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HER2/HER3/NRG1b Heterocomplex Structure and Assembly

by
Devan Diwanji

DISSERTATION

Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

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Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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Committee Members

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by

Devan Diwanji

*Dedicated to my parents, Trupti Ghelani and Chetan J. Diwanji,
and to those who believed in me when I couldn't.*

ACKNOWLEDGEMENTS

Inasmuch as a single 2D projection is insufficient to reconstruct a 3D structure, this thesis is but a mere description of the *scientific* challenges faced and overcome in visualizing receptor-kinases in the full-length context. It does not capture the *human* elements that made this work possible: the camaraderie, the sporadic euphoria, and my evolution in thinking, attitude, and self-respect that paralleled the development of the project. And all this, the scientific and humanistic, wasn't without the assistance of a community of people, of dedicated souls whose steadfast faith in me lifted my gaze upwards.

First and foremost, I would like to thank my parents, Trupti Ghelani and Chetan J. Diwanji. This thesis is dedicated to them because without inheriting their empathy, deductive reasoning, and work ethic, this work would've been impossible. Plain and simple, the world of full-length receptor-kinases is demanding, an equation with almost an infinite number of chief concerns and an even longer differential. Reasoning and work ethic weren't enough for this project as the intellectual challenge presented side effects: anxiety, physical pain, and the occasional lucid dream of HER receptors caught in the act of signal transduction. And my parents witnessed all of this. Who was it on the other side of the line who kept saying, "give it another 6 months, you'll see, it'll start working"? Who patiently listened and contextualized my concerns? Who insisted on driving me to a shoe store so that I could reduce my plantar fasciitis and start walking again, let alone, run? Who brought food over when I didn't have time to cook on the weekend? Mom, Dad, you were there for me. Thank you for listening. Thank you for your presence. Thank you for teaching me to believe in myself, for finding self-respect even at my nadir. Empathy for myself turned out to be the one variable I absolutely needed to solve for through this simulated annealing of self that I call my PhD.

Next, I would like to thank my advisors Drs. Natalia Jura and Kliment Verba for their guidance, support, and just enough rope to...I won't finish that statement. Natalia, from you, I learned scientific resilience. I remember a time when I had a bi-weekly meeting in your office and we both stared at the image of a seemingly empty gel. I thought I could see a band for HER2 but the scanner just didn't capture it. You believed me but more importantly you believed *in me*. You said, "We will do it. I know we will get there." I left your office puzzled by the blind faith. When grants were rejected, you got back up and applied for another one. When I only had negative results to show, you focused on the silver lining. That's resilience and I'm honored to have learned that from you. Klim, from you, I learned scientific tenacity and the pureness of scientific curiosity. There wasn't a group meeting or a HER Team meeting that went by without being awestruck by the depth of your questions and sheer curiosity. I'll admit, it frequently led us down many tangents but rather than shirk away from those tangents you taught me to entertain them. No matter what form my career takes, I will always lean into that infectious curiosity and cherish that in others. I am proud to have learned EM from you and to be your first graduate student.

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get up the next day (with or without a hangover) motivated to figure out these receptors. Thank you for dragging me out of the fluorescent lights, witnessing the lows and highs, and continuously inspiring me to be a better version of myself. I am also grateful for the UCSF community, especially to the additional members of my thesis committee, Drs. Aashish Manglik and William Weiss, administrative staff, Geri Ehle and Demian Sainz, and the UCSF structural biology community, particularly Dr. Feng Wang. This thesis is a testament to the teamwork and collaborative atmosphere at UCSF.

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*Authors contributed equally to the work.

HER2/HER3/NRG1 β Heterocomplex Structure and Assembly

by

Devan Diwanji

ABSTRACT

Cell communication is essential for cellular function and relies on the faithful transmission of signals across the plasma membrane through membrane receptors. Receptor kinases constitute an important class of molecular antennas in which the extracellular signal binding module is linked to an intracellular kinase along one polypeptide chain. Perturbations in this finely coordinated system causes aberrant signaling which lead to pathological states such as cancer or developmental disorders. Despite the disease relevance and extensive therapeutic focus, we fundamentally do not understand how receptor kinases transmit a signal across the plasma membrane in the absence of full-length structures. This is particularly true for the Human Epidermal Growth Factor Receptor 2 (HER2), an orphan receptor, and the Human Epidermal Growth Factor Receptor 3 (HER3), a pseudokinase receptor which form a potent pro-oncogenic heterocomplex upon binding to extracellular ligand. Here, we present three novel high-resolution cryo-electron microscopy (cryo-EM) structures of the extracellular domain of the breast cancer receptor, HER2, engaged with its liganded co-receptor, HER3, solved in the context of near full-length receptors. As the first singly-liganded human HER receptor structures, our findings provide a missing link in the HER receptor field, offer the dimerization arm as an allosteric sensor for ligand binding, visualize HER3 in an extended state for the first time, demonstrate how the most frequent oncogenic HER2 variant, HER2 S310F, exploits dimerization arm dynamics to enhance heterodimerization, and unveil previously unknown details on how

commonly prescribed biologic agents bind the heterodimer. Our studies on near full-length HER2 and HER3, when isolated alone, surprisingly reveal that HER2 is a homodimer that may adopt an autoinhibited state and HER3, contrary to dogma, homodimerizes in the presence of NRG1 β . Taken together, these findings made possible through the lens of full-length receptor biophysics, explain ligand allostery, inform rational drug design, and add nuance to a model of HER2/HER3/NRG1 β heterocomplex assembly.

TABLE OF CONTENTS

Chapter 1: The Structure of the HER2/HER3/NRG1β Heterocomplex	1
Abstract	2
Introduction	4
Results	5
Discussion	32
Acknowledgements	34
Materials and Methods	34
References	43
 Chapter 2: HER2/HER3 Heterocomplex Assembly	 51
Abstract	52
Introduction	53
Results	55
Discussion	65
Acknowledgements	69
Materials and Methods	69
References	73

LIST OF FIGURES

Chapter 1

Figure 1.1. Construct Design, Purification, and Functionality of Near-Full Length HER2, HER3, and HER4.....	6
Figure 1.2. The Ligand Pulldown Affinity Purification Strategy	8
Figure 1.3. Purification and Characterization of the HER2/HER3/NRG1 β	10
Figure 1.4. The LMNG Cryo-EM Strategy.....	11
Figure 1.5. Reconstitution of HER4-NRG1 β Homodimer into lipid nanodiscs and visualization by Cryo-EM	13
Figure 1.6. Initial Cryo-EM imaging of the HER2/HER3/NRG1 β heterocomplex	15
Figure 1.7. Crosslinked sample improves homogeneity	16
Figure 1.8. High resolution reconstruction of the extracellular domains of the HER2/HER3/NRG1 β heterodimer and recovery of micelle density	17
Figure 1.9. Extracellular Domain Structure of the HER2/HER3/NRG1 β Heterocomplex	19
Figure 1.10. A conserved ligand binding mode between HER3 and HER4.....	19
Figure 1.11. HER2 is unchanged upon co-receptor engagement.....	20
Figure 1.12. The HER3 dimerization arm is not engaged.....	21
Figure 1.13. Analysis of HER3 Extracellular Oncogenic Mutations.....	22
Figure 1.14. A spectrum of ligand engagement reveals that the dimerization arm is a finely tuned allosteric sensor for ligand engagement.....	24
Figure 1.15. HER2 S310F Engages the HER3 Dimerization Arm.....	27
Figure 1.16. The HER2/HER3/NRG1 β Heterodimer Accommodates Pertuzumab and Trastuzumab while HER2 S310F/HER3/NRG1 β resists Pertuzumab binding	29

Figure 1.17. The HER2/HER3/NRG1 β Heterodimer bound to Trastuzumab Fab	31
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Chapter 2

Figure 2.1. The Ligand Induced Dimerization Model of HER2/HER3 Heterocomplex Assembly.....	52
Figure 2.2. Characterization of isolated near full-length HER2	55
Figure 2.3. The HER2 homodimer is independent of the asymmetric dimer interface	57
Figure 2.4. Characterization of unliganded near full-length HER3	58
Figure 2.5. Bosutinib alters HER3 oligomerization state	59
Figure 2.6. HER3 forms ligand induced homodimers	60
Figure 2.7. Cryo-EM reconstruction of the NRG1 β bound HER3 homodimer.....	61
Figure 2.8. HER3 ligand induced dimerization is not dependent on activator interface but may rely on kinase interfaces.....	63
Figure 2.9. Scaffold model of heterocomplex activation.....	64
Figure 2.10. Sequestration model of heterocomplex activation.....	65

LIST OF ABBREVIATIONS

ATP	Adenosine Triphosphate
BMP	Bone Morphogenetic Protein
CMC	Critical Micelle Concentration
CTF	Contrast Transfer Function
CV	Column Volume
DDM	n-Dodecyl β -D-maltoside
ECD	Extracellular Domain
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EM	Electron Microscopy
EREG	Epiregulin
Fab	Fragment antigen binding
FGF	Fibroblast Growth Factor
FSC	Fourier Shell Correlation
GO	Graphene Oxide
HEK	Human Embryonic Kidney
HER	Human Epidermal Growth Factor Receptor
IPTG	Isopropyl β -D-1-thiogalactopyranoside
KD	Kinase Domain
LMNG	Lauryl Maltose Neopentyl Glycol
MBP	Maltose Binding Protein
MSP	Membrane Scaffold Protein
NRG1β	Neuregulin-1 β
NS	Negative Stain

OD	Optical Density
PDGFβ	Platelet Derived Growth Factor- β
PEI	Polyethylenimine
PMSF	Phenylmethanesulfonyl fluoride
PTK7	Protein Tyrosine Kinase 7
RMSD	Root Mean Squared Deviation
RTK	Receptor Tyrosine Kinase
TGFα	Transforming Growth Factor- α
TMD	Transmembrane Domain
Trx-A	Thioredoxin-A
TS	Twin Strep

Chapter 1: The Structure of the HER2/HER3/NRG1 β Heterocomplex

ABSTRACT

The Human Epidermal Growth Factor Receptor 2 (HER2), an orphan receptor, and Human Epidermal Growth Factor Receptor 3 (HER3), a pseudokinase receptor, form a potent pro-oncogenic heterocomplex upon binding of the extracellular ligand neuregulin-1 β (NRG1 β) to HER3^{1,2}. The mechanism by which these functionally distinct HER receptors interact remains unknown in the absence of any structures of the heterodimeric extracellular domain complexes between HER receptors. We have solved the cryo-electron microscopy (cryo-EM) structure of the HER2/HER3 extracellular heterodimer at 2.9Å bound to a single ligand, NRG1 β , in the context of near full-length receptors. Our structure represents the first HER receptor heterocomplex extracellular domain structure in the context of near full-length receptor, the first HER3 structure in an active extended extracellular state, and the first singly-liganded HER receptor structure. The structure reveals an unexpectedly weakened active dimeric state in which the dimerization arm of only one receptor, HER2, is engaged, while the dimerization arm of HER3 remains dynamic. The asymmetric dimerization arm arrangement is a result of ligand binding only to HER3 that induces an asymmetric wedged conformation of HER3. In this way, the dimerization arm is an allosteric sensor for ligand binding. The HER3 dimerization arm interface is restored in the structure of the HER3 heterodimeric complex with the HER2 oncogenic variant, S310F, solved to 3.1Å. Our results reveal how the most frequent HER2 disease mutation activates signaling by promoting HER2/HER3 heterocomplex assembly through dimerization arm engagement. Furthermore, we show that this variant, unlike the wild-type heterocomplex, does not bind to the commonly prescribed biologic Perjeta (Pertuzumab) but is recognized by Herceptin (Trastuzumab)³. To elucidate how Herceptin recognizes the heterocomplex, we solved a structure of Herceptin Fab bound to the HER2

S310F/HER3/NRG1 β heterocomplex at 3.5Å and show that Herceptin induces a rotation in HER3 to circumvent a steric clash. These findings have broad implications for understanding HER receptor dimerization, provide explanations for negative cooperativity and ligand allostery, and unveil previously unknown structural details for rational drug design.

INTRODUCTION

To date, our mechanistic understanding of HER receptor ligand-mediated dimerization has been largely informed by crystal structures of doubly-liganded extracellular domain (ECD) dimers and unliganded monomeric ECD species⁴⁻⁸. From these studies, we have learned that the ECDs of two of the four members of the HER receptor family – Epidermal Growth Factor Receptor (EGFR) and Human Epidermal Growth Factor Receptor 4 (HER4) – undergo a significant conformational change upon ligand binding. Each ECD contains four subdomains, two β -helix solenoid subdomains (subdomain I and III) and two cysteine-rich subdomains (subdomains II and IV)⁹. In the unliganded state, the ECDs of EGFR, HER3, and HER4 retain a “tethered” conformation in which an intramolecular interaction between subdomains II and IV obscures the β -hairpin “dimerization arm” in subdomain II¹⁰. When a ligand binds, the ECDs of EGFR and HER4 adopt an extended conformation exposing the dimerization arms and forming a heart-shaped receptor dimer⁹. In the absence of any liganded HER3 structure, it is unclear if HER3 forms a similarly extended conformation upon binding to its ligand, NRG1 β . Interestingly, HER2, an orphan receptor, adopts a constitutively extended conformation as a monomer although the active conformation in which HER2 participates in a heterodimer with another member of the HER receptor family remains to be visualized¹¹. Extracellular dimerization, in turn, brings the transmembrane domains (TMDs) and kinase domains (KDs) in close proximity to enhance kinase catalytic activity through the formation of the rod-shaped allosteric asymmetric kinase domain dimer^{12,13}. The two kinases in the dimer have unique roles: one kinase called the receiver kinase is stabilized in an active conformation by the second kinase called the activator¹².

The HER2/HER3/NGR1 β heterocomplex stands as a unique functionally asymmetric and potentially mitogenic signaling unit. HER2 does not have a known ligand and the pseudokinase

HER3 does not have appreciable catalytic activity nor an intact receiver interface^{1,14,15}. Therefore, the HER2/HER3 heterodimer is a singly liganded species in which liganded HER3 obligately activates unliganded HER2. How such functional asymmetry may be reflected in structural asymmetry and how the heterocomplex assembles in the context of the full-length receptor remains unknown in the absence of any singly ligated HER receptor structure.

Such mechanistic detail is relevant to the rational design of therapeutics specifically targeting HER2/HER3 or the development of HER2/HER3-specific biomarkers. Current FDA-approved treatment options focus on the HER2 ECD (Pertuzumab/Perjeta and Trastuzumab/Herceptin) and on the HER2 KD (Lapatinib/Tykerb)³. Despite the benefits in survival and quality of life these therapeutics afford HER2+ cancer patients, many patients experience side effects (e.g. cardiotoxicity with Herceptin) or resistance that may stem from non-specifically targeting HER2^{16,17}. Furthermore, tumor sequencing has revealed numerous mutations in the HER3 and HER2 ECDs that remain mechanistically unexplained with the current unliganded atomic models of HER2 and HER3¹⁸. Given the outstanding questions of singly-liganded HER receptor asymmetry and the importance of an atomic model for rational targeting, we aimed to isolate stable quantities of the NRG1 β -bound HER2/HER3 heterodimer and solve a cryo-EM structure of the ECD.

RESULTS

Previous biophysical work demonstrated that isolated receptor extracellular domain (ECD) fragments of HER2 and HER3 receptors heterodimerize only weakly *in vitro* upon the addition of NRG1 β and suggested that HER receptor heterodimerization may rely on interactions in the intracellular domains¹⁹. We therefore sought to reconstitute the HER2/HER3 heterocomplex for

structural studies by expressing full-length receptors. Thus far, however, biochemical and biophysical investigations on full-length receptor kinases have been hampered by challenges in expression, heterogeneity, and the relative paucity of tools available to study single-pass membrane proteins. To this end, we developed and employed a suite of tools to efficiently express, stabilize, and visualize near full-length HER receptors.

First, we improved yields by removing most of the unstructured intracellular C-terminal tails on HER2 and HER3, as had been previously seen with EGFR^{20,21}, and placed C-terminal MBP (Maltose Binding Protein) and TS (Twin Strep) tags on HER2, HER3, and HER4 (**Figure 1.1 A**). The modified HER3 construct expressed to moderate levels in adherent HEK293 upon transfection with linear PEI while HER2 expressed to a lesser extent. Given that the initial low yields in adherent mammalian expression hampered optimization efforts for structural and biophysical investigation, we switched to EXPI293 (Life Technologies) transient expression, a suspension expression system which permits high density mammalian cell growth. The EXPI293 system remarkably boosted yields of HER receptors (including HER2, HER3, and HER4) such that a single 30 ml culture expressed for 24 hrs post enhancer induction could produce cryo-EM quantities of each receptor (~ 0.4 mg of purified HER2 from 30 ml of culture). Additionally, we found that kinase inhibitors may be cost-effectively added during expression to further boost yields and/or alter receptor oligomerization state.

Our abilities to express sufficient receptor quantities allowed us to optimize detergent extraction conditions and purification protocols to stabilize receptor complexes. We found that HER2, HER3, and HER4 were most stably extracted in 1% DDM (n-Dodecyl β -D-maltoside) and that HER2 and HER4 retained catalytic activity after purification in DDM (**Figure 1.1 B, C, and D**). We further rationalized that the ligand would induce homo- and heterodimeric HER receptor

complexes and the increased homogeneity would benefit structural pursuits in addition to stabilizing active complexes. Rather than add the ligand to purified receptors, we sought to utilize

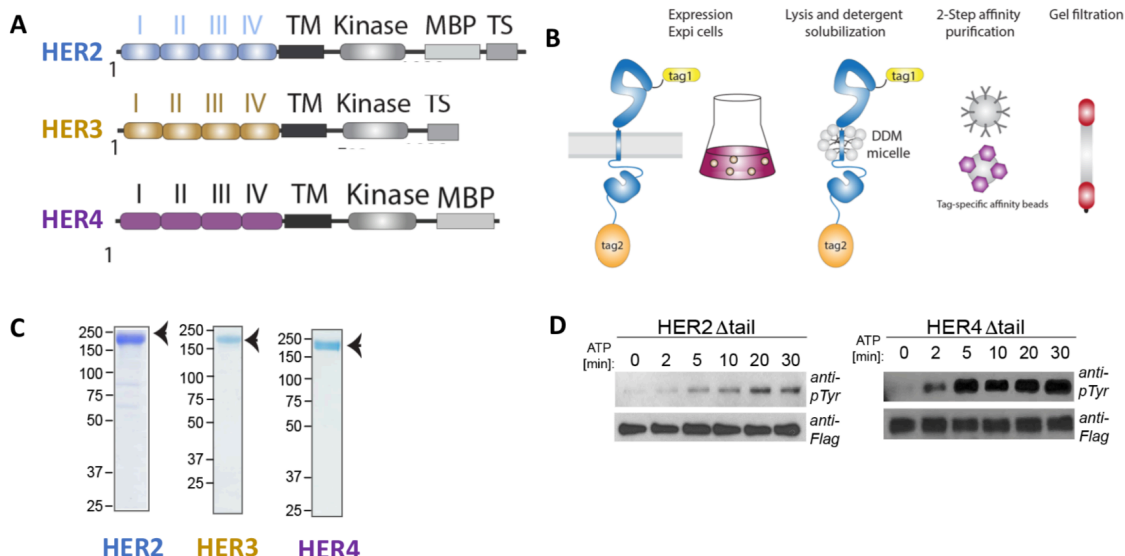


Figure 1.1. Construct Design, Purification, and Functionality of Near-Full Length HER2, HER3, and HER4. (A) Schematic representation of domains and tagging schema of HER2, HER3, and HER4. All three receptors feature the complete extracellular domain (subdomains I-IV), the transmembrane domain, juxtamembrane domain, kinase domain, and a small N-terminal portion of the C-terminal tail. HER2 and HER4 feature the C-terminal MBP tag to enhance solubility, and HER2 and HER3 contain a twin-strep tag on the C-terminus. Various tagged versions of the receptors have been utilized in this work including receptors with an additional N-terminal Flag tag. (B) Schematic representation of dual-tagged receptor purification. Detergent extracted receptors were subjected to two affinity purification steps (Flag and amylose for HER2 and HER4) or 1 affinity purification step (Flag only for HER3) and subsequently applied over gel filtration. (C) Flag elution gel samples indicate high receptor purity for all three receptors (HER3 sample is expressed in the presence of bosutinib). (D) Western Blot shows retained catalytic activity of near full-length HER2 and HER4 in the presence of Mg^{2+} and ATP.

the ligand as an affinity purification tool to specifically pull-down properly folded receptors. Given that HER2 heterodimerizes with HER3 and HER4 homodimerizes in the presence of NRG1 β , we designed a Flag-tagged version of the EGF (Epidermal Growth Factor)-like domain of NRG1 β (**Figure 1.2 A**) and optimized expression in Origami cells, an *E. coli* cell line suited for producing disulfide containing proteins. Milligram quantities of tagged NRG1 β could be obtained for receptor purifications (see Materials and Methods). Our receptor pull-down strategy first includes incubation of Flag-NRG1 β with Flag resin followed by extensive buffer washes and incubation with detergent-solubilized receptor clarified lysate (**Figure 1.2 B**). Initial trials with the HER4 homodimer demonstrated the feasibility and utility of our ligand-pulldown approach to purify a homogeneous sample (**Figure 1.2 B and C**). Our results show that detergent-solubilized HER4 exists in a range of oligomeric states and the addition of ligand through the pulldown strategy stabilizes a more homogeneous dimeric complex as evaluated by negative stain-EM (**Figure 1.2 D**). Curiously, the addition of afatinib, an irreversible EGFR/HER4 inhibitor, during expression homogenized HER4 in the absence of NRG indicating that inhibitor occupancy of the nucleotide binding pocket has the potential to favor certain receptor states without ligand (**Figure 1.2 C**). The elution volume of HER4 with afatinib is consistent with a species larger than a monomer, perhaps an unliganded dimeric state (**Figure 1.2 C**). Interestingly, particles of HER4/NRG1 β + afatinib clustered on negative stain grids which resulted in qualitatively worse class averages despite the relatively monodisperse peak (**Figure 1.2 D**). Overall, our initial HER4 ligand pulldowns proved that we could efficiently purify and stabilize functional receptors by incorporating the ligand into the receptor purification. Such methodology may be readily applicable to other receptor kinases and ligands.

