase domains play a significant role, as the kinase domain is highly conserved across families,^{36,449} and FRET studies of RTK homodimers have shown that the deletion of their IC domains leads to dimer destabilization, in both the presence and absence of ligand.^{13,21,450} Another, not mutually exclusive, possibility is that the TM domains play a key role, as it has been argued in the literature that the interactions between RTK TM domains are promiscuous, forming many weak, nonspecific interactions.^{451–453} Although the EC domains of different subfamilies are often rather different (see Figure 1), they could also play a role, especially if there is a common ligand that binds to them.

There are not many measurements of stabilities of RTK homodimers and heterodimers, but these data suggest that the stabilities of the homodimers and the heterodimers in the absence of ligand may be comparable.^{97,173} In this case, the expression levels of the different RTKs will be the main factor that determines the relative abundance of homodimers and heterodimers. Different cells express different RTKs and do so to different degrees, and thus each cell type will be characterized by its own set of RTK interactions that control its fate. Once a ligand is introduced into the interaction network, it will likely stabilize all dimers to which it binds. Notably, based on the best-estimate calculations in Figure 4, the presence of ligand will likely not abolish the heterodimers, even if the heterodimers do not bind the ligand, except at high ligand concentrations. Accordingly, ligand addition alters the interactions by enriching some dimers and depleting others, and we expect that it will also cause conformational changes which enhance phosphorylation. We cannot currently predict in a comprehensive and quantitative way exactly how this will occur, due to a lack of basic knowledge about RTK and ligand expressions in tissues and about their interaction strengths. Due to the presence of numerous heterointeractions, studies of isolated homodimers are unlikely to be able to predict the complete signal transduction properties of a receptor in cells that express multiple RTKs.

10.1. Biological Function of the Heterointeractions in the RTK Interactome

It is often assumed that the ligand-bound homodimer is the dominant signaling species and the master regulator of the signaling response. In this case, one possible role of the heterodimers is to sequester the receptor, and hence inhibit the signaling that is mediated by the liganded homodimer. As shown in the predictions in Figure 4, the presence of heterodimers decreases the concentration of the liganded homodimer. The more RTK interaction partners that are expressed, the larger the inhibitory effect. For instance, in Figure 4B, we predict that, at physiological ligand concentrations in the presence of only two interaction partners, a greater than 15% decrease in the RTK liganded dimer population will occur relative to the case where there are no homodimers. If more interaction partners are expressed, the inhibitory effect will be much larger.

However, the assumption that the ligand-bound homodimer is the dominant signaling species may not be always correct. There are many possible biological functions of heterointeractions, as depicted in Figure 5. For instance, many of the papers overviewed above demonstrate that the heterodimers are also

