

DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

EFFECTS OF RECURRENT MISSENSE MUTATIONS ON ERBB2 RECEPTOR
ORGANIZATION AND MEMBRANE DYNAMICS

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

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LIST OF ABBREVIATIONS

ADC	Antibody-drug conjugates
ADCC	Antibody-Dependent Cellular Cytotoxicity
AREG	Amphiregulin
ATCC	American Type Culture Collection
BTC	Betacellulin
CDC	Complement classic cascade
CDRs	Complementarity-determining regions
CHO	Chinese hamster ovary
CME	Clathrin-mediated endocytosis
DAG	Diacylglycerol
DIC	Differential interference contrast
DOL	Degree of labeling
ECD	Extracellular domain
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERBB2	Erythroblastic oncogene B2
Fab	Antigen-binding fragments
Fc	Crystallizable fragment
FcRn	Neonatal receptor
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
HER2	Human epidermal growth factor receptor 2
ICD	Intracellular domain
Ig	Immunoglobulins
IgG	Immunoglobulin G
IP3	Inositol triphosphate
JMD	Juxtamembrane domain
KD	Kinase domain

K _d	Dissociation constant
mAbs	Monoclonal antibodies
NGS	Next-generation sequencing
NRG	Neuregulin
NSCLC	Non-small cell lung cancer
PKC	Protein kinase C
PTB	Phosphotyrosine binding
PTPs	Protein tyrosine phosphatases
ROI	Region of interest
RTK	Receptor tyrosine kinase
SH2	Src Homology 2
SNV	Single-nucleotide variants
SPM	Single-particle tracking microscopy
TGF α	Transforming growth factor alpha
TKD	Tyrosine kinase domain
TKIs	Tyrosine kinase inhibitors
TMD	Transmembrane domain
WT	Wild type

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1 PREFACE

Receptor tyrosine kinases are key components of the plasma membrane that act as signal transducers, regulating processes such as cellular proliferation, survival, and metabolism. Among the RTKs, the ErbB family is distinguished by its complexity and its role in homeostasis and development. However, its deregulation has been established as an oncogenic driver for solid tumors.

In particular, the ErbB2 receptor is a notable structural exception within the ErbB family; it lacks ligand-binding capability and adopts a constitutively extended conformation. This characteristic positioned it as the “universal co-receptor” and the preferred dimerization partner for the other family members, maximizing the cellular signaling efficiency. Historically, the pathogenicity of ErbB2 has been understood from a purely quantitative perspective, in which genomic amplification and subsequent massive overexpression of the protein at the plasma membrane drive homodimer formation, triggering sustained cellular signaling that promotes tumor malignancy and evades natural degradation mechanisms.

However, the development of next-generation sequencing has transformed the classical paradigm by revealing that ErbB2 is also susceptible to activating somatic mutations in the absence of genomic amplification. These mutations represent an alternative mechanism for acquiring oncogenic potential, not through membrane saturation, but through structural alterations that destabilize the conformation of the receptor. Functionally, these alterations are not random but cluster at “hotspots” corresponding to key functional domains for receptor regulation: the extracellular domain (ECD), the transmembrane domain (TMD), and the kinase domain (KD). Missense mutations in these regions confer oncogenic competence by destabilizing the inactive, autoinhibitory conformation or by strongly stabilizing a catalytically open conformation, drastically altering the stoichiometry of interaction and promoting ligand-independent hyperactive signaling

In this scenario, this work analyzes three somatic mutations that span distinct functional domains: R143Q (ECD), R678Q (TMD), and V842I (KD). Through a systematic comparison of these missense variants with the wild-type receptor, the work seeks to establish a biophysical framework to elucidate how structural disruptions across different domains affect the organization and dynamics of the receptor in the plasma membrane.

2 LITERATURE REVIEW

2.1 Receptor tyrosine kinase biology and the ErbB family

2.1.1 Overview of receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are vital cell-surface receptors that mediate critical physiological responses. These receptors function as signal transducers for polypeptide hormones, cytokines, and growth factors, thereby regulating essential processes such as cell proliferation, differentiation, survival, metabolism, and migration [1]. RTKs possess a conserved structure consisting of a ligand-binding extracellular region, a transmembrane segment comprising 20 to 25 hydrophobic amino acids, and a functional intracellular domain with intrinsic tyrosine kinase activity [2,3].

Canonical activation of these receptors is initiated by ligand binding to the extracellular domain. This interaction induces a conformational change that facilitates either homo- or heterodimerization of the receptor [4]. This receptor interaction facilitates the cross-transphosphorylation of tyrosine residues within the kinase domain of the intracellular region. Once phosphorylated, they become docking sites for adapter proteins containing Src Homology 2 (SH2) or phosphotyrosine binding (PTB) domains, starting downstream cell signaling [5,6].

Despite the structural homology in their catalytic domains, the RTK superfamily presents a diversity in its extracellular domains (Figure 1), allowing them to recognize a wide variety of ligands [1]. Furthermore, members of the RTK family can activate several secondary signaling pathways. This means that once the receptor is activated, signaling is not linear; it diversifies into complex transduction networks. The recruitment of effector proteins triggers downstream signaling cascades involved in cell cycle progression (MAPK pathway), cell growth, and apoptosis inhibition (PI3K/Akt/mTOR pathway) [7,8].

However, to maintain cellular homeostasis, RTK signaling is regulated through attenuation or negative feedback mechanisms [9]. The main mechanism is clathrin-mediated endocytosis (CME) of the ligand-receptor complex [10], followed by either lysosomal degradation or recycling to the plasma membrane [11]. This process is frequently regulated by receptor ubiquitination through E3 ligases like c-Cbl [12]. Additionally, the protein tyrosine phosphatases (PTPs) play a key role in dephosphorylating the activated tyrosine residues, returning the receptor to its inactive basal state [13].

From the 20 RTK families identified in the human genome [1] the ErbB family stands out for its functional complexity, its essential role in physiological development and homeostasis of organs like the heart, the central nervous system, and the mammary gland [14]. However, the dysregulation of any member due to overexpression, mutation or aberrant signaling has been established as an oncogenic driver in several solid human tumors [15,16].

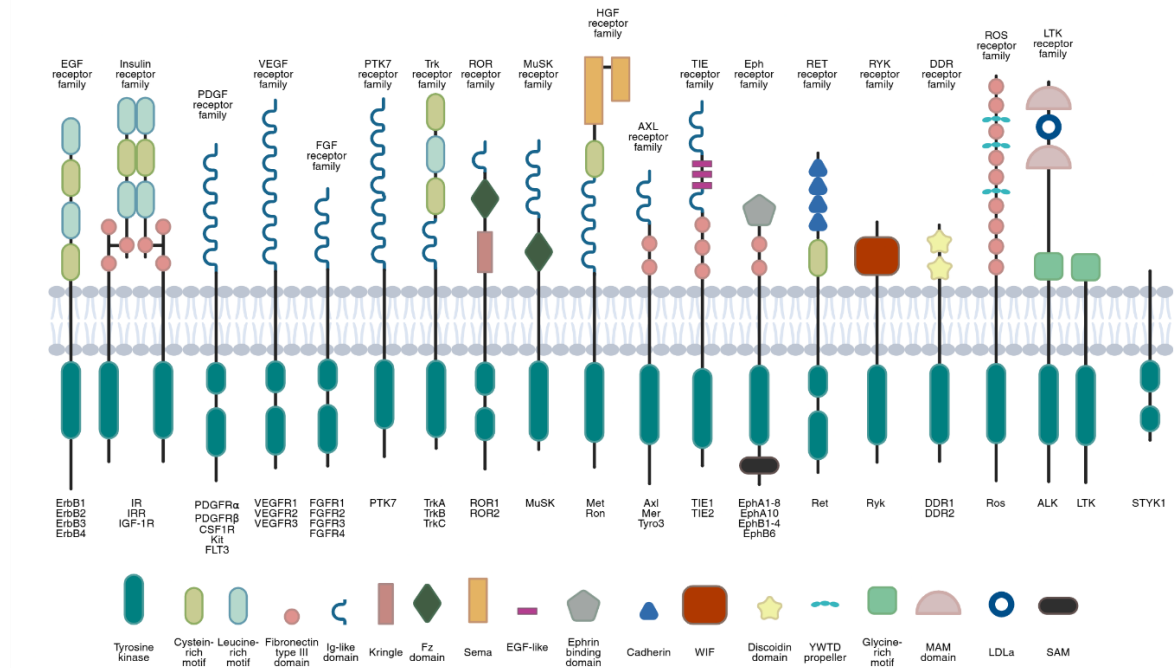


Figure 1. **Overview of the structural domains of the 20 RTK families.** Each RTK is composed of a ligand-binding extracellular domain, a juxtamembrane domain, and a tyrosine kinase intracellular domain. The variability of RTK ectodomains (Ig-like motifs, cysteine- and leucine-rich motifs) enables a highly conserved intracellular catalytic machinery to execute divergent, context-specific biological responses. Modified from [17]. Created in BioRender.

2.1.2 The ErbB family

The ErbB family is composed of four members: ErbB1 (EGFR/HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) [18]. From a genomic perspective, the ErbB family arises from paralogous genes that map to distinct loci, a result of ancient gene duplications [19]. The *EGFR* gene is located in the short arm of chromosome 7 (7p11.2), while *ERBB2*, *ERBB3*, and *ERBB4* are found on chromosomes 17 (17q12), 12 (12q13), and 2 (2q34), respectively [20]. The coding sequence (cDNA) is highly conserved among the four members, with regions organized into a similar number of exons, while the total gene length varies due to differences in intron sizes (Table 1) [21].

Table 1. Genomic organization and structural domain mapping of the ErbB receptor family.

Receptor	Gene, Locus and Genomic Length	Exon Mapping and Structural Domain	References
EGFR (ErbB1)	Gene: <i>EGFR</i> Locus: 7p11.2 Length: ~ 188 kb	Total exons: 28 ECD: exons 1 – 16 TMD: exon 17 ICD: exons 18 – 28 with KD 18 – 24	[20,22],
ErbB2	Gene: <i>ERBB2</i> Locus: 17q12 Length: ~ 40 kb	Total exons: 27 ECD: exons 1–16 TM: exon 17 KD: exons 18–24	[23,24]
ErbB3	Gene: <i>ERBB3</i> Locus: 12q13.2 Length: ~23 kb	Total exons: 28 ECD: exons 1–16 TMd: exon 17 KD: 18–28	[25,26]
ErbB4	Gene: <i>ERBB4</i> Locus: 2q34 Length: >1 Mb	Total exons: 28 JM isoforms: Alternative splicing at exons 15/16. CYT isoforms: Alternative splicing at exon 26.	[21,27]

Structurally, they share a topology comprising a ligand-binding extracellular segment (ECD), a single-pass hydrophobic transmembrane segment, a juxtamembrane domain (JMD), and an intracellular segment containing a tyrosine kinase domain (TKD) and a C-terminal tail rich in tyrosine residues. (Figure 2). The extracellular segment is subdivided into four domains (I-IV), with domains I and III involved in ligand binding and domains II and IV in regulating structural conformation. ErbB2 and ErbB3 stand out within the family for their deficiencies in ligand binding and kinase activity, respectively. Although the corresponding domains are present in both receptors, mutations in key residues or structural rearrangements make these domains nonfunctional [14,20].

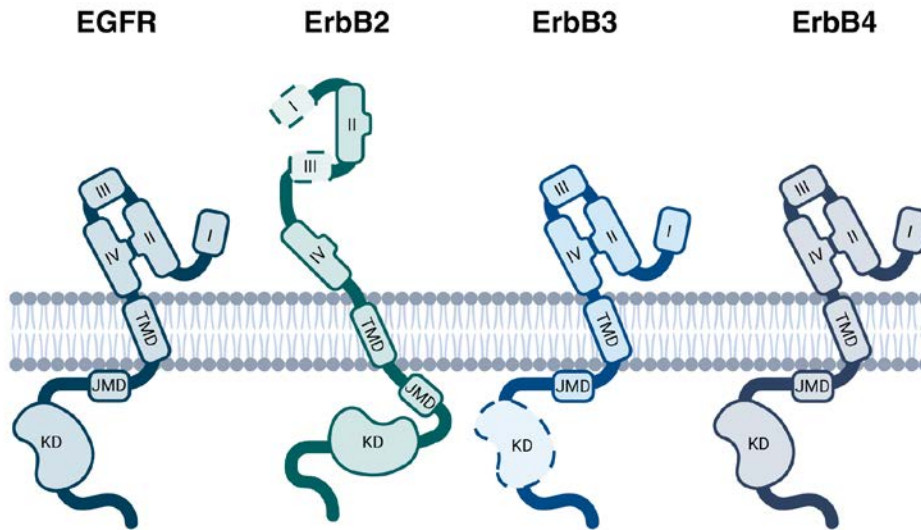


Figure 2: **Schematic representation of the ErbB family members.** The diagram shows the structural organization and the domain differences between the four members of the epidermal growth factor receptor (EGFR) family: EGFR, ErbB2, ErbB3, and ErbB4. All receptors share a modular architecture with an extracellular ectodomain composed of four subdomains (domains I to IV), a transmembrane domain, a juxtamembrane domain, and an intracellular domain that includes a tyrosine kinase domain and a C-terminal tail rich in tyrosine residues. We can differentiate that the ErbB2 receptor has an inactive ligand-binding domain (transparent domain), and the ErbB3 receptor has an inactive tyrosine kinase domain (transparent domain). In the absence of ligand, EGFR, ErbB3, and ErbB4 adopt a tethered conformation in which domains II and IV form a mechanical inhibitory mechanism. On the other hand, ErbB2 is present in a constitutive extended conformation, exposing the dimerization arm in domain II. Modified from [28]. Created in BioRender.

Interestingly, ErbB receptors differ from other RTKs in that direct ligand-ligand interactions are not involved in their dimerization, but use a mechanism mediated exclusively by the receptor itself [20,29]. This means that in the absence of ligand, the receptors ErbB1, ErbB3, and ErbB4 get into a tethered conformation through an intermolecular interaction between domains II and IV (Figure 2), hiding the dimerization arm acting as an autoinhibition mechanism to avoid spontaneous dimerization, preventing receptor activation [30].

The canonical activation mechanism begins with growth factor binding to domains I and III, which induces a conformational change that disrupts the intramolecular interaction between domains II and IV, resulting in an extended receptor conformation (Figure 3). This extension exposes the dimerization arm of domain II, allowing the formation of “back-to-back” homo- or heterodimers [31,32]. This dimerization exerts mechanical torque on the transmembrane segment (TMD), thereby forcing its structural reorientation. In an inactive state, the TMD can interact through the C-terminal; however, the ligand binding

induces a specific helicoidal rotation that shifts the crossing angle towards the N-terminal [33].

This new configuration is stabilized by transmembrane dimerization motifs type GxxxG, inducing the dissociation of the juxtamembrane segment from the lipid bilayer and facilitating the asymmetric dimerization of the kinase domain [34–36]. Within this dimer, the C-terminal lobe from a monomer (“activator” kinase) interacts allosterically with the N-terminal lobe of the second monomer (“receiver” kinase); mimicking the activation mechanism of the cyclin-CDK complex [37]. The allosteric configuration eliminates any intramolecular restriction upon the receiver kinase, activating its catalytic effect of transphosphorylation of specific tyrosine residues in the C-terminal tails, which allows the recruitment of effector proteins with SH2 and PTB domains [9].

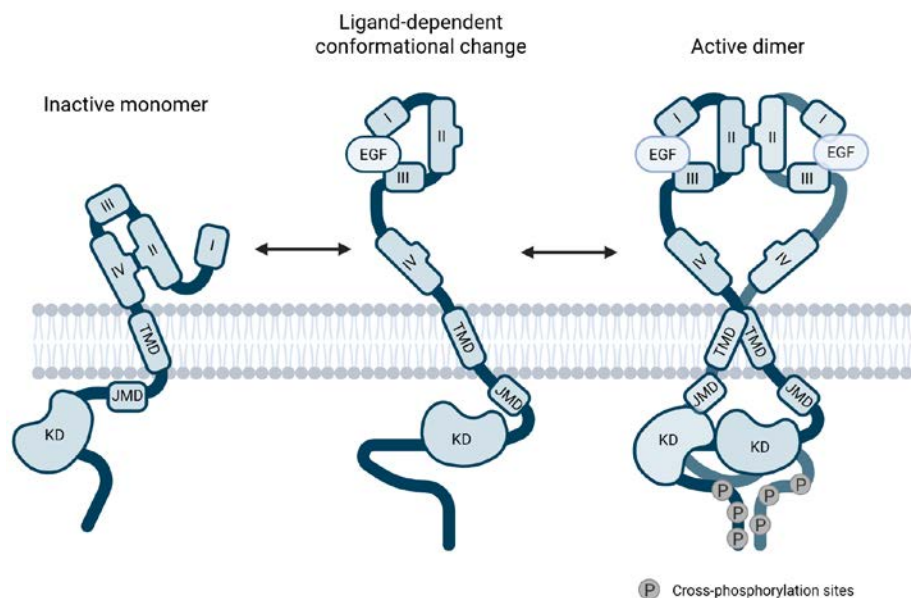


Figure 3: **Canonical activation mechanism of EGFR.** In the absence of ligand, the monomeric EGFR is in a tethered conformation where the dimerization arm, located in domain II, interacts with the pocket of domain IV (left). Upon ligand binding to domains I and III, the receptor ECD undergoes a conformational change, exposing the dimerization arm (center). Once the EGFR finds another extended receptor, the N-terminal dimerization motif of the TMDs interacts to form a stabilized dimer, which induces the dissociation of the JMD from anionic phospholipids in the membrane, thereby forming a “latch” that holds both kinase domains together in an asymmetric dimer. Modified from [38]. Created in BioRender.

However, there are exceptions in the structural biology of this receptor family. The ErbB2 receptor represents an ECD structural exception; it lacks ligand-binding capability, thereby defying the autoinhibition mechanism by exposing the dimerization arm in a constitutively extended conformation, maximizing dimerization potential [39]. This characteristic positions ErbB2 as a “universal co-receptor”, and it is the preferred

dimerization partner of the other members [40]. The ErbB3 receptor is distinct among intracellular domain (ICD) kinases because it lacks key catalytic residues, thereby classifying it as a pseudokinase. Within heterodimers, ErbB3 serves as an allosteric activator [41]. These characteristics facilitate the formation of the ErbB2/ErbB3 heterodimer, in which ErbB3 activates ErbB2, leading to the trans-phosphorylation of the C-terminal tail of ErbB3.[26].