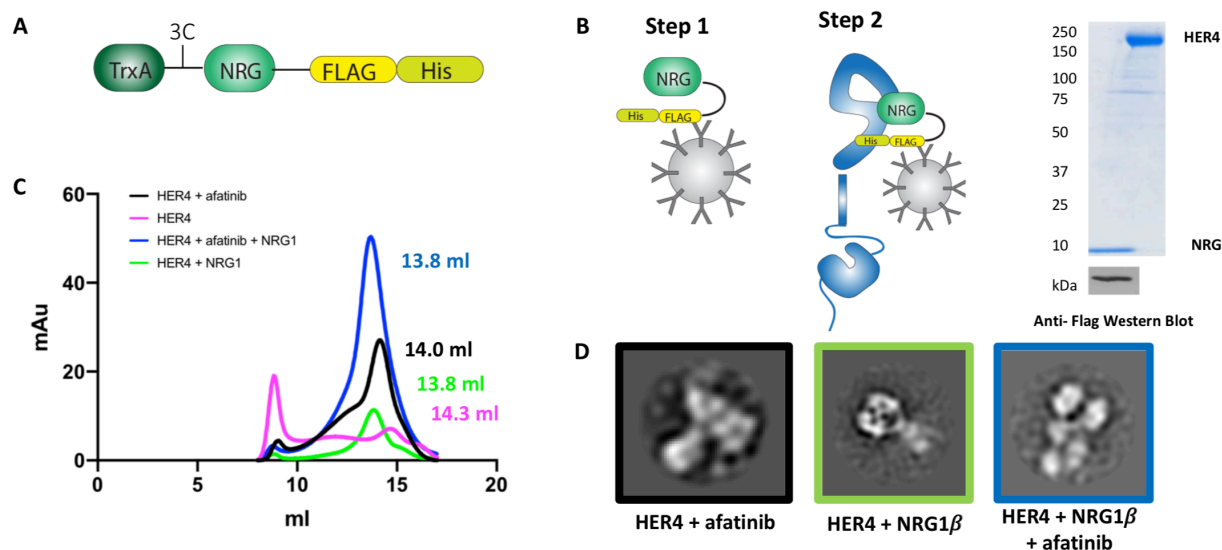


Figure 1.2. The Ligand Pulldown Affinity Purification Strategy. (A) Cartoon of the EGF-like domain of NRG1 β construct used for ligand pulldowns. The construct features a cleavable thioredoxin A (TrxA) domain followed by the EGF-like domain of NRG1 β with C-terminal Flag and His tags. (B) Schematic of the ligand pulldown purification strategy. NRG1 β is first incubated with Flag resin, washed, incubated with detergent solubilized receptor, washed, and finally eluted with Flag peptide. (C) Overlaid gel filtration traces of four different HER4 samples with respective peak elution volumes indicated. (D) Representative negative stain-electron microscopy (NS-EM) 2D class averages from the peaks shown in (C). Extracellular domain density clearly visualized for HER4 + NRG1 β in the absence of afatinib.

For the isolation of the HER2/HER3/NGR1 β heterocomplex, we mixed HER2 and HER3 clarified lysates prior to incubating with the Flag-NGR1 β affinity column. The resulting heterocomplex is eluted from the column and a second affinity step pulling on the MBP tag of HER2 further ensured that both HER2 and HER3 are present in a stable heterocomplex (**Figure 1.3 A and B**) (see Materials and Methods).

Our initial isolation of the heterocomplex did not produce sufficient yields for size exclusion chromatography. To further favor the heterodimeric complex, we introduced two mutations in the kinase domain of HER3 (E928G and Q709R), which we have previously shown

to increase affinity of HER3-containing kinase active dimers through the asymmetric kinase domain dimer interface and juxtamembrane latch^{22,23}. We also introduced a mutation in HER2, G778D, which confers Hsp90 independence^{24,25}, to stabilize HER2. These mutations significantly increased yields of the purified heterocomplexes. The presence of both receptors was confirmed by Western Blotting, mass spectrophotometry, and negative stain-electron microscopy (NS-EM) (**Figure 1.3 B, C, and D**). A final gel filtration step allowed us to isolate fractions of the heterodimer species for structural investigation (**Figure 1.3 E**). Curiously, a higher-order oligomer peak is consistently observed.

We selected single particle cryo-EM as our structural modality to overcome the anticipated structural heterogeneity and reveal structural dynamics in solution through *in silico* image processing²⁶. Our initial screening attempts focused on the HER4 homodimer bound to NRG1 β as that was the most tractable species for troubleshooting. Our initial attempts to freeze DDM solubilized particles on Quantifoil grids resulted in either particle aggregation in thin ice or no particles as the micelle is believed to preferentially coat the air-water interface and thus only sufficiently high protein concentration (>5 mg/ml) may permit visualization in thin ice²⁷. Given that

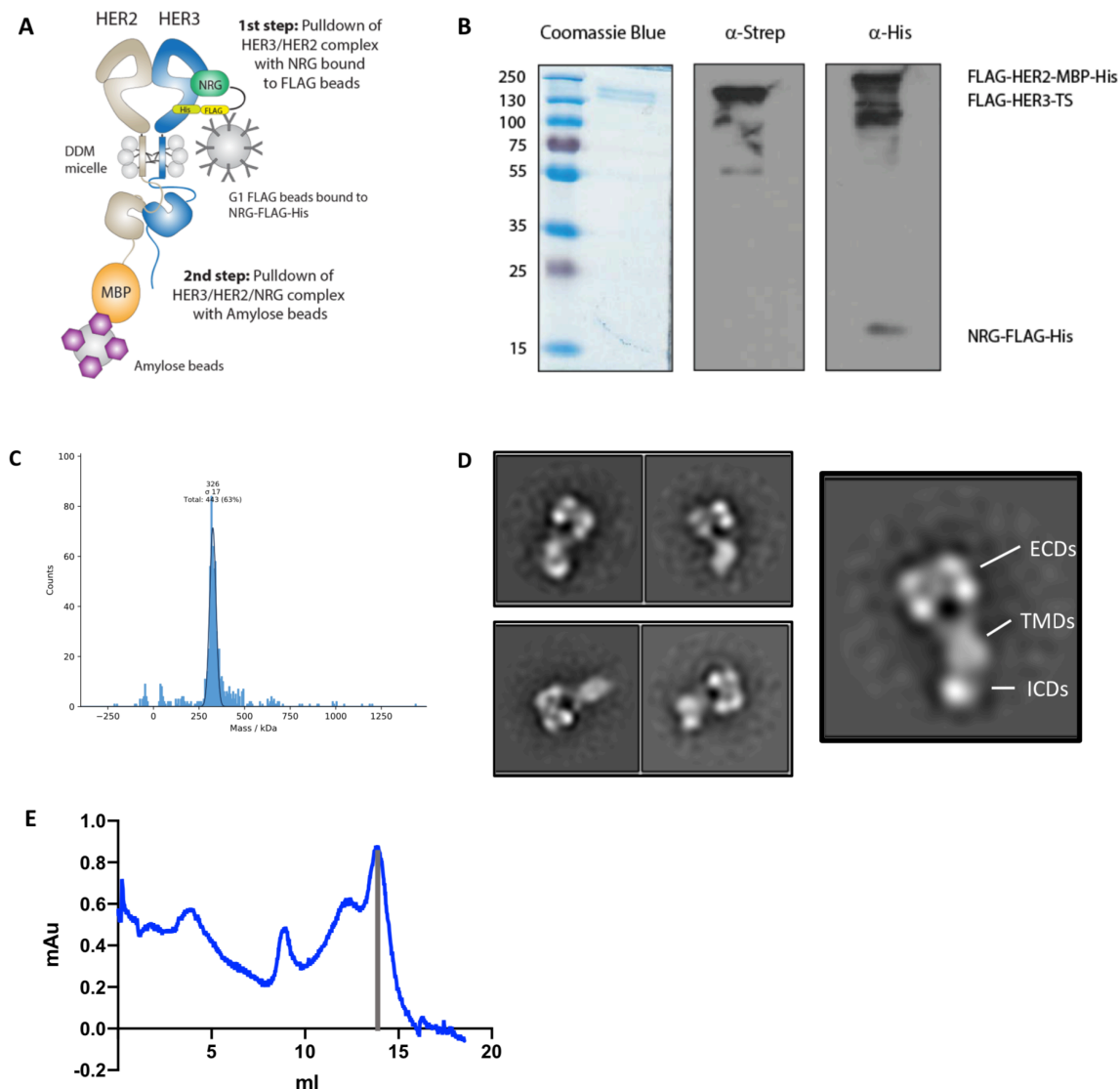


Figure 1.3. Purification and Characterization of the HER2/HER3/NERG1 β Heterocomplex. (A) Purification strategy of the HER2/HER3 heterocomplex through a NRG1 β ligand pulldown and a subsequent affinity purification step pulling on HER2. (B) Coomassie and Western Blot demonstrating the presence of both receptors after second affinity purification step. (C) Average mass from mass spectrophotometry of 326 kDa is consistent with the theoretical molecular weight of a heterodimer. The low standard deviation indicates that the sample has a high degree of homogeneity. (D) Representative negative stain electron microscopy class averages of the heterodimer sample. Sub-micelle density is clearly visible in many class averages (ECD = extracellular domain; TMD = transmembrane domain; ICD = intracellular domain). (E) Representative gel filtration trace on a Superose6 10/300 Increase column. The heterodimer peak is indicated with a gray bar and is consistent in elution volume with other HER receptor dimer species. Curiously, a higher molecular weight peak is consistently observed. The exact nature of this peak is unclear and mass spectrophotometry of this peak shows a heterogeneous mix of trimers and tetramers (data not shown). We routinely obtain only 1-2 mAu peaks from heterocomplex purification which was remarkably sufficient for our cryo-EM methods.

concentrated the receptors may be deleterious to receptor stability and that such concentrations are unattainable for rare species such as the HER2/HER3/NRG1 β heterocomplex, we pursued three alternative strategies: (1) detergent exchange (2) reconstitution receptors in lipid nanodiscs and (3) carbon-supported grids.

Detergent exchange: We exchanged the HER4 homodimer into the ultra-low critical micelle concentration (CMC) detergent, lauryl maltose neopentyl glycol (LMNG), a detergent with a similar chemical characteristic and detergent class as DDM²⁸. Exchange into 0.01% LMNG permitted the final gel filtration step to be run in the absence of detergent without adversely impacting particle quality (data not shown). By eliminating the background detergent micelle, we could effectively reduce freezing concentrations and visualize receptors in thin ice. Curiously, only the HER4 ectodomain could be visualized by this strategy and we observed significant orientation bias with the majority of particles representing the “bowtie” top-down projection. The orientation bias was observed with other LMNG receptor samples and could support the idea that intracellular domains denature at the air-water interface through standard sample preparation techniques. Multiple rounds of 3D heterogeneous refinements and *ab initio* steps produced a more reasonable 3D reconstruction at $\sim 4\text{\AA}$ (**Figure 1.4 A and B**). We also tried exchanging into other detergents

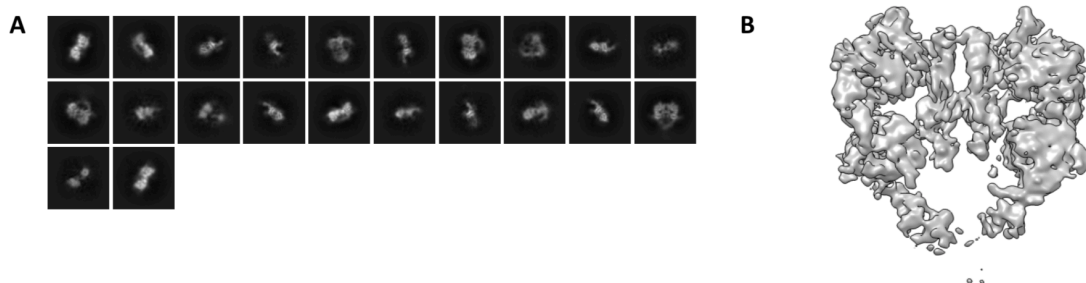


Figure 1.4. The LMNG Cryo-EM Strategy. (A) 2D cryo-EM class averages of the HER4-NRG1 β homodimer particles that went into the reconstruction in (B). (B) Reconstruction $\sim 4\text{\AA}$ of the HER4-NRG1 β homodimer from $\sim 64,000$ particles. Sample features a high degree of anisotropy even after substantial *in silico* efforts to remove orientation bias.

including glyco diosgenin (GDN), Digitonin, MNA-C12, and FA-3. Like other studies, we could successfully solubilize receptor in digitonin (data not shown)^{20,29}.

Reconstitution in lipid nanodiscs: Reconstituting HER receptors in lipid nanodiscs would eliminate the detergent micelle and potentially stabilize the kinase. Reconstituting single-pass transmembrane receptors in lipid bilayers has proved challenging and may be attributed to the low transmembrane energetics favoring reconstitution inherent in single-pass systems. We optimized numerous receptor:membrane scaffold protein (MSP):lipid ratios³⁰ and protocols to reconstitute HER4 homodimer into lipid nanodiscs. We found optimal reconstitution at a receptor:MSP:lipid ratio in SoyPolar lipid extract (see Materials and Methods) and could only visualize the receptor in nanodisc on pegylated graphene-oxide imine (GO-PEG-NH₂) grids (see below) (**Figure 1.5 A, B, C, and D**). Non-carbon supported Quantifoil and bare GO grids did not yield 2D class averages with density for the nanodisc suggesting that grid support is an important consideration in full-length receptor kinase efforts. In fact, only the extracellular domains could be visualized by cryo-EM (data not shown) despite a successfully reconstituted sample. Such a result may further lend credence to the hypothesis of intracellular domain denaturation at the air-water interface which is potentially reduced on GO-PEG-NH₂ grids.

Carbon-supported grids: Hydrophilic GO coated cryo-EM grids were shown to reduce orientation bias and enable particle visualization at NS-EM concentrations³¹. We rationalized that GO grids afforded three major advantages over conventional non-supported grids: (1) protection at the air-water interface, (2) lower sample concentration, and (3) reduction in orientation bias^{32,33}. We optimized numerous freezing parameters and reproducibly found that single particles could be visualized only after freezing with at least a 30 sec wait time and that only 4x negative stain-electron microscopy (NS-EM) receptor concentrations were required for adequate particle density

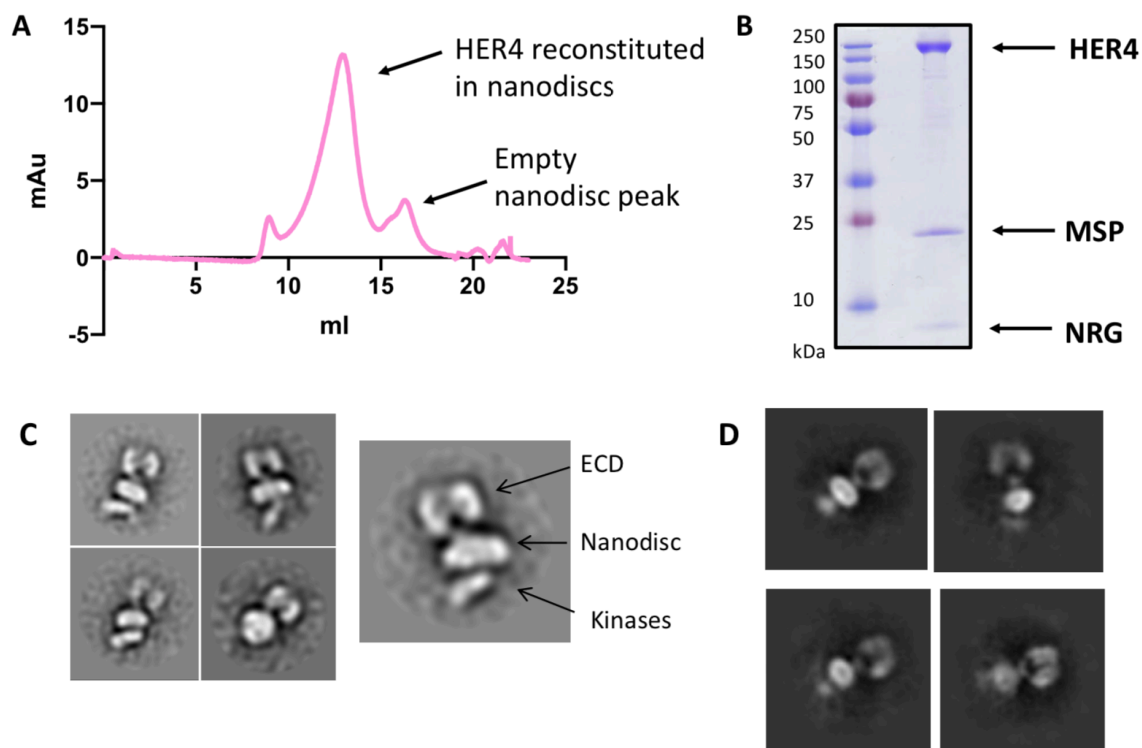


Figure 1.5. Reconstitution of HER4-NRG1 β Homodimer into lipid nanodiscs and visualization by Cryo-EM. (A) Gel filtration trace of reconstituted sample with a peak elution volume ~ 13.1 ml (representing a ~ 0.5 ml decrease in elution volume from detergent-solubilized sample). (B) Concentrated nanodisc peak sample from (A) on a Coomassie gel. Presence of MSP band indicates successful reconstitution. (C) Representative negative-stain EM class averages of nanodisc reconstituted sample. Extracellular, nanodisc, and kinase domain density are all visualized. The rod-shaped nature of the kinase domains is consistent with the catalytically active asymmetric kinase domain dimer. In many class averages, the rod-shaped kinases parallel the nanodisc raising a suspicion that the kinase and/or juxtamembrane domains may interact with the inner leaflet of the plasma membrane. (D) Preliminary cryo-EM 2D class averages of the HER4 homodimer in lipid nanodiscs imaged on GO-PEG-NH₂ grids. Significant extracellular and nanodisc densities are visualized with some density for the intracellular domains. To our knowledge, these are the first cryo-EM classes of a receptor kinase successfully incorporated into nanodiscs with the nanodisc and intracellular densities visualized.

in cryo-EM. Processing of a small dataset of HER4 homodimer on GO revealed 2D class averages consistent with a heart-shaped ECD with some encouraging density for the micelle. We found that ~40-50% of micrographs in any given GO dataset were devoid of particles. In contrast, samples without detergent routinely achieved ~95% coverage indicating that the detergent may lift GO flakes and reduce coverage. The major drawbacks of the GO strategy for detergent samples are therefore the dataset size, collection time, and micrograph curation.

Such encouraging results with the HER4 homodimer on GO were translated to visualizing the HER2/HER3/NRG1 β heterocomplex. Receptor fractions containing the heterodimer (as confirmed by NS-EM and mass spectrophotometry) were applied onto GO grids. Resulting 2D classes from a relatively small dataset (~700 micrographs) offered the first 2D class averages of the heterodimer with significant density for the micelle (**Figure 1.6 A**). An initial 3D model could be reconstructed from ~8,000 particles revealing clearly identifiable densities for HER2 and HER3 (**Figure 1.6 B**). Re-extracting and re-centering a larger particle stack from CryoSPARC³⁴ around the micelle in RELION³⁵ excitingly resulted in a reconstruction with a noticeable rod-shaped sub-micellar density consistent with crystal structures of the asymmetric kinase domain dimer (**Figure 1.6 C**).

