

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Effects of Recurrent Missense Mutations on ErbB2 Receptor
Organization and Membrane Dynamics

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1 LITERATURE REVIEW

1.1 The ErbB family

The ErbB family is composed of four members: ErbB1 (EGFR/HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) [1]. 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. 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.

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 [2,3]. 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, hiding the dimerization arm acting as an autoinhibition mechanism to avoid spontaneous dimerization, preventing receptor activation [4].

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. This extension exposes the dimerization arm of domain II, allowing the formation of “back-to-back” homo- or heterodimers [5,6]. 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 [7]. This new configuration induces the dissociation of the juxtamembrane segment from the lipid bilayer and facilitates the asymmetric dimerization of the kinase domain [8–10]. 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 [11]. 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 [12].

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 [13]. This characteristic positions ErbB2 as a “universal co-receptor”, and it is the preferred dimerization partner of the other members [14]. The ErbB3 receptor is distinct among intracellular domain (ICD) kinases because it lacks key catalytic residues, thereby classifying it as a pseudokinase.

Dimer formation is determined by ligand specificity, receptor affinity, and their relative density at the plasma membrane. 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 (EGF, TGF α , and AREG) [15]; ErbB3 and ErbB4-specific ligands (NRG family) [16], and pan-ErbB ligands (BTC) [2].

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” [17]. 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 [18,19]. Therefore, ErbB2 functions as a “kinetic scaffold” that captures activated receptors, stabilizing their transition states and maximizing signaling efficiency [14].

1.1.1 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 monomer's identity 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 [12,20].

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), the PI3K/Akt/mTOR pathway, and the PLC γ -PKC pathway.

1.2 ErbB2 in oncogenesis

Although ErbB signaling is the key for physiological development, its deregulation is a hallmark of several human carcinomas [16]. 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 receptor's structure [21].

1.2.1 ErbB2 genomic amplification and overexpression

The canonical understanding of ErbB2 pathogenicity is based on the quantitative deregulation of its expression. 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 [16]. 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 [22,23].

The high receptor density in the plasma membrane alters the interaction stoichiometry. The molecular proximity forces the collision and formation of ErbB2-ErbB2 homodimers [24]. 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) [25], starting a sustained downstream signaling primarily mediated by the Shc adaptor [26]. This sustained signaling chronically activates the PI3K/Akt/mTOR and Ras/MAPK pathways [23], promoting uncontrolled proliferation, apoptosis evasion and angiogenesis [27].

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 receptor's conformation.

1.2.2 Activating somatic mutations

The implementation of NGS has enabled characterization of the genomic profile of ErbB2 receptors, undermining the idea that the oncogenic role depends solely on receptor overexpression and that the receptor lacks alterations in its coding sequence. In recent decades,

it has been established that ErbB2 is susceptible to activating somatic mutations. These drivers can sustain cellular transformation and constitutive signaling in the absence of genomic amplification, thereby defining a distinct subset of ErbB2-driven tumors [21,28].

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, the mutation spectrum of *ERBB2* is more complex and distributed than other classic oncogenes [28]. 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 [29,30]. 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 [31].

In the therapeutic area, these mutations have been shown to confer a degree of resistance to standard treatments such as 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 [32,33]. However, this structural deregulation also 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 [28], afatinib [34] and poziotinib [30], that utilize irreversible covalent binding mechanisms [35].

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.

1.3 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 [36] and as high-affinity probes for investigating membrane dynamics [36,37]. Preserving their structural integrity is critical, since even minor conformational alterations can substantially affect affinity and biological efficacy [38,39].

1.3.1 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 [40].

Immunoglobulins are composed of two antigen-binding fragments (Fab) and one crystallizable fragment (Fc), which serve as distinct functional units. The Fc region mediates interactions with the immune system [41]. 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 [42,43].

IgG antibodies are classified as either polyclonal or monoclonal antibodies. Monoclonal antibodies (mAbs) are specific to a single, well-defined epitope of an antigen and are preferred in applications that demand high binding specificity [44].