These structural features are not isolated anomalies. Instead, they constitute the biophysical foundation for an organized signaling system. Although the ErbB2/ErBB3 heterodimer is frequently favored, alternative dimeric combinations also contribute significantly to phenotypic plasticity and signal regulation.

2.1.3 Stoichiometric dynamics of ErbB proteins

Dimer formation is determined by ligand specificity, receptor affinity, and their relative density at the plasma membrane. Although the four receptors (EGFR, ErbB2, ErbB3, and ErbB4) have the theoretical capability of forming ten different dimeric combinations, the real frequency of the combinations followed a very strict hierarchy [42].

Growth factors that interact with ErbB receptors can be specific for a single receptor or bind multiple receptors, thereby restricting the initial dimerization possibilities. They are divided into three main groups:

- EGFR-specific ligands: in this group, we find the epidermal growth factor (EGF), the transforming growth factor alpha ($TGF\alpha$), and amphiregulin (AREG), which induce EGFR/EGFR homodimers in the absence of other co-receptors [42].
- ErbB3 and ErbB4-specific ligands: the Neuregulin (NRG) family (NRG1, NRG2, NRG3, NRG) are bivalent ligands with high affinity for ErbB3 and ErbB4 [25].
- Pan-ErbB ligands: these ligands can activate multiple receptors. In this group, we find betacellulin (BTC) that promotes the formation of heterodimers of all members, and epiregulin, which binds to EGFR and ErbB4 but induces tyrosine phosphorylation in all members [20].

The first level of stoichiometric determination lies in the specificity of the ligand. The ligands exclusive to EGFR (i.e., EGF and $TGF\alpha$) induce conformation changes that favor

the formation of EGFR/EGFR homodimers, while the neuregulins are compulsory for the functional recruitment of ErbB3 and ErbB4. However, dual-specificity ligands like BTC add complexity to receptor interaction mechanisms (Figure 4) [43].

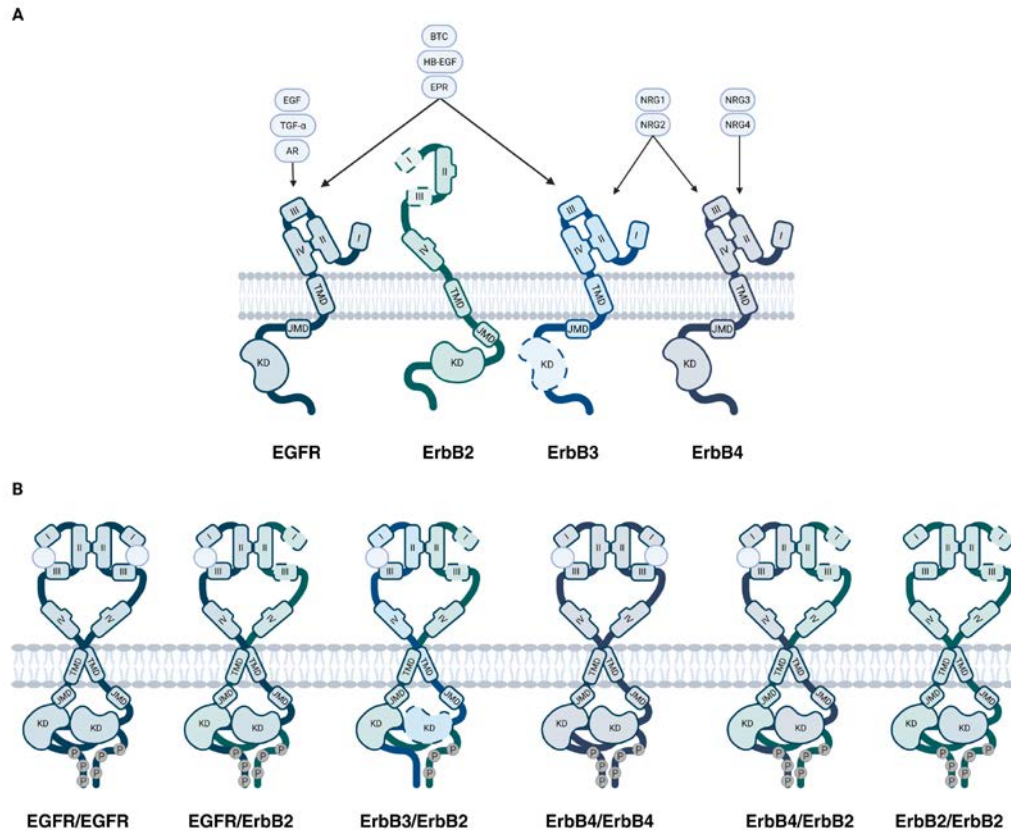


Figure 4: ErbB receptor family ligands and dimerization partners. (A) The EGFR, ErbB3, and ErbB4 show a tethered conformation that extends upon bivalently binding specific ligands (EGF, BTC, NRG1-4, among others). ErbB2 has no known ligand, thereby maintaining an extended, active conformation. (B) The exposed dimerization arm of the active receptors enables multiple dimerization partners, with ErbB2 as the preferred partner. The formed asymmetric dimers stabilize the TMD and dissociate the JMD, activating the KD for the autophosphorylation of the C-terminal tyrosine. Modified from [44]. Created in BioRender.

However, in the presence of ErbB2, the cellular physiology is shifted from a ligand-driven status to be driven by the principle of “lateral signaling” [45]. This model establishes that ErbB2 acts as the universal and preferred dimerization partner due to its constitutively extended dimerization arm, which significantly reduces the activation energy required to form the complex relative to monomers in the tethered state, which require a ligand-induced conformational change [43,46]. Therefore, ErbB2 functions as a “kinetic scaffold” that captures activated receptors, stabilizing their transition states and maximizing signaling efficiency [40]. The previous concept is evident in the competition between EGFR/EGFR homodimer formation and EGFR/ErbB2 heterodimer formation. In cells

where both receptors are co-expressed, the presence of ErbB2 transforms the “low affinity” binding sites of EGFR into “high affinity” sites, showing not only a higher binding efficiency due to a reduced negative cooperativity but also shifts the stoichiometric equilibrium away from EGFR homodimer formation [47].

The relevance of the heterodimerization is exemplified in the ErbB2/ErbB3 complex, where the binding of neuregulins to ErbB3 promotes the interaction with ErbB2, forming what is described as the most functionally dominant oncogenic unit among all the ErbB member pairs [48,49]. The potency of this complex is not only due to the complementarity between the ligand affinity of ErbB3 and the kinase activity of ErbB2, but also to their capacity to form signaling “nanoclusters” in the plasma membrane [50,51]. This spatial organization shields the complex from clathrin-mediated endocytosis and prevents lysosomal degradation, which is essential for cellular survival [29,52].

Although heterodimer formation is generally favored, receptor density significantly influences dimerization. In HER2-positive mammary cancer, ErbB2 overexpression facilitates ErbB2/ErbB2 homodimer formation. Unlike ligand-induced dimers, these homodimers are stabilized by transmembrane domain-specific interactions, primarily involving GxxxG (glycine zipper) motifs. This close helical association induces the conformational change necessary for constitutive kinase activation in the intracellular domain (ICD) [35,53], which is a principal mechanism of ligand-independent oncogenic signaling and is absent in the regulated allosteric activation of heterodimers [54,55]. This affinity hierarchy directs cell-survival signals through ErbB2 heterodimerization, and stoichiometric imbalance may result in pathological conditions [56].

2.1.4 Downstream signaling pathways

The diversity of interactions at the plasma membrane gives rise to a unique intracellular signaling network. Once the dimer is formed, complex activation initiates a phosphorylation cascade. The identity of the monomer determines which tyrosine residues are phosphorylated in the C-terminal tails, thereby determining which adaptor proteins (with SH2 or PTB domains) are recruited. This “phosphorylation code” determines the duration, intensity, and the final cellular response [9,57].

The recruitment of adaptor proteins (i.e., Grb2 or Shc) and enzymes (i.e., PI3K, PLC γ , or Src) initiates one of the three main signaling pathways:

- MAPK/ERK (Ras-Raf-MEK-ERK1/2) pathway: it is the predominant route for mitogenic signaling. The recruitment of the Grb/Sos complex to the pTyr residues of the receptor activates the GTPase Ras [9], starting a kinase cascade that triggers the nuclear translocation of ERK [58] and the activation of transcription factors like c-Myc [59] and Elk-1 [60], promoting the entry into the cell cycle.
- PI3K/Akt/mTOR pathway: it is a key pathway for cellular survival, growth, and metabolism in response to extracellular signals [61]. The activation of AKT promotes cell survival by phosphorylating and inhibiting pro-apoptotic effectors from the Bcl-2 family [7] and regulates protein synthesis through mTOR [62].
- PLC γ -PKC pathway. PLC γ binds directly to phosphotyrosines and hydrolyzes PIP₂, generating diacylglycerol (DAG) and inositol triphosphate (IP₃) [63], which mobilize intracellular calcium and activate protein kinase C (PKC) [42] and regulates cellular migration and chemotaxis [26].

2.1.5 Functional specificity of ErbB dimers

As mentioned before, the combination diversity of the ErbB dimers allows for the generation of a wide range of biological responses, even with a limited amount of ligands, phosphorylating different subsets of tyrosine residues and thus recruiting unique “interactomes” (Figure 5) [64].

In case of the EGFR/EGFR homodimers, after the ligand binding, the receptor autophosphorylates key residues, i.e., Y1068 and Y1173, that are the anchor points for the adaptor protein Grb2 [20,65]. This triggers the classic MAPK cascade (Ras-Raf-MEK-ERK1/2), for the entry to the cell cycle. However, a key characteristic of this homodimer is its strict negative regulation. After activation, the complex is rapidly ubiquitinated by the c-Cbl ligase, which directs the receptor to clathrin-mediated endocytosis and further lysosomal degradation [66]. This mechanism ensures a strong but transitory mitogenic signal, avoiding the uncontrolled cellular proliferation in physiological conditions [11].

In contrast, the formation of the EGFR/ErbB2 heterodimer alters the previously described regulation mechanism. The presence of ErbB2 modifies the intracellular trafficking of the complex due to ErbB2 lacking the tyrosine and di-leucine motifs to recruit the AP-2 adaptor complex [67], and it is resistant to the effects of the c-Cbl ligase [68,69],

evading the lysosomal degradation route, and is preferentially recycled to the plasma membrane [70]. This recycling keeps the signaling capacity for prolonged periods, generating a sustained activation of the ERK pathways and a mitogenic potential higher than the EGFR/EGFR homodimer [26,71].

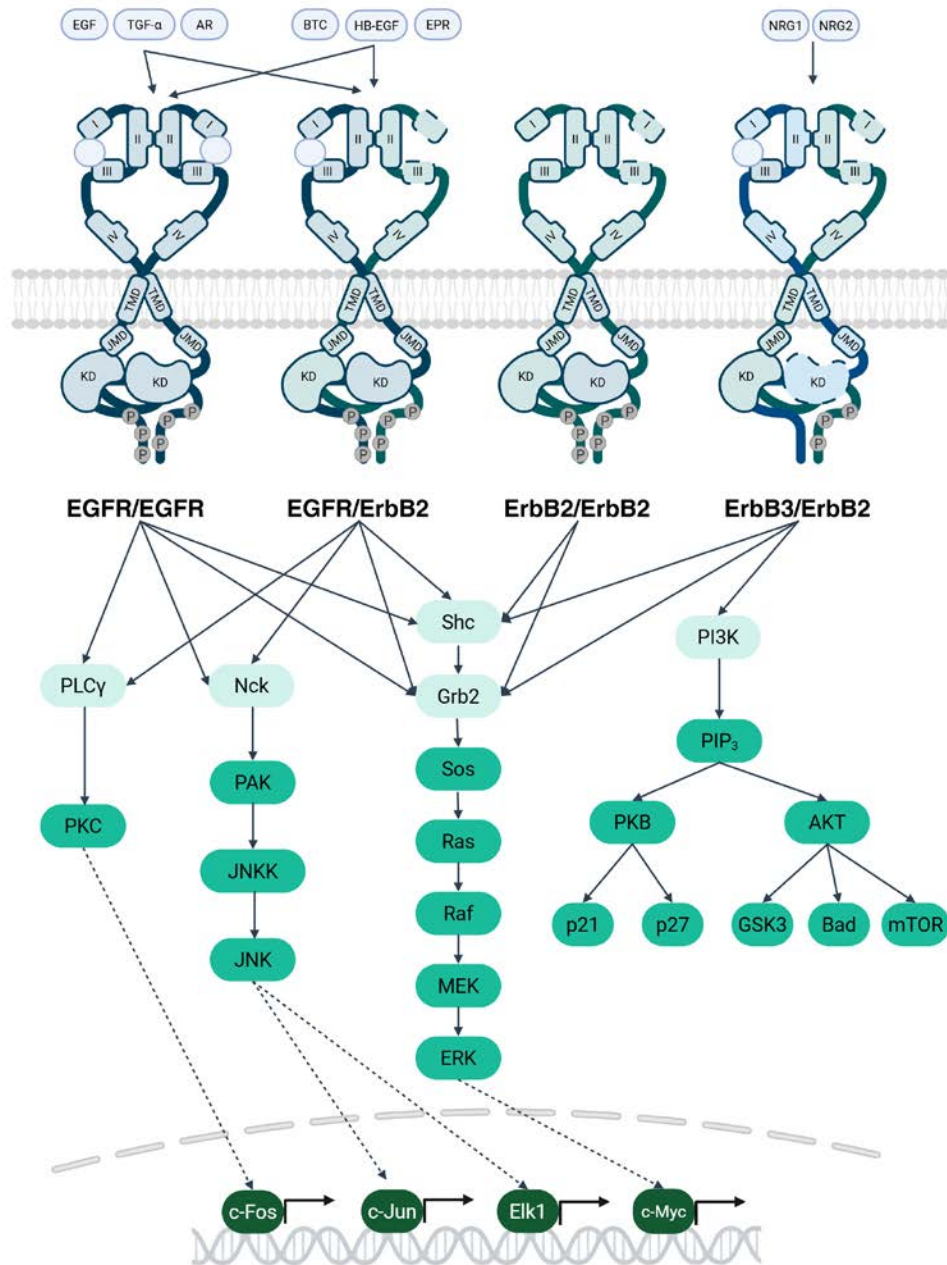


Figure 5. Signaling pathways activated by ErbB family dimer combinations. The specific ligand binding (EGF, TGF α , AR, NRG1-2) promotes homo- and heterodimer formation of the ErbB receptors. The activated KDs trigger the autophosphorylation of C-terminal tyrosine residues, which function as docking sites for adaptor proteins (Shc, Grb2) and enzymes (PLC γ , PI3K). This initiates multiple signaling cascades: the MAPK cascade, involved in cell proliferation; the PI3K/Akt cascade, involved in cell survival, metabolism, and cell cycle regulation through p21/p27, GSK3, Bad, and mTOR; and the PLC γ /PKC and Ncl/JNK cascades, involved in cytoskeletal rearrangement and apoptosis. These signals remodel the transcriptional landscape by regulating the expression of

key genes (c-Fos, c-Jun, Elk1, c-Myc) that determine the cellular fate. Modified from [42]. Created in BioRender.

While the EGFR-based dimers dictate the MAPK pathway, the ErbB2/ErbB3 complex is the most potent activator of the survival pathway, i.e., PI3K/Akt pathway [42]. This potency relies on the ErbB3 C-terminal structure containing six binding sites for the p85 subunit of PI3K, functioning as an oncogenic unit [72,73]. Since ErbB3 lacks any intrinsic kinase activity, it depends solely on the transphosphorylation by ErbB2 to activate those sites. This compulsory cooperation channels the signaling towards the PI3K/Akt/mTOR pathway, leading the cells to avoid apoptosis [7], increase protein synthesis [74], and reprogram the glycolytic metabolism [75], distinctive characteristics of tumor survival.

However, in a pathological context in which genomic overexpression drives the formation of ErbB2/ErbB2 homodimers, signaling is rapid and constitutive. As the dimers form independently of the ligand due to specific interactions of the transmembrane domains [53] and to the high receptor density in the plasma membrane [76], this complex escapes the negative feedback mechanisms. The ErbB2/ErbB2 homodimer is able to simultaneously and strongly activate the MAPK and PI3K/survival pathways through the recruitment of adaptor proteins Grb2 (via Y1139) and Shc (via Y1248), respectively [77]. This constant “activated” state is one of the main molecular mechanisms that promote tumoral malignancy and the metastasis observed in patients [46,78].

Overall, the final cellular response does not depend completely on the presence of the receptors in the plasma membrane, but on the dimer composition and the effector network that interacts with the dimer [9,79].

2.1.6 Clinical and biological relevance of ErbB2

Beyond the intricate mechanistic details of its signaling cascade, the ErbB2 receptor serves as an indispensable biological hub for embryonic development and the maintenance of tissue homeostasis [15,80]. At the physiological level, its signaling network orchestrates fundamental processes of early organogenesis and is essential for the development of the peripheral nervous system and proper cardiovascular organization. Various *in vivo* models have shown that genetic alterations in this pathway result in severe morphogenetic defects, notably embryonic lethality due to abnormalities in ventricular trabeculation architecture and defective neural crest cell migration [81,82]. Today, it is widely recognized that preserving

this specific ErbB2 signaling axis remains absolutely critical for postnatal tissue repair and cardioprotection in adults [83].

From a clinical perspective, the translation of ErbB2 biology has redefined the paradigms of precision oncology. Historically identified as an adverse prognostic biomarker defining a highly aggressive molecular subtype in breast cancer, its functional validation enabled the pioneering development of monoclonal antibodies and selective inhibitors (TKIs) that transformed the natural history of the disease [84]. Currently, the clinical impact of ErbB2 has extended beyond breast cancer; next-generation genomic analyses have established the receptor as a crucial cross-cancer pathological driver in gastric, biliary, colorectal, and non-small cell lung cancers [78]. However, despite the initial proven efficacy of these therapeutic interventions, rapid tumor adaptation and cross-resistance demonstrate that the clinical approach can no longer rely on a purely quantitative view of the receptor on the cell membrane [85]. This makes it imperative to study ErbB2 from a structural perspective to elucidate its biophysical vulnerabilities, laying the groundwork for quantitative diagnosis and the design of the next generation of pharmacological strategies [54,86].