To boost the resolution and potentially stabilize intracellular domains, we opted to (1) mildly crosslink the sample, (2) utilize a HER2 inhibitor, and (3) collect a larger dataset. Based on optimization with the HER4 homodimer (**Figure 1.7 A**), we conducted a glutaraldehyde crosslinking screen and identified the minimal concentration and time needed to empirically crosslink the sample. Efficient heterodimer crosslinking was achieved at 0.2% glutaraldehyde for 40 minutes prior to quenching with Tris buffer. NS-EM of crosslinked sample confirmed the significant improvement in homogeneity and some classes featured sub-micellar densities (**Figure**

1.7 B). We also added the irreversible Type I-like HER2 kinase inhibitor, canertinib, during expression to boost HER2 yields and lock the kinase in an active state³⁶. Merged cryo-EM datasets from Talos-Artica produced a $\sim 3.7\text{\AA}$ reconstruction of the heterodimer showing promise of obtaining a reconstruction at sufficiently high resolution to build an atomic model (**Figure 1.7 C and D**). Data collection and processing of micrographs collected on Titan-Krios with an inserted energy filter produced a final

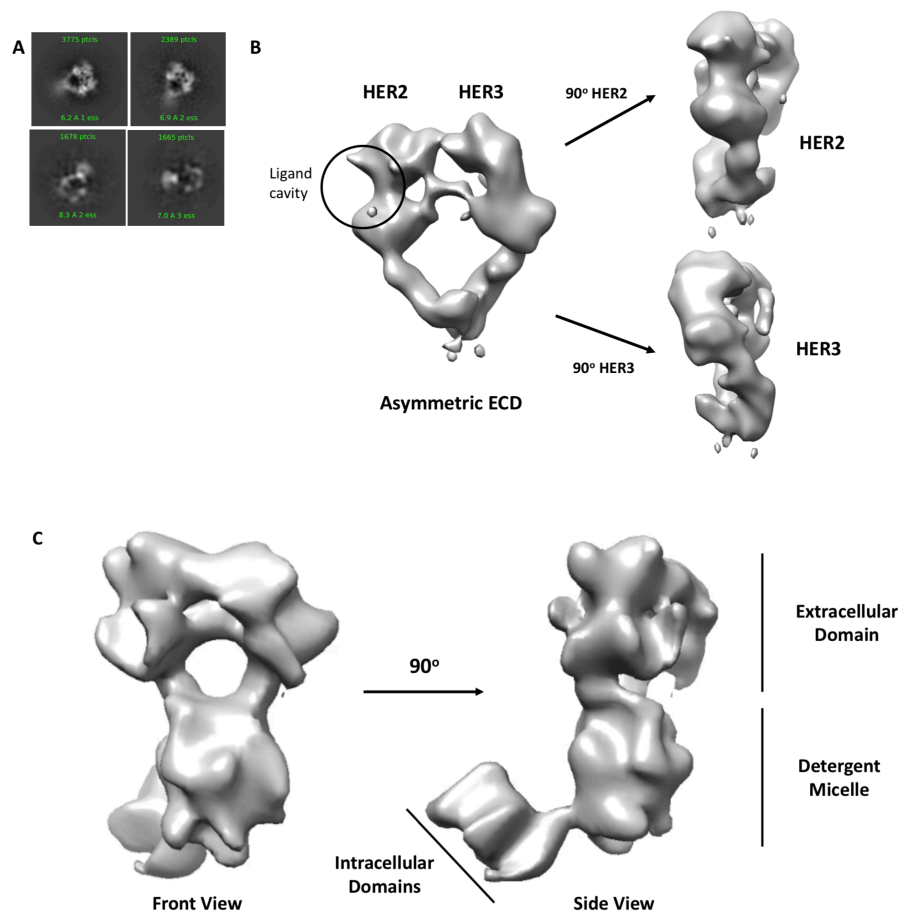


Figure 1.6. Initial Cryo-EM imaging of the HER2/HER3/NRG1 β heterocomplex. (A) Representative 2D cryo-EM class averages of HER2 E928G/HER3/NRG1 β sample on GO. Some classes centered on the extracellular domain (top two) while others centered on the micelle at the cost of extracellular features (bottom two). **(B)** Initial processing in CryoSPARC yielded $\sim 12\text{\AA}$ reconstruction of the extracellular domain from $\sim 8,000$ particles. Even at this resolution, HER2 could be readily identified by the ligand cavity in this inherently asymmetric complex. **(C)** Re-processing of particles in RELION resulted in reconstructions with a visible rod-shaped density below the micelle. To our knowledge, this is the first cryo-EM reconstruction of a receptor tyrosine kinase (RTK) with intracellular density.

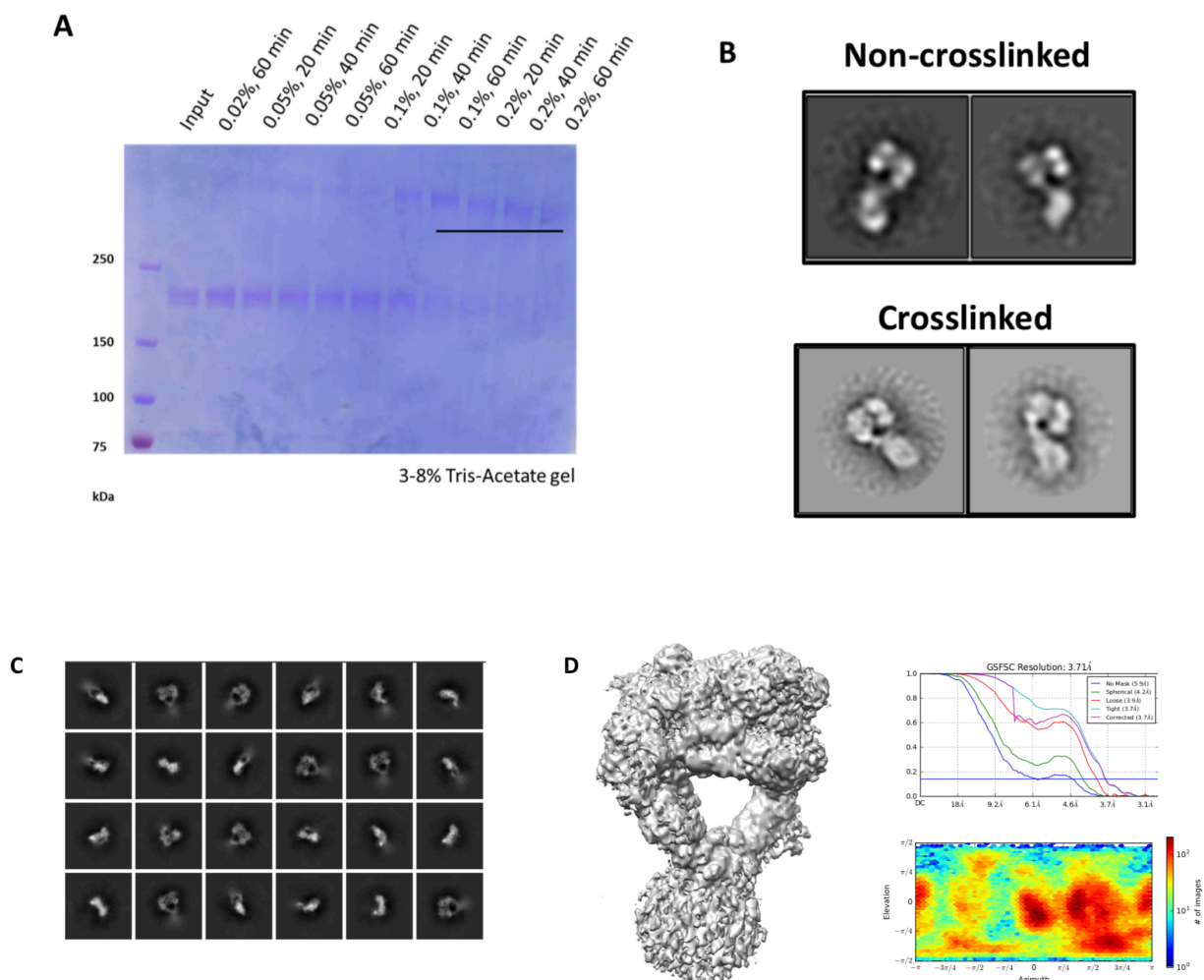


Figure 1.7. Crosslinked sample improves homogeneity. (A) Coomassie stained gradient Tris-Acetate gel of HER4-NRG1 β homodimer. Initial crosslinking screens with glutaraldehyde were conducted with HER4 and a similar assay was performed with limited HER2/HER3/NRG1 β sample. Optimal crosslinking for HER2/HER3/NRG1 β sample was found at 0.2% glutaraldehyde for 40 min as determined by the presence of the upper dimer band. (B) NS-EM class averages of crosslinked samples display more domain-level features compared with non-crosslinked sample. (C) Cryo-EM 2D class averages of crosslinked sample on bare GO shows high resolution features with minimal orientation bias. (D) Low-contoured volume of the HER2/HER3/NRG1 β heterocomplex from an initial merged dataset of just ~1,600 curated micrographs and ~180,000 particles on Talos-Arctica with a resolution of ~3.7 Å. Density for the detergent micelle is apparent. Only minor orientation bias is noted on angular pose map.

~2.9Å reconstruction of the HER2/HER3/NRG1β heterocomplex extracellular domain (**Figure 1.8 A**). Further 3D classification in RELION produced three distinct reconstructions with one reconstruction (with a minor population of particles) featuring a prominent detergent micelle (**Figure 1.8 B**). Processing is underway to recover sub-micelle density through particle re-centering. Freezing with GO approach may therefore represent a superior strategy over freezing on non-supported grids for receptor kinase samples. However, the presence of only a minor population of particles featuring an intact micelle suggests that a significant portion of the receptor may be lost to the air-water interface. Thus, while freezing on bare GO may be a superior strategy to freezing non-supported grids and clearly offers the advantage of solving high resolution structure of sample limited targets (the 2.9Å reconstruction was derived from 1 ml of a 0.7 mAu peak from gel filtration), it may not be the optimal strategy for solving the full-length receptor. For that, additional support layers such as GO-PEG-NH₂ may be required as we have consistently seen sub-micelle/nanodisc density on such grids.

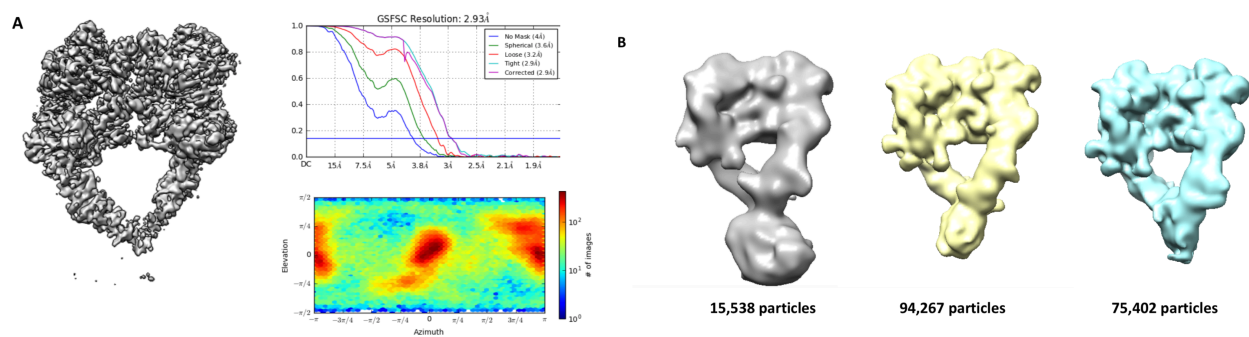


Figure 1.8. High resolution reconstruction of the extracellular domains of the HER2/HER3/NRG1β heterodimer and recovery of micelle density. (A) Reconstruction of the HER2/HER3/NRG1β at high contour where micelle density is sparse demonstrates high-resolution features consistent with a resolution of ~2.9Å. **(B)** Processing the particle stack in RELION through re-extraction and 3D classification (but not with particle recentering) shows a minor reconstruction with clear micelle density (left, gray) with the other two classes are largely (middle yellow and right blue) largely devoid of such density.

The HER2/HER3/NRG1 β structure represents the first of a heterodimeric complex between extracellular domains of two HER receptors and, for the first time, visualizes a human HER ECD complex in a singly-liganded state. All four subdomains of both ECDs are resolved, including two solenoid ligand-binding domains (subdomains I and III) and two cysteine-rich laminin-like domains (subdomains II and IV) (**Figure 1.9 A**). The EGF-like domain of the NRG1 β ligand is wedged between subdomains I and III of HER3 whereas no ligand density is present in the HER2 monomer consistent with its status as an orphan receptor. The majority of the dimerization interface between two ECD monomers is driven by subdomains II containing the dimerization arm, a β -hairpin protrusion, that intimately contacts the opposing dimerization partner^{4,37}. Although subdomains IV of both receptors are brought into close proximity in our structure, they contribute little to dimerization. This observation is consistent with previously solved crystal structures of isolated ECDs^{5,8}.

In our structure, the HER3 ECD is captured for the first time in a ligand-bound state. Previously solved crystal structures of the isolated HER3 ECD correspond to an apo “tethered” conformation in which the dimerization arm intramolecularly tucks into subdomain IV while subdomains I and III remain far apart¹¹. We show that upon ligand binding, HER3 adopts the canonical extended conformation, due to a rigid body rotation about the subdomain IV axis, which was previously observed in ligand-bound EGFR and HER4^{4,5,8}. NRG1 β engages with HER3 primarily through an extensive interaction network at its C-terminus and is locked into place by salt bridges between NRG1 β R33 and D93 in HER3 subdomain I and NRG1 β R46 and D352 in HER3 subdomain III, bringing subdomains I and III into close proximity (**Figure 1.9 B**). Many of the contacts between NRG1 β and HER3 are conserved in the NRG1 β -bound HER4 complex

(**Figure 1.10**). The orphan HER2 receptor, which adopts an extended conformation in the absence of a bound ligand,

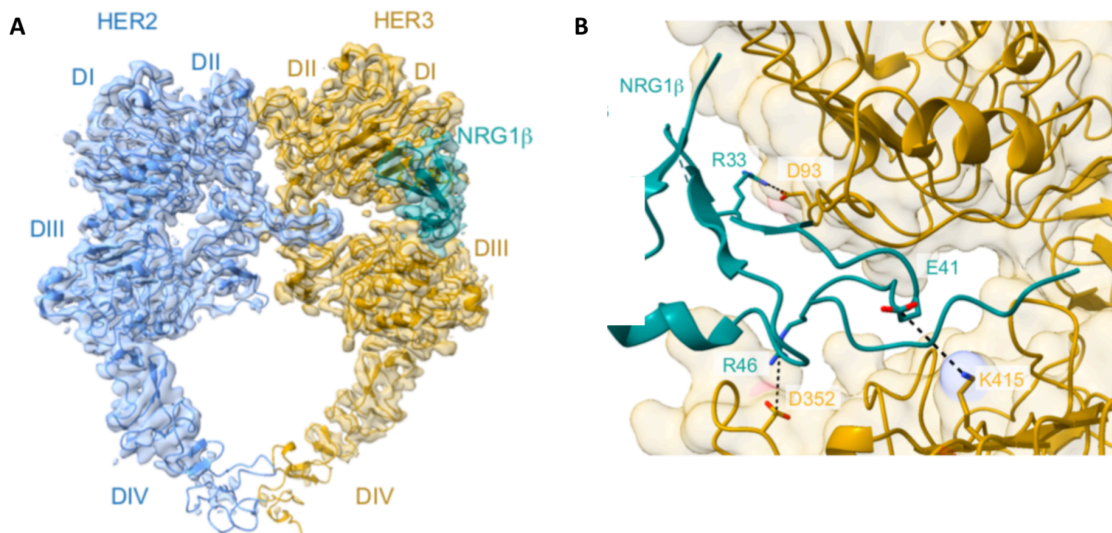


Figure 1.9. Extracellular Domain Structure of the HER2/HER3/NRG1 β Heterocomplex. (A) Atomic model of the extracellular domains of HER2/HER3/NRG1 β is docked within the reconstruction at high contour. HER3, gold, is bound to NRG1 β , teal, while HER2, blue, lacks density for a ligand. (B) Zoomed-in view of NRG1 β interaction with HER3 with key contacts between receptor and ligand shown as dotted black lines.

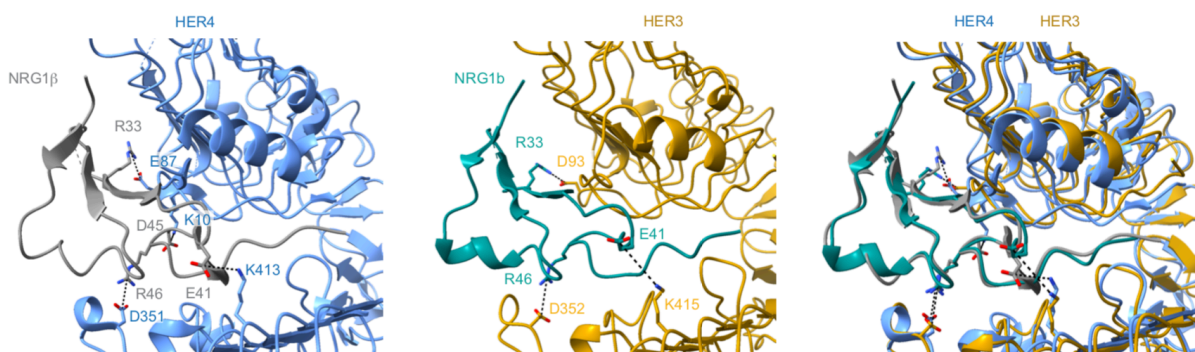


Figure 1.10. A conserved ligand binding mode between HER3 and HER4. Left, HER4 bound to NRG1 β (PDB ID: 3U7U) with notable residues and interactions highlighted. Middle, HER3 bound to NRG1 β with notable residues and interactions highlighted. Right, overlay between the two structures shows that the majority of contacts are shared between HER3 and HER4.

adopts the same extended conformation in our structure. This indicates that the previously published structural state of HER2 alone captures HER2 in an active conformation rather than in an autoinhibited state, as has been previously proposed³⁸ (**Figure 1.11**). Therefore, HER2 is truly primed for heterodimerization on its extracellular domain side with other ligand-bound HER receptors³⁹.

The most striking feature of the HER2/HER3/NRG1 β complex is the lack of any detectable density for the HER3 dimerization arm located in subdomain II (**Figure 1.12 A**). This is surprising because in all other structures of active HER ECD dimers, the dimerization arms of both receptors provide an essential contribution to the dimerization interface^{4,5,8,40-42}. In contrast to HER3, the HER2 dimerization arm is completely resolved at atomic resolution in the heterodimer and packs closely with HER3 through key

sidechain-backbone polar contacts between HER2 Y274 and HER3 G265, HER2 T276 and HER3 G284. We further show that the HER3 dimerization arm is not necessary for heterodimerization (**Figure 1.12 B**) Interestingly, HER3 G284R is a HER3 oncogenic mutation¹⁸ and the mechanistic basis for enhancing heterodimerization may be explained from strengthening the polar interaction with the HER2 dimerization through HER2 D277 (**Figure 1.13 A, B and C**). The result of asymmetric dimerization arm engagement is a reduction in the

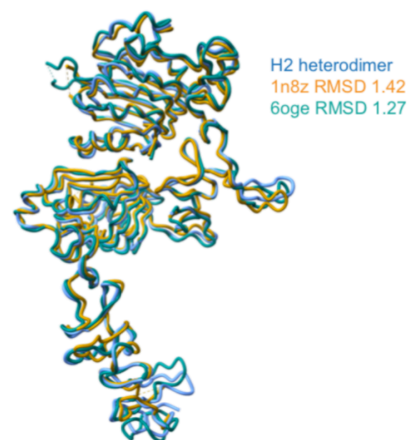


Figure 1.11. HER2 is unchanged upon co-receptor engagement. Structure of HER2 in the heterocomplex is overlaid with a recent cryo-EM structure of extracellular domain of HER2 simultaneously bound to pertuzumab and trastuzumab Fabs (6OGE) and a crystal structure of HER2 bound to trastuzumab Fab (1N8Z). The structures are almost identical with low root mean squared deviations (RMSDs) with only loop rotations in the dimerization arm and subdomain IV indicating that HER2, at least extracellularly, is primed for engagement with co-receptors.