1.3.2 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 [13]. 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 [45].

Additionally, the structural integrity of the Fc domain is essential for the binding of other essential biological mediators [46], 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 [47].

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 antibody's native structure to incorporate external reporters. This bioconjugation can

unintentionally alter the paratope structure, thereby reducing binding strength and compromising analytical accuracy [48].

1.3.3 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 [36].

Historically, fluorescence microscopy and flow cytometry have assumed that stochastic bioconjugation is functionally inert [49]. 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 [50].

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 probe's total brightness and improve the signal-to-noise ratio [48]. 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 [51]. 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 [50].

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 system's biological characteristics.

2 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

3 MATERIALS AND METHODS

3.1 Cells

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

3.2 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 [52]. CHO cells were transiently transfected with wild-type or mutant ErbB2-pcDNA3.1 plasmids using Lipofectamine 2000. For co-expression and dimerization assays, cells were double-transfected in a 1:1 ratio with human EGFR or with pcDNA3.1-ErbB2-YFP and -CFP plasmids, respectively.

3.3 Antibodies and their fluorescent labeling

Trastuzumab was conjugated to Alexa Fluor 546 and 647 via N-hydroxysuccinimidyl esters, with the degree of labeling determined spectrophotometrically. Tyrosine phosphorylation was detected using PY99 (pan-phosphotyrosine), Ab18 (ErbB2 pTyr1248), EM-1 (EGFR pTyr1048), and 1H12 (EGFR pTyr1173) primary antibodies, followed by an Alexa Fluor 647-conjugated secondary antibody.

3.4 Functional tests of antibodies

Antibody binding was assessed by incubating cells with varying concentrations of fluorescent or unlabeled antibodies on ice for 30 minutes. Fluorescence intensity was measured via flow cytometry, and background-corrected data were fitted to single-site saturation or competitive binding models using GraphPad Prism to determine dissociation constants and affinities.

3.5 Staining of cells for measuring protein clustering and lateral diffusion

For homoclustering, cells were stained with AlexaFluor546- and AlexaFluor647-trastuzumab. For heterodimerization, co-transfected cells were starved and labeled with

TAMRA-EGF and AlexaFluor647-trastuzumab, either maintained on ice (unstimulated) or incubated at 37°C for 10 minutes (stimulated). For lateral mobility, starved cells were stimulated with 50 nM unlabeled EGF at 37°C for 10 minutes prior to fluorescence recovery after photobleaching (FRAP) measurements.

3.6 Measurement of protein-protein interactions using FRET

Images were acquired on a Zeiss LSM 880 confocal microscope using a 40× objective. Donor (AlexaFluor546) and acceptor (AlexaFluor647) fluorescence, alongside acceptor-sensitized emission, were recorded using specific excitation and emission spectra. Pixel-by-pixel FRET efficiency was calculated using the custom MATLAB program rFRET [53] after correcting for donor overspill.

3.7 Measurement of lateral diffusion

FRAP measurements were performed at 37°C on a Zeiss LSM 880 confocal microscope with a 40× water-immersion objective. ErbB2-GFP fluorescence was recorded before and after photobleaching a targeted membrane region of interest. Recovery was monitored for 3-4 minutes and analyzed using the custom MATLAB program FRAPfit [52].

3.8 Measurement of tyrosine phosphorylation

Cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100. Double-transfected cells were starved and stimulated with 50 nM EGF prior to fixation. Cells were immunolabeled and visualized using a Nikon N-STORM confocal microscope with a 60× water-immersion objective. Images were analyzed in MATLAB using DipImage; watershed segmentation on the GFP channel defined cell boundaries to quantify background-corrected phospho-tyrosine intensities.

4 RESULTS

4.1 Validation of the fluorophore-conjugated trastuzumab antibody.

Trastuzumab labeled with varying degrees of AlexaFluor546 or AlexaFluor647 labeling (DOL) showed that a high DOL progressively reduces affinity, particularly for Fc recognition by protein A, G, and cellular receptors. Quantitatively, a higher DOL increased the dissociation constant (K_d) for both fluorophores. While unlabeled trastuzumab had a basal K_d of ~2.5-3 nM, highly labeled samples reached ~10 nM. Given the similar detrimental impact of both fluorophores at high densities, a DOL of ~1 was selected for subsequent experiments.