2.2 ErbB2 in oncogenesis

Although ErbB signaling is the key for physiological development, its deregulation is a hallmark of several human carcinomas [25]. As mentioned before, the unmanageable dimer formation and constitutive phosphorylation make ErbB2 a potent oncogene. Two mechanisms have been identified by which ErbB2 acquires its oncogenic potential: gene amplification and consequent protein overexpression [62], and activating somatic mutations that alter the structure of the receptor (Figure 6) [87].

2.2.1 ErbB2 genomic amplification and overexpression

The canonical understanding of ErbB2 pathogenicity is based on the quantitative deregulation of its expression. This receptor was initially identified as the product of the *neu* oncogene, where a point mutation in rat neuroblastoma cell lines was responsible for receptor deregulation [88]. However, when the human homolog (HER2 or ErbB2) was isolated, early genomic screenings failed to identify analogous mutations in human tumors [89,90].

The breakthrough came in 1987, when Slamon et al. showed that the main alteration in mammary cancer was not a mutation in the *ERBB2* gene but amplification, i.e., the tumoral genome contained multiple copies of the gene [91]. Although the gene maintained its “wild-type” sequence, the genomic amplification was directly correlated with an overexpression at the mRNA level and protein amount in the plasma membrane, associated with a lower overall survival and a higher risk of relapse of mammary cancer patients [92].

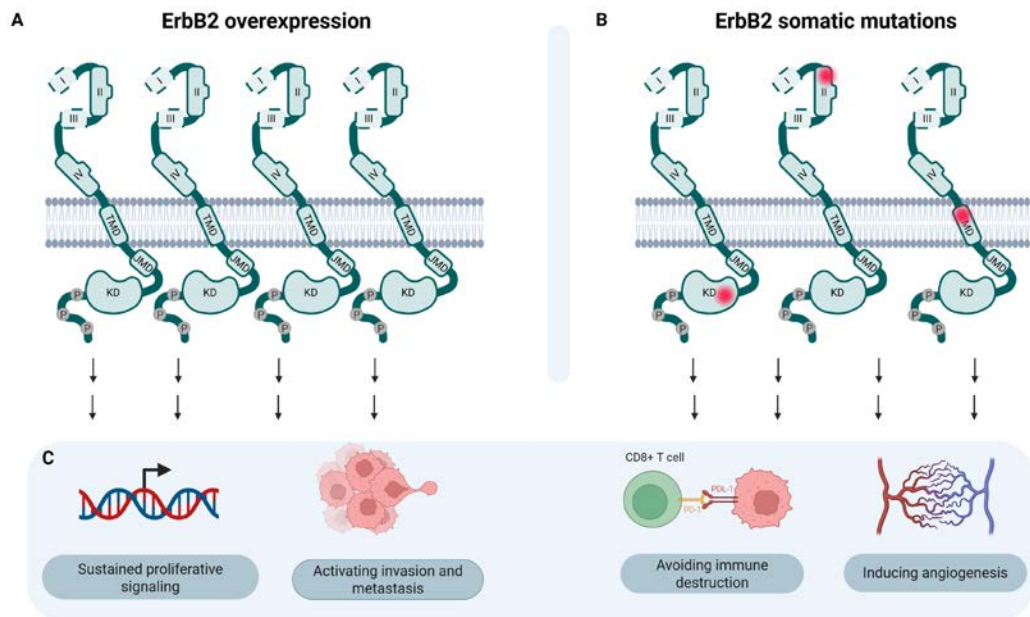


Figure 6. **ErbB2 oncogenic mechanisms.** (A) **ErbB2 overexpression.** The high density of membrane receptors favors spontaneous homo- and heterodimerization, constitutively activating intracellular signaling pathways through autophosphorylation of C-terminal kinase residues. (B) **ErbB2 somatic mutations.** Specific amino acid substitutions (red dots) in the ECD, specifically the dimerization arm, TMD, and KD stabilize the active conformation of the receptor, leading to a hyperactive and dysregulated signaling. (C) Both mechanisms activate signaling pathways that promote the acquisition of several hallmarks of cancer [93], which includes dysregulated cell proliferation, invasive potential, induction of angiogenesis, and reprogramming of the immune microenvironment. Modified from [94]. Created in BioRender.

The direct consequence of the genomic amplification is receptor overexpression in the plasma membrane, a phenomenon directly linked to the constitutive signaling observed in ErbB2-positive carcinogenesis (Figure 6A) [25]. Under physiological conditions, ErbB2 expression is low and tightly regulated, with approximately 20,000 to 50,000 ErbB2 receptors per cell. In contrast, in the presence of genomic amplification, the receptor density can be 50-100 times higher [95,96].

The high receptor density in the plasma membrane alters the interaction stoichiometry. The molecular proximity forces the collision and formation of ErbB2-ErbB2

homodimers [76], overcoming the energetic threshold needed for the dimerization in the absence of ligand [39]. This interaction promotes the constitutive transphosphorylation of the tyrosine residues in the intracellular domain, particularly Tyr1248 (the primary coupling site) and Tyr1221/1222 (in the kinase activation loop) [97], starting a sustained downstream signaling primarily mediated by the Shc adaptor [77].

A crucial aspect of this carcinogenic mechanism is the evasion of the regulatory mechanisms. In a normal condition at physiological ErbB2 expressions, after activation, ErbB2-free homodimers of ligand binding ErbB family members go through an endocytosis process and are directed towards lysosomal degradation after being ubiquitinated by the E3 ligase c-Cbl [66,68]. However, in the context of ErbB2 overexpression, it has been proposed that the endocytic and degradative machinery is saturated or halted. ErbB2 lacks a high-affinity binding site for the c-Cbl ligase and the AP-2 adaptor [98,99], a key characteristic for avoiding the degradation and shifting towards recycling to the plasma membrane; therefore, prolonging the oncogenic signal [71]. This sustained signaling chronically activates the PI3K/Akt/mTOR and Ras/MAPK pathways [96], promoting uncontrolled proliferation, apoptosis evasion and angiogenesis [71].

The convergence of genomic amplification and the subsequent saturation of the plasma membrane clinically defines the pathological phenotype known as “HER2-positive” (HER2+). This tumor subtype is characterized by a state of profound “oncogenic addiction”, in which the viability, proliferation, and invasive capacity of neoplastic cells depend almost exclusively on the hyperactive and constitutive signaling of the ErbB2 axis [100,101]. Clinically, the classification of a tumor as HER2+ is rigorously standardized and requires validation through molecular pathology. According to international guidelines, an HER2+ phenotype is defined by massive overexpression of the protein on the cell surface, detectable with a maximum score (3+) by immunohistochemistry (IHC), or by confirmation of ERBB2 gene amplification using fluorescence in situ hybridization (FISH) techniques, demonstrating a copy number ratio of the gene relative to the centromere of chromosome 17 greater than 2.0 [102].

From a biological standpoint, the HER2+ phenotype alters the stoichiometry of cellular signaling. While in a healthy epithelial cell, ErbB2 expression ranges between 20,000 and 50,000 molecules per cell, in a HER2+ cell this density can reach up to two million receptors [96]. This extreme spatial proximity overcomes thermodynamic barriers,

forcing spontaneous homodimerization (ErbB2/ErbB2) and aberrant heterodimerization, regardless of ligand availability [42]. Consequently, the HER2+ phenotype not only correlates with dysregulated mitosis and evasion of apoptosis via the MAPK and PI3K/Akt pathways but historically dictated one of the most aggressive clinical behaviors with the highest metastatic tropism, until the advent of blocking monoclonal antibodies and targeted therapies[85,103].

Table 2. Clinical distribution and molecular mechanisms of *ERBB2* dysregulation.

Type of Cancer	Alteration Frequency	Primary Alteration	References
Mammary	15-20%	Amplification	[78,104]
Gastric	15-20%	Amplification	[105,106]
Bladder	10-15%	Amplification and mutation	[104,107]
Biliary tract	5-15%	Amplification and mutation	[107,108]
Colorectal	3-5%	Amplification	[104,109]
Lung	2-4%	Mutation	[110,111]
Ovary	2-5%	Amplification	[112]
Cervix	3-6%	Mutation	[104]

Epidemiologically, this phenotype of overexpression/amplification defines a specific molecular subtype, and it is observed in approximately 15-20% mammary and gastric cancer [91,113]. However, next-generation sequencing (NGS) has shown that ErbB2 alterations are relevant oncogenic drivers in a broader spectrum of solid tumors (**Table 2**) [78,107]. This state of “oncogenic addiction”, where the viability of the tumor depends mainly on the signaling started by the receptor overexpression [100], has been the ground for the development of targeted therapies like trastuzumab [84], pertuzumab [114] and antibody-drug conjugates (ADC) [115].

Overall, the classic model shows that genomic amplification can transform a physiological receptor into a constitutively active oncoprotein by overexpressing the protein and saturating the negative feedback mechanisms. However, exclusive reliance on receptor density does not fully account for the pathological spectrum of ErbB2. There is an alternative

mechanism by which oncogenic competence is achieved, not through membrane saturation but through conformational disturbances that destabilize the conformation of the receptor.

2.2.2 Activating somatic mutations

As mentioned before, the implementation of NGS has allowed the characterization of the genomic profile of ErbB2 receptors, undermining the ideas that the oncogenic role is only dependent on receptor overexpression, and that the receptor lacks alterations in its coding sequence. In recent decades, it has been established that ErbB2, as seen with the first identified rat homolog *neu* [116], is susceptible to activating somatic mutations (Figure 6B). These drivers are able to keep a sustained cellular transformation and constitutive signaling in the absence of genomic amplification, defining a distinct subset of ErbB2-driven tumors [87,107].

The epidemiological profile of these mutations differs from that associated with overexpression. While the amplification is a key characteristic of mammary and gastric carcinomas, the somatic mutations present a “pan-cancer” distribution [117]. These somatic mutations have been identified in 2-4% of diverse carcinomas, including non-small cell lung cancer (NSCLC), colorectal, and cervical tumors [107] (Table 3).

A distinctive characteristic of these alterations is the mutual exclusivity with the genomic amplification. Most of the tumors with driver mutations keep a diploid copy number of the receptor, which indicates that the mutations represent an alternative mechanism to promote oncogenic activation (Figure 6C).

In a genomic context, the architecture of these changes is heterogeneous. While the majority of the identified variants correspond to missense-type single-nucleotide variants (SNV, Figure 7), the mutation spectrum of *ERBB2* is more complex and distributed than other classic oncogenes [107]. Besides the point mutations, a wide number of the oncogenic events, especially those found in the KD (exon 20), are insertions or small in-frame deletions [24,118]. And, unlike the nonsense or frameshift mutations that lead to a loss-of-function state, the SNVs and in-frame indels preserve the open reading frame and the general integrity of the protein, creating disruptions that enhance receptor activity [119]. The aforementioned variants function by destabilizing the autoinhibitory “closed” conformation or stabilizing the catalytically “open” conformation [87,119]. This conformational shift significantly reduces the free energy needed to achieve dimerization and catalysis [120].

Table 3: Distribution of hotspot *ERBB2* mutations across protein domains and specific cancer types.

Type of Cancer	Specific mutation	Protein region	Frequency	References
Bladder	S310F, S310Y	ECD	40-50%	[107,121]
	R678Q	JMD	5-8%	[104]
Mammary (Lobular)	L755S	KD (exon 19)	70-80%	[122,123]
Mammary (General)	L755S	KD (exon 19)	20-25%	[87]
	V777L	KD (exon 20)	15-20%	[124]
Lung (NSCLC)	Y772_A775dupYVMA	KD (exon 20)	80-90%	[24,110]
	G776delinsVC	KD (exon 20)	5-10%	[111]
Colorectal	V842I	KD (exon 21)	40-50%	[109,125]
	L755S	KD (exon 19)	20-25%	[126]
Billiar vesicle	S310F	ECD	50-60%	[127]
Cervical	S310F	ECD	30-45%	[128]
Pan-cancer	V659E/G660D	TM	< 2%	[129]



Figure 7: **Spatial distribution and frequency of missense mutations in *ERBB2*.** (A) Complete mutational spectrum comprising exclusively amino acid substitutions (missense) across the 1,255 residues. (B) Isolation of recurrent variants, graphically representing only those mutations documented in at least 10 clinical samples. The panel aligns these high-selective-pressure positions with the conserved functional domains of the protein, its topology (extracellular, transmembrane, cytoplasmic), and the corresponding exons. Visualization generated using cBioPortal with data from the COSMIC database.

On the other hand, functional mapping has revealed that these mutations are not random, but they are clustered in “genomic hotspots” that correspond to key functional domains linked to receptor regulation:

- Mutations in the ECD: mutations in this domain promote the formation of aberrant intramolecular bonds or alter the dimerization interface, allowing the formation of stable dimers through anomalous electrostatic interactions or hydrophobic packing. In specific cases involving cysteine residues, these alterations can lead to covalent dimers stabilized by disulfide bonds [119]. These alterations mimic a pseudo-ligand state, leading to a ligandless dimerization [87].
- Mutations in the TMD: mutations in this domain may force a rotational rearrangement of the transmembrane helices to stabilize the active conformation of the dimer [130].
- Mutations in the KD: this is the most frequently mutated site, with the oncogenic driver mutations concentrated mainly in exons 19 through 21 [107]. Functionally, the mutations in this domain increase the basal catalytic activity by destabilizing the autoinhibitory interactions [87]. Structurally, mutations in this domain alter the plasticity of the receptor, preventing from adopting the inactive configuration, favoring constitutive phosphorylation [118,131].

In the therapeutic area, these mutations have been shown to confer a certain degree of resistance against standard treatments like lapatinib or trastuzumab. In the case of tyrosine kinase inhibitors (TKIs) like lapatinib, resistance is mainly caused by conformation changes that decrease the binding affinity of the drug. Since lapatinib binds to the inactive form of the kinase, mutations that keep the receptor in a constitutively active conformation effectively prevent the drug binding due to the conformational changes [131,132]. However, at the same time, this structural deregulation creates a pharmacological vulnerability. Most activating mutations, particularly those within the KD, enhance oncogene addiction and present sensitivity to next-generation small-molecule TKIs, such as neratinib [107], afatinib [133] and pozoitinib [118], that utilize irreversible covalent binding mechanisms [134].

The identification of somatic driver mutations has redefined ErbB2 as both a quantitative target, due to gene amplification, and a structural target. The functional heterogeneity observed among these variants demonstrates inconsistent activation of

signaling pathways and differential responses to inhibitors. This variability is determined by the specific residue involved.

2.3 Monoclonal antibodies in therapy and molecular research

The involvement of the ErbB family in oncogenesis has established monoclonal antibodies (mAbs) as essential tools for both therapeutic intervention and receptor characterization. These immunoglobulins serve as therapeutic agents that inhibit oncogenic signaling [135] and as high-affinity probes for investigating membrane dynamics [135,136]. Preserving their structural integrity is critical, since even minor conformational alterations can substantially affect affinity and biological efficacy [137,138].

2.3.1 Molecular architecture and dynamics of immunoglobulins

Immunoglobulins (Ig) are special glycoproteins that act as the main sensors in the adaptive immune system. The most common type, IgG, is a glycoprotein composed of two heavy and two light chains joined by disulfide bonds, with a molecular weight of about 150 kDa. Besides the IgG isotype, there are four more types of immunoglobulins: IgA, IgD, IgE, and IgM. Each type is defined by the sequence found in the constant region of its heavy chain [139].

Immunoglobulins are composed of two antigen-binding fragments (Fab) and one crystallizable fragment (Fc), which serve as distinct functional units. Each Fab fragment contains variable domains (V_H and V_L) that encompass the complementarity-determining regions (CDRs), forming the paratope responsible for specific epitope recognition [140,141]. The Fc region mediates interactions with the immune system by binding, depending on the isotype, to specific immune cell receptors such as the $Fc\gamma$ receptor for IgG and the $Fc\epsilon$ receptor for IgE, as well as to complement proteins to initiate effector responses [142]. The hinge region confers segmental flexibility, enabling torsional and swaying movements of the Fab domains. These molecular dynamics facilitate the linkage between antigen recognition and effector response activation [143,144].

IgG antibodies are classified as either polyclonal or monoclonal based on their mode of production. Polyclonal antibodies are produced by multiple B-cell clones and recognize various epitopes on a single antigen; they are frequently utilized for pathogen detection when strong signal amplification is required [145]. In contrast, monoclonal antibodies are specific to a single, defined epitope of an antigen and are preferred in applications demanding high

binding specificity. Monoclonal antibodies (mAbs) are generally produced through hybridoma technology, which entails fusing antibody-producing B cells with myeloma cells to create immortalized cell lines. Hybridoma clones are subsequently screened to identify those that produce the desired antibody [146].

2.3.2 Therapeutic applications of mAbs

In clinical practice, mAbs such as trastuzumab, which target the ECD of ErbB2, exhibit a bimodal mechanism of action. On one hand, the binding of the Fab fragment to domain IV prevents the constitutive activation of the receptor (Figure 8A) [39]. On the other hand, the Fc region mediates critical effector functions by interacting with the complement protein C1q and Fcγ receptors of NK cells and macrophages (Figure 8B) [147]. This interaction is critical for inducing Antibody-Dependent Cellular Cytotoxicity (ADCC), which has been identified as a primary driver of the antitumor efficacy of the drug [148].

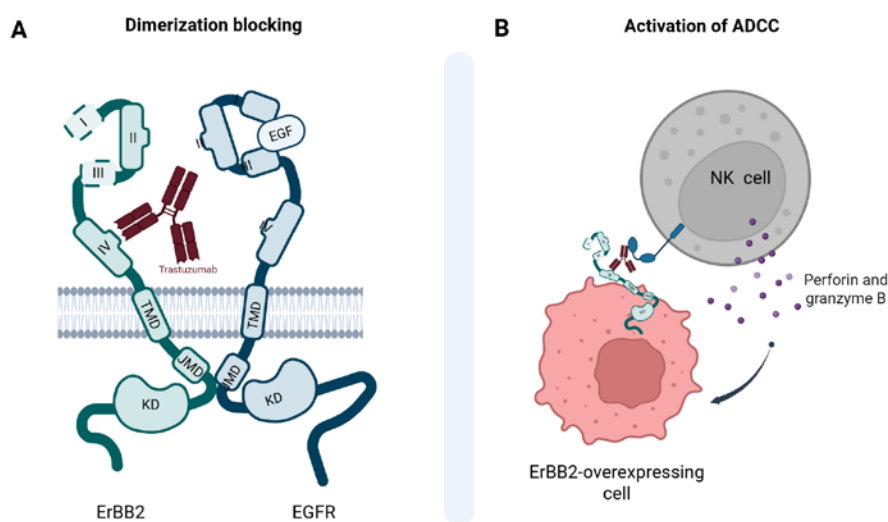


Figure 8. **Mechanism of action of trastuzumab in ErbB2-overexpressed cells. (A) Block of dimerization.** Trastuzumab specifically binds to domain IV of ErbB2, preventing the formation of active dimers through steric hindrance and thus halting constitutive intracellular signaling. **(B) Activation of ADCC.** The Fc fragment of trastuzumab, bound to an ErbB2 receptor in the cancer cell, is recognized by FCγRIIIa receptors from the NK cells. This interaction triggers antibody-dependent cellular cytotoxicity (ADCC), inducing the release of perforin and granzyme B, leading to apoptosis of the cancer cell. Modified from [152]. Created in BioRender.