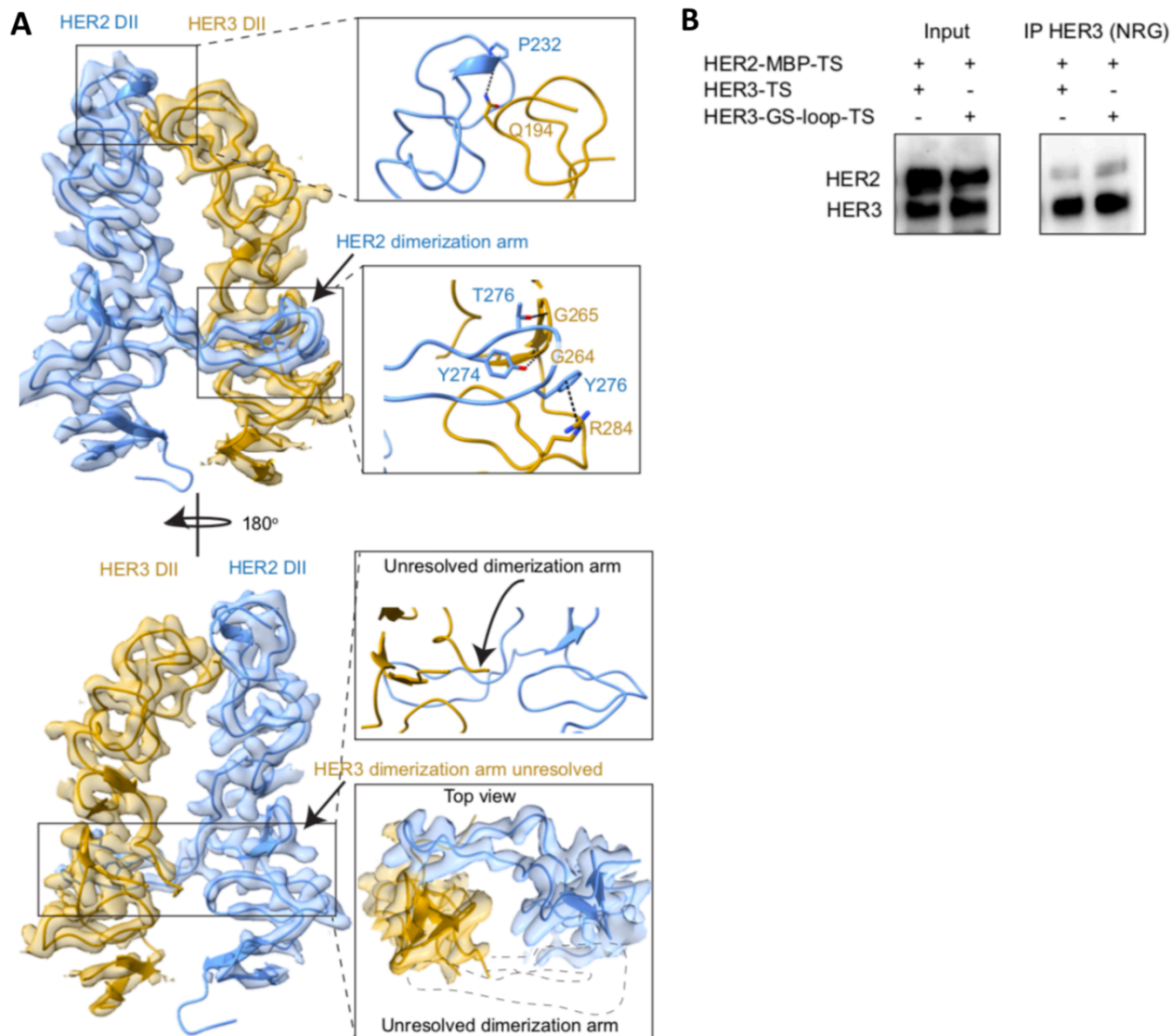


Figure 1.12. The HER3 dimerization arm is not engaged. (A) Top, zoomed in view of subdomain II interactions showing complete engagement of the HER2 dimerization arm. Only one contact is observed outside of the dimerization arm (HER2 P232 – HER3 Q194) while numerous contacts stabilize the HER2 dimerization in a dimerization arm pocket formed by liganded HER3. Bottom, rotating the structure reveals the complete absence of HER3 dimerization arm density at equivalent contour. In the inset, dots represent the expected extent of the HER3 dimerization arm surprisingly absent in our structure. **(B)** Western blot ligand pulldown of the heterodimer in the presence of a non-specific glycine-serine (GS) loop within the HER3 dimerization arm. Successful HER2 pulldown demonstrates that the HER3 dimerization arm is not necessary for heterodimerization.

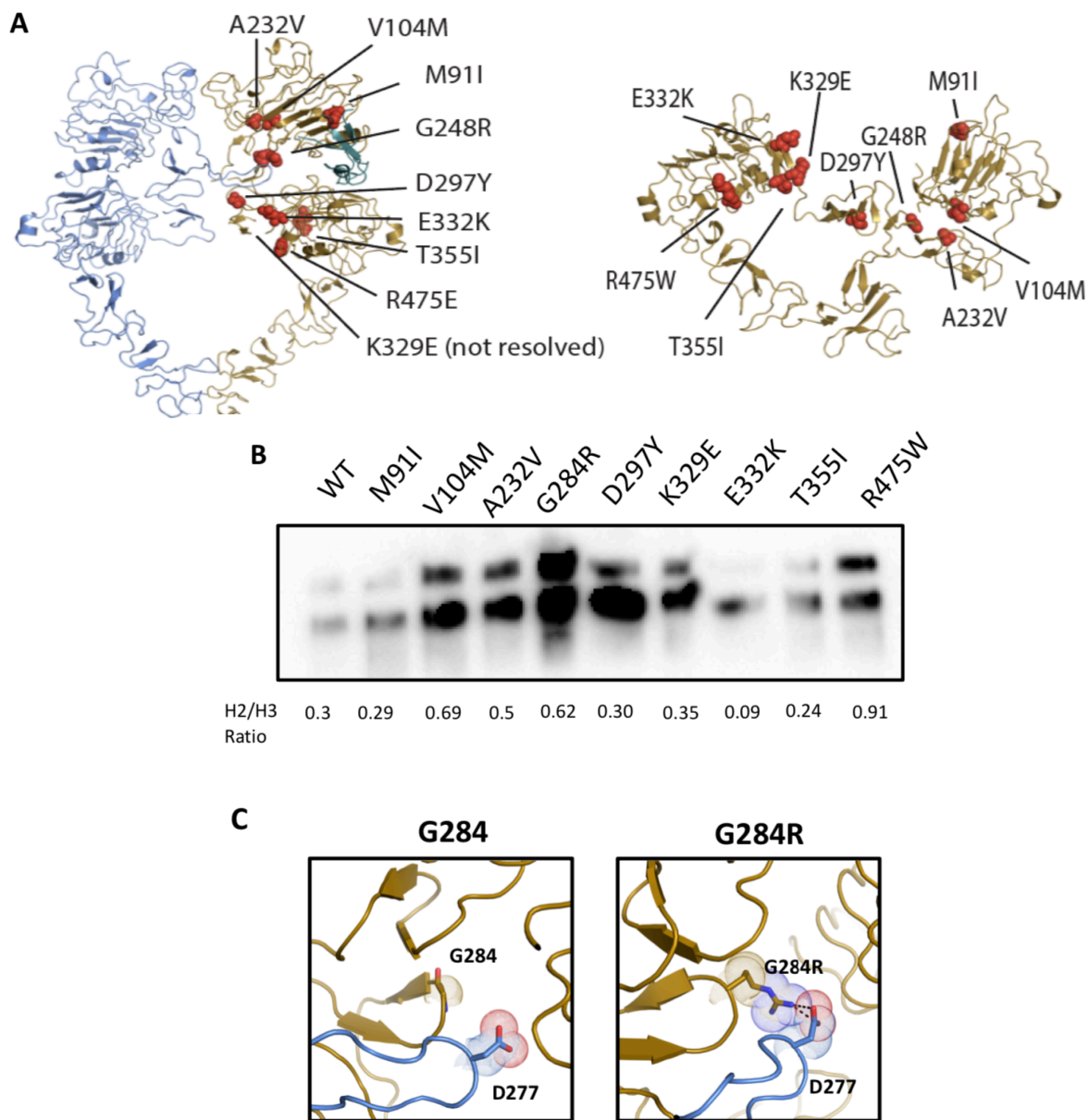


Figure 1.13. Analysis of HER3 Extracellular Oncogenic Mutations. (A) Left, HER3 oncogenic mutations are mapped onto the extended HER3 conformation in the heterodimer structure. Right, the tethered HER3 conformation (PDB ID: 1M6B). Many HER3 oncogenic mutations cannot be explained in the tethered conformation. (B) Ligand pulldown assay in the presence of each oncogenic mutation to detect HER2 pulled down. A ratio of band intensity was calculated for each pulldown, the higher the ratio, the more HER2 that that oncogenic mutant is able to pull down. G284R, R475W, and V104M (the most common HER3 oncogenic mutant) are significantly elevated relative to wild-type. (C) HER3 G284R is predicted to establish a strong salt bridge with HER2 D277 at the tip of the HER2 dimerization arm.

total buried surface area of the HER2/HER3 ECD heterodimer (1,144Å²) which is significantly less than that of HER receptor homodimers (1,633Å² for EGFR homodimer/PDB ID:3NJP and 1670Å² for HER4 homodimer/PDB ID: 3U7U). Additionally, apart from the dimerization arm, only one additional polar side chain interaction exists throughout the entirety of the subdomain II interface (HER2 P232 and HER3 Q194) as opposed to the extensive network of polar interactions seen in other HER receptor complexes^{4,5,40}. Altogether, the HER2/HER3/NRG1β complex uniquely features the asymmetric engagement of a single dimerization arm in the active ECD dimer resulting in a weakened and redesigned interactive surface area.

Comparisons of our cryo-EM HER2/HER3 ECD heterodimer structure with previously determined crystal structures of HER receptor ECD homodimers outline a potential mechanism of HER3 dimerization arm disengagement. Including our structure, there are three types of human HER ECD dimers representing a spectrum of liganded states: (1) doubly liganded symmetric EGFR and HER4 ECD homodimers bound to their respective ligands (EGFR/EGF, EGFR/TGFα, HER4/NRG1β)^{4,5,43}, (2) doubly liganded asymmetric EGFR ECD homodimers in which one of the ligands is less engaged with the receptor (EGFR/EREG, *Drosophila* EGFR/Spitz)^{40,41} and (3) our heterodimer structure in which only one ligand is engaged (**Figure 1.14 A**). The NRG1β-bound HER3 monomer in the heterodimer structure overlays best with both protomers in the symmetric, doubly liganded homodimers (RMSD 1.16). However, when compared to the asymmetric EGFR/EREG ECD homodimer, the NRG1β-bound HER3 monomer is more similar to the EGFR monomer in which the ligand is fully “wedged” between subdomains I and III than the partially “wedged” monomer (**Figure 1.14 B**).

In both overlays significant deviations are observed between HER2 and EGFR. Aligning the HER2 monomer with EGFR/EREG and EGFR/EGF monomers on subdomain I reveals a

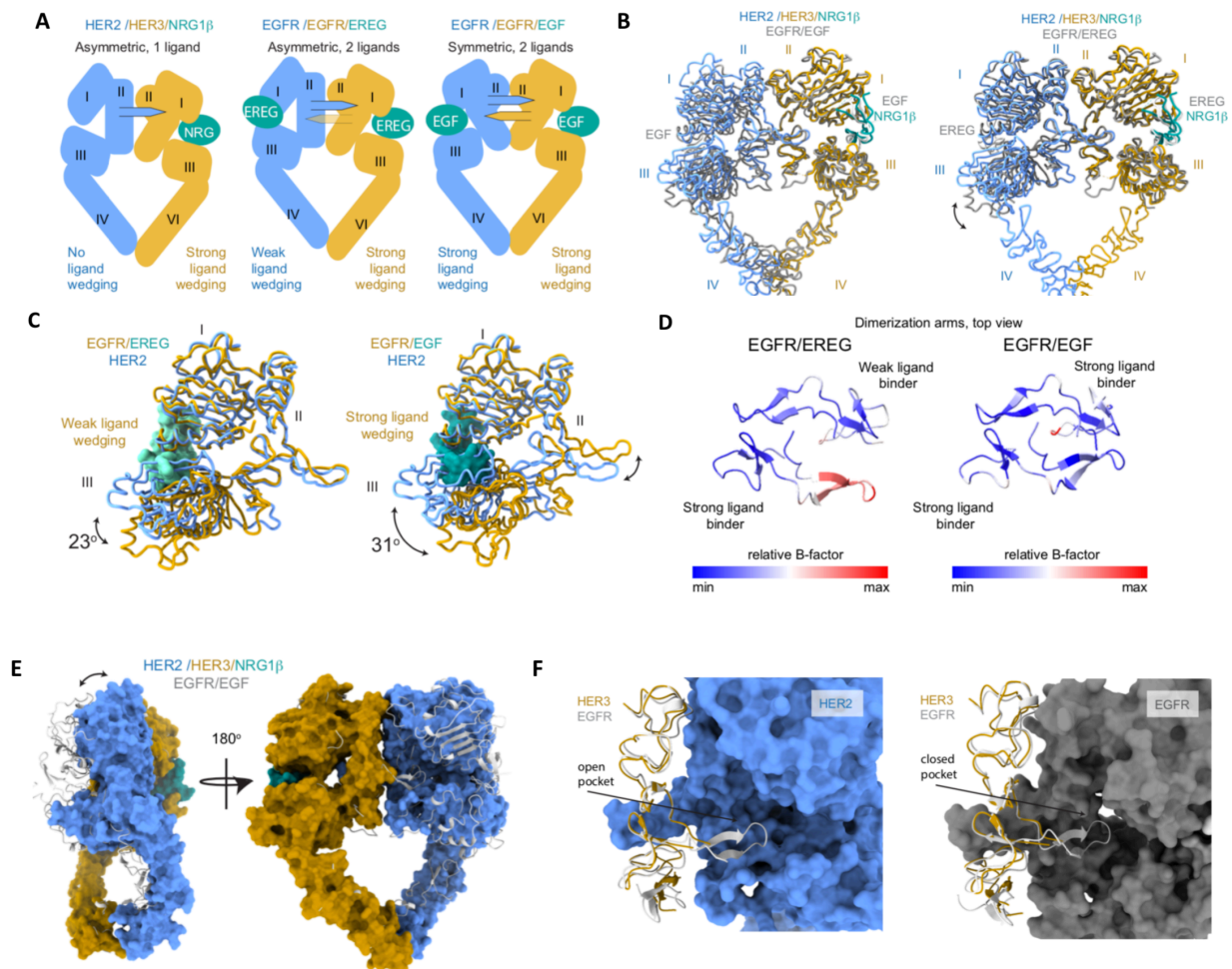


Figure 1.14. A spectrum of ligand engagement reveals that the dimerization arm is a finely tuned allosteric sensor for ligand engagement. (A) Cartoon depictions of three liganded dimeric states. Left, the singly liganded, asymmetric heterodimer presented here, middle, the asymmetric doubly liganded state observed for epiregulin (ERE) bound to EGFR, and right, the doubly liganded, symmetric state observed for EGF bound to EGFR. Ligand wedging between subdomains I and III is characterized as absent, weak, or strong based on the observed ligand interactions with the receptor. (B) Overlays of our structure with EGF bound EGFR (PDB ID: 3NJP) and EREG bound to EGFR (PDB ID: 5WB7) show excellent overlays over HER3 but more deviation on the HER2 side, particularly in subdomains I and III. (C) Subdomains I-III of EREG/EGFR overlaid with HER2 (left) and EGF/EGFR overlaid with HER2 (right) with ligand density showed as teal surfaces. Aligning on subdomain I reveals significant angular movement of subdomain III relative to subdomain I that is dependent on the degree of ligand wedging (weakly wedged EREG: 23°; strongly wedged EGF: 31°). (D) Dimerization arms from EREG:EGFR and EGF:EGFR are color coded by normalized b-factors. (E) Overlay of EGF/EGFR with the HER2/HER3/NRG1 β heterocomplex shown as surface. The absence of ligand wedging in HER2 is apparent in both views. (F) The absence of ligand for HER2 excludes the rotations observed in (C) and (E) thus eliminating the pocket for the HER3 dimerization arm.

rotation of subdomain III relative to subdomain I that is directly attributed to the presence of ligand and the degree of ligand wedging (**Figure 1.14 C**). Without a ligand, the HER2 subdomain III is horizontal relative to its subdomain I. In contrast, subdomain III of EGFR bound to either EREG or EGF rigid-body rotates downwards on the outer aspect of the receptor but upwards on the side closer to subdomain II. The relative subdomain III rotation correlates with the spectrum of ligand wedging now completed with our singly liganded structure. A larger angular deviation (31.3°) is observed between HER2 and the fully wedged EGFR/EGF than that between HER2 and the partially wedged EGFR/EREG (23.4°) (**Figure 1.14 C**). The ligand-induced movement of subdomain III results in the upward positioning of the dimerization arm.

Our data point to a key dependence between the rotation of subdomain III induced by ligand wedging on one receptor monomer and the engagement of the dimerization arm of the opposing monomer in the active HER receptor dimer. While the HER2 dimerization arm is fully engaged by liganded HER3, the HER3 dimerization arm is not by the unliganded HER2 in which subdomain III rotation does not occur. The dimerization arm of the HER3-like/fully-wedged EGFR/EREG protomer is also less engaged than that of the HER2-like/partially-wedged protomer as judged by higher b-factors in the dimerization arm (**Figure 1.14 D**). In contrast, symmetrically fully-wedged EGFR/EGF features equally low dimerization arm b-factors (**Figure 1.14 D**). The EREG/EGFR structure therefore represents an intermediate asymmetric state between symmetric doubly-liganded ECD homodimers and asymmetric singly-liganded dimers. Therefore, we observe a spectrum of ligand wedging that is correlated with the dimerization arm dynamics of the opposing monomer.

Together these observations provide evidence for an allosteric link between ligand binding of one receptor monomer and dimerization arm engagement of another in active HER receptor

dimers. Ligand binding induces a rotation of subdomain I and III around the ligand to generate a receptive pocket for the dimerization arm of the opposing monomer. The lack of HER2 subdomains I and III rotation results in a reduced interactive pocket to stabilize the HER3 dimerization arm whereas the closer positioning of subdomains I and III in fully-wedged EGFR/EGF enables a tighter interaction with the opposing EGFR protomer dimerization arm (**Figure 1.14 E and F**). The partially wedged EGFR/EREG protomer captures the intermediate state through partial rotation resulting in moderate stabilization of the opposite dimerization arm. The dimerization arm is therefore a sensor for ligand engagement.

Our findings also explain how HER2 ECDs do not homodimerize in the solution or in crystal structures even when the dimerization arm is constitutively exposed¹¹. The absence of the ligand-wedged conformation in either monomer of a putative HER2 homodimer does not permit the dimerization arms to engage the opposing monomer. Therefore, HER2 is, in a way, autoinhibited by resisting the formation of a heart-shaped ECD homodimer classically associated with the active conformation.