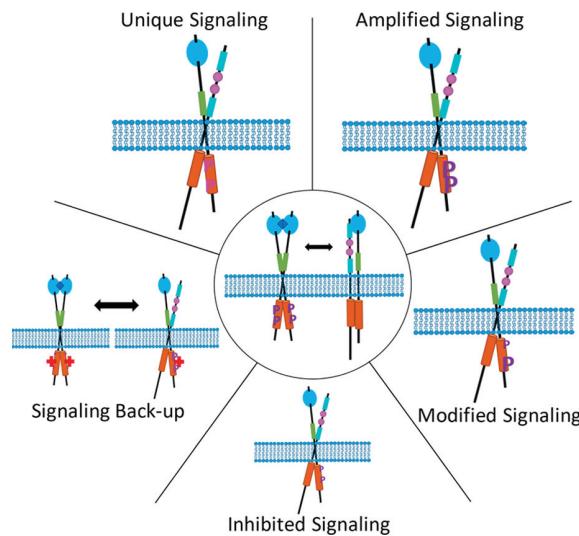


Figure 5. Depiction of possible effects of RTK heterodimerization. The center shows a generic, activated RTK homodimer in equilibrium with an RTK heterodimer (blue diamond, ligand; purple “P,” phosphorylation). Starting from the top left and going clockwise, *unique signaling*, where the heterodimer causes signaling not seen in the homodimers, possibly due to unique tyrosines being phosphorylated (pink “P”). *Amplified signaling*, where the heterodimer has a stronger downstream signal than the homodimer, possibly due to increased phosphorylation or decreased degradation. *Modified signaling*, where the heterodimer has a different probability of phosphorylation or adaptor protein binding than the homodimer, and it is possible that some tyrosines have increased phosphorylation while other tyrosines have decreased phosphorylation. *Inhibited signaling*, where the heterodimer has a weaker downstream signal than the homodimer, possibly due to the heterodimer being inactive or recruitment of a molecule which directly dephosphorylates the RTK. *Signaling backup*, where the homodimer has been inhibited, possibly by a drug (red cross), but signaling can continue due to direct phosphorylation by the heterodimerization partner. Note that the heterodimers are shown as unliganded, which may not always be the case (i.e., some heterodimers could be liganded), and that the heterodimer effects are shown as only affecting signaling of one of the RTKs, although the effects could be bidirectional (i.e., the signaling of both RTKs could be affected).

active. In some cases, the heterodimer signals in a unique way, and thus homodimers and heterodimers mediate distinct downstream signaling cascades. Such unique heterodimer signaling has been observed in several instances, including the cases of the PDGFR α –PDGFR β ^{206,207} and VEGFR1–VEGFR2 complexes.¹⁹⁹ Cross-subfamily heterointeractions resulting in unique signaling have also been seen. For example, the ROR1–ErbB3 interaction results in the phosphorylation of a unique tyrosine on ErbB3, triggering a specific signaling cascade that modulates the Hippo-YAP pathway.³⁰⁹ Mechanistically, this can occur via the phosphorylation of unique tyrosines in the heterocomplex or if the heterocomplex recruits other molecules with unique activities.

There are also examples in the literature where both homodimers and heterodimers work synergistically to activate an RTK, contributing to the same downstream effects. In this case, heterodimerization works to strengthen the response without altering or diversifying it. The RTK can be efficiently phosphorylated by the partner in the heterodimer (and in some cases, the partner is also phosphorylated), and hence the outcome is signal amplification, originating from an increase in the concentration of phosphorylated receptors. Moreover, heterocomplexes can increase signaling by decreasing receptor internalization, and hence increasing the duration of the signal, as seen in the cases of ErbB2–EGFR¹⁷⁷ and ErbB2–AXL²⁹⁷ heterocomplexes.

In some cases, the probability of phosphorylation or the probability of an adaptor protein binding is different within the homodimers and the heterodimers. This leads to quantitative differences in the degree of activation, but it does not cause divergent signaling. It is also possible that in a heterodimer, some tyrosines have increased phosphorylation while other tyrosines have decreased phosphorylation, relative to the homodimer. In these cases, heterodimerization works to modulate the strength of the response. For instance, it has been proposed that the RYK–EphB2 and RYK–EphB3 interactions modulate Eph signaling, and that this interaction becomes dysregulated in craniofrontonasal syndrome.³⁴³ Such modulating effects exert fine control over RTK signaling, enabling small changes in receptor or ligand concentration to alter the population of homo- and heterodimers, resulting in increases or decreases in signaling as needed.

Heterodimerization can also work to inhibit signaling. As discussed above, this can occur if a receptor is inactive within the heterodimer, with heterodimerization working to sequester the receptors and prevent them from forming active ligand-bound homodimers. An example of this is the case of VEGFR1–VEGFR2 heterodimers, where VEGFR1 sequesters both the VEGFA ligand and VEGFR2 in order to tightly regulate the activity of VEGFR2.^{66,197,198} There are also cases where the heterodimer works to recruit a phosphatase which dephosphorylates the heterodimerized receptor. Examples of such inhibition can be seen with the MET–VEGFR2 and PDGFR β –VEGFR2 interactions. In the MET–VEGFR2 case, the heterocomplex results in recruitment of PTP1B, which dephosphorylates MET and hence decreases MET signaling, and this effect is abolished by inhibiting VEGFR2 phosphorylation.³¹⁸ Although an exact mechanism was not identified in the PDGFR β –VEGFR2 case, it was shown that the PDGFR β –VEGFR2 complex inhibits PDGFR β signaling, and this only occurs in the presence of PDGF-BB and VEGFA.^{321,322} This is suggestive of a phosphatase being recruited, although it is possible that in the PDGFR β –VEGFR2 heterodimers, PDGFR β is blocked from

binding adaptor proteins due to steric hindrance, thus failing to activate downstream signaling cascades.