Additionally, the structural integrity of the Fc domain is essential for the binding of other essential biological mediators [149], including the neonatal receptor (FcRn), that regulates the serum half-life of the therapeutic antibody, avoiding its lysosomal degradation; the complement protein C1q, which binds to the CH2 domain to initiate the complement classic cascade (CDC); and the bacterial proteins such as Protein A and Protein G [150].

Adapting these highly specific molecules from clinical to analytical settings poses significant methodological challenges. Visualizing and measuring receptor dynamics, such as ErbB2 expression and lateral signaling, in cell assays or *in vitro* requires chemically modifying the native structure of the antibody to incorporate external reporters. This bioconjugation can unintentionally alter the paratope structure, thereby reducing binding strength and compromising analytical accuracy [151].

2.3.3 Research applications of mAbs

In biological research and quantitative diagnostics, such as studies of membrane dynamics and stoichiometry of the ErbB2 receptor, monoclonal antibodies (mAbs) are typically conjugated with organic fluorophores [135]. The most widely adopted analytical method in modern biotechnology is stochastic labeling of the ϵ -amino groups of lysine residues using N-hydroxysuccinimidyl ester (NHS) chemistry [153]. A typical human immunoglobulin G (IgG) molecule contains 80 to 90 lysine residues, with 30 to 40 being solvent-exposed and sterically accessible, enabling efficient covalent conjugation under physiological conditions [154,155].

Historically, fluorescence microscopy and flow cytometry have assumed that stochastic bioconjugation is functionally inert [156]. This view was based on the premise that attaching a small number of low-molecular-weight fluorophores (less than 1 kDa) to a 150 kDa protein would not significantly alter its native conformation. However, quantitative analyses have shown that random bioconjugation can introduce critical thermodynamic disruptions, including changes in antibody binding kinetics and affinity [157].

The methodological challenge in preparing monoclonal antibodies for microscopy and cytometry lies in optimizing the degree of labeling (DOL). In principle, a higher number of fluorophores per antibody should increase the total brightness of the probe and improve the signal-to-noise ratio [151]. However, the addition of bulky fluorogenic molecules brings strict physicochemical limitations. Theoretically, as the DOL increases, the possibility of generating steric hindrance near or within the CDRs also increases [158]. This structural interference imposes an inherent thermodynamic cost, alters the dissociation constant (K_d), and leads to a systematic reduction in affinity for the target epitope [157].

Beyond reducing recognition capacity, a high degree of labeling (DOL) substantially modifies the protein surface by accumulating predominantly hydrophobic organic molecules. Elevated dye density establishes a microenvironment that favors unfavorable

dye-dye interactions, leading to dimer formation and self-quenching via energy transfer [159]. Consequently, emission intensity exhibits a non-linear relationship with the number of bound fluorophores, introducing significant bias in receptor density quantification, as in the case of ErbB2 [157]. Additionally, the presence of hydrophobic tags generates thermodynamic forces that drive immunoglobulin structural rearrangements to reduce solvent exposure, often leading to localized denaturation or aggregation [160].

In summary, while somatic mutations redefine ErbB2 as a structural target, visualization tools have inherent structural limitations. To accurately interpret ErbB2 dynamics in oncogenesis, it is essential to address these methodological artifacts so that biophysical observations reliably reflect the biological characteristics of the system.

3 AIMS

1. To characterize and evaluate the functional and biophysical impact of the degree of labeling of fluorescent conjugates on the epitope binding capability of trastuzumab.
2. To investigate the effect of selected missense mutations in different domains on the behavior, organization, and dynamics of the ErbB2 receptor at the plasma membrane.
3. To determine whether the biophysical properties of these mutant receptors are directly linked to their cell signaling and oncogenic potential

4 MATERIALS AND METHODS

4.1 Cells

Chinese hamster ovary (CHO), SK-BR-3, and THP-1 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to their specifications. For microscopic experiments, cells were cultured in 8-well chambers (Ibidi, 80826, Gräfelfing, Germany), and for flow cytometry, they were harvested by trypsinization.

4.2 Plasmids and transfection

The constructs expressing mutant ErbB2 were prepared by our collaboration partner Irene Tiemann-Boege at the Johannes Kepler University (Linz, Austria) as previously described [161]. The missense and nonsense mutations in the ErbB2 variants are shown in Figure 9. ErbB2-expressing CHO cells were generated by transient transfection of the wild-type or one of the mutant ErbB2-pcDNA3.1 plasmids using Lipofectamine® 2000 (Thermo Scientific, Waltham, MA, USA). ErbB2 and EGF receptor-coexpressing CHO cells were generated by double transfection in a 1:1 ratio by one of the ErbB2 plasmids and human EGFR cloned into pcDNA3 (from Donna Arndt-Jovin, Max Planck Institute for Multidisciplinary Sciences, Göttingen, Germany) [162]. To measure the trastuzumab effect on ErbB2 dimerization, pcDNA3.1-ErbB2-YFP and pcDNA3.1-ErbB2-CFP plasmids were co-transfected in a 1:1 ratio using Lipofectamine® 2000.

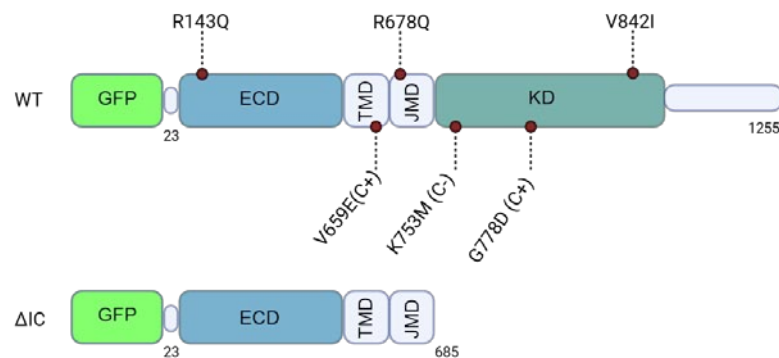


Figure 9. **Schematic representation of the plasmid construct of full-length and the truncated (ΔIC) ErbB2 variants.** Diagram of the structural domains (ECD, TMD, JMD, and KD) fused with GFP in the N-terminal. It displays the mutations of interest distributed in the three functional domains: R143Q in the ECD, R678Q in the JMD, and V842I in the KD. As reference controls, it includes active signaling variants, V659E (TMD) and G778D (KD), and the kinase-dead variant, K753M, which lacks catalytic activity. The ΔIC lacks the ICD (amino acids 685-1255), eliminating the kinase activity and the autophosphorylation sites. The red dots indicate the exact position of every

amino acid substitution. Amino acid numbering in the figure corresponds to the GFP-free version of ErbB2.

4.3 Antibodies and their fluorescent labeling

We used Alexa Fluor 564-trastuzumab and Alexa Fluor 647-trastuzumab for labeling ErbB2. Trastuzumab, marketed as Trazimera (New York City, NY, USA), was obtained from Pfizer as a lyophilized powder intended for preparation of an infusion for human use. The lyophilized powder containing 150 mg antibody, L-His, L-His hydrochloride monohydrate, saccharose, and polyoxyethylene sorbitan monolaurate, was dissolved in PBS at a final trastuzumab concentration of 21 mg/mL. The antibody was labeled with Alexa Fluor 564 (A-20002, Thermo Scientific, Waltham, MA, USA) and Alexa Fluor 647 (A-20006, Thermo Scientific, Waltham, MA, USA) fluorescent dyes using their N-hydroxysuccinimidyl ester derivatives. Briefly, the antibody to be labeled was dissolved to a concentration of 2 mg/mL in PBS, with a total volume of 750 μ L, supplemented with 75 μ L of 1 M carbonate buffer. Depending on the desired degree of labeling, 3-10 μ L of the N-hydroxysuccinimide derivative of the dye was added to the mixture, followed by a 1-hour incubation at 25°C with constant agitation. Unreacted dye was removed by passing the reaction mixture through a Sephadex G-50 column (Sigma-Aldrich, GE17-0043-02, St. Louis, MO, USA), which removed molecules smaller than $\sim$ 30 kDa. The degree of labeling was determined by spectrophotometry. The absorbance of the labeled antibody stock was measured at two wavelengths corresponding to the absorption maximum of the dye and to the absorption of amino acids in the UV range (280 nm):

$$A(\lambda_{dye}) = A_{dye}(\lambda_{dye}) = \varepsilon_{dye} c_{dye,M} L \quad 1$$

$$A(280) = A_{IgG}(280) + A_{dye}(280) = \varepsilon_{IgG} c_{IgG,M} L + A_{dye}(\lambda_{dye}) CF \quad 2$$

where A is the optical density of the species specified in the subscript, measured at a wavelength specified in parentheses. ε_{IgG} is the molar absorption coefficient of the antibody at 280 nm and ε_{dye} is the molar absorption coefficient of the dye at its absorption maximum. $c_{dye,M}$ and $c_{IgG,M}$ are the molar concentrations of the dye and the antibody, respectively, L is the optical path length in cm, and CF is a correction factor specifying the relative optical density of the dye at 280 nm compared to its absorption at its absorption maximum. These correction factors are available from the manufacturer or can be determined by measuring the absorption spectrum of the free dye. If the optical density of the dye at its absorption maximum exceeds 2 OD units, the stock solution must be diluted, and the calculation must

account for the dilution factor (DF). The molar concentrations of the dye and the antibody were determined from the equations above, and their ratio is the degree of labeling. Trace amounts of unreacted dye and residual amino acid additive conjugated to fluorescent dyes may remain in the stock solutions, but they do not interfere with the microscopic measurements because only the fluorescent antibodies bound to their antigen contribute to the measured signals, owing to extensive washing after cell labeling.

In order to quantify phosphotyrosines, monoclonal antibody PY99 (sc-7020, Santa Cruz Biotechnology, Dallas, TX, USA) was used, while ErbB2 phosphorylated at Tyr1248 and EGFR phosphorylated at sites Tyr1048 and Tyr1173 was quantified by mouse monoclonal antibodies Ab18 (MS-1072, Thermo Scientific, Waltham, MA, USA), EM-1 (11-575, ExBio, Vestec, Czech Republic) and 1H12 (2236, Cell Signaling, Danvers, MA, USA), respectively, followed by staining with Alexa Fluor647-conjugated goat anti-mouse IgG (A21236, Thermo Scientific, Waltham, MA, USA).

4.4 Functional tests of antibodies

Antibody binding to cell surface-expressed receptors was investigated by labeling 10^5 freshly harvested cells, washed twice in ice-cold phosphate-buffered saline (PBS; pH 7.4), in 100 μ L PBS supplemented with 1 mg/mL BSA on ice in the dark for 30 min. Unbound antibodies were removed by washing twice in PBS, and the resuspended samples were kept on ice until the measurement. To determine the saturation curve of fluorescently tagged antibodies, a series of cell samples was incubated with varying concentrations of the antibody, and their fluorescence intensity was measured by flow cytometry followed by analysis in FCS Express (Pasadena, CA, USA, developed by De Novo Software). Background correction was performed by subtracting the average intensity of an unlabeled sample from the mean fluorescence intensities of every other sample. The data were then fitted to a single-site binding equation to determine the dissociation constant. Fitting was performed in GraphPad Prism (San Diego, CA, USA, developed by GraphPad Software) by selecting the “One site – Specific binding” model in the “Saturation binding” fitting module. In order to determine the affinity of an unlabeled antibody, 10^5 cells were incubated with a concentration series of the unlabeled antibody in the presence of a constant concentration of dye-conjugated antibody against the same epitope in 100 μ L PBS supplemented with 1 mg/mL BSA on ice in the dark for 30 min. Unbound antibodies were removed by washing twice with PBS, and the resuspended samples were kept on ice until flow cytometric

measurement. After background correction, data analysis was performed by assuming two ligands (labeled and unlabeled antibodies) competing for a single binding site. Fitting was performed by selecting the “One site – Fit logIC50” model in the “Competitive binding” fitting module of GraphPad Prism.

4.5 Staining of cells for measuring protein clustering and lateral diffusion

CHO cells transiently transfected with wild-type or one of the mutant ErbB2 versions were stained with a mixture of AlexaFluor564-trastuzumab and AlexaFluor647-trastuzumab, followed by measuring ErbB2 homoclustering using intensity-based Förster resonance energy transfer (FRET) between the donor and the acceptor on a Zeiss LSM880 confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany). To measure heterodimerization between the EGF receptor and ErbB2, CHO cells were co-transfected with plasmids encoding wild-type EGF receptor and one of the ErbB2 variants. Twenty-four hours after transfection, cells were subjected to overnight serum starvation in medium containing 0.1% serum. For measuring EGFR-ErbB2 heteroassociation under unstimulated conditions, cells were labeled with a mixture of TAMRA-EGF (50 nM, E3481, ThermoFisher Scientific) and AlexaFluor647-trastuzumab (20 µg/mL) for 20 min on ice followed by washing and fixation in 1% formaldehyde for 20 min on ice. Since the cells were kept on ice throughout the procedure, TAMRA-EGF did not stimulate them. To measure heteroassociation between EGFR and ErbB2 in EGF-stimulated samples, cells were labeled with 50 nM TAMRA-EGF for 20 min on ice, then incubated for 10 min at 37°C to allow EGF to stimulate the cells. Afterwards, these samples were labeled by 20 µg/mL AlexaFluor647-trastuzumab for 20 min on ice, followed by washing and fixation. To measure lateral mobility, cells were single- or double-transfected as described above, and were serum-starved overnight for 2 days after transfection. Unlabeled EGF at a concentration of 50 nM was added to the cells on the thermostated stage of the microscope at 37°C for 10 min, followed by measuring the lateral mobility of ErbB2 both in these EGF-stimulated and the unstimulated cells using fluorescence recovery after photobleaching (FRAP) based on the GFP fluorescence of the ErbB2 constructs.

4.6 Measurement of protein-protein interactions using FRET

Images were acquired with a Zeiss LSM 880 confocal microscope (Carl Zeiss, Oberkochen, Germany) using a C-Apochromat 40× (NA=1.2) objective. Fluorescence in the

donor channel (Alexa Fluor546) was excited at 543 nm and recorded in the spectral range 548-628 nm. Directly excited fluorescence of AlexaFluor647 was detected in the range of 638-755 nm after excitation at 633 nm. The donor excitation wavelength (543 nm) and the acceptor emission range (638-755 nm) were used to record acceptor-sensitized emission (FRET channel). Images were analyzed using rFRET, a custom MATLAB program that implements DipImage commands [163]. First, every image was segmented using manually seeded watershed segmentation. All subsequent calculations were performed only on membrane pixels that formed the foreground in the resulting mask images. Overspill parameters of the donor were determined using donor-only samples. Overspill of the donor into the FRET channel was calculated by fitting a line to the FRET channel fluorescence vs. donor channel fluorescence plot using Deming regression. The slope of the line fitted to the graph showing fluorescence in the acceptor channel vs. fluorescence in the donor channel was used as the donor-to-acceptor overspill. A similar principle was used to determine acceptor overspill parameters by using cells labeled with the acceptor only. Furthermore, the α -value corresponding to the ratio of the intensity of an excited acceptor, measured in the FRET channel, to that of an excited donor, measured in the donor channel, was calculated using two microbead samples labeled with equal numbers of donors and acceptors [164]. Once the overspill parameters and the α -value were determined, the FRET efficiency was calculated using the pixel-by-pixel method available in rFRET [165]. Because the laser intensities and detector voltages were adjusted identically in every experiment, the overspill parameters ($S_1 \approx 0.05$, $S_2 \approx 0.03$, $S_3 \approx 9 \cdot 10^{-5}$, $S_4 \approx 0.003$) and the α -value (≈ 0.6) did not change significantly between experiments.

4.7 Measurement of lateral diffusion

CHO cells transfected with GFP-containing constructs of wild-type or mutant ErbB2 were seeded at a density of $2 \cdot 10^4$ cells/well on μ -slide 8-well chambered coverglass (iBIDI) pre-treated with 0.1 mg/mL poly-L-lysine for 15 minutes and dried for 30 minutes. Before measurement, the cells were washed with PBS to remove any residual medium, and preheated Hank's Balanced Salt Solution was added to the wells. Fluorescence recovery after photobleaching (FRAP) measurements were carried out at 37°C using a C-Apochromat 40 $\times$ (NA=1.2) water-immersion objective on a Zeiss LSM 880 confocal microscope. The ErbB2-GFP fusion protein was excited at 488 nm, and its fluorescence was detected between 493 and 630 nm. Fluorescence intensity was recorded before and after photobleaching in a pre-

determined region of interest (ROI) in the cell membrane (bleached ROI). Three images were taken before bleaching to determine the initial fluorescence intensity, followed by bleaching the ROI to 30-60% of the initial intensity using the 543 nm laser. Fluorescence recovery was monitored until fluorescence in the bleached ROI no longer increased (3-4 min). The image series was analyzed in Matlab using a custom-written program, FRAPfit [52]. The stacks were corrected for displacement (registration), and then three ROIs were defined: the bleached ROI, whose coordinates were obtained from the stored stack; the unbleached or total cell ROI generated by watershed segmentation; and the background ROI defined by specifying a cell-free area in the stack. Raw intensity values were triple-normalized, setting the intensity of the bleached ROI to 1 prior to the bleaching pulse and to 0 immediately after. The following double exponential recovery function was fitted to the triple-normalized curves:

$$R(t) = (1-b) \left[f_1 \left(1 - e^{-\frac{t}{\tau_1}} \right) + (1-f_1) \left(1 - e^{-\frac{t}{\tau_2}} \right) \right] \quad 3$$

where $R(t)$ is the time-dependent, triple-normalized recovery of fluorescence, b is the immobile fraction, τ_1 and τ_2 are the empirical recovery time constants, and f_1 is the fractional contribution of the first component to the recovery. By fitting this double exponential function to the experimental FRAP recovery curves, we can mathematically model the kinetics of fluorescence recovery over time. Within this model, the immobile fraction (b) characterizes the proportion of receptors that do not recover their fluorescence during the observation window, reflecting molecules anchored or trapped in the membrane. In contrast, the recovery time constants (τ_1 and τ_2) are inversely related to the speed of lateral diffusion of the mobile receptor pool. The amplitude-weighted time constant of the exponential function was calculated and, together with the immobile fraction, used to characterize the lateral mobility of the ErbB2 variants. The amplitude-weighted time constant was in the range of 10-40 s, whereas τ_1 and τ_2 ranged from 5 to 25 s, and from 60 to 140 s, respectively. The rapid recovery typically accounted for ~50-80% of the total recovery, and both components of recovery responded to EGF treatment. Since no meaningful relationship could be established between the components, the variants, and the treatment, and both changed upon EGF stimulation, only their amplitude-weighted average will be shown.