HER2 S310 is the most frequent mutated residue in non-amplified HER2 cancers but the mechanism for its transforming potential is not understood^{18,44,45}. Given the proximity of the HER2 S310 to the binding interface that is expected to engage the dimerization arm of another receptor in the active dimer, we hypothesized that the most frequent HER2 oncogenic variants, S310Y and S310F, may influence the interactions with the HER3 dimerization arm. The HER2 S310F variant significantly triples yields of the heterodimer yields suggesting that it substantially stabilizes the heterocomplex assembly (data not shown). Using cryo-EM, we solved a structure of the HER2 S310F/HER3 heterodimer bound to NRG1 β at 3.1Å resolution. Overall, the structure closely resembles that of the wild type complex, with the exception of the HER3 dimerization arm, which

now is entirely resolved at atomic resolution (**Figure 1.15 A**). The reduced flexibility of the HER3 dimerization arm can be attributed to a stabilizing π - π stacking interactions between HER2 F310 and HER3 Y265. The HER3 Y265 sidechain hydroxyl group establishes two polar contacts with

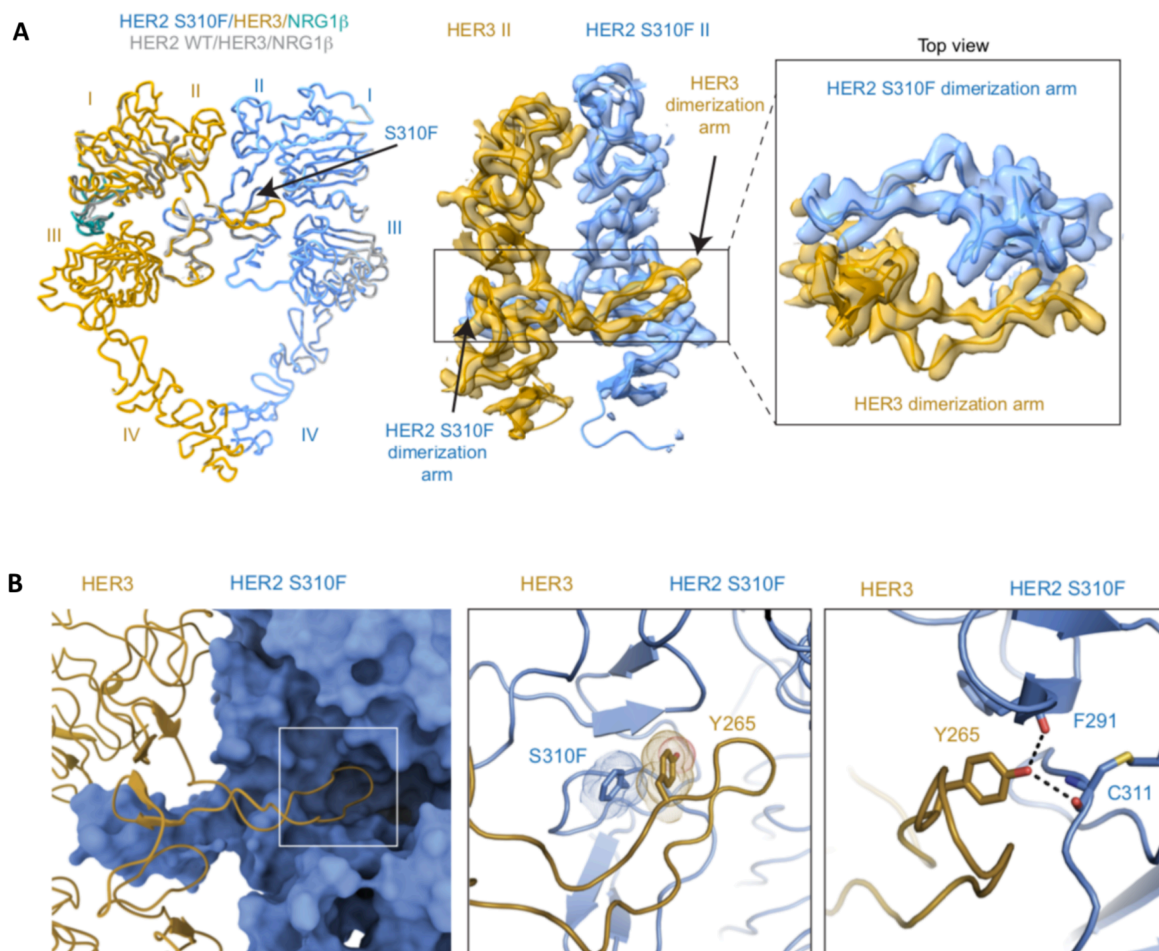


Figure 1.15. HER2 S310F Engages the HER3 Dimerization Arm. **(A)** Left, model of the HER2 S310F/HER3/NERG1 β heterocomplex built from a 3.1Å cryo-EM reconstruction. Middle, subdomain II of HER2 and HER2 shown with the HER3 dimerization arm now resolved. Right, top-down view with both dimerization arms now engaged. **(B)** Left, the HER3 dimerization arm is accommodated in HER2 between subdomains I and III. Middle, HER2 S310F establishes a π - π with HER3 Y265. Right, HER3 Y265 forms backbone polar contacts with F291 and C311.

the backbone of HER2 residues F290 and C311 which concurrently induces a slight upward rotation of the HER3 dimerization arm compared to positioning of the arm in HER4 or EGFR homodimers (**Figure 1.15 B**). The reduced dynamics of the HER3 dimerization arm increases the buried surface area at the HER2/HER3 dimerization interface from 1,144Å² to 1,863Å² which is comparable to the respective interfaces in the HER4 and EGFR homodimer crystal structures. The net energetic stabilization of these interactions combined with the upward rotation of the HER3 dimerization arm into the HER2 pocket likely serves to overcome the unfavorable bulky residues at the tip of the HER3 dimerization arm, K268 and L269. We predict that the same mechanism is employed by HER2 S310Y, which would form an analogous π - π stacking interaction with HER3 Y265 and similarly stabilize the heterocomplex by engaging the HER3 dimerization arm. Thus, our structure provides mechanistic explanations for the activating effects of the HER2 S310F and S310Y cancer mutants.

The increased propensity of HER2-S310F variant to heterodimerize with HER3 could have therapeutic implications. We assessed the ability of the HER2-targeting monoclonal antibodies, trastuzumab and pertuzumab, to disrupt wild type HER2 or HER2-S310F with NRG1 β -bound HER3 especially given conflicting reports in literature over the mechanism of action of trastuzumab and its targeting of heterocomplexes^{46,47}. Recombinant, nearly full-length complexes were incubated with an excess of trastuzumab or pertuzumab Fab, followed by HER2 affinity purification and evaluation of bound Fab levels. We found that wild-type and mutant HER2/HER3 heterodimers interacted equally well with trastuzumab but pertuzumab only bound to the wild type complex and not to the HER2-S310F-containing heterodimer (**Figure 1.16 A**). Our results show how trastuzumab is relatively non-specific in targeting HER2 *and* HER2-

containing heterocomplexes, a mechanistic finding which could explain the cardiotoxic side-effects through non-specific HER2-HER4/NRG1-axis inhibition^{48,49}.

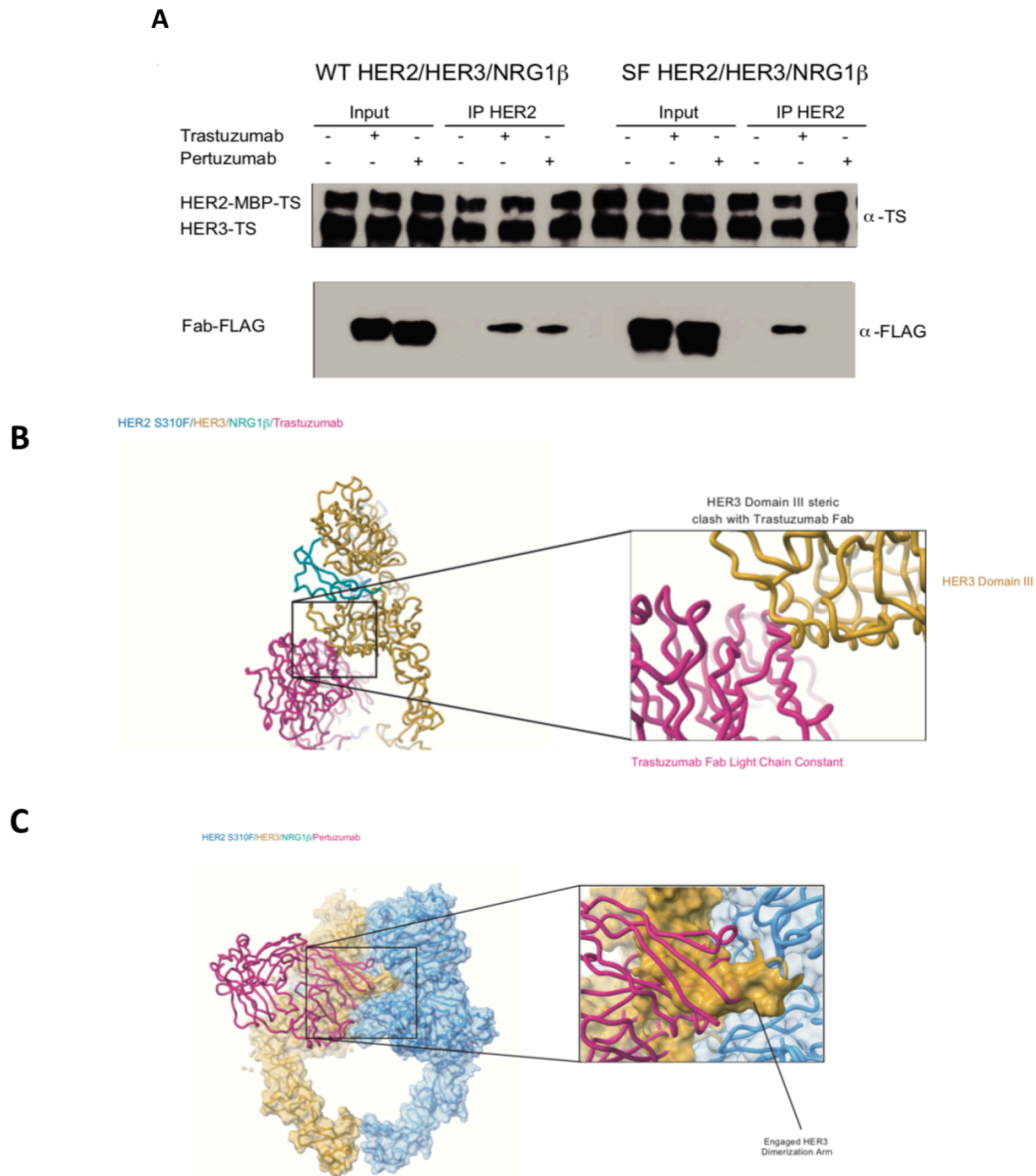


Figure 1.16. The HER2/HER3/NRG1 β Heterodimer Accommodates Pertuzumab and Trastuzumab while HER2 S310F/HER3/NRG1 β resists Pertuzumab binding. (A) Western Blot of heterodimer Fab pulldown assay blotting for twin-strep tagged receptors and flag-tagged Fab. HER2/HER3/NRG1 β pulls down both Trastuzumab and Pertuzumab while HER2 S310F/HER3/NRG1 β only pulls down Trastuzumab. **(B)** Docking Trastuzumab Fab from PDB ID: 6OGE reveals a steric clash of the light chain constant domain with HER3 subdomain III. **(C)** Docking Pertuzumab Fab into HER2 S310F/HER3/NRG1 β from PDB ID:6OGE reveals a steric clash between the Fab variable domains with the HER3 dimerization arm and HER3 subdomain II.

The differences in binding between trastuzumab and pertuzumab can be structurally rationalized. Docking trastuzumab into both wild type HER2/HER3 and HER2-S310F/HER3 heterodimer structures reveals a minor steric clash between Fab and domain III of HER3 (**Figure 1.16 B**). Given the weakened interface between the extracellular domains in the HER2/HER3 heterodimer, domain III could slightly rotate to accommodate trastuzumab without entirely disassociating the heterodimer. Pertuzumab docked into the structure of the HER2/HER3/NGR1 β heterodimer clashes with domain II of HER3 but similarly, the dynamic HER2/HER3 interface likely allows for HER3 to rotate relative to HER2 and present the complete pertuzumab epitope, which includes the HER2 dimerization arm (**Figure 1.16 C**). This HER2 epitope is directly occluded by the HER3 dimerization arm in the same complex that includes the HER2 S310F mutant, explaining why the mutant complex does not bind pertuzumab (**Figure 1.16 C**). Thus, our work suggests that pertuzumab may be less effective than trastuzumab at targeting cancers driven by HER2 S310F/Y owing in part to the engagement of the HER3 dimerization arm in the mutant complexes.

To understand how the heterodimer could accommodate trastuzumab, we incubated the trastuzumab Fab with the HER2-S310F/HER3 heterodimer prior to crosslinking and cryo-EM imaging. Our resulting reconstruction at 3.5Å shows that trastuzumab binds at a different angle to domain IV of HER2 when compared to the crystal structure of HER2 alone with trastuzumab (**Figure 1.17 A and B**). In addition, the Fab pushes the domains IV of HER2 and HER3 further apart, underscoring the inherent flexibility of domain IV. The close proximity of the trastuzumab Fab to HER3 offers exciting avenues for the development of the next generation of biologics and biomarkers with dual specificity for HER2 and HER3.

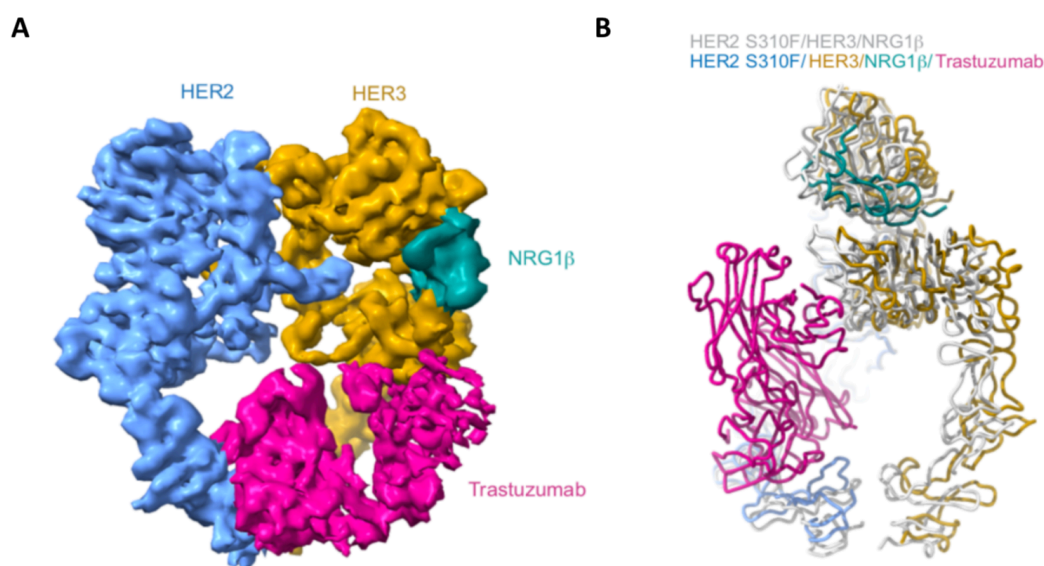


Figure 1.17. The HER2 S310F/HER3/NRG1 β Heterodimer bound to Trastuzumab Fab. (A) Cryo-EM reconstruction of the heterodimer bound to the trastuzumab Fab low-pass filtered to 5Å and zoned. Strong density is evident for the Fab variable chain interaction with HER2 subdomain IV. (B) Overlay of HER2 S310F/HER3/NRG1 β model (light gray) with the trastuzumab-bound model (colored) reveals a conformational shift in HER3 subdomain III and subdomains IV of HER2 and HER3.

The progress on heterodimer structure relied on numerous technical advancements in sample preparation and visualization. We have applied these technologies to isolate the EGFR/HER3/NRG1 β and HER2/HER4/NRG1 β heterodimers and to structurally and functionally interrogate in their full-length forms demonstrating the generalizability of our techniques to studying other challenging and elusive targets beyond HER receptors such as members of the Insulin, PDGF β , FGF, Axl, Trk, PTK7, and BMP receptor families.

DISCUSSION

Our structure of the singly-liganded HER2/HER3/NRG1 β heterocomplex provides the first structural descriptions of the active state of HER2 bound to a liganded, extended HER3 and

expands the repertoire of ligand binding states observed with human HER receptors. The asymmetry in dimerization arm engagement suggests that the extracellular domains across the HER family are able to adopt multiple conformations depending on the mode of interaction with the ligand and that HER2 may be intrinsically autoinhibited in the absence of a known ligand. This differential ligand binding is allosterically linked to the strength of the dimerization interface between the receptors by remodeling the dimerization arm binding pocket between domains I and III. The dimerization arm is therefore not a static structural module but a finely tuned dynamic sensor for ligand engagement. Our allosteric model could extend to HER receptor homodimers in which a single ligand is sufficient for receptor activation and explain biased agonism in which certain lower affinity ligands may physiologically signal predominantly through weaker singly-liganded receptor states^{5,40}.

We furthermore find that HER3 dimerization arm dynamics is exploited by the most frequent HER2 oncogenic mutation, HER2 S310F, to enhance dimerization strength through a fully engaged HER3 dimerization arm. As a parallel mechanism to oncogenic mutations, suspected ligands for HER2, including Mucin 4^{50,51}, which contains EGF-like domains and is correlated to breast cancer invasiveness⁵², could also increase HER2/HER3 heterodimerization by inducing HER2 domain I and III wedging and providing a receptive pocket for the HER3 dimerization arm.

Dimerization arm dynamics may have implications for therapeutic approaches to HER2 driven cancers. Our study shows for the first time *in vitro* that the wild-type HER2/HER3/NRG1 β heterocomplex binds both trastuzumab and pertuzumab which likely results from the inherently labile extracellular interface observed in our wild-type structure. In contrast, the strengthened HER2-S310F heterodimer is less flexible in the pertuzumab epitope and thus resists binding to the pertuzumab Fab. Finally, our structure of trastuzumab-bound HER2-S310F/HER3/NRG1 β reveals

how the heterodimer overcomes a steric clash to accommodate the Fab. The structural models produced in this study may be used in the rational design of therapeutics and biomarkers specific to the active co-receptor bound state of HER2. Small molecule agents, for example, directed at the HER2 dimerization arm pocket on HER3 or bispecific biologics utilizing a trastuzumab scaffold could form a new generation of therapies for HER2 driven cancers.

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MATERIALS AND METHODS

NRG1 β Expression and Purification

A HRV-3C cleavable Thyrodoxin A (TrxA)⁵ was fused to the EGF-like domain of NRG1 β (residue ranges) with C-terminal Flag and 6x Histidine tags and subsequently cloned into a p32A vector. The TrxA-3C-NRG1 β -Flag-6xHis construct was transformed into Origami *E. coli*, grown at 37 °C in Terrific Broth in large scale culture until an optical density (OD) of ~1.0–1.5 was achieved, and induced with 1mM Isopropyl β -d-1-thiogalactopyranoside (IPTG) overnight at room temperature. Cells were harvested the next day, pelleted, flash frozen, and stored until purification.

Cells were resuspended in NRG lysis buffer (50 mM Tris cold pH 7.4, 150 mM NaCl, 1mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors (eOComplete, Roche)) and

sonicated until thoroughly lysed. Lysate was then clarified through ultracentrifugation, syringe filtered through 0.44 micron filters and incubated with Ni-NTA resin overnight. The beads were washed by gravity through 20 column volumes (CVs) of NRG wash buffer (50 mM Tris cold pH 7.4, 150 mM NaCl) + 20 mM imidazole, then 10 CVs of NRG wash buffer + 50 mM imidazole, and finally eluted with 3 CVs of NRG wash buffer + 300 mM imidazole. Imidazole in the eluate was reduced < 30 mM over a 10K concentrator and subsequent dilution with NRG wash buffer. The eluate was cleaved overnight with 3C protease at 4°C. To remove cleaved TrxA, the elution was again applied on equilibrated Ni-NTA resin, incubated, washed, and eluted as described previously. The elution containing NRG1 β was concentrated with a 3kDa cutoff and applied on an S200 10/300 increase column (GE Healthcare). Protein from the major peak was stored in aliquots at -80°C for subsequent receptor purifications. Yields ranged from 5-10 mg/L of culture.

Trastuzumab and Pertuzumab Fab Expression and Purification

The Fragment antigen binding (Fab) heavy chain and light chain sequences encoding for Trastuzumab and Pertuzumab were inserted into the pSVF4 vector. For each Fab, a 1x Flag tag was inserted after the heavy chain constant domain and a 6x histidine tag was inserted after the light chain constant domain. Constructs were transformed into BL21 gold *E. coli* and scaled up to 6L in 2xYT media under Ampicillin antibiotic selection. Cultures were grown at 37°C until achieving an OD of 0.8-1.0, induced with 1mM IPTG for 6 hrs at 37°C, harvested by centrifugation, and stored at -80 °C. Cells were resuspended in 100 ml of lysis buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4 with DNaseI (Roche), 0.5 mM MgCl₂ and 1 mM PMSF). Cells were sonicated until fully lysed and resulting lysate was incubated at 65 °C for 30 minutes. The lysate was cooled on ice and spun down at 40,000 rpm for 60 minutes at 4 °C. The clarified

lysate was loaded onto a Protein A column equilibrated in Buffer A (20mM sodium phosphate, 500 mM NaCl, pH 7.4), washed with 10 column volumes of Buffer A, and eluted in 100mM acetic acid by fractionation into neutralizing 20 mM Tris, 150 mM NaCl, pH 9.0 buffer. Immediately following Protein A purification, eluent was concentrated and loaded onto a Superdex 200 10/300 Increase column (GE Healthcare) equilibrated in 50 mM Tris, 150 mM NaCl, pH 7.4. Fractions corresponding to Fab were pooled and stored at 4 °C until needed.