It is further believed that heterodimerization can work to provide a signaling backup. In this scenario, the main signaling entity is the homodimer. Yet when the homodimer is inhibited, the heterodimer assumes the signaling functions, rescuing the signaling pathway. Although this process is often thought to occur due to two different RTKs having overlapping downstream effects, it can be caused by direct phosphorylation of the inhibited RTK by the other RTK. This is mostly commonly seen in cancer, where a wide range of RTK inhibitors become ineffective or have minimal or inconsistent clinical efficacy. A drug could prevent homodimerization, block ligand binding, or suppress kinase activity, but the RTK can be still activated within a heterodimer and signal normally, since it gets phosphorylated by the partner RTK. It is possible that this signaling backup could sometimes be beneficial, as it could allow normal signaling to occur if a mutation or deficiency reduces the ability of one RTK to autophosphorylate.

In general, the existence of heterointeractions greatly increases the signaling complexity of RTKs and allows for a greater degree of regulation. Based on the current literature, it appears that the major roles of the RTK interactome are to significantly enhance the diversity of the signaling and provide signaling backups. It is likely that the RTK interactome mediates many additional signaling outcomes that have not yet been uncovered. Further comprehensive studies will be needed to understand the full scope of signal diversification through heterodimerization.

10.2. Implications of the RTK Interactome

Studies and mechanistic models of RTK signaling often assume that RTK homodimers are the predominant dimers in the cellular membrane, and experimental data are often interpreted under this paradigm. However, this assumption may lead to incorrect conclusions, especially in cases where other RTKs are expressed at high levels. It is possible that models of RTK activation that solely consider homodimers will never be able to correctly predict activity and cellular outcomes. Instead, we propose that the whole interactome needs to be included in order to arrive at new, comprehensive models of RTK activation with predictive power.

Furthermore, the RTK interactome concept may provide an explanation for differences in experimental data acquired in different cell lines. Usually, the literature only gives information about the expression of the receptor under investigation, and it is generally unknown what other receptors are expressed in a cell. Accordingly, even the types of possible heterodimers are unknown. Yet it is conceivable that the presence of these heterodimers greatly influences the cell signaling outcomes. It is possible that many contradictions in the literature cannot be completely resolved until we understand the entire network of RTK interactions.

The heterointeractions also need to be considered in the design of RTK inhibitors for cancers and other diseases and disorders. Even if a drug successfully inhibits the signaling of an RTK homodimer, if a heterodimer is able to rescue its function, the drug will not have the desired effect. Therefore, the RTK expression pattern in a cell type may be a critical factor in determining the performance of the inhibitor. Since an inhibitor can affect the functions of both homodimers and heterodimers—or even influence the entire RTK interactome—it can have many unanticipated consequences. Without an under-

standing of the biological effects of heterointeractions, it will be difficult to predict all the possible effects of an inhibitor.

There are, of course, many challenges and technical hurdles in determining the strength of heterodimerization. Many RTKs, especially those in the same subfamily, are similar sizes, and hence homo- and heterodimers are indistinguishable by some techniques, particularly western blots. Fluorescence-based experiments can only follow labeled RTKs that have been introduced in the cells, and hence heterodimers may appear as monomers. Detection based on antibodies will only reveal the specific RTK being probed, and heterodimerization partners will accordingly be missed unless they are also individually probed. This is in addition to the fact that different cells express different RTKs and ligands to different degrees, and accordingly, a specific RTK heterointeraction that does not occur under one set of conditions may occur under different conditions. Unfortunately, current biochemical and biophysical experimental techniques are not well-suited to follow the entire RTK interactome in a cell. We look forward to new method development, new computational approaches, and new basic knowledge about RTK heterodimerization strengths and expressions that will move the field forward.

10.3. Beyond the RTK Interactome

There are many documented cases of interactions between RTKs and other membrane proteins, such as cell adhesion molecules, GPCRs, and other signaling receptors. For example, VEGFR2 interacts with VE-cadherin,⁴⁵⁴ and there is evidence of crosstalk between EGFR and E-cadherin.⁴⁵⁵ Numerous RTKs interact with integrins,⁴⁵⁶ including VEGFR2,^{457,458} PDGFR β ,^{459,460} and MET.^{461,462} A few of the RTKs are involved in Wnt signaling; for instance, RYK interacts with Frizzled proteins,³³⁴ ROR2 interacts with Vangl2⁴⁶³ and Frizzled proteins,⁴⁶⁴ PTK7 interacts with LRP6⁴⁶⁵ and Frizzled proteins,⁴⁶⁶ and MuSK interacts with LRP4.^{467,468} Several RTKs interact with semaphoring receptors, including some which interact with plexins, such as MET,^{469,470} ErbB2,^{471,472} VEGFR2,⁴⁷³ and FGFR1;⁴⁷³ VEGFR2 interacts with neurophilin-1^{474,475} and neurophilin-2.⁴⁷⁶ These interactions have a wide range of important biological consequences.