4.8 Measurement of tyrosine phosphorylation

CHO cells were transiently transfected with the wild-type ErbB2 or one of its mutant variants, or co-transfected with one of the ErbB2 versions and EGFR. To measure baseline ErbB2 phosphorylation in single transfected cells 24 hours after transfection, the cells were fixed with 3.7% paraformaldehyde for 30 minutes at 4°C, followed by permeabilization with 0.1% Triton X-100 for 10 minutes on ice. For the double-transfected cells, twenty-four hours after transfection, the cells were cultured in serum-starvation medium (0.1% fetal bovine serum) overnight, followed by stimulation with 50 nM of EGF for 10 min at 37°C. Afterwards, the cells were fixed and permeabilized as mentioned before. In both cases, after permeabilization, cells were labeled with one of the following primary antibodies in 0.1% BSA + 0.1% Triton X-100 in PBS for 30 min at 4°C: 2.5 µg/mL Ab18 (against ErbB2 phosphorylated at Tyr1248), 1 µg/mL PY99 (pan-phosphotyrosine antibody), 3 µg/mL pEGFR Y1048, or with 2 µg/mL pEGFR Y1173. Staining with primary antibodies was followed by staining with a 4 µg/mL solution of goat anti-mouse Alexa Fluor 647 secondary antibody. After antibody labeling, the cells were fixed with 1% formaldehyde for 20 min at 4°C and visualized using a Nikon N-STORM (Tokyo, Japan) confocal microscope equipped with an Apo 60× water-immersion objective. For analysis, images were segmented using manually seeded watershed segmentation in the GFP channel, yielding a mask of cell boundaries. All image analysis was performed in MATLAB (MathWorks, Natick, MA, USA) using custom-written programs that implemented commands from DipImage (Delft University of Technology, Delft, The Netherlands). The GFP channel and the PY99-, Ab18-, pEGFR Y1048-, or pEGFR Y1173-stained images were extracted to measure tyrosine phosphorylation levels, with the GFP image used to define regions of interest and the PY99, Ab18, pEGFR Y1048, or pEGFR Y1173 intensities quantifying tyrosine phosphorylation levels. Background correction was performed using a region of interest placed in a cell-free area of the image, and background-corrected phosphorylation values were exported for subsequent analysis.

4.9 Statistical analysis

Mathematical image processing and data extraction were performed in MATLAB using custom routines (such as rFRET and FRAPfit) based on DipImage commands, while flow cytometry analysis was performed in FCS Express, and general statistical analysis, along with curve fitting, was carried out in GraphPad Prism. Quantitative data are generally

expressed as the mean of three independent experiments $\pm$ standard deviation (SD). For the evaluation of biophysical parameters, data were fitted to single-site and competitive binding models to determine antibody affinity, Deming regression lines to calculate spectral overlap in FRET assays, and empirical biexponential recovery functions to calculate lateral mobility in FRAP. To determine statistical significance between the experimental groups, we applied one-sample t-tests adjusted using the Bonferroni correction, one-way ANOVA with Dunnett's multiple comparison test to compare mutant variants against the wild-type receptor, and two-way ANOVA followed by Sidak or Dunnett post hoc test to analyze experiments with multiple variables and treatments. In the figure legends, it is specified which analysis is done per experiment. Finally, correlation coefficients were calculated to assess trends in bivariate analyses, with a significance threshold of $p < 0.05$ set for all tests, these levels are indicated in the graphical representation using asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

5 RESULTS

To explore how domain-specific amino acid substitutions alter the membrane behavior of ErbB2, we focused on three clinically relevant ErbB2 missense mutations, R143Q, R678Q, and V842I, localized in the extracellular, juxtamembrane, and kinase domains, respectively (Figure 9). These mutations are identified across large pan-cancer cohorts [130,166] or circulating tumor DNA studies [167]. This observation highlights their potential relevance as “pre-sensitized” loci that could lead to functional significance upon somatic selection in tumors. We aimed to determine whether the biophysical properties of these mutant receptors are directly linked to their signaling and oncogenic potential, or whether the mutations instead cause more subtle disruptions in receptor oligomerization and diffusion that can only be detected by correlating multiple parameters.

5.1 Validation of the fluorophore-conjugated trastuzumab antibody.

However, before evaluating the membrane dynamics of ErbB2 variants, it was important to validate fluorophore-conjugated trastuzumab as our primary detection tool. Although it is acknowledged in the literature that the affinity of an antibody to its epitope is impaired by fluorescence labeling [151,157,168,169], it is neither known whether antibody functions are affected in a similar way, nor is the mechanism behind this phenomenon certain. To address the former question, we selected several known IgG interaction partners and tested how their binding depends on the degree of labeling. These selected partners included protein A, protein G, and Fc γ receptors. Protein A and protein G binding is mediated by the junction of the CH2 and CH3 domains [149,170,171], whereas binding to Fc γ receptors takes place through the hinge-proximal region of the CH2 domain [149,172]. Additionally, the overall structural integrity of the Fc portion was assessed by measuring the ability of anti-Fc γ antibodies to bind to unlabeled and labeled antibodies. Trastuzumab was labeled with AlexaFluor546 or AlexaFluor647 fluorescent dyes to achieve a range of different degrees of labeling, followed by measuring the binding of trastuzumab to its epitope (ErbB2 in SK-BR-3 cells), protein A, protein G, Fc γ receptors, or to anti-Fc antibodies. All of these five antibody functions revealed remarkably similar dependence on the degree of labeling for both antibodies and for both dyes (Figure 10), where it can be seen that a high DOL leads to a progressive loss of affinity, especially in the Fc recognition region of protein A, G, and cellular receptors.

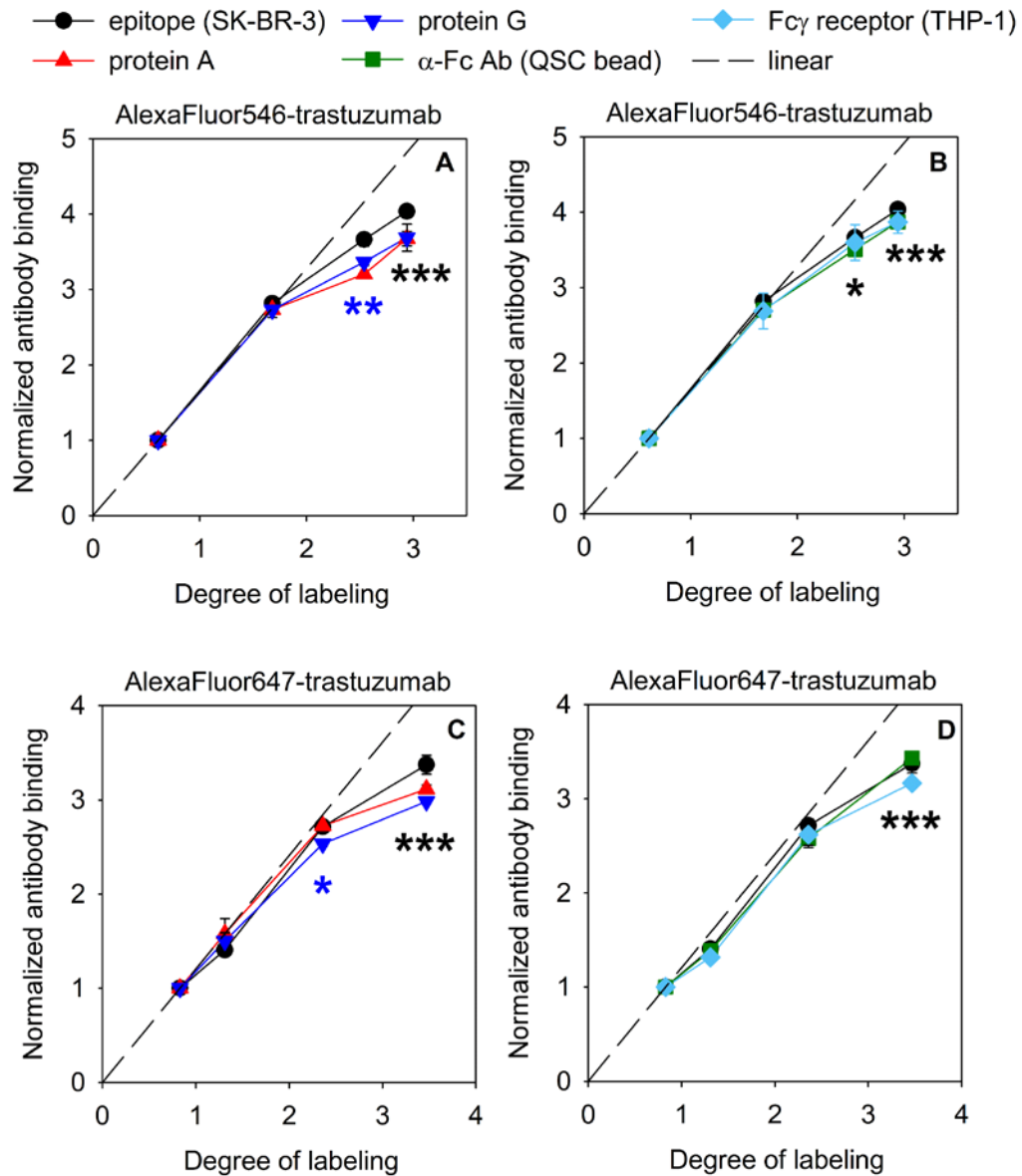


Figure 10. Effect of the degree of labeling (DOL) on the functionality of trastuzumab. Trastuzumab was labeled either with AlexaFluor546 (A, B) or AlexaFluor647 (C, D) fluorophores. Antibody batches with four different degrees of labeling were generated, and their degree of labeling was determined by spectrophotometry. The interaction of trastuzumab with its specific epitope (i.e., ErbB2) in SK-BR-3 cells, with protein A, protein G, anti-Fc antibodies, and Fc γ R expressed** in THP-1 cells was compared. The dashed line indicates theoretical linear behavior. The graphs show the mean of three independent experiments ($\pm$ the standard error of the mean). The asterisks indicate that the normalized antibody binding is significantly different from the linear expectation (the dashed line) that would be observed if the fluorescence intensity of the bound antibody fraction were proportional to the degree of labeling. One-sample t-tests were carried out, and significant differences were declared at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$., with multiple comparisons adjusted using Bonferroni correction.

Since we considered epitope binding the most important antibody function for cell biological applications, we quantitatively determined the dissociation constant characterizing antibody binding to cell-surface-exposed antigens (Figure 11). A higher DOL resulted in an increase of the dissociation constant (K_d) for both conjugated fluorophores,

AlexaFluor546 and AlexaFluor647 (Figure 11A), suggesting a loss in binding affinity induced by the density of fluorophores. While the unlabeled antibody exhibits a basal K_d of ~ 2.5 -3 nM, highly labeled samples showed a significant decrease in binding affinity, reaching a K_d of 10 nM. A representative saturation curve of AlexaFluorA546-conjugated trastuzumab (Figure 11B) and a competitive assay with unlabeled trastuzumab (Figure 11C) demonstrate the fitting procedure. This confirmed that the impact on the stability of the antigen-antibody complex is critical at medium and high DOL levels, as the interference with the epitope recognition sites is more notable. As the impact of A546- and A647-conjugated trastuzumab was similar, it was decided to use conjugated trastuzumab with a DOL of ~ 1 .

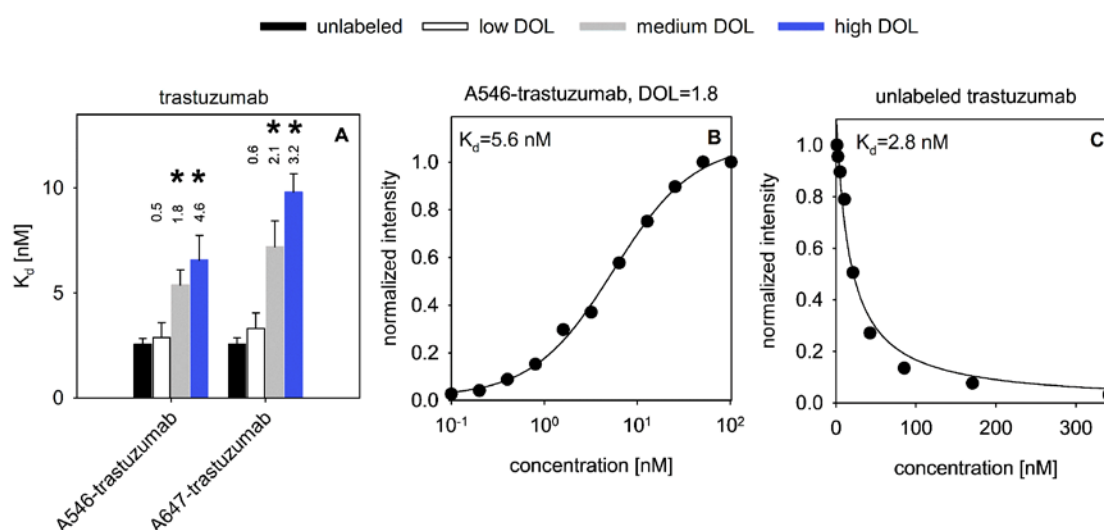


Figure 11. **Deterioration of IgG as a function of the degree of labeling.** Trastuzumab IgG. (A) was labeled by AlexaFluor546 (A546 in the figure) or AlexaFluor647 (A647 in the figure) fluorophores. The numbers above the bars indicate the degree of labeling, as determined by spectrophotometry. The dissociation constant (K_d) of the labeled antibodies was determined by measuring the fluorescence intensity of cell samples labeled by different concentrations of the IgG using flow cytometry, followed by fitting a single-site binding equation to the data. A representative curve of such a measurement is shown in B. The K_d of unlabeled antibodies was determined by incubating cells with a constant concentration of a previously determined labeled antibody and varying concentrations of the unlabeled antibody of the same idiotype, then fitting the data to a competitive binding equation. A representative curve from such an experiment is shown in C. The bars in parts A show the mean of three independent experiments ($\pm$ standard error of the mean). The asterisks indicate significant differences between K_d -s of the labeled and unlabeled antibodies (Dunnett's multiple comparisons test carried out after significant results in 2-way ANOVA, $p < 0.05$).

5.2 Reliability of trastuzumab in ErbB2 labeling

Since the labeling strategy includes labeling control and EGF-treated cells with trastuzumab, we wanted to exclude the possibility that EGF-induced EGFR-ErbB2 heterodimers do not bind trastuzumab. Therefore, we compared the fluorescence intensity

of trastuzumab in control- and EGF-pretreated cells and found no significant EGF-induced change in trastuzumab binding to ErbB2, indicating that EGF treatment does not interfere with trastuzumab binding (Figure 12A). These experiments also showed that the approximate membrane expression level of ErbB2 was comparable in all samples except for the one transfected with the Δ IC variant, in which the surface expression level of ErbB2 was ~3-fold higher than in the other variants, regardless of whether it was co-transfected with EGFR (Figure 12A) or single-transfected (Figure 12B).

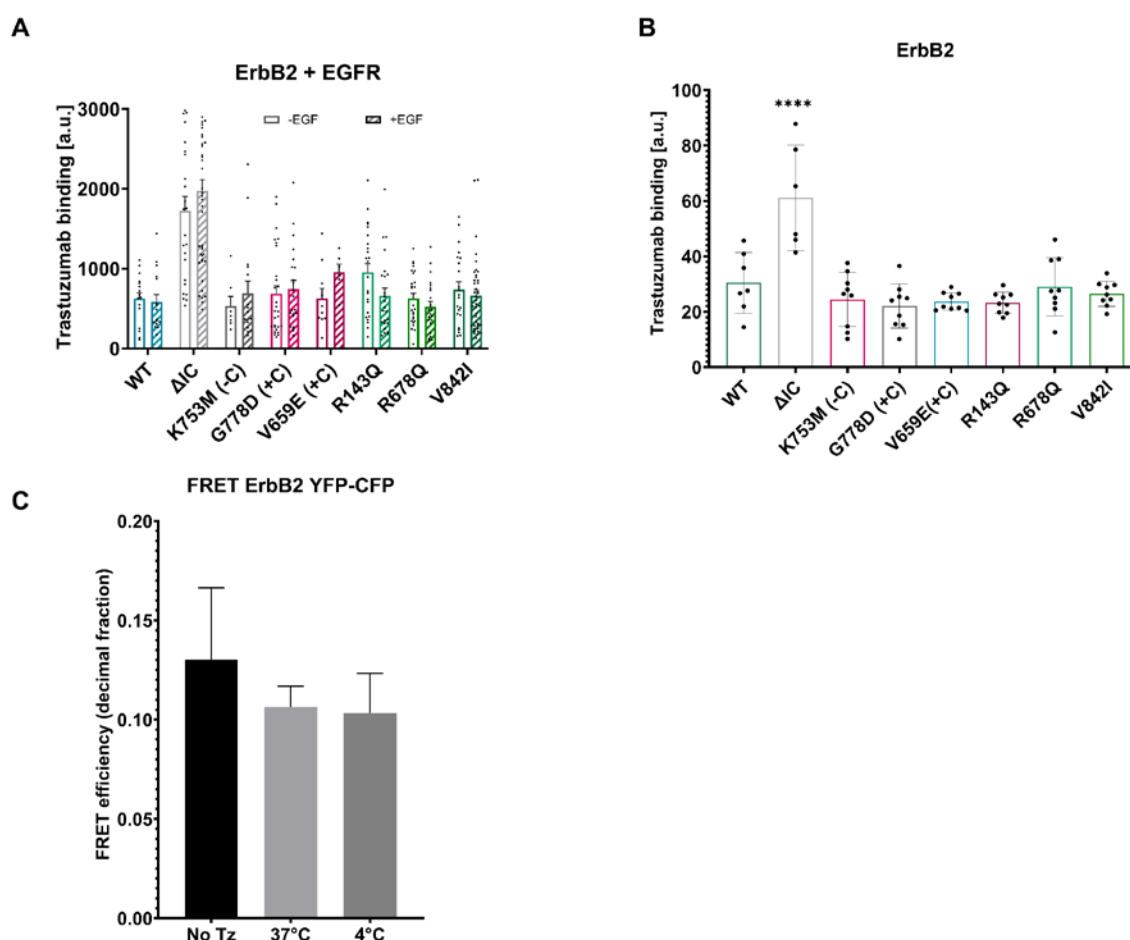


Figure 12: Trastuzumab binding and interaction dynamics with the ErbB2 receptor. (A) CHO cells were double-transfected with wild-type ErbB2 or variants and EGFR, followed by overnight serum starvation twenty-four hours after transfection. The cells were stimulated with 50 nM of unlabeled EGF for 10 min at 37°C. Both the stimulated and non-stimulated cells were labeled with 20 μ g/mL AlexaFluor647-trastuzumab for 20 min on ice with subsequent washing, fixation, and confocal microscopic imaging for measuring trastuzumab binding. The bars show the mean of three independent experiments ($\pm$ SD). Two-way ANOVA was performed and no significant difference between the before and after treatment was observed. (B) CHO cells were transfected with wild-type ErbB2 or variants, followed by labeling with 20 μ g/mL AlexaFluor546-trastuzumab for 20 min on ice with subsequent washing, fixation, and confocal microscopic imaging for measuring trastuzumab binding. The bars show the mean of three independent experiments ($\pm$ SD). Asterisks correspond to significant differences compared to the wild-type determined by Dunnett's test after one-way ANOVA (**** p <0.0001). (C) CHO cells were transfected with plasmid *pcDNA3-ErbB2-YFP* and

pcDNA3-ErbB2-CFP and labeled at 4°C and 37°C for 30 min, followed by washing, fixation, and confocal microscopic imaging for measuring ErbB2-YFP/CFP heteroassociation. One-way ANOVA was performed revealing no significant difference between the treatments.