4.2 Reliability of trastuzumab in ErbB2 labeling

Trastuzumab fluorescence intensity remained unchanged between control and EGF-pretreated cells, confirming that EGF does not interfere with trastuzumab binding. Membrane expression of ErbB2 was comparable across all samples except the Δ IC variant, which exhibited a ~3-fold higher surface expression. To rule out trastuzumab-induced dimerization, CHO cells co-transfected with YFP- and CFP-ErbB2 constructs were labeled at 4°C and 37°C. No significant difference in YFP-CFP heteroassociation was observed between temperatures, confirming trastuzumab does not artificially induce homodimerization.

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

FRET measurements provided no evidence of strong ErbB2 homoassociation in any single-transfected cell lines. However, all variants heteroassociated with EGFR, yielding baseline FRET efficiencies of 20-30%. EGF stimulation significantly enhanced EGFR interaction with wild-type ErbB2, the Δ IC variant, and the V659E mutant. In contrast, the kinase-dead (K753M), mutation-activated (G778D), and cancer-associated variants (R143Q, R678Q, V842I) showed no change in heteroassociation upon EGF treatment.

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

In cells expressing only ErbB2, activated positive controls (V659E, G778D) and cancer-associated variants (R143Q, R678Q, V842I) exhibited ~2-fold faster FRAP recovery times and 10-30% lower immobile fractions compared to WT. The Δ IC variant was highly mobile,

showing a 4-fold lower immobile fraction, likely due to a lack of cytoskeletal tethering. When co-expressed with EGFR, WT ErbB2 basal diffusion was 2-fold faster than in single-expression. Following EGF stimulation, most variants, including WT, Δ IC, and all kinase-active variants, exhibited retarded diffusion (slower recovery, higher immobile fraction). Only the kinase-dead K753M variant failed to respond to EGF, maintaining its baseline mobility.

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

In single-transfected cells, baseline ErbB2 tyrosine phosphorylation was lower in Δ IC (3-fold) and K753M (5-fold) but higher in V659E (2-fold), G778D (2-fold), and V842I compared to WT. General pan-tyrosine phosphorylation was halved in Δ IC and tripled in V659E relative to WT. In EGFR-co-expressing cells under serum starvation, only V659E showed a significant (2-fold) increase in baseline general phosphorylation. Upon EGF stimulation, ErbB2 phosphorylation increased in all variants except Δ IC and K753M. However, K753M showed an EGF-induced increase in overall pan-tyrosine phosphorylation. V659E was so pre-activated it showed no further response, while the cancer variants (R143Q, R678Q, V842I) responded moderately. Notably, none of the cancer-associated variants induced an EGF-stimulated increase in EGFR transphosphorylation at Y1048 or Y1173.

4.6 Correlations

Excluding inactive mutants, baseline ErbB2/EGFR heteroassociation correlated negatively ($R = -0.75$) with its EGF-induced increase; variants with higher baseline activation were less responsive to EGF-induced heterodimerization. Similarly, lateral diffusion exhibited a negative correlation, where lower baseline mobility resulted in a weaker immobilization response to EGF. Activated variants (positive controls and cancer-associated mutants) displayed higher baseline heterodimerization and greater lateral mobility than WT, indicating mutational activation accelerates diffusion regardless of EGFR coexpression. While tyrosine phosphorylation alone did not differentiate mutants from WT, bivariate analysis showed that activated variants achieved similar EGF-induced ErbB2 phosphorylation levels despite lower increases in heterodimerization. Ultimately, correlated comparisons demonstrate that cancer-associated variants mimic other activated mutants, requiring a less pronounced growth factor-induced increase in heterodimerization to achieve comparable phosphorylation responses.