Furthermore, RTKs directly interact with the plasma membrane and the cytoskeleton.^{45,477} There is evidence that the activity of EGFR is strongly affected by lipid composition; in particular, interactions with gangliosides⁴⁷⁸ and negatively charged lipids such as phosphatidylinositol-phosphates (PIPs)^{479,480} have been shown to play a key role. Numerous other RTKs have been found to interact with gangliosides, including the other ErbBs, FGFRs, TrkA, MET, PDGFRs, VEGFR2, and INSR—these interactions can be activating or inhibitory.^{481,482} It has further been proposed that residues in the N-terminal portion of the JM region of all RTKs interact with negatively charged lipids, in particular PIP₂, and this interaction is important for proper RTK dimerization and function.⁴⁸³ Moreover, it has been found that the plasma membrane is sectioned into 40–300 nm compartments or corrals by the associated cortical cytosol mesh, and this can increase local receptor concentration, shifting monomer–dimer equilibria.⁴⁸⁴ The cytoskeleton also directly interacts with RTKs. For instance, EGFR binds to actin,^{485,486} and EphA2 exhibits directed transport due to an actin interaction.⁴⁸⁷ There is also evidence that actin plays a crucial role in the organization of INSR after ligand binding.⁴⁸⁸ These interactions could provide an additional layer of RTK signaling regulation, and they need to

be considered in order to obtain a comprehensive understanding of RTK function.

It is highly likely that the RTK interactome is a part of an extensive membrane protein interactome: a network of interactions between diverse families of membrane proteins and lipids. However, little is known about the scope of these interactions, and much more work needs to be done by cell signaling researchers and membrane biophysicists before we fully understand how these interactions regulate biological function. We are looking forward to the many new discoveries in the years to come.

11. SUMMARY AND FUTURE PERSPECTIVES

The incredible complexity of RTK heterointeractions has only begun to be explored. These interactions can have numerous different effects, and we group these into five general categories (Figure 5): (1) *unique signaling* outputs that increase signal diversity; (2) *amplification of signaling* by direct phosphorylation or by reduced internalization of the receptors, thereby increasing the signaling lifetimes; (3) *modification of signaling* when the probability of phosphorylation or adaptor protein binding is different in a homodimer than in a heterodimer; (4) *inhibition of signaling* of one or both of the downstream pathways of the RTKs which compose the heterocomplex, either by sequestering an RTK in an inactive heterocomplex or by dephosphorylating the components; (5) *signaling backup*, where the signaling of an inhibited RTK is rescued by direct phosphorylation by its heterointeraction partner. Importantly (as discussed under *Using Thermodynamic Models To Understand Heterointeractions*), the understanding of RTK heterointeractions may help in understanding cancer therapy drug resistance and in designing more effective treatments.

A multitude of factors control whether and to what degree RTK heterointeractions occur. These factors include the concentrations of the receptors, the concentrations of the ligands, and whether or not other interaction partners are present. Accordingly, different cell lines, media conditions, the degree of invasiveness of the cells, the degree of drug sensitivity of the cells, and the presence of inhibitor drugs can all affect the formation of these heterocomplexes. In turn, these interactions affect a multitude of downstream biological outcomes. Given the importance of the biological processes mediated by the 58 human RTKs, there is a great need for quantitative, thermodynamic studies that report on the strength of the interactions. As discussed above, such studies can help explain aspects of the complex RTK biology. Furthermore, close attention needs to be paid to the concentrations of all RTKs, as these concentrations determine the identities of the homo- and heterodimers. Such quantitative measurements will allow for the creation of detailed thermodynamic models accounting for all relevant RTK dimers, and can ultimately predict RTK activity and the nature of the biological response.

11.1. Final Thoughts

Our understanding of RTKs has grown tremendously since they were first discovered in the 1970s, and new quantitative, physical–chemical studies of the RTK interactome will contribute to our ever-expanding knowledge of the complexity of RTK signaling. This new knowledge can empower the design of novel RTK targeted inhibitors. Ultimately, a deeper knowledge of the RTK interactome thermodynamics will lead to better understanding of fundamental biological processes in health and disease.

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