Additionally, it has been suggested that trastuzumab induces dimerization upon binding [173]. To rule out this possibility, we co-transfected CHO cells with ErbB2 constructs containing YFP or CFP at the N-terminal. The transfected cells were labeled with trastuzumab at 4°C or 37°C, and the YFP-CFP heteroassociation was measured. There was no significant difference in ErbB2 homodimerization between the two labeling temperatures, indicating that trastuzumab did not induce ErbB2 dimerization (Figure 12C).

5.3 Measurement of homo- and heterodimerization of ErbB2 variants by Förster Resonance Energy Transfer (FRET)

Since receptor activation begins with alterations in receptor clustering and lateral diffusion, we measured homo- and heteroclusters in CHO cells transiently transfected with different ErbB2 variants (Figure 9). We compared the wild-type with two negative controls, a kinase-dead variant (K753M) [174] and a truncated mutant lacking the intercellular domain (Δ IC), two gain-of-function positive controls (+C), V659E [36] and G778D [175], and the three selected ErbB2 variants (R143Q, R678Q, and V842I). Although overexpressed ErbB2 is expected to form homodimers or homoclusters [176–178], FRET measurements did not provide evidence for strong ErbB2 homoassociation in any of the cell lines transfected with one of the ErbB variants (Figure 13B). While uncertainty in the FRET calibration factor α can lead to an underestimation of the energy transfer efficiency [179], the fact that the ErbB2 variants were identical within experimental error indicates that the mutations did not significantly alter their homoassociation tendency.

Since CHO cells are practically devoid of endogenous ErbB receptor expression [168], we co-transfected them with wild-type EGFR and one of the ErbB variants to generate an expression profile more characteristic of the *in vivo* situation. All ErbB2 variants heteroassociated with EGFR, as evidenced by FRET efficiency ranging from 20–30%, with no statistically significant differences (Figure 13C). Stimulation with EGF resulted in a significantly enhanced heterointeraction between EGFR and wild-type ErbB2, and this effect was preserved for the Δ IC variant lacking the whole intracellular domain and the V659E mutant harboring a mutation in the transmembrane domain (Figure 13C). However, neither the kinase-dead (K753M), nor the version with the mutation-activated kinase domain

(G778), nor the three selected, cancer-associated ErbB2 variants (R143Q, R678Q, V842I) showed any change in their heteroassociation with EGFR upon EGF treatment.

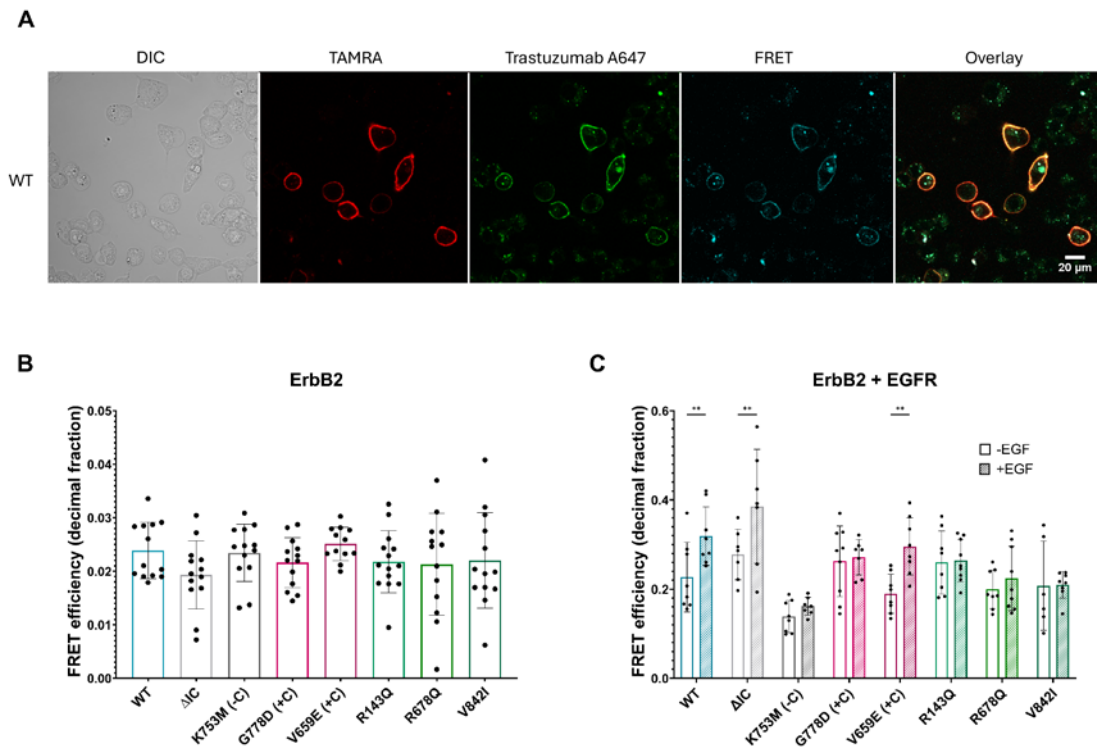


Figure 13. FRET-based analysis of ErbB2 homo- and heteroassociation. (A) Representative images of cells for FRET measurements. A differential interference contrast (DIC) image, as well as images in the donor (TAMRA), acceptor (Trastuzumab A647), and FRET channels, were recorded. **(B)** CHO cells were transfected with wild-type ErbB2 or variants, followed by labeling with a mix of 10 μ g/mL AlexaFluor546-trastuzumab and 10 μ g/mL AlexaFluor647-trastuzumab for 20 min on ice with subsequent washing, fixation, and confocal microscopic imaging for measuring ErbB2 homoassociation. The bars show the mean of three independent experiments ($\pm$ SD). One-way ANOVA was performed revealing no significant difference between the cell lines. **(C)** CHO cells were transfected with one of the ErbB2 variants and wild-type EGFR, followed by overnight serum starvation twenty-four hours after transfection. To measure quiescent cells, double labeling with 50 nM EGF-TAMRA and 20 μ g/mL AlexaFluor647-trastuzumab was performed on ice, preventing fluorescent EGF from stimulating the cells (empty bars). To measure EGF-stimulated cells, they were stained with TAMRA-EGF and warmed to 37°C for 10 min (hatched bars), then labeled with AlexaFluor647-trastuzumab on ice. In both cases, after double-labeling, the cells were washed and fixed with 1% PFA. The bars show the mean of three independent experiments ($\pm$ SD). Pairwise comparisons using the Sidak multiple comparisons test between non-stimulated and EGF-stimulated conditions were carried out after significant results in two-way ANOVA (** p <0.01).

5.4 Lateral mobility of ErbB2 variants measured by fluorescence recovery after photobleaching (FRAP)

We next assessed the lateral diffusion of the ErbB2 variants in living cells to determine how each mutation affects receptor mobility within the membrane. When comparing the lateral mobility of ErbB2 versions expressed as the only member of the ErbB

family, the positive controls (V659E, G778D) revealed ~2-fold faster recovery times (Figure 14B), and the V659E variant also showed an ~25% reduced immobile fraction (Figure 14C) than the wild-type, implying higher mobility. The Δ IC variant lacking the whole intracellular domain was even more mobile with a 4-fold lower immobile fraction (Figure 14C) than the WT variant, most likely due to the absence of potential cytoskeletal interactions. The cancer-associated ErbB2 variants, R143Q, R678Q, and V842I, exhibited ~2-fold faster recovery and 10-30% lower immobile fractions than the wild-type, resembling the positive controls (Figure 14BC). It can be concluded that the mutationally activated positive controls and the cancer-associated ErbB2 versions exhibit accelerated diffusion when expressed as the sole member of the ErbB family.

We also examined the lateral mobility of the ErbB2 variants in an EGFR-expressing background. The lateral diffusion of wild-type ErbB2 co-expressed with EGFR in the absence of EGF stimulation was 2-fold faster than in cells expressing ErbB2 as the only member of the ErbB family (Figure 14D). Stimulation of these cells with EGF led to retarded diffusion of ErbB2 as revealed by a slower recovery time (Figure 14E) and a higher immobile fraction (Figure 14E). The kinase-dead K753M version did not respond to EGF stimulation regarding its lateral mobility, although its baseline mobility did not show any obvious difference from the wild-type before EGF stimulation. All the other versions, including the Δ IC and all the kinase-active variants, responded to EGF by a retarded diffusion without a noticeable correlation between kinase activity and lateral mobility (Figure 14DE).

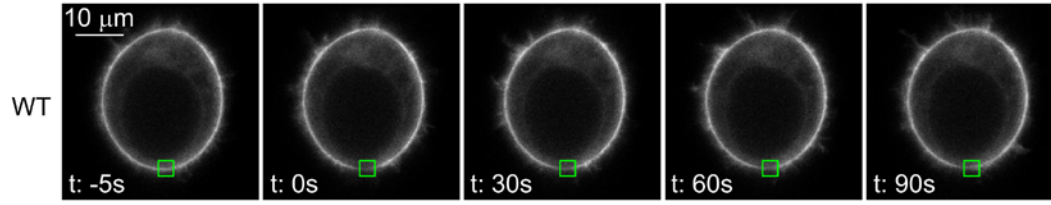
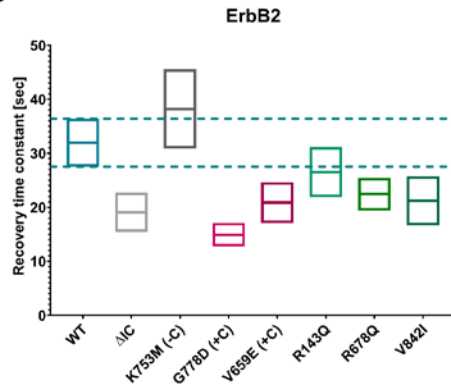
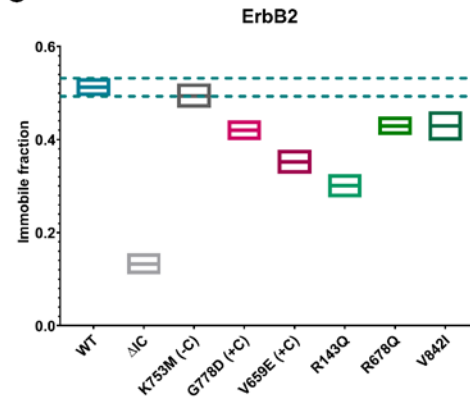
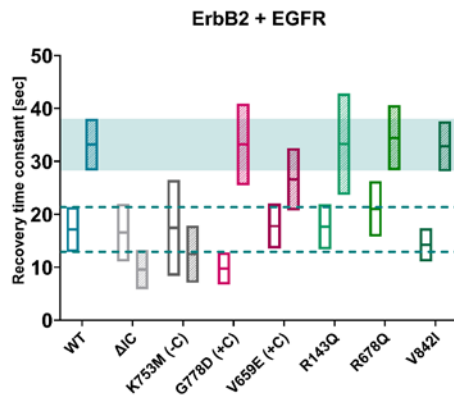
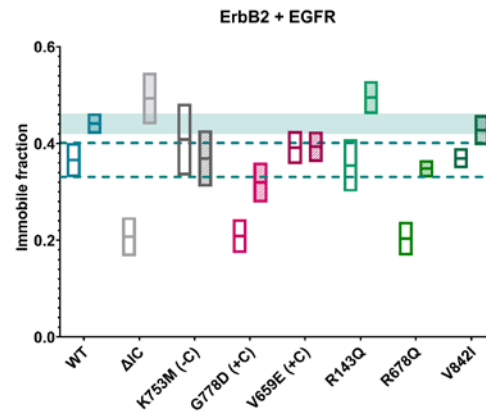
A**B****C****D****E**

Figure 14: Lateral mobility of ErbB2 with or without EGFR coexpression. (A) Representative images of cells for FRAP measurements. The image sequence shows the pre-bleach image (t: -5) followed by three images recorded during the recovery period (t: 30s, 60s, 90s). The green rectangle indicates the bleached region of interest where recovery was monitored. (B-C) CHO cells were transfected with one of the ErbB2 variants, and FRAP experiments were carried out using the GFP tags of the proteins. (D-E) For the investigation of ErbB2 variants co-expressed with EGFR, CHO cells were transfected with one of the ErbB2 variants and the wild-type EGFR. The cells were serum-starved overnight, then, 24 hours after transfection, the lateral mobility of ErbB2 in these unstimulated cells was measured (empty boxes). EGF stimulation was achieved by warming the samples to 37°C on the microscope stage for 10 min, allowing the prebound, unlabeled EGF to stimulate the cells, followed by measurement of ErbB2 lateral mobility (hatched boxes). Recovery curves were averaged and fitted by a double-exponential function from which the amplitude-weighted recovery time constants (B, D) and the immobile fractions (C, E) were determined as described in detail in the Methods section. The fit parameters and their 95% confidence intervals are plotted in the graph. In panels B, C, D and E the mean of three independent experiments is represented.

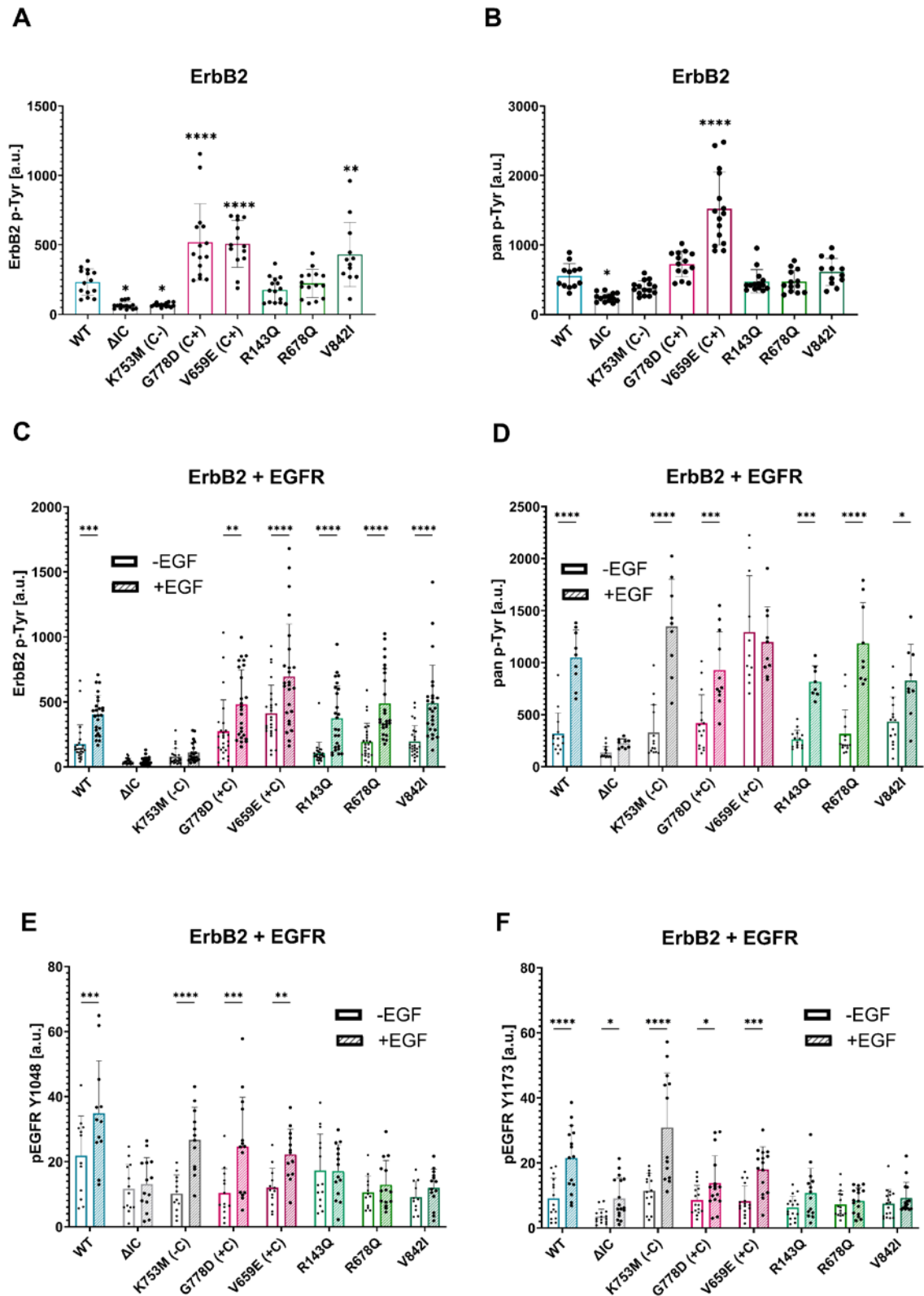


Figure 15: Tyrosine phosphorylation state of cells expressing ErbB2 variants with or without EGFR coexpression. (A-B) CHO cells transfected with wild-type ErbB2 or one of the mutant variants were stained with Ab18 against ErbB2 phosphorylated at Tyr1248 (A) or with an antibody

against any tyrosine phosphorylation (B) followed by secondary staining and confocal microscopy. Asterisks correspond to significant differences compared to the wild-type determined by Dunnett's test after ANOVA (* $p < 0.05$, *** $p < 0.01$, **** $p < 0.0001$). **(C-D)** CHO cells double-transfected with EGFR and one of the ErbB2 variants were serum-starved overnight. ErbB2 phosphorylation at Tyr1248 (C) and pan-tyrosine phosphorylation (D) measured by immunofluorescence, was quantified in quiescent cells (empty boxes) and cells stimulated by 50 nM EGF (hatched boxes). **(E-F)** CHO cells double-transfected with EGFR and one of the ErbB2 variants were serum-starved overnight. EGFR phosphorylation at Tyr1048 (A) and Tyr1173 (B), labeled and measured by immunofluorescence, was quantified in quiescent cells (empty boxes) and cells stimulated by 50 nM EGF. Pairwise comparisons by Sidak multiple comparisons test between the untreated and EGF-treated samples were carried out after significant results in two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$, **** $p < 0.0001$). In all panels, the mean of three independent experiments is represented.