Near Full-Length Receptor Expression

hHER2 with a C-terminal tail truncation (Δ 1030–1255) followed by maltose binding protein (MBP) and twin-strep tags was cloned into pFastBac with a CMV promoter. A single point mutation in the HER2 kinase domain, G778D, which confers Hsp90 independence²⁵, was introduced to improve yields. hHER3 with a C-terminal tail truncation (Δ 1023–1342) followed by a twin-strep tag was cloned in pFastBac with a CMV promoter. Two oncogenic mutations that stabilize the asymmetric kinase domain dimer, Q709R and E928G, were introduced to further improve heterodimer yields^{22,23}. hHER4 with a C-terminal tail truncation (Δ 1029–1308) followed by a maltose binding protein (MBP) and 8x histidine tag was cloned into pFastBac with a CMV promoter. The HER2, HER3, and HER4 constructs were each transfected into 30-60 ml Expi293 mammalian suspension (Life Technologies) cells cultured to 4×10^6 cells/ml at 37°C 8% CO₂ following the standard expression protocol. 10mM canertinib in DMSO was added 16-18 hrs post-transfection to a final concentration of 10uM (for HER2 expression) along with enhancers 1 and 2. Cells were harvested, flash frozen, and stored at -80°C 24 hrs later. The same procedure was followed for HER2 in the presence and absence of S310F mutation.

Heterodimer Purification

Cells were resuspended with lysis buffer (50 mM Tris cold pH 7.4, 150 mM NaCl, 1 mM NaVO₃, 1 mM NaF, 1 mM EDTA, protease inhibitors (eOComplete, Roche), DNase I, and 1% DDM (Inalco)) and lysed for 2 hrs by gentle rocking at 4°C. Lysate was clarified by centrifugation at 4,000g for 20 min at 4°C. Purified EGF-like domain of NRG1 β was incubated with G1 Flag Resin (Genscript) for 1 hr at 4°C and serially washed 3x with Buffer A (50 mM Tris cold pH 7.4, 150 mM NaCl). Clarified HER2 and HER3 receptor lysates were mixed and incubated O/N in batch mode at 4°C with NRG1 β Flag beads. NRG1 β Flag beads were serially 3x washed with Buffer A + 0.5 mM DDM (Anatrace) and eluted with Buffer A + 0.5 mM DDM + 250mg/ml of Flag peptide (SinoBiological). The eluate was then applied to amylose resin in batch mode for 2 hrs, washed serially 3x with Buffer B (50 mM HEPES cold pH 7.4, 150mM NaCl) + 0.5 mM DDM and eluted with amylose elution buffer (Buffer B + 0.5 mM DDM + 10 mM maltose) O/N at 4°C. The eluate was concentrated to 0.4ml with a 100-kDa concentrator (Amicon) and mildly crosslinked in 0.2% glutaraldehyde for 40 min on ice. The sample was loaded on a Superose6 10/300 (GE Healthcare) pre-equilibrated with Buffer A + 0.5 mM DDM collecting 0.5 ml fractions. Peak fractions corresponding to heterodimer sample were pooled, concentrated down to ~20ul with a 100-kDa concentrator, and flash frozen for grid preparation. The same purification protocol was followed for HER2 S310F/HER3 heterocomplex. The HER2 S310F/HER3 + trastuzumab Fab complex sample was generated by incubating a 5x molar excess of Fab with the heterocomplex prior to crosslinking, gel filtration, and imaging.

Qualitative Kinase Assays of Detergent-Solubilized Receptor Samples

Detergent-solubilized receptor samples were incubated with 1mM ATP pH 7.5 and 1 mM Mg^{2+} for 0, 2, 5, 10, 20, and 30 min. The reaction was quenched with SDS loading buffer. Autophosphorylation was qualitatively assessed by Western Blot with anti-phosphotyrosine antibody (Sigma).

Electron Microscopy Sample Preparation and Imaging

Fractions corresponding to heterodimer were applied to negatively glow-discharged carbon coated copper grids, stained with 0.75% uranyl-formate, and imaged on an FEI-Tecnai T12 with a 4k CCD camera (Gatan). The resulting negative stain micrographs were assessed for particle homogeneity and particle density by negative stain was used to determine the target concentration for cryo-EM with graphene oxide grids which typically require 2-5x negative stain concentrations.

3 ul of purified and concentrated heterodimer sample (as empirically determined by negative stain) was applied to graphene-oxide coated Quantifoil R1.2/1.3 300 mesh Au holey-carbon grids prepared as previously described³¹, blotted using a Vitrobot Mark IV (FEI) and plunge frozen with liquid ethane.

Grids were imaged on a 300-keV Titan Krios (FEI) with a Gatan K3 direct electron detector (Gatan) and an energy filter. Data for HER2/HER3/NRG1 β (5228 micrographs) and HER2-S310F/HER3/NRG1 β (4927 micrographs) were collected in super-resolution pixel mode at a pixel size of 0.4175Å/pix with a dose rate of 8.0 e⁻ per pixel per second. Images were recorded in 5.9 s exposures over 118 subframes with a total dose of 0.57 e⁻/Å². Data for HER2-

S310F/HER3/NRG1 β (7566 micrographs) with trastuzumab were collected in super-resolution pixel mode at a pixel size of 0.417Å/pix with a dose rate of 8.0 e⁻ per pixel per second. Images were recorded in 6 s exposures over 120 subframes with a total dose of 0.55 e⁻/Å². All data were collected at 105,000x magnification with a requested defocus range of 0.9-2.0 microns.

Image Processing and 3D Reconstruction

Motion corrected micrographs (MotionCor2⁵³) were initially processed in CryoSPARC³⁴. To account for reduced GO coverage with detergent sample, all datasets underwent strict micrograph curation with a final yield of ~40-50% of the collected micrograph stack. Particles were picked through template picking with the extracellular domain volume low-pass filtered to 25Å, extracted with 2x Fourier cropping with patch Contrast Transfer Function (CTF) parameters, and subjected to iterative rounds of ab initio and heterogeneous refinements. Once reasonable FSC (Fourier Shell Correlation) curves were achieved, unbinned particles were re-extracted run through subsequent rounds of heterogeneous and non-uniform refinements to achieve reconstructions with the highest resolution and healthiest FSCs. The final reconstruction of HER2/HER3/NRG1 β used for model building included 123,173 particles with C1 symmetry and attained a Gold Standard-Fourier Shell Correlation (GS-FSC) of 2.9Å. The final reconstruction of HER2 S310F/HER3/NRG1 β used for model building included 99,755 particles with C1 symmetry and attained a GS-FSC of 3.1Å.

To address incomplete Fab occupancy, a stack containing 330,000 particles from the HER2 S310F/HER3/NRG1 β + Herceptin Fab dataset was imported into RELION³⁵ and subclassified through skip-align classification. Particles classified into reconstructions without Fab density were removed from the particle stack. A final particle stack from Relion containing 243,376 particles

was re-imported into CryoSPARC and subjected to non-uniform refinement to produce a reconstruction with a final GS-FSC of 3.45Å.

Each map was assessed for local and angular resolutions through ResMap⁵⁴ and 3DFSC⁵⁵ respectively. For all reconstructions, extracellular subdomains I-III achieved the highest local resolutions (~3Å) while that of subdomain IV varied from 4-8Å suggesting that a high degree of flexibility exists closer to the transmembrane domains. All reconstructions achieved a sphericity > 0.9.

To recover micelle and sub-micelle densities, 2x binned particle stacks for HER2/HER3/NRG1β and HER2 S310F/HER3/NRG1β were imported into RELION and further 3D-classified. Particles classified into 3D classes with substantial micelle densities were re-extracted with shifted coordinates (PyEM)⁵⁶ on the center of the micelle and refined. Resulting reconstructions featured convincing sub-micelle density with volumes large enough to accommodate transmembrane domains and kinases.

Model Refinement and Validation

An initial model was generated by docking HER2 (PDB ID: 1N8Z) with a homology model of liganded HER3 from its closest homolog, HER4, in SwissProt (PDB ID: 3U7U) into the HER2 S310F/HER3/NRG1β map. Given the substantial variation in domain IV local resolution, domain IV was truncated from the model and domains I-III were torsion relaxed and iteratively rebuilt in Rosetta⁵⁷. Top scoring models were selected and further edited in Coot⁵⁸ and Isolde⁵⁹. Domains

IV were then placed into the model (HER2 PDB ID: 6OGE, HER3 PDB ID: 1M6B) and torsion relaxed.

For HER2 S310F/HER3/NRG1 β + Trastuzumab Fab, the Fab (PDB ID: 6OGE) was torsion relaxed with the HER2 S310F/HER3/NRG1 β model in Rosetta. Model statistics were routinely assessed in Phenix⁶⁰⁻⁶² and glycan geometries were cross validated in Privateer⁶³. All structures were deposited into the Protein Data Bank (PDB).

Small-scale Heterodimer pulldowns and Western Blot

Tagged HER2 and HER3 expression constructs were co-transfected into 2 ml cultures of Expi293 cells as described above. Cell pellets were lysed in 1 ml lysis buffer and clarified lysates were subjected to NRG-pulldown and eluted in 250 μ l FLAG elution buffer as described above. The extent of heterodimer formation was assessed by Western Blot. Samples were boiled in SDS-loading buffer at 95°C for 5 min, run on 4-15% acrylamide gels and transferred onto PVDF membranes. Membranes were blocked in 3% BSA in TBS with 0.1% Tween (TBST) overnight and incubated with Step-Tactin HRP (IBA, 1:5000) in TBST + 3% BSA for 1hr at room temperature. Membranes were washed 5x with TBST and signal was detected using ECL Western Blotting detection reagent (GE) or ECL prime (VWR).

Trastuzumab and Pertuzumab Pulldown Assay

Wild-type HER2 and S310F HER2 heterodimeric complexes with HER3 were expressed and purified as described above until elution from amylose resin with the exception that amylose wash and elution buffers contained 50 mM Tris pH 7.4 instead of 50 mM HEPES pH 7.4. Eluates were

concentrated to 100 μ l and maltose was removed via buffer exchange using 7kDa MWCO Zeba spin desalting columns. 70 nM heterodimer solutions were each incubated with 1 and 10x molar ratios for 30 min and bound to amylose resin overnight. Complexes were eluted as described above and complex formation with trastuzumab and pertuzumab were assessed by Western Blot.

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Chapter 2: HER2/HER3 Heterocomplex Assembly

ABSTRACT

The assembly of the HER2/HER3/NRG1 β heterodimer is thought to follow the ligand induced dimerization model of activation in which Neuregulin-1 β (NRG1 β) binds to a monomeric HER3 receptor to induce its dimerization with monomeric HER2¹. In this study, we examine nearly full-length HER2 and HER3 alone to dissect receptor states leading to the pro-mitogenic HER2/HER3/NRG1 β heterocomplex^{2,3}. To our surprise, we find that HER2 may be isolated from cells as a homodimer that is neither dependent on the formation of a classical heart-shaped extracellular domain nor reliant on the classical asymmetric kinase dimer interface. Similarly, HER3 alone forms stable unliganded complexes that are substantially larger than monomers and may be disassembled into monomers through the addition of the tyrosine kinase inhibitor, bosutinib. In the presence of NRG1 β , HER3 remarkably forms liganded dimers and we present a sub-nanometer resolution cryo-EM reconstruction of the NRG1 β bound HER3 homodimer. Our results add nuance to the ligand induced dimerization model for the assembly of the HER2/HER3/NRG1 β heterocomplex, further support an inherent mode of HER2 autoinhibition, and offer additional receptor states that may be therapeutically exploited.

INTRODUCTION

In the classical ligand induced dimerization model of Receptor Tyrosine Kinase (RTK) activation, a ligand binds a monomeric receptor on the receptor extracellular module to allow two receptors to form a dimeric complex⁴. In turn, the approximation of the two intracellular kinases allows for catalytic activation. In the case of the Human Epidermal Growth Factor Receptor (HER) family, the two kinases form an asymmetric kinase domain dimer in which one kinase, activator, allosterically activates another kinase, the receiver, via a conserved hydrophobic interface⁵. HER3, a pseudokinase without appreciable catalytic activity, features an impaired receiver interface and functions as an obligate allosteric activator⁶. The three other receptors of the HER family, EGFR, HER2, and HER4, may function in either capacity as a receiver or an activator.

Members of the HER family are thought to homo- and heterodimerize when their extracellular domains are exposed to ligands⁴. In this respect, HER2 stands out as an orphan receptor without a known activating ligand. In contrast to the tethered, autoinhibited conformations of EGFR, HER3, and HER4 unliganded extracellular domains, the extracellular domain of HER2 is constitutively extended and closely resembles that of a ligand bound state⁷. However, despite being constitutively extended, the HER2 extracellular module does not homodimerize in solution or in crystal structures. Instead, the activity of HER2 is thought to be regulated by heterodimerization with other members of the HER family^{3,8}. In particular, HER2 forms a potent pro-mitogenic complex with HER3 when HER3 is activated by the ligand NRG1 β and therapeutic resistance depends on HER3 levels^{2,9}. The HER2/HER3/NGR1 β heterodimer is therefore a singly liganded, functionally asymmetric receptor. We present the structure and analysis of the HER2/HER3/NGR1 β heterodimer in the previous chapter.

The mechanism by which this medically relevant heterocomplex assembles in cells is of therapeutic value and is thought to follow the ligand-induced dimerization model. According to the model, in the absence of ligand, HER3 exists as a monomer with the extracellular domain locked in a tethered conformation¹⁰. The binding of NRG1 β to HER3 shifts the equilibrium to the extended extracellular conformation which permits heterodimerization with an already extended albeit unliganded monomeric HER2 (**Figure 2.1**).

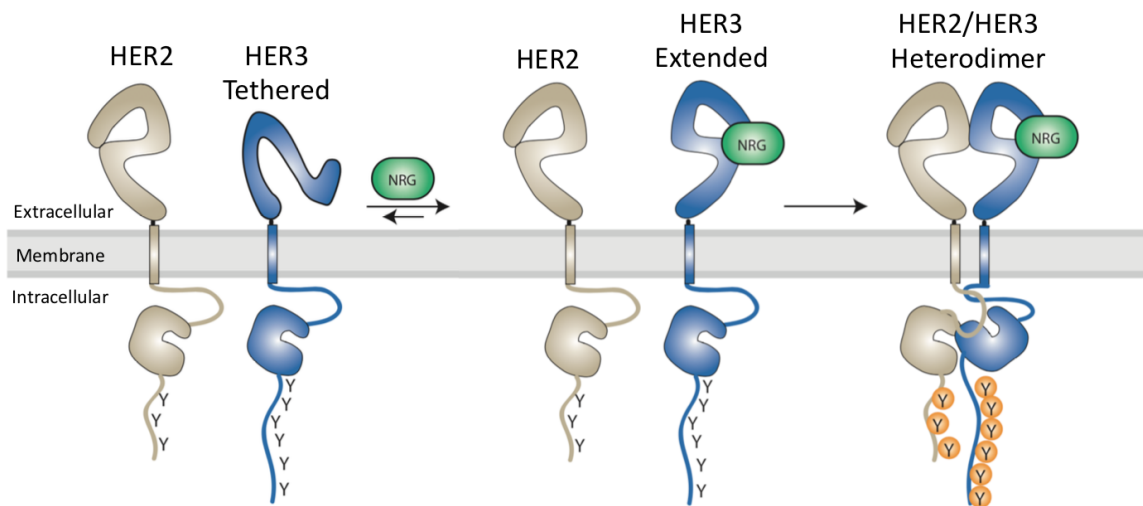


Figure 2.1. The Ligand Induced Dimerization Model of HER2/HER3 Heterocomplex Assembly. (Left) In the absence of ligand stimulus, HER2 and HER3 exist as monomers. The extracellular module of HER2 is constitutively extended whereas that of HER3 is autoinhibited and folds upon itself in the tethered conformation. (Middle) When NRG1 β binds the extracellular module of HER3, a structural transition is induced in HER3 from the tethered conformation to the extended monomer. (Right) The HER3 extended monomer heterodimerizes with the already extended HER2 to form the HER2/HER3 heterodimer. Catalytic activation through the asymmetric kinase domain dimer allows for auto- and transphosphorylation of the C-terminal tails which act like barcodes to recruit downstream signaling components.

This model of heterocomplex assembly is supported by earlier cell-based and structural studies. A chimeric receptor consisting of the HER3 extracellular domain fused to the EGFR intracellular domain failed to demonstrate ligand-induced activation in the presence of NRG1 β ¹¹. Furthermore, the extracellular domain of HER2 in all crystal (and cryo-EM) structures to date

are extended monomers despite the extremely high local concentration in the lattice network of a proteinaceous crystal⁷. However, numerous recent studies have demonstrated that HER2 may exist as a homodimer in certain pathologic states (such as HER2 amplification in HER2 + cancers) and that HER2 homodimers/oligomers could contribute to oncogenesis in addition to promoting the HER2/HER3/NRG1 β heterodimer¹²⁻¹⁵. Furthermore, all HER receptor crystal structures to date are isolated fragments of the receptor and are not in the context of full-length receptors.

The notion that HER3 may form liganded homodimers is also not entirely preposterous despite its impaired allosteric receiver kinase interface. Quantum dot labeling experiments provide evidence in cells that HER3 may homodimerize with ligand and NMR studies of isolated HER3 transmembrane domains show that isolated transmembrane components homodimerize^{16,17}. Given our abilities to express near full-length HER2 and HER3, we decided to isolate these receptors on their own to biophysically interrogate receptor behavior *in vitro* and understand the receptor states that culminate in the HER2/HER3/NRG1 β heterocomplex.

RESULTS

As described in the previous chapter, we designed tagged constructs of near full-length HER2 and HER3 and efficiently expressed the receptors individually in mammalian suspension cell format. In contrast with the heterodimer purification, we utilized HER2 and HER3 constructs with an N-terminal Flag tag, a strategy which permitted us to isolate the receptor sans NRG1 β -Flag pulldown by directly incubating detergent solubilized receptor lysates on Flag resin. The HER2 sample was then subjected to size exclusion chromatography after a second affinity step

on amylose resin (**Figure 2.2 A**). To our surprise, the major peak on a Superose6 10/300 column eluted at ~13.8 ml, an elution volume consistent with that of the HER2/HER3/NRG1 β heterodimer. We utilized mass spectrophotometry to confirm that the major species of wild-type near full-length HER2 is more consistent in mass with a homodimer (~350 kDa fully glycosylated including one detergent micelle) than a monomer (~200 kDa fully glycosylated including one detergent micelle) (**Figure 2.2 B**).

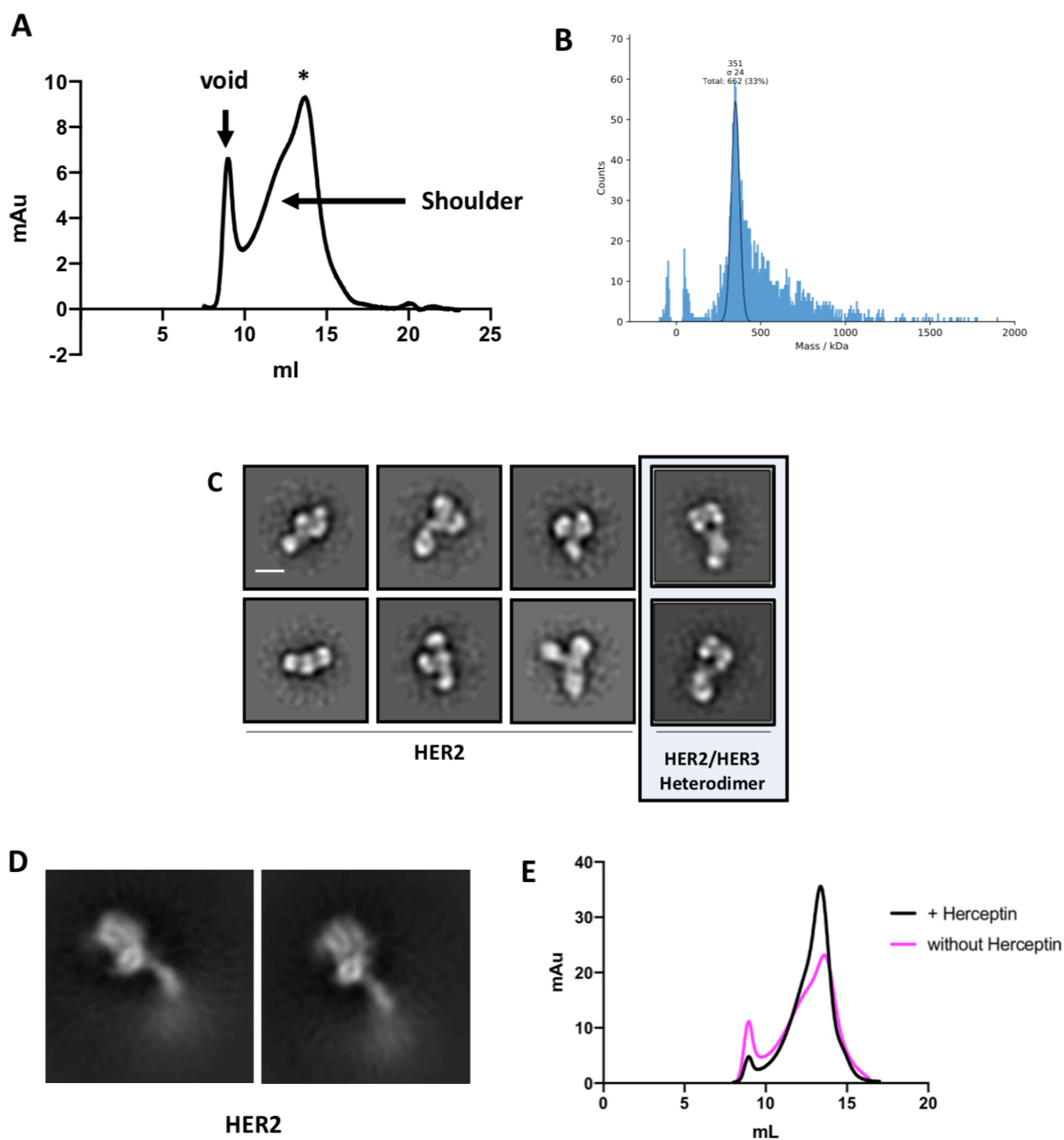


Figure 2.2. Characterization of isolated near full-length HER2. (A) Chromatogram of near full-length HER2 on a Superose6 10/300 Increase (GE Healthcare) gel filtration column. The asterisk (*) indicating major peak corresponds to elution volume of 13.8 ml. (B) Gaussian fitted trace from mass spectrophotometry of near full-length HER2 shows a consistent mass of ~350 kDa. Sample also contains heterogeneous species of higher molecular weight. (C) Negative stain EM 2D class averages of HER2 devoid of heart-shaped extracellular domains compared with HER2/HER3 heterodimer sample. White scalebar is ~10 nm. (D) HER2 cryo-EM class averages with visualized monomeric extended HER2 extracellular module. (E) Chromatogram of Herceptin-pulldown of HER2 overlaid with curve shown in A without a rightward shift upon Herceptin engagement.