5 DISCUSSION

A high degree of labeling (DOL) reduces antibody affinity and alters photophysical properties [48,50] by mediating hydrophobic interactions [54] and causing steric hindrance in the Fc region [55]. A low DOL is necessary to maintain functionality [54,56]. Consistent with previous findings [50], an optimal DOL of ~ 1 for AlexaFluor647 preserved trastuzumab functionality and avoided steric hindrance. Furthermore, EGF stimulation did not prevent trastuzumab binding, as the molecules target different domains (TAMRA-EGF to EGFR subdomain II [14]; trastuzumab to ErbB2 subdomain IV [13]). Comparing ErbB2 dimerization at 4°C (rigid membrane) and 37°C (fluid membrane) [36,57] showed no significant difference, confirming that bivalent trastuzumab [58] does not induce artificial cross-linking or mimic an active homodimerization state [172].

The truncated ΔIC variant exhibited approximately three times higher membrane expression than full-length variants. While ErbB2 has a naturally low endocytosis rate [59], its turnover relies on the intracellular domain (ICD) and C-terminal tail for c-Cbl docking [60,61]. The high ΔIC expression confirms the ICD's essential role in negative regulation. Despite this overexpression, no increased homoassociation was observed. This indicates that ErbB2 homodimerization is strictly regulated by specific lipid microdomains [36,62,63] rather than space saturation [36], and its oncogenic potential likely relies on co-receptor interactions [17,64].

Because ErbB2 functions primarily as EGFR's dimerization partner [15], heterodimerization rates exceeded homoassociation. The ΔIC , WT, and V659E variants retained dimerization capability upon EGF stimulation, confirming the transmembrane (TMD) and juxtamembrane (JMD) domains actively mediate dimerization and asymmetric kinase activation [65,66]. Notably, while the V659E mutation promotes dimerization [66], its maximum FRET matched the WT, highlighting that FRET reports physical proximity rather than catalytic activity. Cancer-associated variants (R143Q, R678Q, V842I) showed basal heteroassociation but became "deaf" to EGF. They likely induce a rigid, pre-activated asymmetric dimer [67] that limits energy transfer [68] and prevents further conformational changes [7], resulting in an allosteric decoupling that bypasses microenvironmental regulation [65,69].

Lateral diffusion (FRAP) analysis showed WT ErbB2 has restricted basal mobility. The hypermobile ΔIC variant confirmed the ICD acts as the primary anchor to the cytoskeleton [70];

without it, the receptor avoids dynamic restrictions (the fence-picket model) [71]. Activated positive controls (V659E, G778D) and cancer-associated variants also showed higher basal diffusion, indicating that the loss of autoinhibition prevents nanodomain confinement [72–74], favoring faster lateral mobility [75]. When co-expressed with EGFR, WT basal diffusion doubled due to spatial interdependence and the sequestration of shared anchoring proteins such as NHERF1 [76,77] or the ERM family [78]. Following EGF stimulation, diffusion slowed as active heterodimers clustered for internalization [61].

The kinase-dead K753M variant retained basal mobility and failed to respond to EGF, suggesting it is trapped in a restrictive conformation [67]. Conversely, the Δ IC and "deaf" cancer variants showed retarded diffusion post-EGF due to a mechanism of passive co-immobilization. Their extracellular and transmembrane domains remain stably bound to aggregating EGFR [79]. Consequently, they are physically retained and laterally immobilized, but they evade internalization [80], acting as constitutive-signaling anchors.

In ErbB2-only cells, Δ IC and K753M had lower basal phosphorylation than WT, while V659E and G778D showed basal hyperphosphorylation. This supports models where the TMD is an active mechanical transducer [81], forcing active interactions even without EGFR [82], and allowing mutants to escape nanodomains to turn catalytically active [83]. In co-expression, K753M paradoxically increased global tyrosine phosphorylation post-EGF via asymmetric allosteric activation [84]. It acted solely as the "activator" for the "receiver" EGFR [11,85,86], leading to strong EGFR Y1048/Y1173 phosphorylation via autophosphorylation [74,84,87,88] without ErbB2 transphosphorylation. This mimics the EGFR-ErbB3 heterodimer [67,89,90] but implies a conformational block restricting C-terminal tail accessibility. Meanwhile, V659E exhibited extreme pre-activation; EGF elicited no additional response, driving sustained constitutive signaling [81,82,91].