5.5 Tyrosine phosphorylation in cells expressing the ErbB2 variants with or without EGFR co-expression

To investigate the biological significance of these ErbB2 mutations at the cellular level, we measured the level of ErbB2 phosphorylation at Tyr1248 and the overall increase in total cellular phosphotyrosine. In CHO cells single-transfected with one of the ErbB2 variants and cultured under normal conditions, i.e., in the presence of serum, ErbB2 tyrosine phosphorylation was 3-fold and 5-fold lower in the Δ IC and K753M mutants, respectively, and 2-fold higher in the constitutively active transmembrane domain mutant (V659E) and the variant harboring a kinase domain with increased activity (G778D) compared to the wild-type (WT) (Figure 15A). From the studied ErbB2 substitutions, only the kinase domain variant (V842I) showed a higher tyrosine phosphorylation (Figure 15A). General tyrosine phosphorylation in cells expressing the Δ IC version was 2-times lower, whereas in cells transfected with the V659E mutant, it was 3-fold higher than in the wild-type (Figure 15B).

In CHO cells expressing both EGFR and one of the ErbB2 variants, both the baseline tyrosine phosphorylation (in unstimulated, serum-starved cells) and the EGF-induced response were determined (Figure 15C). Regarding baseline general tyrosine phosphorylation, only the constitutively active V659E mutant exhibited a significant, 2-fold increase compared to the WT (Figure 15C). The general tyrosine phosphorylation in unstimulated cells only differed in the V659E mutant from the wild type (Figure 15D).

We observed a significant, EGF-induced increase in the tyrosine phosphorylation of ErbB2 in all versions except the Δ IC and K753M mutants (Figure 15CD). As far as the EGF-induced increase in general tyrosine phosphorylation is concerned, we observed a similar pattern with the following notable differences (Figure 15D):

- (i) Although the K753M ErbB2 mutant is kinase-dead, EGF was able to elicit increased pan-tyrosine phosphorylation.
- (ii) The V659E constitutively active version was so greatly pre-activated in serum-starved cells that no further EGF-induced response could be elicited.
- (iii) The three variants R143Q, R678Q and V842I responded to EGF-stimulation with the V842I variant having the least response.

Moreover, in the co-transfected cells, EGFR phosphorylation was measured at two tyrosine phosphorylation sites, Y1048 (Figure 15E) and Y1173 (Figure 15F). Out of the three cancer-associated variants, R678Q and V842I showed a ~2-times lower unstimulated pEGFR Y1048 phosphorylation than the WT (Figure 15E) and a similar phosphorylation state in the Y1173 site (Figure 15F). Upon EGF stimulation, none of the cancer-associated variants (R143Q, R678Q, V842I) showed an increase in tyrosine phosphorylation in any of the analyzed tyrosines of EGFR (Figure 15EF).

5.6 Correlations

Overall, the previously presented data showed the behavior of the ErbB2 variants in the absence or presence of EGFR expression. To create a biophysical framework for how structural changes across different domains influence receptor organization and dynamics, it was necessary to integrate all approaches to highlight the membrane-level impacts of individual missense mutations.

For this, we analyzed the correlation between the baseline association between ErbB2 and EGFR and the EGF-induced response. If the inactive ErbB2 versions were not considered (Δ IC, K753M), there was a negative correlation characterized by a correlation coefficient of -0.75 between ErbB2/EGFR heteroassociation and the EGF-induced increase thereof. Although individual ErbB2 variants vary substantially, these results reveal a general tendency: variants with higher baseline activation are less likely to respond to EGF stimulation by increased ErbB2–EGFR heterodimerization (Figure 16A).

However, when the EGF-induced changes in lateral diffusion parameters were plotted against their baseline values, a clear relationship was evident (Figure 16BC). Although the negative controls (Δ IC, K753M) stood out from this obvious negative

correlation, the remaining variants showed that the more immobile ErbB2 is prior to EGF stimulation, the less immobilization response it exhibits after EGF challenge (Figure 16B).

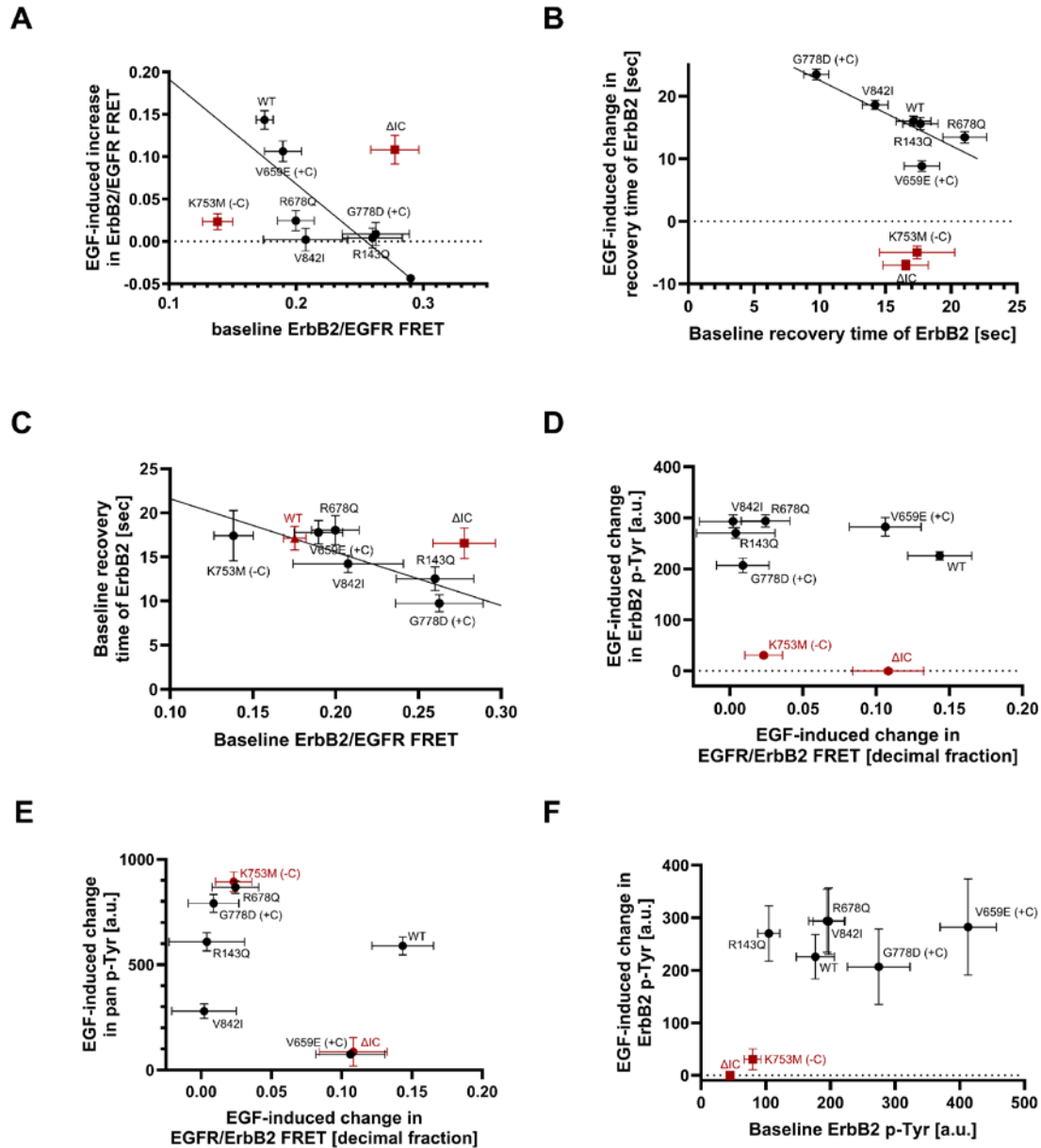


Figure 16: Correlations between the measured biophysical parameters. (A) The EGF-induced increase in the heteroassociation between ErbB2 and EGFR is plotted against the baseline FRET values for their heteroassociation. The correlation coefficient of the line fitted on the black symbols is -0.75. (B) Alternatively, individual curves were also fitted, and the recovery times were averaged to obtain the baseline values and their EGF-induced changes. The EGF-induced change in the mean recovery time constant is plotted against the baseline value before EGF stimulation. The correlation coefficient of the line fitted on the black symbols is -0.91. (C) The FRAP recovery time constants and FRET values measured for the ErbB2/EGFR heterodimerization in the absence of EGF are plotted against each other. A line was fitted to all the points except the one corresponding to the Δ IC. (D) The EGF-dependent increase in ErbB2-specific tyrosine phosphorylation is plotted against the EGF-induced increase in EGFR-ErbB2 heterodimerization. (E) The EGF-dependent increase in general tyrosine phosphorylation (pan p-Tyr) is plotted against the EGF-induced increase in EGFR-

ErbB2 heterodimerization. **(F)** Baseline, ErbB2-specific tyrosine phosphorylation, and its EGF-induced change are plotted against each other. For all panels, the black and red symbols correspond to the active and inactive versions of ErbB2, respectively. The points and the error bars represent the mean and the standard error of the mean, respectively.

Although the activated ErbB2 versions (the positive controls and the cancer-associated variants) did not differ significantly from the wild-type in either heterodimerization or mobility measurements, plotting their baseline FRAP recovery time constants against ErbB2/EGFR heterodimerization revealed another notable correlation. All the activated ErbB2 variants exhibited higher baseline heterodimerization with EGFR than the wild type, and they also tended to show greater lateral mobility (Figure 16C). Although the individual differences are not substantial, an obvious correlation emerges when several ErbB2 mutants spanning a wide range of pre-activation states are investigated. This correlation implies that while growth factor-induced dimerization leads to retarded diffusion of ErbB2 (Figure 14CD), its mutational activation is associated with accelerated diffusion both in the absence (Figure 14BC) and in the presence (Figure 16C) of EGFR coexpression.

Although these investigations aimed to reveal potential differences between the activated mutants and the wild-type ErbB2, no clear correlation was observed in the tyrosine phosphorylation response itself. Therefore, bivariate analyses were performed, and the plot of the EGF-induced increase in ErbB2 tyrosine phosphorylation versus EGFR-ErbB2 heterodimerization showed a remarkable correlation. Although all the ErbB2 versions with a functional kinase domain responded to EGF with a similar change in ErbB2 tyrosine phosphorylation, both positive controls (V659E, G778D) and the three cancer-associated variants achieved this with a lower increase in EGFR-ErbB2 heterodimerization (Figure 16D). This correlation was less discernible in the plot of pan-tyrosine phosphorylation and EGFR-ErbB2 heterodimerization (Figure 16E). Although for EGFR-ErbB2 heterodimerization and ErbB2 lateral mobility, EGF-induced changes negatively correlated with baseline values (Figure 16A), no such correlation could be established for the tyrosine phosphorylation response (Figure 16F).

In conclusion, neither the tyrosine phosphorylation response nor the heterointeraction between EGFR and ErbB2 was suitable for discriminating between activated and wild-type ErbB2; their correlated comparison revealed that the three ErbB2 cancer-associated variants resembled other activated ErbB2 mutants in that the same tyrosine phosphorylation response required less pronounced growth factor-induced increase in heterodimerization than in the wild-type.

6 DISCUSSION

To elucidate the membrane dynamics of the ErbB2 variants, it was necessary to verify that after fluorophore conjugation, the monoclonal antibody trastuzumab maintained its structural integrity and functionality. Although fluorescent conjugation is a standard methodology, previous studies have demonstrated that a high degree of labeling (DOL) leads to a progressive loss of the affinity of the antibody for its epitope and alters its photophysical properties [151,157]. In our results, we observed that this alteration affects not only epitope recognition but also interactions in the Fc region. The excessive conjugation of fluorophores introduces hydrophobic groups mediating hydrophobic interactions between distinct parts of the antibody, modifies the net charge of the antibody [180] and generates a steric hindrance in the CH2 and CH3 domains of the Fc region [155]. Therefore, it is suggested that to keep the structural integrity and functionality of the antibody unaltered, it is necessary to have a low DOL [180,181]. In accordance with this and previous observations by Szabó et al. [157] where AlexaFluor647 showed a greater detrimental effect on antibody affinity than AlexaFluor546, we established an optimal DOL of ~ 1 for AlexaFluor647, thereby preserving trastuzumab functionality and ensuring compatibility with activation assays using TAMRA-EGF while avoiding steric hindrance in subsequent experiments.

Having validated the functionality of both A546-conjugated trastuzumab and A647-conjugated trastuzumab, we wanted to determine three main points: the labeling methodology does not affect the natural dynamics of the receptors that could lead to false negative or positive results, trastuzumab as a labeling probe does not influence the interaction between two receptors, and the membrane expression is similar among the ErbB2 variants to make sure that any observed difference among the variants is due to the receptor itself rather than to variable protein expression.

On first place, we evaluated whether the formation of EGFR-ErbB2 heterodimers induced by EGF stimulation generates a steric hindrance that would prevent the binding of trastuzumab to ErbB2. Our results showed that the fluorescence intensity of trastuzumab remains constant before and after EGF stimulation, indicating that the binding capability of trastuzumab is independent of the oligomerization state of the receptor. This result was expected as both molecules, TAMRA-EGF and trastuzumab, bind to different domains of different receptors, i.e., TAMRA-EGF binds to subdomain II on the ECD of EGFR [40]; while trastuzumab binds to subdomain IV of ErbB2 [39].

On the other hand, it has been suggested that the bivalent nature of type IgG1 monoclonal antibodies, like trastuzumab, can create a cross-linking effect in the plasma membrane [182]. Theoretically, the simultaneous binding of two Fab regions to adjacent ErbB2 monomers could induce artificial homodimerization, mimicking an active state of the receptor, triggering agonist signaling and altering natural lateral mobility [172], thereby greatly complicating the observation of basal receptor dynamics.

However, when evaluating the dimerization of fluorescent protein-tagged ErbB2, no significant difference was observed after incubation with trastuzumab at 4°C compared with 37°C. Biophysically, at 4°C, the lipid bilayer transitions into a rigid-like state that restricts the lateral mobility of the transmembrane proteins, whereas at 37°C, membrane fluidity allows active lateral diffusion [135,183]. Consequently, if trastuzumab were acting as an active cross-linker, the increased receptor collision rate expected at 37°C would have resulted in a significantly higher degree of homoassociation. The absence of this observation indicates that, in our experimental model, trastuzumab used as a probe does not induce an artificial homodimerization state.

Interestingly, the quantitative evaluation of the ErbB2 membrane expression showed that the membrane expression of the truncated Δ IC variant was approximately three times higher than that of the full-length ErbB2 variants. Although ErbB2 is known to have a lower endocytosis rate compared to other ErbB family members [184], it maintains a basal turnover rate essential for cellular homeostasis. This turnover is orchestrated by the intracellular domain, in charge of the kinase activity important for some cellular internalization pathways, and the C-terminal tail, which serves as the primary docking site for clathrin-mediated adaptors and ubiquitin ligases such as c-Cbl [185,186]. The high membrane expression of the Δ IC variant confirms the key role of the intracellular domain in the negative regulation of ErbB2.

Given the high membrane expression observed in the Δ IC variant, it would be expected that a higher rate of homodimer or cluster formation would occur due to physical receptor proximity [135]. However, our clustering results showed that despite the overexpression of the distinct ErbB2 variants, there was no evidence of homoassociation. This strongly suggests that ErbB2 homodimerization is strictly regulated and not directly triggered by space saturation. Recent studies using super-resolution microscopy and single-molecule tracking have shown that ErbB2 exhibits a highly dynamic monomer-dimer

equilibrium transition that does not always generate a stable, macroscopic homoassociation signal unless the receptors are confined within specific lipid microdomains [135,187,188]. The lack of homoassociation signal between the WT and the mutated variants might suggest that its oncogenic potential does not lie in an intrinsic tendency to form homodimers, but rather depends on its interactions as co-receptors [45,48].

Since the ErbB2 receptor lacks its own ligand, its biological function is directly linked to its role as the preferred dimerization partner of EGFR [42]. Cells co-expressing EGFR showed a higher heterodimerization rate between EGFR and all ErbB2 variants than the homoassociation in ErbB2-only-expressing cells. Interestingly, the Δ IC variant retained its dimerization capability upon EGF stimulation, a behavior also observed in the WT and the TMD mutant V659E. This confirms that the rearrangement of the receptor upon ligand binding is controlled by its extracellular and transmembrane domains. Furthermore, although the TMD was considered a passive lipid anchor, recent advances in Cryo-EM and computational modelling have shown that the rearrangement of the TMD and JMD is an essential, active step to mediate the dimerization and support the asymmetric kinase activation [189,190].

Notably, the increase in the catalytic activity is not directly related to an increase in the FRET efficiency. An example is the V659 variant, with a mutation found in the TMD that promotes dimerization by forming atypical hydrogen bonds that alter the helical crossing angle and strengthen the lateral interactions [190]. So, despite its higher basal activity, the maximum FRET efficiency of the V659E-EGFR heterodimer after EGF stimulation was similar to that of the WT-EGFR heterodimer. This highlights that FRET reports on physical proximity, not the catalytic activity rate.