Given that our HER2 sample is predominantly dimeric in a near full-length context and that the HER2 extracellular domain adopts a constitutively extended conformation, we wondered if the HER2 extracellular modules adopt the heart-shaped extracellular domain dimer stereotypical of published liganded HER homodimers and speculated through molecular dynamics¹⁸⁻²¹. We analyzed our HER2 sample by negative stain-electron microscopy (NS-EM) and did not find class averages consistent with the heart-shaped dimer (**Figure 2.2 C**). Cryo-electron microscopy (cryo-EM) on graphene oxide (GO) grids resulted in class averages centered only on monomeric extended extracellular domain (**Figure 2.2 D**). Furthermore, the introduction of trastuzumab or pertuzumab Fabs did not dissociate the HER2 homodimer on gel filtration indicating that their respective epitopes are not necessary for HER2 dimerization (**Figure 2.2 E**). Taken together, our data suggest that while near full-length HER2 may be isolated as a homodimer, the extracellular modules of HER2 do not have a propensity to interact with each other in solution despite the constitutively extended nature. This observation is consistent with (1) crystal structures of HER2 extracellular domain which are not dimeric despite the high local concentration and constitutively exposed dimerization arms and (2) our work presented in the previous chapter suggesting that the absence of ligand wedging in HER2 inherently prevents a receptive pocket for the dimerization arm of the opposing monomer⁷.

Our results raise the intriguing possibility that the HER2 homodimer is not mediated by the extracellular module but rather by the transmembrane and/or intracellular domains. To test if the kinase domains form a dimerization interface through the asymmetric kinase domain dimer, we introduced the HER2 V956R (VR) mutation which was previously shown to ablate the receiver interface and thereby eliminate catalytic activity⁵. Gel filtration and mass spectrophotometry of near full-length HER2 VR sample revealed unchanged elution volume and

mass of the major peak compared to wild-type receptor (**Figure 2.3 A, B**). Notably, the chromatogram profile of HER2 VR deviated from that of wild-type through a reduction in a higher-order oligomer species potentially indicating that a sub-population of higher-order wild-type HER2 is dependent on the asymmetric dimer. Our results suggest that the HER2 homodimer predominantly associates in an asymmetric kinase domain independent manner. It is tempting to speculate that the HER2 homodimer may be naturally autoinhibited by two mechanisms: (1) as previously discussed, the extracellular module that does not associate into the active heart-shaped dimer and (2) kinase domains associated in an autoinhibited dimer such as a putative symmetric or head-to-head dimer^{6,22,23}. Such a model is supported by the inherently low catalytic activity of HER2 kinase domains when concentrated on lipid vesicles as compared to that of EGFR or HER4 (~10% of the catalytic activity)^{5,24}. Elucidating the nature of this potential kinase dimer is an active area of our current research.

Akin to HER2, we isolated near full-length HER3 through a direct Flag pulldown from detergent solubilized cell lysate. Surprisingly, unliganded HER3 co-precipitates with a ~70 kDa

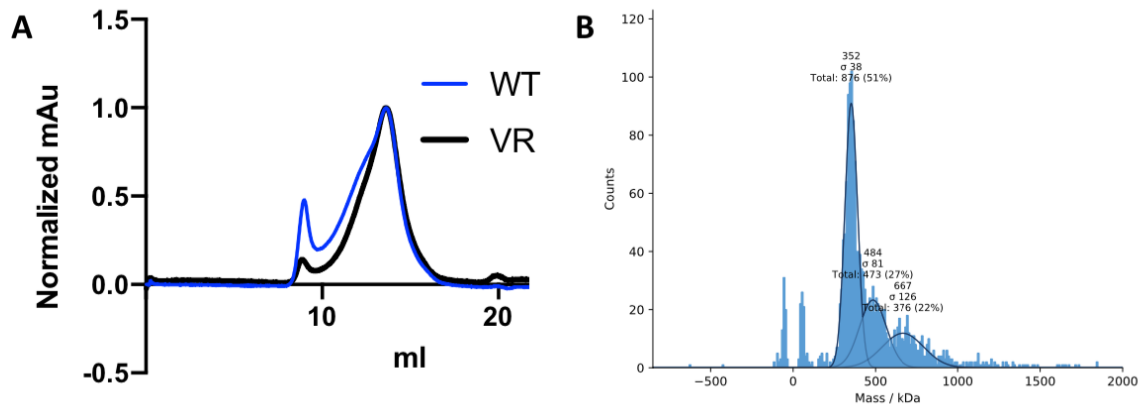


Figure 2.3. The HER2 homodimer is independent of the asymmetric dimer interface. (A) Size exclusion chromatography comparing HER2 WT with VR shows no change in the elution volume although there is a minor reduction in the shoulder. **(B)** Trace from mass spectrophotometry of near full-length HER2 VR shows a major species with a mass of ~350 kDa which is almost identical to HER2 WT and also consistent with a homodimer population. Other higher molecular weight species are also present, although to a lesser extent compared with WT.

species later identified as Hsp70, an ubiquitous chaperone, by mass spectrometry (**Figure 2.4 A**). The identity of the 50 kDa band was not determined by mass spectrometry experiment and may be worth repeating. We do not know if chaperone association is an artifact or biologically relevant. Subsequent affinity purification utilizing the C-terminal twin-strep tag resulted in sample loss in the flow-thru potentially indicating that the stably bound chaperone(s) occludes access to the tag. To avoid loss, we ran the Flag eluate directly on gel filtration which yielded a broad curve and a peak elution volume of ~ 13.8 ml (**Figure 2.4 B**). Mass spectrophotometry on pooled peak fractions revealed a mass of ~ 466 kDa with a substantial standard deviation (**Figure 2.4 C**). The putative chaperone(s) remained stably associated with HER3 throughout the entire

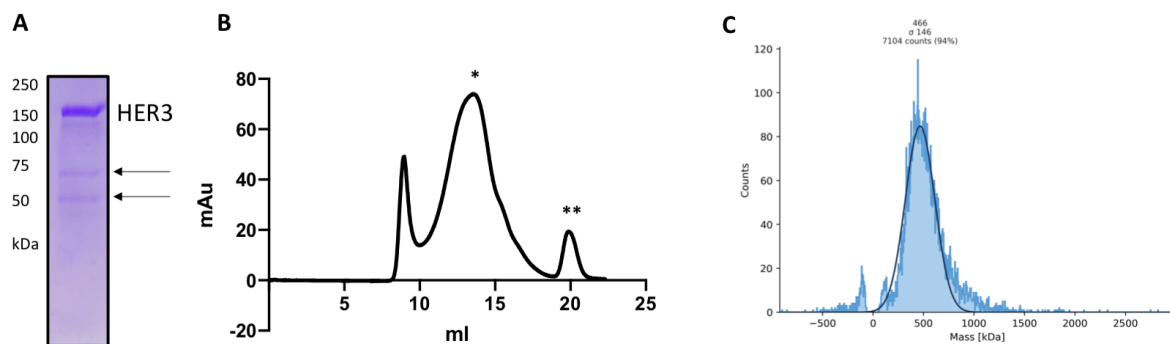


Figure 2.4. Characterization of unliganded near full-length HER3. (A) Coomassie stained gel of HER3 from the elution of the first affinity purification step. HER3 co-purifies with two other species of ~ 70 kDa and ~ 50 kDa in mass. (B) Chromatogram from size exclusion of near full-length HER3. The peak (*) elution volume is ~ 13.7 ml although the peak is notably broad. ** indicates Flag peptide elution. The two lower bands consistently appear through the major peak of gel filtration (data not shown). (C) Trace from mass spectrophotometry of pooled peak fractions indicates mass (~ 450 kDa) consistent with complexes larger than HER3 monomer with predictably large standard deviation.

purification (data not shown). The HER3 species could be dimeric or trimeric depending on the association state of the chaperone. Our results indicate that isolated HER3 may exhibit a range of oligomeric states in the absence of ligand or co-receptor that is not consistent with a monomeric

species. Interestingly, our findings correlate with observations from super resolution imaging of small unliganded HER3 puncta²⁵.

The observation of a HER3 dimer or oligomer calls into question how HER3 stably associates with itself. This is especially intriguing given that HER3 is a pseudokinase with a mutated receiver interface. In order to remove the chaperone, we expressed HER3 in the presence of bosutinib, a Src inhibitor shown to have cross-reactivity with HER3²⁶. Indeed, HER3 expressed with bosutinib did not co-precipitate with the chaperone (**Figure 2.5 A**) and, to our surprise, HER3 expressed with bosutinib eluted at an increased elution volume of ~15 ml (**Figure 2.5 B**). Our results argue that inhibitor occupancy of the nucleotide binding pocket may stabilize a putative HER3 monomer and implicates the HER3 kinase domain as one potential interface for unliganded dimer or higher-order assembly. NS-EM class averages reveal a

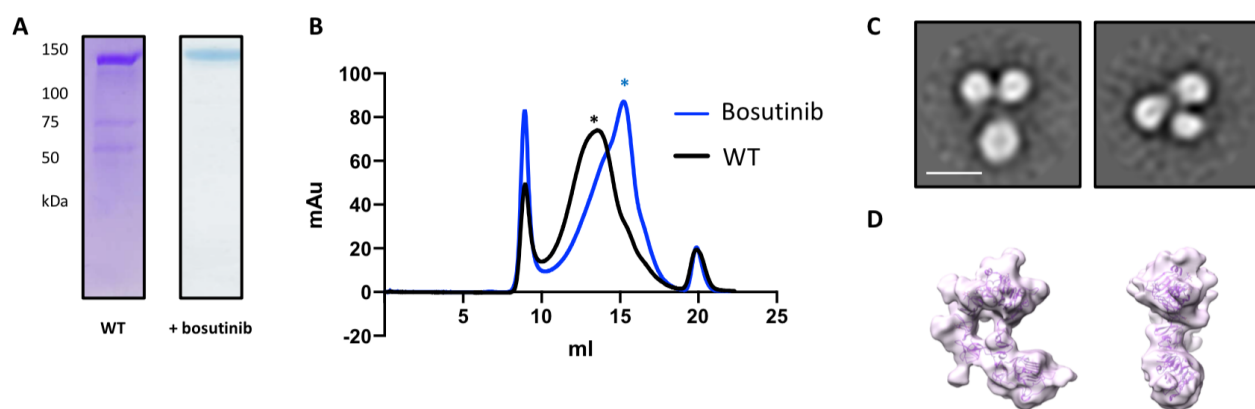


Figure 2.5. Bosutinib alters HER3 oligomerization state. (A) Coomassie stained gel of HER3 expressed in the absence and presence of bosutinib from the elution of the first affinity purification step. The two lower molecular weight bands disappear in the presence of bosutinib. (B) Chromatogram overlay from size exclusion of near full-length HER3 in the presence (blue) and absence (black) of bosutinib. The peak (*) elution volumes noticeably shift from ~13.7 ml to ~15 ml. (C) Negative stain EM 2D class averages show a high degree of sample homogeneity with a tri-lobular shape. Two lobes in the projection may correspond to extracellular domains I and III and the third represent the micelle and/or kinase. (D) Cryo-EM 3D reconstruction (~5.7Å) from ~500k particles of the fully-glycosylated extracellular domain of HER3 in the presence of bosutinib with the previously solved crystal structure of the HER3 tethered conformation (PDB ID: 1M6B) docked in the density. The micelle and kinases are not visible with this imaging strategy although the reconstruction is in the presence of the near full-length receptor.

consistent “tri-lobular” shape and a resulting $\sim 6\text{\AA}$ cryo-EM reconstruction of HER3 solubilized in LMNG is consistent with the tethered monomer extracellular conformation (**Figure 2.5 C and D**). Our results with isolated near full-length HER3 in the absence of drug or ligand are therefore inconsistent with the prevailing notion that unliganded HER3 is a monomer.

An earlier study concluded that liganded HER3 is a monomer despite the high degree of conservation in the extracellular domains across the HER family and the ability of HER3 to bind ligand¹¹. To probe this aspect of HER3 mechanism, we conducted a NRG1 β pulldown of HER3 as described in the previous chapter. Interestingly, like bosutinib, near full-length HER3 with NRG1 β reduced its association with the chaperone and size exclusion chromatography shows a broad peak with elution volumes consistent with dimers and higher-order oligomers (**Figure 2.6 A and B**). We selected fractions at 13.5-14 ml elution volume for mass spectrophotometry and found that the average mass of species in these fractions is ~ 300 kDa (**Figure 2.6 C**), a mass

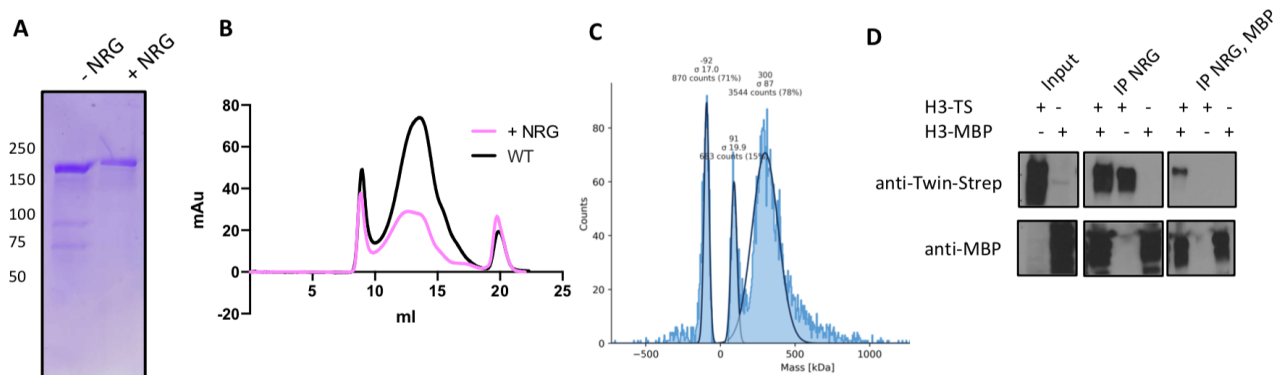


Figure 2.6. HER3 forms ligand induced homodimers. **(A)** Coomassie stained gel of HER3 purified without a ligand pulldown and with a ligand pulldown. The two lower bands disappear through ligand pulldown purification. **(B)** Chromatogram overlay from size exclusion of near full-length HER3 in the presence and absence of ligand. Peak elution volumes did not change and the peak remained equally broad. **(C)** Trace from mass spectrophotometry from a fraction selected between 13.5 – 14.0 ml with a Gaussian estimated mass of ~ 300 kDa. The ~ 90 kDa peak is micelle background and the ~ 90 kDa peak represents micelle unbinding events. **(D)** Western blot ligand pulldown assay of lysate mixed HER3 with two different C-terminal tails (MBP and TS) with subsequent amylose affinity purification. Lysate mixed sample shows both TS and MBP species indicating that liganded HER3-MBP is able to pulldown liganded HER3-TS.

consistent with a receptor dimer (~120 kDa for each monomer + micelle). We also co-expressed HER3 with two different C-terminal tags (MBP and twin-strep) and in the presence of NRG1 β , HER3 MBP dimerized with HER3 twin-strep as shown by Western Blot, adding further support for the dimeric state (**Figure 2.6 D**).

Perhaps most surprisingly, NS-EM class averages clearly show density for the heart-shaped homodimer previously ascribed only to the catalytically active HER family homologs

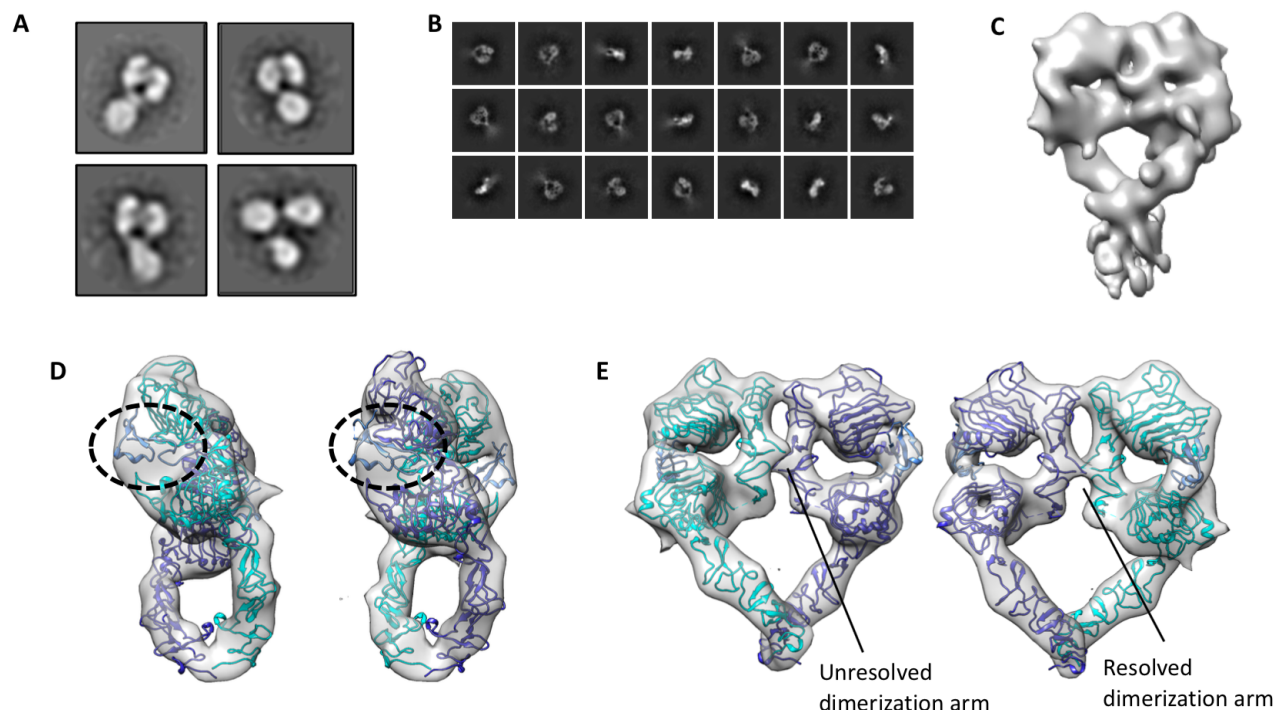


Figure 2.7. Cryo-EM reconstruction of the NRG1 β bound HER3 Homodimer. (A) Representative negative-stain EM 2D class averages with discernable heart-shaped extracellular domain module. Lower right class may have two extracellular domains separated, indicating a degree of appreciated conformational flexibility. (B) Cryo-EM 2D class averages of mildly crosslinked liganded near full-length HER3 homodimer. (C) Cryo-EM 3D reconstruction at ~9.7Å from ~43,000 particles of the liganded HER3 homodimer with strong density for the micelle. (D) The structure of the HER3 extended conformation from our HER2/HER3 heterodimer structure is docked into the density. Dotted circles represent density for ligand of both monomers although at higher contours there appears to be either lower occupancy or a different ligand binding mode of the right monomer compared to that of the left. (E) At higher contours, the dimerization arm of one monomer is more dynamic than that of the other monomer.