The cancer-associated variants (R143Q, R678Q, V842I) exhibited "directional decoupling." Post-EGF, ErbB2 phosphorylation increased, but EGFR Y1048/Y1173 did not. Normally, receptors alternate roles, resulting in bidirectional transphosphorylation [92]. However, these mutations stabilize a rigid asymmetric configuration in which ErbB2 permanently acts as the receptor and EGFR as the activator [75]. Despite altering different domains, ECD surface modification [30,66,93], JMD latch destabilization [94], or KD steric hindrance [21,30,95], all three converge on stabilizing ErbB2 as the active kinase [30]. This unidirectionality resolves the FRAP paradox: EGFR traps the complexes in nanodomains

[185,186], but the mutants block EGFR Y1173 phosphorylation, preventing c-Cbl recruitment and evading endocytic downregulation [27].

This allosteric decoupling and evasion of degradation reflect a convergent evolution paradigm in RTK oncology, mirroring aggressive mutants like L858R EGFR [96] or EGFRvIII [97]. Ultimately, oncogenic ErbB2 substitutions impose rigid, unidirectional, asymmetric conformations that override membrane dynamics and escape cytoskeletal confinement, thereby functioning as permanent catalytic receivers. These conformational dynamics represent a promising alternative target for future therapies.

6 FINDINGS

- **Validation of trastuzumab as a research probe:**

The fluorescent labeling of trastuzumab requires a controlled and low degree of labeling (~1) to preserve its affinity and structure. Under these conditions, trastuzumab does not induce ErbB2 homodimerization nor is it blocked by ligand stimulation.

- **Receptor hypermobility:**

The cancer-associated mutants (R143Q, R678Q, and V842I) of ErbB2 altered the autoinhibitory receptor conformation by inducing a faster lateral diffusion and a lower immobile fraction compared to the wild-type ErbB2.

- **Rigid receptor complexes and “deaf” to ligands**

The mutant ErbB2 versions heterodimerized with EGFR in a basal state, imposing a rigid structure that prevents the heterodimer from undergoing further conformational changes upon EGF stimulation.

- **Unidirectional allosteric decoupling**

Upon EGF stimulation, the cancer-associated ErbB2 mutants show significantly increased phosphorylation; however, they lack the ability to transphosphorylate the tyrosine residues of EGFR (Y1048 and Y1173). Therefore, ErbB2 serves as a permanent activator.

- **Evasion of degradation machinery:**

The inability to transphosphorylate the EGFR tyrosine residue Y1048 prevents recruitment of the ubiquitination machinery. This allows the mutant complex to evade lysosomal degradation and emit constitutive downstream signaling.

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

10 APPENDIX



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List of publications related to the dissertation

1. **Serrano Cano, T. G.**, Yasari, A., Hartl, I., Tiemann-Boege, I., Nagy, P.: Biophysical characterization of recurrent ErbB2 missense mutations reveals alterations in receptor organization and membrane dynamics.
J. Mol. Biol. **438** (12), 1-18, 2026.
DOI: <http://dx.doi.org/10.1016/j.jmb.2026.169770>
IF: 4.5 (2024)
2. Hajdu, T., Rebenku, I., **Serrano Cano, T. G.**, Mocsár, G., Bécsi, B., Erdődi, F., Nagy, P.: Fluorescence labeling-induced structural rearrangement of a monoclonal IgG revealed by biophysical experiments and simulations.
Int. J. Biol. Macromol. **321**, 1-45, 2025.
DOI: <http://dx.doi.org/10.1016/j.ijbiomac.2025.148209>
IF: 8.5 (2024)

Total IF of journals (all publications): 13

Total IF of journals (publications related to the dissertation): 13

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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