It is interesting that the V659E variant retains its ability to respond to EGF with an increased heterodimerization, unlike the other cancer-associated variants R143Q, R678Q, and V842I. Although these mutants showed a heteroassociation rate similar to WT, upon ligand stimulation, they became “deaf”. It has been noted that the basal heteroassociation levels are not higher than the WT even before the EGF stimulation. A possible explanation is that these mutations already induce the receptor into a rigid intracellular conformation in the active state, known as a kinase asymmetric dimer [41]. This would mean that the ErbB2-EGFR dimer is in a pre-activation structure, which positions the fluorophores (TAMRA and AlexaFluor647) in a spatial orientation that limits the energy transfer efficiency [191] and

prevents any additional conformational change after ligand binding, suggesting a type of allosteric decoupling [33].

Taken together, the inability of these cancer-associated variants to respond to EGF stimulation indicates a decoupling of the heterodimers from the *outside-in* cellular control mechanisms [192]. In a tumoral context, these complexes constitutively trigger cell survival signals and completely bypass microenvironmental regulation [189].

Based on the previous structural insights, we analyzed the lateral diffusion of the ErbB2 variants to determine how the observed structural decoupling affects the mobility of the receptor. When ErbB2 was expressed as the sole member of the ErbB family, the WT exhibited a restricted basal mobility, characterized by a high immobile fraction and a slow recovery time. On the other hand, the truncated variant Δ IC showed a four-times reduction in its immobile fraction. This hypermobility confirms that, although the TMD keeps the receptor inserted into the plasma membrane [193], the ICD acts like the main anchor to the cytoskeleton that slows the lateral diffusion of the receptor. By the absence of the ICD, the Δ IC variant avoids the dynamic restrictions imposed by the actin and scaffold protein network, similar to the fence-picket model of membrane confinement [194].

Interestingly, the mutationally activated positive controls (V659E, G778D) and the cancer-associated variants (R143Q, R678Q, V842I) also showed higher basal diffusion and lower immobile fractions than the WT. This suggests that the stabilization of these mutant variants in a rigid, pre-active conformation, as observed in the dimerization results, also alters their interactions with their lipidic and protein environments. Previous studies using single-particle tracking microscopy (SPM) have shown that oncogenic mutations in the TMD and KD in the ErbB receptor family alter the membrane receptor dynamics even in a resting state [195]. In this sense, the lack of autoinhibition mechanisms could prevent the mutant active monomers from establishing low mobility basal complexes or being confined in cytoskeleton-dependent membrane nanodomains [196,197], favoring a faster lateral mobility [198].

Next, we examined how the presence of EGFR alters the ErbB2 receptor dynamics. It was observed that in a non-stimulated state, but in the presence of EGFR, the lateral diffusion of ErbB2 WT was two-times faster than in ErbB2-only expressing cells. This basal alteration highlights the spatial interdependency of the ErbB network [199]. The presence of EGFR and the formation of EGFR-ErbB2 heterodimers probably sequester shared anchoring

proteins such as NHERF1 [200] or ERM protein family [201], or shifts ErbB2 toward a different cytoskeletal interaction. This mechanism highlights that the ErbB2 biophysical nature is not an isolated characteristic, but it is strictly modulated by the availability and co-distribution with its dimerization partners [197].

However, after EGF stimulation, the dynamics observed previously changed. EGF stimulation induced a slower recovery time constant and a higher immobile fraction for most of the ErbB2 variants. This ligand-induced immobilization is a characteristic of the RTK, where the active heterodimers recruit adapter proteins and cluster into clathrin-coated pits for internalization [186].

Additionally, the kinase-dead variant (K753M) retained its basal mobility and failed to respond to EGF. This suggests that receptors with complete intracellular domains are required to form an efficient structural conformation to stabilize these anchored signaling complexes. Unlike receptors like ErbB3, whose pseudokinase domain lacks catalytic activity, but it is structurally capable of forming functional asymmetric dimers [41], probably the missense mutation K753M traps the domain in a restrictive conformation, blocking the allosteric activation of the heterodimer and the recruitment of the endocytic machinery, explaining the inability to downregulate after stimulation. However, despite lacking the kinase domain, the Δ IC variant and all the variants with active kinase, including those that failed to respond to EGF in the FRET assays, showed a retarded diffusion.

This apparent discrepancy between the FRET and FRAP data can be explained by a mechanism of passive co-immobilization. After EGF stimulation, EGFR aggregates to form signaling platforms [202], reducing its lateral mobility. While the cancer-associated variants R143Q, R678Q, and V842I are stabilized in an asymmetric kinase dimer that prevents further conformational changes upon EGF binding, their extracellular and transmembrane domains remain stably bound to the active EGFR. Consequently, when EGFR aggregates into microdomains, it physically retains ErbB2. Therefore, the oncogenic variants suffer from lateral immobilization, as seen in the lower FRAP mobility, but due to its known capability of evading internalization [203], they manage to anchor as constitutive-signaling immobilized complexes.

So far, we have established, through FRET and FRAP assays, that the oncogenic mutations alter the basal dynamics and conformational structure of the receptor after ligand binding; therefore, it is important to evaluate how these biophysical alterations affect the

catalytic activation of the complex. Our phosphorylation results showed a correlation between the cytoskeletal-tethered state of the receptor and its signal-transduction capability, highlighting a structural pre-activation state and a strong decoupling of asymmetric phosphorylation.

Evaluation of tyrosine phosphorylation in ErbB2-only transfected cells confirms that the inhibitory control of the receptor depends mainly on precise structural interactions. The truncated (Δ IC) and kinase-dead (K753M) variants showed a basal phosphorylation level lower than that of WT, while the V659E (TMD) and G778D (KD) showed a basal hyperphosphorylation. These results are in concordance with models in which the transmembrane domain (TMD) acts as an active mechanical transducer rather than merely a lipidic anchor [130]. It has been shown that mutations like V659E alter the helicoidal cross in the TMD, forcing the intracellular kinase domain to free from its monomeric autoinhibitory state to form active interactions, even in the absence of EGFR [129]. Similarly, the V842I variant exhibited a higher intrinsic phosphorylation level than the WT. This is consistent with our previous observation of a higher lateral diffusion in a basal state, i.e., when the autoinhibitory conformation is lost, the mutant variants escape from the cytoskeleton-dependent membrane nanodomains and turn catalytically active, promoting dysregulated cellular proliferation [204].

Interestingly, in a co-expression system, the panorama changed particularly for the K753M variant, which, despite carrying a kinase-dead mutation, responded to EGF stimulation, increasing global tyrosine phosphorylation (pan-pTyr). This paradox can be explained by the asymmetric allosteric activation mechanism representative of the ErbB family [54]. Where in a functional heterodimer, the activation does not require both kinases to possess a catalytic activity simultaneously, i.e., a subunit acts like the allosteric “activator” that binds to the N-terminal lobe of the “receiver” kinase, inducing its active conformation [37,205]. We observed a strong phosphorylation in tyrosine residues Y1048 and Y1173 of EGFR, along with an absence of transphosphorylation of ErbB2. This suggests that the intact structure of its extracellular and transmembrane domains is enough to heterodimerize with EGFR after EGF stimulation [206]. In this scenario, ErbB2-K753M acts solely as the “activator”, allowing EGFR to be the functionally active kinase in charge of phosphorylating the intracellular substrates. The signal of phosphorylated Y1048 and Y1173 might be due to the capability of EGFR, once activated, to phosphorylate its own C-terminal residues by

trans-intermolecular interactions facilitated by the formation of oligomers in the membrane [197,207], or by the intrinsic flexibility of its C-terminal tail [54,208].

It is important to note that the observed asymmetric dynamics observed in the ErbB2-K753M/EGFR heterodimer has a similar activation mechanism than the EGFR-ErbB3 heterodimer [49]. In this natural scenario, ErbB3 acts like a pseudokinase lacking intrinsic catalytic activity, but whose kinase domain is key for the activation of EGFR [41,52]. In this sense, the ErbB2-K753M mimics the roles of ErbB3 by providing an allosteric activation scaffold that induces the active conformation of EGFR. However, while in the EGFR-ErbB3 heterodimer, the active EGFR transphosphorylates the C-terminal tail of ErbB3, in the mutant system, this effect is not observed, limiting EGFR catalytic activity to the autophosphorylation, as seen in the increased signal of tyrosine residues Y1048 and Y1173. This discrepancy suggests that, although the K753M variant keeps the capability of the allosteric activator, the mutation creates a conformational alteration that prevents its C-terminal tail from being accessible to the active EGFR.

In contrast, the V659E variant showed an extreme pre-activation state, in which EGF was unable to elicit an additional response in both ErbB2-specific phosphorylation and pan-Tyr phosphorylation, consistent with its pathogenic nature as a constitutively active mutation [209]. This lack of response is consistent with our observations in the FRET assay, where V659E reached a maximum level of heteroassociation comparable to WT, independent of EGF stimulation. From a functional standpoint, the V659E variant anchors the active heterodimer, leading to increased basal signaling [129]. This constitutive signaling evades ligand-triggered homeostatic controls, leading to the sustained proliferative advantage [130].

However, for the cancer-associated mutants R143Q, R678Q, and V842I, the underlying biological mechanisms appear to differ. Although FRET assays showed that they retain their ability to associate with EGFR, after EGF stimulation, there is an increase in the ErbB2 phosphorylation signal, but they failed to induce any response at the tyrosine sites Y1048 and Y1173 in EGFR. This might suggest the existence of a “directional decoupling” due to an asymmetric alteration of the complex. Under physiological conditions, the EGFR-ErbB2 heterodimer exhibits structural flexibility that allows both receptors to alternate their roles as “activating” kinase and “receptor” kinase, resulting in mutual, bidirectional transphosphorylation [210]. Our data suggest that the R143Q, R678Q, and V842I mutations stabilized the heterodimer in an uncommonly rigid asymmetric configuration, where the

dynamic role alternation does not happen. In this altered state, the ErbB2 mutant makes the EGFR function solely as the allosteric receiver, while ErbB2 assumes the role of the activator [198]. Consequently, the EGFR is not activated and is unable to transphosphorylate its C-terminal residues.

It is interesting to note that the three cancer-associated mutations located in different domains, R143Q in the ECD, R678Q in the TMD, and V842I in the KD, appear to be characterized by the same asymmetrical decoupling. Previous crystallography and biochemical studies have shown that alterations in the ECD, like R143Q, modify the electrostatic or conformational surface in the interacting domains [125], leading to a higher heterodimer stabilization, shifting the equilibrium toward an active signaling state even in the absence of ligand [118,190]. In the juxtamembrane domain, mutations such as R678Q alter electrostatic charges, destabilizing the juxtamembrane latch that anchors the catalytic domain to the cytosolic face of the plasma membrane and keeps it inactive [211]. In the intracellular region, kinase mutations like V842I act through direct steric hindrance in the catalytic core. The substitution of a valine for a bulkier isoleucine residue causes the activation loop to physically clash with the inhibitory conformation of the enzyme, forcing the α C-helix to realign into the active “IN” position, locking the receptor in an open, catalytically open architecture [86,87,118].

Overall, although these three cancer-associated mutants act through different biophysical mechanisms, they all converge on stabilizing ErbB2 as the active kinase receptor [118]. This unidirectionality resolves the apparent paradox observed in our FRAP assays: after EGF stimulation, EGFR retains the complexes within nanodomains [185,186], but the cancer-associated variants block phosphorylation at Y1173, preventing recruitment of c-Cbl and thereby evading endocytic down-regulation [71]. Thus, the complex remains anchored to the plasma membrane as a static cluster with aberrant signaling.

The allosteric decoupling and the observed evasion of degradation in our data do not represent an isolated phenomenon; they reflect the convergent evolution paradigm in the RTK oncology scenario. For example, similarly to the V842I variant, the L858R mutation in EGFR alters the thermodynamics of the intracellular domain and leads to an active conformation of the receptor complex [120]. Furthermore, the failure of transphosphorylating key residues for c-Cbl mimics highly aggressive truncated variants like EGFRvIII, which is a main oncogenic driver due to constitutive evasion of the lysosomal pathway [212].

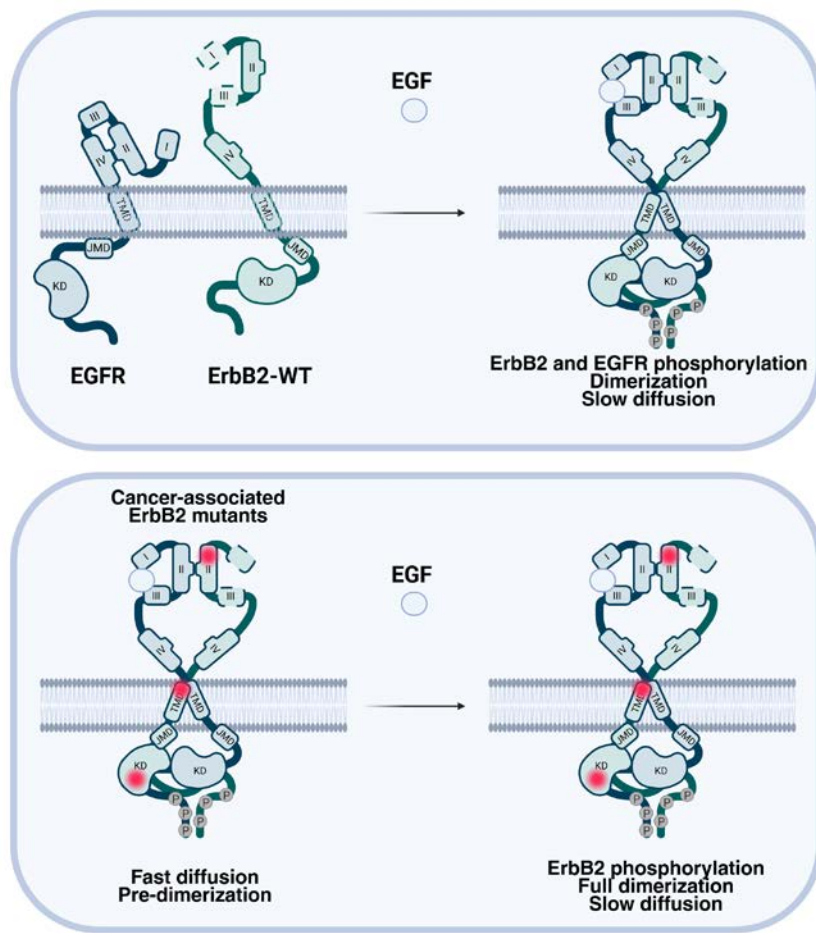


Figure 17. **Proposed mechanistic model for the activation and membrane dynamics of cancer-associated ErbB2 variants.** Under physiological conditions (top panel), EGF stimulation leads to the formation of a flexible EGFR-ErbB2 heterodimer characterized by reciprocal, bidirectional trans-phosphorylation and reduced lateral mobility (slow diffusion) due to cytoskeletal tethering. In contrast, cancer-associated mutants (bottom panel) exhibit a basal state of pre-dimerization and fast lateral diffusion, indicating an escape from basal cytoskeletal confinement. Upon ligand binding, these variants stabilize a rigid, unidirectional asymmetric conformation where the ErbB2 mutant acts as the permanent catalytic receiver, while EGFR trans-phosphorylation is impaired (allosteric decoupling). This complex remains immobilized at the plasma membrane (slow diffusion), sustaining aberrant oncogenic signaling. Created in BioRender.

As illustrated in Figure 17, our findings demonstrate that oncogenic single-amino-acid substitutions in ErbB2 redefine receptor behavior by overriding physiological membrane dynamics and allosteric control. We show that these mutations do not merely “alter” signaling, but impose a rigid, unidirectional asymmetric conformation that favors ErbB2 as the permanent catalytic receiver. Furthermore, the correlation between increased lateral mobility and constitutive activation suggests that the loss of autoinhibitory structural “brakes” allows these variants to escape cytoskeletal confinement. Ultimately, this study aims to position conformational dynamics as an alternative target for designing future targeted therapies that might restore the sensitivity of the receptor to cellular control.

7 SUMMARY

This research focused on elucidating the biophysical and functional impact of three clinically relevant somatic mutations in the ErbB2 receptor: R143Q in the extracellular domain, R678Q in the transmembrane domain, and V842I in the kinase domain, on its organization and dynamics of the plasma membrane.

To ensure precise measurements, the study first evaluated the methodological use of the fluorophore-conjugated monoclonal antibody trastuzumab. It was determined that maintaining a labeling degree of approximately 1 is key to preserving the structural integrity of the antibody and the affinity of its epitope, avoiding steric hindrance or artificial homodimerization that could alter the basal dynamic measurement.

The membrane evaluation revealed that, when expressed in isolation, the cancer-associated ErbB2 variants exhibit higher lateral diffusion and a lower immobile fraction than WT. This increased mobility suggests that the alteration in the autoinhibitory conformation allows the mutant receptor to escape from cytoskeletal constraints and lipid nanodomain clusters. In a more physiological context, when expressed with its main dimerization partner (EGFR), the mutant variants exhibited dimerization, indicating pre-activation. However, upon EGF stimulation, the mutant variants showed an allosteric decoupling. The mutations stabilize the EGFR-ErbB2 heterodimer in a rigid, asymmetrical architecture. In this state, the ErbB2 receptor permanently assumes the role of active kinase, while EGFR functions solely as an activator without catalytic capability, with EGFR being unable to phosphorylate key tyrosine residues in its C-terminal tail. The absence of this phosphorylation prevents the recruitment of degradation proteins. As a result, the heterodimers remain anchored to the plasma membrane in static clusters, sending constitutive signals for cellular survival and proliferation.

In summary, this work shows that single-amino-acid substitutions in ErbB2 can shape receptor membrane dynamics. These mutations not only alter signaling but also seem to induce an active, rigid conformation, conferring the oncogenic advantage described as a hallmark of cancer. Therefore, the study identifies receptor conformational dynamics as a promising new target for designing future targeted therapies to restore cellular homeostasis.

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9 KEYWORDS

ErbB2 Receptor

Somatic Mutations

Membrane Dynamics

Receptor Dimerization

Allosteric Decoupling

Trastuzumab

FRET

FRAP

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*“To the people who look at the stars and wish.
To the stars who listen, and the dreams that are answered.”*

11 APPENDIX



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List of publications related to the dissertation

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