EGFR and HER4 (**Figure 2.7 A**). Cryo-EM class averages of liganded HER3 homodimer on GO further corroborate its heart-shaped nature and the resulting sub-nanometer reconstruction provides direct evidence for HER3 homodimerization in the presence of ligand (**Figure 2.7 B and C**). We docked the HER3 model derived from the high-resolution heterodimer structure presented in the previous chapter into the HER3 homodimer density and we see density for two ligands, thus confirming a 2:2 (ligand:receptor) state (**Figure 2.7 D**). At higher contours, we strikingly find density for only one dimerization arm and weaker density for the ligand of the opposing monomer (**Figure 2.7 E**). This is consistent with our analysis of the singly liganded HER2/HER3/NRG1 β monomer in which insufficient or absent ligand wedging between domains I and III precludes the formation of a receptive pocket for the dimerization arm of the opposing monomer.

Although HER3 is capable of forming a NRG1 β induced homodimer, it is not a strongly stabilized species given the higher resolutions routinely achieved with the HER2/HER3/NRG1 β and HER4/NRG1 β homodimer (see previous chapter) using analogous sample preparation and visualization methods. Such lability may be a byproduct of differences in HER3 kinase domain interfaces and/or dimerization arm and could explain why we did not have structures of HER3 in a liganded extended state. The unique HER3 kinase domain may also play an essential role in stabilizing the homodimer such as via a head-to-head kinase dimer as observed crystallographically⁶. As previously discussed, bosutinib monomerized unliganded HER3 although the mechanism by which the ATP analog induces receptor dissociation remains unclear. A NRG1 β pulldown of HER3 expressed in the presence of bosutinib demonstrates that the receptor is still able to dimerize and provides additional proof that HER3 dimerizes with ligand (**Figure 2.8 A**). Furthermore, ablating the allosteric activator interface of HER3 through the

classical VR activator interface mutation also did not diminish the dimer state (**Figure 2.8 B**). Another interface besides that of the asymmetric kinase domain dimer may be responsible for stabilizing the HER3 homodimer if indeed the kinase domains are necessary for dimerization. Nonetheless, the evolution of HER3 as a pseudokinase in the extracellular and intracellular modules may parallel its mechanistic function as an obligate activator of catalysis.

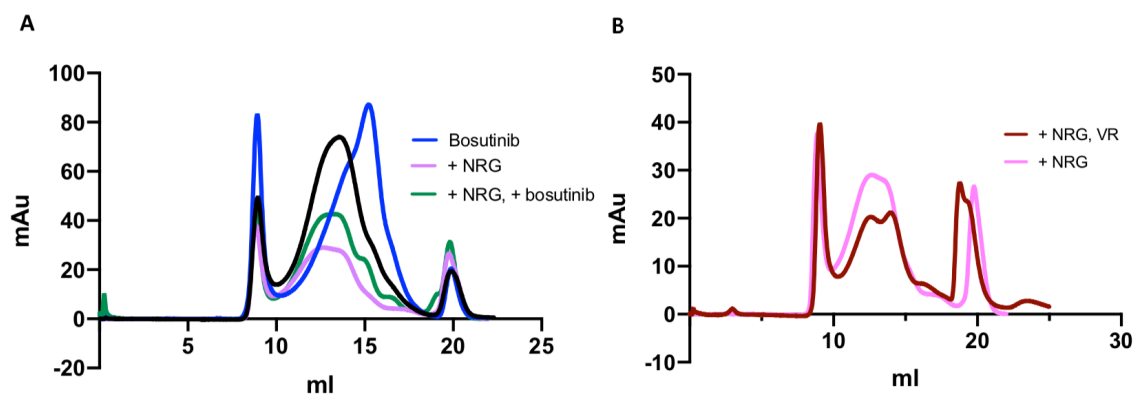


Figure 2.8. HER3 ligand induced dimerization is not dependent on activator interface but may rely on kinase interfaces. (A) Gel filtration chromatogram overlays demonstrating that ligand can induce HER3 dimerization from a monomeric state (+ bosutinib) to a dimeric state (+ bosutinib, + NRG). **(B)** HER3 VR retains ability to homodimerize.

DISCUSSION

Our studies with near full-length HER2 and HER3 indicate that the assembly of the HER2/HER3/NRG1 β heterodimer may involve intermediate receptor states currently not incorporated in the ligand induced dimerization model. Surprisingly, we find that HER2 alone associates in a homodimer which does not rely on a heart-shaped extracellular configuration nor is it dependent on the asymmetric kinase domain dimer. The exact interfaces through which HER2 stably associates with itself in a putative autoinhibited configuration remain an active area

of our research. Additionally, we see that unliganded or liganded near full-length HER3 isn't monomeric suggesting that our current understanding of HER3 mechanism remains incomplete.

We propose two models more consistent with our data thus far: (1) the scaffold model and (2) the sequestration model. In the first model, HER2, especially under pathologic conditions, predominantly exists as a homodimer while HER3 exists as an unliganded dimer or higher-order oligomer (**Figure 2.9**). NRG1 β binds to the extracellular module of HER3 to evoke a heart-shaped extracellular dimer. Unlike EGFR or HER4, the extracellular module of the HER3 homodimer may be relatively weak which could permit the facile exchange of a HER3 monomer for HER2 to form the oncogenic HER2/HER3/NERG1 β heterodimer. In this respect, HER3 may be structurally poised, like its HER2 counterpart, for heterodimerization⁸. The HER3 homodimer could also act in itself as a *scaffold* for HER2 association to form higher-order hetero-oligomers. In fact, we have routinely observed higher-order species when we isolate the HER2/HER3/NERG1 β heterocomplex and these species could consist of multiple copies of HER3

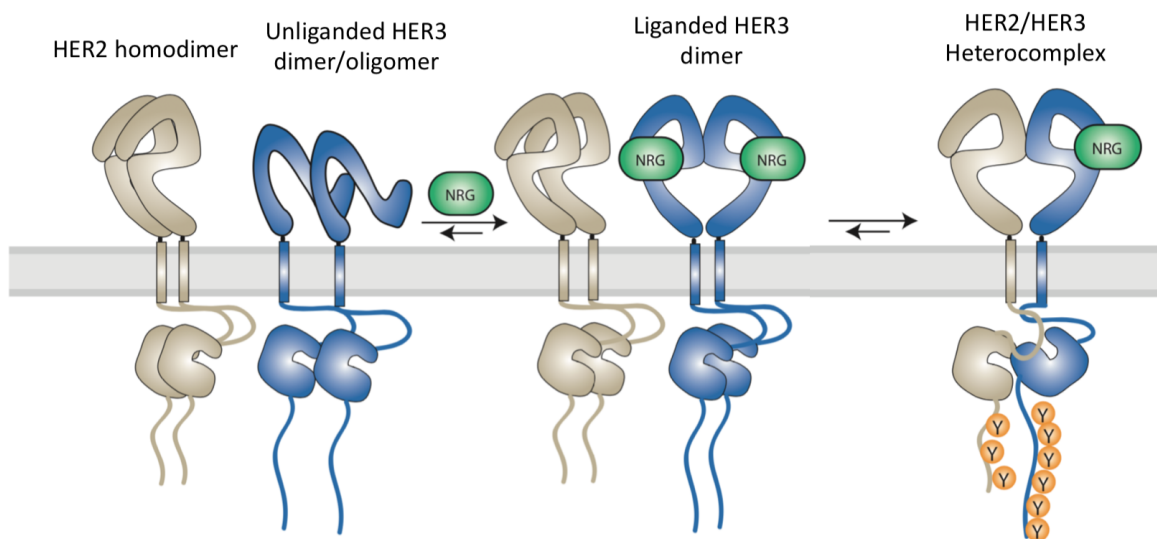


Figure 2.9. Scaffold model of heterocomplex activation. In this model, under conditions of HER2 amplification, HER2 exists as an autoinhibited homodimer and HER3 exists as unliganded dimers and/or oligomers. Upon exposure to ligand (NRG1 β), HER3 forms liganded homodimers which is an intermediate state before assembling the catalytically active HER2/HER3 heterodimer. Although observed *in vitro*, hetero-oligomers are not considered for simplicity.

or HER2 that are nucleated by the liganded HER3 homodimer. If true, the HER3 homodimer would add to the growing list of pseudokinases fulfilling a scaffolding function to recruit signaling partners^{27,28}.

Alternatively, the liganded HER3 homodimer could represent a kinetically slow but thermodynamically stable state. In this model, HER2/HER3/NRG1 β heterocomplex formation is a kinetically fast process and over time the liganded HER3 homodimer competes with the heterocomplex to act as a thermodynamic sink to regulate activity through HER2 or serve as platform to potentiate PI3K-Akt signaling through an extensive network of six YxxM sites on the HER3 C-terminal tail²⁹ (**Figure 2.10**). Our experiences in purifying the HER2/HER3/NRG1 β heterocomplex indicate that the formation of the heterocomplex potentially competes with the HER3/NRG1 β homodimer. In fact, the majority of HER3 is lost in the second affinity step pulling on HER2 quite possibly due to the preponderance of homodimer. If the HER3

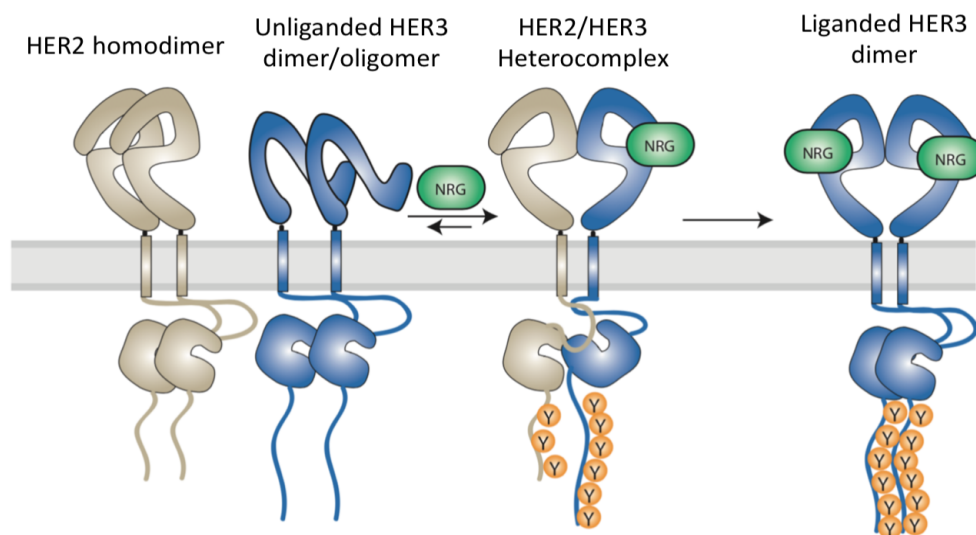


Figure 2.10. Sequestration model of heterocomplex activation. In this model, under conditions of HER2 amplification, HER2 exists as an autoinhibited homodimer and HER3 exists as unliganded dimers and/or oligomers. Upon exposure to ligand (NRG), the catalytically active HER2/HER3 heterocomplex assembles in a kinetically fast step. Over time, a thermodynamically favorable liganded HER3 homodimer state sequesters ligand and HER3 from HER2 to regulate heterocomplex signaling. Alternatively, phosphorylated HER3 homodimer could serve as a potent recruiter of downstream signaling components.

sequestration model is true, inhibitors that could monomerize HER3 such as bosutinib would prove ineffective and only serve to amplify HER2-dependent signaling. Regardless, the liganded HER3 homodimer may not simply be an unproductive state but rather an essential component of heterocomplex regulation. Recent studies provide initial evidence that even HER3 is capable of catalysis and it remains to be seen if our isolated NRG-bound HER3 homodimers are capable of catalysis. The existence and physiologic function of these states require extensive investigation in cells, especially for the liganded HER3 homodimer.

It is important to qualify that our model is supported by evidence from *in vitro* detergent-solubilized near full-length receptors isolated from transient overexpression. Detergents may influence the propensity for receptors to self-associate and receptor expression levels could alter phosphorylation states that may confound our observations. Nonetheless, our abilities to recapitulate *in vitro* the HER2 homodimer, a state observed in cells, and show that a ligand-induced monomer to dimer transition for HER3 boosts confidence in our work.

The identification of intermediate states made possible through near full-length receptor biophysics is not only relevant to elucidating how these receptors function but also may lead to novel therapeutic strategies and biomarkers. If the HER3 homodimer is a physiologically relevant state competing with the HER2/HER3 heterodimer, then stabilizing the HER3/NGR1 β homodimer in an extended state through antibodies or nanobodies may prove therapeutically useful to siphon pools of HER3 away from HER2. However, if the liganded HER3 homodimer is essential for heterocomplex formation, then destabilizing the extended conformation (or stabilizing the tethered conformation as with Patritumab) may prove therapeutically useful³⁰.

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MATERIALS AND METHODS

NRG1 β Expression and Purification: Described in Chapter 1

Trastuzumab (Herceptin) Fab Expression and Purification: Described in Chapter 1

Near Full-Length Receptor Expression: Similar constructs and expression strategies were employed as described in Chapter 1. Unless otherwise indicated, wild-type HER2 and HER3 constructs that were not subjected to NRG1 β -Flag affinity purification contained an N-terminal 1x Flag tag in addition to the standard C-terminal tags described in Chapter 1 Materials and Methods. All HER2 receptors used in this study were expressed in the absence of drug. All HER3 constructs with the exception of HER3 expressed in the presence of bosutinib were expressed in the absence of drug. If bosutinib was used, 30 μ l of 10 mM bosutinib in DMSO (SelleckChem) was contemporaneously added with the enhancers during expression.

Near Full-Length HER2 and HER3 Receptor Purification: Cells were resuspended with lysis buffer (50 mM Tris cold pH 7.4, 150 mM NaCl, 1 mM NaVO₃, 1 mM NaF, 1 mM EDTA, protease inhibitors (eOComplete, Roche), DNase I, and 1% DDM (Inalco)) and lysed for 2 hrs by gentle rocking at 4°C. Lysate was clarified by centrifugation at 4,000g for 20 min at 4°C. Clarified HER2 receptor lysate was incubated O/N in batch mode at 4°C. Flag beads Flag beads were serially 3x

washed with Buffer A + 0.5 mM DDM (Anatrace) and eluted with Buffer A + 0.5 mM DDM + 250mg/ml of Flag peptide (SinoBiological). The eluate was then applied to amylose resin in batch mode for 2 hrs, washed serially 3x with Buffer A + 0.5 mM DDM and eluted with amylose elution buffer (Buffer A + 0.5 mM DDM + 0 mM maltose) O/N at 4°C. The eluate was concentrated to 0.4 ml with a 100-kDa concentrator (Amicon) and loaded on a Superose6 10/300 Increase (GE Healthcare) pre-equilibrated with Buffer A + 0.5 mM DDM collecting 0.5 ml fractions. Peak fractions were either flash frozen or pooled, concentrated down to ~150 ul, and flash frozen for cryo-EM grid preparation.

For unliganded HER3 expressed with bosutinib, we gradually exchanged detergents from DDM to 0.01% LMNG over the Flag wash step. The first wash consisted of buffer with 100% DDM, the second, a 50%/50% v/v mixture of 0.5 mM DDM/0.01% LMNG, the third and fourth washes were in buffer 0.01% LMNG. The final gel filtration step was conducted in 0% detergent conditions. The sample was concentrated to 0.25 uM prior to grid preparation.

Near Full-Length HER2 Pertuzumab and Trastuzumab Pulldowns: Detergent lysis followed the same protocol as described above. Purified pertuzumab Fab or trastuzumab Fab was incubated with G1 Flag Resin (Genscript) for 1 hr at 4°C and serially washed 3x with Buffer A (50 mM Tris cold pH 7.4, 150 mM NaCl). Clarified lysates containing receptor were incubated O/N in batch mode at 4°C with Fab Flag beads. Fab Flag beads were serially 3x washed with Buffer A + 0.5 mM DDM (Anatrace) and eluted with Buffer A + 0.5 mM DDM + 250 mg/ml of Flag peptide (SinoBiological). The eluate was then applied to amylose resin in batch mode for 2 hrs, washed serially 3x with Buffer A + 0.5 mM DDM and eluted with amylose elution buffer (Buffer A + 0.5 mM DDM + 10 mM maltose) O/N at 4°C. The sample was loaded on a Superose6 10/300 Increase (GE Healthcare) pre-equilibrated with Buffer A + 0.5 mM DDM collecting 0.5 ml fractions.

Near Full-Length HER3 NRG1 β Pulldown: Detergent lysis followed the same protocol as described above. Purified NRG1 β was incubated with G1 Flag Resin (Genscript) for 1 hr at 4 °C and serially washed 3x with Buffer A (50 mM Tris cold pH 7.4, 150 mM NaCl). Clarified lysates containing receptor were incubated O/N in batch mode at 4°C with NRG1 β Flag beads. NRG1 β Flag beads were serially 3x washed in Buffer A + 0.5 mM DDM (Anatrace) and eluted with Buffer A + 0.5 mM DDM + 250 mg/ml of Flag peptide (SinoBiological). For HER3 NRG1 β homodimer employed in cryo-EM, 50 mM HEPES cold pH 7.4, 150 mM NaCl + 0.5 mM DDM was used in place of Buffer A during Flag wash and elution steps. For this sample, HER3 was concentrated to 0.4ml, crosslinked with 0.2% glutaraldehyde at 4 °C, quenched by adding 40 ul of 1M Tris pH 8.0 and loaded on a Superose6 10/300 Increase (GE Healthcare) pre-equilibrated with Buffer A + 0.5 mM DDM collecting 0.5 ml fractions. Peak fractions were concentrated ~200 ul and flash frozen for cryo-EM grid preparation. For HER3 NRG1 β homodimer sample not used in cryo-EM, the sample was concentrated to 0.5 ml and loaded directly onto a Superose6 10/300 Increase (GE Healthcare). Peak fractions were flash frozen and stored at -80 °C.

Mass spectrophotometry: Samples from specified fractions were diluted in room temperature buffer 50 mM Tris + 150 mM NaCl cold pH 7.45 to reduce micelle background and applied onto a clean coverslip. Receptor concentrations were approximately equivalent to that of negative stain-EM. Binding and unbinding events were recorded by the Refeyn OneMP mass spectrophotometer and analyzed using corresponding software (Discover MP). Several mass standards including apoferritin and bovine serum albumin (BSA) were used to generate a standard curve for each session. Receptor masses were fit according to the standard curve.

Electron Microscopy Sample Preparation and Imaging: For negative stain-electron microscopy, 3 ul of sample at various dilutions were applied onto negatively glow-discharged

carbon coated copper grids, stained with 0.75% uranyl-formate, and imaged at 52,000x magnification on an FEI-Tecnai T12 120keV LaB6 crystal electron microscope equipped with a Gatan 4k CMOS camera.

For cryo-electron microscopy, 3 μ l of purified HER2 or liganded HER3 homodimer at approximately 5x negative stain concentrations were applied onto graphene oxide coated Quantifoil R1.2/1.3 300 mesh Au holey-carbon grids prepared as previously described using a Vitrobot Mark IV (FEI). 3 μ l unliganded HER3 monomer expressed with bosutinib and solubilized in LMNG was applied onto negatively glow-discharged Quantifoil R1.2/1.3 300 mesh Au holey-carbon grids as previously described using a Vitrobot Mark IV (FEI).

Grids were imaged on a 200-keV Talos-Arctica (FEI) with a Gatan K3 direct electron detector (Gatan). Data were collected at a pixel size of 1.43 Å/pix with a dose rate of 10.4 e⁻ per pixel per second. Images were recorded in 11.5 s exposures over 115 subframes with a total dose of 0.5 e⁻/Å²/frame.

Image Processing: All negative stain datasets were processed in RELION. All cryo-EM datasets were processed in CryoSPARC. The final liganded HER3 homodimer reconstruction of the crosslinked HER3 MBP construct attained a Gold Standard Fourier Shell Correlation (GS-FSC) of 9.7Å composed of ~43,000 particles from 736 curated micrographs out of a total dataset size of 1131 micrographs. The final unliganded HER3 extracellular monomer reconstruction attained a GS-FSC of 5.7Å composed of ~500k particles from 1,119 curated micrographs out of a total dataset size of 1,411 micrographs.

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